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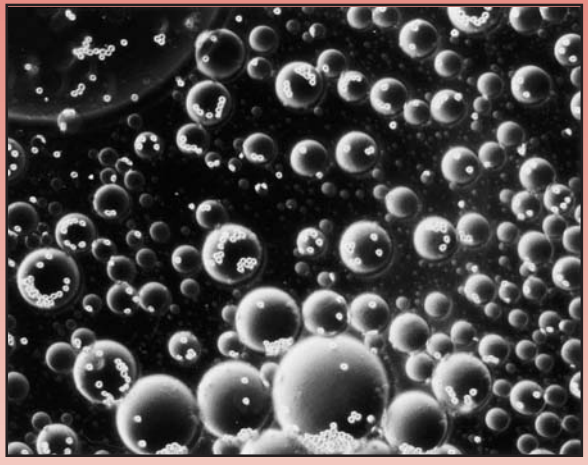
Aqueous Two-Phase Systems

Methods and Protocols

Edited by

Rajni Hatti-Kaul



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Preface

A mixture of two polymers, or one polymer and a salt, in an aqueous medium separates into two phases: this phenomenon is useful in biotechnology for product separations. Separation of biological molecules and particles in these *aqueous two-phase systems* (ATPS) was initiated over 40 years ago by P.-Å. Albertsson, and later proved to be of immense utility in biochemical and cell biological research. A boost in the application of ATPS was seen when problems of separations in biotechnology processes were encountered. Its simplicity, biocompatibility, and amenability to easy scaleup operations make the use of ATPS very attractive for large-scale bioseparations. Despite the advantages ATPS enjoys over other separation techniques, the application of two-phase systems has for a long time been confined to selected laboratories. Recent years have, however, shown a trend in which increasing numbers of researchers employ two-phase partitioning techniques in both basic and applied research.

Aqueous Two-Phase Systems: Methods and Protocols is a collection of cutting-edge methods intended to provide practical guidelines for those who are new to the area of separations in two-phase systems. Besides the established methods, many newly developed techniques with potential applications in biotechnology are also described. As an introduction, the first chapter provides a brief general overview of ATPS and its applications. The remainder of the volume is broadly divided into five sections. The first two sections are basic, describing methods for ATPS preparation and characterization, and the various partitioning techniques that may be employed. Multistage partitioning increases the resolving power of ATPS, allowing separation of materials differing only very slightly in physicochemical properties.

Partitioning applied to soluble molecules and particulates has been dealt with in the third section, where examples of different categories of materials are presented. Once the reader is acquainted with the methodology and the "tricks" to be used to obtain the desired partitioning, the separation technique may then be applied to any material of interest. Separation of particulates—including whole cells, membranes, and organelles—has been a major achievement of ATPS, one that greatly facilitates studies on cells and their properties. Purification of viruses is another successful example. With regard to soluble

molecules, partitioning has been most commonly applied to the separation of macromolecules, since their distribution between the two phases is influenced to a greater extent by a system variation than is the distribution of small molecules. This has enabled the application of ATPS even as an analytical tool to determine, e.g., the concentration and isoelectric point of proteins, molecular interactions, conformational changes of biomolecules, and so on. Lately, applications of ATPS in the separation of small molecules have also emerged. Molecules with defined properties are proving useful for understanding the interactions involved during partitioning, which would be helpful in the selection of appropriate phase systems for specific separation problems.

The main application of ATPS in biotechnology has been the isolation and purification of proteins; hence a significant part of *Aqueous Two-Phase Systems: Methods and Protocols*, compiled as Part IV, is devoted to this subject, including a glimpse of the large-scale handling of the two-phase separations. The real success of this technique has been in the extraction of proteins directly from crude feedstocks, where it has provided clarification, concentration, and even some purification in a single step. The extraction of proteins by spontaneous partitioning alone necessitates optimization of various parameters. The need to improve the selectivity of extractions has also led to exploitation of charge–charge, hydrophobic, and affinity interactions, in which specific binding groups are located in the phase used as the extractant. Integration of ATPS with other separation techniques provides scope for facilitating such selective extractions. Limiting the material costs for large-scale purposes still remains a challenge. The recycling of phase components is thus essential, which is easily done for some phase chemicals, but not for others. New phase materials with easy recyclability are being studied.

There has been interest in using aqueous two-phase systems in another area of biotechnology, i.e., *in situ* product recovery during biocatalytic processes. This concept has been presented in the last section of the volume. Analogous to aqueous systems are the newly developed polymer–polymer systems in organic solvents, which are useful with synthetic reactions.

My hope is that *Aqueous Two-Phase Systems: Methods and Protocols* will not only prove helpful in your research, but will also lead to discovery of the surprises and pleasures of aqueous two-phase systems separations. I wish to thank all the contributors to this volume for sharing their knowledge and practical experience with the reader. Special thanks are due to Associate Professor Göte Johansson, Emeritus Professor P.-Å. Albertsson, and Professor Bo Mattiasson for their useful suggestions.

Rajni Hatti-Kaul

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1

Aqueous Two-Phase Systems

A General Overview

Rajni Hatti-Kaul

1. Basis of Two-Phase Formation

Phase separation in solutions containing polymer mixtures is a very common phenomenon; in fact, miscibility of the polymer mixtures is an exception ***(1)***. Most hydrophilic polymer pairs are *incompatible* in aqueous solutions yielding two coexisting phases in equilibrium with each other, with each of the phases containing predominantly water and one of the polymer types. It is worth noting that the association of unlike polymer species into a polymer-rich phase coexisting with a polymer-poor phase, referred to as *complex coacervation,* can also occur. However, it is the former type of phase systems that form the subject of this book.

The phase separation in polymer mixtures is attributed to the high molecular weight of the polymers combined with interaction between the segments of the polymers ***(1)***. The phenomenon has been treated theoretically by applying theories on the thermodynamic properties of polymers in solution. The driving force for the demixing process in polymer–polymer–solvent systems is the enthalpy associated with the interactions of the components, which is opposed by the loss in entropy associated with the segregation of the components during phase separation ***(2)***. Water as the solvent is able to engage in a number of noncovalent interactions with the polymer, which makes the quantitative description of the phenomenon quite complex. Because the interactions increase with the size of the molecules, the phase separation in the present systems occurs at very low polymer concentrations because of their large size and corresponding small loss in entropy upon demixing ***(3–5)***.

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The phase behavior of aqueous polymer solutions is also influenced by the presence of salts, the effect depending on both their type and concentration. Often, a sufficiently high concentration of salt in a single polymer–water system can induce phase separation to yield a salt-rich, polymer-poor bottom phase that coexists with salt-poor, polymer-rich top phase ***(1,3,5)***. The relative effectiveness of various salts in promoting phase separation is seen to follow the Hofmeister series, which is a classification of ions based on their salting-out ability ***(6)***. The contribution of the anion is more important than that of the cation in determining the effectiveness of a particular salt. The multivalent anions like HPO_4^{2-} and SO_4^{2-} are most effective in inducing phase separation with poly(ethylene glycol) (PEG). The mechanisms through which salts influence the phase separation of aqueous polymer solutions are still poorly understood. A qualitative explanation for phase separation in PEG–salt–water systems relates the observed behavior to the degree to which substitution of water-cation hydration associations occurs by PEG-ether oxygen-cation interactions. Salts with small multivalent anions of high-charge density are constrained from such interactions with the polymer chain, leading to the presence of salt-depleted zones about the polymers and consequent phase formation ***(5,7)***.

2. Aqueous Two-Phase Systems: A Tool for Bioseparations

Both polymer–polymer and polymer–salt aqueous two-phase systems (ATPS) have advantages over conventional extraction using organic solvents. Since the bulk of both phases consists of water, ATPS form a gentle environment for biomaterials ***(1)***. The interfacial tension is extremely low, between 0.0001 and 0.1 dyne/cm compared with 1–20 dyne/cm for water-organic solvent systems, creating a high interfacial contact area of the dispersed phases and thus an efficient mass transfer. Furthermore, the polymers are known to have a stabilizing influence on the particle structures and the biological activities ***(1)***.

It is over 40 years since the potential of both polymer–polymer and polymer–salt systems was first realized by a Swedish biochemist, P. Å. Albertsson, for separations of cells, cell particles, and proteins ***(8–11)***. Separation could be achieved by a batch procedure or by counter-current distribution. Since then, ATPS has become a powerful tool for separation of a range of biomaterials, including plant and animal cells, microorganisms, fungi and their spores, virus, chloroplasts, mitochondria, membrane vesicles, proteins, and nucleic acids. Many of these examples are given in subsequent chapters of this volume. The reader is also referred to the volumes by Walter et al. ***(3)***, Albertsson ***(1)***, and Walter and Johansson ***(12)*** for a general account of ATPS and its various applications. Even polyphase systems containing three or four polymer phases have been constructed and used for bioseparations ***(1)***.

The basis of separation in a two-phase system is the selective distribution of substances between the two phases. Generally, small molecules are more evenly distributed between the phases; the partitioning of macromolecules is extremely variable, whereas that of particles is relatively one-sided. This distribution is governed by a number of parameters relating to the properties of the phase system and the substance, as well as the interactions between the two ***(1)***. This makes the prediction of partitioning, particularly of large molecules, a difficult task. The partitioning can, however, be made selective, e.g., by manipulating the system properties to make a particular kind of interaction predominant. The multiplicity of factors contributing to partitioning also makes the system very powerful, in contrast to the other established separation techniques, like centrifugation, electrophoresis, and others, allowing the fractionation of molecular or particulate species differing very slightly from each other. Thus, separation by partition in ATPS may often be used to substitute these other separation forms.

Aqueous two-phase systems are easy to use, involving two unit operations: equilibration and phase separation ***(13)***. Equilibration is rapid, involving mixing of the components that constitute the phase system with the material subjected to partitioning, and dispersing the phases to obtain equilibrium of phase compositions and partition. This is followed by separation of the liquid phases. The phase separation under gravity is not as rapid as in water-organic solvent systems, varying between a few minutes and a few hours because of a rather low difference in the densities of the two phases (about 0.05–0.15 g/cm^3) ***(13)***, their viscosities, and the time required by the small droplets, formed during mixing, into larger droplets ***(3)***. Centrifugation at low speed is commonly used to hasten the process. A dramatic enhancement in the phase separation process by inclusion of micron-sized magnetic particles in the ATPS has been reported ***(14)***.

3. Analytical Applications of ATPS

The sensitivity of partitioning in ATPS to the surface properties and conformation of both soluble and particulate material has made the technique a very useful analytical tool for a variety of applications. A particular advantage of the partition technique, besides sensitivity and rapidity, is that it can allow analyses on macromolecular and cell structures.

1. The most powerful applications of ATPS have been its ability to fractionate cells into subpopulations, to probe their charge and hydrophobic surface properties, and to trace cell-surface alterations occurring as a result of both normal and abnormal in vivo processes, such as differentiation, maturation, and aging, as well as in vitro treatments ***(3,12)***. The information obtained has been unique and invaluable for research in biochemistry and biomedicine. As the need for prepa-

ration of well-defined cell populations increases, it is predicted that ATPS will find a new unique area of application.

2. Partitioning in ATPS under varying conditions of pH and salt has been useful for estimating surface charges and the isoelectric point of proteins ***(12***; Chapter 18). Determination of surface hydrophobicity and conformational changes in proteins has been made possible by partitioning in the presence of hydrophobic and affinity ligands, respectively, in the ATPS ***(12)***.
3. Phase partition has been used to detect and quantify biomolecular interactions, on the basis of alteration in the partition of the reactants caused by the interaction ***(3***; Chapter 20). The change in partition of one of the reactants as a function of increasing concentration of the other can be used for the calculation of dissociation constants. This concept has been exploited by Mattiasson and coworkers to develop an immunoassay format in ATPS called "partition affinity ligand assay" (PALA) for rapid quantification of antigen ***(15)***. In comparison with conventional enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA), here the separation of free and bound antigen is done by partitioning into different phases. This format has been adapted for both competitive and sandwich-binding assays. If necessary, chemical modification with an appropriate group could be used to maintain the partitioning of one of the reactants in a desired phase ***(16)***.

4. ATPS for Product Recovery in Biotechnology

The aqueous two-phase system provides a technically simple, easily scalable, energy-efficient, and mild separation technique for *product recovery* in biotechnology.

1. Most biotechnological products, soluble molecules, and particles are obtained in very dilute solutions. Hence, the first desirable step for their recovery is *concentration*. A two-phase system is able to carry out such a concentration provided it is constructed in a way that most of the desired substance is transferred to a phase with a small volume compared to the original solution ***(1)***. The particles may also be concentrated at the interface. Since impurities may be concentrated to a lesser extent or not at all, a concomitant purification may also be achieved. A one-step or multistep procedure may be applied depending on the partitioning of the product and the contaminants. Aqueous two-phase separation is today the preferred method for concentration and purification of viruses (*see* Chapter 12). Increasing use of the technique may be foreseen for preparation of different types of cells targetted for various applications.
2. The increasing use of recombinant DNA technology for protein production has brought focus on the downstream processing operations for product recovery. By tradition, protein purification is performed in discrete stages involving clarification by solid/liquid separation techniques and concentration into small volumes, followed by fractionation by high resolution chromatography techniques. A major problem is that a higher number of processing steps results in higher product

loss. Also, many of the separation techniques are not easily scalable. Extraction in ATPS is in many cases a better alternative to existing technology in the early processing stages of large-scale isolation of proteins from crude homogenates, because it circumvents many of the shortcomings of centrifugation and filtration that arise because of high viscosity and heterogeneous distribution of particle size. Moreover, if properly optimized, it also provides integration of clarification, concentration, and partial purification. This has an influence on reducing the number of downstream processing steps and hence in improving the yields and costs of the recovery process.

Besides the intracellular enzymes from microbial cells, proteins from more complex raw materials, like animal tissues (*see* Chapter 24) and mucilagenous plant material ***(17)***, have been conveniently purified by extraction in ATPS. Protein isolation in ATPS is designed so as to suit the system under study. Two extraction steps have been commonly used ***(13)***. In the primary extraction, the cell material and the bulk of nucleic acids, polysaccharides, and contaminating proteins are collected in the denser lower phase, whereas the target protein partitions to the upper phase. A second extraction step may be applied to transfer the target protein into a fresh lower phase. This two-stage extraction procedure can be made continuous and automated. The proteins may alternatively be recovered directly from the PEG-rich phase by ultrafiltration ***(18)***, or direct application of the phase to a chromatography matrix ***(19)***.

3. Isolation of membrane proteins, normally a rather difficult and time-consuming task, has been successfully achieved by the convenient two-phase extraction procedure. Use of nonionic detergents, e.g., Triton X-114, which separate at rather mild temperatures like 20°C into a detergent-rich and detergent-poor phase, provide novel means for obtaining enriched preparations of integral membrane proteins from complex biological systems ***(20,21)***.
4. Two-phase partitioning also holds promise for rapid isolation of DNA. By using novel phase systems made of PEG-salt containing chaotropic agents and detergents, it has been shown that nucleic acids partition with high yields to the salt-rich phase, whereas proteins and other cellular material are concentrated in the other phase or precipitates at the interface ***(22)***.
5. The potential of ATPS for extraction of small-mol-wt bioproducts like amino acids has been demonstrated (*see* Chapter 9). In an unusual two-phase system obtained at high temperatures using PEG and sodium chloride, the amino acids are efficiently extracted into the PEG-rich phase ***(23)***. The product is then recovered in clear solution by precipitating PEG by cooling.
6. Low productivity in biotechnological processes is often a result of inhibition or toxicity of the product itself to the producer organism. Degradation of the product could be yet another cause for low productivity. ATPS has shown potential for improving the productivity of biotechnological processes by creating integration of product removal with that of bioconversion, a concept known as "extractive bioconversion" (*see* Chapter 37). Here the bioconversion takes place in one phase and the product is extracted into the other. Although no large-scale applications

are known, a number of examples have been reported in the literature including production of proteins as well as small molecules.

5. Environmental Remediation Using ATPS

The applications of ATPS are being extended beyond biotechnology separations to include other industrial separations and waste remediations.

1. Microbial growth in water-based cutting fluids, used as industrial lubricants during cutting, drilling, grinding, and other processes are difficult to biodegrade and create occupational hazards for the operators. Use of ATPS for large-scale removal of microorganisms and inorganic particles from cutting fluids has been demonstrated to be superior to alternative techniques, including biocide treatment, irradiation, and so forth ***(24)***.
2. Concern over industrial discharge and its environmental impact has motivated a desire for improved waste management. Elimination of toxic and volatile organic compounds as solvents for product extraction in industry should have an impact on reducing potential downstream pollution and increasing safety. ATPS have the advantage of being nontoxic and nonflammable, and PEG-based ATPS can be modified to match the hydrophobicity and water content of a number of organic solvents. To this end, Rogers and coworkers are developing ATPS as useful systems for the selective batch or chromatographic removal and recovery of a variety of solutes and particulates ***(25,26***; Chapter 8). Some interesting examples of this developing area include removal of color from textile effluents, metal ions and organics from the environment, and aromatics from crude oil.

6. Large-Scale Considerations: Protein Recovery in Focus

Without a doubt, the application of ATPS that has attracted the most interest in biotechnology is its use as a primary recovery operation for isolation of proteins from crude feedstocks. ATPS are being employed today at process scale by a few industries for protein recovery. However, despite the definite advantages of the technique, these applications are limited because of poor understanding of the mechanisms of partitioning which has made the method development wholly empirical, the need for selectivity during protein extraction, and the cost of the phase-forming components and the associated waste water treatment. These limitations form a major focus of study in aqueous two-phase technology.

6.1. Understanding Protein Partitioning

If protein partitioning could be reliably predicted, extraction in ATPS could be optimized by calculation only. However, the quantitative modeling of protein partitioning poses an extremely complex problem because of its dependence on a broad array of factors.

Complementary modeling attempts using lattice-model techniques, virial expansions, UNIQUAC, the scaling-thermodynamic approach, and others have

been successful in highlighting several molecular-level mechanisms influencing protein partitioning ***(4,5)***. The models developed provide a good start for understanding and predicting protein partitioning. However, a model allowing *a priori* calculation of protein partitioning for a wide range of phase polymer molecular weights and polymer and salt concentrations without the measurement of a large number of parameters is not yet available because many of the physical phenomena associated with these complex systems are not well understood ***(4,5)***.

6.2. Selectivity of Extraction

Scientists are trying to increase the selectivity of protein partitioning to make extraction predictable as well as to achieve sufficient purification during this step so that the subsequent downstream processing steps are not needed, or at least are reduced in number. An approach that has been studied for a long time has been the incorporation of affinity ligands, which are able to bind specifically and reversibly with the target protein, in one of the phases of the system (*see* Chapters 29–31). Normally this incorporation involves covalent coupling of the ligand to the phase-forming polymer. Thus, for applications on a large scale, an efficient recycling of ligands will be necessary. Different means for modification of ligands have been suggested to facilitate recycling (*see* Chapters 33–35). The size and/or properties of the polymer to which the ligand is coupled may be varied, thereby changing the partition behavior. Thus, attachment of the ligand to particles (*see* Chapter 33) creates a more predictable system compared to when the ligand is bound to a relatively low-mol-wt soluble polymer. Employing phase-forming components, which would allow for desirable partitioning of the ligand, hence obviating the need for coupling, is also possible ***(23)***. The use of inexpensive phase components and ligands should make affinity extraction economically feasible and will yield a substantially enriched product ***(27)***. A recent approach being studied for enhancing the selectivity of extraction is genetic modification of the target protein by adding certain amino acids to target its partitioning into a desired phase of a predetermined two-phase system ***(28)***.

6.3. Economy

Chemical costs become the dominant cost factor for large-scale protein recovery with extractive cell debris removal. Hence, the use of inexpensive phase components and their recycling has been considered essential. This has favored the choice of PEG/salt systems for industrial use; these systems also have the advantage that the phases have a lower viscosity, thus needing a shorter time for phase separation. PEG recycling has been achieved by direct repeated use of the final polymer phase (remaining after the second extraction in which

the protein is transferred to the salt-rich bottom phase) for the first extraction step by as much as 50–90% depending on the nature of the feed ***(29)***. Processing of the PEG phase prior to recycling increases the cost. Different procedures to clean up the PEG phase have been deproteinization, desalting by ultrafiltration, and extraction of PEG from the phase with an organic solvent followed by evaporation.

Salts, like phosphates or sulfates, also need to be recycled; otherwise, they increase the load on waste-water treatment. Salt recycling has been shown to be possible; however, it has not proved more economical than processes without salt recycling ***(30)***. This has motivated the use of salts that do not create waste-water treatment problems, e.g., citrate, which is easily degraded ***(31)***, and ammonium carbamate, which is volatile ***(29)***.

Efforts have also been put into replacement of the fractionated dextran by less expensive polymers, such as crude dextran, starches, maltodextrins, or pullulans, as the bottom-phase component, which greatly reduces the processing cost ***(13,23,32)***. The drive to eliminate PEG is not so great because it is available at low cost and forms a two-phase system with other neutral polymers as well as salts. In recent years, the use of novel polymers that can be readily recovered from solution by changing an environmental condition, like temperature (*see* Chapter 26) or pH ***(33)***, has been an area of intense research.

Conclusions

ATPS offer extremely simple and powerful media for separations. Their widespread use has been limited because of the complexity of predicting the partitioning of materials. It is hoped that the use of modern analytical methods, e.g., those based on light scattering, calorimetry, and so forth, would help to fill in some of the gaps in the understanding of the forces governing partitioning ***(5)***. The engineering aspects of extraction in ATPS present no obstacles and the method is well adapted for use with commercially available equipment.

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2

The Phase Diagram

Anita Kaul

1. Introduction

The *phase diagram* delineates the potential working area for a particular two-phase system and is a "fingerprint" unique to that system under set conditions of, for example, pH, temperature and salt concentration. Information that can be generated from such a diagram (*see* **Fig. 1**) includes: the concentration of phase-forming components necessary to form a system with two phases that are in equilibrium, the subsequent concentration of phase components in the top and bottom phases, and the ratio of phase volumes. Present on the diagram is a *binodal* curve, which divides a region of component concentrations that will form two immiscible aqueous phases (i.e., above the curve) from those that will form one phase (i.e., at and below the curve). Coordinates for all "potential" systems will lie on a *tie-line*; the tie-line connects two *nodes* on the binodal, which represent the final concentration of phase components in the top and bottom phases. Moving along the tie-line coordinates denote systems with differing *total* compositions and volume ratios, but with the same *final* concentration of phase components in the top and bottom phases. Also present on the binodal is a *critical point*; just above this point the composition and volume of both phases are almost equal (as is partitioned material). A theoretical account of the phase diagram can be found in **refs. *1–3***.

1.1. The Binodal

For scientists new to the field of aqueous two-phase technology, it is advisable to start with construction of a binodal so that a systematic choice of systems can be used for preliminary partitioning experiments, e.g., systems are often chosen by extrapolating through the critical point. If, however, published phase diagrams are used, a few systems should be made up and the resultant

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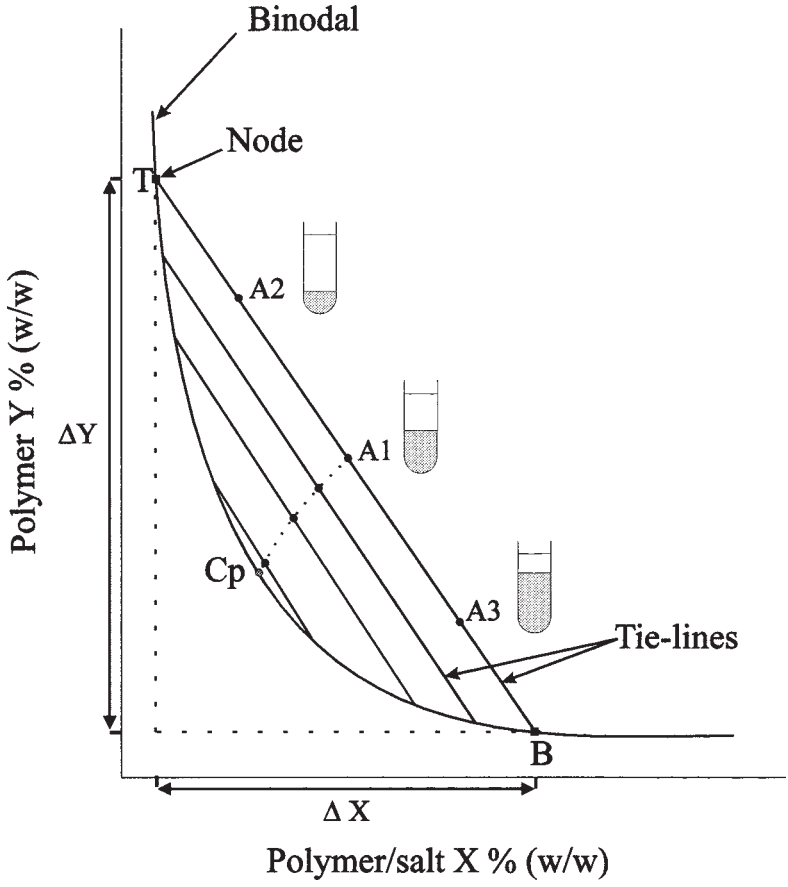

Fig. 1. Illustration of the phase diagram. Bottom phase polymer/salt X (% w/w) is plotted on the abscissa and top phase polymer Y (% w/w) is plotted on the ordinate. A1, A2, and A3 represent the total compositions (●) of three systems lying on the same tie-line with different volume ratios. The final composition of the top and bottom phase is represented by nodes T and B (■), respectively. The ratio of the segments AB (top phase) and AT (bottom phase) can be approximated graphically by the volume ratio of the two phases. The critical point, Cp (○) is determined by extrapolation (------) through the midpoints of a number of tie-lines. The difference in concentration of component X and Y between the two phases is represented by ΔY and ΔX.

volume ratios and/or phase compositions should be compared to those predicted; thus, ensuring that similar experimental conditions have been achieved. Chemicals commonly used for two-phase formation are described in the following chapter. Novel systems that comprise thermoseparating polymers and affinity ligands are also reported in Chapters 26 and 29–31.

Three methods for the preparation of a binodal are described. When phase components are mixed, only when immiscibility occurs does the resultant mixture become "turbid;" this permits a "visual" measurement of the binodal. Turbidometric titration is a relatively quick and commonly used method for determination of the binodal. A series of systems of known total composition and weight are prepared. Upon dilution with the appropriate solvent, the mixture will eventually turn "clear" when one phase is formed. The resultant composition, at the point of transition, is calculated and lies on the binodal. The cloud-point method follows a similar principle. A concentrated stock of component 1 (e.g., dextran or phosphate) is added drop-wise to a known amount of a concentrated stock of component 2 (e.g., PEG). At a critical point—the cloud point—the mixture will become turbid and is indicative of two-phase formation. The composition, just prior to two-phase formation, is calculated and provides a point on the binodal. The mixture is then diluted to below the cloud point and the procedure is repeated. An alternative method is by determination of the nodes for a series of systems, thus, providing points on the binodal. The former methods are relatively inaccurate when using polymers that are polydisperse. Such polymers produce a gradual decrease/increase in turbidity rather than a sharp change, making the point of transition awkward to calculate.

1.2. Tie-Line Length

The tie-line length (TLL) has the same units as the component concentrations (i.e., %w/w) and is often used to express the effect of system composition on partitioned material. The ratio of segments AB and AT (*see* **Fig. 1**) can be estimated graphically by using the weight ratio $V_t p_t / V_b p_b =$ AB/AT where V and ρ are the volume and density of the top (*t*) and bottom (*b*) phase. A more precise method is by analysis of the top and bottom phase composition where the $\mathrm{TLL} = (\Delta X^2 + \Delta Y^2)^{1/2}$, *X* denotes the concentration of component 1 and *Y*, the concentration of component 2 (*see* **Fig. 1**). However, if the total composition and weight ratio are known, then analysis of one phase is sufficient by using, $V_t p_t / V_b p_b = X_b - X_0 / X_0 - X_t$ where X denotes the concentration of component 1 in the top phase (*t*), bottom phase (*b*) and the total system (0) (the same holds true for component 2) ***(1)***. Tie-lines are commonly parallel and hence the slope of the tie-line (STL) can also be calculated, $\mathrm{STL} = \Delta Y_1 / \Delta X_2$ thus, facilitating the construction of further tie-lines ***(3)***. Phase density can be determined using a pycnometer or by weighing a known volume of phase in a volumetric flask using an analytical balance. Analysis of phase composition is routinely carried out by using a combination of either optical rotation (for phase components containing an asymmetric center that can rotate a plane of polarized light), refractive index, dry weight, or conductivity measurements ***(4)***.

1.3. Critical Composition

As tie-lines decrease in length, they ultimately approach a critical point on the binodal where the TLL = 0. At this point the composition and volume of the two phases theoretically become equal. The critical composition can be determined by trial and error where a one-phase system, just below the binodal, forms a two-phase system with approximately equal volumes of the two phases on addition of one component. A less tedious method is by extrapolation through the mid-point of a number of tie-lines near the binodal (*see* **Fig. 1**).

1.4. Operation Point

Once a phase diagram is constructed, the point of operation is dependent on acquiring 1) the desired conditions for partition of the target molecule(s) ***(5)***, cells or cell particles ***(6)*** and 2) an extreme phase volume ratio (if a concentration step is required). However, if systems are to be scaled-up and/or phases are to be recycled, then the working point should also take into account the position relative to the binodal. In such cases, small variations in phase composition are likely to occur and the precision that is achieved at bench-scale may not as easily be achieved at large scale. A system should therefore be "robust," and the consequence of any changes on the physical and chemical characteristics of the system should be considered. Check points should include: 1. dramatic changes in composition may occur near the critical point, and 2. a one phase system may form near the binodal. This may be owing to an unaccounted dilution effect or a variation in concentration of component 1 or component 2. Therefore, systems will differ in stability and the result of an individual change, or a combination of changes, will give a corresponding deviation in the tie-line length and, thus, in the composition and volume of the phases. The behavior of the partitioned material may, as a result, differ from that which would have been obtained in the "selected" system and the separation/purification is rendered ineffective.

2. Materials

1. Chemicals: A variety of phase chemicals and their stock preparation and storage can be found in Chapter 3. Stock solutions are prepared by weight and, therefore, the hydration of any salts used should be accounted for. For polymeric systems, each polymer may be made up in buffer, e.g., 10–100 m*M* phosphate buffer, or double-distilled water. Alternatively, make a concentrated stock of buffer at the required pH and add to the system to obtain the desired final concentration.
   Typical concentrations of stock solutions (*see* **Note 1**) are as follows.
   a. 20–30% (w/w) for various glucose polymers, e.g., dextran (average molecular weights ranging from 3400–460,000) (Amersham Pharmacia Biotech AB, Uppsala, Sweden), Reppal PES 100 (M_r 100,000) and Reppal PES 200 (M_r 200,000) and amylopectins (both from Carbamyl AB, Kristianstad, Sweden).

b. 30–50% (w/w) for PEGs (*see* **Note 2**). A range of molecular weights can be used e.g., 300–20,000 (low molecular weights are in liquid form and may therefore be used as 100%) (Serva, Heidelberg/New York).
c. 20% (w/w) for $MgSO_4$, 30–40% (w/w) for phosphate (mixture of the monobasic and dibasic salt at the required pH, *see* **Note 3**) and 25% (w/w) for citrate (mixture of trisodium citrate and citric acid at the required pH). All salts should be of analytical grade.

2. Apparatus: For phase analysis, a refractometer (Carl Zeiss, Germany), polarimeter (for optically active components) (Optical Activity Ltd, UK) and a conductivity meter (for systems with salt) (Metrohm, Switzerland) are required (*see* **Subheading 3.2.**).

3. Methods

3.1. Determination of the Binodal

By convention, the component predominantly in the bottom phase is plotted as the abscissa and the component predominantly in the top phase is plotted as the ordinate. The three methods are illustrated graphically in **Fig. 2** (*see* **Note 4**).

3.1.1. Turbidometric Titration

1. In test tubes, using the appropriate stock solutions, prepare systems with different compositions of known weight. Account for the additional volume owing to titration, e.g., if 5 g systems are prepared, 10 mL test tubes should be used (*see* **Note 5**). As an example **Table 1** shows systems that can be used for various PEG-phosphate and PEG-dextran systems, and the necessary calculations. This table may be reproduced in a spreadsheet program to allow ease of calculation.
2. Note the weight of the test tube and titrate, drop-wise, with the appropriate diluent (*see* **Note 7**) until the system just turns clear, i.e., one phase is formed. This can be carried out while the system is continually being mixed or by adding a drop, mixing, adding a second drop, and so on. To ensure that a one-phase system has formed, systems should be centrifuged (e.g., 1000–2000*g*, 5 min) (*see* **Note 8**).
3. Note the final weight of the test tube and calculate the weight of diluent added just prior to one-phase formation.
4. Because the number of systems titrated is proportional to the number of points on the binodal, greater accuracy is achieved with a greater number of systems (*see* **Note 9**).

3.1.2. Cloud Point Method

1. Weigh 5 g of a stock solution of component X into a 25-mL conical flask.
2. Weigh the flask and add, drop-wise, a stock solution of component Y until the first sign of turbidity, i.e., the cloud point. Mix as previously noted.
3. Note the weight of component Y necessary for the mixture to cloud. This provides the first point on the binodal. Refer to **Table 2** for calculations.
4. Add a known weight of diluent (*see* **Note 7**) to below the cloud point and repeat as noted.

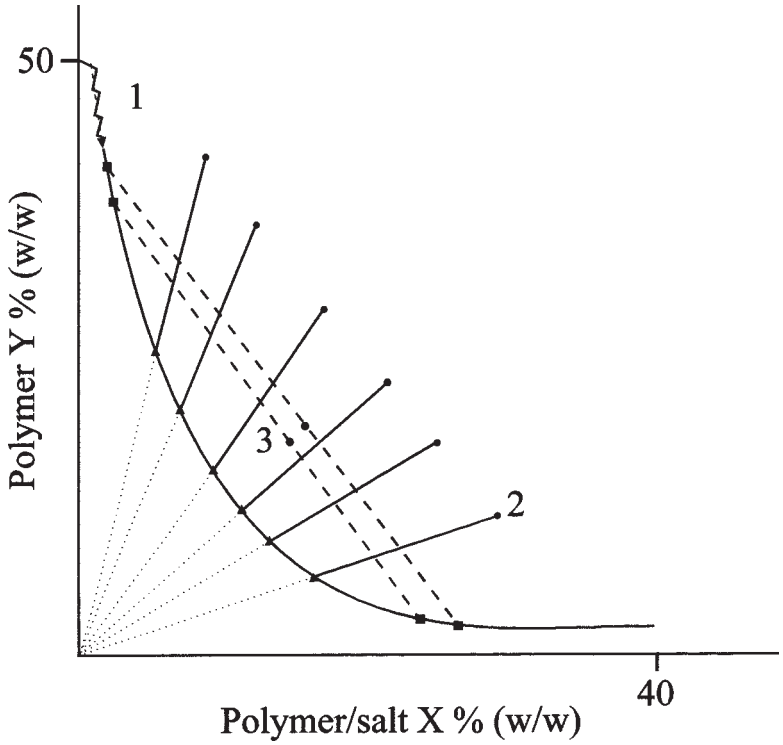

Fig. 2. Graphical representation of three methods used to determine the binodal. **1.** illustrates the cloud point method, where a concentrated stock of component 1, i.e., polymer/salt X is added to a concentrated stock of component 2, i.e., polymer Y. The solution is repeatedly taken above and below the cloud point; the binodal lies between these two points (shown by the "zig zag" line). **2.** illustrates turbidometric titration where a series of systems (●) are prepared and titrated until a one-phase system is formed—the binodal lies just above this point (▲). **3.** illustrates the determination of nodes (■) for systems, which is accomplished by preparing a series of systems lying on different tie-lines (------●------) and analyzing the concentration of components in the top and bottom phase.

3.1.3. Node Determination

1. Prepare a series of systems and analyze the phase composition of the top and bottom phase. (*See* **Subheading 3.2.**).
2. To aid in the selection of systems, start with, e.g., 5% (w/w) of component X and 5% (w/w) of component Y. If the resultant mixture forms one phase, then prepare additional systems in, e.g., 2% (w/w) increments until two phases form. Continue to prepare additional systems in this way for phase concentration analysis.

Table 1
System Composition for Various PEG-Phosphate (bold) and PEG-Dextran (unbold) Systems (*see* Note 6) and the Necessary Calculations for Binodal Determination Using Turbidometric Titration

	A	B	C	D	E	F	G	H	I	J
1										
2		**Stock % (w/w)**								
3	**Component X**	40								
4										
5	**Component Y**	50								
6										
7	**Salt *eg* NaCl**	100								
8										
9	**Final weight**									
10	**of system (g)**	5								
11										
12										
13	**System composition**			**Amount of stock**					**Final composition**	
14										
15	**X**	**Y**	**Salt**	**X stock**	**Y stock**	**Salt**	**Buffer**	**Diluent**	**X *final***	**Y *final***
16	**% (w/w)**	**% (w/w)**	**% (w/w)**	**g**	**g**	**g**	**g**	**g**	**% (w/w)**	**% (w/w)**
17										
18	**7.0** / 1.0	**27.5** / 7.0								
19	**7**	**27.5**	**6.0**	**0.88**	**2.75**	**0.3**	**1.07**	d		
20				=(A19*B10)/B3	=(B19*B10)/B5	=(C19*B10)/B7	=B10-(D19+E19+F19)		=(A19*B10)/(B10+H19)	=(B19*B10)/(B10+H19)
21	**7.5** / 2.0	**25.0** / 6.0								
22	**8.0** / 3.0	**22.5** / 5.5								
23	**8.5** / 4.0	**20.0** / 5.0								
24	**11.0** / 4.5	**17.5** / 4.5								
25	**12.5** / 6.0	**15.0** / 4.0								
26	**13.5** / 7.0	**12.5** / 3.5								
27	**15.0** / 9.0	**10.0** / 3.0								
28	**17.5** / 10.0	**7.5** / 2.5								
29	**20.0** / 11.0	**5.0** / 2.0								
30	**22.5** / 13.0	**2.5** / 1.0								

X, concentration of polymer/salt X; Y, concentration of polymer Y; X, Y final, composition on the binodal; d, amount of diluent required just to pne-phase formation. Example shown has the necessary calculations below the values.

Table 2
Calculations for Binodal Determination Using the Cloud Point Method

	A	B	C	D	E	F	G
1							
2		**Stock % (w/w)**					
3	**Component X**	40					
4							
5	**Component Y**	50					
6							
7							
8	**Amount of stock**			**Final composition**			
9							
10	**X stock**	**Y stock**	**Total Y stock**	**X final**	**Y final**	**Dilutent**	**Total weight**
11	**g**	**g**	**g**	**% (w/w)**	**% (w/w)**	**g**	**g**
12							
13	5	y		=(B3*A13)/(A13+B13)	=(B5*B13)/(A13+B13)	d	
14		*y1*	=B13+B14	=(B3*A13)/(A13+F13+C14)	=(B5*B13)/(A13+F13+C14)	d*1*	=F13+F14
15		*y2*	=C14+B15	=(B3*A13)/(A13+G14+C15)	=(B5*B13)/(A13+F14+C15)	d*2*	=G14+F15
16		*y3*	=C15+B16	=(B3*A13)/(A13+G15+C16)	=(B5*B13)/(A13+F15+C16)	d*3*	=G15+F16
17		*y4*	=C16+B17	=(B3*A13)/(A13+G16+C17)	=(B5*B13)/(A13+F16+C17)	d*4*	=G16+F17

Example shown starts with 5 g of the stock solution of component X. A known amount of the stock solution of component Y is required to cloud the solution. X, Y final: concentration of component X and Y at the point of clouding, y: amount of stock Y required just prior to clouding and d: diluent necessary to reach below the cloud point.

3.2. Tie-Line Determination

Tie-line measurement for polymeric systems containing one optically active component is described in **Subheading 3.2.1.**, e.g., PEG-dextran, PEG-hydroxypropyl starch, PEG-Ficoll, and ethylene oxide-propylene oxide-Reppal PES 100 (*see* Chap. 26 for thermoseparating polymers; *see* **Subheading 3.2.2.** for polymer-salt systems).

3.2.1. Polymeric Systems Containing One Optically Active Polymer

1. Prepare a standard curve for the optically active component, in the range of 0–10% (w/v), i.e., within the linear range (*see* **Note 10**), with the same samples prepare a second standard curve for the refractive index measurement. Prepare a third standard curve for the refractive index of the second polymer also in the range of 0–10% (w/v). If the system is prepared in a buffer then the standard curves for the pure components should be made with the same buffer, because salts also contribute to the refractive index (*see* **Note 11**).
2. Prepare the phase system for analysis, making sure that the phase components are mixed thoroughly; allow phases to separate. To ensure complete separation, centrifuge at a low speed (e.g., 1000–2000*g*, 5 min). The size of the system should be sufficient to allow the removal of at least 5 g of top and bottom phase for phase concentration analysis and a further amount for density measurements.
3. Separate the top and bottom phases making sure not to cause phase mixing.
4. Make appropriate dilutions, e.g., dilute 5 g of phase, with the appropriate solvent, to 25 mL in a volumetric flask.
5. Measure the optical rotation for each phase and calculate the respective concentrations.
6. The concentration of the second component is determined by measuring the refractive index of the top and bottom phase and by subtracting the refractive index contribution made by the optically active component.

3.2.2. Polymer-Salt Systems

1. Prepare a standard curve for the conductivity of the salt within the linear range (in %w/v).
2. Prepare phase systems as previously noted and remove 5 g samples from the top and bottom phase and dilute with water (e.g., 1/5) and freeze-dry. Note the dry weight.
3. Remove a further sample from the top and bottom phase, dilute with water, and measure the conductivity of each phase. Calculate the concentration of salt and subtract the weight contribution from the dry weight of the sample.

4. Notes

1. For greater accuracy, the concentration of all stocks should be analyzed using the appropriate method, i.e., refractive index for PEGs, optical rotation for glucose polymers, and conductivity for salts.

2. PEGs are hygroscopic and in humid conditions will pick up water. When preparing standard curves, take into account any absorbed water by, for example, determining the concentration by freeze drying.
3. K_2HPO_4 and NaH_2PO_4 display greater solubilities than their respective monobasic and dibasic salts.
4. The shape and position of the binodal is dependent on the chemicals used.
   a. Generally, high-polymer molecular weights, the addition of salt, lowering the temperature, and the presence of cell debris *(7)* all serve to decrease the concentration of phase forming chemicals necessary to form two phases.
   b. Polymer-salt systems and an increased difference in the molecular weight of the two polymers in polymer–polymer systems, both contribute to the asymmetry of the binodal.
5. When measuring the volume of the two phases, graduated test tubes are used that are further calibrated for increased accuracy. Alternatively, graduated pipets with the top end cut off and the bottom end sealed can be used.
6. Concentrations in bold face can be used for PEG-phosphate systems for PEG molecular weights ranging from 1500–20,000 at 20°C and pH 7.0. Other concentrations can be used for PEG 6000-Dextran T500 (M_r 500 000) systems at 20°C. Generally, for PEG-salt systems, higher concentrations of components are required to form two phases, when compared to polymer–polymer systems. For this method, compositions should be chosen with this in mind. If systems are prepared and form one phase, then add a known weight of component X until two phases form, calculate the resultant composition, and begin to titrate.
7. When systems are prepared in a buffer or have added salts, e.g., NaCl, the same solvent should be used when titrating/diluting, thus ensuring that only concentrations of component X and Y are decreasing. If, for example, PEG 4000–phosphate with 6% (w/w) NaCl or PEG 6000–dextran in 10 m*M* phosphate is used, then accordingly 6% (w/w) NaCl or 10 m*M* phosphate is required, respectively.
8. Centrifugation is especially advisable when using polymers that are polydisperse.
9. Generally, only the midsection of the binodal is resolved using this method. Extremes of the binodal are difficult to measure because points are almost parallel to the axes and, therefore, the cloudpoint method or determination of the nodes for systems far from the critical point can be used for this purpose.
10. The polymer concentration can also be determined with the following equation, $[P]_v = \frac{\phi}{1[\alpha]}$ where $[P]_v$ is the sample polymer concentration (g/100 mL); ϕ, the optical rotation; l the sample tube length in decimeters and α the specific optical rotation (°mL/dm/g) at 25°C with the sodium D line. The sample concentration can then be converted to % (w/w) (i.e., $[P]_w$ by using the following equation, $[P]_w = [P]_v / \rho$ where ρ is the density of the phase (the same equation is used for $[P]_v$ obtained by refractive index measurement).
11. If systems are made up with buffer or others salts, conductivity measurements should be carried out to ensure that their partition is equal between the two phases (and therefore that the refractive index contribution is the same for the two phases).

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3

Preparation of Aqueous Two-Phase Systems

Daniel Forciniti

1. Introduction

The most common aqueous two-phase systems (ATPS) are created using either two incompatible polymers or one polymer and a salt ***(1)***. These mixtures separate into two liquid phases which are in equilibrium; each phase is enriched in one or the other of the respective phase forming components. Both the phases have a water content of about 85–90%. The most frequently made systems have been those of polyethyleneglycol/dextran (PEG/Dx) and PEG/phosphate. Representative phase diagrams of these two-phase systems are shown in **Figs. 1** and **2**. An extended list including some new phase systems ***(2)*** is presented in **Table 1**.

After choosing the appropriate phase-forming species for a partition experiment, one must choose the species' concentration in each phase (the working tie-line) and the volume ratio. Published equilibrium data ***(1,3,4)*** may be used to make these choices. Unfortunately, there are experimental errors not always reported by the authors and, more importantly, variations in the molecular weight of the polymers, poor temperature control, and uncontrolled or unreported addition of additives (salts, preservatives) that may slightly move the position of the binodial. So, although reported binodals provide a good starting point for the preparation of two-phase systems, they should be used as general reference only.

The preparation of aqueous two-phase systems is quite straightforward. Normally, it involves the preparation of the stock solutions of all the phase components, which are then mixed in appropriate amounts (by weight), and then the emulsion is allowed to separate into two phases under gravity or in a centrifuge. After complete phase separation is achieved, the phases can be sampled. In the laboratory scale experiments, aqueous two-phase systems with

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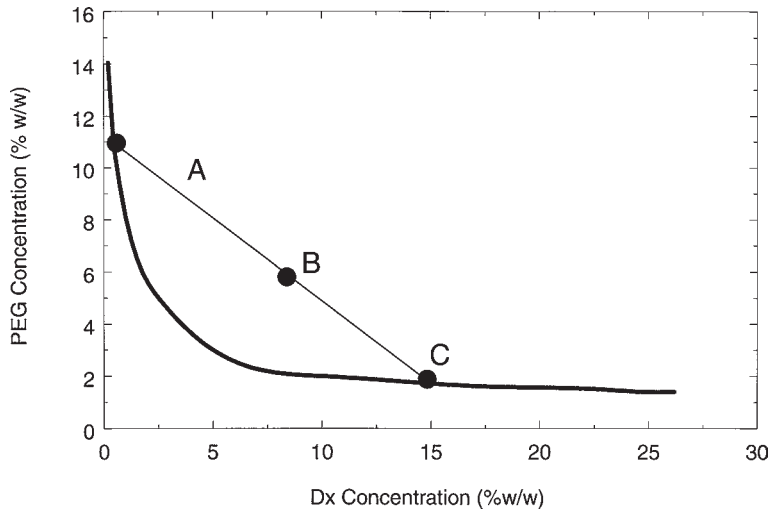

Fig. 1. Equilibrium diagram for Dx-500,000/PEG-4,000 at 20°C (Adapted from **ref. *2***).

a final mass of 5–10 g are usually prepared. At times, it may become necessary to weigh out the phase forming chemicals directly into a tube, and the final weight made up with water, buffer, or the material to be partitioned. In some systems, one of the phase polymers derivatized with, e.g., charged groups or affinity ligands, is also included. The derivatized species often constitute a minor constituent (1–5%) of the total system.

2. Materials

1. Polymers: A variety of polymers have been used to prepare two-phase systems (*see* **Table 1** and **Note 1**). Available molecular weights and some of the sources of a few commonly used polymers are shown in **Table 2**. Polydisperse polymers of good quality can be purchased from specialty chemicals companies like Sigma (St. Louis, MO). For most applications the polymers are used as received (*see* **Note 2**). Polymer batches of narrow molecular weight distributions can also be purchased but at a much higher price (*see* **Note 3**). For example, less polydisperse Dx can be bought from Amersham Pharmacia Biotech (Uppsala, Sweden), whereas narrow fractions of PEG can be purchased from PolySciences (Warrington, PA).
2. Salts: Analytical grade salts can be purchased from any specialty chemicals manufacturer.
3. Buffers: A variety of buffers have been used to regulate the pH in aqueous two-phase systems. The two most commonly used are phosphate and Tris buffers. While phosphate buffers are usually prepared in the laboratory, Tris buffers are

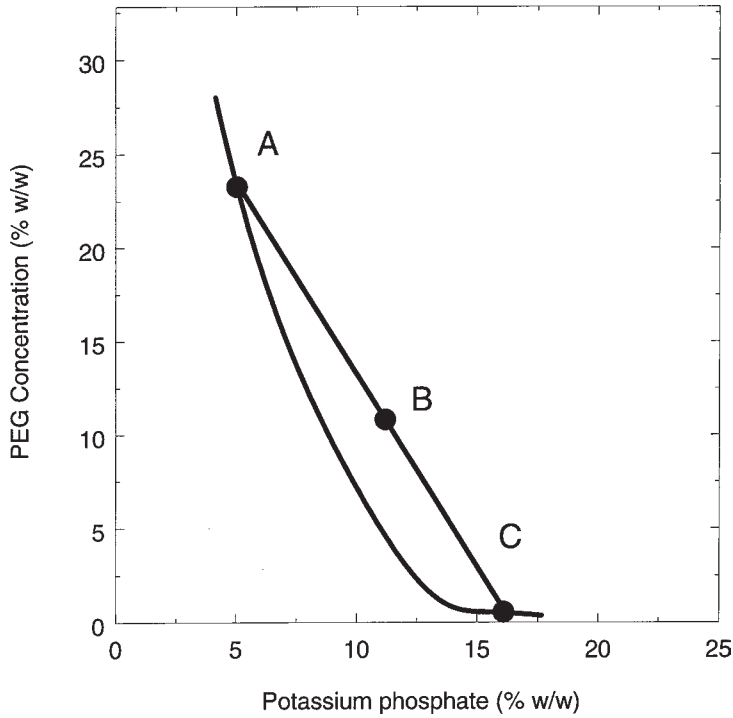

Fig. 2. Equilibrium diagram for PEG/sodium phosphate system at 20°C (Adapted from **ref. *3***).

readily available from some suppliers like Sigma. I have personally used the so called universal buffer (Solution A: 10.2 g of citric acid, 6.1 g of phosphoric acid, 5.6 g of boric acid, 544 mL of sodium hydroxide solution (1 mol/L) and enough water to make up 1 L; Solution B: HCl 0.2 mol/L. This recipe yields a 0.2 *M* buffer). By varying the relative amounts of Solutions A and B, one can obtain a range of pH values from 3–11. It is very convenient for experiments in which even minor changes in salt composition are undesirable.

4. Additives: A series of additives *(see* **Table 3**) are normally used in aqueous two-phase systems. Bactericides are conveniently added to the polymers stock solutions or in solid form to the two-phase system.

3. Methods

3.1. Preparation of Stock Solutions

The stock solutions to be prepared depends on the ATPS to be formed. For a polymer-polymer system, stock solutions of polymers and the appropriate buffer are prepared; the polymer stocks can be prepared in water or buffer. For a polymer–salt system, the stock polymer solution (usually in water), and a

Table 1
Commonly Used Phase-Forming Species

Species 1	Species 2	References
PEG	Dextran	***(1,3,5,9)***
PEG	Ficoll	***(1)***
Dextran	Ficoll	***(1)***
Methyl Cellulose	Dextran	***(1)***
PEG	Benzoil Dextran	***(6)***
Ucon	Reppal (Hydroxypropyl starch)	***(2)***
PEG	Sodium citrate/citric acid	***(4)***
PEG	NaH_2PO_4/K_2HPO_4	***(1,4)***
PEG	Ammonium sulfate	***(1)***
Sodium Dextran Sulfate	PEG (NaCl)	***(1,4)***
PEG	NaCl	***(10)***
Methyl Cellulose	Hydroxypropyl Dextran	***(1)***

Table 2
Sources and Available Molecular Weights of Some Polymers

Polymer	Molecular weights	Source
PEG	600, 1000, 1450, 3350, 8000, 10,000, 20,000	Sigma[a], Union Carbide[b], Merck[c], Polysciences[d]
Dextran	10,000, 40,000, 70,000, 100,000–200,000, 500,000, 2,000,000	Pharmacia[e], Sigma, Pfeifer & Langen[f]
Dextran-Sulfate	5,000, 8,000, 10,000, 500,000	Sigma
Ficoll	70,000, 400,000	Sigma, Pharmacia
Ucon	5000	Union Carbide
Methyl Cellulose	30,000, 80,000, 140,000	Sigma

[a]St. Louis, MO.
[b]Danbury, CT.
[c]Darmstadt, Germany.
[d]Warrington, PA.
[e]Uppsala, Sweden.
[f]Dormagen, Germany.

stock salt solution of the desired pH, are prepared. In the systems for affinity partitioning (Chapters 29–32), stocks of derivatized polymers are also prepared. Sodium azide (1 mmol) can be added to each stock solution as a bactericide; alternatively, chloroacetamide can be added directly to the phase systems (**Subheading 3.2.**). The stock solutions may be stored before use (*see* **Note 4**). Preparation of some representative stock solutions is described below:

Table 3
Some Common Additives Used in Aqueous Two-Phase Extraction

Additive	Comments
NaCl	Changes partition coefficient; used in cross-partitioning experiments
Na_2SO_4	Chages partition coefficient; used in cross-partitioning experiments
NaN_3, 5 mmol	Bactericide
Chloroacetamide 1%	Bactericide
Affinity ligands (PEG-red, Cibacron-blue, etc.)	Changes partition coefficient of target protein
Charged polymer	Changes partition coefficient and phase equilibrium

1. PEG stock of 30–50% (w/w) is prepared by accurately weighing the polymer and the water or buffer in a flask, and stirring for an hour or more on a magnetic stirrer until a clear solution is obtained. For example, to prepare 200 g of a 50% w/w PEG solution, dissolve 100 g of PEG in 100 g of buffer (*see* **Note 5**).
2. Dextran stock of 20–30% (w/w) concentration is prepared by first making a paste of the powder with a small amount of water, and then adding the rest of the water to reach the final mass. Because of the presence of water (5–10%) in commercial Dx, an amount of Dx in excess to that needed may be weighed. For example, for 200 g of a 30% w/w Dx solution, prepare a paste using Dx (66 g) and a minimum amount of water (~ 20 g); add the remaining water (114 g) and stir continuously on a hot plate up to 95°C; this is done to facilitate the dissolution of the polymer and to prolong the shelf-life of the polymer. The final Dx concentration should be checked with a polarimeter or by freeze drying (*see* **Note 6**).
3. Two to four times concentrated stock solutions of salts are prepared using reagent grade chemicals. The solutions may be adjusted to the required pH before making up the final mass of the solution. For example, in case of phosphate and citrate solutions, the acid and the basic salts are weighed in molar ratios determined by the desired pH. Salts can also be used directly in the solid form.
4. Stock solution of the desired buffer, of up to 10X concentration can be prepared. For example, universal buffer (0.2 *M*) at the desired pH is prepared (**Subheading 2., item 3**).
5. Stock solution of affinity ligand, e.g., PEG bound ligand (*see* **Table 3**) can be prepared at a concentration of hundred fold or more, as the final concentration of these species in the two-phase system is quite low.

3.2. Preparation of Aqueous Two-Phase Systems

Phase forming components are chosen depending on the desired two-phase system (*see* **Note 7**). Slightly different approaches for the preparation of the

two-phase systems are followed depending on the equilibration temperature (*see* **Note 8**). At room temperature, the preparation of an aqueous two-phase system is quite straightforward. For example, a Dx-500,000/PEG-4,000 system at room temperature is prepared as follows:

1. Shake the stock solutions well so that there are no density gradients.
2. Place a graduated centrifuge tube of 15 mL total volume on a weighing balance.
3. Weigh out the stock solutions into the tube in order of their increasing densities, and layer them carefully over each other. This facilitates the removal of portions of one stock solution in case of error during weighing. An example of a phase system preparation is 3.33 g of a 30% w/w Dx solution, 1.12 g of a 50% w/w PEG solution, 0.1 g of chloroacetamide, and 5.45 g of buffer. Because of the problem of accurately pipetting the polymer stock solutions due to their high viscosity, they are measured by weight and are easily transferred using a Pasteur pipette with a broken tip.
4. The systems are shaken for 2–20 min by hand or eventually by using a rotary shaker (*see* **Note 9**).
5. The systems are permitted to settle for a period of 30 min up to 24 h depending on the system composition (*see* **Note 10**), or they are centrifuged for 2–15 min at 1500*g*.
6. If instructions are followed carefully, the preparation of aqueous two-phase systems should be routine laboratory work. One may, however, encounter unexpected problems; identification of the source of problem leads to simple solutions (*see* **Notes 11** and **12**).

4. Notes

1. Polymers: PEG, a linear synthetic polymer of ethylene oxide units, and Dx, poly (α-1,6-glucose), are the most commonly used polymers in the preparation of aqueous two-phase systems. Examples of other sugar polymers used are Ficoll (polysucrose), pullulan and maltodextrins; besides use of some derivatized carbohydrate polymers has also been reported including methylcellulose, hydroxyethylcellulose (HEC), Reppal PES (hydroxypropyl starch), benzoyldextran, dextran sulfate, and diethylaminoethyl (DEAE)-dextran.
   Examples of synthetic polymers, besides PEG, are polyvinyl alcohol (PVA), Pluronic, Ucon (a random copolymer of 50% ethylene oxide, 50% propylene oxide; $EO_{50}PO_{50}$), $EO_{20}PO_{80}$, and so forth.
2. Removal of impurities from polymers: For most applications the polymers are used as received. In cases where the material to be partitioned is too sensitive to impurities the polymers may be purified. For example, multivalent ions present in commercial Dx are conveniently eliminated by dialysis, ultrafiltration, or by a desalting step. Impurities in PEG (antioxidants, ethylene glycol, and diethylene glycol) can be eliminated by ether or hexane precipitation of a PEG/acetone solution ***(1)***. For example, 300 g of PEG are dissolved in 6 L of acetone. Three liters of ether are added, the mixture is stirred and allowed to stand overnight. The

precipitate is collected by filtration, washed with a 2:1 acetone-ether mixture and dried under vacuum.

3. Polymer molecular weight: Often, the molecular weight and the molecular weight distribution are given by the manufacturer. The ratio of the weight-average molecular weight, M_w to the number-average molecular weight, M_n is known as the polydispersity of the polymer fraction. The closer the ratio is to 1.0, more monodisperse the polymer is. In the absence of accurate information, the molecular weight can be determined by size exclusion chromatography-low angle light scattering or by size exclusion chromatography using the appropriate standards. Some companies, like Wyatt Technology (Santa Barbara, California), provide determination of molecular weights against a fee. The molecular weights of PEG and Dx can be determined using a Superose 12 column (Amersham Pharmacia Biotech) eluted with a 3% NaCl solution at room temperature. Molecular weight standards for PEG can be bought from Polysciences. Because molecular weight standards for Dx are difficult to obtain, narrow fractions of pullulan (Polysciences) can be used.
4. Preservation of stock solution: Stock solutions must be stored in the dark to prevent UV-induced oxidation and in the refrigerator to retard microbial growth. Stock solutions of the polymers should be consumed within 30 d of being prepared. Oxidation is of particular concern with PEG stock solutions. Although antioxidants are added by the manufacturers, age and exposure to light induces the formation of acidic groups. These acidic groups can be detected since they decrease the pH of the stock solution and/or they change the coloration of it toward yellow.
5. Measuring PEG concentration: Solid PEG, if properly stored, contains very little water (less than 0.5%). In case of doubt when using old PEG, the concentration of the polymer in the stock solution can be tested by refractive index measurements. The concentration of PEG is calculated from:

$$[\text{PEG}] = \text{R} \cdot 1/\text{SR} \cdot \rho$$

where R is the refractive index increment above the buffer, SR is the refractive index increment above water of a 1% PEG solution (0.00139) and ρ is the density of the polymer solution. The latter can be measured very precisely using a U-shaped oscillator densitometer (Antor Paar).

6. Measuring Dx concentration: The concentration of Dx in the stock solution can be measured using a polarimeter with a Na lamp at 589 nm and 25°C. Approximately 5 g of the solution is diluted to 25 mL with water and the optical rotation (a) is measured (the specific optical rotation of Dx is +199° mL/g/dm). The concentration C in % is then calculated from the following formula:

$$C = a \cdot \text{V} \cdot 100/(199 \cdot m \cdot l)$$

where V is the volume in mL (25 in this case), l is the optical length in dm, and m the amount of the original Dextran solution used for the determination. This method is applicable for the determination of concentration of other carbohydrate polymers as well.

Alternatively, freeze drying can be used for concentration determination. A known amount (approx 20 g) of Dx stock solution is added to a freeze-drying flask, the solution is freeze-dried for approx 8 h. The dried polymer is dissolved in 2 mL of water, freeze-dried for another 8 h. The user should check for constant weight at least the first time that this technique is used. We have found that extensive freeze drying followed by rehydration in a small amount of water and subsequent freeze-drying yields results that are identical to those obtained with a polarimeter.

7. Selecting the right system: Although the selection of the appropriate phase system depends on the final application, some rules of thumb can be followed:
   a. Polymers: PEG/Dx is by far the most common pair of polymers used. Other incompatible polymers were already mentioned and summarized in **Table 1**. High molecular weight Dextran (Dx-500,000) is highly recommended because it can be used with low molecular weight PEGs reducing the viscosity of the phases.
   b. Polymer/Salt System: The most popular is PEG/K_2HPO_4-KH_2PO_4. Others, more environmental-friendly systems like PEG/citric acid-sodium citrate have also been used.
   c. Selection of a tie-line: After the phase-forming species have been selected, the next step is to select a particular tie-line. A good source of tie-lines is the monograph by P.Å. Albertsson ***(1)***. Others have also been published ***(3,4,10)***. Some convenient two-phase systems are shown in **Table 4**.
   d. Selection of a volume ratio: As a rule of thumb, those who are using aqueous two-phase systems for the first time should choose equal top and bottom phases volumes to facilitate sampling and protein partition coefficient determination.
   e. Scale: For analytical purposes, 5 or 10 grams systems are very convenient.
   f. Buffer: The selection of the buffer should be based upon the desired pH range. For acidic and neutral pHs use phosphate buffers. For basic pH, use Tris buffers. Buffer concentration should be kept as low as possible, typically 20–50 m*M*. The buffers must be kept in a refrigerator and utilized within 30 d.
8. Temperature: If it is possible, work at room temperature. I personally found working at low temperatures (i.e., 5°C) easier than at higher temperatures (i.e., 40°C). Moreover, since protein partitioning is only marginally affected by temperature changes, low temperatures may be desirable to maintain protein stability.
9. For systems at different temperatures, mixing is done in either a water bath or in a chromatographic chamber. Sedimentation of the phases can be done in a centrifuge that has been previously equilibrated at the desired temperature.
10. The time for phase separation depends on the kind of phase-forming species, on the density of both phases, on their viscosity, and on the interfacial tension between the phases. In general, the time of phase separation depends on the distance of the working tie-line from the critical point. Close to the critical point (where the density difference between the phases is small; 0.022 g/cm^3 at a tie line length of 8.75 compared to 0.067 g/cm^3 at a tie line length of 24.2 for sys-

Table 4
Convenient Aqueous Two-Phase Systems[a]

Concentration of species 1 in %w/w	Concentration of species 2 in %w/w	Buffer
7.6 of PEG 4000	10.0 of Dx 40,000	Universal
5.0 of PEG 6000	8.0 of Dx 40,000	Universal
5.6 of PEG 4000	10.0 of Dx 500,000	Universal
5.0 of PEG 6000	8.6 of Dx 500,000	Universal

[a]The polymers molecular weights are nominal.

tems containing 5.62% PEG 4,000/6.70 % Dx 40,000 and 7.67% PEG 4000/ 10.30% Dx-40,000, respectively) or far from the critical point (because the viscosities are high) the phase separation time is long. At intermediate tie-lines the phase separation time is shorter. If the more viscous phase volume is larger than the volume of the less viscous phase the phase separation time increases. Some of these features are illustrated in the photographic sequence shown in **Fig. 3** for two PEG/Dx systems, A and B, close and far from the critical point, respectively. System A consists of 5.0% PEG and 6.5% Dx in phosphate buffer at pH 7.0 and system B consists of 6.2% PEG and 8.3% Dx, also in phosphate buffer at pH 7.0. Both systems were equilibrated at 20°C. Immediately after mixing, the systems look opaque because of the formation of an emulsion (*see* **Fig. 3A**). After nearly one minute, only the upper part of the solutions is transparent (*see* **Fig. 3B**). After 2 min, both upper and the lower portion of the solution are transparent whereas the interface is not visible yet because of the presence of a fine emulsion (*see* **Fig. 3C**). The size of the emulsified zone continues to decrease (***see* Fig. 3D,E**). After about 30 min (*see* **Fig. 3F**) a clear interface has appeared and only a fine emulsion of the PEG-rich phase into the Dx-rich phase remains. I would take nearly 24 h to obtain two completely transparent top and bottom phases.

11. Lack of Reproducibility: One common source of frustration for those using aqueous two-phase systems for the first time is their apparent lack of reproducibility. As indicated before, systems are normally prepared according to some published binodal. Often, the prepared system differs from the one published. Specifically, the ratio between top and bottom phase volumes of both published and prepared systems may not be the same. The appearance of only one phase after following step by step a published recipe is equally frustrating. These apparent inconsistencies have people believe that the lack of reproducibility is an inherent property of aqueous two-phase systems. Fortunately, this is not true. When all conditions are kept the same (polymer molecular weight distribution, pH, type of buffer, additives, and temperature), the equilibrium composition and volume ratio are uniquely determined by the laws of thermodynamics. The most common reasons for these inconsistencies are:

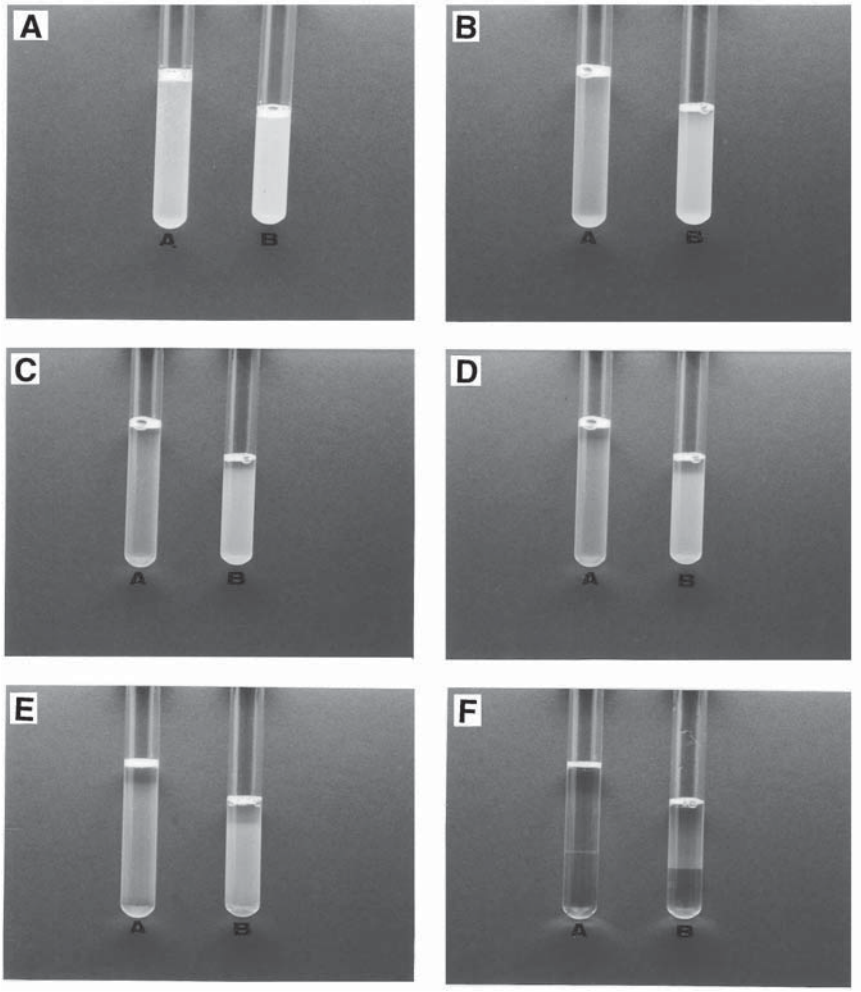

Fig. 3. Photographic sequence of the phase separation phenomenon of two PEG/Dx systems, close to (System A), and far (System B) from the critical point. The polymers used were PEG 4000 and Dx-500 000.

The selected tie-line is too close to the critical point. In this case, small differences in the molecular weight distribution of the polymers, presence of additives, or differences in temperature, are normally the cause. Addition of small amounts of one of the two polymers (I prefer to add PEG) will move the system into the two-phase region.

When working at longer tie-lines, the above differences should not present a problem. In this case, poor mixing is normally the cause. As described in **Subheading 3.**, the denser stock solution is added to the centrifuge tube first. It is

quite difficult to mix the residue of stock solution that is "trapped" in the tip of the tube with the rest of the solution. So, the two-phase system is actually prepared using a considerably smaller amount of this polymer. To assure good mixing proceed as follows. Mix the contents of the tube on a vortex mixer and inspect the tip and walls of the tubes for stock solution residues. Continue mixing for some time, after no deposits are present, on a rotary shaker.

12. *Dirty interface:* Often, impurities that are present in either the polymers or the proteins accumulate at the liquid-liquid or liquid-air interfaces. This is quite normal and it should not cause any problem.

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4

Measurement of Some Physical Properties of Aqueous Two-Phase Systems

Donald E. Brooks and Raymond Norris-Jones

1. Introduction

1.1. Interfacial Tension

The ability of aqueous two-phase systems to support bioseparations obviously depends intimately on the physicochemical properties of the polymers and the phases they form. Measurement of some of these properties can provide insight into the mechanisms responsible for the partition behavior observed. In a more practical sense, measurement of such properties can be used as quality control procedures for industrial separation processes. For instance, if a given separation is to be carried out repetitively and the batch of polymer is to be changed, measurement of the phase diagram and comparing it with that from the previous polymer lot is an obvious requirement. Determination of the interfacial tension might be a preferable procedure for this purpose, however.

Utilizing normal methods, the exact location of the binodial and tie lines are generally not determined with very great accuracy ***(1)***. Measurement of the interfacial tension between the phases is very sensitive to the tie line length (TLL), however, varying as roughly TLL to the 4^{th} power ***(2)***. Because of this relationship, the interfacial tension is roughly four times more sensitive to an uncertainty in concentration of one of the phase polymers than is the TLL. This may be seen as follows:

The fractional change or uncertainty in TLL for a fractional change in the concentration of, say, dextran, C_D, is given by:

$$[(\Delta TLL/TLL)/\Delta C_D/C_D)] \quad (1)$$

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The fractional change in the interfacial tension γ for a fractional change in C_D is given by:

$$[(\Delta\gamma/\gamma)/(\Delta C_d/C_d)] \quad (2)$$

The dependences of γ and TLL on C_D are $TLL^2 = C_D^2 + C_P^2$ and $\gamma \equiv \alpha\ TLL^4$, where α is a proportionality constant that depends on the nature of the polymers. C_D and C_P are strictly speaking the concentration differences between the phases of the two polymers but they can be identified with the highest concentration of each for purposes of this discussion. The ratio, R, of the two terms (1) and (2) contained in the brackets, [ ], is the relative sensitivity of the two parameters to small fractional changes in C_D, $(\Delta C_D/C_D)$. Identifying the Δ terms with differentials and simplifying gives:

$$R = d\gamma/dTLL \cdot TLL/\gamma \quad (3)$$

Performing the differentiation and substituting gives R = 4. Hence, measurement of the interfacial tension provides a significantly more sensitive measure of the effective concentration of a polymer preparation than does determination of the TLL or the phase diagram.

There are various ways to measure the interfacial tension of the boundary between the two phases but the two most popular and best suited to the very low tension values typical of aqueous two phase systems are the rotating drop and sessile drop methods. The former technique has been discussed and described in detail elsewhere ***(1)***. Here we describe the sessile drop method as it is more versatile, allowing the simultaneous determination of the interfacial tension and the contact angle between the phase interface and the solid support. The latter is a useful parameter that is closely related to the partition behavior of solids small enough to suspend ***(2)*** and has the advantage of being interpretable by equilibrium thermodynamics whereas particle partition coefficients in general are not ***(2)***.

The values of γ for aqueous two phase systems are very low, of the order of 1–50 μN/m, much lower than air/water or oil/water interfacial tensions which are of the order of 10–70 mN/m. Classical methods such as the Wilhelmy plate are too insensitive for these measurements. All the successful methods rely on the deformation by gravitational or centrifugal forces, resisted by the tension in the interface, of a drop of one phase in the other. The density difference between the phases must also be determined. The deformation is measured as described below and interpreted via software ***(3)*** or reference to published tables (discussed by **ref. *4***) to obtain γ.

1.2. Electrostatic Potential Difference

The other equilibrium parameter that provides useful information on the determinants of the partition coefficient is the electrostatic potential difference

between the phases, $\Delta\Psi$. It is a type of Donnan potential produced by the unequal partition of some ion species, driven usually by partial exclusion from the phase rich in poly(ethylene glycol) (PEG) or related polymers. Its interpretation has been discussed at length elsewhere ***(2,5)***. It is determined by employing a high input impedance voltmeter connected to reversible electrodes in electrical contact with two salt bridges (actually fine glass capillaries filled with electrolyte solution) that are immersed in the two phase system. The difference between the voltmeter readings when both salt bridges are first immersed in one phase, then one capillary moved into the other phase, is taken as an estimate of $\Delta\Psi$.

The interpretation of the $\Delta\Psi$ values obtained as outlined above is of interest. It is impossible to measure by a thermodynamic (i.e., model-independent) process the inner or Galvani potentials of the type that occurs in two phase systems at equilibrium ***(6)***. However, using salt bridges containing relatively high concentrations of KCl it is possible to get a very good estimate of $\Delta\Psi$. The method is exactly the same as that used to measure the electrical potential drop across biological membranes of either intact cells or membrane patches. That these measurements provide a very good picture of the bioelectric phenomena associated with excitable membranes is no longer questioned. Hence, similar measurements on two phase systems are certainly correct in character. The only difficulty is that the magnitudes are very small, a few mV at most, and in this range uncertainties due to liquid junction potentials necessarily temper interpretation of the potentials measured.

The relationship between the measured values of $\Delta\Psi$ and the partition of salts, believed to be the reason the potential is present, has been discussed at length ***(2,5)***. The two correlate only if a reference system with a common ion is selected and the measured $\Delta\Psi$ and salt partition coefficients related to the reference system, as discussed ***(2,5)***.

1.3. Electrophoretic Mobility of Phase Drops

If electrical properties of the systems are of interest, measurement of the electrophoretic mobilities of drops of one phase in the other can provide insight into the nature of the change in potential across the interface. Interpretation of the mobilities is as yet uncertain, as no complete theory for the low interfacial tension conditions applicable to drops of one aqueous phase in the other aqueous phase has been published to our knowledge (*see* **ref. *7*** for the most complete theoretical examination of the problem to date). The results obtained suggest that the potential drop between the surface of the drop and the outside bulk phase is significant in magnitude only in systems in which a finite potential is measured with electrodes. However, if the sign of the mobility is assumed to reflect the sign of the surface potential, the implied potential is of opposite

sign to that expected from electrode measurements and ion partition coefficients. Typical data is provided in *(5)*. If this observation implies that the potential reverses sign at the phase boundary, it would imply the presence of a potential well on each side of the interface *(5)* that in principle could accumulate material carrying a net charge. This would provide an additional mechanism for accumulation of material at the interface beyond the usual one of reduction of free energy by occupying interfacial area. Hence, further study of the electrophoretic mobility of phase drops is warranted from both a theoretical and more practical perspective.

1.4. Phase Viscosity

Any transport process involving or taking place in a two phase system will depend on the viscosities of the phases. In general the viscosity of a solution of a single polymer will increase strongly with concentration and molecular weight. When the viscosities of typical phases of an aqueous two phase system are measured, however, the results are more complicated, particularly if there is a big difference in molecular weight of the two polymers, as is often the case with dextran/PEG phases. If systems increasingly far from the critical point are examined it is often found that the PEG-rich phase remains at roughly a constant viscosity while the dextran-rich phase increases stongly as the TLL is increased *(1)*. This occurs because the high molecular weight dextran is progressively excluded from the PEG-rich phase as systems farther from the critical point are considered. Because of its molecular weight it has a much bigger effect on the phase viscosity than the PEG so the increase in viscosity provided by increasing PEG is offset by the reduction in viscosity due to the reduction in dextran concentration as more concentrated systems are considered. In high molecular weight dextran/PEG systems, the lower phase viscosity is also independent of PEG molecular weight *(1)*.

2. Materials

2.1. Interfacial Tension

1. Apparatus: A video camera, such as COHU, coupled to a horizontally mounted optical magnification system such as the Wild Apozoom, is focused on a transparent chamber of a few mL capacity. We use a glass- sided plexiglass chamber $1.0 \times 1.5 \times 4.5$ cm long which holds the less dense phase. The chamber is mounted on a plate that can be rotated ±15° around its long axis to allow the drop to be tilted somewhat. The chamber is illuminated with a Nikon fiber optic light source with a frosted glass diffuser between the fiber and the chamber. The more dense phase is contained in a 1.0-mL tuberculin syringe whose lateral location is controlled with a micromanipulator. Instead of a needle, a very fine tip is drawn from a microcapillary over a suitable flame and cut to provide a small

inside diameter tip (~ 0.008 cm) for small drops. Square pieces of glass microscope slide 1.0 cm × 1.0 cm are cut to carry or act as the test surfaces.
2. Software: A standard image-analysis package, such as OPTIMAS™ is used to obtain the drop profile. The software necessary to perform the axisymmetric drop shape analysis by numerical integration of the Laplace equation may be obtained commercially through Dr. A. W. Neumann, Department of Mechanical and Industrial Engineering, University of Toronto (E-mail: neumann@mie.utoronto.ca).
3. Temperature control: Because precise temperature control would be difficult and would require an elaborate chamber, we utilize a laboratory that is thermally controlled to better than ±1°C over the duration of an experiment. If necessary, the temperature can be measured with a thermistor immersed in the less dense phase.
4. Pycnometer: Required to measure phase densities.
5. Phase chemicals: Phase systems are made up as described elsewhere in this volume. Before they are utilized for contact angle and/or interfacial tension measurements they must be carefully equilibrated at the temperature of the experiment.
6. Hexadecane spread on top of the phase system is required to eliminate evaporation.
7. Chromic acid cleaning solution is made from 85% H_2SO_4 and 15% H_2O containing 42 g/L Cr_2O_3.
8. 1.0 *M* HCl is needed for cleaning following chromic acid exposure.

All chemicals should be reagent grade because of the need for maintaining clean surfaces.

2.2. Electrostatic Potential Difference

1. Apparatus: Voltages are measured on a high impedance ($\geq 10^{10}$ Ω) millivoltmeter (most Digital Voltmeters have the required input characteristics). Glass microcapillaries are pulled with an electrode puller to an internal tip diameter of about 30 μm. These instruments, for instance those made by David Knopf Instruments (Tujunga, CA) are commonly found in electrophysiology laboratories.
2. Pure silver plates 1 cm^2 square each are used for the reversible electrodes.
3. Xylene.
4. Acetone.
5. 5 *N* HNO_3.
6. 0.01 *N* HCl.
7. 1.0 *M* KCl.
8. Concentrated ammonia.

Items 3–8 are reagent grade.

2.3. Electrophoretic Mobility of Phase Drops

Apparatus: A temperature controlled microelectrophoresis apparatus is required, such as the Mark I or Mark II from Rank Bros. (Bottisham, Cambridge, UK), in which the drop is observed directly or by video. The more

automated laser doppler instruments will not provide sufficiently accurate individual drop sizes. The instrument must be equipped with an eyepiece filar micrometer such as sold by American Optical (Buffalo, NY) or with a calibrated optical field analyzer for video images.

2.4. Phase Viscosity

Apparatus: A simple capillary viscometer, a controlled temperature bath, a stop watch or electronic timer and a pycnometer are required.

3. Methods

3.1. Interfacial Tension

1. The piece of glass slide on which the sample or drop is to rest is washed in detergent, then acid rinsed in chromic acid cleaning solution (85% H_2SO_4, 15% H_2O containing 42 g/L Cr_2O_3) followed by rinsing in 1.0 *M* HCl then exhaustive washing in distilled water (*see* **Note 1**).
2. If a particulate material is to be tested for its contact angle it is deposited on the glass slide by sedimentation or centrifugation from water or other volatile solvent and drying or by trapping the material on a filter, rinsing in a volatile solvent and drying.
3. The two phase system must be brought to good thermal equilibrium with the room or container in which the measurements are to be made. The upper and lower phases are separated and the volume around the interface discarded. If necessary the upper phase may be centrifuged briefly to remove all lower phase droplets (*see* **Note 2**).
4. The piece of glass slide is placed in the bottom of the chamber with forceps. Lower phase is added to the syringe.
5. Using the micromanipulator, the syringe is positioned over one end of the glass piece. The upper phase is pipetted into the chamber to cover the glass slide and the microcapillary tip. Hexadecane is carefully layered over the entire surface of the upper phase to prevent evaporation (*see* **Notes 2** and **3**).
6. Individual drops of lower phase are formed by gently depressing the syringe plunger and allowing the drops to fall randomly on the slide by moving the micromanipulator or chamber laterally between drops. The drops must then be allowed to come to equilibrium (i.e., achieve a time independent contact angle) (*see* **Note 4**).
7. To determine the tension and contact angle, a good horizontal profile of a series of drops is required. The optics, light source and chamber base angle should be adjusted to produce a reflection of the drop in the glass in order to locate the contact line precisely. The desired orientation is illustrated in **Fig. 1**. The magnification is chosen so the drop image essentially fills the screen. The magnification needs to be known accurately and can be determined with as described in **Note 5** (*see also* **Note 6**).
8. The image is captured as a video frame with any modern frame grabber and taken into an image analysis package to digitize the drop profile, i.e., provide a file

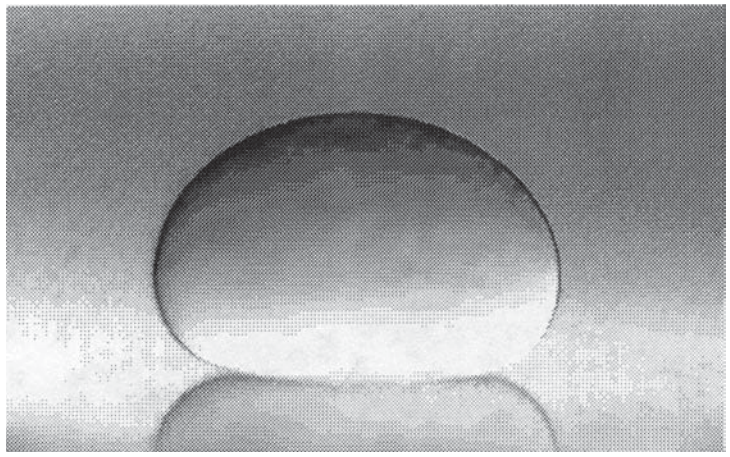

Fig. 1. A drop of a dextran/PEG copolymer/water system with a very small density difference is illustrated, showing the reflection that allows the contact line with the solid surface to be determined accurately.

containing the (x,y) coordinates of a sequence of points around the periphery of the image. Usually 50–75 sets of coordinates are sufficient. This drop profile, along with the difference in density between the phases, is the input required for the software that calculates the interfacial tension and contact angle (*see* **Note 7**).

9. The density difference must be determined at the same temperature as the sample when the profile is recorded. A small volume pycnometer is weighed dry, then filled with distilled water and equilibrated at the desired temperature, filling the bottle initially with water at a lower temperature than the measurement. As the water warms, it is slowly ejected from the capillary in the stopper of the bottle and has to be blotted up with tissue until thermal equilibrium is reached and the ejection ceases. The pycnometer is then removed from the thermostat with tongs and dried, if necessary, before weighing without warming by handling. The process is repeated with the pycnometer filled in turn with each phase.
10. The volume of the bottle is calculated from the weight of the empty bottle, the water filled bottle and the density of water at the measurement temperature, obtained from standard tables. The density of each phase is then calculated from its mass and the volume of the pycnometer.

3.2. Electrostatic Potential Difference

1. The drawn glass microcapillaries are filled under negative pressure with 1.0 *M* KCl.
2. Reversible Ag/AgCl electrodes are made by cleaning two 1 cm^2 squares of pure silver with xylene, acetone and distilled water followed by a brief immersion in 5 *N* HNO_3 until the surfaces appear uniformly white. They are rinsed with distilled water, connected to the anode of a d.c. power supply and immersed in a plating solution of

0.01 *N* HCl, then plated at approximately 1 ma/cm^2 for 2 h with constant stirring of the solution. The electrodes are tapped occasionally to remove bubbles and rotated every 30 min to produce a uniform plum-colored coating of AgCl.

3. The electrodes are then inserted in the KCl-filled microcapillaries (salt bridges) and "conditioned" by shorting them with the ends dipped in the plating solution across a 100 mv a.c. source for approx 1 h. Electrodes that at any time show uneven coloring or signs of wear can be cleaned in concentrated ammonia and replated as before.
4. The phase system is placed in a thermostated water bath and the tips of the two salt bridges immersed in the top phase. We find it convenient to hold one of the micropipettes in a micromanipulator with vertical movement to allow smooth penetration through the interface between the two phases. Once the electrodes are connected to the millivoltmeter via grounded shielded cable, the whole apparatus except the meter is enclosed in a carefully grounded wire cage, avoiding ground loops. The impedance of the circuit with both microcapillaries in one phase should be 1–2 *M* Ω.
5. With both capillaries in the same phase, the meter should not read more than 5 mv. If a higher potential is observed the electrodes should be examined for uneven plating and the capillaries for bubbles, dirt, and so on. It may be necessary to clean and replate the electrodes, or to recondition them with prolonged exposure to the a.c. field.
6. Once the voltage has reached a steady level, one of the capillaries is smoothly lowered through the interface. In systems in which the salts partition unevenly, the voltage will be observed to change, rapidly at first, and asymptotically attain a constant value. The characteristic time over which this will occur will depend on the viscosities and density difference between the phases as it is dependent on the rate at which the film of the opposite phase retracts when the capillary is moved through the interface, dragging along some of the phase in which it was initially immersed.
7. The capillary should be moved in and out of the bottom phase and the voltage difference averaged over at least 10 excursions to provide the electrostatic potential estimate. The values, typically of a few mv, generally have an uncertainty of ±50 μv in our hands (*see* **Note 8**).

3.3. Electrophoretic Mobility of Phase Drops

1. The general procedures for performing microelectrophoresis are described by Seaman *(8)*. The phases should be equilibrated at the temperature of the measurement, preferably in the same bath used to thermostat the apparatus. Suspensions of drops of one phase in the other are made by adding 5–20 μL of one phase to 5 mL of the other and agitating gently. The chamber is filled with the suspension and allowed to come to thermal equilibrium. The drops should be clearly seen with the optics focused at the stationary level in the chamber and should be moving only in the vertical plane with no lateral motion when the electric field is turned off.
2. A drop is selected and the diameter either measured with an eye piece micrometer or the drop image captured if a video microscope is being used.

3. The field is turned on and the time taken for the drop to move across a convenient number of divisions in the eyepiece determined (*see* **Note 9**).
4. The field is then reversed in direction and the measurement repeated with the drop moving in the opposite direction, if it is still in view vertically. The average of the inverse of the times recorded for a given drop should be used to calculate the mobility of the drop, not the average of the times themselves, as it is the velocity that is being averaged (*see* **Note 10**).
5. As large a number of drops as is feasible should be measured, over several fills of the chamber, as the mobilties of these systems cannot be characterized by a single mobility value but must be plotted as a function of drop size and the slope of the line determined ***(1,5)***.
6. It will also be necessary to measure the phase viscosity, described below, if further interpretation is to be made ***(1,5,7)***.

3.4. Phase Viscosity

1. The general procedures are discussed in **ref. *9***. The phases of aqueous two phase systems can be treated like ordinary polymer solutions provided thermal equilibrium is carefully maintained when the phases are separated. They should be isolated as described in **Subheading 3.1.** and the individual phases thermally equilibrated in the same bath as the capillary viscometer. The phases must be optically clear and free of any drops of the opposite phase.
2. The recommended volume for the viscometer (typically 1.0 mL) is used, first with distilled water as the test liquid. The flow time between the two marks on the capillary is recorded for at least three runs and averaged.
3. The viscometer is emptied, dried, and one of the phases added, by weight if the phases do not pipet cleanly due to their viscosity. Once thermal equilibrium has been established the flow time is determined in at least triplicate.
4. The capillary is cleaned thoroughly be rinsing exhaustively with distilled water, dried, and the other phase measured.
5. The densities must also be determined, as described in **Subheading 3.1., item 9**.
6. The viscosity is determined from the fact that it is directly proportional to the flow time multiplied by the density. The known values of the viscosity and density of water at the temperature of the measurement is used to determine the proportionality constant and allow absolute phase viscosities to be calculated.

4. Notes

1. Chromic acid is very corrosive; handle materials to be washed in it only with tongs and be very careful of drips. Following cleaning the slides should be stored in filter-sterilized, distilled water until use.
2. Small changes in the temperature or water content (via evaporation) can change the phase composition, inducing phase separation in previously clear single, isolated phases. The small droplets that result form a haze that make most physical measurements on the separated systems difficult and usually invalid.
3. It is important that the microcapillary tip be placed so that it is immersed when the less dense phase is introduced to the chamber. Once the hexadecane

has been spread the tip must not move through the hexadecane layer floating on the surface.

4. Equilibration times vary with the system composition and type of surface. Most dextran/PEG systems will equilibrate in 30 min but drops can be left overnight to ensure complete equilibration, providing the temperature remains constant. Drop size will vary from less than 0.1 μL to ~1 μL, depending on the phase density difference, interfacial tension, viscosity and microcapillary syringe tip dimension. Larger drops deform too much to be analyzed accurately because of the low interfacial tensions. Drops that are essentially flat on top cannot be analyzed.
5. A convenient magnification standard consists of 18- and 30-gauge injection needles. The diameters can be measured to three significant figures with a micrometer and images of the needle at the magnifications used for that set of drops included in each file.
6. It is easier to find good drops if they are deposited in a horizontal line, not added randomly over the surface, since focusing and tracing the drop profile is more difficult if drop images overlap.
7. The required software is described in detail in ***(3)***. The program is easy to run and quickly converges to provide values for the interfacial tension and provides as well the contact angle, radius of curvature at the origin, surface area, drop volume and contact radius. We have found that the values for γ should be averaged over at least 12 independent drop profiles to provide reliable answers. For a typical aqueous two phase system the values generally have standard deviations corresponding to uncertainties of ±4%.
8. The salt bridges should not be left in either phase any longer than is required for a good reading as the KCl will slowly diffuse out of the tips of each capillary, potentially changing the local phase composition around each tip.
9. The voltage should not be applied too long as changes in temperature due to resistive heating will produce changes in the phase composition and non-equilibrium drops will be formed.
10. In order to determine the sign of the electrophoretic mobility, it must be remembered that the optics typically reverse the direction of motion, so the polarity of movement must be determined with this in mind. A check may be made by diluting a finger prick of fresh blood in physiological saline, pH ~7.0, and observing the movement of the cells at the stationary layer when the field is applied. Red cells have a net negative mobility at pH 7.0 due to the net negative surface charge ***(8)***.

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5

Single-Step Partitioning in Aqueous Two-Phase Systems

Telma Teixeira Franco and Rajni Hatti-Kaul

1. Introduction

Partitioning in aqueous two-phase system (ATPS) provides a rapid and gentle means of separation of soluble as well as particulate biomaterials, e.g. proteins, nucleic acids, cells, viruses, organelles, and membranes. Partitioning between the two phases is a complex phenomenon, guided mainly by the interaction of the partitioned substance and the phase components, e.g., through hydrogen bonds, van der Waals-, hydrophobic-, and electrostatic- interactions, steric-, and conformational effects, etc. ***(1,2)***.

Single-step partitioning is invariably the first stage in determining the separation possible in a two-phase system. To start with, the phase system(s) is often arbitrarily chosen, and partitioning of the material of interest between the two phases (and the interface) is studied. This is followed by further systematic changes in the phase composition either to achieve a desired separation or to study the influence of the changes on partitioning. The changes may be performed by altering the different parameters of the system one by one or by applying an experimental factorial design, in which the parameters are varied simultaneously and at different levels (*see* Chapter 22). A significant difference between the partitioning of the species of interest from the others present in a sample can be used for its separation in a single step; for smaller differences, multistep extraction is required (*see* Chapter 6).

The criteria used for selection of a phase system include:

1. The ultimate goal of partitioning, e.g., if the system is to be selected for large-scale extraction, parameters like cost, availability, and viscosity of the phase components are important;

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2. Sensitivity of the species to be partitioned to e.g. the phase components, intense stirring; and
3. Requirements of pH, temperature, and additives (e.g., metal ions, vitamins, etc.) ***(3–5)***.

Small molecules generally partition nearly equally between the two phases, but there are exceptions which deviate from this rule ***(1,6)***. Unequal partitioning may also be obtained in polymer-salt systems which have greater differences in the physical properties of the two phases ***(7,8)***. With increase in the size, molecules become more sensitive to the phase composition, alterations of which can be used to manipulate their partitioning. The partitioning of soluble molecules occurs between the two bulk phases, top and the bottom. This is normally characterised by the term, partition coefficient, *K*, which is the ratio of the concentration of the solute (e.g., moles /liter, mg/mL or units/mL) in the top phase (C_t) to that in the bottom phase (C_b) ***(1,9)***:

$$K = C_t / C_b \quad (1)$$

K is a function of properties of the phases and the substance, and also temperature; it is independent of the solute concentration and volume ratio of the phases. The mass balance during partitioning is obtained from partition ratio, *G*, which is the ratio of the solute amount in top and bottom phase;

$$G = K \cdot V_t/V_b \quad (2)$$

where V_t and V_b are the volumes of the two phases. Maximal separation of two species having the partition coefficients, K_1 and K_2 is obtained when the product of their partition ratio is 1. The fraction of a solute in either phase, besides indicating separation from other species, provides also a value for its recovery (*see* Chapter 22). The solubility of the molecules in the phases is quite high and hence the partitioning can be performed at rather high concentrations.

In contrast to the soluble molecules, particulates partition between the two bulk phases and the interface, and with increasing size between one bulk phase and the interface. The tendency for adsorption increases with size of the particles and the interfacial tension of the phase system. Adsorption is also favored when the particles display more or less equal affinity for the two phases ***(1)***. PEG-Dextran systems have been the preferred systems for particle partitioning. In contrast to the solubles, requirements of ionic composition and tonicity of the medium are more stringent for cells and organelles. Partitioning of particulates is normally described in terms of *K* (or *P*) value which is the amount of particles in the top phase expressed as percent of the total amount added ***(10***; *see* Chapter 11), while partition ratio is often expressed as the ratio of the quantity in the top phase to that in the interface plus bottom phase.

In case of particulates, partitioning is a surface dependent phenomenon as it is the exposed groups on their surface that interact with the phase components ***(1,10,11)***. In general, most of the factors that influence the partitioning of soluble molecules do so for particles as well. The contributions of these factors to the observed partition coefficient can be summed up in logarithmic terms, according to Albertsson ***(1,2)***, as follows:

$$\ln K = \ln K^0 + \ln K_{elec} + \ln K_{hydrop} + \ln K_{biosp} + \ln K_{size} + \ln K_{conf.} + \ldots . (3)$$

wherein K_{elec}, denotes a term that includes electrostatic effects mainly determined by protein net charge and hence, pH, ion distribution and also the charge of the polymer involved; K_{hydrop}, a hydrophobic term; K_{size}, a term for protein and polymer size; K_{conf}, a term representing conformational effects; K_{biosp}, affinity interactions with a ligand incorporated in the system, while K^0 represents other factors.

This chapter will cover the practical aspects of single-step partitioning experiments. The reader is also referred to **refs. *1*** and ***12*** for a comprehensive review and examples of the partitioning of different molecules and particulates in two-phase systems; Chapters 11–17 of this volume for further details on partition of eukaryotic cells, viruses, membranes and vesicles; and Chapters 22, 23, 28–31 for partitioning of proteins.

2. Materials

1. Stock solution of top phase component. PEG has been the most often used (*see* Chap. 3). The stock solution may be prepared in water.
2. Stock solution of bottom phase component. This could be a polymer or salt. Stock solutions of Dextran 20%; NaH_2PO_4 and K_2HPO_4, (40%); $MgSO_4$ (23%); sodium citrate (28%); Na_2SO_4, and $(NH_4)_2SO_4$ (50%) are made in water. The solutions are made up from reagent grade materials and prepared by weight (*see* also Chapter 3).
3. Stock solution of buffer or salt (0.5–1.0 *M*).
4. Millipore quality water.

3. Methods

1. Into a graduated centrifuge tube of 15 mL total volume, weigh out stock solutions of bottom and top phase forming components, stock buffer or salt solution (if necessary), and water. Equilibrate the mixture at the desired temperature (*see* **Note 1**). Add the material to be partitioned (*see* **Note 2**) (also pre-equilibrated at the same temperature), so that the system comes to the desired composition (*see* **Note 3**). For example, to prepare a system containing 8% (w/w) PEG 8000, 5% (w/w) dextran T500, 50 m*M* sodium phosphate buffer, and 10% (w/w) material, mix stock solutions of 1 g of 40% (w/w) PEG, 1.25 g of 20% (w/w) dextran, and 0.5 g of 0.5 *M* phosphate buffer, 3.25 g water, and 0.5 g material. Each sample may be tested in triplicates.
2. Cap the tube and shake it from end to end 10–20 times, or using a Vortex mixer for 1 min.

3. Allow the phases to separate either under gravity or by low speed centrifugation for approx 3 min at 3000*g*, taking care to maintain the desired temperature (*see* **Notes 1** and **4**).
4. Note the volumes of the two phases (except when the phase volumes are known to be equal). Withdraw a known volume of each phase carefully and empty into a known volume of water, buffer or assay solution (*see* **Note 5**). For particulates such as organelles and membranes, the interface and the bottom phase are usually collected together.
5. Quantify the target species in the phases using specific analytical assay (*see* **Note 6**), and calculate the partition coefficient/partition ratio (*see* **Notes 7** and **8**).

4. Notes

1. Temperature is an important factor in partition. The phase diagram may show variations with temperature. The effect is more significant close to the critical point where small change in temperature will have considerable effect on the polymer composition and thereby on the partitioning of a target substance in the phase system ***(1)***.
2. The material to be partitioned may include standard proteins, fermentation broth, cell homogenate, mammalian cells, microbial cells, organelles, membranes etc. The amount added to the phase system may depend on its availability, sensitivity of the subsequent analytical method used, etc. It may be noted that the components e.g. salts, present in significant amounts in crude preparations of the material may also influence partitioning.

   In case of cells, if the interest is only to achieve separation, their preparation by any means (enzymatic, mechanical) that does not alter the biological property of interest can be used ***(10)***. However, when surface properties of the cells are to be studied, care must be taken not to alter these in the course of cell preparation for partitioning. Standard methods for preparation of organelles and membranes, including homogenization and differential centrifugation for subfractionation, may be used. The use of enzymes for digestion of cell walls etc. is avoided if possible.
3. At least three ways can be used for performing cell partitioning ***(3,10)***:
   a. The cells which can be pipetted even when packed, e.g., erythrocytes, are added (0.01–0.5 mL in 10 mL phase system) into the partition tube, and an equal volume into same volume of saline or water; the latter is used to calculate the total amount of cells.
   b. The cells which cannot be pipetted when packed, are suspended in a salt solution with composition identical to that present in the phase system. This suspension is directly weighed into the phase system, the final composition of which is then adjusted. After mixing the contents of the tube well, a small aliquot is removed to determine the total amount of cells added, prior to phase separation.
   c. A more common procedure is to prepare a phase system, separate the top and bottom phases, and suspend the washed, packed cells directly in a certain

volume of top phase; an aliquot of the latter is used for quantitation of the cell amount, and a known volume of the remaining phase is mixed with an equal volume of the bottom phase (*see* Chapter 11).

4. The phase separation in case of particulates is done under gravity for a definite period of time (15–20 min). It is recommended to use equal volumes of each phase for partitioning particulates so as to eliminate one parameter, i.e., height of the phases, which would otherwise influence the time required for phase settling under gravity. For phase systems close to the critical point, phases may be allowed to settle with the tubes in a horizontal position followed by standing for an additional minute. Reducing the phase column height speeds phase separation ***(3)***.
5. Determination of concentrations of solutes is dependent on the accuracy of pipetting. Sampling is done using automatic or constriction pipette from the middle of the phases. The same pipet should be used to collect the aliquots from each phase. Because of the viscosity of the phases, aliquots are slowly drawn into the pipet and the outside of the pipet is wiped dry. In order to collect the sample from the bottom phase, it is possible to take the tip through the top phase by maintaining a positive air pressure while moving down the upper phase ***(9)***. The interface which acts like a membrane is punctured and the tip is moved to the middle of the bottom phase, and the pressure released. Observe that no upper phase is drawn into the pipet tip which can easily happen because of its lower viscosity.

   After emptying the phase volume into the dilution liquid, rinse the pipet tip by drawing the liquid in and out several times to remove the traces of the polymer adhering to its inner surface. For viscous phases like dextran, the rinsing is done slowly and many times (5–10) because of the slow flow of the solution. Mix the solution properly and gently till it appears homogeneous. It is preferable to change the tips when pipetting several samples.
6. A suitable analytical method is used to determine the concentration of the species of interest. For example for an enzyme, an appropriate enzyme activity assay protocol will have to be followed. Protein concentrations are determined using the dye binding, UV absorption, Bradford or Lowry methods ***(13)***. Particle number can be determined by impedance counting techniques, spectrophotometry, enzyme activity determination, and so on (***11***; *see* Chapters 11, 13, 15, 17).

   Interference by the phase components on an analytical method is invariably a problem. Such interference may be reduced by using a diluted phase for measurements, if possible, and having a similarly diluted phase from a blank phase system as a control. The accuracy may also be improved by having calibration curves of the measurements with both top and lower phases present at appropriate dilutions ***(9)***.
7. Some general comments on the partitioning of soluble macromolecules in two-phase systems ***(1)***:
   a. The concentration of soluble compounds in each of the phases is independent of phase volumes and one can increase the quantity of a substance in one phase by increasing the volume of that phase.
   b. Partition coefficients of nucleic acids change drastically with narrow changes in phase composition.

c. Partitioning of proteins can be made one-sided by choosing a system far from the critical point. Increasing polymer concentrations (tie-line length) result in greater difference between the phases, and *K* usually decreases because of reduced solubility of the protein in the upper phase.
d. The solute partitioning into a phase can be increased by decreasing the molecular weight of the polymer enriched in that phase, or by increasing the molecular weight of the polymer constituting the opposite phase.
e. For charged molecules, pH, type and concentration of salt are important. The effect of salts on the charged macromolecules is most dramatic. DNA can be completely transferred from one phase to the other by minor changes in the ionic composition. The partition is determined by the kind of ions present and the ratio between the different ions, and not really the ionic strength. Ions of salts like sodium acetate are almost equally distributed between the two phases, however, ions of many salts like Li_2HPO_4 display unequal distribution because of different affinities for the two phases leading to generation of an electrical potential between the phases ***(1,9)***. The partition of a charged component is influenced by the interfacial potential, ψ according to the equation derived by Albertsson ***(1,2)***:

$$\ln K_p = \ln K^0{}_p + FZ/RT \cdot \psi \quad (4)$$

where K_p is the partition coefficient of a charged protein in a two-phase system containing ionic species providing an interfacial potential of ψ; Z is the number of charges per protein molecule; $K^0{}_p$ is the partition coefficient of the same protein in the absence of either a net charge ($Z = 0$) or interfacial potential ($\psi = 0$); F, R, and T are Faraday constant, universal gas constant, and absolute temperature, respectively.

For negatively charged proteins, K is increased in PEG-Dextran system containing cations in the order $K^+ < Na^+ < NH_4^+ < Li^+ < (C_2H_5)_4N^+ < (C_4H_9)_4N^+$ and anions, $H_2PO_4^-/HPO_4^-$ (1:1) > SO_4^- > F^- > acetate$^-$ > Cl^- > Br^- > I^- > SCN^- > ClO_4^-. The greatest increase is provided by tetrabutylammonium phosphate, and the greatest decrease by potassium chlorate. For positively charged solutes, the above order is reversed ***(9)***.
f. Replacing a fraction of one of the polymers (often PEG) with the charged polymer derivatives (*see* Chapter 28), polymer with covalently coupled hydrophobic groups (*see* Chapter 29) and affinity ligands (*see* Chapters 30 and 31), respectively, can influence the partitioning of proteins according to their charge, hydrophobicity, and biospecificity.

8. Some important facts about partition of particles:
a. Unlike the soluble molecules, in the case of particulates it is the total amount of material but not the concentration that is constant in the bulk phase ***(3)***. Hence doubling the volume of the phase halves the concentration of the particulate matter.
b. Particle partition and sensitivity to surface differences can usually be increased by decreasing the interfacial tension (i.e., in systems closer to the

critical point). Here the partitioning seems to be influenced by parameters other than surface charge ***(10)***. Nonspecific adsorption of particulate matter to the interface is directly related to the interfacial tension between the phases which in turn is dependent on the tie-line length. By changing the polymer concentrations the particles can be moved from top phase to interface and/or lower phase ***(1,10)***.

c. As for soluble molecules (*see* **Note 7**), alterations in molecular weights of one or both of the polymers may be used to partly direct the partition of the particles into a particular phase. Most mammalian cells partition between interface and PEG rich phase in Dextran T500/PEG 8000 system, and between dextran rich bottom phase and interface in Dx 40/PEG 8000 system ***(1)***. Bacterial cells, depending on the strain, partition between top phase and interface, or bottom phase and interface; some partition between both bulk phases and interface.

d. Salt type, concentration, and pH can also steer partition of cells in the same way as for charged soluble molecules. Altering the salt type will tend to change particle partition in the same direction as that of a negatively charged macromolecule. A varying ratio of phosphate/chloride in a PEG-dextran phase system not close to critical point can vary the *P* value of a particulate in accordance with the change in the electrical potential between the phases ***(10)***.

e. Both hydrophobic and biospecific affinity partition can be applied for facilitating separation of cells, organelles, and membrane vesicles (***1***; *see* Chapters 11, 17).

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6

Partition by Countercurrent Distribution (CCD)

Hans-Erik Åkerlund

1. Introduction

A crucial step in many biochemical studies is the separation and purification of the individual structures of interest. For cells, cell organelles, and membrane fragments, the use of aqueous two-phase partition has become an important tool. The basis for separation in these phase systems is the surface properties of the material, and the method, therefore, nicely complements the more traditional centrifugation techniques. Excellent separations have sometimes been obtained in just one or a few partition steps. However, in most instances, a single partition step only gives a partial or incomplete separation of components. By a multiple-extraction procedure, the resolving power can be drastically increased and components with only small differences in surface properties can be separated.

Countercurrent distribution (CCD) is, in theory (*see* **Note 1**), very similar to chromatography. However, an outstanding feature of CCD is that it is based on several clearly defined steps, each involving an equilibrium partitioning between two liquid phases. This is particularly advantageous when macromolecules are involved, because for these it is thought to be easier to achieve equilibrium between two liquid phases than between a liquid and a solid phase. The lack of steric problems is also an advantage. It is of particular interest that particles can be subjected to CCD and thereby permit their separation and the analysis of complex mixtures such as cells or cell organelles. As examples, the CCD technique has been used to: demonstrate changes in surface properties of red blood cells with age ***(1)***; demonstrate weak interactions between glycolytic enzymes and filamentous actin ***(2)***; separate isoenzymes differing slightly in charges ***(3)***; separate three types of chloroplasts ***(4)***; separate thylakoid vesicles with opposite sidedness ***(5)***; fractionate human serum proteins ***(6)***; affinity frac-

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tionate yeast enzymes ***(7,8)***; fractionate membranes from calf brain ***(9)***; detect changes in surface properties of sperm cells ***(10–12)***; and fractionate wheat proteins ***(13)***.

Two types of apparatus have been constructed for CCD for use with aqueous polymer two-phase systems. Both are designed to reduce the time needed for phase separation. Albertsson constructed an apparatus in which the phases form thin layers (about 2 mm), which reduces the settling time to 5–10 min for common phase systems ***(14,15)***. Åkerlund developed a fully automated apparatus utilizing the increased speed of phase separation obtained in a centrifugal acceleration field ***(16)***. This has allowed the use of the phase systems with high polymer concentrations, which are important in affinity partitioning ***(8)***. To follow, three procedures will be described; manual CCD, thin layer CCD, and centrifugal CCD.

2. Materials

1. Phase system: The choice of a suitable phase composition is governed by the restrictions imposed by the biological material of interest and has to be based on empirical variation of different parameters primarily polymer and salt concentrations (*see* Chapters 5, 11, and 22). The best resolution in CCD is normally obtained when the partition ratio is close to one. For two components, the best resolution will be obtained when their partition ratios are related as $G_1=1/G_2$ (*see* **Note 1**). Prepare a phase system (250 g) in a separatory funnel, by weighing the desired polymers and salts from stock solutions and water up to 250 g (*see* **Note 2**). Mix and allow the phase system to thermally equilibrate at the temperature the experiments will be done. Mix again and allow to settle. Separate the phases, taking care to avoid interface material.
2. Sample system: Prepare an 8 g system by weighing the desired polymers and salts from stock solutions, compensating for salts (if possible) that will come from the sample. Add sample and water up to 8 g. Add sufficient bottom phase (or top phase) from above to give the same volume of each phase.
3. Thin-layer CCD: The countercurrent procedure takes place in a partition cell block consisting of two cylindrical plates constructed of Plexiglas (**Fig. 1**). The lower plate (a) has a shallow annular groove (1), which is concentric with respect to the vertical axis. A number of shallow cavities (2) in this groove form the lower parts of the partition chambers and will contain the bottom phase. The depth of these cavities is normally 2 mm. The upper plate (b) rests in the groove of the lower plate (a). The upper plate can rotate about its axis and is guided by the inner and outer edges of the groove. The lower surface of the upper plate, which is in contact with the groove, also has cavities (3) of the same horizontal cross section as the lower plate cavities. The cavities in the upper plate will contain the top phase. By turning the upper plate (rotor) relative to the lower plate, each of its cavities can be brought into coincidence with a cavity of the lower (stator) plate, resulting in the formation of chambers. A circular sequence of partition chambers thus

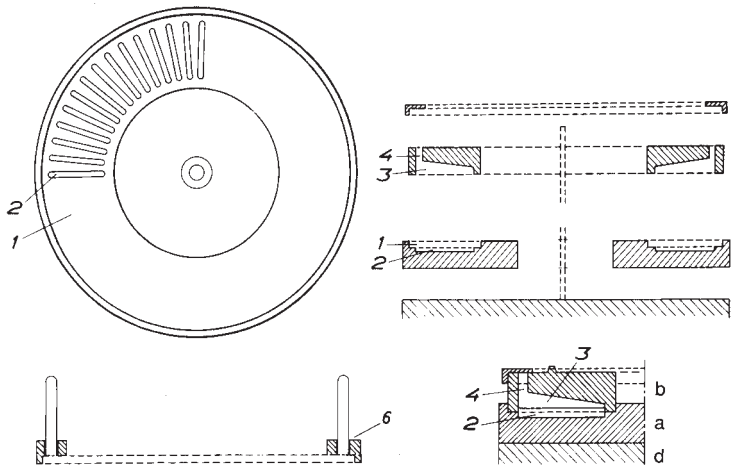

Fig. 1. Details of the partition cell block of the thin-layer CCD apparatus. See text for explanations. (Adapted from **ref. *15*** with permission.)

results. The circular arrangement of the chambers allows recycling procedures so that the number of transfers can be increased to obtain maximum separation. The upper cavities are deeper than the lower cavities to provide air space, which permits efficient mixing in each partition chamber during shaking. Each upper cavity is provided with a hole (4) in the top for filling and emptying. The upper part of the inner surface of each upper cavity slopes to facilitate the latter process. A circular top (c) is used to cover the holes. The partition block rests on a horizontal shaking table (d) with a driving motor and a device for variation in shaking intensity. Mixing of the phase systems is achieved by a planetary movement of the shaking table. In the center of the table there is a unit that drives the rotation of the upper plate while the lower plate remains fixed. All procedures including shaking, settling, and phase transfer are guided automatically by a control unit.

4. Centrifugal CCD: The apparatus consists of a unit for centrifugation (**Fig. 2A–C**), for separation (**Fig. 2D–F**), and for shaking (**Fig. 2N,O**). The separation unit is made up of three Plexiglas parts (*see* **Note 3**): an annular housing (**Fig. 3C**), a cylindrical body (**Fig. 3D**), and a cover plate (**Fig. 3E**). Both the cylindrical body and the annular housing have the same number of cavities. Each cavity has the form depicted in **Fig. 3D**. The cavities in the cylindrical body have a much larger volume to allow changes in the amount of top phase used and to allow movement of liquid during mixing. The cylindrical body can be inserted in the annular housing. The cavities will coincide and form chambers with a close fit to avoid leakage between chambers. A cover plate is fixed to the annular housing to cover the chambers. To avoid leakage from the chambers during centrifugation an O-ring (**Fig. 3F**) is located between the annular housing and the cover plate. When mounted, the cylindrical body can be rotated with respect to the annular housing.

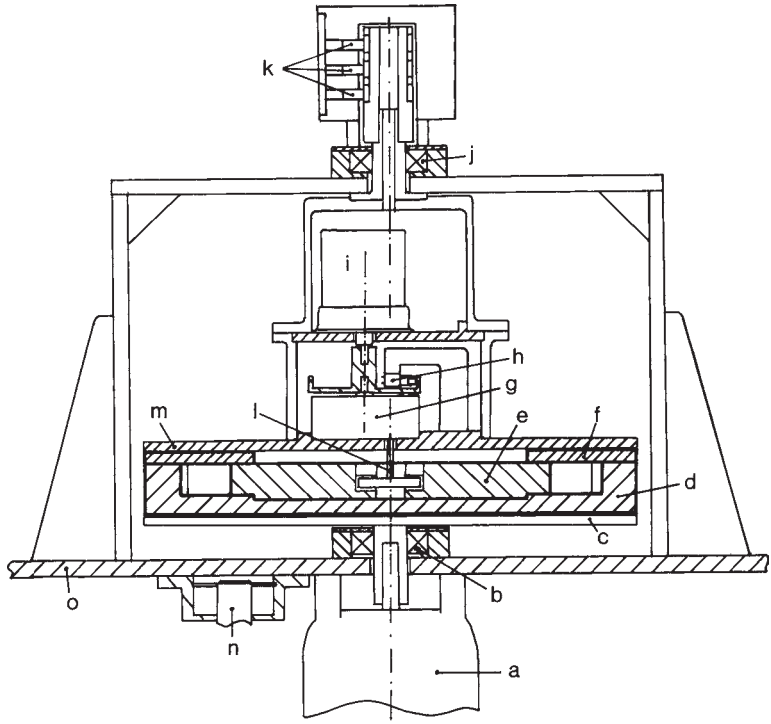

Fig. 2. Cross-sectional view of the central part of the centrifugal CCD apparatus. See text for explanations. (Reproduced from **ref. *16*** with permission.)

The separation unit is connected to the centrifugal unit through the annular housing, and it can be rotated with a speed of up to 3000 rpm, corresponding to an acceleration field of 3000*g*. The annular housing and the cylindrical body rotate with the same speed. In the original construction, a transfer motor (**Fig. 2I**) is mounted on top of the separation unit and fixed to the annular housing. The axis of this motor is connected to the cylindrical body of the separation unit. Power to the transfer motor is obtained through sliding contacts (**Fig. 2K**). The transfer motor is arranged with a gear (**Fig. 2G**) and a micro switch (**Fig. 2H**) in such a way that each time the relay gets a pulse from the steering unit, the cylindrical body turns one step with respect to the annular housing. The units for separation, centrifugation, and transfer are all mounted on a horizontal shaking table (**Fig. 2O**). Mixing is achieved by a planetary movement of the shaking table. All procedures, including shaking, settling, and phase transfer are guided automatically by a control unit. The CCD apparatus described here has been made by the Mechanical Workshop at the Chemical Center, University of Lund (Box 124, S-221 00 Lund, Sweden). A comparable apparatus has been made at the Central Mechanical Workshop, Hicks, University of Sheffield (Hounsfield Road, S10

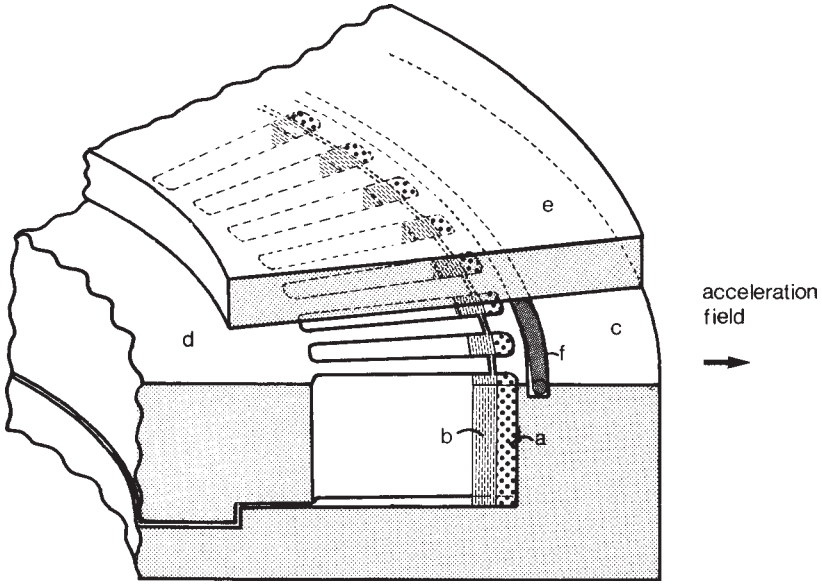

Fig. 3. Section of the separation unit of the centrifugal CCD apparatus. The relative location of bottom phase and top phase during centrifugation is denoted by **A** and **B,** respectively. See text for further explanations. (Reproduced from **ref. *9*** with permission.)

2UN, Sheffield, UK), and Department of Biochemistry and Molecular Cell Biology, Fac. Veterinaria (Miguel Servet 177, 50013 Zaragoza, Spain).

3. Methods

3.1. Manual CCD

This method can be used when CCD machines are not available. The advantage is that it is simple and allows recovery of all material.

1. Prepare 10 tubes; one empty (tube 0) and each of the others with 4 mL of bottom phase (**Subheading 2., item 1**). Number the tubes 1–9 (*see* **Note 4**).
2. Mix the sample system (**Subheading 2., item 2**) and transfer 8 mL to tube 0 (*see* **Note 5**).
3. a. Allow the phases to settle for 30 min.
   b. Alternatively spin the tube for 2 min at 1000*g* (*see* **Note 6**).
4. Transfer 3.6 mL of the top phase of tube 0 to tube 1 (*see* **Note 7**). Add 3.6 mL of new top phase to tube 0.
5. Mix the contents of tubes 0 and 1 separately.
6. Allow the phases to settle.
7. Transfer each top phase to the tube with higher number, and add new top phase to tube 0.
8. Mix the contents of the tubes.

9. Repeat **steps 6–8** as many times as required.
10. Add 8 mL of water to each tube and mix to break the phase systems (*see* **Note 8**).

3.2. Thin-Layer CCD

1. The lower and upper plates (**Fig. 1A,B**) are put together on the shaking table and adjusted so that the cavities of the two plates coincide thus forming chambers (**Fig. 1**, lower right, *see* **Note 9**).
2. The sample, in a two-phase mixture (containing the desired top to bottom phase volume ratio), is added to one or a few of the chambers.
3. A suitable volume (90% of the volume of the bottom cavity) of top and bottom phase is added to each of the remaining chambers (*see* **Note 10**).
4. The cover plate is placed on top and then kept in place by spring-enforced wheels.
5. Set the shaking time to 30 s, the settling time to 10 min, and the number of transfers to 60 (*see* **Note 11**).
6. Start the automatic run.
7. After completion of the CCD, the cover plate is removed.
8. Add a sufficient amount of water to each chamber to break the phase system (*see* **Note 8**).
9. Fractions are collected by use of a collector ring loaded with tubes (**Fig. 1**, lower left). The collector unit is aligned on top of the partition block, and both are jointly and rapidly turned upside down.

3.3. Centrifugal CCD

1. The annular housing and the cylindrical body are put together and adjusted so that the cavities of the annular housing and cylindrical body coincide, thus forming chambers (*see* **Note 9**).
2. The sample, in a two-phase mixture (containing the desired top to bottom phase volume ratio), is added to one chamber (*see* **Note 12**).
3. A suitable volume (90% of the volume of the bottom cavity) of top and bottom phase is added to each of the remaining chambers (*see* **Note 10**).
4. The cover plate is placed on top and then attached with screws, taking care to keep the O-ring in place (*see* **Note 13**). The whole apparatus is then assembled.
5. Set the shaking time to 30 s, centrifugation speed to 500 rpm (~80*g*) and centrifugation time to 1 min, and number of transfers to 60 (*see* **Note 14**).
6. After completion of the CCD, the apparatus is disassembled and the cover plate is removed.
7. A sufficient amount of water is added to each chamber to break the phase system (*see* **Note 8**).
8. Fractions are collected by use of a collector ring loaded with tubes. The collector unit is aligned on top of the partition block, and both are jointly and rapidly turned upside down.

4. Notes

1. A short theoretical description and the relevant formulas will be given here. In CCD, a number of extraction steps are carried out in order to separate substances having different partition coefficients, K, where K is defined as the concentration

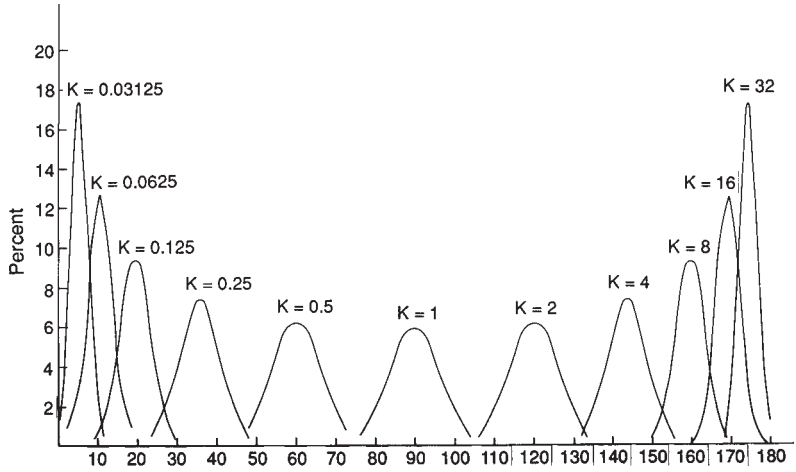

Fig. 4. Theoretical distribution curves as a function of the *K*-value for the substance. The number of transfers is 180 and the volumes of the top and bottom phases are equal. (Adapted from **ref. *15*** with permission.)

ratio, for the partitioned substance, between the two phases. For a large number of transfers each solute, behaving ideally, gives rise to an approximately symmetrical peak. Its position is determined by the number of transfers and by the partition ratio, G (which is the product of K and the volume ratio). **Figure 4** shows examples of some theoretical distribution curves. In the case of cells, cell organelles, and membranes, partition usually takes place between one of the bulk phases and the interface, typically between the top phase and the interface. In this case, the countercurrent principle can also be applied. The bottom phase, together with the interface and a small layer of the top phase just above the interface, is kept stationary in the apparatus while the top phase is the moving phase. If we let the volume of the top phase be V_t, the volume of the layer of the top phase that is kept stationary in the distribution train be v, the concentration of the solute in the top phase be C_t, and the amount of the solute adsorbed at the interface be a, then the partition ratio of interest (G_i) for the CCD is

$$G_i = (V_t - v)C_t / (vC_t + a).$$

When a substance behaves ideally, that is, its adsorption at the interface is reversible and proportional to C_t, it will be transported along the distribution train according to G_i, and, for calculation of theoretical curves, this value may be used in the same manner as G. The following relation holds between G, the ratio between the amount of substance in the top phase (transferred) and the bottom phase (stationary) in the chambers, the number of transfers, n, and the chamber number at peak maximum, r_{max}.

$$r_{max} = nG / (1+G).$$

The width of the distribution curve increases with the square root of n while the r_{max} increases linearly with n. Thus, substances with only small differences in G can be separated by increasing the number of transfers. For a given number of transfers, maximal separation of two substances is obtained if their peaks are symmetrically located on opposite sides of the center of the diagram, that is, $G_1 \times G_2 = 1$.

2. This step needs particular attention, as both temperature and phase composition strongly affect the partition of the biological material.
3. Plexiglas cannot stand organic solvents, but recently a partition block made of alumina and Victrex PEEK has been made ***(13)***.
4. The number of transfers is usually limited by the number of cavities, typically 60 or 120. When sufficient resolution can be obtained with less number of transfers, two or more experiments can be run at the same time.
5. If the phase system is far from the critical point and the biological material can be sedimented, then an alternative way to prepare the sample system is to resuspend a pellet of the biological material in top phase and then to add an equal volume of bottom phase. The polymer concentrations will be somewhat lower, but this may not influence the partition.
6. Although the phase settling can be achieved at unit gravity, centrifugation reduces the time considerably. For larger particles, however, there is a risk for sedimentation of the particles in addition to the settling of the system.
7. When the biological material partition is only between the top and bottom phase, and nothing collects at the interface, then the bottom phase volume can be increased to fill the bottom cavity completely and all of the top phase can be transferred in each step. The volume ratio between the top and bottom phases is usually kept equal, but can be changed thereby changing the G-value without changing the K-value.
8. This step can be omitted, but the yield will be lower because the cavities are more difficult to empty. The water can be changed to a buffer solution that might be required to protect the integrity of the biological material. Also, the volume of dilutant can be changed, but should be sufficient to break the phase system.
9. Maintenance and performance tests. To achieve good results with the equipment described it is important to handle the Plexiglas components with particular care. These parts have been manufactured with high degree of precision to prevent leakage between chambers. They are, however, prone to deformation and should only be washed in luke-warm water and stored horizontally on a planar surface when not in use. To reduce the possibility of leakage, owing to hydrostatic pressure differences, the separation unit should be adjusted horizontally with the aid of a level. Another way to reduce leakage is to use a very thin layer of silicon grease on relevant surfaces. To evaluate the performance of the equipment, the following experiments may be helpful. For testing the efficiency of mixing or the possibility of leakage between chambers, a CCD experiment is run using a pure substance with a K of approximately 1. Insufficient mixing or leakage leads

to a peak broader than the theoretical one. To check whether the settling time for a particular phase system is adequate, experiments may be run with two different settling times. Insufficient settling will lead to a movement of peaks towards the center of the CCD diagram when the settling time is decreased. A more sensitive test for the settling time is to run a CCD experiment with the particular phase system chosen, using a pure substance with an extremely low or high *K*-value.

10. This results in a stationary interface. If the partition of the biological material is purely between the top and bottom phase, then the recommended volume of bottom phase to add would be equal to the volume of the bottom cavity. If the partition would be between the bottom phase and the interface, then the volume of bottom phase should be increased leading to moving interface.
11. Typically, 30 s is a sufficient mixing time, but for very viscous systems, several minutes may be required. The separation time required is typically 10 min, but depends on the phase composition and volume ratio of the phases. In favorable cases, it can be reduced to 2 min, but for very viscous systems or when the density difference between the phases are small, separation times longer than 1 h may be required.
12. When large amount of material is to be separated, it may be difficult to load it in just one chamber. Usually when a phase system becomes overloaded with material, the partition will be influenced by the material itself. To overcome this problem, a larger sample system is prepared and a number of chambers are filled. This does not reduce the resolution seriously, though the number of transfers has to be reduced by the same number as the number of added sample chambers.
13. It is important that the O-ring is clean and has a thin layer of silicon grease to avoid leakage.
14. Typically, 30 s is sufficient mixing time, but for very viscous systems several minutes may be required. Usually an acceleration field of around 100*g* is sufficient. The separation time required is typically 1 min, including approximately 20 s for acceleration, but the time or the acceleration field may need to be increased for very viscous systems.

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Liquid–Liquid Partition Chromatography (LLPC)

Ulla-Britt Hansson and Christer Wingren

1. Introduction

Liquid–liquid partition chromatography (LLPC) is a column chromatographic approach of aqueous two-phase partitioning that offers new and unique possibilites to solve separation and fractionation problems (***1–3***, for review, *see* **ref. *4***). The bottom phase of the two-phase system is adsorbed onto a support and packed into a column, which is eluted with the corresponding top phase (**Fig. 1**). The LLPC method is a rapid (≤ 2 h), sensitive (0.1–100 μg protein is required) analytical method with which even large, flexible, and heterogeneous biomolecules can be analyzed in aqueous solution. The obtained results are highly reproducible (the relative standard deviation of K is ≤ 5%) and conventional chromatographic equipment available in most laboratories can be used (**Fig. 1**).

For a long time, finding materials suitable as support for LLPC was a major problem ***(5–10)***. The problem was finally solved by combining the affinity of polyacrylamide for the dextran-rich bottom phase of the most frequently used phase system, the PEG-dextran system, with the mechanical strength of hydrophilic vinyl (LiParGel), silica (LiChrospher Diol) particles ***(11)***, or dextran-grafted agarose beads (Superdex) ***(12)***. The stationary phase is considered to be adsorbed mainly inside the pores of both LiParGel and LiChrospher ***(11,13)***, whereas the stationary phase may be more evenly distributed on Superdex ***(12)***.

The LLPC setup is schematically illustrated in **Fig. 1**, and the step-by-step procedure for setting up the method is demonstrated in **Fig. 2**. The coated matrix is packed into a column that is eluted with the corresponding top phase. The sample is applied to the column and its retention volume (V_R) is determined and expressed as a partition coefficient, K_C. The latter is defined as the ratio of concentration of the sample in the stationary phase to that in the mobile phase

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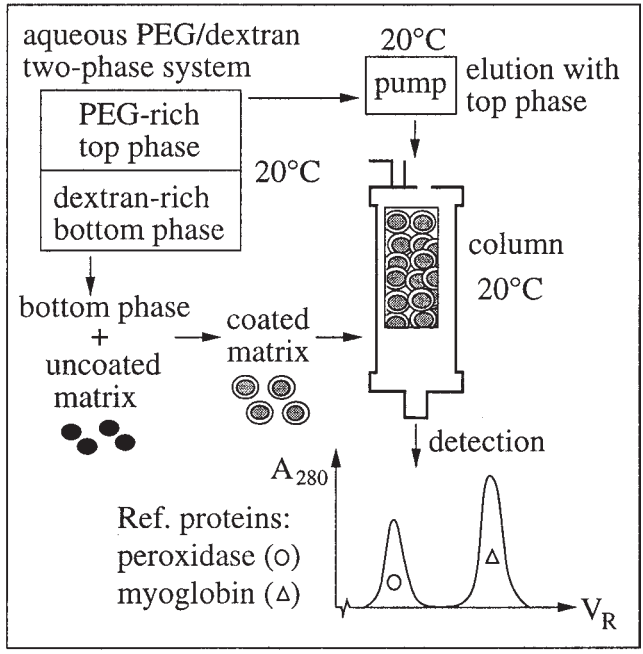

Fig. 1. Schematic illustration of the LLPC setup. A typical chromatogram for the reference proteins, peroxidase and myoglobin, obtained on LiParGel 650 (300 × 8 mm I.D.) in a 4.4% (w/w) PEG 8000/6.2% (w/w) dextran T 500, 50 m*M* sodium phosphate, 0.1 *M* NaCl, 0.1 *M* glycine, pH 7.0, at a flow rate of 0.12 mL/min (20°C) is shown.

$$C_{\text{stationary phase}} / C_{\text{mobile phase}} \tag{1}$$

and calculated from the relation

$$V_R = V_M + (K_C \times V_S) \tag{2}$$

where V_S and V_M, the volume of the stationary and mobile phases, respectively, are calculated from the retention volumes for two reference proteins, peroxidase, and myoglobin. The use of K_C values, instead of retention volumes, allows the comparison of results obtained from different LLPC columns.

The plate number N is calculated from the peak width at half height (w_h) of the myoglobin peak according to

$$N = 5.54 \, (V_R / \mathrm{w_h})^2. \tag{3}$$

The resolution of the peroxidase and myoglobin peaks is calculated as

$$R_S = (\sqrt{N} / 4) \, [k / (1 + k)] \, (\alpha - 1) \tag{4}$$

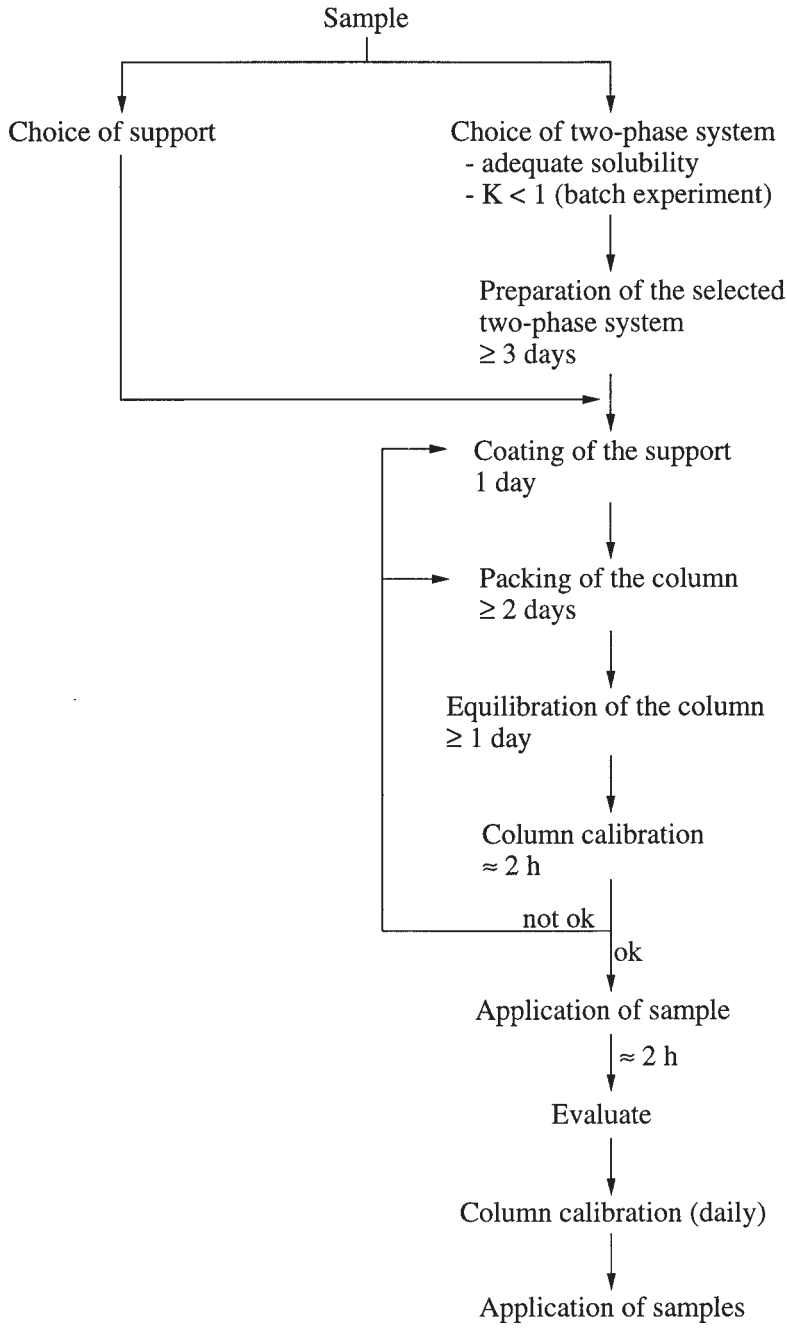

Fig. 2. Schematic step-by-step procedure for setting up the LLPC method.

where k is the capacity factor ($k = (V_S / V_M) K_C$) and α is the ratio of the partition coefficients of the references ($\alpha = K_{\text{batch, peroxidase}} / K_{\text{batch, myoglobin}}$).

The parameters of the LLPC columns (LiParGel 650) should, if possible, be $V_S/V_M = 1.7 \pm 0.2$, $V_S/V_C = 0.42 \pm 0.02$, $V_M/V_C = 0.25 \pm 0.02$, $N = 720 \pm 100$ and $R_S = 3.7 \pm 0.3$ where V_C is the column volume ***(3)***.

Table 1 gives some application examples demonstrating the usefulness of LLPC in the studies of proteins (for review, *see* **ref. *4***). The technique has the capacity not only to separate different proteins, but also to detect and fractionate different conformational isomeric forms of both enzymes and antibodies, as well as to fractionate antibodies with respect to their specificity. Even conformational changes occurring upon ligand-binding may be detected.

2. Materials

1. Gels: Three groups of gels have so far—with respect to phase-binding properties and chromatographic performances—been found suitable to act as supports for LLPC. The particles of LiParGel (polyvinyl) as well as of LiChrospher (silica) are grafted with polyacrylamide, whereas those of Superdex (agarose; Amersham Pharmacia Biotech, Uppsala, Sweden) are grafted with dextran (*see* **Note 1**). The particle size and pore size (exclusion limit) of the matrices differ.

   LiParGel ***(2)***: 80 mL of settled Toyopearl (Tosohaas, Philadelphia) particles are suspended in 250 mL of aqueous acrylamide solution containing 33 g of acrylamide. A stream of nitrogen is passed through the suspension which is stirred magnetically just enough to prevent the gel particles from settling. Fifteen milliliters of solution of 0.2 M cerium(IV) ammonium nitrate in 1 *M* nitric acid, previously placed and deoxygenated in a dropping funnel is added to the particle suspension, which is then stirred gently for about 3 h. The gel particles are collected and washed. Residual traces of Ce(IV) ions are detectable by their yellow color. LiChrospher-Diol ***(2)***: 15 g of dry material of LiChrospher-Diol is suspended in 220 mL of solution containing 30 g acrylamide. The polymerization reaction and rinsing are performed as previously described.
2. Polymers: Polyethylene glycol (PEG) 8000 (Union Carbide, New York) and Dextran T500 (Amersham Pharmacia Biotech) (*see* **Note 2**). Highly pure polymers should be used.
3. Buffer substances: All chemicals used should be of analytical grade (*see* **Note 3**).
4. Reference proteins: Peroxidase (Merck) and Myoglobin (Sigma) (*see* **Note 4**).
5. Chromatography column (*see* **Note 5**).
6. Pump: Should be able to produce a flow rate in the range of 5–2000 (HPLC) or 100–1000 (conventional) µL/min.
7. Ultraviolet (UV)-detector: A detector with a variable wavelength (190–600 nm) and an absorbance range of 0.0001–1 AU is recommended.
8. Valve (injector): equipped with an adequately large sample loop.
9. Control unit: optional equipment.
10. Fraction collector: Optional equipment.
11. Thermostatic equipment (*see* **Note 6**).

Table 1
Some Application Examples Demonstrating How LLPC Can Be Used

Separation and fractionation		Detection of differences in surface properties and conformational changes occurring upon ligand-binding	
Sample	Ref.	Sample	Ref.
Serum proteins	***(14–16)***	Antibodies with different specificities	***(20–22)***
Enzymes from other proteins	***(11)***	Isozymes	***(17,18)***
Isoforms of enzymes	***(17,18)***	Isomerism of antibodies	***(19)***
Monomers and dimers	***(11)***	Ligand binding of enzymes	***(17,18)***
Antibodies of different classes and subclasses	***(1,11)*** ***(19–21)***	Ligand binding of antibodies	***(14,19,23)***

3. Methods

3.1. Preparation of Two-Phase System

The most commonly used aqueous polymer two-phase system, PEG/dextran system, is chosen as a practical example.

1. Prepare the polymer stock solutions, 25% (w/w) dextran and 40% (w/w) PEG solution in distilled water (*see* **Note 7**).
2. To prepare the two-phase system, mix the required amounts of the polymer stock solutions thoroughly with most of the distilled water, followed by dry salt/buffer substances. Adjust the pH (4 *M* HCl or 4 *M* NaOH) before adding rest of the water (*see* **Note 8**).
3. Incubate the stirred mixture in a separation funnel, at the same temperature at which the column is to be run, until the phases are clear (about 4 d).
4. Recover the bottom and top phase; discard the interface (*see* **Note 9**).

3.2. Washing and Coating of Support

1. Choose an appropriate matrix (*see* **Note 1**).
2. Rinse the matrix on a glass funnel equipped with a sintered-glass disc with three volumes of distilled water, three volumes of 0.2 *M* sodium acetate, four volumes of distilled water, and four volumes of stationary phase.
3. Incubate overnight in stationary phase at the selected temperature (*see* **Note 10**).
4. Rinse the coated support with mobile phase in order to remove excess stationary phase (*see* **Note 11**).
5. Suspend in a minimum of mobile phase, deaerate and pour into a thermostatted column with a filling reservoir (*see* **Note 11**).

3.3. Column Packing

Pack the column under gravitational sedimentation for ≤ 24 h and then disconnect the filling reservoir. Further pack the column at a flow rate of 0.6–0.2

mL/min and equilibrate with about three volumes of mobile phase at the selected flow rate until the eluates are clear and the absorbance at 280 nm is constant.

3.4. Column Calibration

1. Determine the performance and capacity of the LLPC column by daily application of two reference proteins (0.02–1.0 mg), peroxidase and myoglobin, in order to minimize the experimental error.
2. Determine the partition coefficient of a reference protein K_{batch} defined as the ratio of the concentration of the protein in the top phase to that in the bottom phase, in batch experiments, i.e., in the absence of any matrix (*see* **Note 13**). $1/K_{batch}$ is then used as K_C (assuming ideal partitioning of the reference proteins also on the column). The relative standard deviation (100[standard deviation/mean]) of K_C may be expected to be ≤5%.
3. Calculate the volumes of the stationary and mobile phases V_S and V_M from the retention volumes for the references V_R using **Eq. 2** (*see* **Note 12**).
4. Calculate the plate number N from the peak width at half height (w_h) of the myoglobin peak according to **Eq. 3**, and the resolution of the two reference proteins according to **Eq. 4** (*see* **Note 12**).

3.5. Sample Preparation

Dissolve the protein sample in mobile phase (*see* **Note 14**). Whenever possible, add mobile phase so that the sample volume constitutes ≤ 70% of the volume to be applied to the column.

To this solution, add the main polymer of the mobile phase (stock solution) so that the concentration of the polymer in the sample solution is the same as that in the mobile phase (*see* **Note 15**).

3.6. Chromatography

1. Apply the sample, dissolved in mobile phase, on to the column via a valve (injector) (*see* **Note 16**).
2. Elute the column with mobile phase (*see* **Note 17**).
3. Monitor the sample continuously by measuring the absorbance of the eluates using a detector and/or by analyzing collected fractions using methods such as enzyme-linked immunosorbent assay (ELISA), enzymatic analysis, etc.
4. Determine the retention volume of the sample and express it as a partition coefficient, K_C (2) (*see* **Note 18**).
5. If necessary, repeat the chromatography by varying some parameter in order to optimize the running conditions (*see* **Note 19**).
6. Follow the troubleshooting guide in case of specific problems (*see* **Note 20**, **Table 2**).
7. Use the mobile phase to "clean" the column prior to storage (*see* **Note 21**).

4. Notes

1. Supports for LLPC: LiParGel is the most frequently used support and the matrix is easy to work with. LiParGel 650 and LiChrospher-Diol 1000 or 4000 columns

Table 2
Guide to Troubleshooting in LLPC

Problem	Remedy
No equilibrium in the system.	Adjust the amount of bottom phase in the mobile phase. Take out the matrix, re-equilibrate it in mobile phase and repack the column. It may also be necessary to recoat the matrix. Use a new batch of top/(bottom) phase.
Sample precipitates.	Determine the solubility of the sample in the mobile phase prior to the application. Decrease the amount of applied sample. Increase the solubility of the protein by adjusting the pH and/or the composition of salts in the system.
Fluctuations in temperature.	Control your thermostatic equipment.
Fluctuations in flow/pressure.	Use a high quality pump.
Poor column packing.	Continue the packing or repack the column.
The selectivity of the system is too low.	Increase the selectivity of the system by changing the composition of phase forming polymers and/or the salt/ buffer composition.
A low plate number.	Increase the length of the column, or decrease the flow rate.
K_C of the sample is too high/low.	Decrease/increase K_C by changing the composition of the phase forming polymers and/or the salt/buffer composition.
Interactions with the support.	Adjust the ionic strength in order to minimize electrostatic interactions with the matrix. Change the support.

(3,11) can be used for the analysis of small as well as of large proteins, whereas LiChrospher-Diol 100 ***(3,11)*** and Superdex ***(12)*** are used for the analysis of small proteins (<100,000 d).

2. Polymers systems: Two-phase systems formed by polyvinylpyrrolidone/dextran, polyvinyl alcohol/dextran, and polyethylene glycol (PEG)/ salt systems have been used for LLPC ***(25–27)*** but the PEG/dextran systems are the most frequently used aqueous two-phase systems ***(4)***. A drawback is the incompatibility between larger proteins and PEG. However, this problem may be at least partly overcome by adding appropriate salts, e.g., betaine ***(11)*** or glycine ***(1)***.

3. Buffer substances: A wide range of ionic strengths, buffer compositions, and pHs have been used, depending on the separation problem at hand ***(4)***.
4. Reference proteins: Besides peroxidase and myoglobin, tRNA and 5sRNA have also been used as reference proteins.
5. Columns: Prepacked LLPC columns (glass or steel) are not, at least for the moment, commercially available. When setting up the LLPC technique for the first time, column dimensions of about 300 × 10 mm I.D. are recommended (a column size also frequently used in analytical runs). Smaller as well as larger columns can be used in other cases, but the actual choice depends on the requirements for column performance and elution time.
6. Temperature control: The experiments should always be performed at a constant temperature (± 0.1°C). The ideal situation is when a thermostatted room is available. The incubation of the two-phase system and of the matrix can, however, be performed in a cooled precision incubator, whereas a column thermostat or a thermostatic jacket allows the column to be run in nonthermostatted rooms.
7. Polymer stock solutions: The polymer solutions should be stored in the dark and at the temperature at which they are to be used (short time) or at 4°C (long time). At temperatures above 30°C and during long-time storage, the polymer solutions should be flushed (saturated) with argon or nitrogen in order to avoid decomposition/oxidation of the polymers. A 0.05% sodium azide (toxic) may be included in all solutions in order to avoid microbial growth.
8. Choice of phase system: Batch experiments are required to find the appropriate system with respect to the size and the concentration of the phase-forming polymers, the pH of the system, the salt to be added, the total ionic strength, and the temperature. By stepwise variation of the listed parameters, the system is adjusted until the solubility of the protein is adequate and its partition coefficient, K ($C_{\text{top phase}}/C_{\text{bottom phase}}$), is <1.
9. Preparation of two-phase system: The two-phase system can also be prepared prior to the addition of salt/buffer substances to the clear top phase, whereas the clear bottom phase is used without any additives.
10. Coating of support: For LiChrospher, use unbuffered stationary phase. For Superdex, the incubation step may be deleted.
11. The coated support: A coated support should never be treated with water or aqueous solutions other than the mobile phase. The columns are packed/equilibrated/eluted with mobile phase that is saturated with stationary phase, i.e., is slightly turbid, in order to assure a constant coating of the columns.
12. Support performance: A ratio of V_S/V_M of 0.6–1.5, a resolution of 1.5–3.6, a plate number of 200–725 and a volume of one plate of 6–78 μL are typical parameters obtained for LLPC columns using LiParGel, LiChrospher, or Superdex as supports, when prepared in the same PEG/dextran two-phase system. Flow rates required to elute the reference proteins within approx 2 h are preferable, although lower flow rates would further improve the column performance.
    The resolution and plate number of a column generally increase with a decreased flow rate and this is also the case for LLPC columns. In the case of LLPC, the

loss in performance caused by an increased flow rate can be partly offset by increasing the temperature; however, the composition of the phase system is then also affected.

13. Determination of K_{batch}: A 4 g amount of the two-phase system is mixed with 0.5–4.0 mg protein and the phases are allowed to separate at the selected temperature overnight. The distribution of the reference protein is determined spectrophotometrically at 280 nm, and K_{batch} calculated.
14. Protein solubility: The solubility of a protein should be examined prior to its application because precipitates will damage the column, i.e., parts of the stationary phase may be eluted.

    Proteins that exists in a dry, salt-free state may in some cases be directly dissolved in mobile phase. Proteins dissolved in aqueous solutions are recommended to be dialyzed against the buffer of the applied two-phase system, although minor deviations in buffer composition (pH ± 1 unit, ionic strength ± 0.3 *M*) may be tolerated.
15. Sample preparation: Assume that you have 200 μL sample and that the mobile phase contains 6.6% (w/w) PEG and 0.2% (w/w) dextran. A 12 μg solid PEG, i.e., 39.5 μL of a stock-solution containing 40% (w/w) PEG should then be added in order to obtain 6.6% PEG in the 239.5 μL solution.
16. Sample volume: The sample volume should not exceed 2–3% of the column volume in analytical runs, whereas it may be twice as large in preparative runs. No cleaning is required before the next sample can be applied.
17. Elution mode: In most cases, the LLPC analyses are performed as isocratic runs. A gradient can, however, be applied in order to reduce the elution time for molecules that are partitioned towards the stationary phase ***(11)***. A ligand (for example procion red), specific for the protein of interest can also be added to the mobile phase ***(13)***, but the usefulness of such experiments remains to be unravelled.
18. Influence of the gel: In order to examine the influence of the support on the partition properties of proteins, the experimentally obtained retention volumes are compared with those calculated according to **Eq. 2**, using $1/K_{batch}$ as K_C. No detectable interaction occurs when the calculated and obtained retention volumes are identical.

    Electrostatic interactions between proteins and charged supports can be eliminated using an ionic strength > 0.05 *M* for LiParGel and LiChrospher ***(2)*** and >0.2 *M* for Superdex ***(28)***.

    Ideal partitioning is observed for proteins ≤ 1 000 kDa on LiParGel 650 ***(1,14)*** and ≤ 500 kDa on LiChrospher-Diol 1000 ***(13)*** (which has a pore size that is four times smaller than that of LiChospher-Diol 4000). Large proteins, > 100 kDa, are strongly retarded on Superdex 200 ***(12)***.
19. Optimization of running conditions: The running conditions can be optimized by changing the support, packing procedure, phase composition, pH (buffer composition), temperature, or flow rate. Use of densely packed columns and LiParGel or LiChrospher-Diol as a support are, in most cases, recommended. The selectiv-

ity can be improved by changing the concentration of one or both the phase-forming polymers and/or of the salt and pH. For example, when a 4.4% PEG/5.2% dextran two-phase system is used, antibodies are eluted as a single component, whereas several components are detected using a 4.4% PEG/6.2% dextran system ***(14)***.

It may be of interest to note that the pH (buffer composition) can be changed in a column equilibrated at one pH, by simply eluting with the top phase in which the pH has been changed. It is then advisable to incubate the column overnight and continue the elution until the absorbance of the eluate is constant.

20. Trouble shooting: *See* **Table 2**.
21. Column cleaning, regeneration, and storage: The matrix must be washed, recoated and repacked if the composition of the phase forming polymers is to be changed, whereas the pH (buffer composition) may be changed when the column is already in place. The column can be stored at the preselected and constant temperature. The "lifetime" of a column may be a year or longer ***(3)***.

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8

Metal Ion Separations in Aqueous Biphasic Systems and with ABEC™ Resins

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1. Introduction

1.1. The Separation of Metal Ions by Liquid–Liquid Extraction in Aqueous Biphasic Systems (ABS)

1.1.1. The Contemporary Challenge of Metal Ion Separations in ABS

Solvent extraction is a proven technology for the recovery of metal ions from aqueous solution ***(1,2)***. Through the application of different diluents, extractants, and aqueous phase conditions, the technique may be adapted to handle a wide variety of solutes. Extraction kinetics are often rapid, enabling high throughput, and the technology may be engineered for selectivity and efficiency through the use of multistage contactors. However, there are disadvantages to the employment of aqueous/organic extraction schemes. In particular, many utilize toxic and flammable organic diluents, which may add significant cost to the overall process design and which are highly regulated owing to their environmental impact.

Aqueous biphasic systems retain all the practical advantages of liquid–liquid extraction, but are also possessed of some unique qualities, not least their wholly aqueous nature. Polyethylene glycol-based aqueous biphasic systems (PEG–ABS) are nontoxic, nonflammable, and inexpensive bulk commodities having favorable phase separation and mass transfer characteristics that are compatible with conventional solvent extraction equipment ***(3,4)***. Significantly, in ABS solute distribution is between two wholly aqueous phases and thus solute dehydration is minimized. Only a subtle reorganization of hydration may be required, which contrasts very favorably with conventional solvent extrac-

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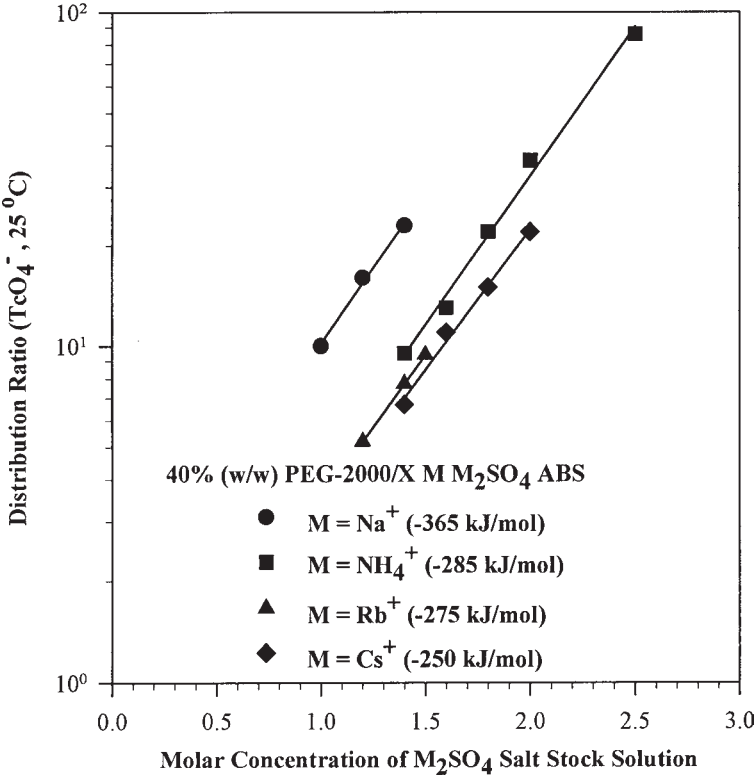

Fig. 1. Distribution of $^{99}TcO_4^-$ in a PEG-2000 ABS as a function of the molar salt concentration of M_2SO_4 salts differing in the free energy of hydration (ΔG_{hyd}) of the salt cation.

tion where near complete dehydration may be necessary. Thus, the application of ABS may represent an opportunity to completely eliminate the need for volatile organic compounds in many separation and waste remediation technologies and thus to revolutionize many industrial separation processes ***(5)***.

1.1.2. The Thermodynamic Basis of Metal Ion Partitioning

The ability of salts to form ABS with PEG has been shown to be directly related to the Gibbs free energy of hydration of the salts. Both cation and anion contribute to this effect, but the anion dominates ***(6)***. The more negative the Gibbs free energy of hydration, the greater the salting-out effect of an ion for PEG. Ions with small negative free energies of hydration have a greater tendency to partition to the PEG-rich upper phase. **Figure 1** shows an example in which cations of sulfate salts, of increasingly negative Gibbs free energy of hydration, increase the tendency of the pertechnetate anion to partition to the PEG-rich phase of a PEG-2000 ABS.

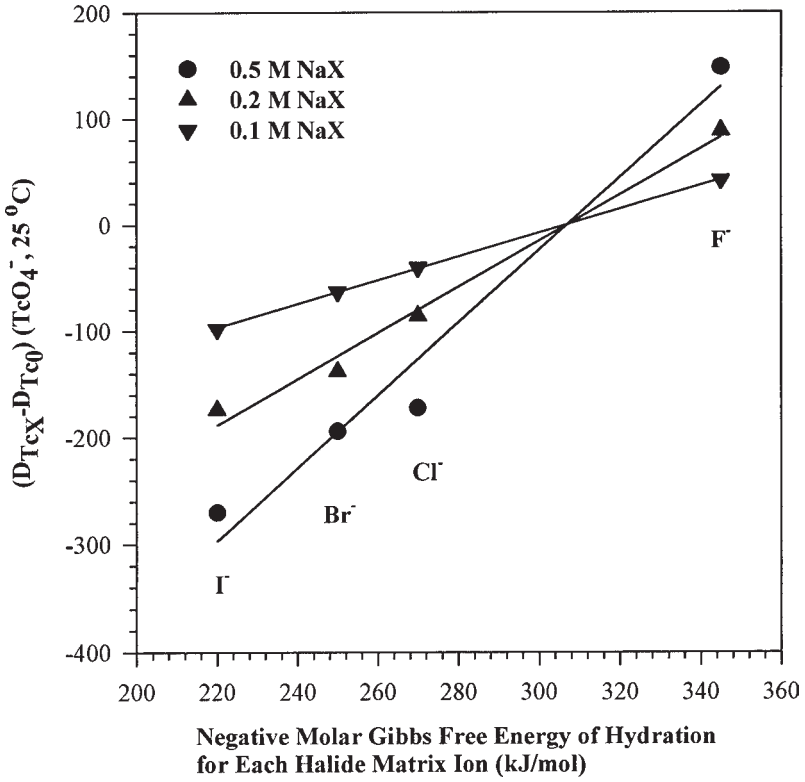

Fig. 2. Difference in the pertechnetate distribution ratio in a 3.5 *M* $(NH_4)_2SO_4$/40% (w/w) PEG-2000 ABS with matrix ions (D_{TcX}) and without matrix ions (D_{Tc0}) versus the negative Gibbs free energy of hydration for each halide matrix ion.

The free energy of hydration appears to be the most important factor in determining:

1. The choice of salt used to form the phase system;
2. The effectiveness of the system for separating a particular ion; and
3. The efficiency of the separation of a given ion from a particular matrix of other ions *(7)*. For instance, **Fig. 2** shows the effect of halide ions on the distribution of pertechnetate in a PEG-2000 ABS. Only fluoride (which also salts-out PEG 2000) results in an increased distribution ratio.

Knowledge of the ionic composition enables the prediction of the performance of ABS separations from a particular matrix. At present, the prediction is more or less qualitative; however, we are currently developing a more quantitative approach. For instance, it can be shown that in an ABS prepared by mixing equal volumes of 3.5 *M* $(NH_4)_2SO_4$ and 40% (w/w) PEG-2000, ions having free energies of hydration (ΔG_{hyd}) equal to –310 kJ/mol have essen-

Table 1
Gibbs Free Energies of Hydration (ΔG_{hyd}) for Selected Anions and Cations *(8)*

Ion	ΔG_{hyd} kJ/mol	Ion	ΔG_{hyd} kJ/mol	Ion	ΔG_{hyd} kJ/mol
PO_4^{3-}	–2835	HCO_3^-	–310	Na^+	–385
CO_3^{2-}	–1300	NO_3^-	–275	K^+	–305
SO_3^{2-}	–1230	Cl^-	–270	NH_4^+	–285
SO_4^{2-}	–1145	Br^-	–250	Rb^+	–285
CrO_4^{2-}	–1120	I^-	–220	Cs^+	–245
SeO_4^{2-}	–1110	MnO_4^-	–195		
OH^-	–345	ClO_4^-	–180		
F^-	–345	ReO_4^-	–170		

tially no effect on pertechnetate distribution ratios. Ions having free energies less negative than this will have the effect of depressing the distribution ratio for pertechnetate, whereas those ions with more negative free energies will enhance the distribution ratio ***(7)***. Salts that enhance the distribution ratio of pertechnetate will also be capable of biphase formation with PEG above a critical concentration. **Table 1** shows the Gibbs free energies of hydration of a selection of important phase-forming ions and common matrix ions ***(8)***.

1.1.3. Important Applications of Metal Ion Separations in Aqueous Two-Phase Systems

Metal ion extractions in ABS are of three main types ***(6,9–11)***: (1) extraction into the PEG-rich phase of the target ion without addition of any other extractant ***(7,12–16)***; (2) extraction of the metal ion mediated by the formation of negatively charged inorganic anion complexes ***(17)***; and (3) extraction utilizing a water-soluble organic complexant ***(18–22)***. The most important application to date, involving the quantitative partitioning and recovery of the pertechnetate anion, falls in the first category. This is of major practical importance because the short-lived ^{99m}Tc ($t_{1/2}$ = 6h) is used in the vast majority of all medical procedures involving radioisotopes ***(23,24)***.

Current production techniques for ^{99m}Tc involve the use of high specific activity ^{99}Mo (produced as a result of the fission of ^{235}U), which is adsorbed onto alumina from which the daughter pertechnetate anion is eluted with a saline solution. The eluate is, however, often contaminated with radiomolybdate and aluminum. Additionally, the fission route to technetium generation is expensive and the number of suppliers is dwindling ***(25)***. An alternative route through low specific activity neutron-irradiated ^{99}Mo requires

extraction using the toxic, flammable organic solvent methyl ethyl ketone. Thus, the PEG–ABS procedure has great potential for the production of concentrated ^{99m}Tc from low specific activity ^{99}Mo (i.e., produced by neutron bombardment as opposed to nuclear fission) in the form of a reduced chelate complex of an imaging agent ***(14,16)***. Potentially, this process could lead to the increased availability of directly injectable medical imaging agents at greatly reduced cost.

Aqueous biphasic systems could also find immediate application in waste remediation. Relatively high levels of $^{99}TcO_4^-$ are present in the alkaline waste storage tanks of Westinghouse Hanford ***(26,27)*** and at Savannah River ***(28)*** in the United States as byproducts of nuclear fission. Similar problems exist in the reprocessing of fission products outside the United States, for instance, in the final discharge of $^{99}TcO_4^-$ into the marine environment in the United Kingdom following treatment to remove other radionuclides. The long half-life and environmental mobility of $^{99}TcO_4^-$ make its recovery a matter of some concern ***(29)***. The applicability of the Aqueous Biphasic Extraction Chromatography (ABEC) variant (*see* **Subheading 1.2.**) of the ABS process to the treatment of simulated Hanford tank wastes has already been demonstrated ***(30,31)***.

A wide variety of metal ion separations could have been considered for illustrative purposes, but in view of its practical importance, the particular experimental example given here concerns the examination of some of the requirements for efficient extraction of technetium as the pertechnetate anion from a solution of NaOH to the PEG-rich phase of an ABS. Detailed experimental instruction is given for the examination of the salt concentration required of a number of different salts in order to promote the partition of the pertechnetate anion to the PEG-rich upper phase. The procedures may easily be adapted to the examination of other salts, to the examination of their effectiveness in particular matrices of other ions, and to their effect on the partition of other metal ions.

1.2. The Separation of Metal Ions by Aqueous Biphasic Extraction Chromatography (ABEC™)

In classical solvent extraction, it is often inconvenient for the extracted species to remain in the solvent phase and it is usual to back-strip into a fresh aqueous phase. This may often be achieved in some simple fashion, such as by reduction or, more commonly, by change in pH to produce a charged moiety that is now more soluble in the aqueous phase. In such cases, albeit after suitable clean-up, spent solvent may usually be recycled and reused, if necessary, after distillation. For ABS, this is much more difficult to achieve. Back-stripping steps may be more difficult to design—especially where the forward

extraction has been most effective. Recycle of the extracting PEG-rich phase may be difficult and expensive to achieve because it necessitates the removal of water.

Covalent attachment and immobilization of PEG onto a solid support appears to overcome the limitations of ABS, because the biphasic conditions necessary for the forward extraction of a given species may be created or destroyed at will simply by addition of the appropriate solvent, i.e., salt solution or water, without any loss of the phase-forming polymer ***(11,32)***. In the simplest case, extraction to the immobilized PEG phase may be made under appropriate conditions of salt, pH, and so forth, and then recovery effected by the simple expedient of adding water overcoming in large part many of the potential drawbacks of adopting aqueous biphasic extraction technology. All those applications outlined in **Subheading 1.1.** for ABS thus may be simply and directly transferred to the ABEC mode of operation, with considerable advantage to the overall efficiency of the process.

Figure 3 shows the distribution ratios for ABEC and ABS of the pertechnetate anion for a variety of different salts. The distribution ratio *(D)* referred to in the figure is simply the ratio of the concentration of the solute between the two phases (PEG-rich/salt-rich). An analogous distribution ratio may be defined for partition to a solid phase (D_W, *see* **Subheading 3.**). The similarity in the effect of changing salt type and/or concentration in ABS and ABEC may be judged from **Fig. 3**.

Again, for illustrative purposes, and because of its high practical potential, the example given here concerns the extraction of technetium in the form of the pertechnetate anion onto an ABEC resin. The two procedures may thus be directly compared. **Figure 3** shows such a comparison for the distribution of pertechnetate in ABS and ABEC with increasing concentration of ammonium sulfate. As in the case of the ABS procedure, the technique may easily be adapted to the examination of other conditions and ions. Additionally, the ABEC procedure as outlined may be adapted to packed bed or column mode of operation, thus permitting the examination of performance criteria pertinent to industrial scales of operation ***(32)***. A simple procedure for the operation of ABEC adsorbents in chromatographic mode is also presented here.

2. Materials

2.1. Separation of Metal Ions by Liquid/Liquid Extraction in ABS

2.1.1. Chemicals

1. 40% (w/w) polyethylene glycol MW 2000 (reagent grade) in deionized water (*see* **Note 1**).
2. Solutions of NaOH (reagent grade) in the range 4–6 *M* as the phase-forming salt (*see* **Note 2**).
3. Ultima Gold scintillation cocktail (Packard Instrument Co., Meriden, CT).

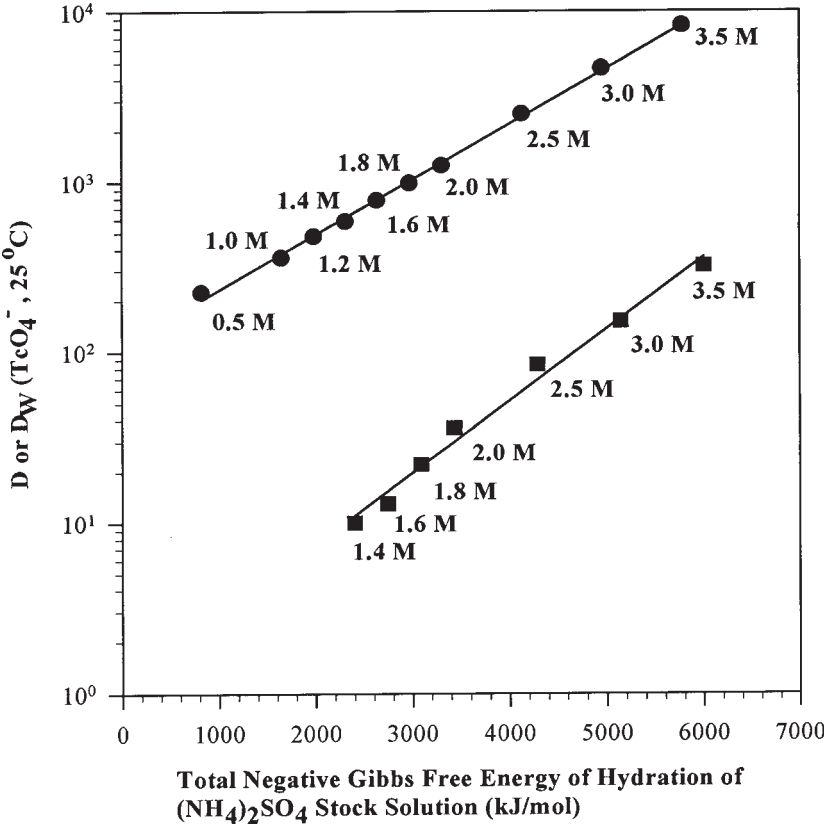

Fig. 3. Distribution (D) and dry weight distribution ratios (D_w) of $^{99}TcO_4^-$ with 40% (w/w) PEG-2000 (■) or ABEC-5000 (●) and $(NH_4)_2SO_4$ in relation to the total negative Gibbs free energy of hydration of the salt stock solution.

2.1.2. Metal Ion Tracers

1. A stock solution of $NH_4{}^{99}TcO_4$ (Isotope Products Laboratories, Burbank, CA) is diluted with deionized water to an activity of approx 0.06–0.08 μCi/μL for use as the "spike" in the partitioning experiments.

2.1.3. Equipment

1. Liquid scintillation counter (LSC) (e.g., Packard Tri-Carb 1900 TR Liquid Scintillation Analyzer).
2. Laboratory vortex mixer.
3. Laboratory bench top centrifuge capable of delivering 2000*g*, and of holding the 13 × 100 mm tubes.
4. Plastic disposable Pasteur pipets.

5. Eppendorf pipets and pipet tips (or equivalent).
6. Glassware: 13 × 100 mm borosilicate glass culture tubes with caps, 1 dram (3.7 mL) shell vials and 6 mL scintillation vials (Fisher Scientific, Pittsburgh, PA).

2.2. The Separation of Metal Ions by ABEC™

2.2.1. Chemicals

1. EIChroM Iodine Resin (EIChroM Industries, Darien, IL): In order to condition the ABEC resin, suspend about 3 g in deionized water and pour into a filter funnel containing 42.5 mm diam. Whatman No. 2 qualitative filter paper, to which vacuum (ca. 5 in. Hg or 10^3 Pa) is applied. The resin is washed three times in the funnel with fresh deionized water with the vacuum applied. In order to avoid excessively dehydrating the resin, the apparatus is arranged so that the air that is pulled through the funnel under vacuum is bubbled through a sidearm vacuum flask containing deionized water. The resin is finally dried to an approximately constant final moisture content by drawing moist air through the resin on the funnel in this manner for 5 min.
2. Salt solutions are required for promotion of the ABEC interaction analogous to those used in the liquid–liquid procedure (i.e., tripotassium phosphate, potassium carbonate, ammonium sulfate, or sodium hydroxide), which should be of reagent grade or better. For the purposes of example, 4–6 *M* NaOH solutions should be prepared as in **Subheading 2.1.** (*see* **Note 2**).
3. Liquid scintillation cocktail: We use Ultima Gold scintillation cocktail (Packard Instrument).

2.2.2. Metal Ion Tracers

A stock solution of $NH_4{}^{99}TcO_4$ (Isotope Products Laboratories), for use as the "spike" in the partitioning experiments, is diluted as in **Subheading 2.1.2., item 2.**

2.2.3. Equipment

1. LSC (e.g., Packard Tri-Carb 1900 TR Liquid Scintillation Analyzer).
2. Automatic pipets and disposable pipet tips (e.g., Eppendorf, Gilson, and so forth).
3. Magnetic stir bars (1.5 × 8 mm), which should be cleaned between use in 5 *M* HNO_3 and rinsed three times with deionized water.
4. Glassware: 13 × 100 mm borosilicate-glass culture tubes, 1 dram (3.7 mL) shell vials, and liquid scintillation vials.
5. 0.45-μm pore-size filters to fit 1 mL Eppendorf pipet tips and of a diameter suitable to insert into the shell vials during sampling. We use filters from Lida Manufacturing Kenosha, WI.

2.3. Separation of Metal Ions using an ABEC™ Column

2.3.1. Chemicals

1. EIChroM Iodine Resin (EIChroM Industries): *See* **Subheading 2.2.1., item 1**. for the detailed procedure for conditioning this resin.

2. Salt solutions: *See* **Subheading 2.2.1., item 2**.
3. Liquid scintillation cocktail is required when using ^{99}Tc as the spike. We use Ultima Gold scintillation cocktail (Packard Instrument).

2.3.2. Metal Ion Tracers for Use with ABEC™ Columns

1. A stock solution of $NH_4{}^{99}TcO_4$ (Isotope Products Laboratories) is diluted with deionized water to an activity of approximately 0.06–0.08 μCi/μL and is used at this concentration to prepare the load solution for the column. (Alternatively, the medical isotope, ^{99m}Tc, a gamma emitter, may be used.)
2. For the free column volume measurements, a stock solution of ^{22}NaCl (Isotope Products Laboratories) is diluted with deionized water to an activity of approximately 0.06–0.08 μCi/μL and is used at this concentration to prepare the load solution for the column (*see* **Subheading 3.3., step 18**).

2.3.3. Equipment

1. A suitable glass chromatography column (e.g., Isolab Practi-column 10 × 100 mm with 0.45 μm frits.): Columns can be run under gravity flow, but peristaltic pumps can also be used and, because of the decontamination issues presented by the presence of radionuclides, these are more suitable than other pump types.
2. LSC (e.g., Packard Tri-Carb 1900 TR Liquid Scintillation Analyzer).
3. Gamma counter (e.g., Packard Cobra II Auto-Gamma counter).
4. A fraction collector is desirable, but not essential.

3. Methods

All procedures should be carried out in places designated for the use of radioactive materials and in accordance with all relevant local safety procedures. The procedures outlined here are routinely performed in a stainless steel radiological safety hood equipped with high-efficiency particulate air (HEPA) and charcoal filters. The experiments are laid out in such a way as to minimize time and extent of exposure by arranging that the maximum possible prepreparation of nonradioactive materials is performed prior to commencing the tracer work. Disposal of all radiologically contaminated materials should conform to all local regulations in force. Consult your own health and safety/environmental protection/radiological safety office for detailed considerations pertinent to the performance of these procedures.

3.1. Separation of Metal Ions by Liquid–Liquid Extraction in ABS

1. For each sample or partitioning condition to be tested, add 1 mL of 40% (w/w) PEG-2000 (or other molecular weight, concentration, or polymer to be tested, *see* **Note 1**) followed by 1 mL of salt stock solution (*see* **Note 2**) to each of two culture tubes. (This will be sufficient to provide duplicate conditions.)
2. The system is vortex mixed for 2 min to equilibrate the components and then centrifuged for 1 min at 2000*g* to separate the phases (*see* **Note 3**).

3. Prepare four scintillation vials containing 3 mL of Ultima Gold scintillation cocktail (*see* **Note 4**).
4. Spike each biphase with 2 µL of tracer (prepared as outlined in **Subheading 2.1.2.**) and recap the tube.
5. Centrifuge for 2 min to ensure contact of the entire spike with the ABS.
6. Vortex and then centrifuge the samples twice more for 2 min each to ensure equilibrium and finally to separate phases.
7. Carefully pipet from the tubes containing biphase, a sample of each separated phase into a fresh shell vial using a disposable Pasteur pipet. This is a nonquantitative step and the exact volume is unimportant, but should be greater than 100 µL (*see* **Note 5**).
8. From each sample of separated phase in the shell vial, quantitatively withdraw by pipet 100 µL of the sample and add to the prepared scintillation cocktail vials (*see* **Note 6**).
9. Close scintillation vials, vortex to mix, carefully remove any dust, dirt, or fingerprints from the glass vial, and measure the activity using a LSC (*see* **Note 7**).
10. The distribution ratio *D* may be determined directly from the radiation counts in the phases as counts per minute (cpm) in the top PEG-rich phase divided by the cpm in the bottom salt-rich phase. This is equivalent to the ratio of the concentration in the PEG-rich phase vs the concentration in the salt-rich phase (*see* **Note 8**).
11. It is also possible to examine back extraction conditions (stripping, i.e., repartition of the $^{99}TcO_4^-$ from the PEG-rich upper phase to the salt-rich lower phase). To test stripping of pertechnetate from the PEG-rich phase, 1 mL of the pertechnetate-loaded PEG-rich phase (produced in **step 6**) is contacted with 1 mL of a fresh salt solution (e.g., 2.5 *M* sodium citrate) containing 0.01–0.05 *M* $SnCl_2$ as reductant. This mixture is then vortexed and centrifuged and the resultant biphasic system treated as for the forward extraction outlined previously by performing **steps 6–10.** The resultant reduced pertechnetate-citrate complex partitions strongly to the lower salt-rich phase.

3.2. Separation of Metal Ions by ABEC

1. Accurately weigh 15–20 mg of adsorbent to each of two capped 13 × 100 mm glass vials to provide duplicates of each condition to be tested. Record the actual weight of resin added to each vial (*see* **Note 9**).
2. Add 2.25 mL of each salt solution to be tested to an equal number of fresh 6 mL scintillation vials and cap (*see* **Note 10**).
3. 3 mL of Ultima Gold scintillation cocktail is added to three scintillation vials ready to receive the test samples (*see* **Note 11**).
4. Spike each 2.25 mL salt solution with 4 µL of ammonium pertechnetate tracer prepared according to the outline given in **Subheading 2.2.2.**, and vortex after capping tightly.
5. From each 2.25 mL spiked salt solution, quantitatively transfer 100 µL to a scintillation vial containing scintillation cocktail and remove from the hood (*see* **Note 12**). Prepare and count this sample (*see* **Subheading 3.1., step 9**) to determine the initial activity (A_i) present in the spiked solution.

6. Add a magnetic stir bar to each tube containing adsorbent.
7. Transfer 1 mL of spiked salt solution to each of two duplicate tubes containing adsorbent.
8. Briefly centrifuge the tubes now containing adsorbent and adsorbate to pull all liquid and absorbent to the bottom of the tube.
9. Place the tubes containing adsorbent and adsorbate onto a magnetic stirrer and allow to equilibrate for 30 min.
10. Centrifuge the adsorbent containing tubes (2000*g*) for 2 min.
11. Place the tubes containing adsorbent and adsorbate back onto the magnetic stirrer once again and allow to equilibrate for 30 min (*see* **Note 13**).
12. Centrifuge the adsorbent containing tubes (2000g) for 2 min.
13. Transfer the contents of the adsorbent containing tubes to shell vials using disposable Pasteur pipets. This is a nonquantitative step designed to facilitate the removal of the adsorbent particles by filtration.
14. Remove a sample of about 1 mL from each shell vial using a 0.45-μm filter attached to an Eppendorf pipet tip. This serves to remove the adsorbent from contact with the equilibrium solution. After removing the filter, transfer the contents of the pipet to a new shell vial. Again this is a nonquantitative step.
15. Quantitatively transfer 100 μL of this adsorbent-free equilibrium adsorbate solution from the shell vials to a vial containing scintillation cocktail (*see* **Note 12**).
16. Load vials to a LSC and count (*see* **Note 7**).
17. The partition of pertechnetate may be estimated from the D_W value calculated as

$$D_w = \frac{A_i - A_f}{A_f} \cdot \frac{\text{contact volume (mL)}}{\text{wt of resin (g)* } dwcf}$$

where A_i is the activity in counts/min in the initial sample and A_f is the activity in cpm after contact with the adsorbent. The contact volume is the total volume of the adsorbate, and *dwcf* is the dry weight conversion factor of the resin. The latter is determined from the weight of the resin dried to constant weight at 100°C in a suitable drying oven or by thermogravimetric analysis (*see* **Note 14**).

3.3. Separation of Metal Ions Using an ABEC™ Column

1. Weigh out ca. 0.5 g of the ABEC resin prepared as outlined in **Subheading 2.2.1., item 1.**
2. Suspend the resin in ca. 30 mL of deionized water in a 50-mL beaker.
3. Sonicate the sample for 20 min to break up any clumps to facilitate packing the column.
4. Slurry pack the suspended resin into a small glass chromatography column. 0.5 g of resin will yield a bed height of about 2 cm in a column of the currently suggested dimensions.
5. Place a small filter frit (0.45-μm pore size) on top of the bed to prevent disturbance to the top of the bed by the addition of eluants.
6. If possible, place the column in the sonicator and sonicate for an additional 20 min. This will also help to produce an evenly packed column.

7. Equilibrate the column by washing with 5 mL of load solution without tracer (*see* **Note 15**).
8. Spike the load solution with tracer. Typically, 10 mL of load solution is spiked with 20 μL of tracer (2 μL/mL of load).
9. Remove a 100 μL sample from the spiked load solution and add to a vial containing scintillation cocktail. This will be used to determine the total pertechnetate challenge to the column.
10. Wash the column with the spiked load solution and collect fractions of the eluant in liquid scintillation vials. A flow rate of 2–4 mL/min is suitable. Samples are subsequently taken from the collected fractions and diluted into liquid scintillation cocktails and counted using a LSC.
11. Progress may be followed, at the time, by making quick 1 min counts on selected fractions and comparing the activity in the breakthrough fraction to that of the initial load sample.
12. At the desired end point (e.g., 50 or 100% breakthrough) the column is rinsed with unspiked load solution to remove unbound pertechnetate.
13. Once the activity of the effluent has fallen, the column may be eluted simply by washing with water. Because the pertechnetate is not retained on the resin in the absence of a high concentration of salt, it is released rapidly from the column and small fractions should be collected. 10 mL of wash water should be more than sufficient to strip this small column. Once again, progress may be monitored by performing quick 1 min counts on the collected fractions.
14. Each collected fraction is then sampled by taking 100 μL, which is added to 3 mL of liquid scintillation cocktail in a scintillation vial, and counted to determine the activity per mL of each sample.
15. A chromatogram of the column behavior may be constructed from the activity/mL (cpm/mL) of the collected fractions as the total eluant volume increases, as illustrated in **Fig. 4** for ^{99m}Tc. The free column volume parameter (fcv) used in **Fig. 4** may be determined as follows.
16. The packed column is equilibrated with ca. 5 mL of unspiked load solution.
17. Allow this solution to pass through the column until its meniscus is level with the top of the bed, i.e., do not allow the bed to run dry.
18. Approximately 1 mL of a ^{22}NaCl spiked load solution (prepared by adding 1 μL/mL of a solution of ^{22}NaCl having an activity of 0.06 μCi/μL) is passed through the column, collecting dropwise. Sodium ions are not retained on the column.
19. Elute the $^{22}Na^{+}$ solution into about 15 preweighed gamma tubes, collecting every 1–2 drops.
20. Determine the volume in each tube by weight difference and count each tube (*see* **Note 16**).
21. The sum of the volume up to the first occurrence of activity is the free column volume.
22. Repeat **steps 18–21** using deionized water as the load solution. As a result of bed swelling, the free column volume determined with water will be larger than that determined for a salt containing load solution. The appropriate free column volume determination should be used to plot the chromatogram.

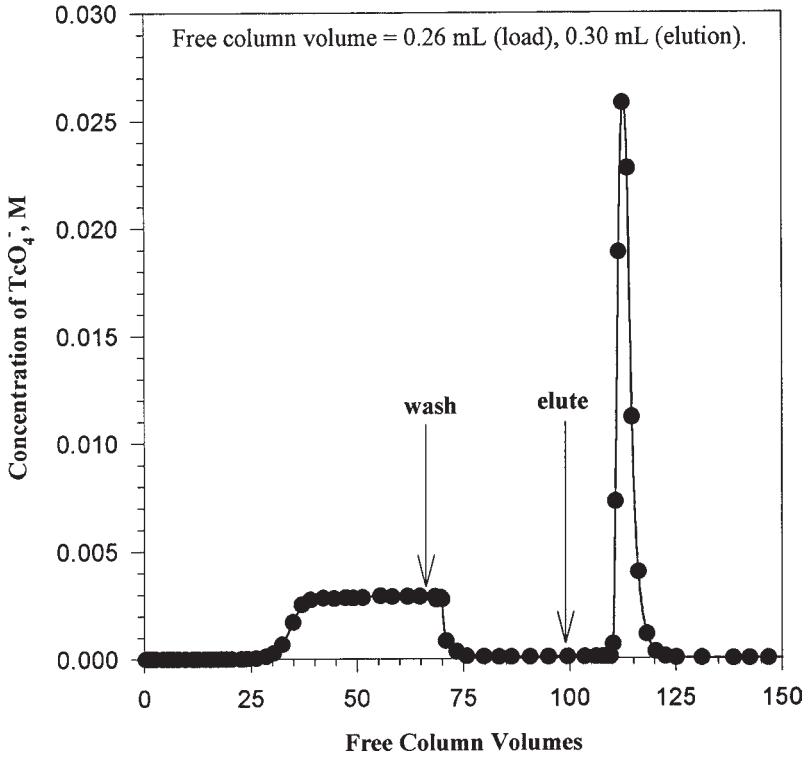

Fig. 4. Chromatogram of the TcO_4^- concentration as a function of the eluant volume over three stages; load, wash, and elution, of a 1-mL ABEC column contacted with simulated nuclear waste solution containing greater than 2 *M* sulfate.

4. Notes

1. Phase forming polymers are usually confined to PEGs of reagent grade of molecular weights between 1500 and 5000, but other molecular weights and other polymers may on occasion be of interest ***(15)***. Polymer stock solutions are prepared on a weight/weight basis (e.g., to prepare 40% w/w, weigh 40 g PEG-2000 and dissolve in 60 g deionized water).
2. Sodium hydroxide is here selected as the phase-forming salt for its particular relevance to the separation of pertechnetate both in the $^{99}MoO_4^{2-}/^{99m}TcO_4^-$ separation and in the remediation of $^{99}TcO_4^-$ waste solutions. Other salts may be equally applicable as indicated in **Subheading 1.** on the basis of their free energy of hydration ***(6,11)***. Typical phase forming salts that may be used include tripotassium phosphate, potassium carbonate, ammonium sulfate, and so forth. Typically, such salts would be suitable in reagent grade and are prepared as molar solutions in the range (which varies from salt to salt depending on the critical concentration for phase separation) 0.5 to ca. 3 *M* or above. Matrix ions may be

included to simulate particular solutions from which extraction of technetium or other metal ion is proposed. This can be achieved either by adding phase-forming salts as previously outlined to a solution of the matrix ions provided that overall solubility can be maintained or, in order to test the effect of particular ions, they may be added to the phase forming salt stock solution in varying amounts. The results of such an experiment are shown in **Fig. 2**.

3. The polymer and salt solutions added to the tube are not at equilibrium; hence, they are mixed and the equilibrium mixture separated to ensure that the spike will be added to an equilibrated biphasic system.
4. The amount of cocktail required depends on the cocktail, the volume of the sample, and the composition of the sample. The procedure outlined here is suitable for the 100 µL samples of pertechnetate at the activity levels outlined in **Subheading 2.** See the manufacturers instructions pertaining to particular scintillation cocktails and LSCs. Avoid the use of fluorescent markers on the tube sides. These may be used only on the caps. These systems are biphasic and thus one vial containing scintillation cocktail is required for each phase. Thus, with duplicates, four scintillation cocktails are required for each test condition. Duplicates, once counted, should agree within 5%. (The radiochemical method itself should be accurate to within 5%.)
5. A number of points are worth noting regarding the separation of the phases of the biphasic system. Aspirate the top phase first using a plastic disposable Pasteur pipet. There may be a tendency for bubbles to be included in samples from the viscous top phase (the PEG-rich phase), which will interfere with volume measurements. When removing the bottom phase, slowly push the pipet through the top phase while expelling bubbles of air from the pipet to prevent ingress of the top phase. On removal, wipe the outside of the pipet with a tissue in order to avoid contamination of the lower phase with material from the upper phase. Try to avoid disturbing the phases. Entrainment of one phase in another can occur depending on which phase was the disperse phase. This could be overcome by further brief centrifugation, however, there may also be a tendency for the phases to cloud again after separation if there is an increase in temperature. This may be avoided by cooling the separated phase or ensuring that it is well mixed and a representative sample is taken. Careful attention to this step is a prerequisite of reproducible results.
6. Take care over these quantitative pipetting steps. Use a tissue to wipe excess material from the outside of the pipet tip and ensure that the pipet tip is properly filled and dispensed, because the density and viscosity of these phases can be very high.
7. The ^{99}Tc is a β^- emitter with an energy of 292 keV**.** The main window of the LSC is set to count from 5 to 320 keV. The 2σ coincidence level is set to shut off at 70%. A first vial background count is performed using a 3-mL scintillation cocktail alone. Quenching is compensated using the built in tSIE Transform Spectral Index of the External Standard Spectrum. Often it is helpful to perform a quick count of the samples, especially with the addition of samples that might decom-

pose or react slowly with the cocktail. It is also helpful when counting large numbers of samples when, for instance, the sample separates from the cocktail over time, in which case the samples can be remixed and counted again.

8. Because the activity of the $^{99}TcO_4^-$ is directly proportional to its concentration and equal aliquots of the phases are sampled, the distribution ratio may be determined as the cpm in the PEG-rich (upper) phase divided by the cpm in the salt-rich (lower) phase. Deviations from the outlined procedure may require adjustment to the way the distribution ratio is calculated.
9. The procedure adopted is intended to produce material of a uniform moisture content and size range. As supplied, the material is predominantly made up of particles whose size ranges from *ca* 80–120 μm diam. (to some extent, this is ionic-strength-dependent), but there is a fraction in current preparations much smaller than this (<5 m diam.) that is removed by the outlined washing procedure. Typically the ABEC resin contains 80–90% water and it is important that this should not be removed. An oven-dried sample of the ABEC resin will perform poorly. New samples of resin may contain aggregates. These can be removed by sonication for 20 min. As supplied, the resin is suspended in 10% methanol to inhibit microbial growth and this requires removal by washing. After washing, the resin may have a tendency to float owing to gas adsorption, in which case it should be degassed by application of gentle vacuum to an aqueous suspension of the resin. Our conditioning procedure has been devised to ensure uniform moisture content in the resin and this is crucial to its experimental performance. It is important that the rather small amount of resin used in these experiments not be allowed to stand in the culture vials for a long time (> 2 h) to avoid further dehydration.
10. One mL of salt solution is required to contact each duplicate resin sample. 100 μL is required to determine the initial activity present. The remainder, 0.15 mL, simply enables pipeting, making 2.25 mL in total.
11. The number of vials of scintillant required is equal to one plus the number of duplicates multiplied by the number of test conditions in order to determine the concentration for initial and final sample concentrations. Thus, for one salt condition measured in duplicate, three vials of scintillant are required. Avoid the use of fluorescent markers on the tube sides. These may be used only on the caps.
12. Transfer of scintillation vials to and from the hood is via a clean beaker placed inside the hood. This ensures that materials entering and leaving the hood for analysis cannot become contaminated inside the hood**.**
13. By mixing and centrifuging twice, an attempt is made to ensure that all the adsorbent contacts the adsorbate. The adsorbate has a high density and some adsorbent may become trapped at the top of the tube, hence this procedure.
14. The dry-weight conversion factor used in the determination of the distribution coefficient may be obtained by drying to constant weight several samples (ca. 0.2 g) of the same resin as used in the adsorption studies. This may be achieved by drying at 100°C for several days in a suitable oven. Greater efficiency and accuracy may be achieved by the use of a thermogravimetric analyzer to determine

the dry-weight conversion factor. In the latter case, 10–20 mg of resin is weighed into a platinum pan and heated to 400°C at a rate of 5°C/min while purging with nitrogen. The dry-weight conversion factor may be determined from

$$dwcf = \frac{\text{wt of sample} - \text{wt of dry sample}}{\text{wt of sample}}$$

15. Changes in the concentration of eluant solutions should be made cautiously because the resin shrinks in high concentrations of salt and expands in low ionic-strength solutions. Thus, for example, in changing eluant strength from water to 4 *M* NaOH, it is best to proceed in stages by washing first with 1 *M* NaOH followed by 2 *M* NaOH, and so on. Rapid changes in concentration can cause channeling in the bed.
16. The $^{22}Na^{+}$ tracer is a γ emitter with an energy of 1274.5 keV**.** The window of the gamma counter is set to count from 433 to 1417 keV. The 2σ coincidence level is set to shut off at 70%. Count times of 2 min are typically used.

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9

Partition of Amino Acids and Peptides in Aqueous Two-Phase Systems

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1. Introduction

Partition behavior of amino acids and peptides in the aqueous two-phase (ATP) systems is of academic and practical importance for the following reasons.

1. Amino acids and peptides are more simple than the larger protein molecules, in that their partition behavior can be studied in a more systematic fashion ***(1,2)***, so that further understanding the fundamental thermodynamics of the partition of more complex proteins in these systems can be obtained.
2. Amino acids and peptides are also very important bioproducts that can be made from biological or biochemical processes, so their recovery via ATP extraction may represent realistic alternatives to more traditional recovery methods ***(3)***.
3. The partition between two liquid phases has already been shown to be a capable analytical method for determining the relative hydrophobicities of the distributed amino acid, macromolecules, or particle surface ***(4,5)***. Thus, the study of the partition behavior of amino acids and peptides in the ATP systems can enhance the understanding of surface hydrophobicity of various surfaces in relation to their composition, charges, sizes, and conformation.

In studies of ATP separation mechanisms, hydrophobic forces have attracted the most attention because the hydrophobic interaction between peptide or protein and water can provide net favorable free energy for the formation of the native structure in an aqueous solution. Charge and its relation with solution pH also plays an important role in determining the partition of peptides and proteins, although pH may have an effect on the ATP system as well ***(6)***. Studies of amino acid and DNP-amino acid partition in ATP system by Zaslavsky et al. ***(7–9)*** concluded that the hydrophobic effect is the determining factor for separation. Some amino acids like lysine and glutamic acid have relatively low

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hydrophobicity, and they partition favorably in the salt-rich phase in the PEG/salt ATP systems ***(3)***, whereas tryptophan and phenylalanine, with their aromatic side group, have relatively high hydrophobicity and partition to the PEG-rich phase predominantly. In comparison, partition behavior of peptides and proteins is much more complex. Eiteman and Gainer ***(2)*** attempted to relate the hydrophobicity with partitioning of peptide, but the amino-acid sequences of the peptides were not discussed. However, the relation between the partition coefficient in ATP with the surface hydrophobicity of protein and the hydrophobic factors of different partition systems has been reported by Tanaka et al. ***(10).*** Because the primary structure determines the properties of the peptide, the amino-acid sequence should account for the discrepancy of peptide partition in ATP system. The discrepancy might be caused by the charges, the charge distribution, the varying distribution of hydrophilic or hydrophobic segments of amino-acid residues and the conformation of peptide. One of the major limitations of correlation studies for bioactive peptides lies in the lack of a reliable physicochemical amino-acid side-chain descriptor ***(4)***. Also, the summation of individual amino-acid properties in order to account for the properties of peptide or protein has not been proved. Thus, to recognize the primary structures of peptide hydrophobicity by determining the partition coefficient of peptide in ATP is the major reason for doing experiments in peptide partition in ATP systems.

Extraction of amino acids from the fermentation broth may be a viable recovery process if the amino acid in question is difficult to concentrate. Besides, the ability of ATP to remove cell mass is an advantage when cell removal presents technical problems. The difference between ATP extraction of amino acids from simple solution as compared to fermentation broth lies in the vastly complex nature of the latter, which in turn depends on the media used for the fermentation.

Diamond et al. ***(1,11,12)*** have studied the effect of the sequence of amino acid on the partition of peptides in ATP systems. Chen et al. ***(13)*** have extended this to longer and synthetic peptides with specific amino-acid sequences and residue numbers. The study included eight sets of dipeptides and eight sets of tripeptides and chose the following 11 synthetic peptides for the study:

1. Phe-Phe-Phe-Phe-Phe-Phe-Phe-Phe-Phe,
2. Tyr-Tyr-Tyr-Tyr-Tyr-Tyr-Tyr-Tyr,
3. Lys-Lys-Lys-Lys-Lys-Lys-Lys-Lys-Lys-Lys,
4. Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg(Lys-Bradykinin),
5. Phe-Ser-Pro-Arg-Pro-Arg-Pro-Arg-Lys-Gly-Pro-Phe,
6. Tyr-Tyr,
7. Tyr-Tyr-Phe,
8. Tyr-Tyr-Gly-Phe-Ile,

9. cytochrome-c,
10. Tyr-Leu-Glu-Asn-Pro-Lys-Lys-Thr-Ile, and
11. Tyr-Lys-Met-Ile-Phe-Ala-Gly-Ile-Lys.

Also, biologically active peptides, Angiotensin II and its analogue, [Val5]-Angiotensin II, were selected for the study.

With due consideration to hydrophobicity, the polyethylene glycol (PEG)/ Na_2SO_4/ water system was chosen for this study rather than PEG/dextran/water system, because a possible consequence of the copartition of sodium sulfate itself in these phases may result in the top phase being even more hydrophobic than the bottom phase.

2. Materials

1. PEG: PEG of various molecular weights can be purchased from, e.g., Merck (Darmstadt, Mannheim, Germany).
2. Salts: Several salts of reagent grade are frequently used in preparing PEG/salt ATP systems, such as sodium sulfate, magnesium sulfate, potassium phosphate, and ammonium sulfate.
3. Amino acids: These can be obtained from Sigma (St. Louis, MO) or Merck. Store at 4°C and protect from moisture.
4. Peptides: All of the dipeptides, tripeptides, and Angiotensin II and its analog in the following discussion study are purchased from Sigma. The synthetic peptides are synthesized by a solid phase peptide synthesis method using an LKB Biolynx 4175 peptide synthesizer (UK). Store at 4°C and protect from moisture.

3. Methods

1. Amino acid or peptides tested are first dissolved in water at concentrations larger than the final concentrations (0.4 g/L) in ATP systems desired (*see* **Notes 1** and **2**). When studying whole-broth extraction by ATP, the fermentation broth replaces water and amino acids.
2. Predetermined amount of dry powder of PEG of specific molecular weight and salt are then added, so the final composition and pH (*see* **Note 3**) desired is achieved.
3. The solution is then vortexed till all solids are dissolved.
4. The solution is placed at defined temperature for 24 h to reach equilibrium. With fermentation broth, the equilibrium time is shortened to 1 h to prevent changes in the chemical composition of the broth.
5. The solution is centrifuged at 1000*g* for 10 min to ensure complete phase separation. *See* **Note 4** for special consideration in whole broth extraction.
6. Samples from both phases are withdrawn and analyzed for their composition.
7. The concentrations of amino acids, peptides, and protein are measured by reverse-phase high-pressure liquid chromatography (RP-HPLC) (using C18 Column with an refractive index detector) and UV/Vis spectrophotometer.

8. The amount of PEG in each phase is weighed, after samples are extracted by acetone and then freeze-dried.
9. The concentration of ions is detected by ion-exchange chromatography (IEC) (DIONEX Series 4500I).
10. The partition coefficient, K (defined as concentration of solute of top phase divided by the concentration of solute of bottom phase) of amino acids (*see* **Notes 5** and **6**) and various peptides (*see* **Notes 7–9**) previously mentioned can be measured as well as predicted.

4. Notes

1. Care must be taken to avoid saturation of the amino acid or peptides tested. For example, Trp has low solubility in aqueous solution, thus when it is concentrated to the top phase because of large partition coefficient, its solubility limit may be passed. Observe for any precipitation formation following the centrifugation operation.
2. Some peptides may be difficult to dissolve, so raising the water temperature may be helpful to accelerate this process. Again, any precipitation should be carefully monitored.
3. When adjusting the pH of the ATP systems, HCl and NaOH can be used, except for the potassium phosphate-containing ATP where pH is adjusted by changing the ratio between K_2HPO_4 and KH_2PO_4.
4. In whole-broth extraction experiments, the results depend heavily on the broth properties, such as solid content and ingredients in the broth. Thus, it is vital that the condition of the broth be carefully characterized in terms of cell mass content, product (i.e., amino acid) concentration, and pH. Solids in the ATP systems after centrifugation may distribute in the two phases as well as at the interfacial region, depending on the ATP composition and broth conditions. It is recommended that centrifugation force or duration be increased to ensure that solids are centrifuged to the bottom of the solution, so they won't interfere with sampling of the two phases.
5. The partition coefficients of amino acids in PEG 6000/Na_2SO_4/H_2O system are listed in **Table 1**. Amino acids in the extraction solution at pH value between 5–6 are predominantly dipolar ions. The dramatic discrepancy of K values might be contributed to by hydrophobic interaction between amino acids and the aqueous two-phase system. Notice that two amino acids, Asp and Glu, are negatively charged in the system and in the situation of higher electrical potential of the top phase of this case, the K value would be greater than 1.0 provided that electrostatic interaction is the dominating force for distribution of solutes. The results of K values of 0.04 and 0.08 for Asp and Glu, respectively, support the assumption that hydrophobic interactions between solutes and system is pronounced in partitioning in ATP system.
6. Partition coefficients of amino acids depend on the difference between the composition of the two phases, as described by the tie-line length in the phase diagram or other equivalent measures (*see* Zaslavsky et al. ***[9]***). As shown in **Figs. 1** and **2**, the partition coefficients of tryptophan and aspartate vary with the difference in PEG content of the two phases. The phase systems used are taken from

Table 1
The Partition Coefficient of Amino Acids in PEG 6000/Na_2SO_4/H_2O System

Amino Acid	M.W.	K
Trp	204.23	4.40
Tyr	181.19	1.24
Leu	131.17	1.13
Ile	131.17	1.11
Phe	165.19	1.10
Gly	75.07	0.90
Thr	119.12	0.42
His	155.16	0.36
Met	149.21	0.34
Ala	89.09	0.31
Arg	174.20	0.29
Cys	121.16	0.18
Pro	115.13	0.14
Val	117.15	0.09
Glu	147.13	0.08
Ser	105.09	0.07
Asn	132.12	0.06
Asp	133.10	0.04
Lys	146.19	0.03

PEG 6000/Na_2SO_4/amino-acid solution (0.4g/L)=10/14/76 (w/w/w).

ref. ***14*** and are shown in **Table 2**. The concentration of both amino acids in these systems are 0.4 g/L. Note that the trend of the partition coefficients are different for these two amino acids.

7. In the dipeptide experiments, dipeptide formed by hydrophobic amino acids has a stronger hydrophobicity than individual amino acids, as shown in **Table 3**. This table presents several observations. For different amino terminal residues, increasing hydrophobicity by adding hydrophobic amino acids to the carboxyl terminal results in the hydrophobicity pattern Phe>Ala>Gly, whereas individual amino acids are in the sequence of Phe>Gly>Ala. The phenomena suggests that an individual amino acid gives a similar contribution to partition in different peptides, but the loss of a carboxyl and amino group from an amino acid in peptide contributes noticeably to the hydrophobicity of the peptide. Accordingly, the simple additive method of amino-acid properties to peptide properties, such as the partition coefficient of the dipeptide, presented by Nozaki and Tanford ***(15)*** has to be corrected with the contribution of amino and carboxyl groups. In observing the hydrophobicity of carboxyl and amino groups, the results in **Table 3** are provided to illustrate that the A-B and B-A type of dipeptides have different K

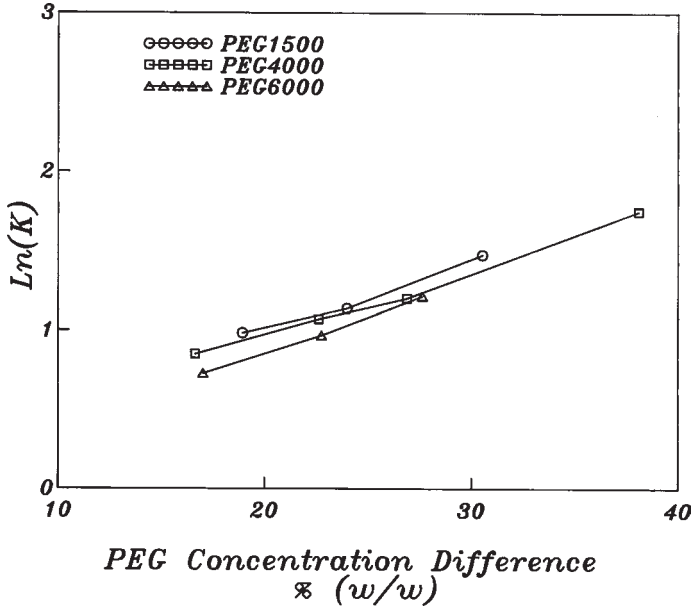

Fig. 1. The partition coefficient K of tryptophan in ATP systems listed in **Table 2** plotted against the difference of the PEG % (w/w) between the top and bottom phases, respectively.

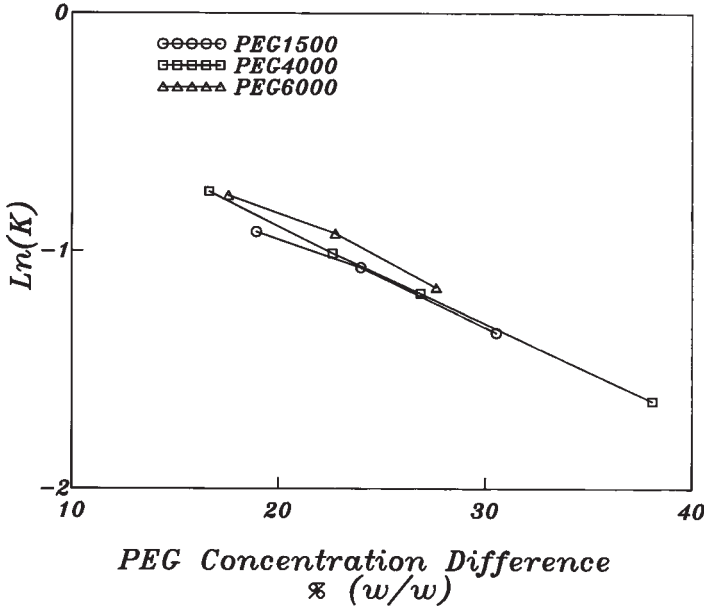

Fig. 2. The partition coefficient K of aspartate in ATP systems listed in **Table 2** plotted against the difference of the PEG % (w/w) between the top and bottom phases, respectively.

Table 2
The Phase Systems Used in Figs. 1 and 2

Top phase		Bottom phase	
PEG %(w/w)	*Phosphate %(w/w)	PEG %(w/w)	Phosphate %(w/w)
21.86[a]	7.31	2.94	18.34
25.64[a]	6.11	1.68	20.13
31.00[a]	4.62	0.48	23.17
12.65[b]	9.67	7.09	12.35
15.96[b]	8.19	4.23	14.06
19.16[b]	7.01	2.54	15.46
23.90[b]	5.56	1.30	17.41
28.15[b]	4.55	1.27	19.41
38.92[b]	2.68	0.85	26.26
15.45[c]	6.13	0.90	13.84
23.28[c]	5.63	0.55	15.53
28.00[c]	4.13	0.37	17.66

*: K_2HPO_4 : KH_2PO_4 = 306.9 : 168.6 to make the solution pH = 7.0 at 20°C.
[a, b, c]: PEG used are PEG 1500, PEG 4000 and PEG 6000, respectively.
These systems were selected, and adapted with permission, from **ref. *14***.

Table 3
The Partition Coefficient of Dipeptides in PEG 6000/Na_2SO_4/H_2O System

Dipeptide	K	E
Trp-Trp	109.00	4.691
Trp-Phe	25.50	3.239
Trp-Ala	3.81	1.338
Trp-Gly	3.12	1.138
Tyr-Phe	8.86	2.182
Tyr-Ala	1.30	0.262
Tyr-Gly	1.09	0.086
Phe-Ala	1.45	0.372
Gly-Gly	0.14	–1.959
Gly-Ala	0.11	–2.198
Ala-Gly	0.22	–1.505
Gly-His	0.17	–1.772
His-Gly	0.50	–0.701
Gly-Phe	1.13	0.124
Phe-Gly	1.32	0.278
Phe-Leu	3.74	1.319
Leu-Phe	3.01	1.101

PEG 6000/Na_2SO_4/peptide solution (0.4 g/L)=10/14/76 (w/w/w).

Table 4
Dimensionless Transfer Energy of Amino Acid, Carboxyl Terminals, and Amino Terminals

Amino acid	E	∂^c	∂^n
Gly	–1.105	2.589	–1.840
His	–1.016	0.420	–1.938
Ala	–1.168	1.072	–1.997
Phe	0.095	0.552	–2.723
Leu	0.124	1.841	–1.652
Tyr	0.213	0.862	—
Trp	1.482	1.079	—

∂^c, C-terminal dimensionless transfer energy.
∂^n, N-terminal dimensionless transfer energy.
PEG6000/Na_2SO_4/H_2O/amino acid solution (0.4g/L)=10/14/76 (w/w/w).

Table 5
Comparison of Experimental and Predicted Partition Coefficient for Dipeptides in PEG 6000/Na_2SO_4/H_2O System

Dipeptide	Experimental K value	Predicted K value
Trp-Phe	25.50	25.05
Trp-Ala	3.81	3.43
Tyr-Phe	8.86	8.75
Tyr-Ala	1.30	1.20

PEG 6000/Na_2SO_4/peptide solution (0.4g/L)=10/14/76 (w/w/w).

values. A carboxyl group is more hydrophilic than an amino group from the energy point of view as explained in the following discussion on dimensionless transfer energy. Also, the amino acid with a higher molecular-weight side chain in dipeptide contributes a higher weighting factor for distribution. As a result, in dipeptides, the same carboxyl terminal peptide would have a higher K value whenever the molecular weight of the amino terminal is larger. The above qualitative descriptions fit our experimental results and also fit Diamond and Hsu´s results of 23 sets of dipeptides ***(1)***.

8. The dimensionless transfer energy between phases of Diamond's work ***(11)*** were also adapted in the present study. The ∂^c and ∂^n (**Table 4**) values are obtained by the E value (E=lnK) of Gly, Ala, His, Phe, Leu, Tyr, Trp. The energy loss of C-terminal is positive, whereas N-terminal is negative. This echoes the aforementioned description that a carboxyl group is more hydrophilic than an amino group since the positive value of ∂ would lower the E and K value according to Diamond's equation and also a lower value of K means a lower hydrophobicity with our system design. The K value of other dipeptides are calculated here using the value of **Table 4** and accurate predictions have been obtained as shown in **Table 5**.

Table 6
The Partition Coefficiens of Designed Peptides and Biologically Active Peptides in PEG 6000/Na_2SO_4/H_2O System

	Peptide	K
I	nonaphenylalanine	1.65
II	octatyrosine	>>1
III	declysine	0.21
IV	Lys-Arg-Pro-Pro-Gly-/ Phe-Ser-Pro-Phe-Arg	3.43
V	Phe-Ser-Pro-Arg-Pro-/ Arg-Lys-Gly-Pro-Phe	10.88
VI	dityrosine	4.75
VII	Tyr-Tyr-Phe	6.03
VIII	Tyr-Tyr-Gly-Phe-Ile	9.42
IX	Cytochrome C	<<1
X	Thr-Leu-Glu-Asn-Pro-/ Lys-Lys-Thr-Ile	0.41
XI	Thr-Lys-Met-Ile-Phe-/ Ala-Gly-Ile-Lys	4.59
Angiotensin II	Asp-Arg-Val-Tyr-Ile His-Pro-Phe	4.01
[Val^5]Angiotensin II	Asp-Arg-Val-Tyr-Val-/ His-Pro-Phe	1.70

PEG 6000/Na_2SO_4/peptide solution (0.4 g/L)=10/14/76 (w/w/w).

9. The purpose of using Peptide I to Peptide III in **Table 6** is to inspect the K value of different homopolyamino acids. The results illustrate the same trend of hydrophobicity in amino acids as in polypeptides with molecular weight being roughly the same. Peptide IV and Peptide V possess the same amino-acid composition but in different sequences. With the additive property proposed by Nozaki and Tanford ***(15)***, Peptide IV and Peptide V would have the same hydrophobicity as would be expressed in their K values. The results have shown that not only the additive property of amino acid to protein is not true in this case, they also support the assumption that the terminus residue has a greater contribution toward peptide property, because the hydrophobicity of Phe is greater than that of Arg and Lys. However, for longer peptides or whenever the secondary structure of oligopeptides is concerned, the conformation effect should also be considered. Burley and Petsko ***(16)*** have shown previously that proteins may possibly be more hydrophobic, with strong hydrophobic interactions between amino acids that are embedded in the protein backbone. This means that the structure additivity rule fails when two or more hydrophobic groups are within a certain distance. Again, the relationship between the partition coefficient in ATP system with the protein surface hydrophobicity proposed by Tanaka et al. ***(10)*** proved that sur-

face amino acids play more pronounced roles in the partitioning than the amino acids inside of the protein structure. Peptide VI to VIII, in order of increasing number of hydrophobic amino-acids residues, exhibit increases in K values in **Table 6**. Peptide X and Peptide XI are hydrophilic segment (67–75) and hydrophobic segment (78–86), respectively, of cytochrome-c with about the same molecular weight. The results were as expected by considering hydrophobic effects for peptide partitioning in the ATP system. For biologically active peptide, the partition coefficient of Angiotensin II and its analog with amino-acid sequences shown in **Table 6** were chosen. The exchange of hydrophobic amino acid Ile with hydrophilic amino acid Val actually makes the analog relatively hydrophilic.

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10

Predicting Partition Coefficients of Small Solutes Based on Hydrophobicity

Mark A. Eiteman

1. Introduction

Aqueous two-phase systems form when an aqueous solution exceeds specific threshold concentrations of two water soluble, but mutually incompatible, components (at least one of which is a polymer). A biomolecule, such as a peptide or protein, added to these systems will distribute between the two phases. Ever since Albertsson recognized the potential of aqueous two-phase systems for the separation of cells and biomolecules ***(1)***, there has been a desire to predict with minimal effort how molecules will distribute between two given phases. The important parameter describing this phenomenon is the partition or distribution coefficient, denoted *K*, and it is defined as that molecule's upper phase concentration divided by its lower phase concentration. Aqueous two-phase systems have more degrees of freedom than, for example, organic solvent-water because three substances (i.e., two dissolved substances and water) form the two phases. Aqueous systems are moreover markedly altered by changes in the solution such as temperature ***(2,3)***, pH ***(4–10)***, the presence of additional salts ***(10–16)***, and the polymer's molecular weight ***(3,17,18)***. The term *component* will be reserved for those compounds which participate in the phase-forming phenomenon, whereas *solute* connotes an additional dissolved species sufficiently dilute not to influence the phase-forming components. Care must be taken in using these terms because a small quantity of a solute may alter the system itself. Many pairs of soluble polymers, such as poly(ethylene glycol) (PEG), poly(propylene glycol), dextran, ficoll, methyl cellulose, poly(vinyl alcohol), and various copolymers, when dissolved together in water, form aqueous two-phase systems. The combination of one polymer and a water-

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soluble salt such as the sodium, potassium, or ammonium salts of sulfate, phosphate, or citrate, will often also form a biphasic system.

Two types of models exist to aid the selection of an aqueous two-phase system. The goal of one type of model is to predict the phase diagram of the particular mixture, or specifically, the binodal, that boundary between the one-phase and the two-phase regions on axes of component concentrations. Such models are naturally founded on thermodynamic principles, and can become quite complex. Examples of such models include ones considering fluctuation solutions theory ***(19–21)***, polymer lattice theories ***(22)***, UNIQUAC ***(23,24)***, osmotic virial expansions ***(25–27)***, polymer scaling ***(28)***, statistical geometry ***(29)***, and others ***(30,31)***. In general, a difficulty in using these models can be a deficiency of experimental data necessary to evaluate the model parameters for numerous possible systems. Fortunately, phase diagrams are available in the literature (e.g., **ref. *2***), and therefore predicting the existence of a two-phase system is often unnecessary.

The goal of a second type of model is to predict the partition coefficient, the focus of this chapter. Those thermodynamic models used to predict the binodal can often be extended to the prediction of a solution's partition coefficient by considering the solution to have four constituents rather than three. One might prefer to estimate the partition coefficients of many substances without lengthy procedures to determine model parameters. Another approach toward the ultimate goal of selecting a phase system for a particular separation has been merely to accumulate many observations and develop practical heuristics, observations that might be used to develop neural networks ***(32)*** or fuzzy systems for selecting appropriate phase systems and conditions.

Specific quantifiable factors of the solute that influence its partition coefficient include solute hydrophobicity ***(33–35)*** and solute charge ***(9,10)***. The method described herein focuses on the role of solute hydrophobicity, particularly for low molecular weight solutes, and how this phenomenon can aid in the *a priori* selection of an aqueous two-phase system for the goal of separating solutes. The reader is cautioned that other phenomena also affect partitioning, and some of these are discussed briefly in the **Notes** section.

The simple equation used by Diamond and Hsu ***(36)***, which can also be derived from a simplification of the osmotic pressure virial expansion ***(25)***, is a good starting place:

$$RTlnK = k\Delta w_2 \tag{1}$$

This simplification is possible by recognizing that the tie-lines of binodals for the vast majority of aqueous two-phase systems are nearly parallel. This equation states that the logarithm of a solute's partition coefficient is proportional to the tie-line length or the concentration difference between the

phases of component 2 (by convention, the component which is most concentrated in the upper phase, often PEG). The goal now is to quantify the proportionality constant k in **Eq. 1**.

Among other factors, the proportionality constant k in **Eq. 1** depends on the solute's hydrophobicity (as measured by some relative scale), and the degree to which solutes' having different hydrophobicities can be distinguished in a particular phase system. This latter concern is unique to aqueous two-phase systems, wherein both phases are uniquely rather waterlike. Nevertheless, some phase systems can more readily be used to "discriminate" between solutes on the basis of hydrophobicity, and this method allows quantification of this effect.

Like in octanol-water systems, uncharged solutes of differing hydrophobicity partition differently in a given aqueous two-phase system. If present, the PEG-enriched phase is generally the more hydrophobic of the two phases. Thus, if the PEG-enriched phase is the upper phase (as generally is the case), then butanol (a comparatively hydrophobic solute) will have a greater partition coefficient than propanol. In these cases, the proportionality constant in **Eq. 1** therefore increases with increasing solute hydrophobicity. However, the partition coefficients of an uncharged solute in two different two-phase systems having identical Δw_2 in general are unequal. That is, the partition coefficient of butanol will generally be different in two systems having the same tie-line length. This observation implies that some intrinsic hydrophobicity difference exists between the two aqueous phases, and that this intrinsic nature of a phase system itself affects the value of the proportionality constant in **Eq. 1**. In other words, not only does the proportionality constant depend on the particular solute, but it depends on the phase system. This notion is supported by the extended virial equation ***(25)*** from which **Eq. 1** is a simplification: the virial coefficients for each concentration difference term are interaction parameters between the solute and the components.

For a given two-phase system, we can find an uncharged solute that partitions equally between the phases for all tie-line lengths. The hydrophobicity of this solute provides a convenient means to quantify the hydrophobicity of the phases themselves. Thus, a system in which butanol partitions equally between the phases may be thought of as intrinsically more hydrophobic than a phase system in which propanol partitions equally between the phases. In this case, **Eq. 1** can be written as ***(39)***:

$$\mathrm{RTlnK} = j(\alpha + \Delta f)\Delta w_2 \qquad (2)$$

Here Δf is a solute's hydrophobicity on some consistent scale found in the literature. The parameter j is a discrimination factor, indicating the difference in partition coefficients between two solutes partitioned in systems having different tie-line lengths. The parameter α, the phase constant, is related to the

phase system's intrinsic hydrophobicity. The partition coefficient will be one for all values of Δw_2 for an uncharged solute having a hydrophobicity equal to $-\alpha$. To obtain a different partition coefficient for such a solute, one must either select a different system with a different phase constant, change the solute's charge if possible, or rely on specific interactions (e.g., ligand partitioning).

Eq. 2 permits phase systems to be classified by the values of the two parameters, j and α, both of which can readily be determined from a series of simple experiments. Moreover, **Eq. 2** has the advantage of permitting one to estimate the partition coefficient of larger solutes (e.g., peptides) from the partition coefficients of constituent solutes (e.g., amino acids) ***(34,37)***.

The method described is not only for the estimation of partition coefficients of neutral amino acids and peptides, but also for any small solute for which a value of the hydrophobicity can be estimated. The method is incomplete for estimating the effect of other phenomena such as charge ***(8–10)***.

2. Materials

2.1. Apparatus

The method requires small volumes (10 mL usually is sufficient) of phase systems to be held at constant temperature, separated, and the partitioned solutes analyzed.

1. A constant temperature bath or incubator is required which maintains temperature, ideally within 0.1–0.2°C.
2. Pasteur pipets are used for separation of the phases.
3. Analytical instrumentation is necessary to determine the concentration of solute in each phase, and hence by division the partition coefficient. Normal alcohols may be readily analyzed by gas chromatography. Amino acids may be quantified by their reaction with ninhydrin (Sigma Chemical Co., St. Louis, MO), or by liquid chromatography. Amino acids and peptides with aromatic side chains may alternatively be quantified by ultraviolet absorbance.

2.2. Chemicals

1. Phase forming chemicals or "components": The method described is most suited to PEG and salt systems. Any molecular weight of PEG may be selected. Also, any soluble phase-forming salt (ammonium sulfate, sodium citrate, potassium phosphate, magnesium sulfate, and so forth) may be selected.
2. Chemicals for quantification of phase component(s): If phase systems are used for which literature values of the component 2 concentration difference, Δw_2, are not available, a means to quantify this component's concentration in each phase will be necessary. For phase systems containing PEG, this component may be quantified by the absorbance of a barium-iodide complex at 535 nm ***(38)***. This analysis uses a Titrasol iodine solution (E.M. Merck, Darmstadt, Mannheim, Germany).

3. Chemicals for partitioning, i.e., "solutes": A series of normal alcohols, such as ethanol, propanol, butanol, and pentanol, is suitable to determine the value of the discrimination factor *j* in **Eq. 2**. However, any series of solutes differing by methylene groups in their structure may be used (*see* **Note 1**), such as glycine, alanine, α-aminobutyric acid, norvaline, and norleucine. One solute with a known hydrophobicity will be necessary to partition to determine the phase constant α in **Eq. 2**. Ideally, this solute will distribute close to equally between the phases, and thus have a partition coefficient near one.
4. Chemicals for quantification of solutes: For example, many amino acids are most readily analyzed by their reaction with ninhydrin (Sigma).

3. Methods

3.1. Determining the Discrimination Factor

1. Form three or four concentrations of one phase system in test tubes by varying the relative amount of salt and/or PEG (*see* **Note 2**). This can be easily be accomplished by altering the quantity of one component such as PEG. Alternatively, form phase systems described in the literature. For example, one concentration of a PEG/potassium phosphate system (*see* **Fig. 12.44** in **ref. *2***) may be formed with 12.00 mL of water, 1.19 g potassium phosphate dibasic trihydrate ($K_2HPO_4 \cdot 3H_2O$), 0.499 g potassium phosphate monobasic anhydrous (KH_2PO_4) and 1.32 g PEG-6000. A second concentration on the same phase diagram may be formed with 11.84 mL water, 1.35 g dibasic phosphate, 0.567 g monobasic phosphate, and 1.24 g PEG-6000. If necessary, the pH should be brought in a consistent manner to a value for which the solutes will be neutral (*see* **Note 1**).
2. Place a few drops of one alcohol (or another series of solutes that vary in methylene groups) into each solution for the phase system, and seal the test tubes to prevent evaporation of solute or water. Just enough solute should be added for quantification. Repeat this process for each alcohol.
3. Equilibrate systems together in a constant temperature bath (*see* **Notes 3** and **4**).
4. Separate from the test tube first the upper and then lower phase for each system with a Pasteur pipet and analyze each phase for alcohol concentration. Calculate the partition coefficients (*see* **Note 5**). Examples of the data collected are shown in **Figs. 1** and **2**. Figure 1 shows the results for three solutions of a PEG/potassium phosphate aqueous two-phase system, while **Fig. 2** shows the results for three solutions of a PEG/dextran aqueous two-phase system.
5. Regress the alcohol partition coefficient data to the following equation:

$$\ln K = \frac{j}{RT} \Delta w_2 (N \Delta f_{CH_2} + \alpha + \Delta f_{MeOH}) \quad (3)$$

The independent variables in **Eq. 3** are Δw_2 and N, the number of methylene groups the alcohol has beyond methanol. A value of 500 cal/mol may be used for Δf_{CH_2} ***(39)***. Nonlinear regression will result in best-fit values for j and for the term $\alpha + \Delta f_{MeOH}$. From the example data shown in **Fig. 1**, the discrimination factor

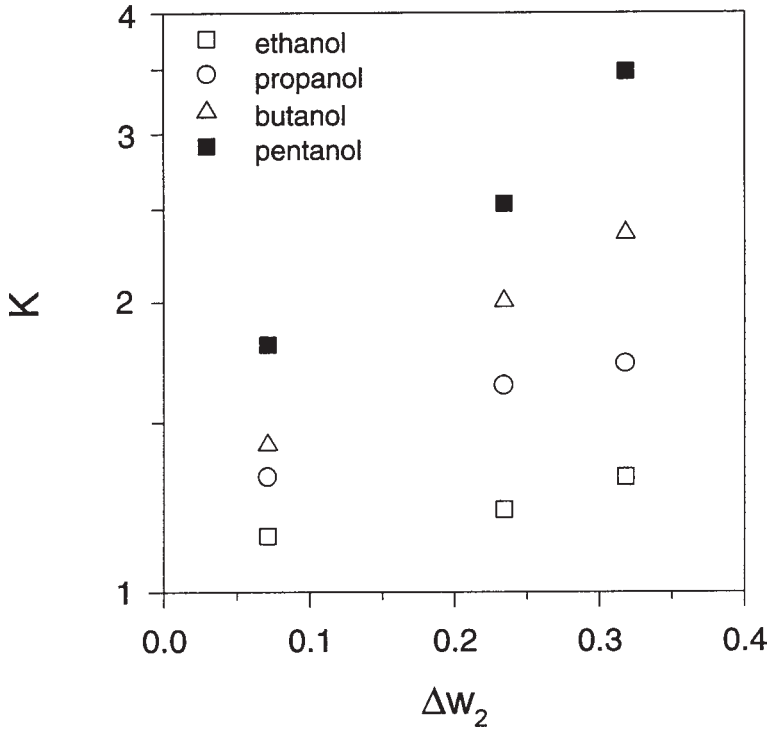

Fig. 1. Partition coefficients of normal alcohol in three PEG-6000/potassium phosphate aqueous two-phase systems at 20°C (*see* **Fig. 12.44** in **ref. *2***).

was determined to be 1.22. From the example data shown in **Fig. 2**, the discrimination factor is about zero since essentially all partition coefficients are one. **Fig. 2** demonstrates that the method cannot be used for aqueous two-phase systems in which partitioning is governed by phenomena other than hydrophobicity. In other words, the results indicate that this PEG/dextran phase system would not be well suited to discriminate between solutes based on hydrophobicity.

3.2. Determining the Phase Constant

1. The same three- or four-phase system solutions must again be prepared, and into each of them, an additional solute partitioned that will allow determination of the phase constant. An ideal solute is one which distributes equally between the phases, can be very accurately quantified, and a value of the hydrophobicity is available (*see* **Note 5**). For many two-phase systems, phenylalanine is a good choice for a solute to partition, and it has a hydrophobicity, Δf, equal to 2500 cal/mol ***(39)***. **Figure 3** shows the partition coefficients of phenylalanine in the PEG/potassium phosphate aqueous two-phase system.
2. The phase constant may be determined by regressing the phenylalanine partition to **Eq. 2**. For the three data points in **Fig. 3**, the best fitting curve is $\ln K = 0.347\Delta w_2$. Thus, 202 cal/mol = $j(\alpha+\Delta f)$, and the phase constant α is –2330 cal/mol.

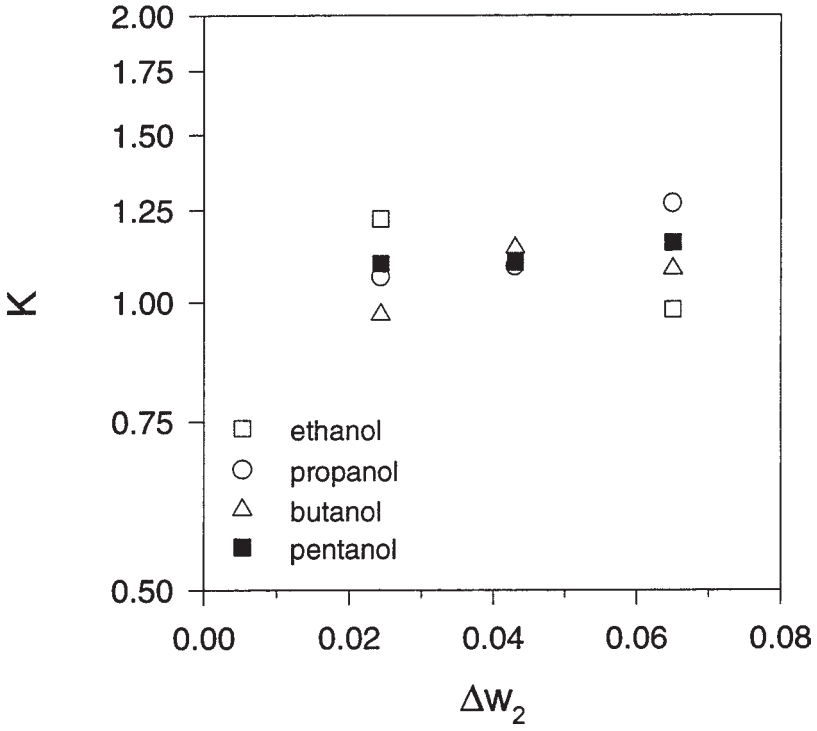

Fig. 2. Partition coefficients of normal alcohol in three PEG-6000/dextran 48 aqueous two-phase systems at 20°C (*see* **Fig. 12.6** in **ref. *2***).

3.3. Prediction of Partition Coefficients

1. For other solutes, a value for the hydrophobicity is required on the same scale as the one used to determine the phase constant. For example, tryptophan has a hydrophobicity of 3400 cal/mol, methionine 1300 cal/mol and alanine 500 cal/mol ***(39)***. Using these values in **Eq. 2** yields equations which predict the partition coefficients for these three solutes in the example PEG/potassium phosphate system:

   For tryptophan: $\ln K = \frac{j}{RT}(\alpha + 3400)\,\Delta w_2$ or $\ln K = 2.24\,\Delta_{w2}$

   For methionine: $\ln K = -2.19\,\Delta_{w2}$

   For alanine: $\ln K = -3.84\,\Delta_{w2}$

   Figure 3 shows the predicted partition coefficients for each of these three amino acids in addition to the observed values in the example PEG/potassium phosphate system.
2. Partition coefficients may be estimated for larger solutes, such as peptides, by two methods. One way to estimate the partition coefficients of peptides is by summing together the literature values of the hydrophobicities of the individual

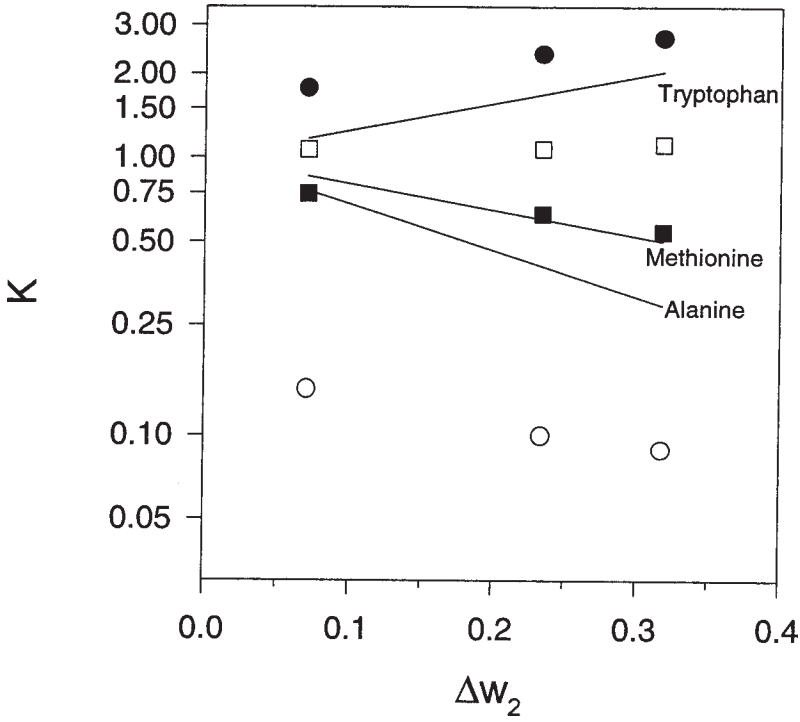

Fig. 3. Observed partition coefficients of tryptophan (●), phenylalanine (□), methionine (■), and alanine (○) in three PEG-6000/potassium phosphate aqueous two-phase systems at 20°C (*see* Fig. 12.44 in **ref. *2***). Lines indicate the model predictions for tryptophan, methionine and alanine (*see* **Subheading 3.3., step 1**).

amino acids (*see* **Note 6**). With this method, the hydrophobicity of the dipeptide tryptophan–tryptophan is calculated to be 3400 + 3400 or 6800 cal/mol, while the hydrophobicity of tryptophan–phenylalanine is calculated to be 3400 + 2500 = 5900 cal/mol, and the hydrophobicity of tryptophan–glycine is calculated to be 3400 + 0 = 3400 cal/mol. These values may each then be used in **Eq. 2** to predict the partition coefficients. Alternatively, the hydrophobicities of peptides may be estimated by summing together the observed values for amino acid hydrophobicities measured by partitioning in the phase system itself. For example, the observed hydrophobicity of tryptophan from **Fig. 3** was calculated to be 3900 cal/mol (based on fitting the actual partitioning data to **Eq. 2**), phenylalanine was the reference solute and was taken to have a hydrophobicity of 2500 cal/mol, whereas glycine can be assumed to have a hydrophobicity of 0 cal/mol. Using this approach, the hydrophobicity of the dipeptide tryptophan–tryptophan is estimated to be 3900 + 3900 = 7800 cal/mol, tryptophan–phenylalanine to be 3900 + 2500 = 6400 cal/mol, and tryptophan–glycine to be 3900 cal/mol. These hydrophobicities may now be used in **Eq. 2** for the prediction of partition coefficients.

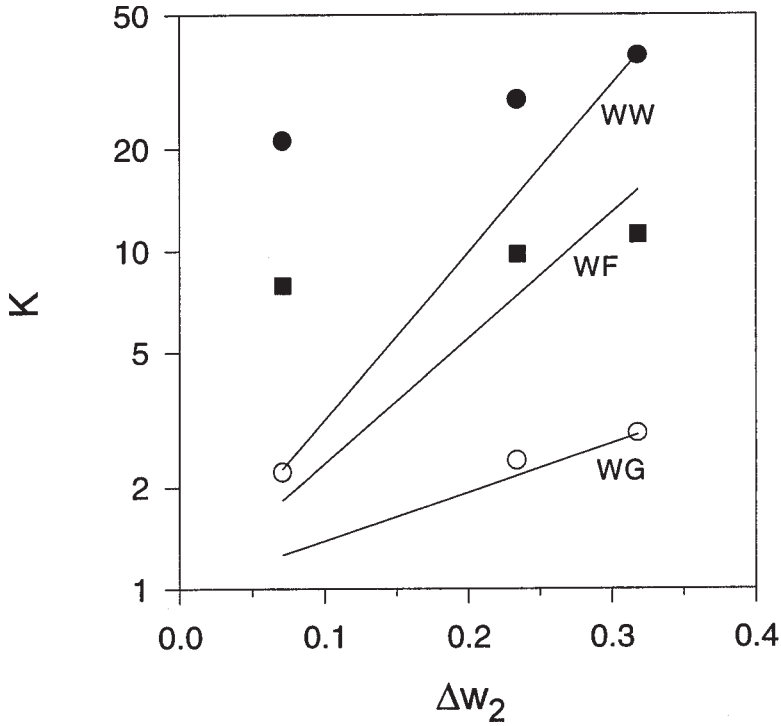

Fig. 4. Observed partition coefficients of tryptophan-tryptophan (WW, ●), tryptophan-phenylalanine (WF, ■), and tryptophan-glycine (WG, ○) in three PEG-6000/potassium phosphate aqueous two-phase systems at 20°C (*see* Fig. 12.44 in **ref. *2***). Lines indicate the model predictions (*see* **Subheading 3.3., step 2**).

This second method of calculating peptide hydrophobicities usually gives the better prediction of partition coefficients. **Figure 4** shows the partition coefficients of these three peptides and the model prediction using this second method.

4. Notes

1. The method requires the solutes to be neutral. Therefore, if amino acids and peptides are used, the pH of the phase systems must be near the isoelectric point. The method is therefore not directly suitable for charged amino acids such as lysine without additional procedures ***(40)***.
2. The value of the concentration difference at the lower component concentration is subject to the greatest error. Being near the binodal (i.e., phase boundary), these phase systems may be slightly shifted by temperature, polymer molecular weight distribution, and so forth. Moreover, the model is less valid at the lowest concentration differences. The results in **Figs. 1**, **3**, and **4** show these effects.
3. It is important to reach chemical equilibrium in the phase systems. This is usually accomplished by periodically agitating (i.e., dispersing) the phase systems over 2

or 3 d, and then allowing the phases to settle for another 2 or 3 d. Some unpublished reports indicate that such a duration is necessary to ensure equilibration. Methods which hasten the phase separation process (e.g., centrifugation) do not hasten solute equilibration.

4. It is important to reach thermal equilibrium. The phases are most effectively stored between agitation and during settling time in a constant temperature bath.
5. The method relies heavily on the accuracy of experimental data. The phase systems must be consistently prepared, and improved consistency is obtained by drying some components (particularly PEG) in order to minimize water absorbed. **Equation 2** requires a solute partition coefficient calculation, which involves the (mathematical) division of raw concentration data, a practice that increases experimental error. **Equation 2** also requires the measurement of the concentration difference between the phases (Δw_2) by some analytical method. Often, literature hydrophobicities for other solutes are based on others' experimental results.
6. Some error is introduced by merely summing hydrophobicities of amino acids to obtain hydrophobicities of peptides, because such a summation does not take into account the water of hydrolysis. Also, amino acids on peptides of larger size would be more likely to interact, and hence alter the solute's true hydrophobicity in an aqueous solution.

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11

Eukaryotic Cell Partition

Experimental Considerations

James M. Van Alstine

1. Introduction

Literature related to the partitioning of biological materials in aqueous polymer two-phase systems encompasses over two thousand articles and almost half a century of experience ***(1)***. Much of this effort has involved eukaryotic cells or other biological particles. Over the past 15 years the methods and results related to these articles have been summarized in a number of books dedicated to partitioning ***(2–6)***, as well as in various book chapters, such as **ref. *7***. In addition, papers from the last three Biannual International Partition Meetings have been published in the *Journal of Chromatography* ***(8–10)*** and a Partitioning "website" exists on the world wide web ***(11)***. As such, it is easy to find a plethora of methods and results on the partitioning of various cells ***(12)***. What is perhaps harder to come by is appreciation for various experimental factors that influence cell partition. Such appreciation is necessary for researchers who wish to read critically the partition literature, and develop their own partition experiments. A challenge made more difficult because, for a variety of historical and practical reasons, many of the methods used in various aspects of partitioning have not been standardized. This includes methods for phase-system preparation, cell preparation, and the interpretation of results; methods that often involve only minor modification of standard cell biology procedures, or can be found elsewhere in this book. It therefore seems wise that this chapter should not concentrate on one partition method used with one cell type, but rather provide a general introduction followed by a brief outline of protocols, so as to enable the reader to evaluate the literature in relation to their own cell-

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Table 1
Composition of Some Buffer Systems Used in Phase Systems[a]

System	A	B	C	D	E	F	G	1	2	3	4	5
Salt (m*M*)												
Na_2HPO_4	109	91	73	55	34	18	7.3	55	45	30	15	5
NaH_2PO_4	35	29	23	17	11	6	2.3	55	45	30	15	5
NaCl	0	25	50	75	100	125	150	0	30	75	120	150

[a]Systems 1–5 buffered to pH 6.8 have often been used by Walter et al., e.g., ***19***,***21***,***36***, whereas systems A to G buffered to pH 7.2 have often been used by Van Alstine et al., sometimes with the abbreviations I, II, and V corresponding to buffers A, C, and G (e.g., ***13***,***14***,***20***).

separation challenge. In addition to **ref. *3***, which provides a thorough introduction to "Partitioning," novitiates are encouraged to read other chapters in this book particularly Chapters 1–6, as well as those dealing with the fractionation of other particles.

As detailed in the aforementioned references, partition methods can be generally categorized on the basis of various criteria. One is the use of two-phase systems containing two neutral, hydrophilic polymers (such as poly[ethylene glycol] or PEG, and the polyglucose dextran®) vs those containing a single polymer, such as PEG plus a high concentration of a salt like phosphate or sulfate. The latter are less expensive to produce and typically possess higher interfacial tensions than two polymer systems (**see** Chapter 4). As a result they are often used in larger scale biotechnical applications where the survivability of shear sensitive bioparticles is not of primary concern (*see* Chapter 23). This chapter only concerns itself with two polymer phase systems, however the basic information provided will be generally applicable to both types of systems. Other system criteria include the use of aqueous polymer two-phase systems containing added organic solvents (*see* Chapters 21 and 38) or polymer pairs other than PEG and dextran (*see* Chapters 26 and 27). Many such pairs are known ***(3)*** and some show real promise as regards bioparticle partition. Yet, to date, most research involving eukaryotic cells has utilized the aqueous PEG and dextran two-phase systems this chapter is limited to. Partitioning is often categorized in relation to the salts used in phase system compounding, and their effect on phase system physicochemical properties (*see* **Table 1** as well as Chaps. 3, 4, and 18). Another major category is phase systems which contain polymer-coupled affinity ligands (***2***,***3***,***5***,***13***; *see* Chapters 17, 29, 30, and 31).

Partitioning experiments may also be sorted as to the use of single-step (*see* Chapter 5) vs multiple-step extraction procedures. The latter, which are difficult to scale, are typically carried out using countercurrent distribution (CCD)

(***3***,***14***; *see also* Chapter 6) countercurrent chromatography (CCC) ***(15,16)*** or liquid–liquid partition chromatography (LLPC) (*see* Chapter 7; *see also* **refs. *17*,*18***). CCD has traditionally been more useful for eukaryotic cells than CCC ***(14)*** or LLPC ***(18)*** although these and other multiple-step methods are not without promise. Countercurrent distribution in the "thin-layer" device invented by Albertsson ***(3)*** has yielded a number of amazing results, such as the separation of different cell types ***(2,3,5)*** or distribution of cancer cell samples into viable subpopulations of different pathogenic capability ***(19,20)***. The ability of CCD to achieve such results can often be evaluated by single-step partitions ***(14,20–22)***. Methodologies related to eukaryotic cell partition via CCD have recently been detailed by Walter and Larsson ***(22)***. This chapter will primarily concern itself with single-step partition and only briefly touch on CCD.

1.1. Why and When Partitioning Is Used For Cell Separation

The ideal method for cell separation should involve biocompatible reagents that are inexpensive and recyclable, require little energy, generate little waste material, easily adapt to various lab facilities, exhibit sensitive and often unique discriminatory capability, are capable of fractionating cells on the basis of a variety of operator-influenced criteria (including use of affinity and immunoaffinity interactions), have diagnostic potential, and are gentle enough to isolate fragile cell types without altering their surface structure. It should work over a wide range of temperature, be easy to scale (cm^3–m^3 vessels) without loss of either resolution or cost-efficiency, and handle concentrated (1–10% w/w) product solutions. It should be understood theoretically, be amenable to predictive modeling, and not overly burdened with patents. These attributes describe Partitioning and why it is gaining interest as a separation technique ***(1)***.

Partitioning offers a number of distinct advantages for cell separation. It is relatively inexpensive, both in terms of reagents and apparatus, which makes it easy to initiate studies to evaluate the method's potential to solve a particular separation challenge. Members of a functional subpopulation of cells often possess similar surface properties but differ in size and shape. Separation techniques sensitive to cell-surface properties are required to isolate such subpopulations. Phase partitioning is one of the few techniques that separates cells on the basis of surface properties, and is easily scaled.

Partitioning allows cells to be fractionated on the basis of known surface features, such as surface-displayed molecules (which are targets for phase polymer-coupled affinity ligands or immunoaffinity reagents). Separations may also be attained on the basis of unknown surface differences, which often relate to physiologically significant properties. In such cases knowledge of the physicochemical properties of the phase system used may provide insight to the underlying cell-surface differences ***(2–7,19,20)***. This is because cell partition is

sensitive to surface properties which determine particle interaction with the phase solutions and their liquid–liquid interface. Interactions which can be controlled by varying temperature ***(2,3)*** or system composition ***(1,2)***. A noteworthy advantage of partitioning is that it is a relatively gentle procedure carried out in polymer-containing phases buffered and rendered isotonic via the additional salts such as in **Table 1**. Such systems actually protect cells better than the corresponding buffers without added polymers. Cells can typically be exposed to phase systems and repeated partitioning without any loss of native surface features or normal physiological function ***(2–5)***. Another noteworthy advantage of partitioning is that it can fractionate cells in a manner exponentially related to their surface differences ***(3,5)***. By comparison electrophoresis and many other surface-sensitive methods separate cells in a manner related linearly to their surface differences. Studies suggest that affinity partitioning with PEG-modified antibodies could allow for the immunological screening and selection among 1 billion cells (preexposed to 1 mg of polymer modified antibody in a 1 mL incubation) in 30 min using only 10 mL of phase system in a single test tube containing a few cents worth of phase system ***(7,23–25)***.

1.2. Overview of Cell Partitioning

What follows is somewhat simplistic and the reader wishing a more accurate theoretical description is referred to **refs. *26–28***. Note that the author's experience suggests that the partition behavior of many cell and particle types (e.g., gram-negative bacteria, liposomes, polystyrene latex particles, platelets, and white blood cells, plus various tissue-culture cells) is often similar to erythrocytes in regard to partition effects related to alteration in cell-surface properties; via exposure to enzymes, fixatives, surfactants, affinity ligands, and so forth; or system composition and physicochemical properties.

When pairs of polymers such as 500 kDa dextran and 6–8 kDa PEG are dissolved in aqueous biological buffers (*see* **Table 1**) at approx 5% (w/w), two immiscible liquid phases often form. Such systems possess low liquid–liquid interfacial tensions (*see* **Table 2**) and readily emulsify upon gentle agitation (most of the mechanical energy of mixing going into interface formation). The phase in which the droplets are suspended is determined by several factors, including relative phase volume ratios, and (particularly at equal phase volumes) phase viscosity. These may vary, with time, from position to position in a partition chamber. The systems discussed here, and described in **Table 2**, are abbreviated in the fashion "(X,Y) Z" where the first number represents the total system (not phase) concentration of dextran as % w/w; the second number refers to PEG concentration and the third number of letter corresponds to the buffer (*see* **Table 1**). Systems composed with Dextran T40 instead of T500 may be indicated by an asterisk (*see* **Table 2** refs.). In these systems, the PEG-

Table 2
Physicochemical Properties of Phase Systems Used in Cell Separation

Number	Abbreviation	Buffer type	Dextran mol wt (kDa)	PEG mol wt (kDa)	Phase viscosity (cP)		Interfacial potential (mV)	Interfacial tension (μ*N*/m)	Phase Density (g/cc)		Tie-line length (% w/w)
					Dextran	PEG			Dextran	PEG	
1	(7*,5)	I = A	40	8	14.6	5.7	—	14.3	1.0642	1.0289	—
2	(5,3.5)	I	500	8	24.3	3.9	1.93 ± 0.21	—	1.0517	1.0239	9.41
3	(5,4)	I	500	8	32.4	4.0	2.10 ± 0.13	—	1.0529	1.0195	10.77
4	(5,4)	B	500	8	—	—	1.48 ± 0.07	—	—	—	—
5	(5,4)	II = C	500	8	—	—	1.35 ± 0.08	—	—	—	—
6	(5,4)	D	500	8	—	—	1.14 ± 0.05	—	—	—	—
7	(5,4)	E	500	8	—	—	0.78 ± 0.03	—	—	—	—
8	(5,4)	F	500	8	—	—	0.50 ± 0.03	—	—	—	—
9	(5,3.5)	V = G	500	8	—	—	—	0.70 ± 0.28	—	—	—
10	(5,4)	V	500	8	—	—	0.08 ± 0.14	4.99 ± 0.06	—	—	10.26
11	(5,4)	V	500	8	—	—	—	10.87 ± 0.07	—	—	12.40
12	(6.5,4)	V	500	8	—	—	—	14.36 ± 0.06	—	—	13.26
13	(7,4)	V	500	8	—	—	0.20 ± 0.04	17.22 ± 0.12	—	—	14.14
14	(8,4)	I	500	8	69.1	4.4	—	34.7	1.0697	1.0239	15.68
15	(8,4)	V	500	8	—	—	—	26.55 ± 0.21	—	—	15.68
16	(5,4)	1	500	8	—	—	1.57 ± 0.16	—	—	—	—
17	(5,4)	2	500	8	—	—	1.44 ± 0.10	—	—	—	—
18	(5,4)	3	500	8	—	—	0.65 ± 0.06	—	—	—	—
19	(5,4)	4	500	8	—	—	0.26 ± 0.05	—	—	—	—
20	(5,4)	5	500	8	—	—	0.05 ± 0.05	—	—	—	—

Measurements (***31***; Chapter 4) at 22°C typically represent mean of 10 determinations ***(28–32)***. Missing entries not determined. For typical phase-system diagrams, *see* **refs. *3*** and ***30***. Above systems contained Dextran T40 lot 18974, or T 500 lot 7830 (Pharmacia Fine Chemicals) and PEG 8000 Sentry Grade lot B529-9104 or M68548 (Union Carbide) and are expected to vary somewhat with lot. PEG mol wt stated as 8 kDa by manufacturer but estimated from HPLC against PEG standards as 6.65 kDa. Electrostatic potentials measured with open tipped capillaries, top phase positive relative to bottom phase. Systems enriched in NaCl typically exhibit a chloride ion concentration phase ratio of 0.99 ± 0.02 ***(31,32)***.

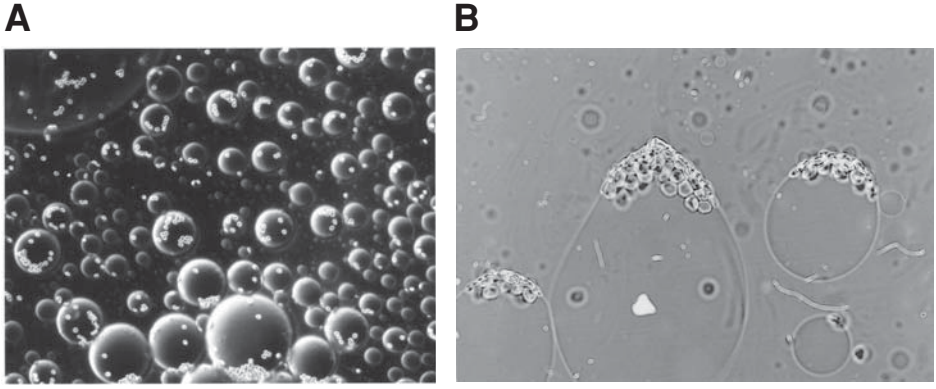

Fig. 1. Microscopy of fresh erythrocytes and dextran-rich phase droplets in (5,4) V phase system during the **(A)** initial and **(B)** later stages of demixing (*see text)*.

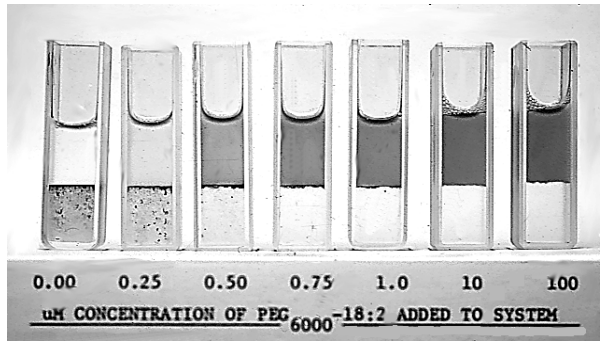

Fig. 2. Partition of fresh erythrocytes in 10 mL (5,4) V phase systems (*see* **Table 2**, system 10) containing various micromolar amounts of PEG 6000—18:2 *linoleate* ester approx 20 min after mixing.

rich phase is typically the suspending phase. Phase droplets in the emulsion rapidly coalesce (**Fig. 1A**) and grow to a size where these denser-phase droplets quickly sediment (**Fig. 1B**). As a result the phases demix more rapidly, and with more complicated hydrodynamics, than by coalescence alone ***(28,33)*** yielding an aqueous polymer two-phase system of a PEG-enriched phase floating on top of a denser dextran-enriched phase (**Fig. 2**).

Figure 3 indicates a typical single-tube, cell-partition experiment flow chart. It consists of preparing a phase system, allowing the phases to equilibrate at the temperature they will be used at (typically in a cylindrical separatory funnel) isolating the phases, mixing cells in the PEG-rich upper phase (often with polymer-derivatized affinity ligands) to make a "load mix," letting the cells and

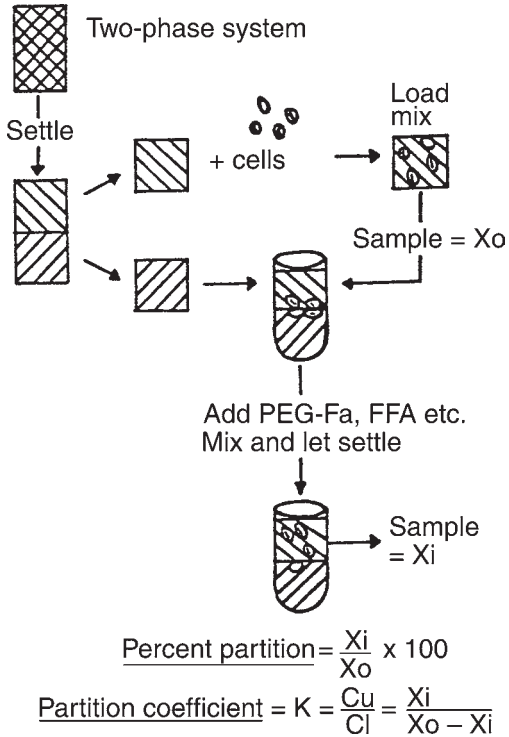

Fig. 3. Typical single-tube cell partition involving isolation of equilibrated phases, adding cells and other material (e.g., PEG-linked fatty acyl [PEG-FA] hydrophobic affinity ligands, or free fatty acids [FFA]) to PEG-rich top phase, remixing the phases in 1:1 vol ratio, and sampling at a specific time following phase demixing.

phases equilibrate for an hour, recombining equal volumes of upper-phase load mix and dextran-rich lower phase, mixing the phases, letting the phases demix, and determining the ratio of cells repartitioning back into the upper phase. The partition ratio is then expressed as either % partition or as a partition coefficient K (**Fig. 3**). The data may then be plotted as partition vs the variable studied, such as system alteration, cell type or source, cell treatment, partition time, or concentration of included affinity ligand (**Figs. 4–7**).

The partition of substances between the phases should ideally be based solely on their chemical interactions with the phase-system components, especially the polymers ***(3,26,27)***, and thermodynamically follow the Boltzmann equation:

$$K = \exp\,[-\Delta E/kT] \qquad (1)$$

where ΔE is the energy necessary for solute to transfer from one liquid-phase compartment to the other, k is the Boltzmann constant, and T is the absolute

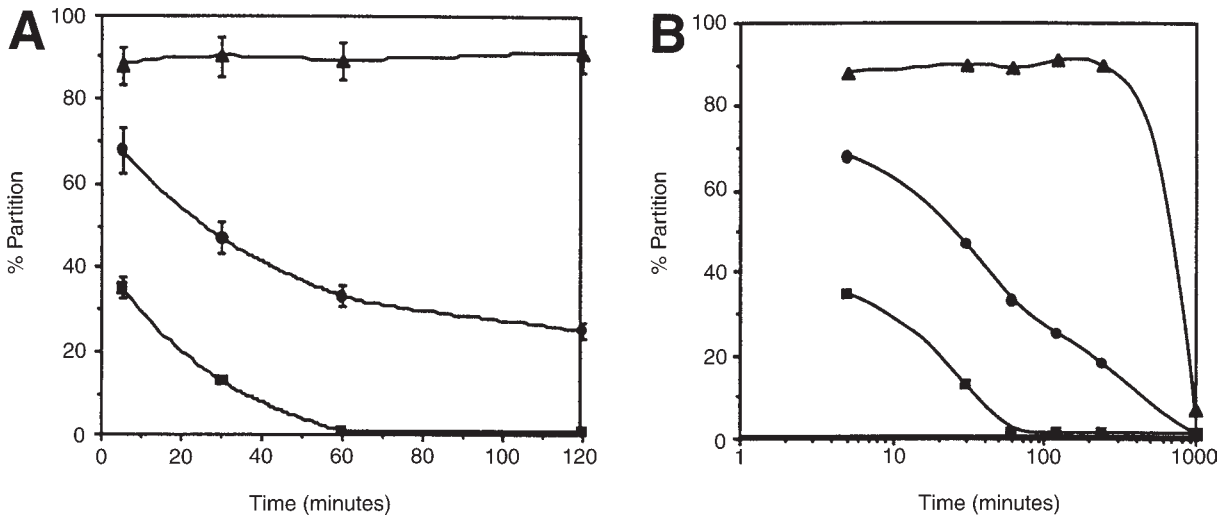

Fig. 4. Influence of settling time on the partition of 1% glutaraldehyde-PBS fixed human erythrocytes in (5,4) V system containing 0.25, 0.5 or 2 μ*M* PEG—18:2 *linoleate* fatty acid (**Fig. 2**) to generate low (■) medium (●) and high (▲) partition coefficients ***(29)***. Data shown with regard to both **(A)** time and **(B)** log time.

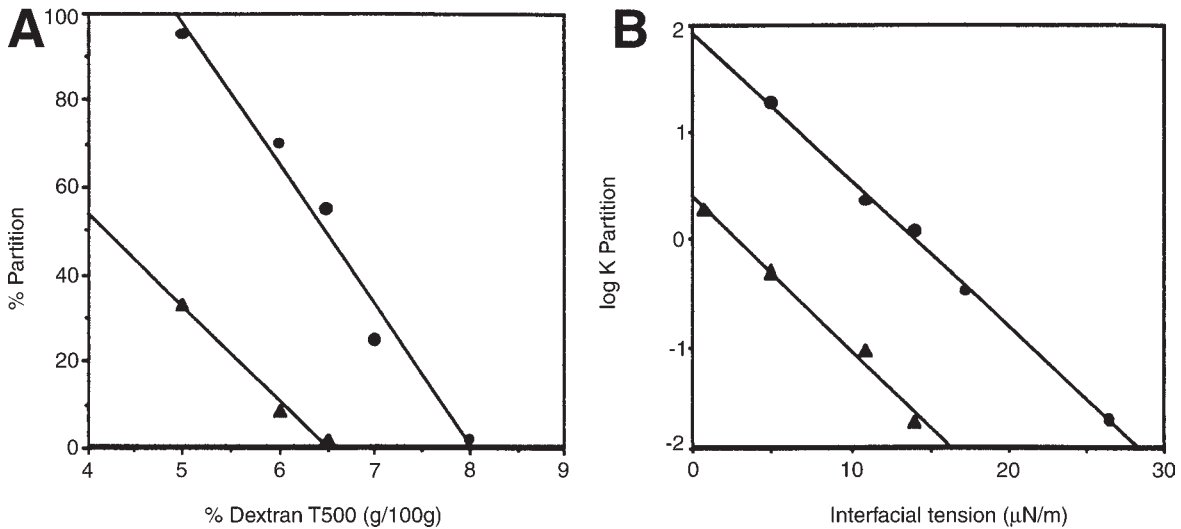

Fig. 5. Influence of **(A)** phase system polymer composition and **(B)** corresponding increase in interfacial tension on partition of rat erythrocytes (▲) and palmitate enriched *Acholeplasma laidlawii* cells (●) in (X,4) V systems of variable dextran T500 concentration (i.e., 9–13 plus 15, *see* **Table 2**). **(B)** also includes one point for rat erythrocytes in a 0.7 μN/m, (5,3.5) V system (*see* **Table 2**). The lines have r^2 correlation coefficients of 0.980–0.997 ***(29)***.

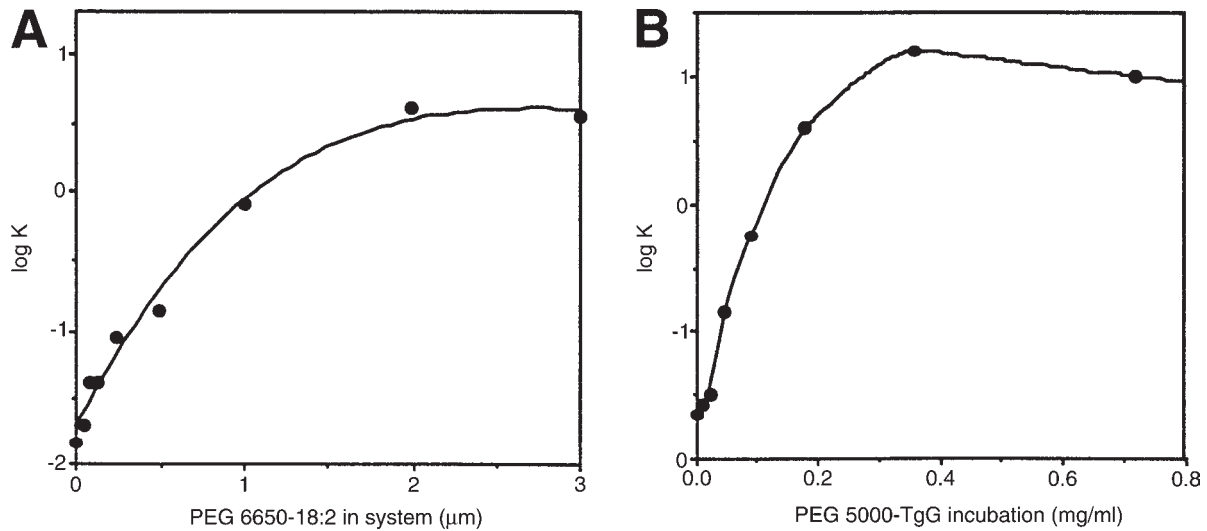

Fig. 6. Affinity partition of fresh human erythrocytes in **(A)** (5,4) V system containing PEG 6650-18:2 *linoleate* hydrophobic affinity ligand ***(13,29)***, or **(B)** (5,4) V system following cell incubation with PEG 5000-modified IgG (rabbit anti-human erythrocyte) (***23***; *see also* ***7,24,25***).

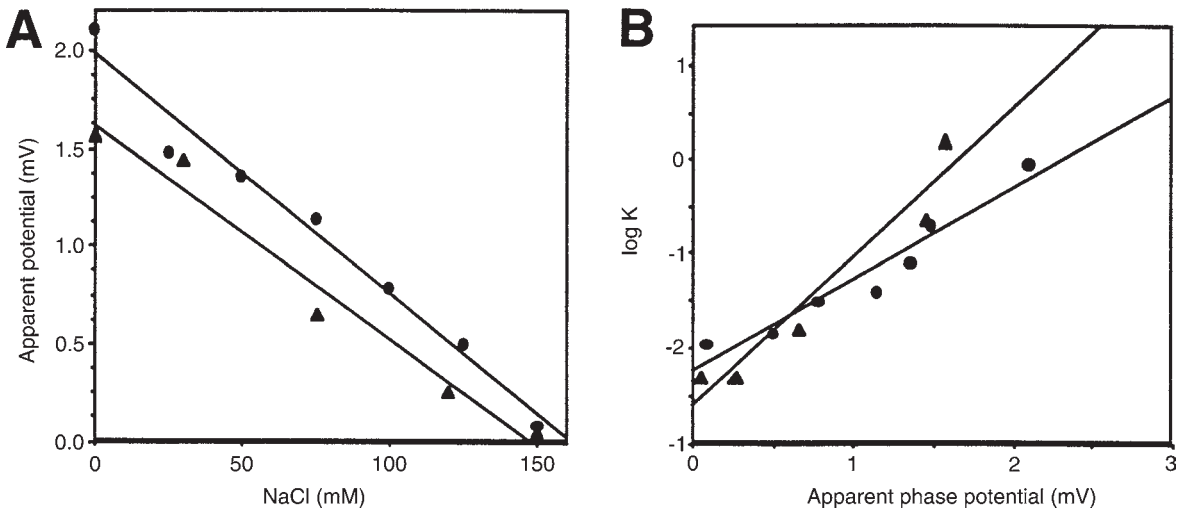

Fig. 7. Influence of **(A)** NaCl vs NaPhosphate concentration (*see* **Table 1**) and **(B)** corresponding alteration in apparent phase system interface potential (*see* **Table 2**), on log K partition of fresh human erythrocytes in (5,4) systems of pH 7.2 (●) or pH 6.8 (▲) (*see* **Table 2**). Lines relate to two r^2 correlations of 0.980 in **(A)** and 0.940 in **(B)** ***(29)***.

temperature. From this it follows that log *K* should vary directly with the system and solute properties determining the partition. For many solute types, including most biological macromolecules, log *K* has been shown to vary linearly with solute physical properties such as charge, or system properties related to polymer or salt composition ***(2–6)***. Under normal circumstances, such as absence of molecular aggregation, equilibrium partition coefficients should be independent of time and other factors such as partition vessel geometry. However, cells and similar particles are large enough to sediment, even in the relatively viscous phase systems. Their measured partition coefficient is a function, to some extent, of the time between phase mixing and sampling. This is illustrated in **Fig. 4** for erythrocytes fixed in 1% glutaraldehyde, 150 m*M* NaCl, 10 m*M* NaPhosphate, pH 7.2 (*see* **Table 1**) so that their surface properties do not alter with time. In most systems, phase demixing (to a defined planar interface) generally occurs at a more rapid rate than cell sedimentation. However, it is important to carefully reproduce sampling times in cell partition experiments.

Owing to sedimentation, which is exacerbated by cell aggregation, and complex phase-demixing hydrodynamics (influenced by factors such as phase system total volume, phase-density differences, phase volume ratios, and phase viscosities), cell-partition coefficients may vary from one experimental set up to another. However, results are very reproducible when performed under identical conditions, and general partition trends, such as one cell type having a greater partition than another in a certain system, are also typically reproducible.

The data in **Fig. 4**, like that for fresh human erythrocytes shown in **Figs. 5–7**, is for cells obtained from normal healthy volunteers, made up with upper phase to a load mix concentration of 1×10^7 cells/mL. Equal 2.5 mL volumes of load mix and lower phase were then combined in 13 × 100 mm glass or plastic tubes at 22°C, capped, mixed 20 times by inversion, and given 30 min (45 for [5,3.5] phase systems) to demix to a planar interface. The "load mix" and middle section of the upper phase were then counted so as to determine *Xo* and *Xi*, respectively (*see* **Fig. 3**). More detail is provided below. Data related to **Fig. 2** is shown in **Fig. 7A** as "log K vs affinity ligand concentration." When comparing literature results and **Figs. 4–7**, it should be noted that plots of % partition vs the corresponding log *K* yields a reasonably straight line between approximately 10% and 90% (log *K* –1 to 1).

In addition to sedimentation, cell partition is influenced by a number of other "nonthermodynamic factors." Cells are generally large enough to exhibit negligible diffusion and possess significant surface area. As a result, they show a tendency to partition to, and remain at, the phase interface which has significant, albeit relatively small, interfacial tension (*see* **Table 2**). In many systems, including most of the Dextran T500-containing systems in **Table 2**, cells tend to partition between the upper phase and the phase interface. Only in the latter

stages of phase demixing do large aggregates form at the interface and hang down into the lower phase (**Fig. 2**). Cells distribute to the interface when the system is mixed and in the emulsion stage (**Fig. 1A**) and cell-partition coefficients vary directly with both the fraction of cells attached to emulsion interfaces and the energy of this interaction as it relates to cell-phase droplet contact angles ***(27)***. Cells may remain attached to the interface as droplets coalesce to become larger droplets (**Fig. 1B**). This has several practical implications.

Interfacially localized cells may secondarily affect droplet-fusion rates and significantly influence phase droplet-sedimentation rates. The cell–droplet complexes (**Fig. 1B**) can be more hydrodynamic and denser than either cells or droplets so they tend to sediment faster. Compare the sedimentation-related decrease in partition associated with the cell types of different partition character (relative phase vs interface affinity) in **Fig. 4**. To some extent such effects vary with cell size, geometry, density, concentration, and tendency to localize at the interface, or be sheared off the interface during rapid phase demixing. The significance of such effects will also be a factor of phase demixing kinetics (as influenced by the factors noted above) and system interfacial tension ***(3,28,33)***. As a result, the reproducibility of results often depends on the reproducibility of experimental conditions and protocols, including phase-system volumes, volume ratios, and phase (vessel) geometries. In practice these factors often tend to balance each other. For example, although some cell-concentration effects are noticeable, erythrocyte partitions tend to be reasonably similar between 10^5 and 10^7 cells/mL of system. Results obtained at lower cell concentrations may differ with those obtained at higher concentrations (e.g., $>1 \times 10^8$/mL, or >1% cell mass to system volume) but trends in the data generally will be reproducible.

Scientists are still investigating the aforementioned phenomena whose understanding is crucial to chemical-engineering aspects of phase partitioning ***(5,10)***. Experiments carried out in the absence of appreciable gravity have shed considerable light on the role of cell and droplet sedimentation effects ***(28,33)***. The "take home message" is that, in addition to cell-specific phenomena, cell partition appears to be determined by nonthermodymanic and thermodynamic factors, which can be manipulated by the operator via alteration of system composition and other experimental variables. Let us look at some more of these factors.

Although the Boltzmann relationship in **Eq. 1** holds for solutes it is not expected to hold for particles ***(27)***. **Equation 1** can be rewritten in regard to the free energy of interfacial adsorption (ΔG°) ***(3,27,28)*** with

$$K = \exp(\Delta G^\circ / kT) \tag{2}$$

It should be possible to calculate ΔG° by measuring the equilibrium contact angle θ formed between the line tangent to the surface and the two-phase

boundary. For a spherical particle of radius r_c in a system with liquid–liquid interfacial tension of γ_{TB}, Young's equation holds ***(27)***, and

$$\Delta G^\circ = -\gamma_{TB} r_c^2 \, (1 - \cos \theta)^2 \tag{3}$$

Equations 2 and **3** suggest that all other system factors being equal log K should vary linearly with interfacial tension, or various other factors that affect the free energy of phase-cell interaction (as reflected by θ). **Table 2** describes a number of phase systems compounded from dextran and PEG polymers and the isotonic buffers (A–G) given in **Table 1**. Systems 9–13 and 15 indicate a series of NaCl-enriched systems, which differ over almost two orders of magnitude in interfacial tension while only varying dextran system concentration between 3.5 and 8%. The related phase-composition differences will somewhat affect θ, via alteration of system properties other than γ_{TB} (*see* **Table 2**); however, the more significant alteration in interfacial tension allows for a partial validation of **Eq. 3**, plus insight into the strong role interfacial tension plays in cell partition ***(27)***. This is shown, for two representative particle type, including erythrocytes, i.e., a representative eukaryotic cell types, in **Fig. 5** ***(29)***.

Another prime determinant of cell partition is the relative interaction of phase polymers with cell surfaces. The partition of solutes, such as small molecules or even macromolecules, into one of the phases can be dramatically enhanced by covalently linking the solute to the polymer enriched in the phase of interest. Polymer-coupled molecules may include small molecular-weight affinity ligands, such as enzyme-substrate analogs or molecules with hydrophobic groups. If the coupled molecule has affinity for the cell surface, cell partition will also be altered. "Affinity Partition" ligands are generally coupled at one terminal polymer group, typically on PEG, to yield an affinity partition ligand whose partition is similar to that of the free polymer. This partition will increase similarly with system tie-line length, e.g., increasing phase-polymer concentration (*see* **Table 2**), or decreasing temperature ***(2,3,5,7,13,20)***. Larger "affinity" substances such as immunoglobulins or other proteins are typically grafted with many polymer molecules ***(7,23–25,34)***. PEG grafting typically enhances the operational behavior of such macromolecules (e.g., reduces nonspecific adsorption, and antibody-induced cell agglutination) even at high levels of grafting ***(23,34)***. At such levels, the partition coefficients of the polymer-protein conjugates can exceed those of the corresponding free polymers ***(13,27)***.

Figure 6 indicates the ability of PEG-grafted hydrophobic affinity ligand **(Fig. 6A)** and PEG-antibody (**Fig. 6B**) to increase the partition of model eukaryotic cells (human erythrocytes). Similar results have been seen with other cell types (e.g., ***5,7,20***). The hydrophobic ligand, which adsorbs reversibly, was present in the phase system at μ*M* concentrations (*see also* **Fig. 2**) where it does

not appear to affect system physical properties ***(27)***. The immuno-affinity ligand used in **Fig. 6B** only had to be incubated with cells which were washed free of weakly bound ligand prior to their partitioning ***(23)***. The latter shows some agglutination-mediated sedimentation effects but only at higher concentrations (**Fig. 6B**). For such cases, the work of Sharp et al., ***(27)*** and other investigators has shown that log *K* varies directly with cell-surface concentration of affinity-localized polymer (which in turn affects θ). As can be seen in **Fig. 6**, the relationship between log *K* and affinity ligand is reasonably linear at lower ligand concentrations ***(7,13,20)***. Recent studies ***(13)*** suggest that the cell-surface orientation of affinity ligand-localized polymers may explain why they alter partition to a greater extent than the unmodified polymers abundant in the systems.

Some of the earliest observations of note made in regard to cell partition were that the partition of erythrocytes from different species, or those surfaces altered by various chemical or enzymatic means, varied directly (often linearly) with cell-surface charge differences detected electrophoretically ***(5,21)***. In collaboration with Brooks and other investigators, Walter ***(21)*** showed, using systems 16–20 in **Table 2**, that the "charge sensitive" nature of cell partition increased with the isotonic system ratio of sodium phosphate to sodium chloride, which in turn appeared to vary the asymmetric partition of negatively charged ions between the phases. Such asymmetric ion partition apparently gives rise to an electrochemical (Donnan) potential across the phase interface (*see* **Table 2**), which can be measured with high impedance, open-ended, microelectrodes. (***21***; *see* also ***27,31,32,35***). Although these experimental observations have been verified by many investigators, and are very useful in regard to practical applications of cell partition, there is still much debate about the existence of such potentials and their significance in partition, especially cell partition ***(10)***. Many scientists believe that because the phases are in equilibrium, the asymmetric partition of ions as measured in terms of concentration (as previously described) may not fully describe the system ***(7,26,35)***. This author believes it could be related to asymmetric activity of the ions in the two phases compensating for any concentration differences. As such, the "charge-sensitive" nature of cell partition in such systems may reflect how the ionic composition of the phase systems affects solvation interactions with cell-surface groups, or phase–polymer interaction with the cell surface. Regardless, alteration of salt concentration, especially phosphate to chloride ratio has dramatic effects, which appear to relate to cell-surface charge ***(21,36)***.

Figure 7A shows a plot of apparent potential (top phase positive) relative to increasing NaCl concentration (i.e., decreasing Na Phosphate concentration) for two series of (5,4) systems of pH 7.2 or 6.8 (systems 3–9 plus 16–20 in **Table 2**). In both cases, there is a reasonably inverse linear relationship. In **Fig. 7B**, fresh erythrocyte partition (log K) is shown to vary directly with the appar-

ent potential. Alteration of ion composition in these systems affects many phase properties, e.g., interfacial tension increases with phosphate concentration (*see* **Table 2**), and the linearity suggested by **Fig. 7B** should be taken with some caution.

Comparing results for the pH 7.2 and pH 6.8 phase systems, composed of different buffer solutions (*see* **Table 1**) indicates that varying ion type and concentration also has a strong effect on cell partition. The cation or anion groups that are used in phase compounding decrease or increase cell partition in a sequence similar to the Hofmeister series (***3***,***36***). Reducing overall ion concentration tends to increase cell partition, perhaps by decreasing interfacial tension. Many other compositional factors affect cell partition. For example, compounding phase systems using a lower mol wt polymer, for the phase into one wishes to shift cell partition. Consider system 1 in **Table 2**. Such "(7,5) phosphate-rich" systems made with PEG 8000, and dextran T40 vs T500, exhibit greater lower-phase cell partition. In the analogous "NaCl-rich" (7,5) V system erythrocyte partition is primarily between the lower phase and the interface. Generally cell partition is increased by using phosphate-rich systems, instead of NaCl-rich systems ***(21)***.

Systems enriched in NaCl appear to be sensitive to what has been described as "noncharge-related" cell surface characteristics. Thus, NaCl-enriched systems that contain lower concentrations of PEG, such as the (5,3.5) V system described in **Table 2** appear to be sensitive to erythrocyte lipid composition ***(21***,***36)*** although the latter does vary with many other cell-membrane properties ***(37)***. Other cell-partition factors have been reviewed by Walter ***(36)*** in the same volume, in which he described methods related to countercurrent distribution ***(22)***. In regard to the latter, the reader is also advised to consult **refs. *2***, ***3***, and ***14***. Some general problems related to various methods of affinity cell separation have recently been reviewed by Hubble ***(38)***.

The aforementioned material provides the reader with a framework for initiating eukaryotic cell-partition studies in terms of compounding a series of basic PEG and dextran phase systems (*see* **Tables 1** and **2**) that can be used to partition a variety of different cell samples in order to subfractionate them, or to evaluate their surface differences (e.g., charge-related properties, existence of affinity ligand targets, and so forth). It indicates how to alter cell partition with regard to phase composition and its effect on system properties such as interfacial tension (**Fig. 5**), buffer salt type and concentration (**Fig. 7**), as well as application of polymer-coupled affinity ligands (**Fig. 6**). Although the physicochemical data given in **Table 2** only applies to the systems studied (whose characteristics are expected to vary with temperature, as well as phase polymer source, mol wt, lot number, and concentration) it also provides a starting point for interpreting results obtained with similar systems. The author suggests physically characterizing the phase systems used in a series of experiments, so as to support data

interpretation and reproducibility. Such characterization may simply involve determination of phase polymer compositions (i.e., system tie-line lengths; *see* Chapters 2 and 4; *see also* ***30***).

2. Materials

Experiments related to the figures previously described—with the exception of those involving affinity ligands—can be reproduced for a variety of cell types using systems compounded from the stock solutions listed below. Stock solutions may be stored for one week at 4°C and at that time can be used to prepare systems which can be stored 1 wk at 4°C. Longer storage times result in larger standard deviations in partition data.

1. 4× NaCl stock (600 m*M*). Provides for final system buffer concentrations given in **Table 1**.
2. 2× sodium phosphate buffer, pH 7.2 stock (218 m*M* Na_2HPO_4 and 69 m*M* NaH_2PO_4) or pH 6.8 stock (110 m*M* Na_2HPO_4 and 110 m*M* NaH_2PO_4). The stock solutions provide for final system buffer concentrations given in **Table 1**.
3. 20% (w/w) Dextran T500 (Amersham Pharmacia Biotech, Uppsala, Sweden). If possible, concentration of dextran should be verified using polarimetry (*see* Chapter 3 or **ref. *31***).
4. 30% (w/w) PEG 8000 (Union Carbide Sentry Grade). If possible, concentration of PEG should be verified by refractive index measurements (*see* **Note 1**, and Chapter 3 or **ref. *31***).
5. Affinity ligand stock solution (*see* **Note 2**).
6. Tissue-culture medium (*see* **Note 3**).

3. Methods

3.1. Preparation of Phase System

1. To make 110 g of (5,4) I system (*see* **Table 2**), mix 55 g of pH 7.2 phosphate buffer stock, 27.5 g of 20% dextran stock, 14.67 g of PEG stock, and 2.83 g of water. To make 110 g of (5,4) V system mix 27.5 g of NaCl stock, 3.68 g of NaPhosphate pH 7.2 stock, 27.5 g of 20% dextran stock, 14.67 g of PEG stock, and 36.65 g of water.
2. The phase systems should be mixed well and, depending on volume, left in a separatory funnel for 4–12 h to equilibrate at the temperature at which they will be used (*see* **Notes 4** and **5**). Wherever possible, sterile technique should be used (*see* **Note 6**).
3. The phases are then separated, care being taken to discard the 10% of system volume associated with the interface, which often contains polymer lot contaminants (see **Note 4**).

3.2. Cell Preparation and Enumeration

1. Cell preparation (**Fig. 8**) is similar to that used in many experiments where cells are isolated, washed with centrifugation in buffer, and suspended in a defined medium (in this case upper phase) prior to being further processed (*see* **Note 7**).

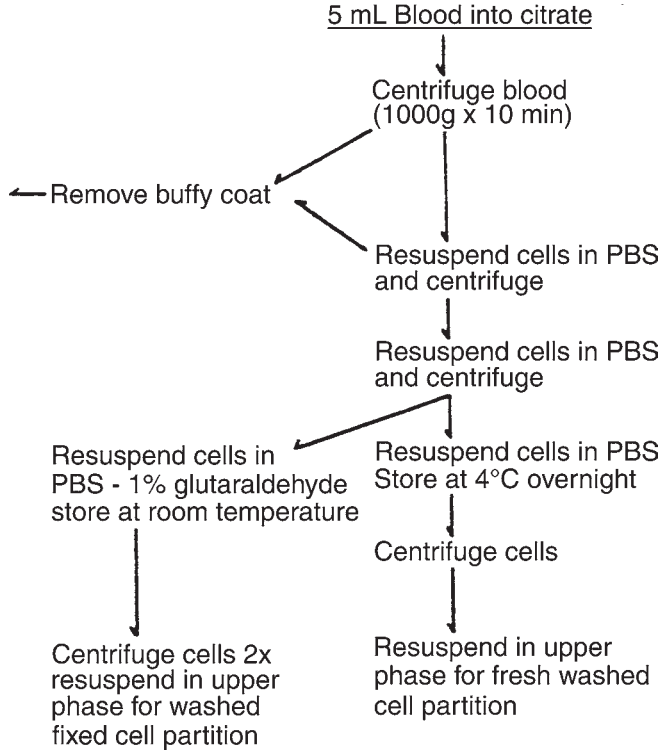

Fig. 8. Typical flow chart related to preparation of cells, in this case erythrocytes from blood treated with citrate anticoagulant, for cell partitioning *(29)*. Less dense cells, cell debris, etc., are discarded in the supernatant and "buffy coat" on top of the cells of interest. Fixation (if undertaken) is generally allowed to progress for 24 h with cell storage in the same fixative buffer. Similar procedures have been used for a variety of other cell types *(13,20)*.

2. Cell enumeration and characterization, with regard to viability or specific functions of interest, should be performed using a method with which the operator is familiar (e.g., impedance cell counting). Cells may need to be centrifugally washed to remove polymers that may interfere with some chemical assays.

3.3. Single-Tube Partition

The type of methodology used to enumerate cells will fix the number of cells per mL of load mix and the volume of load mix (**Fig. 3**) required in order to ascertain partition coefficients. It should generally be high enough such that one can discern a 1–2% alteration in partition. For erythrocytes or similar blood or tissue-culture cells counted via impedance cell counting, this is a load mix concentration of 1×10^7 cells/mL. According to the above experiments:

1. Combine equal 2.5 mL volumes of "load mix" and lower phase in 13 × 100 mm glass or plastic tubes at 22°C (*see* **Note 8**).
2. Cap the tubes, mix 20× by inversion, and leave for 30 min.
3. Remix by inversion and after another 30 min (45 min for [5,3.5] or other phase systems near the critical point [Chapter 2]) to demix to a planar interface.
4. The middle of the upper phase is then sampled (250 μL) three times and the sample placed in 10 mL PBS and counted 3–5 times. The load mix is similarly sampled and counted (*see* **Notes 9–11**). This gives a partition based on 9–15 determinations (**Fig. 3**).

3.4. Defining Partition Characteristics of a Cell Type

One may wish to characterize a specific cell type or sample, and compare it to another cell type or to experimentally treated cells from the same source. Such comparisons often relate to gaining knowledge of the sample cells' surface properties (*see* **Subheading 1.2.**). Such applications work best when the control sample has a partition in the range 10–50% (i.e., log K = 0.1–1) and the experimental sample partition is greater. From the Introduction and **Tables 1** and **2** the reader should be able to develop rapidly a series of single-tube partitions that provide some information with regard to cell-surface charge (I buffer systems) or noncharge-related characteristics (V buffer systems). The most useful systems will often be the "(5,4) systems" of **Table 2**; however, in some cases (e.g., **Fig. 5**) one may need to go to systems with higher or lower polymer concentrations. In such cases, one may first characterize the control cells in a series of systems of varying polymer concentration, produced by varying the dextran concentration as per **Fig. 5**. These do not have to be limited to NaCl-enriched systems.

3.5. Single-Tube Partitions with Affinity Ligand

Phase systems can easily and often dramatically indicate cells' ability to interact with a certain affinity ligand derivatized with a polymer. **Figures 2** and **3** illustrate a typical experiment. If it involves 10 tubes of 2.5 mL of each phase, it will require at least 25 mL of each phase, plus some extra upper phase for "load mix" determinations. It is typical to work with 100 mL of system because this allows for alterations in phase volume ratios during equilibrium. As previously noted in regard to **Fig. 6**, some affinity ligands may be added to the phase systems in buffer. If they will be added in a volume <1% of the system (<50 μL for a 5-mL system) in PBS or some other solution, it will generally not affect the noted partition. If they must be added in higher volumes it is wise to make up the stock with upper phase. The ideal phase system should give a cell partition of a 1–3% in the absence of affinity ligand. The (5,4) V or (5,3.5) V systems are generally fine for such applications. However it is prudent to first characterize the cell partition in series of V buffer systems of varying polymer composition (*see* **Fig. 5**).

3.6. Special Considerations

This chapter has mentioned, and in some cases illustrated, a wide range of factors that affect cell partition. Many of these relate to nonthermodynamic factors such as partition settling time, cell concentration, phase volume ratio, phase vessel geometry (height and interfacial area during demixing). When undertaking single-tube partitions to characterize or preparatively separate cells, or to evaluate phase systems or affinity ligands in regard to special applications, the aforementioned factors need to be taken into account. Thus, for example, when developing phase system for use with countercurrent (CCD) or similar apparatus one should undertake the single tube (transfer) experiments in a manner that mimics the experiment as closely as possible; ideally, in the apparatus itself.

4. Notes

1. Experience suggests good reproducibility with phase systems compounded with Dextran® from Amersham and Pharmacia Biotech (formerly Pharmacia) (Uppsala, Sweden). Samples normally come with molecular weight data. Several sources exist for PEGs (Union Carbide, BDH, Fluka, and so forth) each with their own product ranges of molecular weights and added antioxidants ***(34,39)***. Experience suggests testing several samples to find one that works well, characterizing it, and buying enough for a large series of studies. Union Carbide Sentry grade has proven to be reliable.
2. There is no reliable commercial source for affinity ligands although activated PEGs for producing such ligands can be purchased from several suppliers, e.g., Shearwater Polymers (Huntsville, AL). Most affinity partition references contain information regarding polymer-ligand synthesis, purification, and characterization ***(2,5,13)***. If using a relatively high concentration ($> \mu M$) of affinity ligand in a system, one should take into account the increase in system polymer concentration associated with the ligand. If ligands are added to existing systems (e.g., **Figs. 2** and **3**), aliquots should be $\leq 1\%$ (v/v) of system volume.
3. In order to enhance the viability of delicate cell types, phase systems may be compounded with tissue-culture media. Serum-free media is best so that there is no protein precipitation owing to the presence of PEG. In some cases, one may also have to work with custom media containing reduced calcium and magnesium. In most cases, the media will yield a system similar to a NaCl enriched system (e.g., buffer V in **Tables 1** and **2**). As a result, (5,4) systems made with such buffers may be useful in affinity-based separations ***(13,20)***, whereas (5,3.5) systems may separate cells in a manner related to lipid composition ***(21)***.
4. Phase systems should be allowed to equilibrate initially in a cylindrical separatory funnel, at the temperature at which they will be used, until they have a planar defined interface. This is typically 12–24 h for a 500–1000 mL sample; smaller samples require less time. If centrifugation is used to speed up the process, care should be taken with regard to temperature control. Cylindrical separatory funnels allow for easy isolation of upper and lower phase and interface. The latter often contains polymer lot contaminants and should be discarded.

5. Given some variation in phase-system physicochemical properties, especially with variation in polymer lot and source, the reader is encouraged to undertake chemical characterization of their phase systems (***31***; Chapter 4). This greatly aids both the reproducibility of phase systems and results, as well as the interpretation of such results.
6. Phase-system sterility can be achieved via ultrafiltration through 0.22- or 0.45-μm media filter units (of mixed phases prior to the first separation). Such filtration is more difficult once the viscous phases have been separated. Phase systems can also be autoclaved (although this may affect polymer concentrations) or for some cell types various additives such as antibiotics or, in the case of erythrocytes, sodium azide can be added.
7. When cells are washed and centrifuged prior to partitioning, the final wash should be in the PEG-rich upper phase in which they will be partitioned. This will reduce the possibility of trapped buffer diluting the phase system. Cell-partition coefficients can be affected by prior exposure to polymer-containing solutions (including the phases). This is particularly true for solutions containing higher molecular-weight polymers, such as the dextrans used in system compounding. Cells preincubated in dextran-rich phase exhibit lower K values.
8. Clear plastic polypropylene centrifuge tubes provide an ideal way to partition cells and then sample cell partition to the top phase directly. Bottom-phase partition can be sampled via needle puncture at the bottom of the tube. This allows calculation of cell partition to the interface, which can be verified by letting the phase system slowing drain out, as from a small separatory funnel so that a small fraction of interface localized material can be sampled. Use of such tubes, as opposed to glass tubes, also tends to reduce tube wall adsorption.
9. The polymers used in phase systems may interfere with some assays, such as protein or carbohydrate assays. They may also interfere with post partition processing of cells, such as labeling. For that reason, cells should be resuspended in medium and thoroughly washed following partition.
10. Whenever possible, cells should be enumerated by two or more methods that do not rely on similar physical phenomena. For example electronic impedance counting and spectroscopy, or ^{51}Cr labeling and direct microscopic visualization (hemocytometry), or total protein or nucleic acid assay combined with ^{14}C label counting. Operators should use assays with which they are most familiar in regard to the cells being partitioned.
11. In some cases cell aggregates, which interfere with enumeration of single cells and their partition, can be reduced by exposing the samples, following partition, to micromolar concentrations of a PEG-linked C16-saturated alkyl surfactant, which may not harm the cells. Examples can be found in the Brij or Myrj surfactant series. Hydrophobic affinity ligands also tend to reduce cell aggregates.

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12

Concentration and Purification of Viruses

Lena Hammar

1. Introduction

Two-phase extraction has found its platform in several aspects of virus research. With the rapid development of rational design systems for the production of biotechnology products ***(1)***, development of large-scale systems (*see* Chapter 36) and methods to recycle the polymers (*see* Chapter 32), the extraction in aqueous two-phase systems appears as one of the more attractive methods for recovery of viruses and other biological particles. Among available methods for virus concentration and purification, there are some fields where this technique is one of the most powerful of those available. It needs no elaborate equipment and adapts well to scaling up. Close to physiological conditions can be maintained, and at the same time, this system has a strong resolving power with just a few steps in the procedure.

Undoubtedly, ultracentrifugation in density gradients is very efficient in the concentration and purification of virions from culture fluids. However, it does sometimes yield a low recovery of infectious particles. This is mainly because the loosely attached components of the virus surface do not withstand the shearing forces involved in that procedure. For such cases, the two-phase extraction provides an alternative, separating as it does without this stress. Further, risk factors as aerosol formation and leakage owing to high *g*-forces or high pressure are minimized. This should have impact on the choice of methods for the handling of hazardous infectious material. For the concentration from large volumes, the two-phase extraction with aqueous polymers provides a technically, as well as economically, interesting alternative to the more commonly used techniques like tangential flow filtration and continuous flow centrifugation.

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Two-phase extraction in water soluble polymer systems was used early for the concentration and purification of viruses from culture supernatant, as reviewed by several authors during the years *(2–7)*. It provides a method for the concentration of virus particles that is useful for their detection and typing in dilute solutions *(8–11)*. At the same time, it may provide a means of hazardous waste clearance.

1.1. Concentration from Sewage Water

Maini and Piva tested a series of different concentration methods for measuring adenoviruses in sewage water *(10)*. They found no advantage in using dextran sulfate-polyethylene glycol systems, compared to alumina adsorption, for that purpose *(10)*. Quite the opposite was found with a similar system for porcine enteroviruses by Hazlett *(12)*, who obtained about 100-fold concentration with from 37% to full recovery. In the latter study, the concentration or adsorption, obtained with cobalt chloride, polyethylene glycol precipitation, or adsorption to aluminium hydroxide or calcium hydroxyphosphate were not satisfactory *(12)*. Pöyry used a dextran-polyethylene glycol system for the concentration of polioviruses with successful results in a study on polio vaccination in Finland *(11)*. This method is described in **Subheading 3.1.**

1.2. Preparation of Purified Virus Material

The aqueous polymer extraction method has been utilized for the preparation of a Foot-and-Mouth disease virus vaccine *(13,14)*. It was used for purification of Venezuelan equine encephalitis virus (VEE) and the material used to establish an enzyme-linked immunosorbent assay (ELISA) for detection of VEE antibodies in human and animal sera *(15,16)*. The high purity was obtained by a three-step extraction procedure in which the virus was alternatively partitioned to the bottom and top phase. A similar procedure has been discussed by Philipson et al. *(17)* and Norrby and Albertsson *(18)*.

The external glycoprotein of retroviruses are essential for their binding to the target cell. However, these are usually not recovered to a high extent with conventional virus purification protocols. To overcome this problem, we have studied the behavior of several retroviruses in polymer phase systems. Feline and bovine leukemia viruses *(19,20)*, as well as human immunodeficiency viruses (HIV-1 and HIV-2), and simian immunodeficiency virus (SIV_{mac}), were all recovered from culture supernatant with retained external glycoproteins *(19–22)*. With HIV-2 (unpublished results) and SIV_{mac}, the method was scaled up to 25 L of culture supernatant *(7,22,23)* and the purified viruses used as source for the preparation of highly purified external glycoproteins *(22,23)*. One may also mention the report by Andrews, Huang, and Asenjo *(24)*, which points to the usefulness of the polymer phase-extraction method for the separa-

tion and purification of recombinant virus core particles. Within virus research, the two-phase extraction technique has also been applied to the isolation of a plasma membrane virus receptor ***(25)***.

1.3. Analytical Approaches

Even small differences in distribution coefficient may be enough to allow separation of related viruses, if applied to repeated extractions or counter-current distribution. Thus, Bengtsson et al. separated several strains of poliovirus by counter-current distribution ***(26,27)*** and Walter et al. showed that a single amino-acid substitution in the coat protein of phage ϕX174 was enough to alter its partition ***(28)***. Similarly, an antibody bound to poliovirus particles effects their distribution between the top and bottom phase ***(29,30)***. Further, the selective partition aspect has been utilized in molecular biology assays ***(31)***.

1.4. Virus and Polymer Properties

Table 1 gives a summary of the different animal viruses that have been subjected to two-phase extraction in aqueous polymer systems. The different genera of the virus group include a field of members, greatly varying in size and shape, as well as in surface properties. They may also differ in their requirements for stability and in adaptability to the surrounding medium. In spite of these variations among viruses, there are some polymer two-phase systems that work well with most viruses and only require minor variation in the system parameters for optimization. These are the systems containing polyethylene glycol in combination with dextran or dextran sulfate, or a high concentration of phosphate. The starch-based polymer Reppal PES (*see* Chapter 3) in combination with PEG works fine with HIV and SIV_{mac} and gives similar results as the dextran-PEG systems. Parameters like pH, ionic strength, and osmotic pressure, requirement for metal ions, and so forth, has to be considered in all handling of biological particles. With the majority of the water soluble polymers it is possible to establish systems of close to physiological conditions to suit a particular virus. Further, the selection of available polymers would make it possible to define working systems for most virus applications.

For a particular virus application, the choice of polymers may be considered in relation to the final usage of the concentrated and purified virus, because traces of polymer may cause unwanted problems. Some observations on polymer properties in this respect are given in **Table 2**.

The methods described in this paper are collected from the literature and from our own laboratory. They include systems based on dextran–PEG, and dextran sulfate–PEG. The examples include the concentration of polio virus from sewage water ***(11)***, the preparation of a small enveloped virus in a three-step extraction procedure ***(15,16)*** and the concentration of retroviruses from 3–25 L culture supernatant ***(7,22,23)***.

Table 1
Animal Virus Subjected to Partitioning in Aqueous Two-Phase Systems
Updated from *(7)*

Type	Size of genome	Virion (nm)	Classification	Virus
Naked viruses				
Site of capsid assembly:				
Cytoplasm	ps RNA, *ss*	28–30	Picorna	Enteroviruses: Coxsackie virus ***(9)*** ECHO virus ***(9,17,18,32,33)*** Poliovirus ***(9,11,18,26,27,29,30,32)*** Porcine enterovirus ***(12)*** Foot-and-mouth disease virus ***(14)***
	DNA, *ss*	33–38	?	Parovirus; similar virus from rabbit liver causing hemorrhagic disease ***(34)***
Nucleus	DNA, *ds*	80–110	Adeno	Adenovirus ***(17,33)***
Enveloped viruses				
Membrane type:				
Intracytopl. vacuoles	psRNA, *ss*	40–50	Flavi	Japanese encephalitis virus ***(35)***
ER or Plasma membrane	ps RNA, *ss*	50–70	Toga	Venezuelan equine encephalomyelitis virus ***(15,16)***
Plasma membrane	ns RNA, *ss*	80–120	Orthomyxo	Influenza virus ***(9,17,33)***

Plasma membrane	ps RNA, *ss*	80–150	Retro	Feline leukemia virus ***(19)*** Bovine leukemia virus ***(20)*** Human immunodeficiency virus ***(21)*** Simian immunodeficiency virus ***(22,23)***
Plasma membrane	ns RNA, *ss*	150–300	Paramyxo	Measles virus ***(36,37)*** Newcastle Disease virus ***(33)*** Parotitis virus ***(33)***
Nuclear membrane - ER inclusions- exocytosis	DNA, *ds*	150–200	Herpes	African malignant catarrhal virus ***(38)***
Perinuclear area - plasma membrane		188–212	ASF-like ***(39)***	African swine fever virus ***(40)***
Intracytoplasmic inclusions		230–300	POX	Vaccinia virus ***(32,33)***

ps, positive sense; ns, negative sense; *ss*, single-stranded; *ds*, double-stranded.

Table 2
Effects of Some Commonly Used Polymers on Virus Detection and Analyses (*see* also Notes 3 and 5–8)

Dextran
α-D-glucose polysaccharide, predominantly linked α-1–6.
Usually inert and harmless to test systems ***(9)***, but was found to inhibit reversibly HIV-reverse transcriptase in vitro ***(7,21)***

Dextran Sulfate
A polycationic derivative of dextran with sulfate groups linked to the dextran glucose residues by ester linkages. Usually provided as a sodium salt.
May inhibit plaque formation and cell cytopathic effect ***(9,41)***. Toxic to H9 cells above 400 g/mL ***(21)***. Blocks HIV infection at concentrations down to at least 1 μg/mL ***(21,42)***. Immunostimulant ***(43)***.

DEAE-dextran
A polyanionic derivative of dextran with diethylaminoethyl groups linked to the dextran glucose residues by ether linkages. Usually provided as hydrochloride salt.
May enhance virus infectivity in culture ***(44–46)*** and increase sensitivity in plaque assays ***(47,48)***. Immunostimulant, may be used as adjuvant ***(13,49,50)***

PEG
Polyoxyethylene glycol
Bio-compatible. Immunosuppressive ***(43,51,52)***

Reppal PES
Hydroxypropyl starch, Hydroxypropyl α1-4-D-glucan. By a low degree of hydroxy-propyl substitution the natural polymer starch is converted into a water soluble, nongelling product. Nontoxic and biodegradable.

2. Materials (*see* Note 1)

2.1. Concentration of Polioviruses from Sewage Water

1. Virus containing start material: Sewage water specimens not older than one day should be taken for this procedure.
2. 20% (w/w) Dextran T-40 (Amersham/Pharmacia Biotech, Uppsala, Sweden) (*see* Chapter 3 for preparation).
3. 30% (w/w) PEG-6000 (Fluka, Buchs, Switzerland).
4. 5 *M* NaCl.
5. Equipment: Sterile separation funnels or similar types of bags as used in **Subheading 3.3. (Fig. 1)**.

2.2. Purification of VEE Virus from Tissue-Culture Fluid

1. Polyethylene glycol 4000 (PEG-4000, LOBA Chemie, Austria).
2. 10% (w/w) PEG-4000 in water.
3. 10% (w/w) Dextran sulfate (MW 500,000, from Serva, Heidelberg) (*see* **Note 2**). Prepared in the same way as Dextran (**Subheading 2.1., item 2**).

4. Solid NaCl.
5. 0.5 *M* NaCl.
6. 50 m*M* Tris-HCl, pH 9.0, containing 8% (w/w) PEG-4000 and 5 *M* NaCl.
7. 3 *M* KCl.

2.3. Purification of Retroviruses

1. Viruses:

   SIV: SIV_{mac} 251 32H isolate from the 11/88 virus bank, held at Center for Applied Microbiology and Research (Porton Down, UK). The virus is produced in roller bottle cultures (500 mL/flask) of the human T-cell line C8166. The growth medium is RPMI 1640 supplemented with 5% fetal bovine serum (FBS) and 2 m*M* L-glutamine.

   HIV: The $HIV\text{-}1_{IIIB}$ is obtained from the culture supernatant of persistently infected H9 cell line and the $HIV\text{-}2_{6669}$ is propagated in the cell-line U937. The cells are grown in roller bottles (500 mL) at 37°C and 5% CO_2 in RPMI-1640 medium supplemented with 10% FBS, 2 m*M* L-glutamine, penicillin (100 IU/mL), streptomycin (100 g/mL), and 20 m*M* HEPES buffer, pH 7.2.
2. Cell-free supernatant solution containing virus: Leave the culture flasks standing upright in the incubator overnight. The cells will sediment and 400 mL of the 500 mL culture fluid can be collected, with the aid of a peristaltic pump, as a cell-free solution. The remaining culture fluid is included after centrifugation at 800*g* for 10 min.
3. PBS(10×): 100 m*M* Na-phosphate, pH 7.4, 1.55 *M* NaCl.
4. 10% Dextran-T500 stock solution: For the preparation of 100 g of this stock solution, mix 10 mL PBS(10×) and 80 mL H_2O and heat to 100°C in a water bath. Add 10 g Dextran T-500 (Pharmacia) while stirring until it is completely dissolved. Store at room temperature and use within a week.
5. 50% Polyethylene glycol stock solution (50% PEG-6000): For 1000 g of this solution, mix 100 mL PBS(10×) (*see* **item 3**) and 400 mL H_2O. Heat to 100°C as aforementioned. Add 500 g PEG-6000 (E. Merck-Schuchardt, Darmstadt, Germany) while stirring until completely dissolved. Store at room temperature and use within a week.
6. Phosphate buffered saline (PBS): 10 m*M* sodium phosphate, pH 7.4, 154 m*M* NaCl
7. 8% PEG-6000: Prepare fresh from the 50% PEG-6000 stock (*see* **item 5**) and PBS (*see* **item 6**) and add dithiothreitol to 1 m*M*.
8. Tris-buffered saline (TBS): 10 m*M* Tris-HCl, pH 8.0, 155 m*M* NaCl.
9. 30% (w/w) and 60% (w/w) sucrose in TBS (*see* **item 8**) with 1 m*M* dithiothreitol.
10. Plastic bags (5 L) with attached tubing (Transfer bags, Baxter Medical AB, Bromma, Sweden).
11. A peristaltic pump: allowing a flow of about 500 mL/min, with sterile tubing of appropriate strength and dimensions and *secured fittings*.
12. A balance: 4000 g capacity, for weighing the bags while filling them.
13 Support for the bags in the lamellar-flow hood (*see* **Note 3**).

3. Methods

3.1. Concentration of Polioviruses from Sewage Water

The method described here is in current use at the National Public Health Institute, Helsinki (Dr. M. Stenvik, personal communication). It was originally devised for the screening of sewage for cytopathic viruses ***(11)***. Other enteroviruses detected were Coxsackievirus B and echoviruses ***(11)***. The system contains 1% Dextran T-40, 10% PEG-6000, and 0.2 *M* NaCl. About 100-fold concentration is obtained.

1. Add 30 g of 20% Dextran T-40, 200.4 g of 30% PEG-6000, and 24 mL of 5 *M* NaCl to the sewage specimen to make a total volume of 600 mL.
2. Adjust the pH to between 7.0 and 8.0.
3. Agitate vigorously for 1 h at room temperature.
4. Leave overnight at 4°C for the phases to separate.
5. Collect the small bottom phase of 2–3 mL and likewise the small interphase into separate tubes and extract with chloroform (*see* **Note 4**) before tests for cytopathic effect and virus typing.

3.2. Purification of VEE Virus from Tissue Culture Fluid

This three-step extraction method was adopted to the Venezuelan equine encephalitis viruses by Pomelova et al. ***(15)*** and has been used for the preparation of antigen material in ELISAs for VEE virus in human and animal sera ***(16)***. The use of dextran sulfate may restrict the analyses (*see* **Table 2** and **Notes 2**, **5–7**). The work is performed with cold solutions (4–8°C) and the purification followed by electron microscopy inspection of samples (*see* **Note 5**). The first system contains 0.7% dextran sulfate (500,000), 8.6% PEG-4000 and 0.5 *M* NaCl.

1. Start with 1000 mL of cell-free virus-containing culture fluid and add 105 g of PEG and 36 g of NaCl as dry powder and mix to dissolve.
2. Add 86 mL of 10% dextran sulfate stock solution.
3. Mix end-over-end for 30 min.
4. Allow the phases to settle for 1 h in the cold.
5. Sharpen the separation by centrifugation at about 1000*g* for 20 min.
6. Collect the bottom phase (about 25 mL) and add 3 vol of 50 m*M* Tris-HCl, pH 9.0 containing 8% (w/w) PEG-4000 and 5 *M* NaCl, which makes final concentration of NaCl as 3 *M*.
7. Repeat **steps 3–5**. The virus now distributes to the PEG containing top phase.
8. Collect the top phase (about 30 mL) containing the virus and add 5 vol of 10% PEG-4000 to decrease the concentration of NaCl to 0.5 *M*.
9. Add 10% dextran sulfate stock solution to get a final concentration of 0.7%.
10. Repeat **steps 3–5**.
11. Collect the small bottom phase (about 4 mL) containing the purified virus.

12. Remove the dextran sulfate by precipitation (*see* **Note 7**). This is performed by adding 700 μL of 3 *M* KCl per mL of the bottom phase. Allow the precipitate to stabilize in the cold and spin down at 1000*g* for 10 min.

3.3. Purification of Retroviruses from 3–25 L of Culture Supernatant Solution

This procedure is based on a system containing 0.24% Dextran T-500 and 7.2% PEG-6000 which will give a volume ratio between the bottom and the top phase of about 1 to 500 (*see* **Note 8**). In summary, the culture medium containing the virus is mixed with polymer stock solutions in a plastic bag **(Fig. 1)**. Material from a 25-L culture will make eight bags. You may need about 20 min/bag to fill it with the virus- and polymer solutions. The bags are left for the phases to separate (4–5 h, or overnight) and the virus collected from the interphase. Work with the human pathogenic retroviruses is performed under Lab-PII conditions.

1. Prepare the polymer solutions in advance. Make sure all solutions, including the virus, are equilibrated to the same temperature (room temperature).
2. The phase-system mix (4000 g total weight) for each bag requires 3330 mL (g) virus-containing cell-free culture supernatant, 96 g of 10% dextran T-500, and 576 g of 50% PEG-6000.
3. Weigh out the amounts of stock solutions needed for each bag in polypropylene flasks or tubes. Alternatively, if it is possible to arrange a balance in the hood, the amounts needed are monitored "on line."
4. Set up a pump in a lamellar-flow hood. Attach the inlet via a silicon tubing to a 10-mL sterile pipet (with cotton plug removed) and the outlet to the plastic bag with *secured fittings*.
5. Transfer most of the virus containing solution to the bag by using the pump.
6. Pump in the required amount of the dextran stock solution, then some more of the virus medium, so that the two polymers do not mix in the tubing.
7. Pump in the PEG stock solution. Rinse the tubing with the remaining amount of virus medium (and/or PBS). Assure that all PEG solution has been completely introduced into the bag and that the total weight of the system is 4000 g.
8. Agitate the mixture while filling the bag by tapping. Finally, secure the tubing of the bag with a clamp. Disconnect it from the pump and cover the fittings securely. Turn the bag upside down a couple of times and leave for 3–4 h, or overnight, to allow the phases to settle. Hook up the bags to allow the bottom phase, and the virus containing interphase, to collect in the bottom "funnel." This takes less than 1 h. The bags may also be directly left hanging overnight with practically the same result.
9. Open the outlet tubing from the separation bag and remove the bottom phase. Collect the bulky interface fraction, containing the virions, in a Falcon tube.
10. Wash the interface fraction, which contains also the bottom phase, by adding an equal volume of 8% PEG-6000. Mix without agitation by turning the tube upside down a couple of times and centrifuge at 800*g* for 10 min. This sharpens the virus band at the interface that forms. Replace the top phase with new 8% PEG-6000 and repeat centrifugation. Repeat this washing once more.

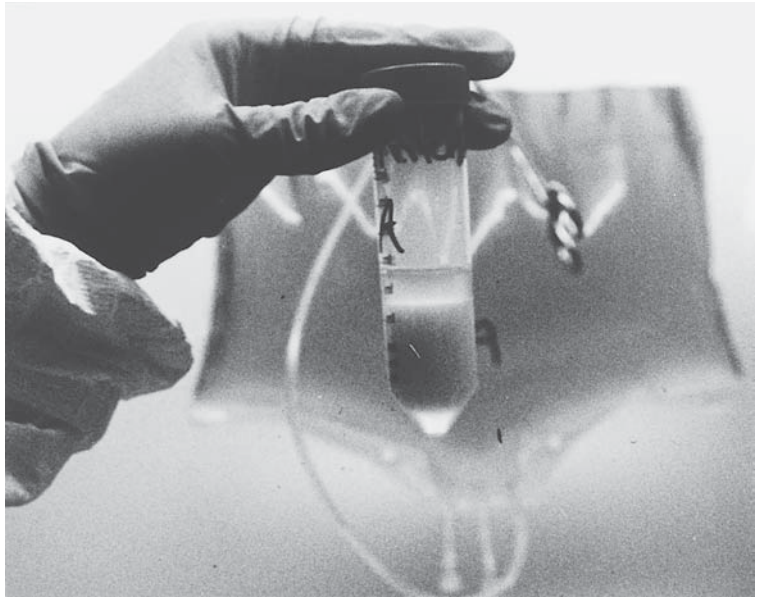

Fig. 1. A Falcon tube with the pooled HIV-2, from a preparation starting with 25 L of cell-culture supernatant, is held in front of one of the eight plastic bags, used as separation funnels for the two-phase system. The virus was collected in the interphase.

11. Remove the virus at the interphase with a plastic Pasteur pipet. The virus fraction now represents about 0.3% of the initial culture fluid volume.
12. To proceed for removal of polymers and soluble proteins, dilute the virus fraction, obtained from a 25-L preparation, with PBS to a final volume of 120 mL to match 10 tubes (one rotor) in the following centrifugation. The material may be too viscous if the dextran bottom phase is incompletely removed. In such a case suspend the material in more PBS, and allow time for equilibration before proceeding.
13. Layer 3 mL of 60% sucrose under 15 mL of 30% sucrose in a centrifugation tube (30 mL), and 12 mL of the virus suspension on top of the sucrose.
14. Centrifuge at 12,000*g* for 16 h at 4%C.
15. Collect the virus on top of the 60% sucrose layer. The sucrose may be removed by chromatography on a small desalting column (*see* **Notes 9** and **10**).
16. The membrane and capsid proteins of the virus may be separated by detergent treatment (*see* **Note 11**).

4. Notes

1. Maintenance of sterility: It is essential that the virus samples are handled with sterile equipments and that added solutions are autoclaved or sterile filtered.
2. Dextran sulfate, or just dextran: If the method is used mainly for concentration, to make detection and classification possible, the dextran in combination with PEG or

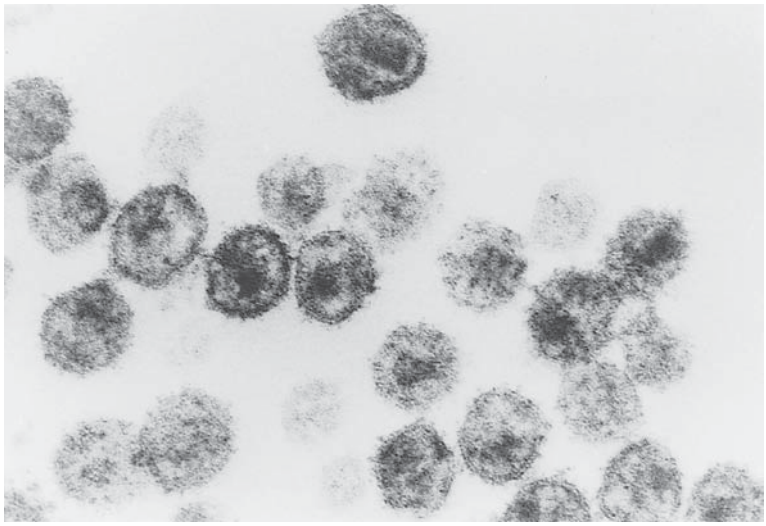

Fig. 2. HIV-2 concentrated in the system 0.2% Dextran T500–7.0% PEG 6000. Material from the interphase was fixed by the addition of glutaraldehyde to 1.25% (v/v) and formaldehyde to 1% (v/v), final concentrations. The specimen was postfixed in 2% (w/v) osmium tetroxide and treated with 1% tannic acid before embedding in Spurr epoxy resin. After sectioning the material was further contrasted by uranyl acetate and lead citrate. (Electron micrograph kindly supplied by Dr. Sigrid Eriksson, The Walter and Eliza Hall Institute, University of Melbourne, Victoria, Australia.)

the starch polymers may be a better choice than dextran sulfate. The latter most often has negative effects on analyses as plaque formation test ***(9)*** (*see* **Table 2**). However, this is not always the case. With the porcine enteroviruses studied by Hazlett, there were no serious effects on plaque formation with the dextran sulfate ***(12)***. In systems with dextran sulfate and PEG, the virus partition can be drastically changed by manipulating the salt concentration, as demonstrated in **Subheading 3.2.**

3. Supports for hanging separation funnels or bags: Most hoods have a T-profile around the ceiling. Suspend a stainless steel rod, about 1 cm in cross section, over the T-profile as support for S-hooks to hold the bags, *see* **Fig. 1**.
4. Chloroform extraction: Small, naked viruses, as the picorna viruses, are usually stable towards chloroform extraction. It may be included as a step to destroy and remove cellular membranous material. Chloroform extraction is included in the preparation of a highly purified, inactivated hepatitis A vaccine to remove cellular contaminants and PEG, remaining after precipitation step ***(53)***.
5. Electron microscopy: Many polymers block the binding of the virus to the grids. The solutions may also be too viscous to permit easy penetration of negative contrast media. For embedding and sectioning the presence of Dextran and PEG in the sample causes no problem when conventional fixing and contrast media are used (**Fig. 2**).

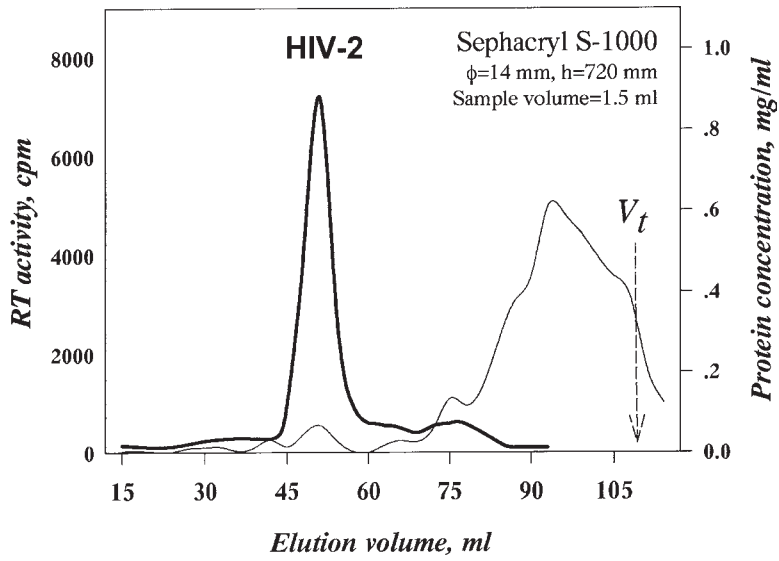

Fig. 3. Chromatography of HIV-2 virions on Sephacryl S-1000. Virus, concentrated by a system containing 0.2% Dextran T500 and 7% PEG 6000 and collected at the interphase, was diluted with 2 vol of phosphate-buffered saline and applied to the column. The gel was equilibrated with the same buffer, which was also used for elution at a flow rate of 0.25 mL/min. Reverse transcriptase (RT) activity (heavy line) indicates the position of the virus in the chromatogram.

6. ELISA and blots: Polymers like dextran sulfate, PVA, and methyl cellulose block protein binding to polystyrene or blotting membrane surfaces. In ELISA, this is overcome by coating the plates with a capturing antibody or lectin.
7. Electrophoresis: Charged polymers in the samples should be removed in order not to distort fractionation. Dextran sulfate can be removed from the sample by precipitation with potassium or barium ions ***(17)***. Note that an excess of these ions will precipitate sodium dodecyl sulfate (SDS). Alternatively, for analytical purposes, the virus peptides could be precipitated with trichloroacetic acid or similar, prior to electrophoresis.
8. Phase diagram. In the design of an application the phase diagram for the polymer system should be consulted so as to find suitable concentration ranges and appropriate phase volume ratios (*see* Chapter 2). Advice on the preparation of two-phase systems are found in Chapter 3. Phase diagrams can usually be obtained from the supplier of the polymers. Quite a selection of such diagrams are given by Albertsson ***(54,55)***. Note that the molecular weight of the polymers greatly affect the phase diagrams and the partition of the analyte. Different manufacturers may use different conventions for defining their products.
9. Albumin: One of the major protein constituents of the cell culture medium, and therefore also a major protein contaminant in most virus preparations. This is

removed to a large extent by centrifugation through a sucrose cushion, or by size exclusion chromatography (SEC) **(Fig. 3)**. We have also used a column with immobilized antialbumin antibodies (HiTrap-Albumin-adsorption gel, Amersham Pharmacia Biotech) to remove albumin from the "gag-fraction" after detergent treatment of SIV_{mac} ***(22)*** and HIV-2. One would assume that the Cibacron Blue derivatized PEG ***(56)***, or polymers with other albumin binding ligands, would be useful tools to remove contaminating albumin in virus preparations.

10. Chromatography of concentrated virus: If traces of cell organelles are still present in the virus preparation after the two-phase extraction these contaminants may be removed by chromatography on Sephacryl S-1000. The exclusion limit of this gel is suitable for the fractionation of cell organelles and viruses **(Fig. 3)**.
11. Detergents partition in the aqueous polymer systems: This effects the distribution of the analyte in the phase system. The effect of Triton X100 on the partition of HIV-1 gp120 was earlier discussed ***(7)***. The detergent Triton X114 provides in itself a temperature sensitive two-phase system that nicely separates the membrane glycoproteins from the core proteins of small enveloped viruses, as, for instance, the Semliki Forrest virus (unpublished results). (*See also* Brusca and Radolf, ref. ***57***).

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13

Isolation of Plant Plasma Membranes and Production of Inside-Out Vesicles

Christer Larsson and Susanne Widell

1. Introduction

The plant plasma membrane (PM) is the outermost membrane of the cell and constitutes the cell border across which nutrients are imported and metabolic products exported. It is also the site for receptors recording the environment and the interphase between the cell wall and the cytoskeleton. This makes the PM a key membrane for cell functioning and explains the great interest in its characterization. However, as the PM only constitutes 5–20% of the total membranes of a plant cell, a strong demand is placed on the isolation procedure to obtain pure PM. Furthermore, for studies on transport, there is a need for vesicles of defined sidedness, i.e., PM preparations where the vesicles expose either the apoplastic side (right-side out vesicles) or the cytoplasmic side (inside out vesicles) of the membrane to the medium. These vesicles should also be sealed to allow gradients to build up across the membrane. In this chapter, we describe how the PM is separated from all intracellular membranes resulting in a PM preparation with high yield and purity ***(1)***, and consisting of mainly sealed, right-side out vesicles ***(2)***. We also describe how these right-side out vesicles are everted to produce tightly sealed inside-out vesicles ***(3,4)***.

2. Materials

2.1. Preparation of a Microsomal Fraction

1. Homogenization medium: 0.3 *M* sucrose, 50 m*M* 3-(-*N*-morpholino) propanesulfonic acid (MOPS)-KOH, 5 m*M* Na-EDTA, pH 7.5, 0.2% (w/v) casein (enzymatic hydrolysate; Sigma Chemical Co., St. Louis, MO, USA; C 0626) (*see* **Note 1**).

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2. Insoluble polyvinylpyrrolidone (PVPP) to bind free phenols.
3. Dithiothreitol (DTT), reducing agent (*see* **Note 2**).
4. Ascorbic acid, reducing agent (*see* **Note 2**).
5. 100 m*M* Phenylmethylsulfonyl fluoride (PMSF) in isopropanol (*see* **Note 3**).
6. Resuspension medium: 0.3 *M* sucrose, 5 m*M* potassium phosphate, pH 7.8 (*see* **Note 4**), 0.1 m*M* Na-EDTA, 1 m*M* DTT (*see* **Note 2**).
7. Kitchen blender.
8. Nylon cloth with about 0.2-mm pore size.
9. Refrigerated centrifuge equipped with an angle rotor for 50-mL tubes.

2.2. Isolation of Plasma Membranes

1. 20% (w/w) Dextran T 500 (Amersham Pharmacia Biotech, Uppsala, Sweden) (*see* **Note 5**).
2. 40% (w/w) Polyethylene glycol 3350 (PEG) (Sigma; P 4338).
3. Sucrose (solid).
4. 0.2 *M* Potassium phosphate, pH 7.8 (*see* **Note 4**).
5. 2 *M* KCl.
6. 0.5 *M* DTT in 50 m*M* EDTA, pH 7.8 (*see* **Note 2**).
7. Phase mixture: 11.70 g of Dextran solution (*see* **item 1**), 5.85 g of PEG solution (*see* **item 2**), 2.77 g of sucrose (*see* **item 3**), 0.675 mL of potassium phosphate, pH 7.8 (*see* **item 4**), 0.090 mL of KCl (*see* **item 5**), and water to a final weight of 27.00 g in a 50 mL centrifuge tube (*see* **Notes 6** and **7**).
8. Bulk-phase system: 97.5 g of Dextran solution (*see* **item 1**), 48.75 g of PEG solution (*see* **item 2**), 30.8 g of sucrose (*see* **item 3**), 7.50 mL of potassium phosphate, pH 7.8 (*see* **item 4**), 0.75 mL of KCl (*see* **item 5**), and water to a final weight of 300 g (*see* **Notes 7** and **8**).
9. Centrifuge with swinging bucket rotor for 50-mL tubes and temperature control (4°C).
10. Ultracentrifuge with angle rotor for 70-mL tubes.

2.3. Inside-Out Plasma Membrane Vesicles Produced by Freeze/Thawing

1. Plasma membranes (5–10 mg protein/mL) in resuspension medium (*see* **Subheading 2.1., item 6**) containing 50 m*M* KCl.
2. Liquid nitrogen.
3. Waterbath (room temperature).

2.4. Inside-Out Plasma Membrane Vesicles Produced by Brij 58 Treatment

1. 1% (w/v) Brij 58 (polyoxyethylene-20-cetyl ether; Sigma P 5884), made fresh in 10 m*M* MOPS-KOH, pH 7.0.

2.5. H^+-ATPase Assay

1. 20 m*M* MOPS-KOH, pH 7.0.
2. 1 *M* KCl.

3. 48 m*M* Na-ATP, pH 7.0 (store frozen).
4. 67 m*M* Na-EDTA, pH 7.0, 67 m*M* DTT (*see* **Note 2**).
5. 2.5 m*M* acridine orange.
6. 10 mg/mL Lactate dehydrogenase (Boehringer 127 221, solution in glycerol).
7. 10 mg/mL Pyruvate kinase (Boehringer 109 045, solution in glycerol).
8. 50 m*M* NADH in 20 m*M* MOPS-KOH, pH 7.0 (*see* **item 1**) (made fresh).
9. 100 m*M* phosphoenolpyruvate in 20 m*M* MOPS-KOH, pH 7.0 (*see* **item 1**) (store frozen).
10. 270 m*M* $MgCl_2$ in 20 m*M* MOPS-KOH, pH 7.0 (*see* **item 1**).
11. Assay medium: 10.00 mL of 20 m*M* MOPS-KOH, pH 7.0 (*see* **item 1**), 3.36 mL of KCl (*see* **item 2**), 1.00 mL of ATP (*see* **item 3**), 0.36 mL of EDTA/DTT (*see* **item 4**), 0.19 mL of acridine orange (*see* **item 5**), and 4.77 mL H_2O.
12. Plasma membranes diluted in resuspension medium (**Subheading 2.1., item 6**) to a final concentration of 1.0 mg protein/mL (keep on ice).
13. Coupling enzymes: 0.195 mL of MOPS-KOH (*see* **item 1**), 0.065 mL of lactate dehydrogenase (*see* **item 6**), 0.130 mL of pyruvate kinase (*see* **item 7**) (keep on ice).
14. Substrate: 0.840 mL of MOPS-KOH (*see* **item 1**), 0.12 mL of NADH (*see* **item 8**), 0.24 mL of phosphoenolpyruvate (*see* **item 9**) (keep on ice).
15. Disposable 1-mL plastic cuvets.
16. A spectrophotometer (Shimadzu UV-1601, or similar).

3. Methods

3.1. Preparation of Microsomal Fraction

1. All steps should be conducted at 4°C using precooled media and equipment.
2. Homogenize 125 g of plant tissue in 275 mL medium [made up of 275 mL homogenization medium, 1.5 g PVPP, 0.212 g DTT (final concentration 5 m*M*), and 0.242 g ascorbic acid (final concentration 5 m*M*)] for 3 × 20 s in a kitchen blender.
3. Filter the homogenate through a nylon cloth, gently squeezing out the remaining liquid, and immediately add 2.5 mL of PMSF to the filtrate.
4. Centrifuge the filtrate at 10,000*g* for 10 min, discard the pellets.
5. Centrifuge the supernatants at 50,000*g* for 30 min; save the pellets.
6. Resuspend the pellets in a total volume of 10 mL resuspension medium to yield the microsomal fraction (MF).

3.2. Isolation of Plasma Membranes

1. Add 9 mL of MF (*see* **Subheading 3.1., item 6**) to the phase mixture (**Fig. 1**, **step 1**, loading).
2. Mix the contents thoroughly by 20 inversions of the tube (**Fig. 1**, **step 1**, mixing).
3. Centrifuge at 1500*g* for 5 min to get phase separation (**Fig. 1**, **steps 1–2**, separation).
4. Transfer ca 90% of the upper phase (use a disposable transfer pipet), without disturbing the interface, to a 50-mL centrifuge tube containing ca 15 mL lower phase obtained from the bulk-phase system (**Fig. 1**, **step 2**).
5. Mix and spin, i.e., repeat **steps 2** and **3** above.

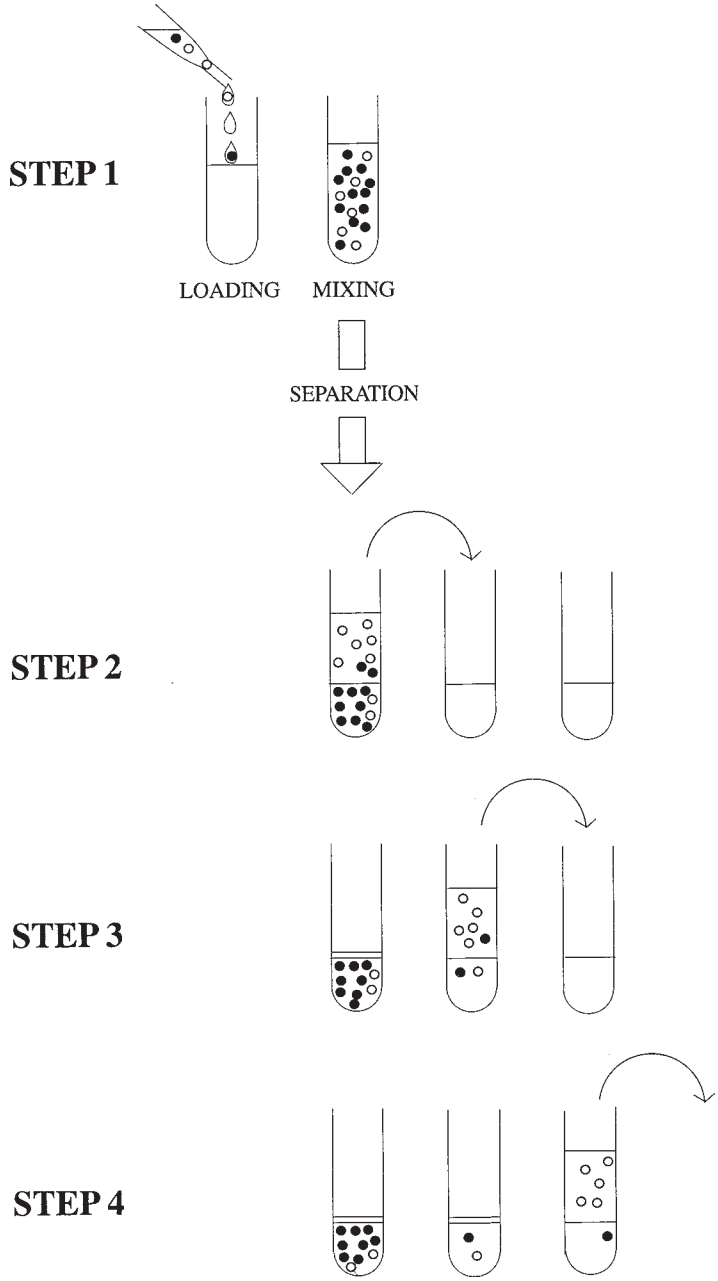

Fig. 1. Separation of plasma membranes (○) from intracellular membranes (●) by three phase-partitioning steps. The microsomal fraction is loaded to a phase mixture (**step 1**). After mixing and separation, the upper phase is transferred to a fresh lower phase (**step 2**). The procedure is repeated (**steps 3** and **4**) to produce a final upper phase enriched in plasma membranes. The lower phase left in the first tube is enriched in intracellular membranes.

6. Repeat **steps 4** and **5** (**Fig. 1**, **step 3**).
7. Withdraw the final upper phase (**Fig. 1**, **step 4**) and dilute it to ca 70 mL (*see* **Note 9**) with resuspension medium (*see* **Subheading 2.1.**, **item 6**) in a 70-mL ultracentrifuge tube. This fraction is highly enriched in plasma membranes (PM), and these PM are mainly right-side out (apoplastic side out) vesicles.
8. Dilute the lower phase left in the first tube (**Fig. 1**, **step 4**) to ca 100 mL with resuspension medium (*see* **Subheading 2.1.**, **item 6**), and transfer 20 mL of these 100 mL to a 70-mL ultracentrifuge tube (*see* **Note 9**). This fraction is enriched in intracellular membranes. Discard the remaining 80 mL.
9. Transfer the remaining ca 1 mL of the MF that was not added to the phase system to a 70-mL ultracentrifuge tube. Dilute with resuspension medium (*see* **Subheading 2.1.**, **item 6**).
10. Pellet all membranes by centrifugation at 100,000*g* in an ultracentrifuge for 1 h.
11. Resuspend the pellets (use a small [ca 2 mL] glass homogenizer) in resuspension medium (*see* **Subheading 2.1.**, **item 6**) to a final volume of 0.5–1 mL and store the samples in liquid nitrogen until use (*see* **Notes 10** and **11**).

3.3. Inside-Out Plasma Membrane Vesicles Produced by Freeze/Thawing

1. The plasma membranes are frozen in liquid nitrogen and thawed in water of room temperature. This procedure will result in a PM preparation containing 40–50% inside-out vesicles (**Fig. 2**; *see* **Note 12**).
2. Further enrichment of inside-out vesicles may be obtained by subjecting the freeze/thawed plasma membranes to phase partitioning (*see* **Note 13**).

3.4. Inside-Out PM Vesicles Produced by Brij 58 Treatment

1. Brij 58 is added to the plasma membranes to reach a final concentration of 0.05% (w/v). This procedure will result in a plasma membrane preparation containing 100% inside-out vesicles (**Fig. 2**; *see* **Note 14**).

3.5. H^+-ATPase Assay

1. Mix in a cuvet: 0.82 mL of assay medium, 0.05 mL of plasma membranes (*see* **Subheading 2.5.**, **item 12**), 0.015 mL of coupling enzymes, 0.05 mL of substrate, and 0.05 mL of water (*see* **Note 15**).
2. After 5 min incubation at assay temperature, start recording the baseline.
3. About 1 min later, add 0.015 mL of $MgCl_2$ (*see* **Subheading 2.5., item 10**) to start ATP hydrolysis and H^+-pumping (*see* **Note 16**).

4. Notes

1. The casein hydrolysate is first dissolved in a small volume of water and boiled for 10 min under stirring. The casein hydrolysate serves as a competing substrate for proteases.
2. DTT and ascorbate are unstable in water (slowly oxidized) and are therefore added fresh at the time of usage.

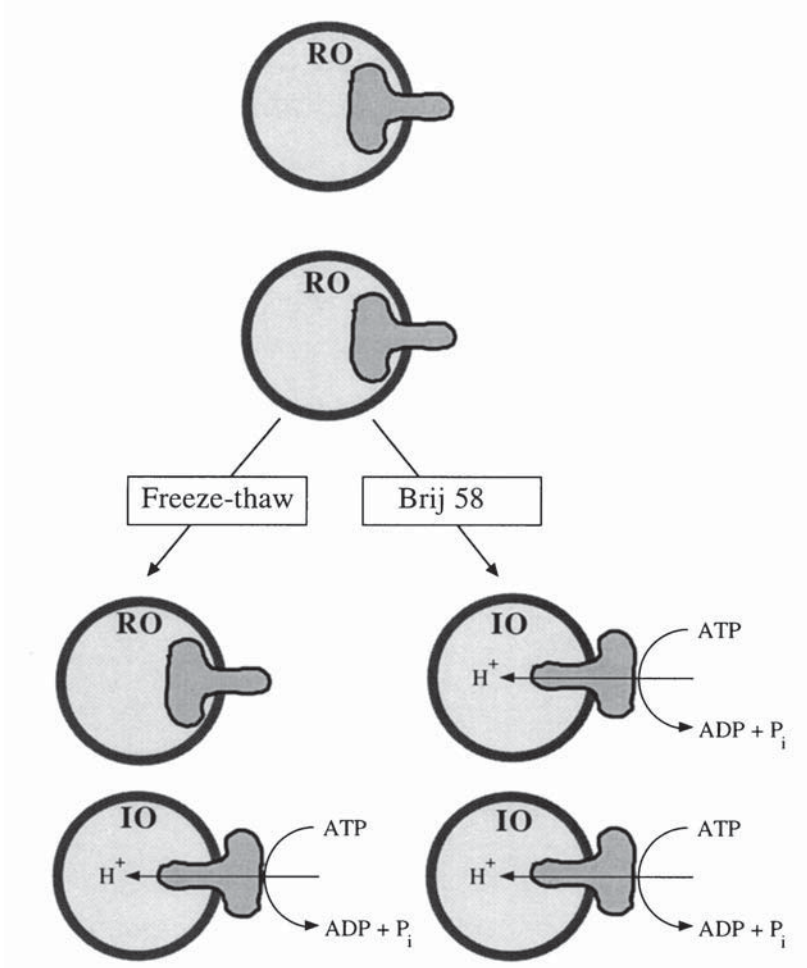

Fig. 2. Production of inside-out plasma membrane vesicles (IO) from right side-out vesicles (RO). With freeze/thawing, a mixed population of inside-out and right side-out vesicles is obtained. With Brij 58 treatment, all vesicles are turned inside out. The vesicles produced by both treatments are sealed and transport-competent.

3. PMSF is a protease inhibitor, and is poisonous. It is unstable in water solution but stable in isopropanol at room temperature for several months.
4. Add 0.2 *M* dipotassium phosphate to 0.2 *M* potassium diphosphate (ratio about 1/10) to obtain a 0.2 *M* stock solution of potassium phosphate, pH 7.8 .
5. See Chapter 3 for a detailed description on the preparation of the 20% (w/w) Dextran solution.
6. Phase partitioning is temperature-dependent, therefore, all solutions should be equilibrated at 4°C before use, and the manipulations done in the cold-room (or on ice, if a cold room is not available).

7. The final polymer concentrations of the described phase system are 6.5% (w/w) for both Dextran T500 and PEG 3350, and the chloride and phosphate concentrations are 5 m*M* each. This system is useful for many tissues, but the optimal concentrations for separation should be determined for each tissue studied. Usually, varying the polymer concentrations between 6.1 and 6.5%, and the KCl concentration between 1 and 5 m*M* is sufficient to find the optimal phase composition for yield and purity (as long as the phosphate concentration is held at 5 m*M*).
8. The bulk-phase system is made in a separating funnel, temperature equilibrated over night at 4°C, thoroughly shaken, and left for 4 h for phase separation to occur. The upper and lower phases are withdrawn (discard the interface) and stored at 4°C.
9. The polymers make the phases viscous, and particularly the dextran in the lower phase. Therefore the phases should be well-diluted (about three times for the upper phase, at least 10 times for the lower phase) to ascertain that all membrane vesicles will be sedimented in the subsequent ultracentrifugation step.
10. If the preparation will be used for production of inside-out PM vesicles by freeze/thawing (*see* **Subheading 3.3.**) the resuspension medium should also contain 50 m*M* KCl (*see* **Subheading 2.3.**, **item 1**).
11. Omit DTT in the resuspension medium if you intend to measure oxidoreductase activity. Note that for calculation of recoveries after phase partitioning, the volumes of all fractions have to be recorded. Purity, recovery, and contamination by other membranes in the PM fraction are judged by using appropriate markers. For an evaluation of these, see ref. 6. Vanadate inhibition of the H^+-ATPase (*see* **Subheading 3.5.**) (measured as, e.g., H^+ pumping in the presence of Brij 58) is a useful marker for the PM. The vanadate inhibited activity is determined by assaying ± 0.1 m*M* orthovanadate (made fresh and heated just before use).
12. The proportion of inside-out vesicles is determined by measuring the H^+-ATPase activity ± Brij 58 (*see* **Subheading 3.5.** and *see* **Notes 14** and **16**). With some material, several freeze/thaw cycles are necessary to obtain 40–50% inside-out vesicles.
13. The freeze/thawed PMs are subjected to phase-partitioning by essentially following the scheme previously outlined (*see* **Subheading 3.2.**). A smaller phase system (8–12 g final weight) is used and the lower phase in the first tube is successively extracted with a second and a third upper phase obtained from the bulk-phase system (*see* **Subheading 2.2.**, **item 8**). In this case, the lower phase left in the first tube will be enriched in inside-out vesicles and the right-side-out vesicles will be removed with the upper phases ***(3)***.
14. A concentration of Brij 58 of 0.05% is useful in the H^+-ATPase assay where the protein concentration is 0.05 mg/mL. If a higher protein concentration is used, the detergent/protein ratio of 10/1 should be kept. Brij 58 does not interfere with the H^+-ATPase assay but may interfere with other assays. Excess Brij may be removed by pelleting and resuspending the vesicles, with the inside-out orientation retained ***(4)***. Freezing the vesicles after removal of excess Brij will, however, result in a partial reversal of sidedness.

15. The assay medium should be kept at assay temperature (e.g., room temperature) and is stable for 2–3 h (ATP is the unstable component).
16. The true substrate for the ATPase is the Mg^{2+} complex of ATP. The reaction can be started by the addition of either ATP, if Mg^{2+} is already present, or Mg^{2+}, if ATP is present. We chose to have ATP present during incubation because ATP protects the ATPase from inactivation. In this assay, ATPase activity and H^+ pumping may be monitored simultaneously in the same cuvet ***(5)***. ATP hydrolysis is coupled enzymatically to oxidation of NADH, and the rate of ATP hydrolysis is measured as the absorbance decrease at 340 nm. H^+ pumping is measured as the absorbance decrease at 495 nm of the ΔpH probe acridine orange. By using a spectrophotometer capable of collecting data in the photometric mode at two different wavelengths with 6 cycles/min (e.g., a Shimadzu UV-1601), ATP hydrolytic activity and H^+ pumping may be monitored simultaneously. Alternatively, H^+ pumping is recorded during the first minute at 495 nm (the reaction is not linear, and the initial rate should be measured) and then ATP hydrolysis at 340 nm (linear for several minutes). The 0.05 mL water in the assay mixture may be replaced by Brij 58 to obtain 100% inside-out vesicles, or by some other effector (vanadate, ionophores, and so forth). Several effectors, including Brij 58, may be added during the run (after, e.g., 2 min recording of ATP hydrolysis). Final concentrations in the assay are: 10 m*M* MOPS/KOH, pH 7.0, 140 m*M* KCl, 2 m*M* ATP, 4 m*M* $MgCl_2$, 1 m*M* EDTA/DTT, 20 m*M* acridine orange, 0.25 m*M* NADH, 1 m*M* PEP, 25 μg mL^{-1} lactate dehydrogenase, 50 μg mL^{-1} pyruvate kinase.

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14

Isolation of Inside-Out Thylakoid Vesicles

Hans-Erik Åkerlund

1. Introduction

To understand the function of a biological membrane like that of chloroplast thylakoids, it is important to understand the arrangement of its different protein and lipid components. Preparations that have proven to be particularly suited for such studies are those consisting of membrane vesicles that are turned inside-out. Inside-out vesicles from the thylakoid membrane were first obtained from spinach chloroplasts by a combination of mechanical fragmentation and separation by aqueous two-phase partition ***(1,2)***. By the same or very similar procedures, inside-out thylakoid vesicles have now also been obtained from other plant sources such as pea ***(3)***, barley ***(4)***, mangrove *(Avicennia marina)* ***(5)***, lettuce ***(6)***, *Euglena gracilis* ***(7)***, cyanobacteria ***(8,9)*** and the photosynthetic bacteria *Rhodopseudomonas viridis* ***(10)***. Because the isolation procedure does not involve the use of detergents, the inside-out thylakoids have a preserved membrane structure and are ideally suited for structure–function studies. They have been used extensively in studies on thylakoid membrane topography ***(11–14)*** and for the identification of proteins associated with oxygen evolution ***(15,16)***. An important finding was that the inside-out vesicles are only formed from appressed (not exposed to stroma) thylakoid membranes, whereas right side-out vesicles derive from nonappressed (stroma exposed) membranes ***(17)***. As a result, the usefulness of inside-out vesicles could be extended to include studies on the lateral organization of the thylakoid membrane. The use of inside-out thylakoids in studies on structure and function of the thylakoid membrane has been reviewed by Andersson et al. ***(18)***.

The isolation of inside-out thylakoid vesicles can be divided principally into two steps; fragmentation and subsequent separation. The ionic conditions imposed during fragmentation have a profound influence on both the proper-

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ties and relative proportions of inside-out thylakoids and right side-out thylakoids *(17)*. Basically, three different fragmentation procedures have been described. The original procedure (*1*,*2*; *see* **Note 1**) gives rise to inside-out vesicles originating from the appressed thylakoid region, highly enriched in photosystem II, but with relatively low yield. The procedure that will be described here *(19)* gives inside-out vesicles in a high yield and somewhat lower enrichment in photosystem II. The inside-out vesicles obtained by these two procedures are particularly suited for studies on the lateral distribution of thylakoid components and for studies on the organization and function of photosystem II and its oxygen evolving system. The third procedure (*17*, *see* **Note 1**) gives inside-out vesicles with the same relative amounts of photosystem I and II as whole thylakoids and is therefore especially suited for studies on transverse arrangements of all components of the thylakoid membrane.

The key step for the isolation of inside-out vesicles from right side-out vesicles is aqueous polymer two-phase partition. This method separates membrane particles according to differences in surface properties such as charge and hydrophobicity. It is, therefore, ideally suited for separation of membrane vesicles that only differ in sidedness. This separation step is essentially the same for all three preparation procedures. The procedures described here were developed for thylakoids from spinach, but should be suitable for most other species too.

2. Materials

2.1. Thylakoid Isolation

1. Knife blender.
2. Nylon mesh (25-μm mesh size).
3. Preparation medium: 50 m*M* sodium phosphate buffer, pH 7.4, 10 m*M* $MgCl_2$, 300 m*M* sucrose.
4. 5 m*M* $MgCl_2$.
5. Wash medium: 50 m*M* sodium phosphate buffer, pH 7.4, 10 m*M* NaCl, 50 m*M* sucrose.
6. Press medium: 10 m*M* sodium phosphate buffer, pH 7.4, 5 m*M* NaCl, 2 m*M* $MgCl_2$, 100 m*M* sucrose.

2.2. Generation of Inside-Out Thylakoid Vesicles

1. Yeda-press (Yeda, Rehovot, Israel) and a nitrogen gas tube (*see* **Note 2**).
2. 100 m*M* ethylene diamine tetraacetic acid (EDTA), adjusted to pH 7.4 with NaOH.

2.3. Phase Partition

1. Polyethylene glycol (PEG) 3350 (earlier named PEG 4000), 40% (w/w) stock solution: Dissolve 400 g of PEG (obtainable as Carbowax 3350 from Union Carbide, NY) in 600 g of distilled water.

2. Dextran T500, 20% (w/w) stock solution: 220 g of dextran powder (obtainable from Amersham Pharmacia Biotech, Uppsala, Sweden) is layered on 780 g of water. This is then heated in a boiling water bath with occasional gentle stirring until the dextran has dissolved. Because the dextran powder contains some water, the concentration of this solution will not be 22% but approx 21% (w/w). The concentration of the solution is determined polarimetrically (*see* Chapter 3), and the stock solution can then be adjusted to 20% with water. If a polarimeter is not available, the approximate value of 21% given previously can be used. However, in this case, care should be taken to minimize the dissipation of water vapor during the heating. A frozen dextran solution is stable for at least 1 yr.
3. Phase buffer: 50 m*M* sodium phosphate buffer, pH 7.4; 25 m*M* NaCl; 100 m*M* sucrose.
4. Basic phase system: Mix in a tube for a swingout centrifuge rotor 7.13 g of 20% Dextran T500 stock solution, 3.56 g of 40% PEG 3350 stock solution, and 4.8 mL of the phase buffer. Add water up to 24 g.
5. Pure upper and lower phases: The pure phases required for the washing steps are obtained as follows. A 250-g two-phase system is prepared in a separatory funnel by mixing 71.3 g of 20% Dextran T500 stock solution, 35.6 g of 40% PEG 3350 stock solution, 50 mL of the phase buffer, and water up to 250 g. The final concentrations of the phase components are 5.7% (w/w) dextran, 5.7% (w/w) PEG, 5 m*M* NaCl, 20 m*M* sucrose, and 10 m*M* sodium phosphate, pH 7.4.
   The phase system is adjusted to 4°C, mixed and allowed to settle. The two phases are collected separately and the interface discarded (*see* **Note 3**).

3. Methods

3.1. Thylakoid Isolation

All work is performed at 0–4°C unless otherwise stated.

1. Homogenize spinach leaves (lots of 25 g) for 5 s at maximum speed in a knife blender containing 100 mL of preparation medium (*see* **Note 4**). Each lot of 25 g will give the material roughly required for one basic phase system.
2. Filter the slurry through four layers of nylon mesh (25 μm).
3. Collect the chloroplasts by centrifugation at 1000*g* for 1 min.
4. To concentrate the chloroplasts, resuspend each pellet in 5–10 mL of preparation medium and centrifuge at 1000*g* for 10 min.
5. Resuspend the pellet in 5 m*M* $MgCl_2$ (same volume as above) to hypotonically break the envelope of intact chloroplasts. Homogenize the suspension (glass-glass homogenization) and centrifuge at 2000*g* for 5 min.
6. Wash the pellet twice with wash medium to free the released thylakoids from stroma components and centrifuge at 2000*g* for 5 min.
7. Resuspend the pellet in wash medium (same volume as above) once more. Note the volume and determine the concentration of chlorophyll (*see* **Note 5**) and centrifuge at 2000*g* for 5 min.
8. Resuspend the thylakoids in press medium to a concentration of 4 mg chlorophyll/mL, using the value obtained above under **step 7** and assuming that the volume of the pellet is roughly 0.06 mL/mg chlorophyll in this medium (*see* **Note 1**).

3.2. Generation of Inside-Out Thylakoid Vesicles

1. Pass the thylakoid suspension dropwise through the Yeda press at 10 MPa (*see* **Note 6**). Repeat once.
2. The homogenate is made 2 m*M* with respect to EDTA.
3. Pass the material twice more through the Yeda press (*see* **Note 7**).
4. Centrifuge the material at 1000*g* for 10 min to remove starch grains and unfragmented material (*see* **Note 8**). The supernatant (Yeda-press homogenate) is used for phase partition.

3.3. Phase Partition

1. Add 1 mL of the Yeda-press homogenate to each tube of the preformed basic phase system. Keep the temperature of the phase system at 4°C to obtain reproducible results (*see* **Note 9**).
2. Cover and tighten the tubes with flexible plastic film (e.g. Parafilm or Nescofilm) or use tube caps and mix the phase system by 50 inversions.
3. Centrifuge at 1500*g* for 3 min in a swing-out rotor (*see* **Note 10**). The inside-out vesicles will partition predominantly to the lower phase while the right side-out material prefers the upper phase. The amount of material found in the lower phase and interface together should be roughly (by eye) 60% on a chlorophyll basis. If this is not the case, adjust the polymer concentrations (*see* **Note 9**).
4. Improve purity:
   a. To improve the purity of the inside-out thylakoids, remove the upper phase and add 13 mL of new pure upper phase to the lower phase. Repeat the mixing and settling procedure (**steps 2–3**). This washing step is usually repeated twice (*see* **Note 11**). After the last partition step, remove the material at the interface together with the upper phase.
   b. The right side-out material partitioning to the upper phase can be purified in a manner corresponding to that for the the inside-out material. Remove the upper phase after the first partition step and transfer to 9 mL of a new, pure lower phase. Repeat the mixing and settling procedure (**steps 2–3**). This washing step is usually repeated twice.
5. Removal of polymers: Although the thylakoid vesicles in the phase system can be used directly for activity measurements, most studies require the removal of the polymers and the thylakoid material has to be concentrated. Dilute the lower phase at least threefold with buffer solution, and the upper phase at least twofold with buffer to reduce the viscosity, and then centrifuge at 100,000*g* for 60 min to sediment the vesicles (*see* **Note 12**).
6. In order to determine whether the isolation has been successful, the properties of the isolated thylakoid vesicles are determined (*see* **Note 13**).

4. Notes

1. With slight modification, two alternative types of fractionations can be made.
   a. Inside-out vesicles highly enriched in photosystem II: Thylakoids isolated according to **Subheading 3.1.**, **step 1–7** are resuspended in 50 m*M* sodium

phosphate, pH 7.4, 150 m*M* NaCl, at a concentration of 0.2–1 mg chlorophyll/mL, and passed dropwise twice through a Yeda press at a nitrogen gas pressure of 10 MPa. The press homogenate is centrifuged at 40,000*g* for 30 min. The supernatant, which contains stroma lamella membranes highly enriched in photosystem I, is carefully removed. The sediment, containing 90–95% of the starting material, is drained carefully to reduce carryover of excess salt and is resuspended in 10 m*M* sodium phosphate buffet, pH 7.4, 5 m*M* NaCl, and 100 m*M* sucrose to a concentration of 1 mg chlorophyll/mL, and then passed twice more through the Yeda press. Centrifuge the material at 1000*g* for 10 min to remove starch grains and unfragmented material. The supernatant is used for phase partition. However, in this case, 5 mL sample should be added to the basic phase system (**Subheading 2.3., item 4**) modified by taking 4 mL of phase buffer (**Subheading 2.3., item 3**), instead of 4.8 mL, and water to 20 g instead of 24 g. Other steps are the same.

b. Inside-out vesicles with both photosystem I and II: Thylakoids isolated according to **Subheading 3.1., step 1–7** are resuspended in 10 m*M* Tricine, pH 7.4, 100 m*M* sucrose and incubated in this medium for 1.5 h to allow complete destacking and lateral randomization of membrane components. The pH is adjusted to 4.7, using 0.01–0.1 *M* HCl, to reduce electrostatic repulsion and create artificial membrane pairing. The material is then passed twice through the Yeda press at 10 MPa, followed immediately by readjustment of the pH back to 7.4 using 0.1 *M* NaOH. The homogenate is centrifuged at 40,000*g* for 30 min. The sediment is resuspended in 10 m*M* sodium phosphate, pH 7.4, 5 m*M* NaCl buffer, 100 m*M* sucrose to a concentration of 1 mg chlorophyll/mL, and then passed twice more through the Yeda press. Centrifuge the material at 1000*g* for 10 min to remove starch grains and unfragmented material. The supernatant is used for phase partition. Also, in this case, 5 mL sample should be added to the basic phase system (**Subheading 2.3., item 4**), modified by taking 4 mL of phase buffer (**Subheading 2.3., item 3**), instead of 4.8 mL, and water to 20 g instead of 24 g. Other steps are the same.

2. In the Yeda press, the liquid is forced through a needle valve by a gas pressure and the thylakoids are broken by shearing forces. Alternatives to the Yeda press for the thylakoid fragmentation is the use of a French pressure cell ***(4)*** or sonication. However, these methods appear less reproducible and care must be taken to avoid extensive fragmentation.
3. If only inside-out thylakoids are to be isolated, an alternative way to obtain pure upper phase, which consumes less dextran, is to make a phase system of 1.5% (w/w) dextran; 7.5% (w/w) PEG;10 m*M* sodium phosphate, pH 7.4; 5 m*M* NaCl; 20 m*M* sucrose. This phase system has a large upper phase with the same composition as the former one.
4. Any other isolation procedure for thylakoid isolation may be used. However, if the medium used for isolation of the intact thylakoids contains divalent cations it is necessary to wash the thylakoids in a medium composed of 50 m*M* sodium

phosphate, pH 7.4; 10 m*M* NaCl; 50 m*M* sucrose prior to the resuspension for Yeda press treatment. This is done to avoid carryover effects, owing to the enrichment of divalent cations at the negatively charged membrane surface, which may alter the balance between Mg^{2+} and EDTA.

5. A crude estimate of chlorophyll is obtained by measuring the absorbance at 680 nm (1 cm) in water and multiplying with the factor 15 (μg/mL). A more accurate method is to make a determination in 80% (v/v) acetone ***(21)***. However, polymers have to be removed before addition of acetone to avoid precipitation.
6. At the end of the press treatment, the nitrogen gas will flow very quickly out of the Yeda press. The outlet should therefore not be directed straight down into the collecting tube, otherwise the gas stream may empty the collecting tube.
7. The first pair of Yeda press treatments in each procedure is the key step for the formation of the inside-out thylakoids. The idea behind the second pair of press treatments is to convert material, that was only partly fragmented in the first treatment, into right-sided vesicles. This improves the following separation steps.
8. The time has to be reduced if partly filled tubes are centrifuged.
9. The partition can be controlled through changes in (1) polymer concentrations; (2) type and concentration of salt; and (3) the temperature at which the partition is performed.

   These are the most critical factors in the procedure described and it is therefore important to know how they influence the partition. Increasing the concentration of both Dextran and polyethylene glycol generally favors the collection of particles at the interface or in the lower phase. Conversely, lowering the polymer concentrations will favor the collection of material in the upper phase. Different ions have different affinities for the two phases, and inclusion of salt in the phase system therefore creates a Donnan type of electric potential between the two phases. The interfacial potential is determined by the dominating salt and is usually different for different salts. For the phase system applied to isolate inside-out thylakoids, an increase in NaCl concentration or a decrease in sodium phosphate concentration will favor the partition of the material to the lower phase. A change in pH will also have an influence because both the ratios between different ionic species and the membrane charge will change. An increase in the temperature at which the partition is performed generally favors collection of the material in the upper phase.
10. The centrifugation is used to speed up the settling of the phases, and the best result is obtained with a swing-out rotor. However, if not available, the tubes can be allowed to settle at 4°C without centrifugation, which will take about 10–30 min. Alternatively, an angle rotor could be used, but the speed or time should be reduced compared to the swing-out case as the settling distance will be shorter.
11. The purity of the inside-out thylakoids can be further improved at the expense of yield by repeated extraction of the lower phase with fresh upper phase. Lowering the concentration of both polymers, to 5.55%, for example, will have similar effect.
12. An alternative method is to add 15 mL of pure upper phase adjusted to room temperature. In this case, a phase system at 10–15°C is created in which, after

mixing and settling, the thylakoid material is collected in the upper phase. The upper phase is removed, diluted at least twofold, and centrifuged as noted previously. The advantage is that the thylakoid material is obtained in a smaller volume, which facilitates the centrifugal step. The sedimentation of the thylakoid material can be obtained already at 40,000*g* for 30 min if 10 m*M* $MgCl_2$ is included in the medium.

13. Test of result:
    a. As an indicator of sidedness of the isolated vesicles, the direction of pH changes associated with photosynthetic electron transport reaction is determined. The membranes are first freed from buffer by two consecutive centrifugations at 100,000*g* for 30 min using unbuffered 10 m*M* NaCl. Light-induced pH changes are measured in a thermostatted vessel (20°C) using a combined glass electrode with a medium composition of 10 m*M* NaCl, 0.4 m*M* phenyl-*p*-benzoquinone (electron acceptor, 10 m*M* dissolved in dimethyl sulfoxide [DMSO]), and thylakoid material corresponding to 50–100 μ*M* chlorophyll/mL. A magnetic stirrer can be used for homogenous mixing. The initial pH is adjusted to 6.5 with diluted NaOH or HCl. Light of sufficient intensity is obtained if two projectors are placed one on each side of the reaction vessel. For inside-out thylakoids a light-induced pH decrease in the external medium is expected. A limitation with this method is that it only indicates whether right side-out or inside-out thylakoids are dominating the preparation. A quantitative determination can be made by freeze-fracture electron microscopy ***(20)***.
    b. Because the inside-out vesicles obtained by the methods described should be enriched in photosystem II, this can be used as an indirect criterion for a successful isolation of these vesicles. The most convenient assay for this enrichment is to measure the chlorophyll *a*/*b* ratio in 80% acetone (*see* **Note 1**). The inside-out vesicles should have chlorophyll *a*/*b* ratios 0.8–0.9 units lower than the original thylakoids, i.e., 2.3–2.4 if the original thylakoids have a value of 3.2 (For the third procedure, the chlorophyll *a*/*b* ratio should not be more than 0.1 units lower than for the original thylakoids).

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Two-Phase Partitioning as a Method for Isolation of Tight Plasma Membrane Vesicles from *Saccharomyces cerevisiae* and from *Chlamydomonas reinhardtii*

Birgitta Norling

1. Introduction

Plasma membranes from various sources, e.g., higher plants ***(1)***, green algae ***(2)***, cyanobacteria ***(3),*** and yeast ***(4,5)*** have, during the last two decades, been isolated in pure state by the two-phase partitioning method. The aqueous two-phase method is, in addition to being mild, rapid, and reproducible, unique in its ability to separate organelles and membrane vesicles according to surface properties. By this method, membrane vesicles with the same density but different orientation can be separated. These vesicles can be used to establish model systems for studies of transport processes across the plasma membrane. In such membrane vesicles, the mechanism of energy coupling to secondary transport of solutes can be studied in the absence of cellular metabolism, which may otherwise influence the transport mechanism in a complex manner. Furthermore, individual transporters can be characterized in plasma membrane vesicles derived from different mutant strains. By selective disruption of the genes encoding various transporters, the functional properties of the remaining transporters in the membrane can be characterised without laborious purification and reconstitution into liposomes. Thus, isolated plasma membrane vesicles allow for characterization of the individual transporters devoid of their genetic and metabolic regulation.

This chapter decribes how two-phase partitioning can be used effectively for isolation of pure and well-sealed plasma membrane vesicles from yeast and from green algae. The method can be exploited for separation of inside-out and right side-out vesicles.

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1.1. Saccharomyces cerevisiae *Membrane Vesicles*

The difficulties of preparing well-sealed plasma membrane vesicles of defined orientation from yeast have been recognized for a long time ***(6)***. The two-phase partitioning procedure is based upon spheroplasts prepared from wild type *S. cerevisiae* by zymolyase treatment followed by an osmotic shock. After differential centrifugation the membrane fraction enriched in plasma membranes is added to a two-phase system, the composition of which has been optimized for purification of the plasma membranes. It has been found that in a two-phase system of relatively low polymer concentration, 5.4% of both Dextran T-500 and polyethylene glycol 3350, and at relatively high temperature 7–8°C, inside-out-oriented plasma membrane vesicles have a high affinity for the lower phase. Two repartitionings of the lower phase with pure upper phase result in plasma membranes completely free of other cellular components ***(5)***.

1.2. Chlamydomonas reinhardtii *Membrane Vesicles*

The algae *Chlamydomonas reinhardtii* offers an excellent unicellular eukaryotic system, in which genetical analysis of mutants can be combined with biochemical and biophysical techniques. It has been much used as a model system in molecular plant research. The procedure presented here is based on cell breakage, differential centrifugation, and aqueous two-phase partitioning ***(2)***. The *CW15* strain of *C. reinhardtii* is a cell-wall mutant, which can be easily broken mechanically. Two low-speed centrifugations remove most of the chloroplasts and mitochondria, the low-density membranes, including plasma membranes, are collected by centrifugation and added to a two-phase system. The two-phase system used is similar to the one used for isolation of plasma membranes from higher plants ***(1)*** and consists of 6.5% (w/w) of both polymers, DextranT-500, and polyethylene glycol 3350, in the presence of potassium chloride and is performed at 4°C. Well-sealed plasma membrane vesicles of right side-out orientation have a high affinity for the upper phase and can be recovered, after repeating the two-phase partitioning twice, in a state virtually free of other membranes and organelles, as judged by measurements of marker enzymes and chlorophyll ***(2)***.

The results presented here on yeast and green algae, as well as what is known for higher plants ***(1,9)***, show that two-phase systems based on Dextran T-500, polyethylene glycol 3350, and potassium phosphate buffer offer an excellent method for separation of inside-out and right side-out plasma membrane vesicles. Regardless of the eukaryotic organism, right side-out plasma membrane vesicles have a very high affinity for one extreme of the two-phase system, i.e., the upper phase at very high polymer concentration whereas the inside-out plasma membrane vesicles have a high affinity for the other extreme, i.e., the lower phase at low polymer concentration.

2. Materials

2.1. S. cerevisiae *Membrane Vesicles*

2.1.1. S. cerevisiae: *Cell Growth and Preparation of Total Membrane Fraction*

1. *S. cerevisiae*: wild-type strain (*CW04* or equivalent) or mutant strains to be characterized.
2. Growth medium: commonly used rich yeast growth medium of choice (*see* **Note 1**).
3. Buffer 1: 5 m*M* Tris-HCl, pH 7.5, 700 m*M* sorbitol.
4. Zymolyase: 20,000 units/g (ICN Biomedicals, Costa Mesa, CA, USA).
5. Dithiothreitol (DTT).
6. Buffer 2: 15 m*M* MES-Tris, pH 6.5, 500 m*M* sorbitol, 100 m*M* glucose.
7. 0.2 *M* phenylmethylsulfonyl fluoride (PMSF) (*see* **Note 2**).
8. Buffer 3: 25 m*M* MES-Tris, pH 6.5, 5 m*M* ethylene diamine tetraacetic acid (EDTA), 0.2% bovine serum albumin (BSA) (fatty-acid free), 0.2% casein hydrolysate (Difco, Detroit, MI), 1 m*M* DTT and 1 m*M* PMSF.

2.1.2. S. cerevisiae*: Two-Phase Partitioning*

1. Two-phase buffer (4 × concentrated): 1.32 *M* sucrose, 20 m*M* potassium phosphate buffer, pH 7.8, 4 m*M* EDTA.
2. Two-phase buffer: 0.33 *M* sucrose, 5 m*M* potassium phosphate buffer, pH 7.8, 1 m*M* EDTA.
3. 20% (w/w) Dextran T-500 (Amersham Pharmacia Biotech, Uppsala, Sweden).
4. 40% (w/w) polyethylene glycol 3350 (PEG) (Union Carbide, New York).
5. 0.1 *M* DTT.
6. Dilution buffer: 0.33 *M* sucrose, 15 m*M* MES-Tris, pH 6.5, 1 m*M* DTT and 1 m*M* PMSF.
7. One sample tube and two repartitioning tubes are prepared according to **Table 1** (*see* **Notes 3** and **4**).

2.2. C. reinhardtii *Membrane Vesicles*

2.2.1. C. reinhardtii*: Cell Growth and Preparation of Total Membrane Fraction*

1. *C. reinhardtii* strain *CW15*.
2. Growth medium: Tris-acetate-phosphate medium (TAP-medium) (*see* **Note 5**).
3. 20 m*M* HEPES-KOH, pH 7.5.
4. 0.2 *M* PMSF in methanol (*see* **Note 2**).
5. Breaking medium: 0.25 *M* sucrose, 50 m*M* MOPS-KOH, pH 7.8, 3 m*M* EDTA, 0.5 m*M* EGTA, 5 m*M* $MgCl_2$, 1% (w/v) BSA, 5 m*M* mercaptoethanol, and 0.5 m*M* PMSF.
6. Waring blender.

2.2.2. C. reinhardtii*: Two-Phase Partitioning*

1. Two-phase buffer (4 × concentrated): 1.0 *M* sucrose, 20 m*M* potassium phosphate buffer, pH 7.8.

Table 1
Preparation of Phase Systems for Purification of Plasma Membrane Vesicles from *S. cerevisiae*

Stock solution	Sample tube (10 g)	Repartitioning tube (10 g)
20% Dextran	2.700 g	2.700 g
40% PEG	1.350 g	1.350 g
Two-phase buffer (4 × conc.)	1.400 g	2.500 g
0.1 *M* DTT	0.100 g	0.100 g
H_2O	—	3.300 g
0.2 M PMSF	0.050 g	0.050 g
Membranes (in two-phase buffer)	4.400 g	—

2. Two-phase buffer: 0.25 *M* sucrose, 5 m*M* potassium phosphate buffer, pH 7.8.
3. 20% (w/w) Dextran T-500 (Amersham Pharmacia Biotech).
4. 40% (w/w) polyethylene glycol 3350 (PEG) (Union Carbide).
5. 0.5 *M* KCl.
6. Diluting buffer: 0.33 *M* sucrose, 50 m*M* MOPS-KOH (pH 7.0), 4 m*M* $MgSO_4$ and 1 m*M* PMSF.
7. One sample tube (20 g) and two repartitioning tubes (20 g) are prepared according to **Table 2** (*see* **Notes 3** and **6**).

3. Methods

3.1. S. cerevisiae *Membrane Vesicles*

3.1.1. S. cerevisiae*: Cell Growth and Preparation of Total Membrane Fraction*

1. *S. cerevisiae CW04* is grown aerobically at 30°C to an A_{600} from 0.7 to 0.9 (*see* **Note 7**).
2. Cells from a 2-L culture are harvested by centrifugation at 3000*g* for 5 min and the cell mass (gram wet weight) is determined.
3. The cells are suspended in buffer 1 to a volume corresponding to an absorbance at 800 nm of 0.6 when diluted 10 times.
4. Zymolyase, 4 mg/g cells, and DTT, final concentration 6.5 m*M* are added.
5. The cell suspension is incubated with shaking for 1 h at 30°C.
6. After having verified the completion of spheroplast formation (*see* **Note 8**), the spheroplasts are washed twice by suspension in 50 mL of buffer 2 and centrifugation at 5000*g* for 5 min.
7. The washed spheroplasts are suspended in 20–30 mL of buffer 2 and incubated for 10 min at 30°C and then placed on ice.
8. The spheroplasts are subjected to osmolysis by the addition of three volumes of ice-cold buffer 3 (60–90 mL) and homogenization in a Potter Elvenhjelm-type homogenizer by five gentle strokes.

Table 2
Preparation of Phase Systems for Purification of Plasma Membrane Vesicles from *C. reinhardtii*

Stock solution	Sample tube (20 g)	Repartitioning tube (20 g)
20% Dextran	6.500 g	6.500 g
40% PEG	3.250 g	3.250 g
Two-phase buffer (4 × conc.)	3.438 g	5.000 g
0.5 *M* KCl	0.160 g	0.160 g
H_2O	0.302 g	4.990 g
0.2 *M* PMSF	0.100 g	0.100 g
Membranes (in two-phase buffer)	6.250 g	—

9. By centrifugation at 3300*g* for 10 min unbroken cells, cell debris, and a large fraction of the mitochondria are obtained as a pellet.
10. The supernatant is centrifuged at 73,000*g* for 30 min for collection of the lighter membrane fraction, consisting of plasma membranes and other subcellular membranes. The pelleted membranes are homogenized in 4.4 mL of two-phase buffer (*see* **Note 9**).

3.1.2. S. cerevisiae*: Two-Phase Partitioning*

1. For a 2-L culture, one tube of a 10 g two-phase system is needed for the sample and two tubes for repartitioning. Add the membranes, 4400 g, (from **step 10**, **Subheading 3.1.1.**) to the sample tube.
2. The tubes are placed in a water bath of 7±1°C to be equilibrated (*see* **Note 10**).
3. Turn the tubes gently upside-down 35 times (*see* **Note 10**).
4. Centrifuge at 1000*g* for 15 min in a rotor cooled to 7±1°C.
5. Two phases are obtained in the sample tube containing the membranes as well as the two repartitioning tubes (*see* **Note 11**).
6. The upper phase of the sample tube is discarded, and to the lower phase, enriched in inside-out plasma membrane vesicles, is added a new upper phase from the repartitioning tube.
7. Partition is performed according to **steps 2–4**.
8. One more repartitioning of the lower phase is performed in a similar way.
9. The third lower phase containing pure tight inside-out plasma membrane vesicles is diluted at least eightfold with dilution buffer and centrifuged at 125,000*g*, 4°C, for 30 min.
10. Homogenize the pellet consisting of inside-out plasma membrane vesicles in a small volume of the dilution buffer (*see* **Note 12**).

3.2. C. reinhardtii *Membrane Vesicles*

3.2.1. C. reinhardtii*: Cell Growth and Preparation of Total Membrane Fraction*

1. *Chlamydomonas* cells are grown at 25°C with magnetic stirring and supplied with a mixture of 5% CO_2 in air under continuous light (*see* **Note 13**).

2. The cells (10 L) are harvested at approx 20 μg chlorophyll/mL by centrifugation at 3000*g*, 20°C, for 8 min (10–18 g wet weight).
3. The cells are resuspended once in 20 m*M* HEPES-KOH , pH 7.5, and centrifuged as above (*see* **Note 14**).
4. The cells are resuspended in 320 mL of ice-cold breaking medium.
5. The cells are broken mechanically in a precooled Waring blender at 0°C for 2 × 5 s (*see* **Note 15**).
3. By centrifugation at 1200*g* for 2.5 min unbroken cells, cell debris, and a large fraction of the chloroplasts are obtained as a pellet.
4. The supernatant is centrifuged at 13,000*g*, 4°C, for 5 min, resulting in a pellet containing mainly mitochondria and broken chloroplasts.
5. The supernatant from **step 4** is centrifuged at 125,000*g*, 4°C, for 1 h resulting in a pellet containing the low density membranes, including plasma membranes.
6. The pellet is homogenized in about 6.5 mL of two-phase buffer (*see* **Note 9**).

3.2.2. C. reinhardtii*: Two-Phase Partitioning*

1. For a 10-L culture, one tube of a 20-g two-phase system is needed for the sample and two tubes for repartitioning. Add membranes, 6.250 g, (from **step 6**, **Subheading 3.2.1.**) to the sample tube.
2. The tubes are placed in a water bath of 4 ± 1°C to be equilibrated (*see* **Note 10**).
3. Turn the tubes gently upside-down 35 times (*see* **Note 10**).
4. Centrifuge at 1000*g* for 4 min in a rotor cooled to 4 ± 1°C.
5. Two phases are obtained in both the sample tube containing the membranes and the two transfer tubes (*see* **Note 16**). Remove the upper phase from the repartitioning tube.
6. The upper phase of the sample tube, which is highly enriched in right side-out plasma membrane vesicles, is transferred to a repartitioning tube containing the lower phase and repartitioned as described in **steps 2–4**.
7. Another repartitioning of the upper phase is performed in a similar way.
8. The purified right side-out plasma membrane vesicles in the third upper phase is diluted 7–10 times with diluting buffer, and centrifuged at 125,000*g* for 1 h.
9. The pellet consisting of right-side out plasma membrane vesicles is homogenized in a small volume of diluting buffer (*see* **Note 17**).
10. The isolated tight right-side-out vesicles can be inverted to tight inside-out membrane vesicles by incubation of the vesicles (0.05–0.07 mg protein/mL) with 0.05% Brij 58 for 5 min (*see* **Note 18**).

4. Notes

1. Both high and low phosphate medium can be used in this work *(7)*.
2. Phenylmethylsulfonyl fluoride (PMSF) should be dissolved in methanol, freshly prepared and added to all solutions just before use.
3. Plastic tubes with a cap should be used. If tubes with conical bottom are used be aware that the concentrated polymers do not form a plug in the bottom. The repartitioning tubes and sample tube (without membranes) can be prepared in advance and kept in the refrigerator. PMSF is added just before use.

4. The final concentration of the two-phase system is 5.4% (w/w) of both Dextran T-500 and PEG 3350, 0.33 *M* sucrose, 5 m*M* potassium phosphate buffer, pH 7.8, 1 m*M* EDTA, 1 m*M* DTT, and 1 m*M* PMSF.
5. TAP-medium (per liter): 0.4 g NH_4Cl, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.05 g $CaCl_2 \cdot 2H_2O$, 0.108 g K_2HPO_4, 0.056 g KH_2PO_4, 2.42 g Tris, 1 mL glacial acetic acid, 50 μg NaEDTA, 22 μg $ZnSO_4 \cdot 7H_2O$, 11.4 μg H_2BO_3, 5.06 μg $MnCl_2 \cdot 4H_2O$, 4.99 μg $FeSO_4 \cdot 7H_2O$, 1.61 μg $CoCl_2 \cdot 6H_2O$, 1.57 μg $CuSO_4 \cdot 5H_2O$, 1.10 μg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 16 μg KOH ***(10)***.
6 The final concentration of the two-phase system is 6.5% (w/w) of both Dextran T-500 and PEG 3350, 0.25 *M* sucrose, 5 m*M* potassium phosphate buffer, pH 7.8, 4 m*M* KCl, and 1 m*M* PMSF.
7. A 2-L culture is grown in a 5-L Erlenmeyer flask on a shaker.
8. Spheroplast formation is verified by removing an aliquot from the suspension, diluting 10-fold in 10% sodium dodecyl sulfate (SDS), and microscopically examining the suspension for the presence of cells.
9. Remove all the supernatant carefully before homogenizing the pellets in two-phase buffer.
10. The whole two-phase partitioning is performed in a cold room. While turning the tubes upside down, hold cap of the tube to avoid heating the tube with your fingers. It is very important to maintain the temperature at which the partitioning is decided to be performed. Check the temperature with a thermometer inside the tube and allow only the range of ±1°C of the chosen temperature.
11. The volume of the lower phase is about 4.5 mL. If another volume is obtained, this may be owing to incorrect composition or temperature of the two-phase system. The lower phase of the repartitioning tubes is usually a little cloudy.
12. The purity of the isolated plasma membranes can be verified by use of marker enzyme activities of other cellular components. The isolated plasma membrane vesicles show ATPase activity in the absence of detergents indicating an inside-out orientation. The vesicles are demonstrated to be tight, since they catalyze ATP-dependent H^+-pumping as shown by the ΔpH sensitive optical probe acridine orange ***(5)***. Both the ATPase activity and the ATP-dependent H^+-pumping are inhibited by vanadate demonstrating that the enzyme is a P-type ATPase.
13. Cultures are grown as 5 L batches in flasks with a diameter of 17 cm.
14. Use a small paint brush to resuspend the cells.
15. This should be done in the cold room. Allow to cool for 1 min between the two treatments.
16. The volume of the lower phase is about 7 mL and the upper phase 11.5 mL. If other volumes are obtained, this may be the result of incorrect composition or temperature of the two-phase system. The lower phase of the repartitioning tubes are usually a little cloudy.
17. The isolated plasma membrane vesicles contain P-type ATPase as demonstrated by Western blots carried out with antibodies prepared against *Arabidopsis* P-type ATPase. The ATPase activity of the isolated plasma membrane vesicles is latent and can be stimulated 10–20-fold with detergents, indicating that the vesicles are

tightly sealed and of right side-out orientation, making the ATPase inaccessible to the hydrophilic substrate ATP. The detergent acivated activity is inhibited by vanadate, a specific inhibitor of P-type ATPase. The mechanical breaking of *C. reinhardtii* cells also produces inside-out plasma membrane vesicles, which are partitioned into the lower phase (together with the cell organells), because nonlatent vanadate sensitive ATPase activity can be found in the first lower phase. By comparing the total vanadate-sensitive ATPase activity, it is found that mechanical breaking produces about equal amount of inside-out and right side-out plasma membrane vesicles ***(2)***.

18. In the presence of the detergent Brij 58, the isolated plasma membranes catalyze ATP dependent H^+-pumping as shown by the ΔpH sensitive optical probe acridine orange ***(2)***. Addition of Brij 58 has been shown to produce 100% sealed inside-out vesicles of plant plasma membranes ***(8)***.

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Purification of Cyanobacterial Thylakoid, Plasma, and Outer Membranes by Two-Phase Partitioning

Birgitta Norling

1. Introduction

Cyanobacteria, as gram-negative bacteria, are surrounded by a cell envelope consisting of the outer membrane and the plasma membrane with a peptidoglycan layer in between. However, cyanobacteria, is a unique prokaryote, because in addition to the cell envelope, there is an inner cytoplasmic membrane system—namely the chlorophyll-containing thylakoid membranes—which perform oxygenic photosynthesis, the most fundamental life process on the earth ***(1)***. This organism is also interesting from the evolutionary point of view, because it arose in a very ancient age and has survived in various environments. Furthermore, chloroplasts are believed to have evolved from cyanobacterial ancestors that developed an endosymbiotic relationship with an eukaryotic host cell.

Preparation of membranes from cyanobacterial cells is accomplished mostly by disruption of spheroplasts (after lysozyme treatment of the cells) by French press. The major part of the total membranes are the thylakoid membranes, which, in many species of cyanobacteria, are seen as concentric layers in the cytoplasm. The outer membrane and the plasma membrane constitute only a minor part. In sucrose gradients, total cyanobacterial membranes have been shown to contain membranes of different density and pigment content ***(2–4)***. In addition to the chlorophyll-containing thylakoid membranes, a lighter and a heavier membrane fraction containing not chlorophyll, but carotenoids, are found. The lighter membrane fraction, as well as the chlorophyll-containing thylakoid membranes, contain a large number of different proteins but with different polypeptide compositions. This is revealed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, whereas the heavier membrane

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fraction contains only very few proteins. It was concluded that the lighter and heavier membrane fractions constitute the plasma and the outer membranes, respectively. Plasma membranes *(5)* and—to a lesser extent—outer membranes *(2,6)* from different cyanobacterial strains have been characterized. In order to be able to separate the three different membranes, the optimal conditions for spheroplast formation by lysozyme, as well as the optimal composition of the density gradient (mostly discontinous), had to be found for each species. Also, the pressure used with the French press varied for each strain.

Although it is possible to obtain pure plasma membranes from sucrose gradients, the preparation procedure has obvious disadvantages: it displays low yield, involves a time-consuming equilibrium centrifugation step, and cannot easily be performed in large scale. These disadvantages have been overcome by a method based on partitioning in aqueous two-phase system, which has turned out to be an excellent method for isolation of the different membranes from cyanobacteria *(7)*. This chapter describes the separation of pure outer-, plasma-, and thylakoid membranes of the filamentous cyanobacterium *Phormidium laminosum* by the two-phase partition method, using a system based on Dextran-T-500, polyethyleneglycol 3350 and phosphate buffer. This is similar to the system used for separation of plant membranes *(7)*. Cells grown to late linear phase are used for spheroplast formation and subsequent disruption using a French press cell. The total membranes are collected by ultracentrifugation and thus separated from the soluble phycobilisomes. The three membrane types isolated by sucrose gradient centrifugation *(4)* are used for determination of partition behavior in a two-phase system. **Figure 1** shows that the three membranes have very different partition behaviors: the outer membranes having a high affinity for the upper phase, the plasma membranes a high affinity for the lower phase, and the thylakoids are intermediate. These differences in partition properties are exploited in the isolation procedure. Two polymer concentrations (5.6% and 6.2%) are used in the purification of the three cyanobacterial membranes from total membranes. First 5.6%, at which the plasma membranes can be purified from total cyanobacterial membranes by three to four repartitionings of the original lower phase with fresh upper phase. The original upper phase, containing more then 90% of the thylakoid and outer membranes, is also repartioned one to two times with fresh lower phase to be free from residual plasma membranes. In order to separate the thylakoid and outer membranes, the polymer concentration has to be raised to 6.2%. At 6.2%, the lower phase will contain most of the thylakoid membranes, whereas the upper phase will contain most of the outer membranes. By repartitioning the upper and lower phase separately two to three more times, pure thylakoid and pure outer membranes will be obtained in the final lower and upper phase, respectively. If outer membranes are the only membranes of

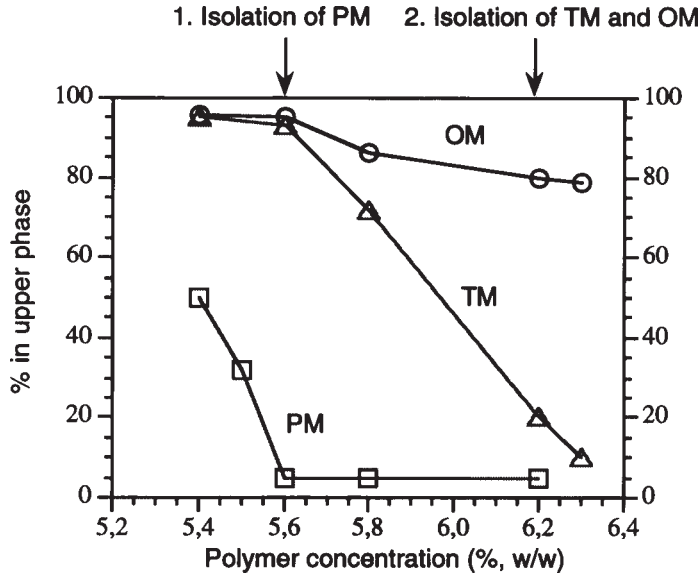

Fig. 1. Partition behavior of thylakoid, plasma, and outer membranes from *Phormidium laminosum* in two-phase systems (PEG 3350–Dextran T500) of increasing polymer concentrations. Both polymers are present in equal concentration.

interest, it is possible to purify them by a combination of sucrose gradient and two-phase partitioning *(7)*.

Recent studies on *Synechocystis* 6803 reveal that the two-phase partitioning method can also be used for isolation of the different membranes from this species *(8)*, suggesting that the method is applicable for various cyanobacteria. Studies on *Synechocystis* 6803 are very useful, because recently its complete genome sequence has been deduced. Furthermore, *Synochocystis* is readily transformable and highly amenable to molecular genetic manipulation. Genetical analysis of mutants can be combined with biochemical and biophysical studies to understand structural/functional relationships. Therefore, the ability to isolate pure plasma, thylakoid and outer membranes is of utmost importance. A very critical step in the purification of cyanobacterial membranes is the procedure for cell breakage, because this can lead to membrane vesicles of different orientation that will affect the partition properties of the membranes. Our preliminary studies on the isolation of the different membranes from *Synochocystis* 6803 show that depending on how the cells are broken—lysozyme treatment followed by French press, or breakage of cells directly using glass beads—the partition properties are drastically different. In general terms, when the proper conditions for spheroplast formation and cell

breakage are settled, the polymer two-phase method offers an excellent method for isolation of the different membranes from most cyanobacterial species, and offers a complement or alternative to traditional centrifugation techniques.

2. Materials

2.1. Cell Growth and Preparation of Total Membrane Fraction

1. Cell strain: *Phormidium laminosum*.
2. Growth medium: BG-11 (*see* **Note 1**).
3. Buffer 1: 5 m*M* NaCl, 7 m*M* Na_2EDTA, 10 m*M* Hepes/NaOH, pH 7.4, 28% (w/w) sucrose.
4. Hematocrit tube.
5. Lysozyme, from hen egg white, approx. 100,000 units/mg (Boehringer Mannheim, Mannheim, Germany).
6. Buffer 2: 5 m*M* NaCl, 10 m*M* HEPES/NaOH, pH 7.4, 28% (w/w) sucrose.
7. 20 m*M* phosphate buffer, pH 7.8, 20% (w/w) sucrose.
8. DNase I, from bovine pancreas, grade II, 2000 units/mg (Boehringer Mannheim).
9. 0.2 *M* phenylmethylsulfonyl fluoride (PMSF) in methanol (prepare fresh).
10. French press cell.
11. 20 m*M* phosphate buffer, pH 7.8.

2.2. Two-Phase Partitioning

1. Two-phase buffer (4 × concentrated): 1.0 *M* sucrose, 20 m*M* potassium phosphate buffer, pH 7.8.
2. Two-phase buffer: 0.25 *M* sucrose, 5 m*M* potassium phosphate buffer, pH 7.8.
3. 20% (w/w) Dextran T-500 (Amersham Pharmacia Biotech, Uppsala, Sweden).
4. 40% (w/w) Polyethylene glycol 3350 (PEG) (Union Carbide, New York).
5. 0.2 *M* PMSF in methanol.

3. Methods

3.1. Cell Growth and Preparation of Total Membrane Fraction

1. *Phormidium laminosum* is grown in BG-11 medium under white light and air/CO_2 atmosphere (5% CO_2) at 45°C to late linear phase (*see* **Note 2**).
2. Cells from 5 L culture are harvested by centrifugation at 4000*g*, 20°C , for 10 min (*see* **Note 3**).
3. The cells are washed once in distilled water and once in buffer 1 and each time collected by centrifugation as noted previously.
4. The cells are suspended by homogenization in buffer 1 (*see* **Note 4**) to a cell concentration of 50–80 μL/mL of packed cells (*see* **Note 5**).
5. Solid lysozyme is added to a concentration of 0.1% (w/v) and the suspension is incubated for 1–1.5 h at 37°C with gentle agitation (*see* **Note 6**).
7. The lysozyme treatment is terminated by diluting the suspension threefold with ice-cold buffer 2 followed by centrifugation at 4000*g*, 4°C, for 10 min.

8. The pellet, containing spheroplasts, is resuspended (*see* **Note 4**) in ice-cold 20 m*M* potassium phosphate buffer, pH 7.8, containing 20% sucrose to a cell concentration of approximately 100 μL/mL calculated from the cell concentration in **step 4**.
9. 0.01% (w/v) DNase and 1 m*M* PMSF final concentrations are added and the suspension is incubated for 30 min on ice.
10. The suspension is passed through a precooled French press cell operated at 50 MPa.
11. The suspension obtained is centrifuged at 4000*g*, 4°C, for 10 min to remove unbroken cells and cell debris.
12. The resulting dark blue-green supernatant is diluted threefold with ice-cold 20 m*M* potassium phosphate buffer, pH 7.8, containing 1 m*M* PMSF and centrifuged at 125,000*g*, 4°C, for 1 h.
13. The pellet containing total membranes is resuspended in 48 mL of two-phase buffer containing 1 m*M* PMSF with a glass homogenizer (*see* **Notes 4** and **7**).

3.2. Two-Phase Partitioning

3.2.1. Isolation of Plasma Membranes

1. For a 5-L cell culture, three tubes of 45 g two-phase systems are needed for the sample (membranes from **step 13** in **Subheading 3.1.**). Two 210 g systems are needed for repartitioning. The sample tubes and repartitioning bottles are prepared according to **Table 1** (*see* **Note 8**).
2. The tubes (bottles) are placed in a water bath of 3–4°C for equilibration (*see* **Note 9**).
3. Turn the tubes (bottles) gently upside-down 35 times (*see* **Note 9**).
4. Centrifuge at 1000*g* for 4 min in a rotor cooled to 3–4°C.
5. Two phases are obtained in both the sample tubes containing the membranes and the repartitioning bottles (*see* **Note 10**). Separate the upper and lower phases of the repartitioning bottles.
6. The dark green upper phase of the sample tubes contains most of the thylakoid membranes and outer membranes and the light green lower phase contains most of the plasma membranes (*see* **Fig. 1**). The upper phase is removed from each sample tube and transferred to similar tubes. These tubes are to be used for separation of the thylakoid and outer membranes as described in **Subheading 3.2.2.**
7. For purification of plasma membranes, new upper phase from the repartitioning system is added to the sample tubes with the original lower phase.
8. Partitioning is performed according to **steps 2–4**. The new upper phase is regarded as a washing phase and discarded.
9. Two more repartitionings of the original lower phase are performed in a similar way.
10. The fourth lower phase contains yellow plasma membranes with no contamination of thylakoid membranes. The lower phase is collected and diluted at least eightfold with 20 m*M* potassium phosphate buffer, pH 7.8, containing 1 m*M* PMSF, and centrifuged at 125,000*g*, 4°C, for 1 h.

Table 1
Preparation of Phase Systems for Purification of Outer-, Thylakoid-, and Plasma-Membrane Vesicles from *Phormidium laminosum*

Stock solution	Sample tube (45 g)	Repartitioning bottle (210 g)
20% Dextran	12.600 g	58.800 g
40% PEG	6.300 g	29.400 g
Two-phase buffer (4 × conc.)	7.250 g	52.500 g
Distilled water	2.625 g	68.250 g
0.2 M PMSF	0.225 g	1.050 g
Membranes (in two-phase buffer)	16.000 g	—

11. Homogenize the membranes in a small volume of two-phase buffer containing 1 m*M* PMSF (*see* **Note 11**).

3.2.2. Isolation of Thylakoid and Outer Membranes

1. To the tubes containing the original upper phase (from **step 6** in **Subheading 3.2.1.**) a new lower phase from the repartitioning system is added.
2. Partitioning is performed according to **steps 2–4** in **Subheading 3.2.1.** Most of the thylakoid and outer membranes are partitioned to the upper phase, whereas most of the residual plasma membranes are partitioned to the lower phase. The lower phase is discarded and a new lower phase is added from the repartition system (*see* **Note 12**).
3. For separation of thylakoid and outer membranes the polymer concentration has to be raised as shown in **Fig. 1**. In order to get a two-phase system of the final polymer concentration of 6.2% for both polymers, Dextran and PEG are added from the stock solutions. When a 45-g system is used, 2.60 g of 20% Dextran and 1.30 g of 40% PEG solutions are added to make the final polymer concentration 6.2%.
4. Partition is performed as in **steps 2–4** in **Subheading 3.2.1.** The upper phase contains most of the outer membranes and the lower phase most of the thylakoid membranes (*see* **Fig. 1**).
5. For purification of the outer membranes, the upper phase is transferred to a new tube and a new lower phase from a repartitioning system of 6.2% is added (*see* **Note 13**).
6. For purification of thylakoid membranes, a new upper phase from a repartitioning system of 6.2% is added (*see* **Note 13**).
7. Partitioning in both tubes is performed according to **steps 2–4** in **Subheading 3.2.1.** (*see* **Note 12**).
8. Collect the final upper phase containing the outer membranes and dilute at least threefold with 20 m*M* potassium phosphate buffer, pH 7.8, containing 1 m*M* PMSF. Collect the final lower phase containing the thylakoid membranes and dilute at least eightfold in the same buffer. Centrifuge at 125,000*g*, 4°C, for 1 h.
9. Homogenize the membranes in the two-phase buffer containing 1 m*M* PMSF.

4. Notes

1. Composition of BG-11 medium : 17.65 m*M* $NaNO_3$, 0.18 m*M* K_2HPO_4, 0.30 m*M* $MgSO_4$, 0.25 m*M* $CaCl_2$, 0.19 m*M* Na_2CO_3, 0.003 m*M* Na_2-EDTA, 0.031 m*M* citric acid, 6 mg/L ferric ammonium citrate, 46 μ*M* H_3BO_3, 0.17 μ*M* $CoCl_2$, 0.32 μ*M* $CuSO_4$, 9.2 μ*M* $MnCl_2$, 1.6 μ*M* Na_2MoO_4, 0.77 μ*M* $ZnSO_4$ ***(9)***.
2. Growth of a culture of 5 L is performed in a cylinder (12 cm in diameter and 50 cm high) on a thermostatted heating plate with magnetic stirring.
3. The cells form very loose pellets and the supernatant should therefore be removed carefully with water suction.
4. Use a loosely fitted glass homogenizer.
5. The concentration of cells is determined by centrifugation of aliquots of the suspension in hematocrit tubes (1200*g*, 10 min).
6. The formation of spheroplasts can be followed in the light microscope. Before lysozyme treatment long, thin filaments of the cells are seen. The lysozyme treatment is finished when the cells are seen as swollen entities mostly one by one, but also up to mostly five cells still connected to each other. It is critical for the optimal separation of thylakoid and plasma membranes that the lysozyme treatment is completed.
7. The membranes can be left on ice until the next day, when two-phase partitioning is performed.
8. Plastic tubes (or bottles) with a cap should be used. If tubes with conical bottom are used, be aware that the concentrated polymers do not form a plug in the bottom. The final concentration of the two-phase system is 5.6% (w/w) of both Dextran T-500 and PEG 3350, 0.25 *M* sucrose, 5 m*M* potassium phosphate buffer, pH 7.8, and 1 m*M* PMSF.
9. The whole two-phase partitioning is performed in the cold room. While turning the tubes upside down, hold the cap of the tube to avoid heating the tube with your fingers. It is very important to maintain the temperature at which the partitioning is decided to be performed. Check the temperature with a thermometer inside the tube and allow only the range 3–4°C.
10. In the 45 g system (5.6%), the volume of the lower phase is about 18 mL and the upper phase 24 mL. If other volumes are obtained, this may be due to incorrect composition or temperature of the two-phase system. The lower phase of the repartitioning bottles is usually a little cloudy. Use the same volumes of the separate phases upon repartitioning. However, this is not critical; the same two-phase system will be obtained even if another volume of upper/lower phase is added for repartitioning. When an additional polymer concentration is going to be used, however, as in the case of the further procedure to separate thylakoid and outer membranes (*see* **step 3**, **Subheading 3.2.2.**), the volumes should be kept as accurate as possible.
11. When the isolated plasma membranes from the two-phase procedure are subfractionated in a discontinuous sucrose gradient, it is found that in addition to the light plasma membranes, a previously undescribed plasma membrane

subfraction with the same density as the thylakoid membranes, is found. Obviously, this plasma membrane fraction cannot be resolved by the direct conventional sucrose gradient centrifugation. Moreover, this fraction is the major plasma membrane fraction in *Phormidium laminosum*, constituting as much as 65%, on a protein basis, of the total plasma membranes obtained in the phase partitioning. It means also that thylakoid membranes isolated by the conventional gradient method are contaminated with this plasma membrane fraction.

12. Another partitioning may be performed in a similar way. If not, continue with the next step.
13. Repartitioning systems of 6.2% of varied quantity can be prepared depending on if all material (or part of it) is going to be used for isolation of thylakoid and outer membranes. A 45 g system of 6.2% is prepared in the following way: 13.950 g Dextran, 6.975 g PEG, 11.250 g two-phase buffer (4 × conc), 0.225 g 0.2 *M* PMSF, and 12.600 g distilled water.

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17

Purification of Plasma Membranes by Affinity Partitioning

Bengt Jergil and Lars Ekblad

1. Introduction

This chapter describes the purification of rat liver plasma membranes by affinity partitioning in an aqueous polymer two-phase system using the lectin wheat germ agglutinin (WGA) as affinity ligand. Affinity partitioning is advantageous for conventional membrane fractionation techniques in being highly selective, allowing the rapid and high-yield purification of membranes. In addition, the aqueous polymer environment is gentle to membrane structure and function, which is of importance when studying labile structures and components.

A two-phase system will form when aqueous solutions of two structurally different polymers are mixed at sufficiently high concentrations ***(1)***. A commonly used polymer pair is polyethylene glycol 3350 (PEG) and Dextran T500. These polymers will form a two-phase system at concentrations above 5.4% (w/w) of each, the top phase being enriched in PEG and the bottom phase in Dextran. A conventional two-phase system like this may be used for the fractionation of biological material, including membranes. The distribution of material between the phases is modulated by altering polymer concentration and salt contents of the system ***(2)***, but the selectivity is often insufficient for the ready separation of membranes.

A better selectivity may be attained by introducing an affinity ligand into one of the phases. Thus, a suitable ligand coupled to one of the phase polymers may selectively pull the target membranes into this polymer phase. Ideally, separation conditions should be chosen to partition all membranes present into one of the phases in the absence of ligand. When introduced, the ligand coupled to the second phase polymer should selectively pull the target membranes into this phase *(see* **ref. *3)***.

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The methods presented describe the conjugation of WGA to Dextran and the use of the WGA-Dextran adduct for affinity purification of plasma membranes. WGA specifically binds N-acetylglucosamine and sialic-acid residues exposed on the extracellular face of plasma membranes, thereby discriminating them from other cellular membranes. Two purification schemes are presented, one describing the affinity purification of plasma membranes from crude rat liver membranes ***(4)***, the other dealing with the rapid purification of plasma membranes from rat liver tissue by a combination of conventional two-phase partitioning and affinity partitioning ***(5)***. Although elaborated upon for rat liver membranes here, these schemes should be easy to adopt for the purification of plasma membranes from other animal tissues as well.

2. Materials

2.1. Preparation of WGA-Dextran

1. Dextran T500 (Amersham Pharmacia Biotech, Uppsala, Sweden) is dissolved in water and freeze-dried before use. This makes it readily soluble and more convenient to use than Dextran obtained directly from the manufacturer, which dissolves very slowly. Freeze-dried Dextran retains its solubility for several months when stored dry.
2. Dichloromethane (Merck, Darmstadt, Germany): Dried with molecular sieves (KEBO Lab, Stockholm, Sweden) prior to use.
3. Dimethyl sulfoxide (DMSO; Merck): Dried as **item 2**.
4. Triethylamine (Merck): Dried as **item 2**.
5. WGA: from Boehringer Mannheim, (Mannheim, Germany).
6. 2,2,2-Trifluoroethanesulfonyl chloride (tresyl chloride): Synthelec AB (Lund, Sweden).
7. 0.4 *M* Tris-HCl buffer, pH 7.5.
8. A Filtron Omegacell 150™, supplied with an ultrafilter with 100 kDa cutoff, obtained from Filtron Technology Corp. (Northborough, MA, USA).

2.2. Purification of Plasma Membranes from Crude Membranes

1. Dextran T500 stock solution, 20%, by weight (use freeze-dried Dextran, *see* **Subheading 2.1.**, **item 1**) is made up in distilled water (*see* **Note 1**). Store in suitable aliquots at –20°C.
2. PEG 3350 stock solution, 40%, by weight (Union Carbide, NY) is made up in distilled water (*see* **Note 1**). Store in suitable aliquots at –20°C.
3. 0.2 *M* Borate-Tris buffer: Dissolve the required amount of boric acid in distilled water and adjust to pH 7.8 with a concentrated aqueous solution of Tris base.
4. WGA-Dextran: as prepared in **Subheading 3.1.**
5. Two-phase affinity system (2 g): Weigh out an amount of WGA-Dextran (*a* mg) containing 100 µg conjugated WGA into a clear 3-mL test tube fitting a benchtop centrifuge. Add 600-5×*a* mg Dextran stock solution, 300 mg PEG stock solution, 150 mg of 0.2 *M* borate-Tris buffer and distilled water to altogether 1.80 g. Temper the two-phase system at 4°C, preferably overnight (*see* **Note 1**).

6. Pre-equilibrated top phase is obtained by weighing out 600 mg of Dextran stock solution, 300 mg of PEG stock solution, 150 mg of 0.2 *M* borate-Tris buffer, and 950 mg of distilled water, all tempered at 4°C, into a clear test tube. Mix thoroughly and leave at 4°C for at least 8 h. Siphon off the top phase prior to use.
7. 5 m*M* Tris-HCl buffer, pH 8.0.
8. 0.1 *M* N-acetylglucosamine in 0.25 *M* sucrose and 5 m*M* Tris-HCl, pH 8.0.

2.3. Rapid Purification of Liver Plasma Membranes

1. Dextran T500 stock solution (as in **Subheading 2.2.**, **item 1**).
2. Polyethylene glycol 3350 stock solution (as in **Subheading 2.2.**, **item 2**).
3. 0.2 *M* Tris-H_2SO_4, pH 7.8: Weigh out the required amount of Tris base and adjust pH to 7.8 with H_2SO_4.
4. A homogenization two-phase system is made by weighing out 11.4 g of Dextran stock solution, 5.7 g of PEG stock solution, 3.0 g of 0.2 *M* Tris-H_2SO_4, pH 7.8, and 14.9 g of distilled water in a 40-mL Dounce homogenization tube. The phase system is mixed and left to equilibrate at 4°C overnight. The total weight of the phase system will be 40 g after the addition of 5 g of tissue sample (*see* **Subheading 3.3.**, **item 1**), the final concentration of each phase polymer 5.7% (by weight) and the Tris-H_2SO_4 concentration 15 m*M*.
5. Preequilibrated top phase I is obtained by weighing out 11.4 g of Dextran stock solution, 5.7 g of PEG stock solution, 3.0 g of 0.2 *M* Tris-H_2SO_4, pH 7.8, and 19.9 g distilled water in a 50-mL measuring cylinder. The phase system is mixed thoroughly and left at 4°C overnight. The top phase is siphoned off immediately before use.
6. Pre-equilibrated affinity bottom phase is obtained by weighing out enough WGA-Dextran (*a* mg) to contain 200 μg of bound WGA, 3000-5×*a* mg of Dextran stock solution, 1.5 g of PEG stock solution, 0.75 g of 0.2 *M* borate-Tris buffer (*see* **Subheading 2.2.**, **item 3**), and distilled water to 10.0 g in a clear 35-mL centrifuge tube. The phase system is mixed thoroughly, also to dissolve the WGA-Dextran, and left at 4°C overnight. The bottom phase is collected by siphoning off the top phase prior to use.
7. Pre-equilibrated top phase II is obtained by weighing out 15.0 g of Dextran stock solution, 7.5 g of PEG stock solution, 3.75 g of 0.2 *M* borate-Tris buffer (*see* **Subheading 2.2.**, **item 3**), and 23.75 g of distilled water in a 50-mL measuring cylinder. Mix the phase system thoroughly and leave at 4°C overnight. Collect the top phase prior to use.
8. 0.1 *M N*-acetylglucosamine in 0.25 *M* sucrose and 5 m*M* Tris-HCl, pH 8.0.

3. Methods

3.1. Preparation of WGA-Dextran

1. Dissolve 5 g of freeze-dried Dextran T500 in 25 mL DMSO at room temperature. Add dropwise 1 mL of triethylamine followed by 5 mL of dichloromethane (additions should take ca 10 min altogether; *see* **Note 2**). Chill on ice and add dropwise 0.35 g of tresylchloride while stirring vigorously at 0°C with a mag-

netic stirrer. Stir gently for another 30 min on ice, for 60 min at 4°C, and overnight at 20°C.

2. The following steps are performed at 20°C (room temperature). Add 50 mL of dichloromethane to terminate the reaction and precipitate the tresyl Dextran formed. Collect the precipitate and wash, until the precipitate is firm, with several 25-mL portions of dichloromethane, each time thoroughly kneading with a glass rod. Dissolve the washed tresyl Dextran in 30 mL of distilled water and dialyze twice against 5 L water, at least for 4 h each time. Freeze-dry and store dry at –20°C (*see* **Note 3**).
3. To conjugate WGA to Dextran, dissolve 2 g of tresyl Dextran in 10 mL of 0.1 *M* sodium phosphate buffer, pH 7.5, also containing 0.5 *M* NaCl. Ten mg of WGA is dissolved in 1 mL of the same buffer mixture. Add dropwise the WGA solution to the tresyl Dextran while stirring vigorously. Incubate overnight at 4°C with gentle stirring. Then add 10 mL of 0.4 *M* Tris-HCl, pH 7.5, and incubate for at least 1 h to inactivate unreacted tresyl groups.
4. Uncoupled ligand and salts are removed from the WGA-Dextran adduct by repeated ultrafiltrations in a Filtron Omegacell. Add the incubation mixture to the cell and fill with distilled water to 150 mL. Ultrafilter to a volume of ca 15 mL. Add water to the cell and ultrafilter again, repeating this cycle five times. Freeze-dry the WGA-Dextran and store dry at –20°C (*see* **Note 4**).
5. Determine the amount of WGA conjugated to Dextran by protein analysis using WGA as standard, and test the affinity properties of the conjugate (*see* **Note 5**).

3.2. Purification of Plasma Membranes from Crude Membranes

1. Add 0.20 g of a crude rat liver membrane suspension (containing up to 2 mg membrane protein) in a suitable buffer to a well-tempered affinity two-phase system (*see* **Note 6**) and mix thoroughly by inverting the tube 20 times, vortexing and inverting the tube another 20 times at 4°C. Centrifuge the tube gently (150*g* for 5 min in a bench-top centrifuge) to separate the phases. Siphon off the PEG-rich top phase immediately, saving the interface and the plasma membrane-containing bottom phase.
2. Reextract the interface plus bottom phase by adding an equal amount of fresh preequilibrated top phase as the top phase removed in **step 1**. Mix thoroughly and separate the phases as described in **step 1**. Mix the resulting bottom phase with a 10-fold volume of 0.1 *M N*-acetylglucosamine in 0.25 *M* sucrose and 5 m*M* Tris-HCl, pH 8.0, and centrifuge at 100,000 g for 60 min to sediment membranes (*see* **Note 7**). Resuspend the sediment in 5 m*M* Tris-HCl, pH 8.0, or some other suitable buffer, to the desired concentration.
3. Follow the purification by analyzing key fractions for protein contents and plasma membrane marker enzymes as well as markers for other subcellular compartments (*see* **Note 8**).

3.3. Rapid Purification of Liver Plasma Membranes

1. A rat liver is perfused with 0.25 *M* sucrose in 5 m*M* Tris-HCl, pH 8.0, to remove as much blood as possible. Squash the liver in a garlic press and transfer maxi-

mum 5 g to a homogenization two-phase system in a Dounce homogenizing tube. Make up to 40 g with distilled water in case less than 5 g liver is used. Homogenize by 20 up and down strokes with a loose-fitting Dounce A pestle (*see* **Note 9**).
2. Transfer the system to a clear centrifuge tube and spin at 150*g* for 5 min in a swing-out rotor to speed up phase separation. Siphon off the top phase, measure its volume, and keep on ice.
3. Reextract the bottom phase by adding the same volume of fresh preequilibrated top phase I as the top phase siphoned off in **step 2**. Mix thoroughly by inverting the tube 20 times, vortexing and inverting the tube another 20 times. Separate the phases as aforementioned and combine the two top phases (*see* **Note 10**).
4. Affinity-purify plasma membranes by adding preequilibrated affinity bottom phase to the combined top phases, mix the system thoroughly as in **step 3**, and separate phases by centrifugation as in **step 2**. Siphon off the top phase and measure its volume.
5. Reextract the bottom phase with fresh preequilibrated top phase II, the same volume as that removed in **step 4**. Mix and separate phases as before (*see* **Note 7**).
6. Dilute the final bottom phase tenfold with 0.1 *M N*-acetylglucosamine in 0.25 *M* sucrose and 5 m*M* Tris-HCl, pH 8.0, or some other suitable buffer (*see* **Note 7**). Mix and pellet membranes by centrifugation at 100,000*g* for 60 min. Suspend the pellet in a suitable buffer.
7. Follow the purification procedure by analyzing the fractions for protein and markers for plasma membranes as well as for other subcellular compartments (*see* **Note 11**).

4. Notes

1. The partitioning process is extremely sensitive to salt concentration (even changes in the millimolar range may redirect membranes from one phase to the other, *see* **ref. *2***), polymer concentration, and temperature. Therefore, stock solutions, buffers, and phase systems, have to be prepared with great accuracy and the temperature should be kept constant during the partitioning process to ensure reproducibility. It is advisable to work in a cold room that has a constant temperature.
2. Triethylamine and dichloromethane are added slowly to avoid the precipitation of Dextran. Because the mixture is rather viscous after these additions, it should be ascertained that the tresyl chloride is admixed properly when added.
3. Tresyl Dextran is stable for several months when stored dry at –20°C.
4. The removal of salt is crucial as salt remaining in the WGA-Dextran preparation may affect the distribution of membranes in the affinity partitioning step, two-phase partitioning being extremely sensitive to salt conditions. Uncoupled WGA, having an Mr of 36,000, also passes through the ultrafilter. WGA-Dextran retains its separation properties for at least six months when stored dry at –20°C.
5. The amount of WGA conjugated to Dextran should be determined for each batch as the coupling efficiency may vary. The Bradford procedure ***(6)*** is suitable as long as WGA is used as standard; WGA yields much less color than many other

proteins, e.g., bovine serum albumin (BSA). Usually, 4 mg WGA is found per g freeze-dried product with the recovery of Dextran exceeding 90%. The affinity properties of each batch of WGA-Dextran should be tested as well. This is done by two-phase partitioning in a series of 2 g two-phase affinity systems (described in **Subheading 2.2., item 3**) where the amount of WGA-Dextran is varied between 0–100 μg WGA. Such a series is illustrated in **Fig. 1**. Crude membranes, e.g., microsomes, are used as membrane source, and the distribution in top and bottom phases of a plasma membrane marker enzyme as well as markers for other membrane compartments (*see* **Note 8**) should be followed to test the selectivity of the affinity system. In a well-functioning 2 g affinity system 100 μg WGA as WGA-Dextran will attract more than 90% of plasma membrane markers to the Dextran-rich bottom phase, whereas more than 90% of the markers for other membranes remain in the PEG-rich top phase.

6. Crude rat liver membranes may be prepared in any of a number of ways. The membranes should be suspended in a low concentration buffer because the salt conditions are crucial for a successful affinity partitioning. Borate is used as buffer in the two-phase system to cause membranes in general to partition in the PEG-rich top phase. WGA-Dextran will attract plasma membranes selectively into the Dextran-rich bottom phase within a rather narrow borate concentration interval. Additional salts should be avoided as far as possible because they might disturb the WGA-dependent redistribution of plasma membranes or cause membranes to partition in the bottom phase unselectively.
7. Reextraction of the bottom phase usually increases plasma membrane purity by removing contaminants. A second re-extraction performed in the same way may be included, but is usually not necessary. N-acetylglucosamine is added to facilitate dissociation of WGA-Dextran from membranes, but may be omitted if the presence of WGA-Dextran does not disturb.
8. Protein is preferably analyzed by the method of Bradford ***(6)***. The following marker enzymes are suitable in affinity-partitioning procedures: Plasma membranes, apical domain, 5'-nucleotidase ***(7)***, and alkaline phosphodiesterase I ***(8)***; plasma membranes, basolateral domain, asialoorosomucoid binding ***(2,9)***; endoplasmic reticulum, arylesterase ***(10)***; Golgi membranes, *N*-acetylglucosamine galactosyl–transferase ***(11)***; mitochondria, succinate-cytochrome *c* reductase ***(12)***; lysosomes, *N*-acetyl-β-glucosaminidase ***(13)***; cytosol, lactate dehydrogenase ***(14)***.
9. The method of homogenization is not critical, and more vigorous methods can be used if necessary.
10. Re-extraction increases the yield of plasma membranes by *ca* 30%, but may be omitted when a rapid preparation is important.
11. The purification procedure thus includes conventional two-phase partitioning followed by affinity partitioning and pelleting of the membranes by centrifugation ***(5)***. The conventional two-phase step is designed to partition more than 95% of mitochondrial and endoplasmic reticulum markers in the bottom phase. Plasma membranes are enriched in the top phase together with much of Golgi membranes and lysosomes. These latter membranes are removed in the affinity step,

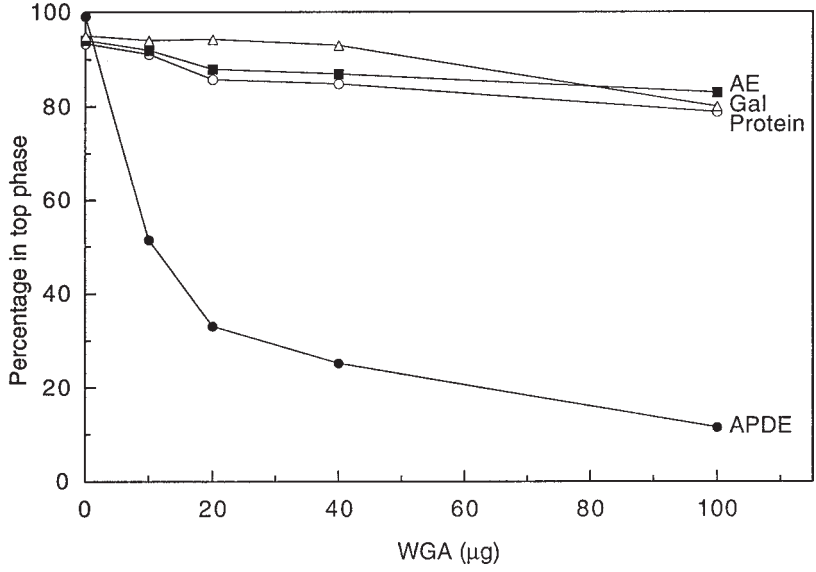

Fig. 1. WGA-Dextran affinity partitioning of rat liver microsomes. Microsomes were partitioned with increasing amounts of WGA-Dextran in a 2-g affinity system containing 6.0% (by weight) each of PEG and Dextran in 15 m*M* borate-Tris buffer, pH 7.8. Protein content and the following membrane marker enzymes were measured: plasma membranes, alkaline phosphodiesterase I (APDE); endoplasmic reticulum, arylesterase (AE); Golgi membranes, galactosyltransferase (Gal). (Adapted with permission from **ref. *4***).

whereas the centrifugation step removes soluble material still remaining after the extractions. The distribution of membranes of interest as well as of unwanted membranes should be followed by marker enzyme analysis for each separation step to ascertain that each step works properly. The final preparation should be enriched 30–40-fold in markers for both the apical and basolateral plasma membrane domains compared to the homogenate, whereas only traces of markers for other membranes should remain ***(5)***.

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18

Studying the Influence of Salts on Partitioning of Proteins

Isoelectric Point Determination

Daniel Forciniti

1. Introduction

In addition to its application as a separation process, aqueous two-phase partitioning often yields information on important physical properties of proteins, replacing other more cumbersome analytical techniques. For example, aqueous two-phase partitioning can be used to measure the hydrophobicity and charge of a protein ***(1–3)***, to calculate dissociation constants between enzymes and substrates ***(4)***, to fractionate cell populations ***(5)***, or to characterize cell surfaces ***(6,7)***.

One of the most popular pairs of phase-forming species are Dextran and poly(ethylene)glycol (PEG). Although these two polymers are uncharged, salts have unequal affinities for the top and bottom phases of aqueous two-phase systems made with them. The uneven partition of salts between the two phases affects the chemical potential of the protein in each phase and thus its partition coefficient (K= [top]/[bottom]). The following mental exercise helps to understand this phenomenon. Consider two phases at equilibrium in which a salt has been previously partitioned. The different affinity of the salt for the bottom and top phases creates two distinct ionic atmospheres in both phases. Next, bring a charged protein molecule from infinite and try to insert it in each of the two phases. Because the protein is going to see different ionic atmospheres in both phases, the work needed to insert it in one or the other phase will be different. This difference in the work of insertion of a charged protein in each of the two phases at equilibrium is proportional to the electrochemical potential difference of the protein between them. Consequently, the partition coefficient

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(which is a function of that potential difference) is strongly affected by the type and concentration of salts and by pH.

The effect of salt type and concentration on protein partitioning in aqueous two-phase systems is well known *(2)*. In general, the partition coefficient of proteins away from their isoelectric point depends on both the type and concentration of cation and anion. For example, for positively charged proteins, the partition coefficient in PEG/Dextran systems is higher in potassium chloride than in potassium phosphate; the reverse is true for negatively charged proteins. The effect of the cation on the partition coefficient of positively charged proteins is K~Cs>Na>Li, whereas the reverse order holds for negatively charged proteins. On the contrary, the partition coefficient of proteins at their isoelectric point is quite insensitive to salt type and concentration.

Manipulations of the pH have been of primary importance in partitioning studies of proteins. Although a fundamentally based theory that explains pH effects remains elusive, it is convenient to split the partition coefficient into two contributions, one that is independent of pH and the other that is proportional to the net charge of the protein. In so doing, electrostatic and nonelectrostatic contributions to the partition coefficient can be conveniently (but artificially) separated. This approach predicts that the logarithm of the partition coefficient is a linear function of the charge of the protein.

Cross-partitioning *(3,8)* is a simple method by which a protein's isoelectric point can be determined. If the protein of interest does not interact with contaminating materials in the solution, its isoelectric point can be determined without prior protein purification (other than desalting). The method is based on both the relative insensitivity of the partition coefficient of proteins at their isoelectric point to the type and concentration of salts, and on its dependence on salt type and concentration away from the isoelectric pH.

The determination of isoelectric points of proteins by cross-partitioning is straightforward. Two sets of two-phase systems (usually PEG/Dextran) covering a wide pH range are prepared. One set contains NaCl, whereas the other set contains Na_2SO_4. The pH dependence of the partition coefficient of proteins in the presence of NaCl is usually different from that in the presence of Na_2SO_4. Thus, the partition coefficient vs pH curves cross each other at a pH (crosspoint) that usually agrees with the isoelectric point of the proteins **(Table 1)**. Still, some discrepancies have been observed *(9)*.

2. Materials

1. Polymer stock solutions. The polymers can be used as received, because they are usually salt free. In cases where the material to be partitioned is too sensitive to impurities, the polymers may be purified. For example, Dextran is conveniently purified by dialysis to eliminate multivalent ions.

Table 1
pH at the Cross-Point of Some Proteins *(8)*

Protein	pH at the crosspoint	Isoelectric pH
Human serum albumin	4.7	4.7
Papain	8.6	8.7
Human A hemoglobin	7.0	7.0
Pig hemoglobin	6.7	—

   a. 30% (w/w) Dextran T500 (Sigma, St. Louis, MO).
   b. 50% (w/w) polyethylene glycol (PEG) 6000 (Sigma).
2. Stock solutions of a series of 0.04 *M* buffers (glycine or sodium phosphate) spanning the pH range from 3.5–11.5.
3. Stock solutions of alkali (i.e., lithium, sodium, potassium) chlorides: (0.33 *M*).
4. Stock solutions of alkali sulfates: (0.167 *M*).
5. 1 g/L Protein stock solution: Proteins should be desalted before use. Dialysis against a buffer made in nanopure water, ultrafiltration operated in dialysis mode, or a desalting column can be used for this purpose. For example, to desalt a protein by dialysis, prepare 50 g of a 1 g/L protein solution and dialyze it against 50-m*M* Tris-HCl buffer, pH 7.0, for 24 h with three buffer changes.

3. Methods

1. Prepare two sets of PEG/Dextran systems spanning a pH range from 3.5–11.5 (*see* **Note 1**). One set contains alkali chloride (Set A) and the other alkali sulfate (Set B).
2. In preparing the two-phase systems, the following stock solutions are added to a 10-mL centrifuge tube: 2.5 g of Dextran stock solution, 1.0 g of PEG stock solution, 3 g of sodium chloride or sodium sulfate solution, 2.5 g of buffer, and 1 g of the protein stock solution. The final phase system composition is 7.5% (w/w) Dextran, 5.0 (w/w) PEG, 0.1 *M* alkali chloride or 0.05 *M* alkali sulfate, and 0.04 *M* glycine or sodium phosphate buffer. Blanks of the phases without added protein are also prepared.
3. Special care has to be taken in mixing the systems. This has to be vigorous enough to allow distribution of the proteins between the two phases but gentle enough to prevent protein denaturation (a rotary shaker is highly recommended).
4. The phase systems are centrifuged at 1500*g* for 20 min to speed phase settling.
5. Pipet carefully 1 mL of top phase and 1 mL of bottom phase from each partitioning tube. The sample from the bottom phase should be pipeted using a small positive pressure to avoid contamination with the top phase (*see* **Note 2**). Blank phase systems are sampled in the same manner.
6. The samples are diluted with buffer. The actual dilution depends on the particular protein and on its partition coefficient (*see* **Note 3**). Because of the relatively high adsorbance of the blanks at 280 nm, an adsorbance reading of protein containing samples between 0.5 and 1 is recommended (*see* **Note 4**).

7. The partition coefficient is calculated from K = [Adsorbance$_{sample}$ – Adsorbance$_{blank}$]^{top}/[Adsorbance$_{sample}$ – Adsorbance$_{blank}$]^{bottom} (*see* **Notes 5** and **6**).
8. The pH in each phase is measured with a microelectrode (*see* **Note 7**).
9. The partition coefficients of Sets A and B are plotted vs pH. The pH and the partition coefficient values at which one K vs pH line (Set A) crosses the other one (Set B) are read from the axes (*see* **Notes 8** and **9**). These values are the pH and partition coefficient at the cross-point. Examples of crosspartitioning of ribonuclease A and hemoproteins are discussed in **Notes 10** and **11**.
10. The experimental conditions may be modified to improve sensitivity (*see* **Note 12**).

4. Notes

1. Two runs are highly recommended for precision work. In the first run, four or five different pH values are enough. In the second run, five or six points should be obtained in the neighborhood of the crosspoint.
2. Impurities may accumulate at the liquid–liquid or liquid–air interfaces. They do not constitute a problem unless they are pipetted during sampling. Thus, a positive pressure on the pipet as it enters the phases is always recommended.
3. The dilution of samples from the phases must be done very carefully. Because the viscosity of the phases is very high, improper mixing may result. Uncontrollable scattering from regions of different densities within the sample produces erroneous absorbance readings. As a general rule, mix the sample of the phase with the dilution buffer and stir in a vortex mixer. Leave the solution resting and stir again. Inspect the solution to detect density differences along the axial direction of the test tube. Continue stirring until the solution is completely transparent.
4. To minimize errors owing to scattering, a wavelength scan from 250–350 nm should be done. The absorbance reading at 350 nm should be close to zero (0.010 absorbance units or smaller). If it is not, the solution is scattering light. Additional dilution of the sample normally eliminates this problem. If further dilution of the sample is not feasible because the absorbance at 280 nm is already too small, the absorbance at 280 nm can be corrected for scattering as follows. Fit the absorbance reading from 320 to 350 nm with a polynomial and extrapolate to 280 nm. Use this extrapolated value to correct the absorbance at 280 nm, i.e.,

$$A_{280}^{corrected} = A_{280} - A_{280}^{extrapolated}$$

5. Since the volumes of the phases are very easy to measure and the density of each phase are well-correlated with polymer concentration (the densities of PEG and Dextran in g/mL at 25°C are given by $\mathbf{D}_{PEG} = [0.997 + 0.169\ C_{PEG}/100]$ and $\mathbf{D}_{Dx} = [0.0997 + (0.391\ C_{Dx}/100]$, respectively with C in g/100 mL), a protein mass balance can be easily performed. If the mass balance does not agree (within 5%) check for the formation of a precipitate at the liquid/liquid interface. If a precipitate is present one should use more diluted protein solutions (a decrease of 50% in protein concentration is usually enough). If no precipitate is present, poor sampling is probably the source of error.
6. Partition coefficients should be reported with no more than two decimal figures. To improve accuracy, every point of a cross-partitioning curve should be measured in triplicate.

7. The pH is measured directly on the undiluted phases using a microelectrode. Because of the high viscosity of the phases, the pH measurements must be done during a relatively long period of time.
8. Lines of K vs pH do not cross each other. If the pH range is wide enough the sources of error are either the values of the partition coefficients or the pH. One must be sure that the pH has been measured long enough to reach equilibrium. The pH of both phases should agree within the experimental uncertainty (±0.05 pH units). Erroneous values of K are generally owing to poor sampling. A protein mass balance should be done to assure that sampling has been done correctly.
9. In the analysis of partitioning data at different pHs, we must consider any change in the physical properties of the proteins owing to changes in the pH and how these changes may affect the protein partition coefficient. For example, lysozyme does not change its conformation over the pH range 1.2–11.3 in dilute salt solutions at moderate temperatures; however, it polymerizes reversibly at pH above 5.0 ***(13)***. This change in the molecular weight of the protein will change the partition coefficient. A good example of a protein that is greatly affected by changes in pH is bovine serum albumin ***(14)***. At pH 9.0 and low temperatures, albumin partially denatures. As the pH is lowered down to 8.0, albumin undergoes a transition between two forms with different helix content. From pH 5.0–8.0 the native form of the protein is stable. From pH 4.0 to 4.5 albumin partially opens and below four it becomes fully uncoiled.
10. When bovine pancreatic ribonuclease A is cross-partitioned in PEG/Dextran systems a pH region of equal partition coefficient values rather than a cross-point is found ***(9,10)***. In addition, its partition coefficient at the cross-point depends on the concentration of salt. These results indicate salt concentration-dependent conformational changes in the ribonuclease molecule that affect the molecule's surface charge and/or hydrophobic-hydrophilic surface properties (*see* **Note 9**).
11. The crosspartitioning of hemoproteins ranging from cytochrome C (MW 12,000) to catalase (MW 240,000) in PEG/Dextran systems ***(9)*** yields partition coefficients at the cross-point that do not show the clear dependence on protein molecular weight found with nonhemoproteins. Even though the molecular weights of human hemoglobin variants (A, F, S, C) and hemoglobins from different species are essentially the same, the partition coefficient at the crosspoint of hemoglobins A and F and those of hemoglobins from different mammalian species show measurable differences. Although the four human hemoglobin variants differ in charge, adult hemoglobins A, S and C have the same partition coefficient, whereas the fetal hemoglobin (F) has a lower partition coefficient. An example of the effect of temperature on crosspartitioning of one hemoprotein (pig hemoglobin) is shown in **Fig. 1**. The pH at the cross-point at 4°C is approx 6.9, whereas at 22°C it is around 6.7. The partition coefficient at the crosspoint decreases from ~0.04 to ~0.03 as the temperature decreases from 22° to 4°C, as expected.
12. The sensitivity of crosspartitioning depends on the angle at which the two lines intersect (**Fig. 2**). If the lines are perpendicular the sensitivity is at a maximum, whereas parallel or nearly parallel lines yield no crosspoint or a "crosspoint

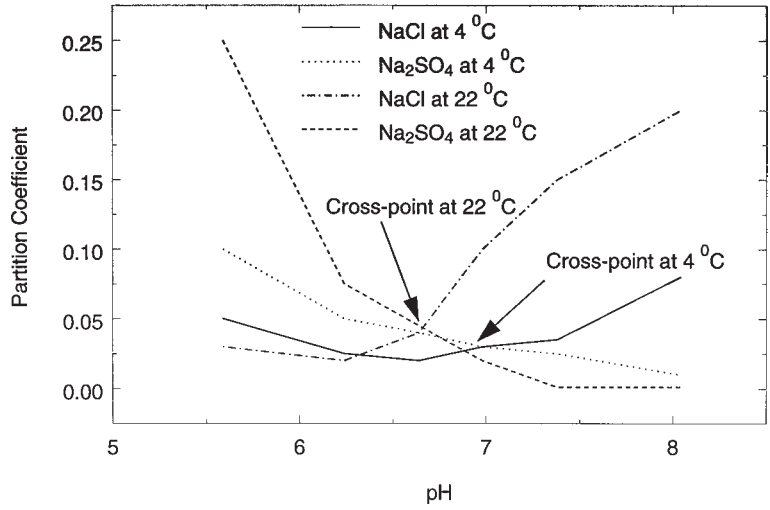

Fig. 1. Cross-partitioning of pig hemoglobin at two temperatures.

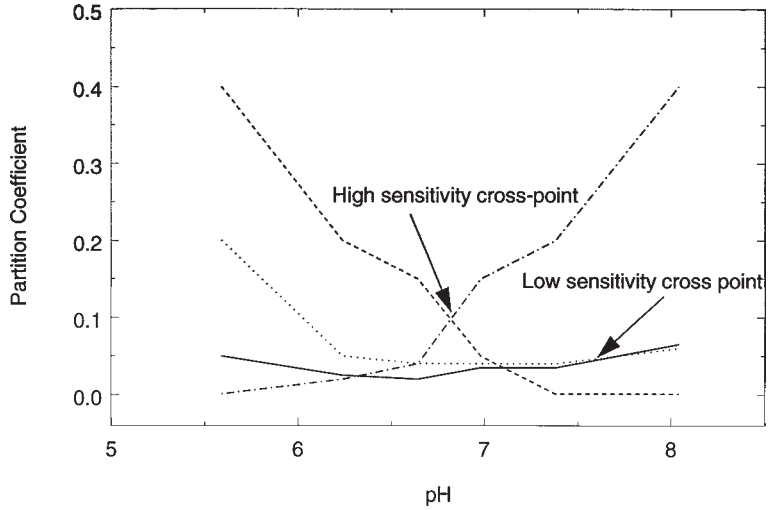

Fig. 2. Two cross-partitioning curves showing different sensitivities.

range." The slope of the lines depends on the activity of the protein in both phases. This depends on the type of salt, the change in net charge of the protein with pH, the specific interactions between the ions and the proteins, and the salt-induced changes in the interactions between polymer and protein. The sensitivity can be manipulated by varying the molecular weight of the polymers, the temperature, the concentration of the polymers, and the type of salt. Unfortunately, the partition coefficient of proteins does not follow a clear trend with polymer molecular

weight or concentration and pH ***(11,12)***. Thus, cross-partitioning should be done using the lowest possible PEG molecular weight to minimize problems associated with the high viscosity of the phases. If the sensitivity is not good enough, the experimentalist needs to explore different conditions until a good sensitivity is found.

Generally, the partition coefficient increases with increasing temperature in PEG/Dextran systems between 4° and 40°C. The partition coefficient of small and hydrophilic proteins is only slightly affected by changes in temperature, whereas the partition coefficient of bigger and more hydrophobic proteins is strongly affected by temperature changes. High temperatures (around 40°C) may be used to minimize protein association whereas low temperatures may be desirable to maintain protein stability.

The pH and the partition coefficient at the cross-point are only marginally dependent on the combination of salts used and on their concentration. For example, NaCl can be replaced by potassium chloride and/or sodium sulfate by lithium sulfate without affecting the results. Still, some small differences in pH values at the crosspoint with different salts have been observed. These differences are similar to those encountered in the electrophoretic determination of isoelectric points which can also be slightly affected by the salt used.

This independence of cross-partitioning on the type and concentration of salt, makes cross-partitioning a viable option for determining the isoelectric point of proteins that are stable only at high salt concentrations. In contrast, the type and concentration of salts have a strong influence on the shape of the lnK vs pH curves. For example, the combination of KCl with either Na_2SO_4 or K_2SO_4 generates the most sensitive cross-partition plot for ovalbumin.

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19

Partitioning of Chemically Modified Proteins

A Method for Detection of the Resolution of Aqueous Two-Phase Systems

Telma Teixeira Franco

1. Introduction

Partitioning in aqueous two-phase systems (ATPS) is mainly a process where the exposed groups of molecules come into contact with the phase components, and is therefore surface-dependent ***(1)***. Among the main protein properties, hydrophobicity and charge appear to be the most important parameters in ATPS. The interactions between the properties of the surface of the proteins and the properties of the systems determine the partitioning behavior. A protein interacts with the surrounding molecules within a phase via various bonds such as hydrogen, ionic, and hydrophobic interactions, together with other weak forces. The net effect of these interactions is likely to be different in the two phases and the protein will partition preferentially into one phase, in which the energy is most favorable ***(2)***.

A number of different ATPS were initially classified according to their hydrophobic/hydrophilic nature, arranged in a scale of standards from aqueous salt solution to acetone ***(1)***. Recently, other ATPS have been classified according to their resolution for charge and for hydrophobicity ***(3–5)***. It was observed that poly(ethylene-glycol) (PEG)/phosphate systems provided highest resolution in terms of protein surface hydrophobicity, and that PEG/dextran systems had some resolution for different surface charged protein when buffered with phosphate salts, but not with Tris salts, and that this resolution was improved by the addition of low concentrations of KCl or Li_2SO_4. Eiteman and Gainer ***(6)*** found that the partition coefficient of peptides varied linearly with the hydrophobicity of the solute in a defined system with a constant difference in

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the concentration of one of the phase components (e.g., PEG) between the two aqueous phases. They developed an empirical correlation that describes the effect of solute hydrophobicity on partitioning at a constant phase concentration difference between top and bottom phases, however the correlation has only been mainly tested on small molecules (alcohols, peptides) (*see also* Chapter 10).

In order to understand better how ATPS are able to separate proteins according to surface hydrophobicity and charge, chemical modification of proteins can be used to change one property only while maintaining the other protein properties almost unchanged ***(3–5)***. The properties that are required to be maintained after chemical modification are size and protein conformation, and additionally isoelectric point in case of hydrophobically modified proteins, and hydrophobicity for charge-modified proteins. The characterized modified "families" of proteins are then partitioned in ATPS to investigate how surface hydrophobicity and charge independently affect protein partitioning.

In this chapter, relatively conservative modifications—under mild conditions—of three chosen proteins are presented. For the investigation of resolution of the ATPS for protein charge and for protein hydrophobicity, different system compositions will be presented.

2. Materials

2.1. Chemical Modification of Proteins

1. Proteins. For example, Thaumatin (Tate and Lyle, Reading, UK), bovine serum albumin (BSA), and bovine β-lactoglobulin (both from Sigma, UK).
2. Acetic-, butyric-, hexanoic-, and succinic-anhydride (Sigma).
3. 2.0 *M* sodium acetate solution in 0.1 *M* Na_2HPO_4, pH 6.5.
4. 1 *M* NaOH and 1 *M* HCl for pH adjustments.
5. Phenyl Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden).
6. Mobile phases for hydrophobic interaction chromatography (HIC), Solvent A: 20 m*M* Tris-HCl (or 20 m*M* sodium phosphate buffer), and Solvent B: 20 m*M* Tris-HCl (or 20 m*M* phosphate buffer) + 2.0 *M* $(NH_4)_2SO_4$. The pH values of the mobile phases are adjusted to 7.9 for thaumatin and BSA, and to 5.3 for β-lactoglobulin chromatography.
7. XK 26/100 column (Amersham Pharmacia Biotech) for HIC.
8. Equipment and materials for preparative isoelectric focusing (IEF).
9. PD Sephadex mini-columns (Amersham Pharmacia Biotech).

2.2. Partitioning of Proteins in ATPS

1. Poly(ethylene-glycol) (PEG 1500 and 4000) stock solutions, 50% (w/w) in water (*see* Chapter 3 for preparation).
2. Dextran T500 stock solution, 25% (w/w) in water (*see* Chapter 3 for preparation).
3. Salt stock solutions (w/w): NaH_2PO_4-K_2HPO_4, 40%; $MgSO_4$, 23%; sodium citrate, 28%; Na_2SO_4 and $(NH_4)_2SO_4$, 50% in deionized water (*see* Chapter 3).

3. Methods

3.1. Chemical Modification of Proteins

3.1.1. Modification of Surface Hydrophobicity

1. Dissolve 370 mg of a protein like BSA or lactoglobulin in 16 mL of 2.0 *M* sodium acetate solution in 0.1 *M* Na_2HPO_4 (*see* **Note 1**).
2. Keep in an ice bath (3°C) and adjust the pH to 8.2–8.6 with 1 *M* NaOH.
3. Add the reagent anhydride in three portions to each protein solution, at 15 min intervals. Four different molar ratios (protein:anhydride) 1/0.8, 1/1.6, 1/3.1, 1/6.2 can be chosen (*see* **Notes 2** and **3**).
4. Allow to stand for 15 min and stop the reaction by lowering the pH to 5.2 with HCl.
5. Salts and any byproducts of the reaction are removed by dialysis against water with several water changes (usually 6 changes in 2 L each) or by ultrafiltration.
6. Salt removal is checked by conductivity, determined against a standard curve of sodium acetate, using a conductivitymeter.
7. After salt removal (to 5 m*M*), freeze-dry the protein solution.
8. Apply the above protein sample, dissolved in about 2 mL of solvent B, to the XK 16/100 preparative column packed with Phenyl-Sepharose (bed vol: 80 mL), which is also equilibrated with solvent B.
9. Separate the fractions by using a concentration gradient of 2.0–0 *M* $(NH_4)_2SO_4$ (Solvent B-Solvent A) in a total vol of 700 mL (*see* **Note 4**).
10. Collect fractions of 12 mL vol and pool them in groups. Dialyze and freeze-dry prior to characterization and partitioning studies in ATPS.
11. Characterization is performed with respect to isoelectric point (pI) (*see* **Note 5**), molecular weight (*see* **Note 6**), and conformational analysis (*see* **Note 7**).

3.1.2. Modification of Surface Charge

1. Dissolve 500 mg of protein in 20 mL of 2.0 *M* sodium acetate in 0.1 *M* Na_2HPO_4 (*see* **Note 8**).
2. Keep in an ice bath during the course of the reaction (T = 3°C).
3. Add three portions of 5 mL acetic anhydride at intervals of 5 min. The pH is kept constant at 8.2 by the addition of 1 *M* NaOH (*see* **Note 9**).
4. Stop the reaction by adding triethanolamine after 15 min (*see* **Note 9**).
5. Dialyze the samples against water and/or ultrafilter, and freeze-dry the protein solution, in the same way as in **Subheading 3.1.**
6. Separate the charge-modified proteins by preparative isoelectric focusing (*see* **Note 10**).
7. Remove the ampholytes from the fractions obtained from preparative IEF (*see* **Note 10**) by gel filtration using PD 10 columns; their absence is confirmed by SDS-PAGE (PhastGel®).
8. Measure protein content of the fractions by reading the optical densities at 280 nm, and pI values by analytical IEF. Titration curves are constructed to find the net charge and the average charge density (charge/mol wt) of the various fractions (*see* **Notes 5** and **11**).
9. The size and conformation of the protein fractions are also determined (*see* **Notes 6** and **7**).

3.2. Partitioning of Proteins in ATPS

1. Stock solutions of PEG, Dextran or other materials (phase forming salts) are weighed into flasks with solid NaCl being added when necessary to give the desired composition (described in **Tables 1–3**).
2. Fine adjustments of pH are made if required with NaOH or HCl to give the desired values (*see* **Note 12**).
3. Protein solutions are mixed with the ATPS using a Vortex mixer for 1 min. Protein concentration in the systems should be approx 1 mg/mL of total volume. At least three replicates are done for each sample.
4. The volumes are then made up (5.0 mL) with distilled water as required.
5. Phase separation is achieved by centrifugation for 3 min at 3000*g*.
6. Phases are carefully separated and the interface of each tube is discarded.
7. Determine the concentration of the proteins in the phases (*see* **Note 13**).
8. Partition coefficients (K) of the modified proteins are found by calculating the ratio of protein concentration in the top phase to that in the bottom phase.
9. Resolution of the ATPS for protein surface hydrophobicity (R_h) is defined as the slope of the lines obtained by plotting the values of log *K* (log of the partition coefficient of the hydrophobically modified proteins) vs log *P* (the unit of hydrophobicity used: 2 *M* minus the value of $(NH_4)_2SO_4$, or [2 *M* – M_e]) (*see* **Notes 4** and **14**; **Fig. 1**).
10. Resolution of the ATPS for protein surface charge (R_c) is defined as the slope of the lines obtained by plotting the values of log K vs the charge density of the charged modified proteins (*see* **Note 15** and **Fig. 2**).

4. Notes

1. For hydrophobic modification, it is preferred to choose proteins with low surface hydrophobicity, as measured by HIC, because the method of chemical modification used increases protein hydrophobicity and it is desirable for all the modified products to elute from the HIC column in a suitable decreasing concentration linear salt gradient. The proteins for this study, BSA and β-lactoglobulin, have also the additional advantage of having average (i.e., not extreme) isoelectric points (4.8 and 5.1, respectively).
2. In order to have proteins with different hydrophobicities, four different groups were inserted: one with 6 carbons (hexanoic), one with 4 carbons (butyric), and one also with 4 carbons and a free carboxylic group (succinic), and acetic anhydride with only 2 carbons. According to reactions 1 and 2 (below) some of the free amino groups of the protein polypeptide chains are substituted by an aliphatic chain donated by the anhydride.

   Protein + Monocarboxylic anhydride (e.g., hexanoic)

$$P\text{-}NH_2 + (CH_3(CH_2)_4CO)_2O \Rightarrow PNH\text{-}CO(CH_2)_4CH_3 + CH_3(CH_2)_4COOH \quad (1)$$

   Protein + Dicarboxylic acid anhydride (internal anhydride, e.g., succinic)

$$PNH_2 + (RH\text{-}COO\text{-}CO)_2O \Rightarrow PNHCO\text{-}R\text{-}COOH \quad (2)$$

   This method can be used for most proteins by considering specific requirements in each case.

Table 1
Composition of ATPS Used for Partitioning of Hydrophobically Modified β-Lactoglobulin (Systems pH 5.3) *(4)*

Phase component 1, %		Phase component 2, %	NaCl, %	R[a]
PEG 1500	15.0	Phosphate, 10.0	—	10.2
	15.0	Phosphate, 10.0	6.0	25.5
	15.0	$(NH_4)_2SO_4$, 15.0	—	9.1
	15.0	$MgSO_4$, 15.0	—	3.5
	15.0	Na citrate, 15	—	11.4
PEG 4000	15.5	Phosphate, 15.2	—	22.6
	14.7	Phosphate, 14.7	6.0	29.0
	7.0	Dextran, 7.0	—	6.0
	7.0	Dextran, 7.0	3.0	5.0
	7.0	Dextran, 7.0	9.0	8.0

[a]Values of resolution of ATPS for differences in protein surface hydrophobicity.

Table 2
Composition of ATPS Used for Partitioning of Hydrophobically Modified BSA (Systems pH 5.3) *(4)*

Phase component 1, %		Phase component 2, %	NaCl, %	R[a]
PEG 1500	20.0	Phosphate, 14.0	—	20.7
	20.0	Phosphate, 14.0	6.0	44.0
PEG 4000	11.0	Phosphate, 15.0	—	12.0
	11.0	Phosphate, 15.0	3.0	11.0
	11.0	Phosphate, 15.0	6.0	20.4

[a]Values of resolution of ATPS for differences in protein surface hydrophobicity.

Table 3
Salt Composition of the ATPS Systems in 7% PEG 4000/ 7% Dextran T500, pH 7.9, for Measuring the Resolution for Differences in Protein Surface Charge *(5)*

Buffer	Salt
50 m*M* phosphate	50 m*M* Li_2SO_4
50 m*M* phosphate	None
50 m*M* phosphate	50 m*M* Na_2SO_4
50 m*M* phosphate	200 m*M* NaCl
50 m*M* phosphate	50 m*M* $MgSO_4$
50 m*M* Tris	None
50 m*M* phosphate	500 m*M* NaCl
50 m*M* phosphate	100 m*M* Na_2SO_4
50 m*M* phosphate	100 m*M* KCl

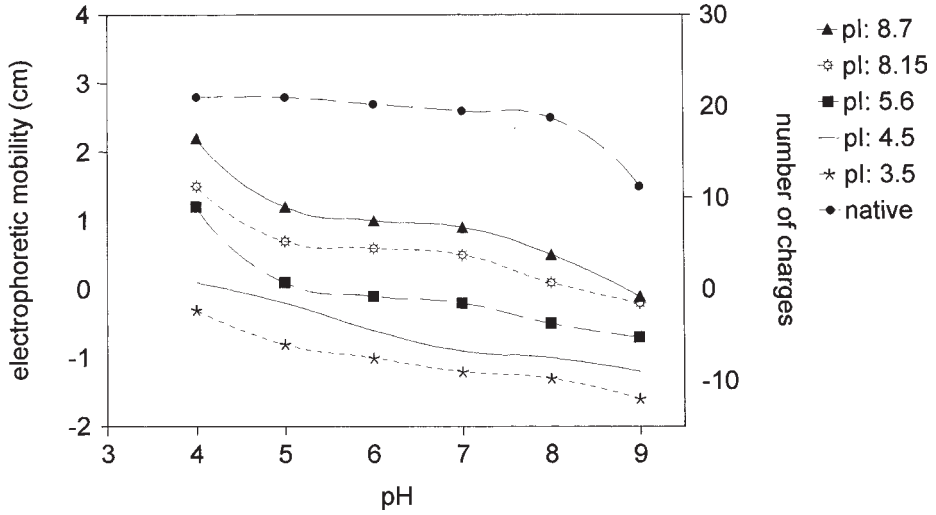

Fig. 1. Partitioning of hydrophobically modified BSA as a function of surface hydrophobicity in 20% PEG 1500–14% phosphate, pH 7.9, in the absence and presence of 6% NaCl. (Reproduced with permission from **ref. *4***.)

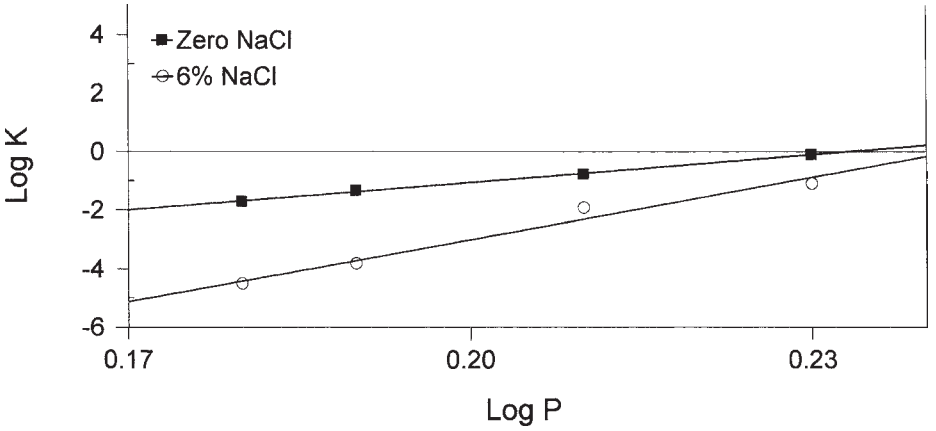

Fig. 2. Partitioning of modified thaumatin in 7% PEG 4000–7% dextran T500 systems with 50 m*M* phosphate buffer at pH 7.9, and addition of 50 m*M* Na_2SO_4, 50 m*M* $MgSO_4$ or 100 m*M* KCl. (Reproduced with permission from **ref. *5***.)

3. It is observed that the reactivity of the anhydrides decreases with increasing chain length, with the consequence that anhydrides of short chain lengths lead to more extensively substituted protein products than longer-chain anhydrides, under the chosen conditions.

4. The unit "P" to describe surface hydrophobicity of the proteins and modified fractions used is based on a linear relationship between the highest salt concentration used for starting the chromatographic run and the final salt concentration. As the start salt concentration for HIC is usually 2.0 *M* $(NH_4)_2SO_4$, the unit of hydrophobicity used is 2 *M* minus the value of $(NH_4)_2SO_4$, [2 *M* – M_e], at which a particular protein fraction eluted in the decreasing gradient. Varying values of P are obtained depending on the type and the amount of reagent anhydride used. For example, BSA when modified with acetic anhydride resulted in a product with a P of 2.0, and with hexanoic acid a maximal P value of 1.68 was obtained in contrast to 1.5 for a control (unmodified) sample ***(3)***.
5. The pI is determined by analytical IEF in polyacrylamide gels (PAGIF; pH range 3.0–9.0) using PhastSystem™ and prepackaged PAG plates (both from Amersham Pharmacia Biotech). Some decrease in pI values of the proteins is observed after hydrophobic modification owing to blocking of some of the free amino groups of the proteins. During modification of surface charge, fractions of highly varying pI values are obtained ***(3)***.
6. Molecular weight determination is performed by size exclusion chromatography on Superose 12HR column using a mobile phase of 100 m*M* sodium phosphate buffer containing 50 m*M* NaCl, with pH adjusted at 7.9 for BSA and thaumatin, and to 5.3 for β-lactoglobulin, at a flow rate of 0.5 mL/min ***(3)***. A standard curve is made with mol wt markers. No notable change in the size of the proteins is seen after chemical modification.
7. Conformational analysis of the modified protein is done by intrinsic fluorescence methods ***(9)*** using standard curves of each protein dissolved in solutions of increasing concentrations of urea vs fluorescence reading. Excitation wavelength is 297 nm and emission wavelengths are 290 nm and 350 nm. The change in conformation is monitored by following the ratio in absorbance readings at 350 and 290 nm. Changes in protein conformation, such as unfolding, very often lead to large changes in intrinsic fluorescence. Increase or decrease in fluorescence intensity can occur upon protein unfolding, the magnitude depending on the overall effect produced by the denaturation, e.g., exposure of fluorescent groups, proximity of quencher groups which decrease protein emission, etc. It seems that the conditions during the chemical modification (pH changes, dialysis, freeze drying steps and hydrophobic interaction chromatography) generate some conformational change, even in the absence of a modification reaction ***(3)***.
8. For charge modification, thaumatin is chosen because the native material has a very high pI (10.3–10.5) which facilitates the preparation of a series of modified proteins with lower pI values.
9. The extent of amino group blocking of thaumatin is controlled by the reagent concentration and time of the reaction.
10. Charge-modified proteins are separated by IEF in a preparative gel bed (Multiphor electrophoresis system, Amersham Pharmacia Biotech). Suspend 10 g of Sephadex IEF in 200 mL of water. Add 10 mL of Pharmalyte solution (pH range 3.0–10.0). Dissolve 500 mg of the modified protein in water and add 1.0 mL of

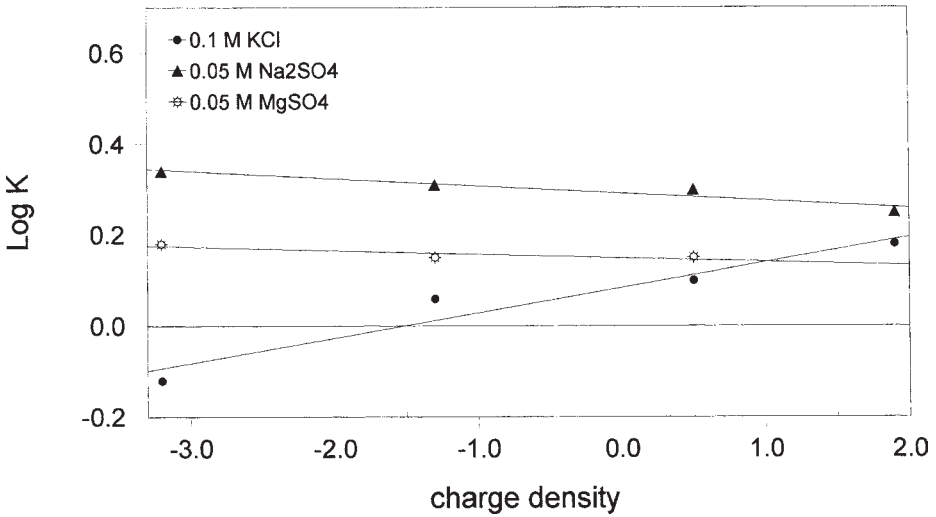

Fig. 3. Electrophoretic mobility and net charge of modified thaumatin fractions along a pH gradient of 3–9. (Reproduced with permission from **ref. *3***.)

the Pharmalyte solution. Prefocusing is carried out for 20 min at 30 W. A narrow trough is cut out of the gel bed with the sample applicator, the gel is removed and mixed with the dissolved protein sample. Return the mixture to the flat-bed cavity. Isoelectric focusing is then carried out with initial conditions of 530 V, 98 mA, 50 W at constant power for 3.5–4.0 h until the voltage stabilizes. Strips of the gel bed are cut out with the help of a fractionating grid, collected into separate tubes and their pH values measured. The fractions are filtered through Sintered filters and washed through with distilled water ***(10)***. The volume of each fraction collected from isoelectric focusing is made up to 15 mL. Protein content and pI values are determined for the proteins in the different fractions.

11. Titration curves are performed in IEF gels (pH range 3.0–9.0) using the PhastSystem. The electrophoretic mobility (cm) for the modified thaumatin samples of differing pI vs pH is represented in **Fig. 3** ***(3)***. Since the distance between the electrodes, the length of the run and the molecular weight of all samples were the same, the net charge of a protein is directly related to its electrophoretic mobility on the titration curve. The samples of modified thaumatin selected for later ATPS partitioning were those which showed only one main band of protein in a IEF gel ***(3)***. **Table 4** shows the properties of the selected charge-modified thaumatins. There appears a surface hydrophobicity variation, consistent with the blockage of some surface amino grouping, but a much more dramatic effect is seen on pI and on electrophoretic mobility.
12. All systems used for partitioning experiments with modified β-lactoglobulin should be adjusted to pH 5.25–5.35 where native β-lactoglobulin is mostly in the dimeric form and its conformation is more stable. The systems used for partition-

Table 4
Properties of Charge-Modified Thaumatins[a]

Fraction	Electrophoretic mobility	Net charge	Charge density	pI	P
1	0.53	4.2	1.9	8.70	1.66
2	0.14	1.1	0.5	8.15	1.80
3	–0.47	–3.7	–1.70	5.60	1.84
4	–0.99	–7.9	–3.6	4.50	1.86

[a]Mobility (cm) in an electrophoretic titration curve in IEF gel at pH 7.9; net charge/protein molecule; charge density (net charge/mol wt, × 10^{-4}); isoelectric point (pI); protein surface hydrophobicity (P) ([2 M – M_e]. Hydrophobicity units are 2 M minus the concentration of $(NH_4)_2SO_4$ at which the protein eluted in a decreasing gradient of salt from 2 M to 0 M in a phenyl sepharose column ***(3)***.

ing modified BSA should be adjusted to pH 7.9, at which the net molecular charge of the various modified BSA samples is most similar.

13. Protein concentrations are determined using the dye binding technique ***(11)***. A standard curve for each protein is prepared. When the protein concentration in the phase is above 0.1 mg/mL, protein can be determined by absorbance at 280 nm against a blank of the same phase composition.
14. By plotting the K values of each series of hydrophobically modified protein vs the values of surface hydrophobicity, values of R and log P_o are found, where R represents the resolution of the system to separate proteins based on their hydrophobicity while log P_o is the "intrinsic hydrophobicity" of the system (**Fig. 1**). Proteins partition preferentially to the top phase in systems with lower values of log P_o.

    Negative slopes of the resolution for protein surface hydrophobicity represent systems where proteins partition towards the bottom phase with the increase in the hydrophobicity (increase of log P) and positive slope values represent systems where proteins behave in the opposite way (**Fig. 1**). Increase in protein surface hydrophobicity cause increase in K for all the ATPS studied. All PEG/phosphate systems give high values of R. The addition of NaCl to the phase systems, especially PEG/phosphate, increases the resolution of the systems for protein surface hydrophobicity ***(4***; *see* **Tables 1** and **2***)*.
15. The partition coefficient of charge modified thaumatin vs charge density is shown in **Fig. 2**. Negative slopes of the resolution for protein surface charge represent systems where proteins partition toward the bottom phase with increase in the electrophoretic mobility (increase of pI also) and positive slope values represent systems where proteins behave in the opposite way.

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20

Detection and Analysis of Interactions by Two-Phase Partition

Lars Backman

1. Introduction

All biological life is dependent on interactions. On the molecular level, interactions between proteins, nucleic acids, and lipids are required for formation of the organelles, as well as other cellular structures such as cytoskeletons, ribosomes, and multienzyme complexes. In addition, usually weaker interactions between receptors and low molecular weight molecules such as hormones, guanosine triphosphate (GTP) or ions, are involved in many regulatory events. The importance of interacting systems for biological life as well as the abundance of interacting molecules, has led to development of a large number of techniques for detecting as well as defining the interaction in terms of binding strength and stoichiometry.

In general, any technique that is able to differentiate between bound and free forms of the interacting molecules should be appropriate for binding studies. Because properties such as size, net surface charge, or hydrophobicity of the formed complex most probably will differ from those of the free molecules, complex formation can be measured by any technique that can separate molecules according to these properties.

In solid-phase-based (overlay) techniques, one component is attached to a surface (often a nitrocellulose membrane), whereas the other component is overlaid on the membrane. After incubation, unbound material is washed away and the bound amount is determined ***(1,2)***. In addition, chemical crosslinking and similar techniques often have been used for identifying interacting molecules ***(3)***. Although overlay assays can give quantitative results, mass-transfer techniques like sedimentation, dialysis, and gel filtration ***(4–7)*** are classical

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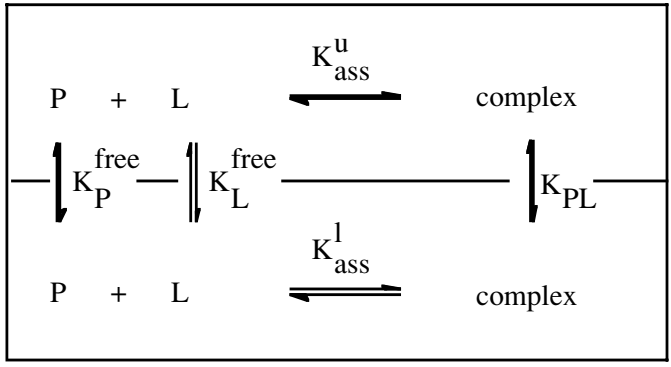
techniques for determining binding strength and number of sites. Various spectroscopic methods can also be used to determine binding ***(8,9)***.

A powerful yet simple technique for obtaining qualitative as well as quantitative data is phase partition ***(2,10)***. This technique has several advantages:

1. It measures binding at equilibrium,
2. Any buffer systems can be used,
3. Ordinary laboratory ware is sufficient and no elaborate equipment is required, and
4. The aqueous two-phase systems used usually have a stabilizing effect on biological macromolecules.

1.1. Basic Theory

In a two-phase system the equilibria occurring in each phase as well as between the phases is described by



where K_P, K_L, and K_{PL} represents the partition coefficients of free components and the formed complex, respectively. K^{u}_{ass} and K^{l}_{ass} are the association constants in the upper and lower phases, respectively.

The partition coefficients of the two components, P(rotein) and L(igand), are defined by

$$K_P^{free} = \frac{[P]^u}{[P]^l} \text{ and } K_L^{free} = \frac{[L]^u}{[L]^l}$$

where $[...]^u$ and $[...]^l$ represents the concentrations in the upper and lower phases, respectively. If there is a ligand-binding protein present, a certain fraction of the ligand (as well as of the protein) will be bound and the partition can be described by an apparent partition coefficient, defined as

$$K_L^{app} = \frac{[L]^u_{total}}{[L]^l_{total}} = \frac{[L]^u_{free} + [L]^u_{bound}}{[L]^l_{free} + [L]^l_{bound}}$$

The apparent partition coefficient of the protein is defined similarly. By measuring the concentration of ligand in the two phases, the total concentration of ligand in each phase can be determined, and from these values the apparent partition coefficient is calculated. The determined apparent partition coefficient will depend on the presence of the ligand-binding protein. In the absence of such a protein, the apparent partition coefficient will equal the partition of free ligand. If the concentration of the binding protein is increased (keeping the ligand constant), an increasing fraction of ligand will be bound and the apparent partition coefficient will approach that of bound ligand. Similarly, if the concentration of protein is kept constant while the concentration of ligand is increased, the partition of the ligand will approach that of free ligand.

Because the partition behavior or isotherm is dependent on the partition properties of free and bound ligand, as well as on the number of sites and the affinity of each site, it is possible to extract stoichiometry and binding affinities from the partition data.

In order to determine the binding by partition, it is obvious that the partition of free and bound forms of either ligand or protein must differ. It is an advantage if the partition of ligand and protein differs as much as possible, as this will enhance the sensitivity and facilitate the data analysis. It is also advantageous if either ligand or protein partitions exclusively to one of the phases. In such a case, the binding equilibria will only occur in one of the phases and not only the actual measurements of concentration, but also the data analysis is facilitated.

2. Materials

1. 20% (w/w) Dextran T-500: Dissolve 100 g of Dextran in 300 mL of water by boiling. Be careful that no Dextran is stuck to the bottom since this may discolor the final Dextran solution. Let the solution cool to room temperature with the flask covered with a watch glass. The exact concentration of the Dextran solution can be determined from the optical rotation (*see* Chapter 3). Dilute the Dextran stock solution to 20% (w/w) and store refrigerated at 4°C (*see* **Note 1**). If done properly, the Dextran solution can be stored for several weeks at 4°C. However, as soon as there are any traces of bacterial growth, the solution should be discarded.
2. 50% (w/w) Ficoll 400: Dissolve 100 g of ficoll in 100 mL of water as previously described. Measure the optical rotation and calculate the concentration of ficoll using a specific optical rotation of +54.9°. Dilute the stock to 50% (w/w) and store similar to Dextran (*see* **Note 1**).
3. 40% (w/w) Polyethylene glycol (PEG) 8000: Dissolve 40 g of PEG 8000 in water to give exactly 100 g solution. Store as the other polymer solutions.
4. 200 m*M* HEPES, pH 7.0 (*see* **Note 2**).
5. 2 *M* KCl.
6. 100 m*M* $MgCl_2$.

7. 100 m*M* $CaCl_2$.
8. Isolated and purified biological material: The biological material of interest must be available in such quantities that it will be possible to measure its concentration in the two separated phases. In the binding experiments used to illustrate the technique brain spectrin and ^{14}C-labeled calmodulin are used ***(2)***.

3. Methods

3.1. Choice of Phase System

The preferred two-phase system for studying interactions should be such that one of the components only partitions to one of the phases. Since it is not always possible to vary the composition of components of the two-phase system, a one-sided partition may not be attainable. Although the sensitivity of the method may be reduced slightly, any phase system in which a change in apparent partition of one (or both) of the components is detectable will be sufficient. When selecting a phase system, one approach is to choose the phase system that gives the largest difference in partition behavior of the components. Such a phase system can be obtained in several ways: by trial and error or by applying experimental design such as simplex ***(11)*** (*see* **Note 3**).

3.2. Preparing the Bulk Phase System

1. Make a stock two-phase system by adding 30.0 g of 50% Ficoll, 25.0 g of 20% Dextran, 0.5 g of 40% PEG, 5.0 mL of 200 m*M* HEPES, pH 7.0, 5.0 mL of 2 *M* KCl, 1.0 mL of 100 m*M* $MgCl_2$, 1.0 mL of $CaCl_2$, followed by water to give a final weight of 80.0 g, in a 125 mL separatory funnel placed on a weighing balance. This gives a phase system composed of 18.75% Ficoll, 6.25% Dextran, 0.25% PEG, 12.5 mmole/kg HEPES, pH 7.0, 125 mmole/kg KCl, 1.25 mmole/kg $MgCl_2$, and 1.25 mmole/kg $CaCl_2$.
2. Mix the stock phase system thoroughly and leave overnight at 20°C to allow the phases to separate.
3. Next day (or when a distinct interface has developed), collect the two phases (*see* **Note 4**). If not used immediately, the separated upper and lower phases can be stored at 4°C for several weeks.

3.3. Preparing the Analytical Phase System

Dispense 0.4 mL of each phase into disposable test tubes (*see* **Note 5**). By using a repetitive pipet a large number of these analytical two-phase systems can quickly be prepared (*see* **Note 5**). These two-phase systems can be stored at 4°C.

Dispense 4 mL of each phase and 2 mL of buffer into a 10 mL measuring cylinder. Cover with parafilm and mix thoroughly. Set aside and let the phases separate. After phase separation, read the volume of upper and lower phase, respectively (*see* **Note 6**).

3.4. Partition of Individual Components

The partition experiment is done in two series; the first series is used to establish the partition behavior of the free components, especially to check that the partition of free components is independent of the concentration, whereas the second series is used to measure the interaction.

1. Make a serial dilution of both components, ranging from the lowest detectable concentration to the highest possible concentration (*see* **Note 7**).
2. Let a sufficient number of test tubes with the analytical phase systems attain 20°C.
3. Add 0.2 mL of sample (from each dilution) to an analytical phase system, to make the final volume to 1.0 mL. Make two or three systems at each dilution. Repeat with the other sample.
4. Mix the phase systems carefully and incubate the phase systems for 2 h with end-over-end rotation, at the proper temperature.
5. Separate the phases by centrifugation in a table-top centrifuge. During centrifugation the temperature should not increase more than a few centigrades otherwise the partition may be affected (*see* **Note 8**).
6. Remove 0.1 mL from each phase and measure the concentration of the respective components. This step is probably the most critical in the whole experiment. First, use an automatic pipet to withdraw an aliquot from the upper phase and transfer it to a clean test tube. Then change tip, and very slowly stick the tip through the interface down to the bottom of the test tube; wait 10 s before allowing the lower phase to enter the tip. Remove the pipet slowly and use soft paper tissue to remove any traces of solution on the outside of the tip. If the lower phase is very viscous, it may help to use tips with wider openings (*see* **Note 9**).
7. Calculate the partition coefficient of each component (together with an estimate of the error of measurement) and plot these values vs the concentration. The partition behavior of both components, or at least one of them, should be independent of its concentration (*see* **Note 10**).

3.5. Binding Experiment

In this series the binding equilibria is investigated, initially to obtain evidence that there is an interaction between the two components, but also to obtain data for the final numeric analysis of the binding.

1. Add 0.1 mL of one of the components (from the serial dilution) and 0.1 mL of the other sample at a constant concentration (*see* **Note 11**).
2. Mix the phase systems carefully and incubate the phase systems for 2 h with end-over-end rotation.
3. Remove 0.1 mL from each phase and measure the concentrations of the two different components.
4. Calculate the apparent partition coefficient of each component and plot these values versus the concentration.

5. The measurements should be repeated, both at different constant concentrations and by keeping the other component constant.

3.6. Qualitative Analysis of Partition Results

An interaction is indicated if the partition of one of the components is affected by the presence of the other component, as illustrated for the interaction between spectrin and calmodulin shown in **Fig. 1**. The positive identification of an interaction is strengthened if the apparent partition approaches the partition of free component either at very high concentrations of that component or at very low concentrations of the other component.

3.7. Quantitative Analysis of Partition Results

The extraction of binding strength and number of sites from the partition isotherm is much more complicated and, in general, requires fitting of plausible binding models to the experimental data.

A binding model can be derived by setting up the relevant equations describing the binding equilibria as done below.

The total concentration of *P* is expressed as

$$[P]_{tot} = r \cdot [P]^{u}_{tot} + q \cdot [P]^{l}_{tot} \quad (1)$$

where *r* and *q* are the fractional volumes of the upper and lower phases, respectively, defined by

$$r = \frac{\text{volume}_u}{\text{volume}_u + \text{volume}_l} \text{ and } q = \frac{\text{volume}_l}{\text{volume}_u + \text{volume}_l} \quad (2)$$

Assuming a single binding site, the total concentration of P is

$$[P]_{tot} = r \cdot \left([P]^{u}_{free} + [PL]^{u}\right) + q \cdot \left([P]^{l}_{free} + [PL]^{l}\right) \quad (3)$$

The concentrations of *P* and *PL* in the upper phase are related to the corresponding concentrations in the lower phase by

$$[P]^{u}_{free} = K_P^{free} \cdot [P]^{l}_{free} \quad (4)$$

$$[PL]^{u} = K_{PL} \cdot [PL]^{l} \quad (5)$$

Combining **Eqs. 3–5** gives

$$[P]_{tot} = r \cdot \left(K_P^{free} \cdot [P]^{l}_{free} + K_{PL} \cdot [PL]^{l}\right) + q \cdot \left([P]^{l}_{free} + [PL]^{l}\right. \quad (6)$$

The concentration of the formed complex (in the lower phase) is given by

$$[PL]^{l} = K^{l}_{ass} \cdot [P]^{l}_{free} \cdot [L]^{l}_{free} \quad (7)$$

Inserting **Eq. 7** in **Eq. 6** gives

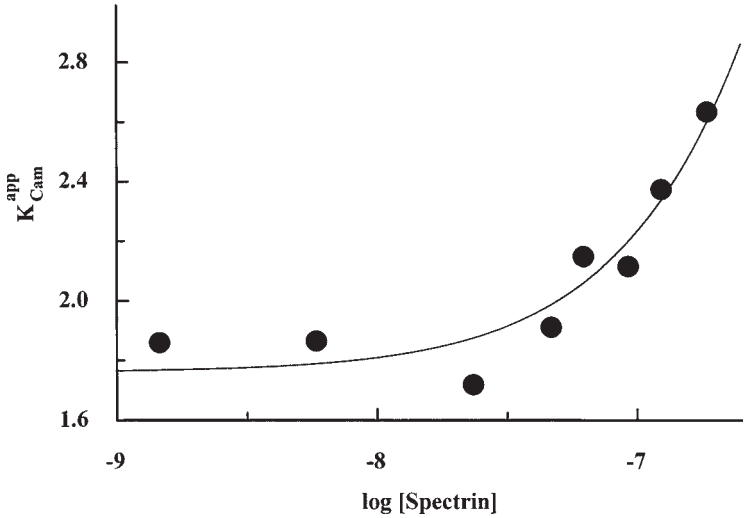


Fig. 1. Binding of calmodulin to brain spectrin. The apparent partition coefficient of calmodulin (21.1 n*M*) was measured at the indicated concentrations of brain spectrin (●). The full drawn line represents the best-fitting single-site model. The partition coefficients of free spectrin ($K_P^{free} = 10$) and calmodulin ($K_L^{free} = 1.76$) were used in the fitting procedure. Because the partition of spectrin was not affected by the presence of calmodulin, it was assumed that all bound forms of calmodulin (K_L^{bound}) also was 10. The volumes of the upper and lower phases were 0.44 and 0.56 mL, respectively. For a single-site model, a best fit was obtained with an association constant of $3.15 \times 10^6\ M^{-1}$.

$$[PL]_{tot} = r \cdot \left(K_P^{free} \cdot [PL]_{free}^{l} + K_{PL} \cdot K_{ass}^{l} \cdot [P]_{free}^{l} \cdot [L]_{free}^{l} + q \cdot ([P]_{free}^{l} + K_{ass}^{l} \cdot [P]_{free}^{l} \cdot [L]_{free}^{l}\right) \tag{8}$$

which is reduced to

$$[P]_{tot} = [P]_{free}^{l} \cdot \left(q + r \cdot K_P^{free} + K_{ass}^{l} \cdot [L]_{free}^{l} \cdot (q + r \cdot K_{PL})\right) \tag{9}$$

The total concentration of L can be expressed similarly

$$[L]_{tot} = [L]_{free}^{l} \cdot (q + r \cdot K_L^{free} + K_{ass}^{l} \cdot [P]_{free}^{l} \cdot [P]_{free}^{l} \cdot (q + r \cdot K_{PL} \tag{10}$$

From **Eqs. 9** and **10**, it is possible to express $[P]_{free}^{l}$ and $[L]_{free}^{l}$ in terms of total concentrations, partition coefficients, and an association constant

$$[P]_{free}^{l} = \frac{[P]_{tot}}{\left(q + r \cdot K_L^{free} + K_{ass}^{l} \cdot [L]_{free}^{l} \cdot (q + r \cdot K_{PL})\right)} \tag{11}$$

$$[L]_{free}^{l} = \frac{[L]_{tot}}{\left(q + r \cdot K_L^{free} + K_{ass}^{l} \cdot [P]_{free}^{l} \cdot (q + r \cdot K_{PL})\right)} \tag{12}$$

From these expressions it is also possible to calculate the apparent partition coefficients

$$K_P^{app} = \frac{K_P^{free} \cdot [P]_{free}^{l} + K_{PL} \cdot K_{ass}^{l} [P]_{free}^{l} \cdot [L]_{free}^{l}}{[P]_{free}^{l} + K_{ass}^{l} [P]_{free}^{l} \cdot [L]_{free}^{l}} = \frac{[P]_{free}^{u} + [P]_{bound}^{u}}{[P]_{free}^{l} + [P]_{bound}^{l}} \quad (13)$$

$$K_L^{app} = \frac{K_L^{free} \cdot [L]_{free}^{l} + K_{PL} \cdot K_{ass}^{l} [P]_{free}^{l} \cdot [L]_{free}^{l}}{[L]_{free}^{l} + K_{ass}^{l} [P]_{free}^{l} \cdot [L]_{free}^{l}} = \frac{[L]_{free}^{u} + [L]_{bound}^{u}}{[L]_{free}^{l} + [L]_{bound}^{l}} \quad (14)$$

To fit the calculated apparent partition coefficients to the experimentally determined partition coefficients, the variable parameters K_{PL} and K_{ass} are varied to minimise the error of sum of squares given by

$$ess = \sum \left(K_P^{app} - K_P^{exp}\right)^2 + \left(K_L^{app} - K_L^{exp}\right)^2 \quad (15)$$

For this purpose, **Eqs. 11** and **12** are solved iteratively for each data point in Microsoft Excel, by using the add-in tool Solver (or any similar program). When this is done for the data in **Fig. 1**, assuming a single binding site for calmodulin on spectrin, an association constant of $3.15 \times 10^6\ M^{-1}$ gives the best fit to the experimental data (*see* **Note 12**).

Models for more complex interacting patterns can be derived similarly.

4. Notes

1. There is no real need to dilute the polymer solution to 20% or 50%, this is only done for convenience.
2. Any buffer system as well as any ions compatible with the polymers can be used. Addition of ions and other molecules may create an excellent growth media for bacteria. Therefore, care should be taken, both when the phase system is prepared and during storage to reduce possible contamination.
3. The finding of a useful two-phase system is often the most tedious part of the experiment. In my laboratory we have found that the simplex technique is very useful for obtaining a "good" two-phase system ***(11)***. Often the buffer and salt composition cannot be adjusted but must remain fixed. This means that the partition can only be influenced by the polymer concentration and composition, which will limit the testing of suitable phase compositions.
   It should be noted that even a two-phase system in which both components partition equally may be useful, because it is only necessary that the partition of formed complexes must differ from the partition of the free components.
4. When separating the two phases, it is easiest first to collect slowly the lower phase into a flask. As the interface is approaching the valve, the flow is stopped to allow the interface to reform before the final drops of lower phase are collected. Then let 1–2 mL of upper phase slowly flow through the outlet to remove the interface. Collect the upper phase by pouring through the top of the funnel

into a flask. If the interaction is to be measured at a different temperature, the phase separation of the bulk system should also be done at this temperature.

5. Using 0.4 mL of each phase will allow the addition of 0.2 mL of sample. If larger volumes must be added, owing to low concentration or any other reason, the bulk two-phase system can be made more concentrated; however, it should be noted that a higher polymer concentration leads to a higher viscosity of the phases which may lead to less accurate dispensing. Repetman from Gilson is very suitable for dispensing the upper and lower phases.
6. The volumes of the upper and lower phases, respectively, are required for the formal analysis of the binding.
7. The relative large amount of material required for a series of experiments is probably the major disadvantage of the partition technique. However, the amount required for each analytical two-phase system is also dependent on the sensitivity of the assay method; if a very sensitive assay is available, lower concentration can be used and less material is required. At the same time, it is advantageous to use as large in excess as possible of one of the components because this will cause a greater change in partition behavior on binding.
8. A centrifugal force of 2250*g* for 15 min was sufficient to separate the phase system used for analysis of the interaction between spectrin and calmodulin. Because the ficoll/Dextran/PEG phase system give very viscous phases, the centrifugation can probably be shortened considerably for the more common Dextran/PEG systems. The rise in temperature can be controlled by blowing cold air on the table-top centrifuge or by using a temperature-controlled centrifuge.
9. If care is taken larger aliquots can be withdrawn from each phase. The concentration can be determined by any technique. The phase polymers do not interfere with most general methods for determining protein concentrations. In my laboratory, the concentration has been determined spectrophotometrically (usually at 280 nm), by radioactivity, enzyme activity, and so forth.
10. If the partition of one of the components shows a concentration-dependence, this component should be kept at a constant concentration in the binding experiments to avoid unnecessary complications in the analysis of the results. If the partition of both components is concentration-dependent, this must be accounted for, which complicates the qualitative as well as the quantitative analysis. For the qualitative interpretation, it is probably enough to compare the apparent partition coefficient with the determined free partition of the component, at least if the change in apparent partition is much larger than the error of measurement. The effect of increasing concentrations can also be investigated by adding a nonbinding component.
11. The volumes of added sample can be changed as long as the total added volume is 0.2 mL.
12. MS Solver is set up such that the program adjusts $[P]^{l}_{free}$ and K^{l}_{ass} in order to find the values giving the minimal error of sum of squares. The initial value of $[P]^{l}_{free}$ is set to the highest possible (i.e., the total concentration of P). These trial values are then used to calculate $[L]^{l}_{free}$. Another set of $[P]^{l}_{free}$ is calculated

from $[L]^1_{free}$ and K^1_{ass}; these values are then used to calculate the apparent partition coefficients and the error of sum of squares. This process is repeated until the minimal error of sum of squares is found that also satisfy the constraint that the two sets of $[P]^1_{free}$ should be equal. MS Solver often reports that a solution cannot be found. Ignore this message and keep the calculated results and then restart Solver.

To speed up the fitting procedure, it is advisable to start with a low precision (i.e., 1E-10), and after the program has found a best fitting set of data, increase the precision and redo the fitting procedure (using the found parameters as start values) until the sum of error no longer improves.

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21

Cryopartitioning in Two-Phase Systems Containing Organic Solvents

Göte Johansson

1. Introduction

Aqueous two-phase systems, based on two polymers, usually freeze at temperatures below –1°C. The freezing point can be considerably lowered by exchanging part of the water for a water miscible organic solvent such as ethylene glycol or glycerol ***(1)***. Partitioning of proteins and particles has been carried out in such systems using temperatures down to –20°C ***(2,3)***. The low temperature, in combination with the solvent, gives the phases high viscosity, which influences the time for equilibration and especially the time for phase separation. Sometimes centrifugation at the equilibration temperature is advisable.

The partitioning at subzero temperatures offers the possibility to stabilize sensitive enzymes as well as structures of fluid membranes. Also the interactions between protein and ligand or between a receptor and its ligand may be strengthened by the low temperature as well as the interactions between protein molecules of possible weak enzyme complexes.

This chapter describes the preparation of such modified two-phase systems and their use in partitioning of proteins and membranes at subzero temperatures.

2. Materials

2.1. Partitioning of Enzymes

1. 50% (w/w) aqueous solution of polyethylene glycol (PEG) with a mean molecular weight of 8000, PEG 8000: Dissolve 50 g of PEG in 50 g of water at room temperature.
2. 25% (w/w) aqueous solution of Dextran T 500 (M_w = 500,000): Layer 26.2 g of Dextran powder over 74 g of water in a 250 mL Erlenmeyer flask and heat the

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mixture under occasional shaking to gentle boiling. When cool, the extract concentration may be determined polarimetrically (*see* Chap. 3).
3. Ethylene glycol, water-free (*see* **Note 1**).
4. 0.1 *M* Tris-HCl buffer, pH 8.0: The concentration is based on Tris. Mix 50 mL of 0.2 *M* Tris, 29 mL of 0.2 *M* HCl, and 21 mL of water.
5. Coomassie Brilliant Blue G (100 mg/L) in 8.5% phosphoric acid and containing 5% ethanol (Bradford reagent) (*see* **Note 2**).

2.2. Partitioning of Membranes

1. 50% (w/w) aqueous solution of PEG 3400 with a mean molecular weight of 3400 (*see* **Subheading 2.1.**, **item 1**).
2. 25% (w/w) aqueous solution of Dextran T40 (M = 40,000) (*see* **Subheading 2.1.**, **item 2**).
3. Glycerol, water-free (*see* **Note 1**)
4. 0.1 *M* Tris-HCl buffer, pH 8.0 (*see* **Subheading 2.1.**, **item 4**).

3. Methods

The systems suggested here for partitioning of proteins, at –15°C, and of membranes, at –10°C, contain ethylene glycol and glycerol, respectively.

3.1. Partitioning of Enzymes

1. Make systems by weighing out 1.26 g of 50% (w/w) PEG 8000, 3.72 g of 25% (w/w) Dextran T500, and 3.3 g of ethylene glycol in 10-mL graduated centrifugation tubes.
2. Equilibrate the systems and put them in a thermostat bath at –18°C (*see* **Note 3**).
3. Add 1.62 g of protein sample also containing 40% (w/w) ethylene glycol and 25–50 m*M* buffer (e.g., Tris-HCl) and cooled to –18°C.
4. Mix each tube carefully by inverting them five times avoiding increase in temperature of the systems (*see* **Note 4**). Put the tubes back in the bath. Repeat this mixing at least twice with 5-min intervals.
5. After 30 min, each tube is mixed again quickly (*see* **Note 4**).
6. Let the phases settle at –18°C for 2.5–3 h (*see* **Note 5**).
7. Take out samples from both top and bottom phase, respectively, and analyze for the content of activities of interest (*see* **Note 6**).
8. Analyze both top and bottom phases for the concentration of protein using the Bradford method (*see* **Note 2**). Mix 250 µL of the sample with 3.00 mL of reagent solution and measure the absorbance at 595 nm after 10 min. Prepare a blank in the same way from the phases of a system not containing protein.

3.2. Partitioning of Synaptic Membranes

Membrane fragments can appear in the two phases as well as at the interface (*see* **Fig. 1**). They are negatively charged and their partition is therefore strongly dependent on the kind and concentration of salt present in the system. A salt concentration of even 1 m*M* or less may effect the partitioning.

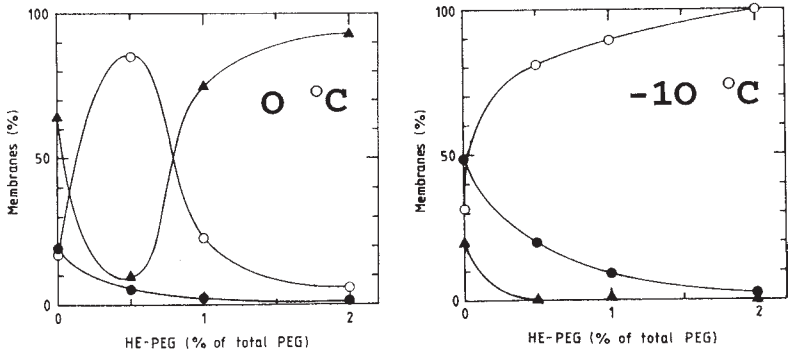

Fig. 1. The partitioning of synaptic membranes, isolated from calf brain cortex, between top phase (○), interface (▲) and bottom phase (●) at 0 and –10°C, respectively. The systems contained 10.1% (w/w) Dextran T500, 6.9% (w/w)PEG 8000 (0°C) or 9.0% (w/w) Dextran T500, 6.1% (w/w) PEG 8000 (–10°C) together with 20% glycerol and 2 m*M* Tris-HCl buffer, pH 7.8. Various amounts of the PEG were replaced with a ligand-PEG, hexaethonium-PEG (HE-PEG) with affinity for nicotinic cholinergic receptors. (Adapted with permission from **ref. *3***.)

1. Mix 3.44 g of 25% Dextran 40, 1.17 g of PEG 3400 (eventually containing ligand-PEG, *see* **Note 7**), 2.00 g of glycerol, and 0.39 g of water in a 10-mL graduated centrifugation tube. Instead of water, various concentrated salt solutions can be added.
2. Add 3 mL of membranes suspended in 10 m*M* Tris-HCl buffer (0.1 *M* buffer diluted 1:10) and mix (*see* **Note 8**).
3. Cool the system to –10°C in a thermostat bath under occasional mixing (*see* **Note 3**).
4. Leave the system for half an hour in the bath and mix again (*see* **Note 4**). Take out a sample of the mixed system for analysis and dilute it with the same volume of buffer.
5. The systems are left for 2 h in the bath to let the phases settle. Note the volumes of the two phases.
6. Take out samples from top and bottom phase, respectively, for analyses and dilute them with the same volumes of 10 m*M* Tris-HCl buffer.
7. Analyze the samples for enzyme activities of interest (*see* **Note 6**).
8. Analyze the protein using Bradford method by mixing 200 μL of diluted sample with 50 μL of 2.5 *M* phosphoric acid and incubating it for 1 h in a 50°C bath. After cooling to room temperature, add 3 mL of Bradford reagent solution and measure the absorbance at 595 nm after 10 min. Samples from a system not containing membrane, but otherwise treated in the same way, are used as blanks (*see* **Note 9**).
9. Measure the absorbance at 400 nm (A^{400}) to determine the membrane concentration using diluted mixed blank system and diluted separated phases, respectively, as blanks.

10. Calculate the percentage of particles, enzyme actitvities, and (membrane-bound) proteins recovered in top and bottom phase, respectively. The difference from total is assumed to be collected at the interface (*see* **Notes 10** and **11**).

4. Notes

1. Anhydrous glycerol and ethylene glycol are very hygroscopic and should be kept in well-closed bottles.
2. The Bradford reagent ***(4)*** is obtained by dissolving 100 mg of Coomassie Brilliant Blue G in 50 mL of ethanol and adding 100 mL of 85% phosphoric acid. Dilute to 1 L with water and filter after 1 d. Precipitate may form with time but will sediment (do not shake). A calibration curve using bovine serum albumin (BSA) as standard is used for evaluating the protein concentration.
3. The thermostat bath (e.g., Grant LTD6) is supplied with a nonfreezing liquid like 40% glycerol. Ready-to-use nonfreezing cooling liquid for cars is a cheap alternative.
4. Take out the tube only for very short periods from the bath to avoid increase in temperature.
5. If a refrigerated centrifuge is available the settling can be achieved by low-speed centrifugation for 15 min. Check that the temperature does not change during centrifugation.
6. Normally, any enzyme activity can be measured directly in the phases or after dilution. Some enzymes may temporarily lose or reduce their activity at subzero temperatures and it can appear again after some incubation at 0°C or at room temperature for 1–2 h.
7. Ligand-PEG, 0.1–5% of total PEG, may be introduced as part of the 50% PEG solution. The molecular mass of ligand-PEG may be 3400 Da or higher. Examples of ligand-PEGs are given in ***(3,5,6)***.
8. The membranes can be prepared from tissue by standard methods. A preparation of synaptic membranes from brain used for partitioning is found in ***(5)***. It is important that the membrane fragments are small enough in size to avoid aggregation and sedimentation during the time for phase settling. For this purpose the membrane suspension can be passed twice through a Yeda press (using 100 atm pressure of nitrogen) or sonication.
9. To determine the concentration of membrane-bound protein, the treatment with phosphoric acid liberates protein without any flocculation that would otherwise disturb the measurements.
10. If a linear relation between the absorbance at 400 nm and the concentration of membrane particles is assumed, the percent of material in top phase (m_T), in bottom phase (m_B), and at the interface (m_I) can be calculated from the absorbance of the diluted mixed system (A_{mix}), top phase (A_T), and bottom phase (A_B), respectively. The volume ratio, top phase/bottom phase = V_r, has also to be known. $m_T = 100 \cdot A_T \cdot V_r / (A_{\text{mix}} \cdot (1+V_r))$; $m_B = 100 \cdot A_B / (A_{\text{mix}} \cdot (1+V_r))$; $m_I = 100 - m_T - m_B$. The same calculations are used to obtain the partitioning of protein and enzyme by replacing A for protein concentration and enzyme activities (in U/mL), respectively.

11. The partitioning of synaptic membranes at 0 and –10°C, respectively, using a PEG-bound ligand for cholinergic receptors is shown in **Fig. 1**. At 0°C the membranes are extracted towards the top phase at low concentration of ligand-PEG while higher concentration causes aggregation and a resdistribution of the membranes to the interface. This is avoided by carrying out the partitioning at –10°C.

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22

Optimization of Extractions in Aqueous Two-Phase Systems

Ulrich Menge

1. Introduction

The extraction of proteins in aqueous phase systems is one of the most rapid purification methods available, and very useful for separation of cell debris. However, for each separation problem, the composition of phase systems must be optimized separately to achieve the individual optimum purification and recovery of a desired protein product. The factors controlling phase partitioning are as follows:

1. Top-phase component, predominantly poly(ethylene glycol) (PEG);
2. Bottom-phase component, predominantly Dextran or phosphate;
3. The concentrations of these phase components or the length of the corresponding tie-line, respectively;
4. pH;
5. Additives (salts, organic compounds);
6. The volume ratio; and
7. Sample concentration.

In view of the multiplicity of these factors, the selection of phase systems is a search in a multidimensional space, requiring a precise "orientation device" to achieve optimization efficiently. If each of these factors would be analyzed at three levels, $3^7 = 2187$ experiments would be necessary. A reduction of this large number requires a systematical approach, e.g., factorial design of experiments. The benefit of this methodology as well as of a three-dimensional (3D)-graphical presentation of results will be depicted in this chapter.

During factorial design of experiments, factors controlling extraction of sample constituents are systematically varied at distinct levels. The principle of variation during such experiments is shown in **Table 1** where two factors

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Table 1
Principle of Factorial Design of Experiments

Experiment no.	Level of factor 1	Level of factor 2	Level of factor 3
1	–	–	–
2	0	–	–
3	+	–	–
4	–	+	–
5	0	+	–
6	+	+	–
7	–	–	0
8	0	–	0
9	+	–	0
10	–	+	0
11	0	+	0
12	+	+	0
13	–	–	+
14	0	–	+
15	+	–	+
16	–	+	+
17	0	+	+
18	+	+	+

Factors 1 and 3 are varied on three levels (codes -, o, +) and factor 2 on 2 levels (codes -, +).

are varied at three levels and one at two levels (factor 2). This scheme can be freely extended or reduced.

In practice, the number of experiments can be reduced to 9–18 during a first approach to optimization. For example, using PEG/phosphate systems it can be sufficient to test PEG of different molecular weights, e.g., 1550, 4000, and 6000, and to vary the concentration of the phase components and the pH. Such a set of experiments repeatedly enabled the elaboration of systems of a high purification factor (up to several hundred-fold) and high recovery yield (*see* **Subheading 3.6.**).

The identification of factors controlling extraction requires several analyses and calculations. All concentrations of product and of accompanying protein to be removed (total protein) have to be determined precisely in all phases as well as their volumes. From these data partition coefficients of product (K_a) and of total protein (K_p) are calculated, as well as the volume ratio (Q_{vol}), the partition ratio ($G = Q_{\text{vol}} * K$), the recovery yield of product in top and bottom phases ($Y_{\text{top, bottom}}$), and the purification factors ($n_{\text{top, bottom}}$). As a rule, it is suf-

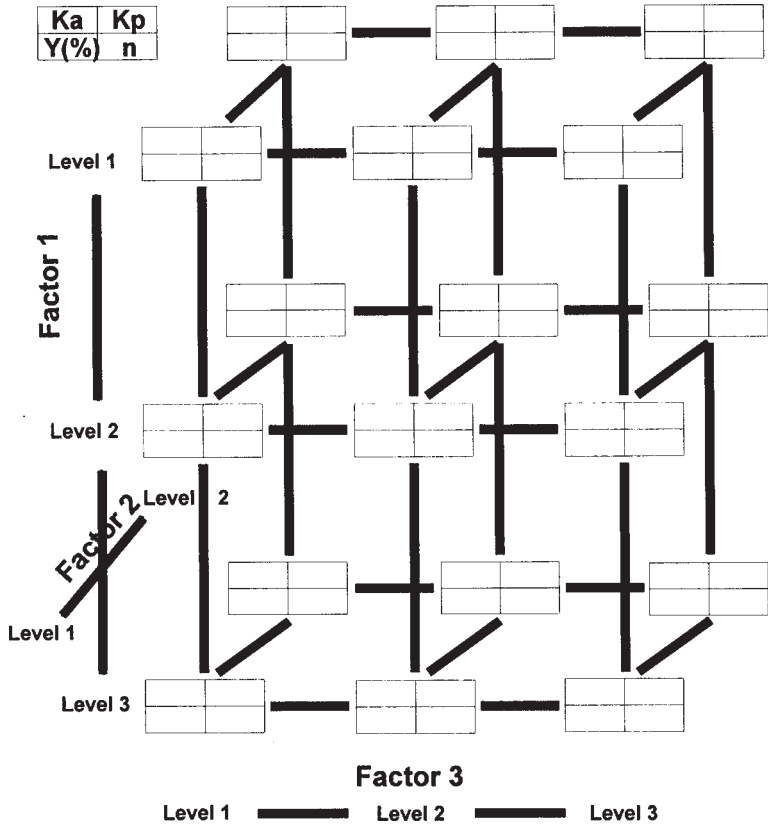

Fig. 1. 3D-graphical presentation of results for the evaluation of factorial-designed experiments (*see* **Table 1**).

ficient to evaluate K_a, K_p, yield, and purification factor. However, a documentation of these data in a table gives no chance to identify quickly decisive factors and their interdependency because at least four data must be analyzed in this example, in 18 different combinations.

But, a 3D-graphical presentation of data, practically a 3D-table, enables a quick survey of the results in all possible arrangements (**Fig. 1**). Each coordinate of this graph represents one factor and the different planes its varied levels. Thus, data observed at a distinct level of any factor can be looked at in a distinct plane and a comparison of different planes facilitates a quick differentiation of trends of experimental factors. Because several data must be considered, these are documented in appropriate small boxes drawn for each system in the grid.

Table 2
A Spreadsheet for Factorial-Designed Partitioning Experiments: Selection of Factors and Levels, Compositions, Analyses, and Calculations

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U
1		Factor 1 (and 2)		(Factor 2)	Factor 3																
2	No.	Phase components			pH	Sample (S)			System	System composition (% w/w)				Stock solution (% w/w)			Weighing in (of stock solutions) (g)				
3		Top phase (TP)	Bottom phase (BP)	Additive (AD)		Name	Activity (U/ml)	Protein (mg/ml)	Total mass (g)	TP	BP	AD	S	TP	BP	AD	TP	BP	AD	Water	S
4	1																=J4*I4/N4	=K4*I4/O4	=L4*I4/P4	=I4-Q4-R4-S4-U4	=M4*I4/100
5	2																=J5*I5/N5	=K5*I5/O5	=L5*I5/P5	=I5-Q5-R5-S5-U5	=M5*I5/100
6	Add as necessary																				

	A	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI
1		Analyses of phase systems							Calculations 1						
2	No.	Volumens			Concentrations				Sample		Partition coefficients		Vol.ratio	Partition ratio	
3		Vt	Vz	Vb	Ct, a	Cb, a	Ct, p	Cb, p	Uo	Po	Ka	Kp	QVol	Ga	Gp
4	1								=G4*U4/densitiy	=H4*U4/density	=Y4/Z4	=AA4/AB4	=V4/X4	=AE4*AG4	=AF4*AG4
5	2								=G5*U5/densitiy	=H5*U5/density	=Y5/Z5	=AA4/AB4	=V5/X5	=AE5*AG5	=AF5*AG5
6	Add as necessary														

	A	AJ	AK	AL	AM	AN	AO	AP	AQ
1		Calculation 2							
2	No.	Yields (%)				Spezific activities		Purification factors	
3		Total	Top phase	Bottom phase	Total protein	sat	sab	nt	nb
4	1	'=(Y4*V4+Z4*X4)*100/AC4	=Y4*V4*100/AC4	=Z4*X4*100/AC4	=(AA4*V4+AB4*W4)*100/AD4	=Y4/AA4	=Z4/AB4	=Y4*AD4/(AA4*AC4)	=Z4*AD4/ (AA4*AC4)
5	2	'=(Y5*V5+Z5*X5)*100/AC5	=Y5*V5*100/AC5	=Z5*X5*100/AC5	=(AA5*V5+AB5*W5)*100/AD5	=Y5/AA5	=Z5/AB5	=Y5*AD5/(AA5*AC5)	=Z5*AD5/ (AA5*AC5)
6	Add as necessary								

Additional advantage of 3D-graphical presentation results from the possibility to compare trends of all factors on 4–6 parallel axes. These trends are in most cases uniform, and thus they are confirmed to some extent statistically. The quick survey of results is of large benefit above all, if data are burdened by errors. Such occur easily because partition coefficients are quotients of experimental data. If one of the determined concentrations is very large or very small, a small error in analysis results in a pronounced deviation of the calculated partition coefficient. This can even be unavoidable as in the case of imprecise and complex biological tests ***(1)***.

The graphical presentation of data permits not only an easier identification of deviant data, but also an identification of individual systems or a group of systems exhibiting peculiar partitioning, e.g., a nonlinear or a reversal of the common trend. Such systems can be found even in a small set of experiments and they can be utilized for a two-step purification extracting a desired protein into opposite phases ***(2,3)***. The complete advantage of a 3D- graphical presentation of results will become obvious below (*see* **Subheading 3.6.**). This chapter describes the guidelines for selection of parameters for the factorial design of experiments.

2. Materials

1. Format for the compilation of data: A spreadsheet as shown in **Table 2** is used for a compilation of the factorial design of experiments following the principles outlined in **Table 1** and the guidelines described ahead (*see* **Subheading 3.**). After selection of the compositions of phases, the weights of components are calculated by a simple table calculation program with the equation documented in columns Q–U. The analytical data are documented in columns G, H, V–AB, and the equation for the calculation of the several evaluation parameters are presented in columns AC–AQ.

   Some columns include data that are varied seldom only, for example, U_o or P_o. An exact calculation of these data from the sample weight requires an additional field to list the sample density. The small effort to edit such a spreadsheet is very worthwhile in view of the multiplicity of calculations, even if these are very simple.
2. Format for presentation of results: A 3D-graph as shown in **Fig. 1** is used for presentation of results and its grid can be adapted to any combination of factors

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Table 2 *(continued)* $V_{t/b}$, volume of the top/bottom phase; V_z, volume of a possible interphase; $c_{t,a/b,a}$, concentration of the product (enzyme activity) in the top/bottom phase; $c_{t,p/b,p}$, concentration of total protein in the top/bottom phase; U_o, total activity/product in the sample; P_o, total protein in the sample; density, density of the sample, for the calculation of U_o and P_o from its volumes; $K_{a/p}$, partition coefficient of the product (enzyme activity)/total protein; Q_{vol}, volume ratio; $G_{a/p}$, partition ratio of a product (enzyme activity) / total protein; $sa_{t/b}$, specific activity of the product/enzyme in the top/bottom phase; $Y_{t/b}$, yield of the product (enzyme activity) in the top or bottom phase; $n_{t/b}$, purification factor of the product (enzyme activity) in the top/bottom phase.

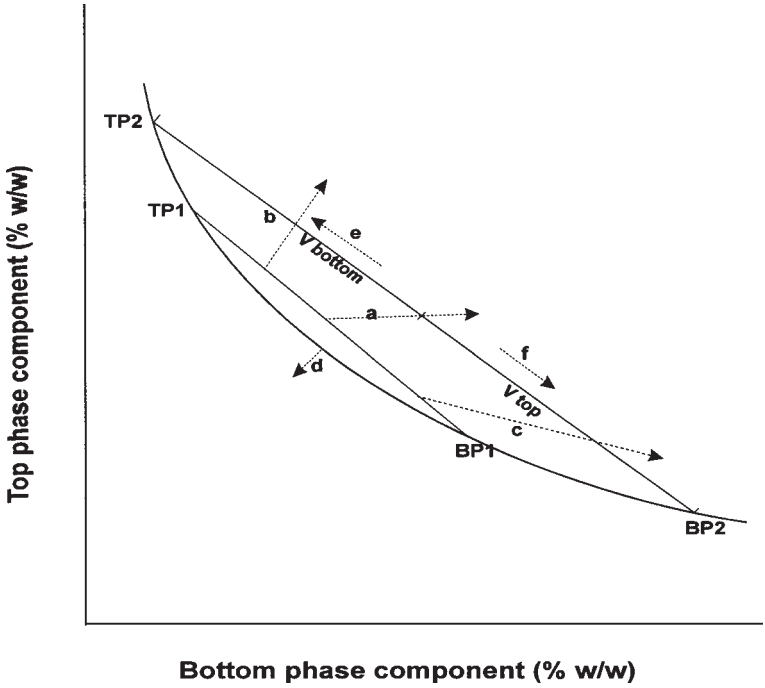

Fig. 2. Hypothetical phase diagram with arrows indicating possible changes in phase composition (*see text*).

and of levels. The graph as shown is designed for optimization of experiments with two factors varied at three levels and a third one at two levels. The different evaluation parameters are documented in small boxes drawn for each system.

3. Method

Optimization of phase partitioning aims at systems that extract product and contaminants most effectively into opposite phases and which result in high recovery. In many cases, balancing these parameters becomes the main problem. From practical experience in the preparation of seven different proteins, the following guidelines for variation of experimental factors proved to be reasonable:

3.1. Selection of Phase Compositions

1. Selection of phase components: This is normally based on the cost and convenient handling of the two-phase systems (*see* **Note 1**).
2. Molecular weight of polymers: The molecular weight of the polymers used controls partitioning of proteins, e.g., in PEG/phosphate systems the partition coeffi-

cient increases by lowering the molecular weight of the polymer significantly (*see* **Subheading 3.6.** and **Note 2**).

3. Concentration of phase components: The selection of different concentrations of phase compounds should orientate at the corresponding phase diagram (***4***; Chaptert 2). The general effects changing these concentrations in different directions on a phase diagram are explained by a hypothetical phase diagram (**Fig. 2**).

   The arrows a–c indicate possible shifts of system compositions to increase the difference in composition of top and bottom phase. The different arrows show reasonable changes in phase composition for systems having similar-sized phases (a), a large top phase (b), or a large bottom phase (c). In the direction of these arrows the composition of phases gets more different and, as a consequence, the partition coefficients of proteins should deviate more and more from one. In fact, increasing the concentrations of the phase components by only a few percent can increase partition coefficients, e.g., fivefold and sometimes even 50-fold (*see* **Subheading 3.6.** and **Note 3**). In a few other systems, a partition coefficient may remain constant or even decrease at higher concentrations.

   Further, increasing the molecular weight of PEG or the pH the binodials shift in phase diagrams as indicated by arrow d in **Fig. 2** ***(4)***. Consequently, if the influences of these factors are studied, systems should be selected which have tie-lines of similar length to reduce effects of different concentrations of phase compounds (*see* **Subheading 3.6.**).
4. Volume ratio: The volume ratio of a phase system can be adjusted by shifting system compositions along the tie-line in direction of arrows e (larger top phase) or f (larger bottom phase) in **Fig. 2**. Its value equals the ratio of the tie-line sections. All systems on a tie-line result in top and bottom phases of identical composition and, in the case of an ideal partitioning, identical partition coefficients. The partition ratio reflecting the mass ratio, however, change with the volume ratio (*see* **Note 4**).
5. pH: The pH value of a phase system is another very essential factor controlling partitioning of proteins. Its selection is restricted by the stability of a desired protein and should be orientated at its isoelectric point. One pH value should be equal to the isoelectric point and one value each about one unit above and below it, respectively.
6. Addition of salts or organic compounds: By addition of salts, such as 2 *M* NaCl, KCl, or $(NH_4)_2SO_4$, large changes of partition coefficients are possible, caused, for example, by charge differences between the opposite phases ***(4)***. Alternatively, chaotropic salts reducing hydrophobic interactions, or such with a salting-out effect and thus increasing hydrophobic interactions can be tested. Further additives of choice are organic solvents, glycerol, ligands, carbohydrates, or protective agents.

3.2. Preparation of Phase Systems

Preparation of aqueous two-phase systems are described in Chapter 3. A convenient size of phase systems used for optimization work is 10 g, but because partition coefficients are independent of the total size, restrictions on its selection result only from the practicability and the availability of the sample. A set of systems should be prepared in parallel and the most promising sys-

tems should be repeated more than twice. A spreadsheet, as shown in **Table 2**, can be used for a comfortable calculation of the masses of the different phase components, the documentation of the analytical data and, finally, for the calculation of the evaluation data.

3.3. Analyses

As mentioned, a reliable evaluation of factorial-designed partitioning experiments requires a precise determination of all concentrations, because partition coefficients are quotients. Thus, checking all applied analytical methods for disturbances by phase components is very important (*see* **Note 6**).

3.4. Evaluation of Partitioning Experiments

The calculated partition coefficients, yields, purification factors are documented in a 3D-graph as **Fig. 1**. Starting an evaluation of partitioning experiments, data and trends can easily be checked from such a graph on consistency. Because each experimental factor has been varied systematically, its effect on partitioning can be examined comparing the corresponding axes and parallel planes. However, nonuniform or opposite trends of partitioning coefficients may also be observed either on a single axis or even only in a single phase system.

The evaluation of phase systems orientates primarily at the partition coefficients of a product and the accompanying proteins, which should be as different as possible to achieve efficient separation and, thus, a maximum purity of a product.

Besides the purification factor, a high recovery yield in the product phase is essential. Therefore, the loss of product by extraction into the counterphase should be minimal. However, the yield can be reduced also by precipitation or instability of a product in a phase system at an unfavorable pH. Subsequently, an optimum combination of purification and yield should be found and often there are domains of systems of similar efficacy.

Another interesting feature is a reversal of partitioning, i.e., a partition coefficient changes above or below 1. In this case phase systems extracting a product into opposite phases can be combined to an effective two-step extraction procedure (*see* **Subheading 3.5.**).

Finally, if the strategy previously described does not disclose acceptable phase systems, optimization should be tried in systems of completely altered composition, for example, such as with affinity ligands ***(5)***. Low molecular additives, as described in **Subheading 3.1.6.**, offer another alternative to optimize phase partitioning ***(2)***.

3.5. Two-Step Partitioning

Additional phase extractions of a product can be favorable for different reasons. Already a repetition of an extraction under identical conditions can result

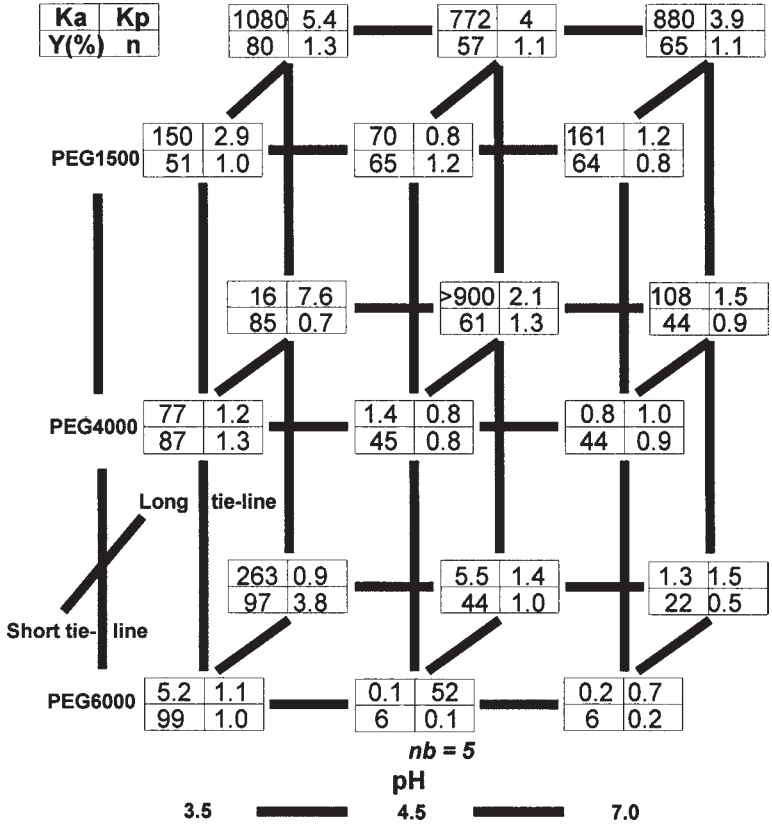

Fig. 3. Partitioning of lipase from *Mucor miehei* in aqueous phase systems containing PEG_{1550} 9/16 (short tie-line) and 4/22% (w/w) (long tie-line), PEG_{4000} 8/14, and 4/20% (w/w) PEG_{6000} 4/18 and 7/12% (w/w).

in a significant increase of product purity. Thus, critical contaminants, which interfere even in spurious amounts, for example endotoxins or degradation enzymes, can be removed. Further, a product can be extracted into a phase of low viscosity, or in view of a subsequent chromatographic step into a phase whose constituents can be easily separated, e.g., by dialysis.

As can be calculated (*see* **Note 4**), a sufficiently large (or small) partition coefficient and volume ratio is necessary to minimize loss of product during repeated extractions.

3.6. Examples of Factorial Designed Experiments

1. Partitioning experiments with lipase from *Mucor miehei*: The partitioning of lipase from *M. miehei* was studied in 18 factorial-designed PEG/orthophosphate

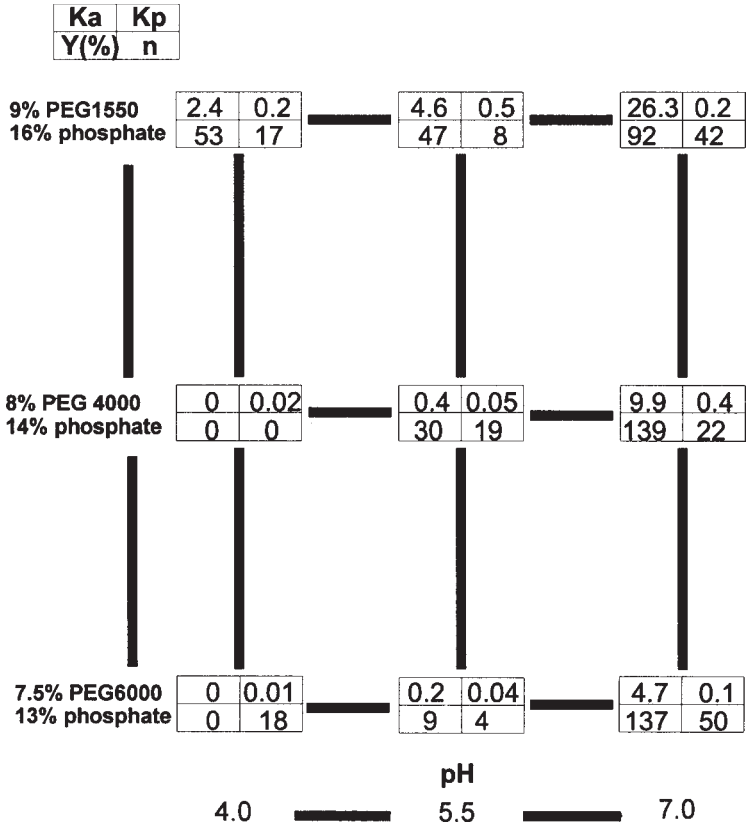

Fig. 4. Partitioning of glutathionylspermidine synthetase of *Chritidia fasciculata.*

systems (**Fig. 3**) using PEG 1550, 4000, and 6000 ***(2)***. As a second factor, the length of the corresponding tie-lines was varied for each kind of PEG [PEG_{1550} 9/16 and 4/22% w/w, PEG_{4000} 8/14 and 4/20% (w/w), PEG_{6000} 4/18 and 7/12% (w/w)]. As a third factor, three different pH values were selected, pH 3.5, 4.5 (the isoelectric point of this lipase), and 7.0 (*see* **Note 7**).

2. Partitioning experiments with glutathionylspermidine synthetase of *Chritidia fasciculata*: A similar series of partitioning experiments was performed with glutathionylspermidine synthetase of *C. fasciculata* (*see* **Fig. 4**) ***(6)***. Because the analysis of the crude enzyme was very laborious, the length of the tie-line is not varied so as to halve the number of experiments (*see* **Note 8**).
3. Partitioning experiments with lipases from different sources: The partitioning of lipase from three different microorganisms was performed (**Fig. 5**) using a set of experiments similarly designed as in **Fig. 3** (*see* **Note 9**).

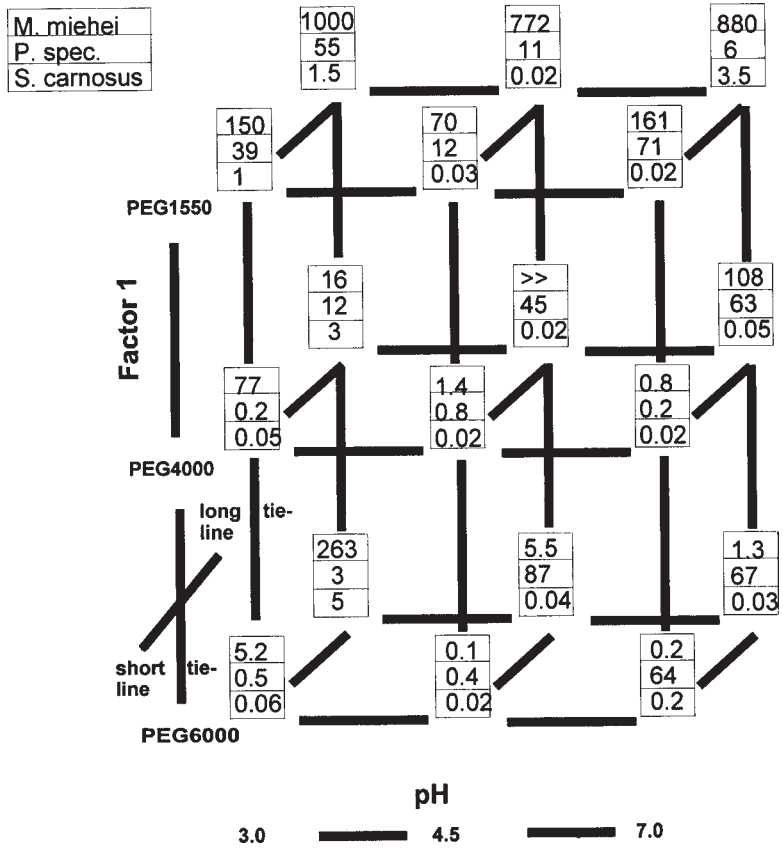

Fig. 5. Partitioning of three different lipases from *Mucor miehei*, *Pseudomonas spec*., and *Staphylococcus carnosus* (phase systems as described in **Fig. 3**).

4. Notes

1. All phase systems, which form the base of this article, contain PEG and phosphate as main constituents. Such systems are low-priced and can be handled conveniently. However, these guidelines should also be applicable for all other kinds of phase components ***(4)***.
2. There are some proteins showing an opposite trend and the partition coefficient of the total protein mixture depends much less on the molecular weight of the polymeric phase constituent.
3. Cell debris to be separated by phase-partitioning limits the range of feasible concentrations significantly, as observed in a system containing PEG/phosphate. At concentrations exceeding the corresponding binodials by only a few percent, emulsions form that prevent separation of phases (**Fig. 6**) (*see* **Note 9**).

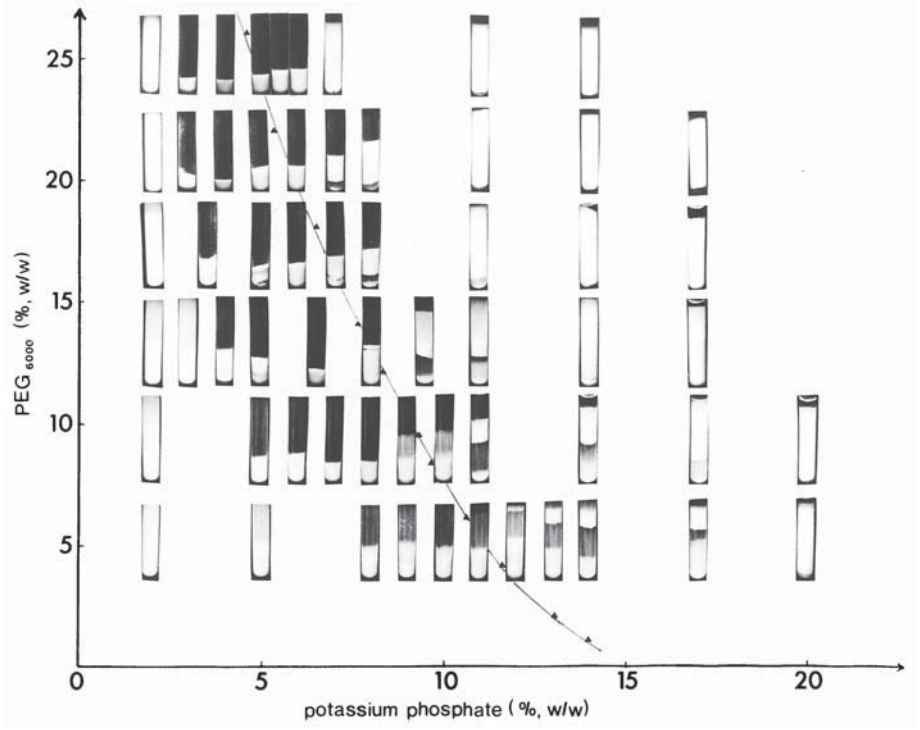

Fig. 6. Separation of cell debris in aqueous two-phase systems with dependence on the concentration of PEG_{6000} and potassium phosphate, at pH 7.1. After centrifugation for 20 min at 1300*g* clear top phases could be observed only in a small range of concentrations below and above the tie-line in the corresponding phase diagram. Size and shape of this "clearing field" depend on the phase composition and the type of cell debris (not shown). Its extension below the binodial and close limits above the binodial demonstrate a strong influence of cell compounds on the phase separation.

4. The volume ratio of a two-phase system can also be optimized to achieve, e.g., a higher degree of purification or to reduce the volume of the product phase. This controls not only the yield but also the purity of a product, because it increases with the mass of contaminating proteins extracted into the counter phase. Theoretical purification factors can be calculated from the following equations:

$$n_t = Y_t\,(\%) \quad [1 + (1 / (K_p \cdot Q_{vol})]/100$$

$$n_b = Y_b\,(\%) \cdot (1 + K_p \cdot Q_{vol})/100$$

Figure 7 is a graphical presentation of the first equation and shows the dependence of achievable purification factors on the partition coefficient of total protein and on the volume ratio. These equations are valid only if the product is a minor part of the total protein and, thus, K_p is independent from K_a and further, if

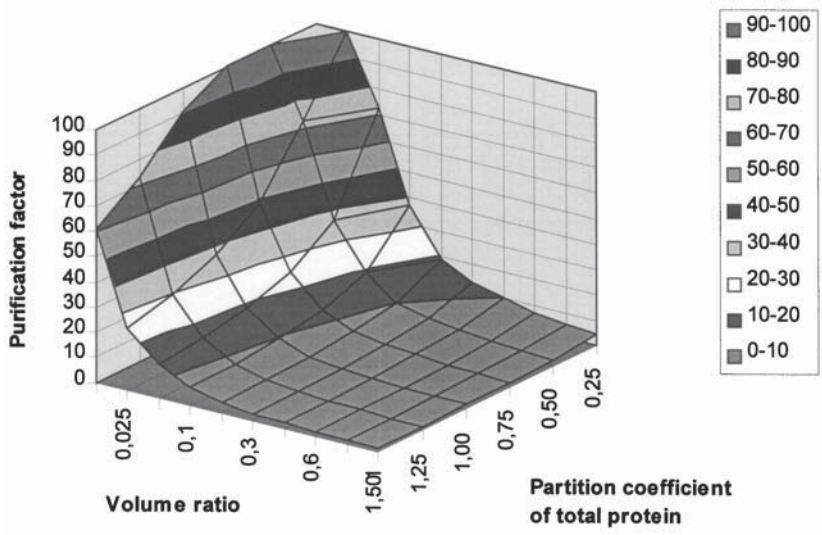

Fig. 7. Purification factor of a protein product extracted into a top phase with dependence on the partition coefficient of the total protein and the volume ratio.

no losses of product are caused by the phase components during the extraction procedure. The corresponding data during extractions into bottom phases can be taken from the same diagram using the reciprocal of the volume ratio and the partitio coefficient.

The reduction in the recovery yield of the product by a smaller volume can be neglected, if large or small partition coefficients of a product can be realized, respectively. This loss can be calculated from the subsequent equations that describe the theoretical yield of a product with dependence on its partition coefficient and the volume ratio.

$$Y_t\,(\%) = 100 / \{1 + [1/(K_a \cdot Q_{vol})]\} \text{ or}$$

$$Y_b\,(\%) = 100 /(1 + K_a \cdot Q_{vol})$$

From the graphical presentation of the first equation, **Fig. 8**, it becomes evident that, for example, at an unfavorable partition coefficient of 3, 60% of the product can be recovered at a volume ratio of 0.5 and at a volume ratio of 1.0, only an additional 15%. However, at a partition coefficient of 100 the volume ratio can be reduced to 0.1 at a yield of 91%.

5. The effects resulting from different pH values on the partitioning properties of proteins can also not be predicted, however, often higher pH values raise partition coefficients. Sometimes, even an opposite trend may be observed comparing different axes in the graph. The criteria for the selection of an optimum pH is the purity of a product and the achievable yield.

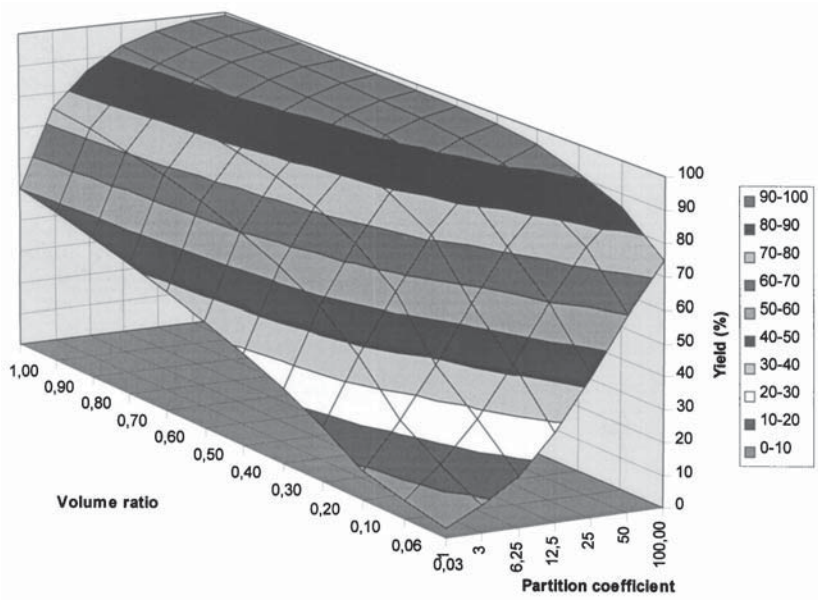

Fig. 8. Yield during an extraction into top phases with dependence on the volume ratio and the partition coefficient of the product. For estimation of the yield in bottom phases, the reciprocal of volume ratio and partition coefficient can be used.

6. As an example of interference of phase components during analyses, the extinctions during protein determination following the method of Bradford *(7)* are significantly dropped by PEG, Dextran, or NaCl. As the concentrations of these phase constituents may significantly vary from system to system, different calibration curves would be necessary. This trouble can be avoided by addition of a surplus of an interfering component to the analytical reagent, which eliminates any influence of its different concentration in samples. For instance, add 3% of PEG of middle molecular weight to the protein reagent. Alternatively, the volume of samples has to be reduced as much as possible.
7. As shown in **Fig. 3**, the partition coefficient of lipase from *M. miehei* increases considerably with decreasing molecular weight of PEG; for example, from 0.2–162 or 1.3–880. In contrast, the partition coefficient of the accompanying total protein changes little. Yields of up to 99% are achievable at pH 3.5, but only a single system (PEG_{6000}/phosphate 4/18% w/w, pH 3.5) results in an acceptable purification factor of 3.8-fold. Surprisingly, in a similar system at slightly increased pH (PEG_{6000}/phosphate 7/12% w/w, pH 4.5) the lipase is extracted into the bottom phase and purified fivefold. These systems were combined to a two-step extraction procedure that resulted in a degree of purity of 69%.
8. **Figure 4** demonstrates that yield and purification factor of glutathionyl-spermidine synthetase increase significantly with the pH and, further, the parti-

tion coefficient of the synthetase increases with decreasing molecular weight of PEG much more than that of the accompanying proteins. Some yields exceed 100% because an interfering enzyme, here an ATPase, is separated from the crude glutathionylspermidine synthetase. Best reproducibilty was found in systems containing PEG_{6000}/phosphate (7.5/13% w/w, pH 7.0) and this extraction was repeated once to remove additional contaminants ***(3)***.

At pH 5.5, the synthetase is extracted into the bottom phase and this feature can be utilized during a third partitioning step. This can be easily performed by acidifying the secondary top-phase and mixing it with the bottom phase of an acid blank system. By this procedure, the synthetase is purified 66-fold and can be dialyzed for subsequent column chromatography ***(3)***.

9. Lipase of *M. miehei* is characterized by highest partition coefficients of up to 1000 and a lowest one of 0.1. In contrast, that of the lipase from *Pseudomonas spec.* ranges only from 0.4–87 and that of the lipase of *Staphylococcus carnosus* exceeds only in one system a value of one, i.e., it is extracted predominantly into bottom phases. At longer tie-lines and at pH 4.5 and 7.0 the partition coefficient of lipase of *Pseudomonas spec.* does not decrease with increasing molecular weight of PEG, as commonly seen, but increases. These data demonstrate a significant influence of the protein source on the partitioning behavior of related enzymes.

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Two-Phase Extraction of Proteins from Cell Debris

Kristina Köhler Van Alstine and Andres Veide

1. Introduction

An important use for aqueous two-phase systems is in protein purification processes for the removal of cell disintegrate and nucleic acids in combination with partial protein purification. When a phase system is to be applied in the primary purification of an intracellular protein, the operating phase composition has to be determined with the cell disintegrate present. This is owing to the fact that the phase diagram and the position of the binodal is affected significantly by the presence of components of the cell disintegrate in the aqueous two-phase system. The experiments described here address such cell disintegrate effects on the phase diagram when extracting β-galactosidase ***(1)*** and β-galactosidase fusion proteins ***(2)*** produced intracellularly by the bacterium *Escherichia coli* (*E. coli*). The phase systems used are based on poly(ethylene glycol) (PEG) 4000 and potassium phosphate. In order to determine an operating window in the phase diagram, experiments in test tubes are performed to evaluate the effects of cell disintegrate load on the position of binodal of the phase diagram and the effects this will have on partitioning of the target protein and the cell disintegrate. The operating window could be defined as the area in the phase diagram that represents compositions at which cell disintegrate and nucleic acids partition strongly to the lower phase, (here, the potassium phosphate-rich phase), and the target protein is recovered with a desired yield in the upper phase, (here, the PEG-rich phase). The top-phase yield (*Yt*) of a protein is a function of both its partition coefficient (*K*) and the top to bottom phase volume ratio (*R*) according to

$$\mathrm{Yt}\ (\%) / [100 - \mathrm{Yt}\ (\%)] = \mathrm{K} \cdot \mathrm{R}$$

In process operation, a continuous centrifugal separator is often used to speed up phase separation. In order to limit problems with operation of the

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separator, as well as cell disintegrate loading capacity in the lower phase, *R*-values are kept at 5 or below. This means that for a 90% yield of target protein in the top phase a *K*-value of at least 1.8 is needed. On the other hand, if the target protein has a *K*-value of around 50, as is the case of, e.g., β-galactosidase, an *R*-value of about 0.2 may be used to achieve a *Yt*-value of 90%. Thus, the selection of representative phase compositions for studies of cell disintegrate effects on the phase diagram and the partitioning will depend strongly on the protein of interest.

2. Materials

2.1. Effect of Cell Disintegrate Load and Cell Debris Components on the Position of the Binodal

1. The phase-forming components polyethylene glycol (PEG) 4000 and potassium phosphate [K_2HPO_4/KH_2PO_4 molar ratio of 1.42 (≈pH 7.0)] are kept as 40% weight/weight (w/w) stock solutions at 4°C for 1 mo.
2. Homogenization buffer (PPM) consists of 50 m*M* potassium phosphate (pH 7.0) and 10 m*M* $MgCl_2$. Buffer is stored at 4°C for 1 mo.
3. Bacterial cells for partition experiments are stored at –70°C in 10-mL aliquots of, e.g., 50% wet weight (ww) cell suspension in PPM. Cells are used within 3 mo.
4. Deoxyribonuclease I (D-4527, EC 3.1.21.1, Sigma, St. Louis, MO), DNase, is dissolved to a concentration of 2.5 mg/mL in a buffer consisting of 10 m*M* Tris-HCl, pH 7.4; 10 m*M* $CaCl_2$; 10 m*M* $MgCl_2$; and 50% weight/volume (w/v) glycerol. It is stored at –20°C for 1 mo.
5. Ribonuclease A (R-4875, EC 3.1.27.5, Sigma), RNase, is dissolved to 50 mg/mL of buffer consisting of 10 m*M* Tris-HCl and 15 m*M* NaCl, pH 7.5. It is stored at –20°C for 1 mo.

2.2. Effect of Binodal Change on Protein and Cell Debris Partitioning

1. Phase components used are as described in **Subheading 2.1., item 1**.
2. Homogenization buffer (Tris-HCl) consists of 50 m*M* Tris-HCl, pH 7.2, 100 m*M* NaCl, and 10 m*M* $MgCl_2$. Buffer is stored at 4°C for 1 mo.
3. Harvested cells are suspended to, e.g., 50% (ww) in an appropriate buffer (here, Tris-HCl), and stored in volumes of 5 or 10 mL at –70°C and used within 3 mo.

3. Methods

3.1. Effect of Cell Disintegrate Load and Cell Debris Components on the Position of the Binodal

1. Frozen cells are thawed and mixed with an appropriate buffer (here, PPM buffer), to 25% (ww). For cell disintegrate load experiments, frozen cells are thawed and mixed to 50% (ww) without prior dilution (*see* **Note 1**).

2. The cell suspension is subsequently homogenized with a French press high pressure homogenizer (SLM Instruments, Inc., USA) (*see* **Note 2**).
3. For cell debris free crude extract, the homogenized cell suspension is centrifuged at 4°C and 35,000*g* for 25 min. The supernatant is decanted and used for partition experiments.
4. For preparation of DNA-free crude extract, the cell debris-free crude extract is incubated with DNase to hydrolyze the DNA. Add 50 μg DNase/mL crude extract and incubate at 37°C for 2–4 h (*see* **Notes 3** and **4**).
5. For preparation of RNA-free crude extract, the cell debris free crude extract is incubated with RNase to hydrolyze the RNA. Add 2 mg RNase/mL crude extract and incubate at 37°C for 2–4 h (*see* **Notes 3** and **4**). The RNase solution should be incubated at 100°C for 15 min to inactivate any DNases present prior to use.
6. For preparation of DNA and RNA free crude extract, the cell debris free crude extract is incubated with DNase and RNase. Add 50 μg DNase and 2 mg RNase/mL crude extract and incubate at 37°C for 2–4 h (*see* **Notes 3** and **4**). The RNase solution should be incubated at 100°C for 15 min to inactivate any DNases present prior to use.
7. In order to investigate how cell disintegrate load affects the position of the binodial, titration experiments are performed. A number of phase systems with desired compositions, spread out along the binodal of the pure system (*see* **Note 5**), are prepared by mixing appropriate amounts of PEG and salt stock solutions on weight basis in graded centrifuge tubes. When using 15-mL graded tubes, total phase system weight should be approximately 10 g. After addition of phase components, water is added to adjust the weight to 5.00 g. Note that the concentrations of PEG and salt at this point are double compared to final concentrations.
8. For a final 25% (w/w) cell disintegrate concentration, 5.00 g of 50% (w/w) cell disintegrate is subsequently added to the phase components. After 5 min incubation at 20°C, the test tube is quickly mixed by inversion (1 min) and again placed in a 20°C water bath for a 5 min incubation.
9. After incubation, the tubes are centrifuged for 5 min at 1000*g*. After centrifugation, the tubes are incubated at 20°C for another 5 min. If phase separation has occurred, the top and bottom phase volumes can be read. Top phase volume divided by total volume gives the percentage of top phase.
10. By titrating the systems stepwise with small additions (0.05*g*/cycle) (*see* **Note 6**) of 25% (w/w) cell disintegrate, a small decrease in PEG/salt composition is achieved. The procedure in **steps 8–9** is performed for each addition until the switch from two- to single-phase system occurs.
11. The exact phase system composition after each cell disintegrate addition is calculated using data from weighing up the phase systems.
12. In the same titration manner the position of the binodal in systems containing different cell disintegrate loads, crude extracts at different concentrations or DNase- and RNase-treated material can be assessed. **Figure 1** shows the result of a cell-disintegrate load experiment. **Figure 2** illustrates how the position of the binodal as a function of cell disintegrate constituents may vary.

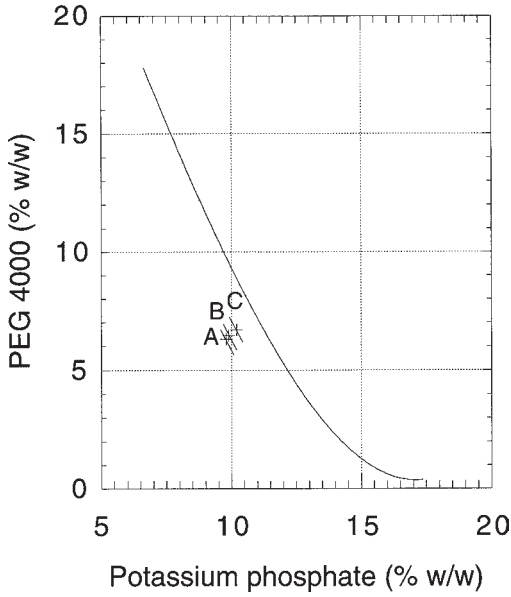

Fig. 1. Displacement of phase binodal of pure system (20°C, pH 7.0) by different loads of *E. coli* cell disintegrate. **(A)** 25% (ww); **(B)** 20% (ww); **(C)** 15% (ww) ***(2)***. Reproduced by kind permission from Elsevier Science Inc.

3.2. Effect of Binodal Change on Protein and Cell Debris Partitioning

1. Frozen cells are thawed and diluted to an appropriate wet weight concentration, e.g., 25% (w/w), in the same buffer as was used for storage (*see* **Note 1**).
2. The cells are disintegrated by a desired method, e.g., a glass bead mill or a French press high pressure homogenizer (*see* **Notes 2** and **7**). Save enough sample for analysis (e.g., enzyme activity, total protein, DNA, RNA, endotoxin).
3. A set of experiments is performed by varying the concentration of disintegrated cells in the system with each selected phase composition (*see* **Note 8**). For example one can select three different cell disintegrate concentrations, e.g., 5, 10, and 15% (w/w). (The highest concentration possible will be determined by the cell disintegrate stock concentration.)
4. Phase systems are made up on a weight basis from stock solutions of phase forming components and disintegrated cells, and finally balanced with distilled water to give the correct final weight (typically 5 or 10 g total system weight).
5. Partitioning is performed by incubating the phases at the selected temperature for 5 min. Then the systems are mixed by inversion (1 min) and incubated again at the appropriate temperature for 5 min.
6. Phases are separated by centrifugation at 1000*g* for 5 min. After centrifugation, the phases are incubated at the desired temperature for another 5 min. Phase volumes

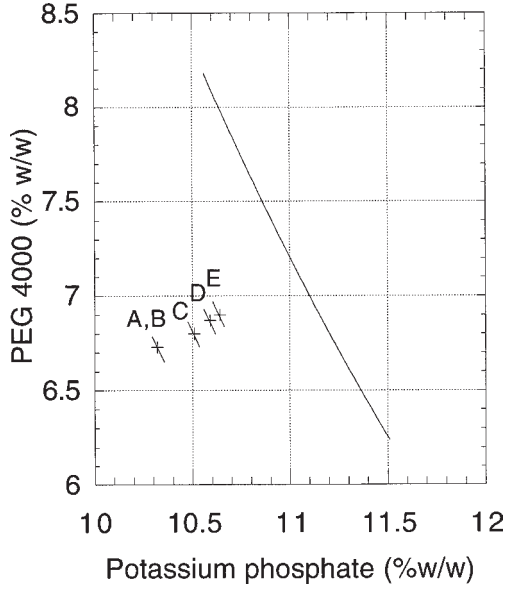

Fig. 2. Displacement of phase binodal of pure system (20°C, pH 7.0) by different cell constituents from a 12.5% (ww) cell suspension. (A) disintegrated cells; (B) cell disintegrate free crude extract; (C) crude extract hydrolyzed with RNase; (D) crude extract hydrolyzed with DNase; (E) crude extract hydrolyzed with RNase and DNase ***(5)***.

are read and cell-disintegrate partitioning is visually estimated from the turbidity of the two phases. Samples for analysis are withdrawn from the phases (*see* **Note 9**).

7. In **Table 1** an example is shown of partitioning of *E. coli* cell disintegrate, the enzyme β-galactosidase and total protein at different cell disintegrate loads and phase system compositions (*see* **Note 4**). The phase compositions are shown in **Fig. 3**.

4. Notes

1. An appropriate buffer for cell storage and homogenization, as well as cell concentration, has to be selected in each case.
2. Cell-disruption methods such as high-pressure homogenization or glass bead milling are selected because they can be scaled up.
3. Incubation time should be optimized for each crude extract system (e.g., crude extracts containing different recombinant proteins or originating from different *E. coli* strains). The concentrations of DNase and RNase may also be varied to obtain complete hydrolysis.
4. In order to verify the absence of DNA and RNA respectively, analysis of crude extract after treatment with nucleases should be performed. These methods (a and b) may also be used to optimize the conditions for the hydrolysis.
   a. The increase in absorbance at 260 nm upon nuclease treatment could be followed. The absorbance will increase until all polynucleotide has been hydrolyzed and then remain constant with time.

Table 1
β-Galactosidase Partition, Yield in Top Phase, and Purification Factor, Together with Biomass and Total Protein Partition at Different Biomass Loads and Phase-System Compositions *(1)*

Phase-system comp.[a]	Biomass conc. % (ww)	Cell-disintegrate partition[b]	*K* β-gal.	β-gal. yield (%)	*K* Total protein	Purification factor
1	6.9	One phase	–	–	–	–
	11.5	One phase	–	–	–	–
	16.0	C	74	88	0.35	17
2	6.9	C	91	99	0.24	20
	11.5	C	84	90	0.26	15
	16.0	C	47	83	0.28	14
3	6.9	C/T	75	95	0.29	12
	11.5	T	32	86	0.52	7
	16.0	T	14	71	0.70	4

[a] Phase compositions are shown in **Fig. 3**.
[b] *C*, clear top phase; *T*, turbid top phase with cell disintegrate.

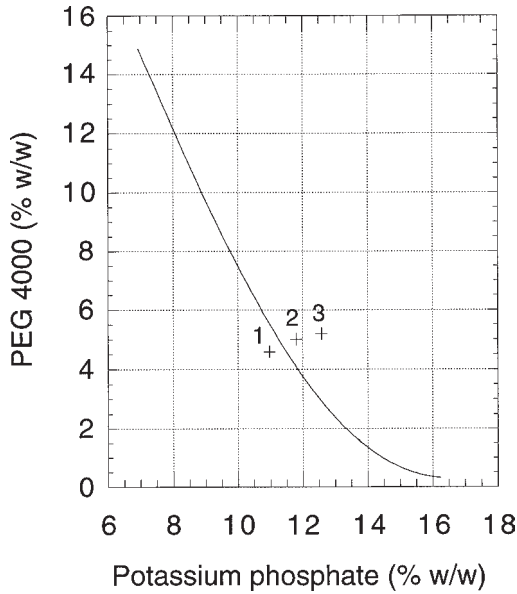

Fig. 3. Binodial of a pure phase system (25°C, pH 7.0) supplemented with 26.3 m*M* Tris-HCl, pH 7.2. Phase compositions are shown for partitioning experiments with *E. coli* cell disintegrate at different biomass loads (*see* **Table 1**) ***(1)***.

b. Electrophoresis of samples on 1% agarose gels is a convenient way to analyze samples before and after nuclease treatment ***(3)***.

Two alternative methods to investigate DNA content after DNase treatment, but also to assess DNA partitioning, are suggested. They are referred to as the fluorescence ***(6)*** and threshold ***(7,8)*** methods, respectively.

5. If the phase diagram for the pure phase-forming components is not available, it can also be determined by the titration method described here (using pure water for titration) or other methods as described in this volume (*see* Chapter 2) and elsewhere ***(4)***.
6. In order to get an accurate determination of the binodal position when total phase system weights are around 10 g, the added amount at the phase switch should be kept at about 0.05 g. In order to find the approximate position of the binodal and to speed up the procedure, a screening round could be performed with larger amounts added (0.2–0.5 g/cycle).
7. It is essential that the same disintegration method is applied as when the effect of cell disintegrate on the binodal position was studied.
8. As the binodal position is changing with the load of cell disintegrate in the system, it is advisable that the maximum cell disintegrate concentration (cdc_{max}) to be used in the experiments is decided. Now the phase compositions to be used in the experiments can be selected from the binodal curve obtained with cdc_{max} in previous experiments (**Subheading 3.1.**). The phase composition for the first point is chosen in such a way that it is positioned very close to the binodial on a relatively short tie-line. The following points are positioned further out from the binodial on separate tie-lines of increasing length. Partition experiments at the selected phase compositions are then performed with cell disintegrate concentrations ≤ cdc_{max}. If, e.g., the binodal in **Fig. 1** for 25% (w/w) cell disintegrate is considered as cdc_{max} phase compositions indicated by B and C could be chosen. If cell disintegrate concentrations larger than cdc_{max} are to be used additional phase compositions below the binodal curve should also be selected. In **Fig. 3**, the selection of phase compositions are shown for a partition experiment with different cell disintegrate loads where only the binoial for the pure system was available.
9. Careful sampling can be performed with a Pasteur pipet in order to avoid contamination of one phase in the other owing to low interfacial tensions in the systems. When the top-phase volume is relatively large, it is not so difficult to remove enough volume of pure top phase. Bottom phases are sampled by putting the tip of the pipet through the top phase into the bottom phase, and here it is important that the bottom phase is sucked into the pipet slowly enough to avoid contamination. Another way to sample bottom phase is to remove the remaining top phase completely (i.e., after sample recovery) and then sample from the bottom phase.

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24

Aqueous Two-Phase Extraction of Proteins from Animal Tissue

Michael J. Boland

1. Introduction

1.1. Scope

This chapter covers the use of aqueous two-phase systems for extracting and purifying enzymes and other proteins of interest from animal tissue. This area was the subject of a general review by the author in 1990 ***(1)***.

1.2. Why Use Aqueous Two-Phase System?

One of the major differences between animal tissue, and most other sources of industrially important enzymes and other proteins, is that the animal tissue generally occurs as a solid mass. To extract the protein(s) of interest invariably requires massive tissue disruption to overcome both impermeable membrane barriers and mass transfer problems. Further preparation of the protein then necessitates separation from the insoluble particulate material that arises from such disruption. Although such operations present little problem at laboratory scale (superspeed centrifugation or ultracentrifugation being the norm), such separation cannot be easily achieved at industrial or pilot scale. Traditional methods such as centrifugation are difficult because the particulate material is usually of a similar density to the surrounding solution. Similarly, filtration almost invariably leads to blinding, owing to the wide size range and deformable nature of the insoluble material. A common solution to this problem has been to use a rotary vacuum filter, which continually replaces the filtration surface. This process is messy, has considerable cost and waste generation from use of filter medium (typically diatomaceous earth), and causes considerable foaming that can lead to interfacial denaturation. The resulting product may not always be completely clear and may need polishing in a clarifier.

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Table 1
Some Examples of Use of Aqueous Two-Phase System to Prepare Proteins from Animal Tissue

Protein	Tissue	Comments
Alkaline phosphatase	Bovine	Lab scale *(2)*
d-Aminoacid oxidase	Porcine kidney	Lab scale *(3)*
Catalase	Bovine liver	Lab scale *(3,4)*
Insulin	Porcine pancreas	Lab scale *(5)*
Lactate dehydrogenase	Rabbit muscle	Affinity method, lab scale *(6,7)*,
	Porcine muscle	and pilot scale *(8)*, part of sequential affinity process *(9)*
	Bovine liver	Lab scale *(3)*
Malate dehydrogenase	Porcine muscle	Part of sequential affinity process *(9)*
Myokinase	Porcine muscle	Part of sequential affinity process *(9)*
Pyruvate kinase	Porcine muscle	Part of sequential affinity process *(9)*
Superoxide dismutase	Bovine liver	Lab scale *(3)* and pilot scale *(10)*

Aqueous two-phase processing offers a separation of the protein of interest from the particulates based on completely different criteria, with the possibility of a concurrent purification of the protein with respect to total soluble protein. The ability to take a crude homogenate, and after a single unit operation deliver a clear solution of partially purified protein, makes this technology in principle an attractive alternative to traditional methods.

1.3. Examples

There are no examples of use of aqueous two-phase systems for commercial preparation from animal tissue known to the author. There are a small number of demonstrations of potential commercial processes in the literature, and a rather greater number of examples of use at laboratory scale. **Table 1** lists some examples.

1.4. Overview of the Process

Typically, extraction of a protein from animal tissue will involve coarse disruption, usually by grinding or mincing, followed by fine disruption in the presence of extraction medium, by homogenization. Phase-forming components are added to the resulting slurry and the phases are separated by centrifugation. The aqueous two-phase system is designed so that the protein(s) of interest will partition into the upper phase, leaving the particulate material in the lower phase. A second phase separation may be induced in the separated upper phase to effect further purification and sometimes to allow recycling of the upper phase components. Following this, further purification, for example, by chromatographic methods, is usual. The process is shown schematically in **Fig. 1**.

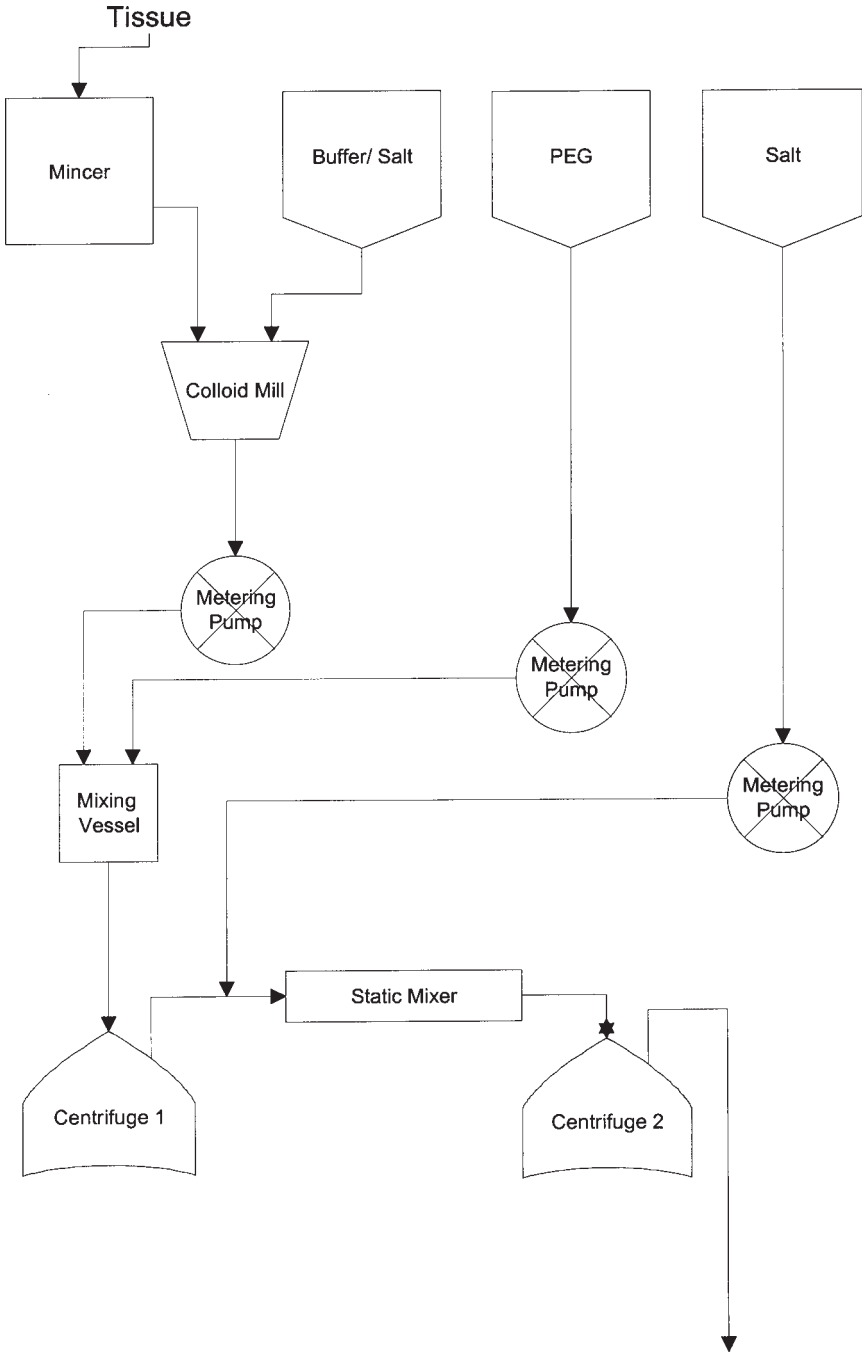

Fig. 1. Overview of the process.

2. Materials

1. Animal Tissue: A wide range of animal tissue has been traditionally used for extraction of enzymes and other animal proteins. Such tissue is usually obtained from an appropriate slaughterhouse or butcher. Prices can range from a few dollars a kilo for larger tissues (such as liver), to more than $1000 a kilo for small tissues that are difficult to collect, such as pituitary glands. Tissue must be as fresh as possible. (*see* **Note 1**)
2. Phase Forming Material: Polyethylene Glycol (PEG): Most phase forming systems use PEG. This is available from many chemical suppliers and is a product of the petrochemical industry (*see* **Note 2**). The chain length of the PEG is of critical importance, and is one of the key variables when designing an aqueous two-phase system. If following a recipe, it is important to match the chain length; when developing a system, it is advisable to have a range of PEG sizes available. For animal tissues, PEGs in the range of 500–2000 have been found to be particularly useful.
3. Second Phase Material: Salts: The second (lower) phase will generally involve a salt such as sodium, potassium or ammonium phosphate, citrate, sulfate, and so forth. Such salts are generally available from most chemical suppliers. Decisions on the salt to be used will relate to considerations of cost, effectiveness, and rate of use in phase formation, partition coefficients for the desired protein and costs of disposal in waste.
4. Second Phase Material: Polymers: The most common polymer used for the lower phase is Dextran, although many alternatives are available (*see* elsewhere in this volume). These polymers are usually much more expensive than salts, but a lower rate of use may make them viable alternatives. Recycling of the lower phase of the first separation is not usually possible owing to the high load of cell debris, so this may rule out the use of an expensive lower phase component.
5. Affinity Phase Materials: Affinity media can also be used to achieve selective separations of proteins from animal tissue. Such media have traditionally used triazine dyes bound to a polymer ***(7–9)***. Such media are expensive and generally must be custom synthesized. Although of potential use on laboratory scale, their application in commercial processing is likely to be limited by cost.
6. Laboratory Equipment: Laboratory equipment for aqueous two-phase system extractions from animal tissue is simple, involving some sort of homogenizer and a centrifuge capable of 1000*g*.
7. Process Equipment:
   a. Tissue disruption: Initial disruption may be done by a supplier, or using an industrial meat grinder (mincer). Finer disruption is necessary to achieve good extraction in short timescales. Normally a colloid mill is used for this. We have typically used a Fryma (Model MZ-50 or MZ-80) set at minimum gap.
   b. Mixing: In-line mixing is needed to form the phase mixture during processing. In-line static mixers, such as those used in industrial separations from cells, are not reliable because they have a tendency to clog with the fibrous material present in tissue extracts. We have been successful in using a home-

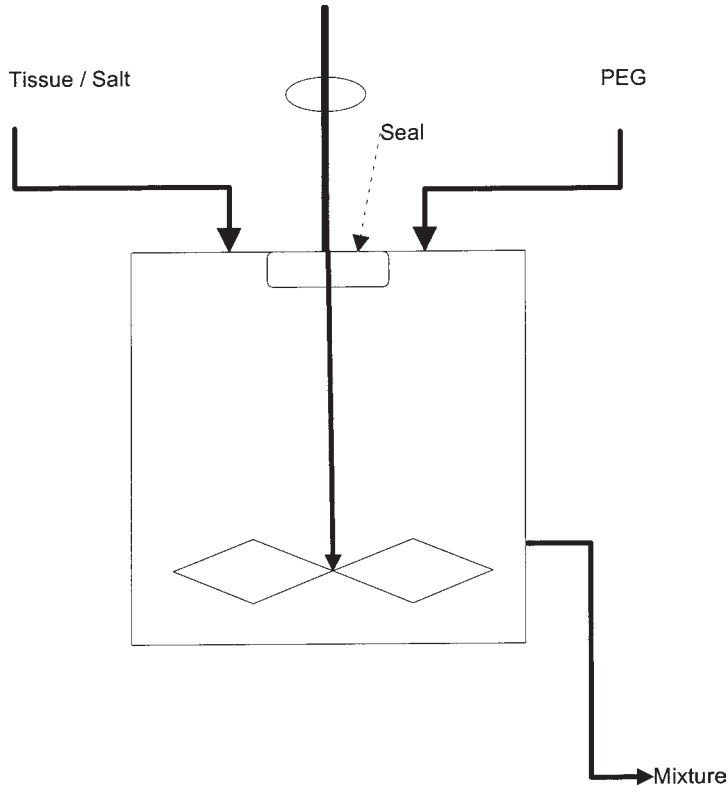

Fig. 2. The stirred mixer.

made 1 L sealed cylindrical container with a mechanically rotated stirrer, rotating at 100–200 rpm. (**Fig. 2**).

c. Separation: Phase separation can be achieved in many cases just by standing for several hours in a large glass cylinder (volume 50–100 L) but this method is slow, and activity can be lost on standing owing to proteolysis and other adverse reactions. Preferable is the separation by a disk stack centrifuge. It is essential that the centrifuge has a desludging capability and is regularly desludged, as build-up of the larger particulates can interfere with the separation ***(10)***.

The stand-alone system developed by Westfalia Separator (described in **refs. *11*,*12***) is a useful system, but will require replacement of the first static mixer with a mechanical mixer as previously described.

Trials with decanters have generally been unsuccessful, mostly because the deformable nature of the insoluble material makes it difficult for the scroll to remove it, and heating caused by the scroll can cause the two phases to become miscible. Other types of equipment have been described for phase separation, but many are not suitable for biomass containing fibrous material.

3. Methods

1. Selection of phase-forming systems: There is a wide range of phase-forming systems available, and selection of the appropriate system can involve much trial and error. A typical choice for extraction of animal proteins would be a PEG–salt system. Some of the considerations that need to be taken into account are listed in **Note 3**. The system will need to be tested at laboratory scale before any scale up is attempted. Ten-mL systems in 15-mL graduated conical centrifuge tubes have been found to be particularly suitable for this (*see* **Note 4**).
2. Disruption of tissue and particle size reduction: Initial disruption can be done by the supplier if the tissue is obtained from a butcher; alternatively, the tissue can be passed through a commercial or domestic meat grinder (mincer). Further size reduction and initial extraction is achieved by homogenization at laboratory scale, or milling through a colloid mill at pilot or industrial scale.

   For this step, the tissue is mixed with an appropriate amount of water or buffer (typically an equal weight or a twofold excess). If a buffer salt is used, allowance must be made for this in the later phase-forming system. At laboratory scale, homogenization can be achieved using a Potter–Elvenhjem type of homogenizer for small volumes, or a blender type homogenizer (Waring blender or Ultra Turrax homogenizer). At larger scale, the mixture can be poured or pumped through a colloid mill with the gap set at minimum (typically 5–10 μm).
3. Effect of tissue on phase diagrams: Phase diagrams are normally developed for a particular two-phase system using model solutions of the specific phase-forming components only; however, because animal tissue contains considerable levels of protein (as well as other soluble components) and is being added at a relatively high addition rate, these components will play a part in phase formation as well. In particular, the soluble proteins will act as additional water-binding polymers in both phases. It has been widely observed that the presence of proteins from the animal tissue will cause phase separation at lower concentrations of the phase-forming components than would be expected in their absence ***(3)***. This can be used to advantage in developing separations, both in increasing the solubility of the proteins of interest in the upper phase, and in minimizing the use of phase-forming components.

   Typical systems have used a tissue loading of between 10% and 25% of the weight of the total system, although loadings as high as 35% have been tried ***(3)***. The choice of level is a compromise between the desire to load the system as much as possible (to minimize cost), and increased difficulty in getting good separation as the system becomes more heavily loaded ***(3)***. A tissue weight that is 20% of the total system is a good starting point.
4. Mixing: Proteins have been shown to equilibrate very rapidly between aqueous phases, and provided mixing is thorough, it need not occur over a long period. At laboratory scale, 1 min of mixing using a vortex type test tube mixer has been found to be adequate.

   At pilot or industrial scale, in-line mixing is usual. A mixer of the type described in **Subheading 2.7.** will usually be satisfactory. The size of the con-

tainer should be such as to give a mean residence time of the order of at least half a minute.

5. Separation: Phase separation at laboratory scale is trivial: centrifugation at 500–1000*g* for one minute will normally suffice to give a clean separation of the phases. There will almost always be a layer of insoluble material at the bottom of the tube, and layers of insoluble or lipid material may occur at the interface, and at the top of the upper phase. This is quite usual, depending on the type of tissue. Phases can be separated by decanting, or transferred using a Pasteur pipet.

   Separation at larger scale is usually by using a disk stack separator. The author has had considerable experience using the Westfalia type SA-1 ***(10)***, either as a stand-alone unit, or as part of the integrated system by Westfalia ***(11,12)***. At this scale, a flow rate of around 1 L/min was found to be appropriate for best results, and the high loading of tissue needed periodic desludging (*see* **Note 5**).

   When setting up the separator, it is important that the holes in the disks are at an appropriate radius to for maximum separation efficiency. This means that the smaller the relative volume of the lower phase, the further out the holes should be. Disk sets with holes at different radii are available from manufacturers.
6. Second phase separation: Often a second phase-forming step will be used. This will usually be a PEG–salt system if PEG has been used in the first phase separation. This step has the benefit of removing the protein in question from the PEG phase into an aqueous salt-containing phase that is immediately suitable for further purification. If appropriately chosen, the second separation can also achieve substantial purification with respect to other proteins. In the case of a PEG-containing upper phase from the first separation, it is simply a matter of mixing with an appropriate stock solution of concentrated salt. This is a trivial matter even at process scale, and can be achieved by injection of the salt solution into a process stream, followed by mixing, and separation in the disk stack centrifuge. If an in-line static mixer is used just after the injector, the top phase from the first separator can be put directly through the mixer and into a second separator; thus the second separation is a very rapid in-line step of the process.
7. Suitability for further separations: The solution of the required protein from the second separation is usually a clear solution, containing a high level of salt. This can either be desalted using a method such as dialysis or diafiltration prior to further processing, or the solution can be directly loaded onto a hydrophobic interaction chromatography system ***(14,15)***.
8. Recycling of phase-forming materials: Phase-forming chemicals can be a considerable proportion of the cost of an aqueous two-phase system purification ***(16)***, and can also incur costs in waste disposal. It is possible in process scale to recycle the phase-forming components to save these costs. Extractions of animal tissue are not suitable for recycle of the lower phase of the first separation owing to the high loading of cell debris; however, if a second separation is used, the upper phase can often be recycled, as for separations from other systems ***(17)***. The reuse of this phase may involve simply recycle of this phase because it is into the mixture for the first separation. In principle, the phase could be cleaned by heat precipitation of proteins or by some sort of membrane processing.

Table 2
Economics of Pilot and Process Scale Aqueous Two-Phase System of Animal Tissue (Data from ref. *10*)

	Cost (in 1988) of extractive purification of superoxide dismutase from bovine liver, DM/kg liver	
	100 kg batch	1000 kg batch
Capital costs	0.61	0.25
Labor	5.85	1.36
Materials	5.22	5.22
Utilities	0.11	0.09
Total	11.80	6.92

9. Scale effects/economics: Because aqueous two-phase system is a continuous process, and the main process operations, mixing, and centrifugal separation are very scalable (very large throughputs can be achieved using dairy-type separators), scaling up can be simple. There will always be debate about the cost of aqueous two-phase separations. In one example in which the author was involved, the costs of processing were estimated as shown in **Table 2**, based on a two-separation system for the extraction of superoxide dismutase from bovine liver ***(10)***:
   Although the costs are somewhat dated, the relativity between the various cost components should be relatively unchanged. The key point is that the major cost component is for materials. This includes the source tissue and the phase-forming components. The model assumes no recycling.
10. Future prospects: aqueous two-phase system extraction has not been embraced by the industry for products from animal tissue. Some of the reasons are given in **Note 6**. However, it is important for the processor to keep the possibilities of this method in mind because it has two distinct advantages, namely the ability to operate as a continuous process, thus requiring a relatively small plant for high throughput, and the fact that the separation is based on a different principle from most other protein separation methods.

4. Notes

1. It is often helpful to have the supplier do the initial tissue disruption, particularly at larger scale, as they usually have access to industrial meat grinders. However, be sure to advise the supplier that the material is for scientific purposes. (In one case, the author was rather surprised to be presented with 50 kg of ground liver, nicely dressed with chopped parsley!!).
2. Some suppliers of PEG fractions are: BDH (has agencies in most countries), Hythe Chemicals (Hythe, England), Merck (Darmstadt, Germany, has agencies in many countries). For large-scale operation, users are recommended to contact petrochemical companies directly. Because PEG is fractionated to give the different nominal molecular weights, and different suppliers use slightly different

methods, phase systems should be rechecked if a supplier of PEG is changed.

3. In designing an aqueous two-phase system extraction and purification, there are several considerations that need to be taken into account.
   a. In the initial separation, the protein of interest must partition substantially into the upper phase. In this separation, almost all the insoluble material will go into the lower phase, both because of its density and because it normally partitions there (in the case of PEG–salt systems).
   b. The relative volumes of the phases. This is important particularly if the partition coefficient for the desired protein does not strongly favor the upper phase. By having a favorable phase volume ratio, it is often possible to get good yields of protein in the top phase when coefficients are close to one.
   c. The relative cost of the phase-forming components must be weighed up against the value of the product and the difficulty in obtaining it. For an enzyme from a relatively cheap tissue source, only an inexpensive PEG–salt system would be considered; however, if extracting a valuable protein from an expensive tissue, then more sophisticated systems, such as affinity media, can be considered.
   d. The higher molecular weight PEGs often give poorer partitioning of proteins into the upper phase; however, they can have cost advantages because less PEG is usually needed to effect phase separation.
   e. If the process is to be carried out at industrial scale, the impact of phase-forming material on waste streams should be considered. It is for this reason that phosphate-containing systems have recently fallen into disfavor, being replaced by biodegradable salts such as citrate ***(13)***.
4. For laboratory-scale development, it is convenient to operate using concentrated stock solutions of phase-forming components. The following system has been used by the author for PEG-phosphate systems and originates from the laboratory at Gesellschaft fur Biotechnologische Forschung mbH (GBF), Braunschweig, Germany: Solution 1 is 50% (w/w) PEG in water. Solution 2 is 40% (w/w) potassium phosphate, made up according to **Table 3**.

   Both solutions are quite stable for several weeks at room temperature, but should be kept out of strong light such as direct sunlight. Systems are made up in 15-mL graduated conical glass centrifuge tubes by *weighing* all components into the tube.
5. Desludging (self-cleaning) of the centrifuge will be needed at regular intervals during pilot or process scale operation. The frequency of this will be a function of the biomass loading, the fineness of the homogenate, and the flow rate. Generally, a finer homogenate and a faster flow rate will carry more insoluble material out in the lower phase. In the case of bovine liver homogenate at a 20% biomass load, and a flow rate of around 1 L/min, it was necessary to desludge about every 20 min. The interval can only be found by trial and error.
6. There are several reasons why the aqueous two-phase system is not more widely used in processing today.
   a. Animal tissue is a traditional, but expensive, source of proteins, and animal proteins are increasingly being replaced by recombinant proteins produced in

Table 3
Composition of Stock Phosphate Buffers (40% w/w)

pH of buffer	g KH_2PO_4 per 1000 g solution	g $K_2HPO_4 \cdot 3H_2O$ per 1000 g solution
6.0	260	183.4
6.48	200	262
7.0	160	314.4
7.70	80	419.2
8.78	40	471.6

microbes. Examples of this include chymosin, used in cheese making, for which the traditional calf-stomach source has been substantially replaced by the recombinant enzyme; and insulin, most of which is now the recombinant product that uses the human gene.

b. The system is perceived as being expensive. This depends very much on the type of system used and the possibility of recycling components. The process could certainly be competitive with other processes in many cases.

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Chaotropic Aqueous Two-Phase Systems

Preparation and Uses

Daniel Forciniti

1. Introduction

The expression of eukaryotic proteins in a foreign host is frequently accompanied by the formation of aggregates or inclusion bodies of inactive proteins that accumulate within the cell ***(1,2)***. Although inclusion bodies have been found mainly in *Escherichia coli*, they have also been found in other bacteria and even in insect cells.

Protein aggregation is a side product of protein folding; as a protein folds toward its native structure, it can also go through alternative folding pathways forming intermediates that aggregate. As in the folding process, the aggregation process depends strongly on temperature, pH, salt type, and salt concentration. In addition, the aggregation process depends on protein concentration, high concentrations favoring the formation of aggregates. Protein folding is catalyzed in some bacteria by enzymes (chaperones) that bind intermediates along the folding pathway, thereby avoiding protein association. Hydrophobic probes and poly(ethylene)glycol (PEG) also reduce the formation of protein aggregates during in vitro protein refolding by a similar mechanism.

The first step in the recovery of recombinant proteins from inclusion bodies is their dissolution using chaotropic salts. After the inclusion bodies are dissolved, the proteins need to be refolded. It has been found that the recovery of native protein increases by performing the refolding at very low concentrations, or in the presence of rather large amounts of denaturant (1–2 mol/L urea or guanidine hydrochloride). (A good review about the recovery of proteins from inclusion bodies can be found in **ref. *3***). Inclusion of a hydrophobic probe, e.g., a polypeptide, in the refolding mixture to enhance protein recovery has

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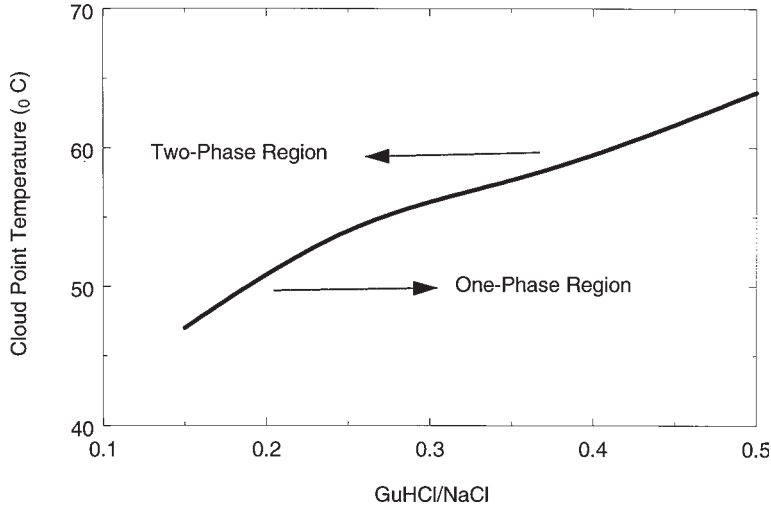

Fig. 1. Cloud-point temperature of PEG/GuHCl/NaCl system vs GuHCl/NaCl ratio at pH 7.2 (phosphate buffer 67 mmol). These 10 g systems contain 3.75 g of 40% (w/w) PEG stock solution, 0.7–1.8 g of a 6-*M* GuHCl stock solution, and 2.6–2 g of NaCl. The reader is referred to **Note 7** for some features of these systems.

also been investigated. The hydrophobic probe reduces the number of aggregates by interacting with intermediates (e.g., molten globules) in the folding reaction. Unfortunately, hydrophobic probes are specific for each particular protein. Cleland and Wang ***(4)*** have found that the formation of aggregates is minimized and the recovery of the active protein is enhanced by adding a cosolvent like PEG to the refolding mixture. This approach is especially appealing because it seems independent of the kind of protein.

The need of chaotropic salts for the disruption of inclusion bodies and the use of PEG as a chaperon suggest that aqueous two-phase systems containing chaotropic salts may be an interesting alternative for the recovery of recombinant proteins that are produced as inclusion bodies ***(5,6)***.

Nonchaotropic salts such as phosphate, sulphate, or citrate, which form two liquid phases with PEG at room temperature, cannot dissolve the inclusion bodies. Thus, the scope of this chapter is limited to PEG/chaotropic salt systems. Chaotropic salts form two-phase system with PEG only at relatively high temperatures. This inconvenience can be circumvented by spiking chaotropic salts/PEG systems with NaCl. For example, the cloud-point temperature for PEG/LiBr/NaCl and PEG/$CaCl_2$/NaCl systems decreases with increasing substitution of the chaotropic salt by NaCl. **Figure 1** shows the change in cloud-point tem-

perature of PEG/GuHCl/NaCl system with change in ratio of the two salts. On the contrary, NaSCN and PEG form two liquid phases at reasonable temperatures even if a small quantity of NaCl is added.

PEG/chaotropic salt systems can be used to attempt the recovery of recombinant proteins from inclusion bodies, or simply to study the partition behavior of proteins under non-native conditions; hence, they offer another possibility to understand better the solution properties of a particular protein. The preparation of chaotropic salt/PEG two-phase systems is very similar to that of "standard" aqueous two-phase systems, however, most of these systems are in the one phase region at room temperature, and are driven into the two-phase region by increasing the temperature.

2. Materials

To facilitate the preparation of a series of two-phase systems, stock solutions of the phase forming species in the buffer of choice are prepared.

1. Polymers: PEG of molecular weight 3000, 8000, or 10,000 (Sigma Chemical Co., St. Louis, MO) are used as received. Typical PEG stock solutions range from 30–50% w/w.
2. Chaotropic salts: NaSCN, Guanidine hydrochloride (Sigma). Stock solutions of the chaotropic salts are prepared at concentrations compatible with their solubility limit. For example, a 6-*M* GuHCl stock solution should be prepared if this salt is chosen. Stock solutions are kept in the refrigerator until ready to be used.
3. NaCl.
4. 2-Mercaptoethanol.
5. Oxidized glutathione (GSSG) (Sigma).
6. Buffer: Phosphate or Tris buffer are usually used. The concentration of the stock solution is lower than 100 mmol.
7. Proteins: Proteins are used as received. The proteins may also be used in denatured or aggregated forms (*see* **Note 1**). Proteins refolded after their denaturation may also be used for partitioning studies (*see* **Note 2**).

3. Methods

3.1. Preparation of the Chaotropic Phase System

1. Choose a phase system to be prepared (*see* **Note 3**).
2. Place a centrifuge tube on a balance and add stock solutions of the salt, PEG, and buffer (in that order) to complete either 5 or 10 g. NaCl is added as a solid. For example, to prepare 10 g of a system containing 15% PEG, 5.7% GuHCl, and 20% NaCl, add to a centrifuge tube: 3.75 g of a 40% PEG stock solution, 1 g of a 6 *M* GuHCl stock solution, 2 g of NaCl, and 3.25 g of 67 mmol phosphate buffer.
3. Mix the system well using a vortex mixer and/or a rotary mixer for 20 min.
4. After mixing is completed, the system is driven into the two-phase region by increasing the temperature (*see* **Note 4**).

3.2. Determination of Phase Diagram

Phase diagrams may need to be determined because only a few are available in the literature for PEG/chaotropic salt/NaCl systems (*see* **Note 5**). The sytems for which phase diagrams have been determined include PEG/NaSCN/NaCl and PEG/GuCl/NaCl systems (*see* **Notes 6** and **7**). A phase diagram for the former system at 20°C is shown in **Fig. 2** (*see* **Note 6**).

Three different protocols can be followed to determine the phase diagram.

3.2.1. Protocol 1

1. Prepare 5 g phase systems as previously indicated (**Subheading 3.1.**).
2. Place the tubes containing the phase systems in a water bath and increase the temperature of the water bath in intervals of 2° or 3°C. The systems must remain in the water bath at each temperature for about 30 min to allow stabilization. The systems should be shaken every 5 min.
3. The temperature at which the systems become cloudy is recorded as the cloud-point temperature, which indicates the onset of the liquid–liquid phase transition.
4. The temperature is set just above the cloud-point temperature, the phases are allowed to separate during 12 h, and salts and PEG concentrations in each phase are measured (*see* **Notes 8–10**).

3.2.2. Protocol 2

1. The phase systems are prepared as previously indicated (**Subheading 3.1.**).
2. They are placed into a stirred cell of a temperature controlled spectrophotometer and the absorbance at 450 nm is measured as a function of temperature against a blank of the same system at room temperature.
3. First, the temperature is increased until a sharp increase in the absorbance is observed, indicating that the system has become cloudy (one reaches the onset of a liquid–liquid phase separation from the one-phase region). The temperature at which the systems become cloudy is recorded as the cloud-point temperature (forward experiments).
4. Thereafter, the temperature is decreased until the absorbance reading is zero. The temperature at which the systems become transparent is recorded as the "cloud-point temperature" (backward experiments). The cloud point temperature obtained by the backward and forward experiments should agree within 1°C.
5. The temperature is set just above the cloud-point temperature, the phases are allowed to separate during 12 h, and salts and PEG concentrations in each phase are measured (*see* **Notes 8–10**).

3.2.3. Protocol 3

1. Place a test tube on a balance and add 3 g of a 30% stock solution of PEG.
2. Place in a microburet a concentrated solution of a chaotropic salt/NaCl mixture.
3. Add the salt solution to the PEG solution dropwise until the PEG solution becomes turbid (cloud point) (*see* **Note 11**). Write down the weight of salt stock solution added.

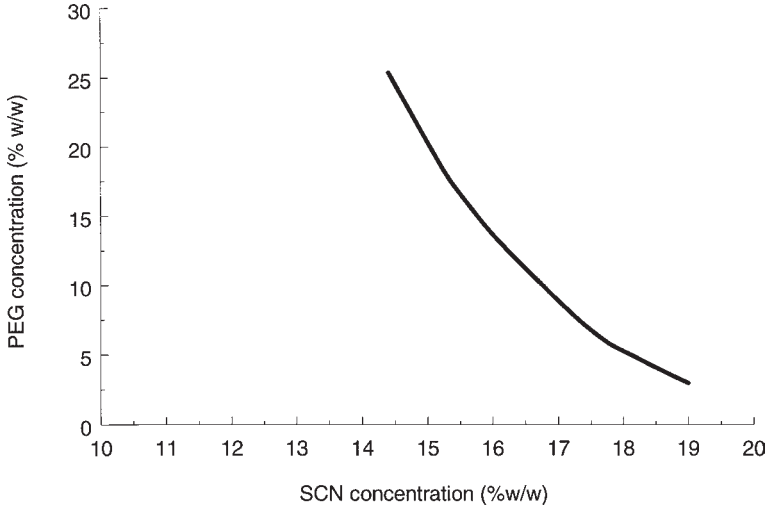

Fig. 2. Phase diagram for PEG/SCN/NaCl/ system. The total salt concentration is 15% (w/w); 60% of the total salt is NaCl and 40% is NaSCN (*see* **Note 5**).

4. Calculate the composition of the system at the cloud point.
5. Add buffer until the system becomes clear. Keep track of the grams of buffer added.
6. Repeat **step 3**.

3.3. Partition Experiments

1. For the partition experiments, 0.5 or 1 g of a 2.5 mg/mL protein stock is added to the phase systems replacing an equal amount of buffer (*see* **Note 12**). For example, 0.5 g of the 3.25 g of buffer used in **Subheading 3.1.** are replaced by 0.5 g of protein stock solution. The protein may be pretreated, e.g., denatured, aggregated, or refolded for partitioning studies (*see* **Notes 1** and **2**). A phase system without added protein is also prepared and used as a blank.
2. The systems are stirred for 20 min.
3. Place in a water bath to induce the phase transition.
4. After complete phase separation (5–12 h), samples from top and bottom phase of the sample and the blank are taken carefully (so as not to disturb the liquid–liquid interface) with a micropipet. A small positive pressure is needed to sample the bottom phase (*see* **Note 13**).
5. After suitable dilution of the respective phases and intense mixing, the absorbance at 280 nm is measured (*see* **Note 14**). The enzymatic activity is also measured in each phase (*see* **Note 15**).

3.4. Recovery of Refolded Proteins from PEG Phase

Ultrafiltration can be used to recover refolded proteins from the PEG-rich phases of the various systems. The procedure is described as adapted to lysozyme.

1. One mL of the PEG rich top phase is diluted with 2 mL of phosphate buffer.
2. Two mL of this solution is placed into Ultrafree-20 (Sigma) centrifuge tubes with a nominal molecular weight cutoff of 10,000. Centrifuge at 5000*g* for 30 min.
3. After 30 min, most of the 2-mL sample had penetrated the membrane and *lysozyme* is retained on the membrane.
4. The filtrate is then assayed for PEG concentration (*see* **Note 8**).

4. Notes

1. Use of denatured protein: For partitioning studies, a denatured protein can be prepared by the treatment of the native protein with GuHCl. For proteins containing disulfide bonds, denaturation is effective under reducing conditions. Quick refolding of the denatured protein can be used to create "inclusion bodies."

   Example of a protein without disulfide bonds: Carbonic anhydrase II denatures by incubation of the enzyme for 24 h in 5 *M* GuHCl. Under these conditions most proteins that do not contain disulfide bonds are denatured. Dilution of 10 mL of a 2.5 mg/mL of denatured carbonic anhydrase II solution with 25 mL of Tris buffer to decrease the concentration of GuHCl to 0.174 *M* results in protein aggregation ***(5)***.

   Example of a protein with disulfide bonds: Lysozyme can be denatured by adding a 1-mL aliquot of a 25-mg/mL native protein stock solution to 0.05 mL of 2-mercaptoethanol and 8.95 mL of 6 *M* guanidine hydrochloride in a 10-mL volumetric flask. The solution is allowed to stand at room temperature for approx 5 h to ensure complete protein denaturation.
2. Use of refolded protein: We have found that the partition behavior of native and refolded proteins in chaotropic aqueous two-phase systems may not be the same. Therefore, the partitioning of refolded proteins may yield additional information about the refolding process. Denatured proteins can be refolded by dilution of the denaturing salt.

   Example of a protein without disulfide bonds: Denatured carbonic anhydrase II (*see* **Note 1**) can be refolded by diluting the 5 *M* GuHCl solution used to denatured it to 0.32 *M* with phosphate buffer, pH 9.4, containing 3 g/L PEG. Without the addition of PEG to the refolding buffer, only a fraction of the original activity is recovered. Refolding of the protein is completed after 1 h.

   Example of a protein with disulfide bonds: Denatured lyzosyme can be refolded by diluting the concentrated GuClH solution used to denature it to 0.8 *M* with 1 m*M* GSSG phosphate buffer. The addition of PEG to the refolding buffer marginally affects the recovery of activity. Refolding is completed after 6 h.
3. Choice of two-phase system: If the goal is to concentrate the refolded protein in a small volume to simplify the subsequent purification steps, phase systems whose top phases represent a small fraction of the total volume need to be identified. We have found, for example, that the volume of the bottom phase decreases with increasing amounts of GuHCl. Therefore, high total GuHCl concentrations in the systems increase the volume of the top phase, which is undesirable. Also, the volume fraction of the bottom phase increases with increasing pH for a given GuHCl-NaCl : PEG ratio; therefore, basic pHs should be used.

4. Precipitation of salts: A residue of salt at the bottom of the test tube may be observed in some phase systems. It should be left undisturbed.
5. Phase diagram: The data can be, within experimental error, plotted in a PEG/total salt or PEG/NaCl plane to represent phase diagrams. We refrain to present the data in this form because it is not possible to move along the experimental tie lines to change the volume ratio between top and bottom phases. Moreover, the volume of the PEG-rich phase increases with increasing amounts of PEG, which is contrary to well established observations. Also, the resulting "binodal" does not separate the plane into one and two phase regions. i.e., we can only use these curves when the concentrations are close to the determined cloud points.
6. PEG/NaSCN/NaCl system: As with conventional PEG/salt systems, an increase in PEG concentration in PEG/NaSCN/NaCl system facilitates phase separation. The amount of PEG and NaSCN in the PEG-rich phase increases with increasing temperature, in contrast to the amount of NaCl in the phase which decreases.
7. PEG/GuHCl/NaCl system: Except at low total salt concentrations, the concentration of PEG needed to obtain two liquid phases increases with increasing GuHCl to NaCl ratio. With phosphate buffer, the cloud-point temperature decreases with increasing pH; however, there is almost no difference in systems containing Tris buffer at two different pH values. The extremely high salt concentration in both phases and the dependence of the cloud-point temperature on pH (basic pHs favoring phase separation), and buffer type and concentration differentiate these systems from the more traditional PEG/salt or Polymer 1/Polymer 2 phase systems.
8. Measurement of PEG concentration: PEG concentration is determined using a colorimetric assay. To 1 mL of PEG solution is added 5 mL of 0.5 *M* perchloric acid. After 15 min, the precipitate (not always present) is discarded, and to 4.0 mL of the solution are added 1.0 mL of 5% $BaCl_2$ and 0.4 mL of 0.1 *M* iodine. After 15 min, the absorbance at 535 nm is measured against a blank that does not contain PEG. A calibration curve is made using PEG solutions of known concentration.
9. Measurement of NaSCN concentration: NaSCN concentration is determined using a colorimetric assay. Dilute 4.0 mL of 1000 ppm iron reference solution (1 mg/mL of ferric nitrate in 2% nitric acid solution) to 40 ppm with water. Add 3.0 mL of this solution to 1.0 mL of NaSCN containing solution and measure the absorbance at 480 nm against an appropriate blank. A calibration curve is made using NaSCN solutions of known concentrations. No interference by PEG or NaCl has been detected in the range of concentrations studied.
10. Measurement of chloride concentration: To a 25 mL flask are added 0.2 mL of sample, 2 mL of water, 1 drop of 2/3 *N* sulfuric acid, and two drops of indicator (one tablet of diphenylcarbazone indicator in 2 mL methanol). The solution is stirred gently and titrated with mercuric nitrate solution (0.010 *N* in dilute nitric acid) until a slight and permanent violet color appears (a kit is available from Sigma). The standard solutions of Cl^- and SCN^- are also titrated in the same way. The amount of Cl^- in the sample is calculated by subtracting the amount of SCN^- determined by the method previously described.

    Chloride and thiocyanate can be separated and quantified by ion exchange chromatography. Alltech (Deerfield, IL) offers a column of hydroxyethyl meth-

acrylate copolymer with quaternary amine functional group that can be used to separate these anions. 1.5 m*M* sodium octane sulfonate is used as the eluant (1.0 mL/min) and the anions are detected by conductivity.

11. Preparation of phase diagrams by titration: The contents of the tube must be thoroughly mixed after the addition of each salt solution drop. Titration is a simple method to determine phase diagrams when working at room temperature, but it becomes very cumbersome to use when a phase diagram at higher temperatures is desired. At high temperatures, the test tube needs to be equilibrated at the desired temperature in a water bath after the addition of each drop.
12. Proteins may change phase equilibrium: PEG/GuHCl/NaCl systems appear quite stable upon addition of moderate amounts of proteins, i.e., no observable changes in cloud point temperature or in phase compositions were detected. However, PEG/NaSCN/NaCl systems have been seen to become very unstable upon addition of small amounts of protein. An example of the latter is the partitioning of carbonic anhydrase (0.625 g/L) in PEG/NaSCN/NaCl systems containing 20% PEG and 15% total salt (with variable amounts of NaSCN), buffered with Tris-HCl, pH 7.0, at 40°C. For systems containing 50% NaSCN and 50% NaCl, 40% NaSCN and 60% NaCl or 30% NaSCN and 70% NaCl the protein partition coefficients, $K=C^{top}/C^{bottom}$, are 2, 1.3, and 3.6, respectively. The interface of the system containing a high concentration of NaSCN, which remains clear when 0.5 mL of the stock protein solution is added, becomes turbid and very broad with increase in the volume of protein solution used to 1 mL in a 10 g system.
13. Sampling of the phases: The accumulation of protein at the liquid/liquid interface, creates difficulties in the sampling of the phases. We found that it is better, whenever possible, to prepare these systems in separation funnels and remove the bottom phase directly from the bottom of the funnel.
14. Protein precipitation in the top phase: The amount of PEG in the top phase is rather high (from 30% w/w to 50% w/w). In this range of PEG concentrations, proteins may precipitate and accumulate at the interface. The interface should be isolated and the amount of protein and its activity at the interface quantified. To avoid precipitation of the proteins at the interface, the amount of PEG needed to obtain two phases may be decreased by manipulating the pH and temperature. Changes in pH and temperature may also be beneficial to increase the solubility of the protein in the PEG-rich phase.
15. Examples of protein partitioning in PEG/GuHCl/NaCl systems:
    a. Ribonuclease and Hemoglobin: **Table 1** shows the partition coefficients of bovine ribonuclease and pig hemoglobin at six different pHs in a system containing 10% w/w PEG, 4.3% w/w GuHCl, and 20.8% w/w NaCl. Both proteins (used at a concentration of 1 g/L) partition preferably into the top phase and the partition coefficients of both ribonuclease and hemoglobin in phosphate buffer are higher than in Tris-HCl buffer of a similar pH (*see also* **Note 12**).
    b. Carbonic anhydrase: The partition coefficient of native and refolded protein in the presence of PEG (*see* **Note 2**) in two-phase systems containing 10% PEG 8000, 4.2% GuHCl, 20.8% NaCl, 50 mmol phosphate buffer, pH 9.4, is 11 and

Table 1
Partition Coefficients of Ribonuclease and Hemoglobin in a PEG/GuHCl/NaCl System at Different pH Values *(5)*

	Partition coefficient	
pH	Ribonuclease	Hemoglobin
5.5[a]	2.33	7.47
7.6[a]	1.51	1.62
8.8[a]	1.70	3.01
9.4[a]	1.72	4.11
7.4[b]	1.37	2.25
8.9[b]	1.35	2.78

[a]In phosphate. [b]In Tris buffer. The two-phase system was comprised of 10% PEG, 4.3% GuCl, and 20.8% NaCl.

15, respectively. It can be argued that PEG does not completely detach from the protein surface after the protein is folded and therefore, acts as a ligand driving the protein into the top phase. This suggests that refolding in the presence of PEG can be used to attach noncovalently PEG to the protein and, therefore, to change its partition coefficient. Once the protein is in the top phase, the amount of PEG is so large that the protein precipitates (*see* **Note 14**).

c. Lysozyme: Partitioning of lysozyme has been studied using the native protein, the denatured protein, the protein refolded in the presence of PEG, and the protein refolded in the presence of PEG and GSSG (a disulfide shuttle; *see* **Note 2**). Partitioning of 2.5 mg of all forms of lysozyme in 10 g two-phase systems containing 11% PEG-8000, 2.6% GuHCl, 29% NaCl, and 50 mmol phosphate buffer, pH 7.6, at 50°C result in strong precipitation of the protein in the top phase. Analysis of the precipitate reveals the presence of 80% of the activity of the native protein. About 80% of the total protein is present in the top phase. The protein that was refolded in the presence of PEG and the native protein showed different partition behavior, as in case of carbonic anhydrase previously described.

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Temperature-Induced Phase Partitioning for Protein Purification

Anita Kaul, Josefine Persson, and Folke Tjerneld

1. Introduction

Ethylene oxide (EO)-propylene oxide (PO) random copolymers are water-soluble and separate into two phases above a lower critical solution temperature (LCST), i.e., the cloud point, a polymer-rich phase, and a water phase depleted of polymer. Traditionally, the removal of polymer from protein in an aqueous two-phase separation requires either back extraction into a salt-rich phase, ultrafiltration, diafiltration, or a chromatographic step, e.g., ion exchange (*see* Chapter 32). Thermoseparating polymers can be used in place of the commonly used polyethylene glycol in polymer–polymer two-phase systems and, therefore, offer an obvious advantage for the cost-effective purification of target protein into a "clean" water phase and for the recycling of polymer ***(1,2)***.

A purification scheme will consist of a primary extraction system composed of, for example, EO-PO copolymer and either Dextran, hydroxypropyl starch, or other starch derivatives. Target protein is partitioned to the top phase enriched with thermoseparating polymer, whereas the contaminants are partitioned to the bottom phase. A secondary recovery system is formed by removal of the top phase, which is heated to above the cloud point, thus enabling the recovery of protein in one phase and EO-PO copolymer in the other phase (*see* **Fig. 1**).

The phase diagram of a system containing an EO-PO copolymer is constructed in the same way as for other polymer–polymer systems (*see* Chapter 2, **Subheading 3.2.1.**). However, a second-phase diagram is required in order to describe the cloud-point temperature (CPT) of the thermoseparating polymer, as a function of concentration. This is carried out by making an aqueous

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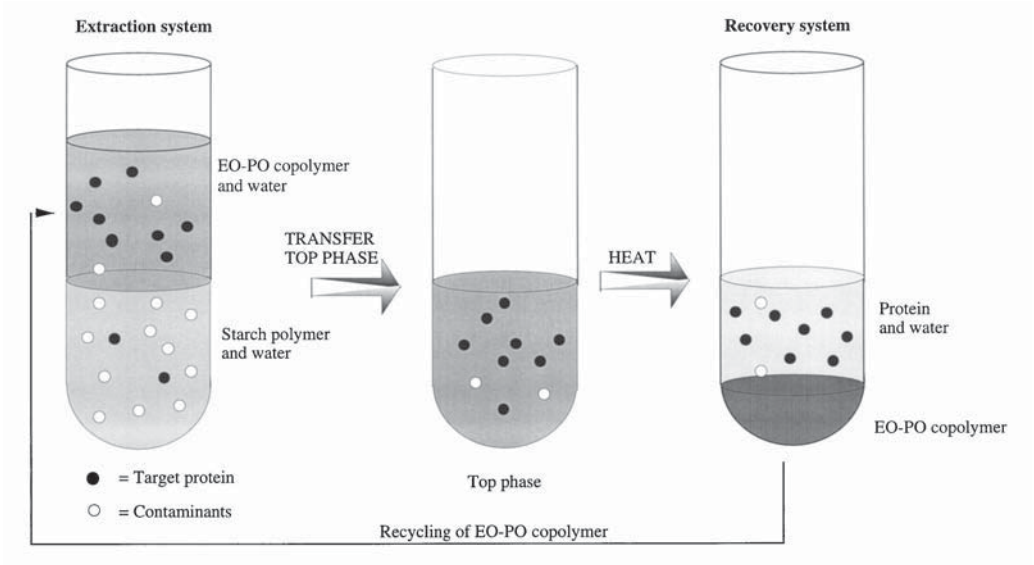

Fig. 1. Schematic diagram for the phase recycling of an aqueous two-phase system containing a thermoseparating polymer. First, target protein is partitioned to the top phase in the *extraction system* (i.e., the EO-PO copolymer phase). This phase is removed and the temperature is raised above the cloud point to produce a *recovery system*, where target protein is recovered in the top phase and polymer is recovered in the bottom phase.

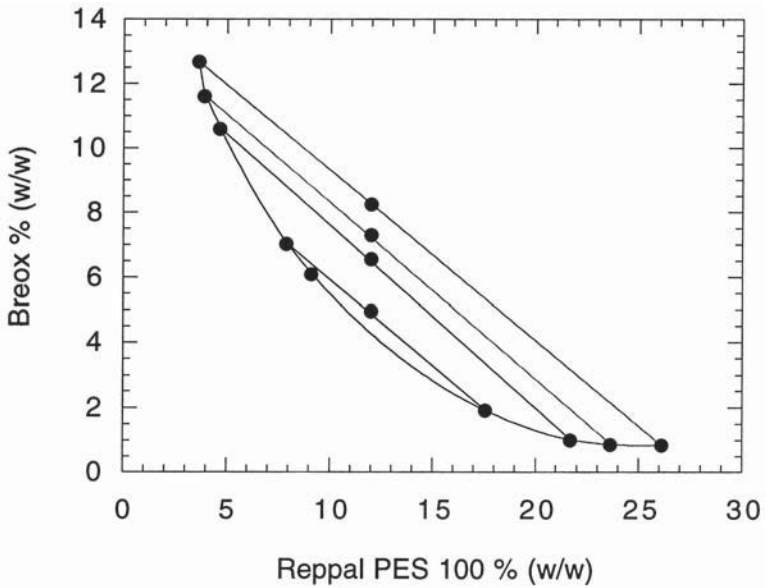

Fig. 2. Phase diagram for an aqueous two-phase system composed of $EO_{50}PO_{50}$ (Breox 50A 1000, M_r 3900) and Reppal PES 100 (M_r 100,000) in water at 22°C.

solution of the EO-PO copolymer at different concentrations and gradually raising the temperature, e.g., 1°C/min, until the first signs of clouding appear ***(3)***.

Thermoseparating copolymers are available with differing percent weight ratios of EO:PO and molecular weights. Those that have been studied to date include: $EO_{20}PO_{80}$ (M_r 3400, CPT: 30°C for a 10% solution), $EO_{30}PO_{70}$ (M_r 5400, CPT: 40°C for a 10% solution) and $EO_{50}PO_{50}$ (M_r 3900 or 4000, CPT: 50°C for a 10% solution) (i.e., Breox 50A 1000 or UCON 50-HB-5100, respectively). For the bottom-phase polymer Dextran or, alternatively, cheaper hydroxypropyl starch (e.g., Reppal PES 100 and PES 200) or hydroxyethyl starch (e.g., Solfarex A85), can be used. **Figure 2** shows a phase diagram for $EO_{50}PO_{50}$ (Breox 50A 1000)—Reppal PES 100 systems and **Fig. 3** shows a cloud-point diagram for $EO_{50}PO_{50}$ (Breox 50A 1000) (*see* **Note 1**).

A desired partition of the target protein can be achieved by manipulation of system variables, which include: choice and concentration of phase components, pH, and the type and concentration of added salt ***(3–6)***. To optimize recovery of protein and polymer during thermoseparation, the ratio and M_r of EO-PO, the type and concentration of salt ***(1)***, and the temperature and time for thermoseparation are all important variables. We have found that the higher the M_r and PO content, the greater is the amount of polymer is recovered. The

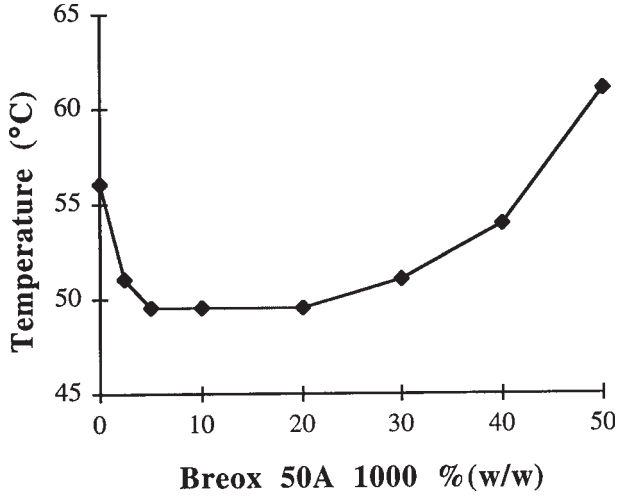

Fig. 3. Cloud-point diagram for $EO_{50}PO_{50}$ (Breox 50A 1000, M_r 3900) in water.

temperature and time of thermoseparation have to be chosen judiciously according to the stability of the target protein, and the process requirements, i.e., higher temperatures and shorter times or lower temperatures and longer times, are likely to result in similar recoveries.

2. Materials

1. Thermoseparating polymers are available from Union Carbide (NY), (i.e., Ucon 50 HB 5100, i.e., $EO_{50}PO_{50}$); Shearwater Polymers, Inc. (Huntsville, AL), (i.e., $EO_{20}PO_{80}$, $EO_{30}PO_{70}$ and $EO_{50}PO_{50}$); and International Speciality Chemicals Ltd. (Southampton, UK), (Breox 50A 1000, i.e., $EO_{50}PO_{50}$). EO-PO random copolymers are in liquid form, and therefore can be added as 100% or prepared at the required stock concentration.
2. Reppal PES 100 (M_r 100,000) or PES 200 (M_r 200,000) can be obtained from Carbamyl AB (Kristianstad, Sweden), and Solfarex A85 from AVEBE (Veendam, The Netherlands). Starches are used as 20–30% (w/w) stock solutions and can be prepared in water or buffer (*see* **Note 2**) or, alternatively, can be added straight to the system in solid form (except for Solfarex).
3. If polymer is added straight to the system in solid form or if stock solutions are made in water, to buffer the systems, make a concentrated stock (e.g., 100 m*M* sodium phosphate) at the required pH and add to the system to obtain the desired final concentration. All salts should be of analytical grade.

3. Methods

3.1. System Selection and Preparation

1. The "working point" in the phase diagram, for the extraction system, will depend on the concentration of chemicals and the volume ratio of phases required. For example, to assess the effect of polymer concentration on the partition coefficient, systems with differing tie-line lengths can be chosen by extrapolating through the critical point. Alternatively, a single system can be selected (of intermediate concentration) and the effect of variables such as pH and salt type and concentration can be studied (*see* **Note 3**) ***(3,6)***. Either way, once the desired partition has been achieved, an appropriate volume ratio can be selected to maximize the purification and yield of the target protein (*see* **Note 4**).
2. **Table 1** shows the calculations required to prepare a Reppal PES 100-Breox 50A 1000 system with intermediate concentration, with added salts and protein (either pure or crude extract; *see* **Note 5**). For further system compositions, this table should be reproduced in a spreadsheet program for ease of calculation. Systems may be prepared in a graduated test tube (*see* **Note 6**). Phase components should be mixed well to ensure a homogeneous mixture; this can be carried out simply by inverting the test tube several times or using a vortex mixer. A "blank" system (i.e., containing all the phase components except protein), as well as a system for the partitioning should be prepared.
3. To assay for protein concentration take a sample of the top and bottom phase and make appropriate dilutions (*see* **Notes 7** and **8**).

3.2. Thermoseparation of EO-PO Phase

1. Once the required partition has been accomplished, note the volume ratio of the extraction system. Remove the top phase, making sure not to disturb the interface, and place in a separate vessel (*see* **Note 9**).
2. Temperature-induced phase separation is carried out by placing the vessel in a water bath at the required temperature. We have found that for a 10% (w/w) solution of EO-PO, the thermoseparation should be carried out for 5 min at a temperature 15°C above the cloud point to obtain optimal recovery of both polymer (bottom phase) and protein (top phase) (*see* **Note 10**). However, similar recoveries may be obtained at lower temperatures for longer periods of time. For example, for a 10% (w/w) solution of $EO_{30}PO_{70}$, an optimal polymer recovery of 96% is obtained at 55°C for 5 min, however, a recovery of 94% can be achieved at 50°C for 10 min.
3. To ensure the efficient removal of polymer, centrifuge at a high speed for a short period of time (i.e., 4000*g* for 0.5 min). If a temperature equilibrated centrifuge is available, separation of the phases can be carried out in the centrifuge itself.
4. Note the volume ratio, remove the top phase and place in a separate vessel (*see* **Note 11**). To assay for protein concentration make appropriate dilutions of the top and bottom phase (*see* **Note 12**).

Table 1
Calculations Required to Prepare a Reppal PES 100-Breox 50A 1000 System (Volume Ratio ≈ 1)[a]

	A	B	C	D	E	F	G	H	I	J	K
1											
2		**Stock**									
3	**Breox 50A 1000**	100% (w/w)									
4											
5	**Reppal PES 100**	30% (w/w)									
6											
7	**Salt**	1 M									
8	*eg* **NaClO4**										
9											
10	**Protein**	20 mg/mL									
11											
12	**Buffer**	100 mM									
13											
14	**Final weight of**	**20 g**									
15	**system**										
16											
17	**System composition**					**Amount of stock (g)**					
18											
19	***Breox***	***Reppal***	***Salt***	***Protein***	***Buffer***	***Breox***	***Reppal***	***Salt***	***protein***	***Buffer***	***water***
20	**% (w/w)**	**% (w/w)**	**M**	**mg/ml**	**mM**						
21											
22											
23											
24											
25	**7**	**13**	**0.1**	**5**	**10**	**1.4**	**8.67**	**2**	**5**	**2**	**0.93**
26						=(A25*B14)/B3	=(B25*B14)/B5	=(C25*B14)/B7	=(D25*B14)/B10	=(E25*B14)/B12	=B14-(F25+G25+H25+I25+J25)
27											
28											
29											

[a]For a blank system, water should be added in place of the protein solution. Reppal and salt can be added in solid form if required. The dilution of the salt, buffer and protein stock solutions is carried out by weight and therefore the final concentration is an approximate value as the density of stock relative to the polymers is not taken into account. The column referring to protein, can be either pure protein or crude extract with and without cells. *See* **Notes 3**, **4**, and **5** regarding the size of the system, type of salt added, and addition of protein.

5. To determine the amount of protein recovered, calculate the amount of protein in the top phase of the extraction system and the total protein in the top phase after thermoseparation. Generally, with the EO-PO copolymers presently studied, we have not been able to detect any protein in the concentrated copolymer phase after thermoseparation.

3.3. Recycle of Thermoseparating Polymer

1. To calculate the amount of copolymer that can be recycled, assay for polymer concentrations in the top and bottom phase in a blank extraction system (i.e., a combination of optical rotation for Dextran and starch derivatives, refractive index measurement for EO-PO copolymer, and conductivity if a buffer/salt is present, *see* Chapter 2). Multiply the concentrations by the volume of the respective phase, to give the total amount (g) of each polymer in the top and bottom phase; a certain amount of copolymer will be lost to the bottom phase in the extraction system and the rest will be carried forward for thermoseparation in the top phase.
2. After thermoseparation of the top phase, assay for polymer concentration in the top and bottom phase. Once again, calculate the total amount of copolymer and Dextran or starch polymer in the top and bottom phase as above (*see* **Note 13**).
3. Taking into account the amount of recyclable polymers (mostly EO-PO, and small amounts of starch and salt), calculate the amount of fresh EO-PO copolymer, starch polymer, buffer, and/or salt required to prepare a second extraction system with the same initial concentration.

4. Notes

1. The cloud-point temperatures in this diagram are for $EO_{50}PO_{50}$ in water. It should be noted that the presence of salts and a second polymer will affect the cloud point and, therefore, this diagram should be used as a guideline only.
2. For the preparation of Reppal stock solutions, add the water/buffer to the powder and stir. For Solfarex A85, dissolution requires heating at *ca* 70°C until the mixture turns "clear."
3. The partition of protein can be affected by various salts, where positively charged proteins partition to the phase for which negatively charged ions have affinity. A series similar to the Hofmeister, can be used for the selection of salts where chaotropic ("hydrophobic") salts have an affinity for the EO-PO copolymer phase and salting out ("hydrophilic") salts have an affinity for the Dextran or starch phase. For cations the order of decreased hydrophobicity is as follows: triethylammonium$^+$ > $NH_4^+ \geq K^+ \geq Na^+$ and for anions, also in order of decreased hydrophobicity, is as follows: $ClO_4^- \geq SCN^- \geq I^- > Br^- > Cl^- > NO_3^- > HPO_4^{2-} \geq SO_4^{2-}$ ***(3,6)***.
4. Provided a high value for the partition coefficient K is obtained, a concentration in the top phase can be achieved in the extraction system by selecting a small top-phase volume. For increased yield, a larger volume of top phase should be used. The following equation is useful when deciding an optimal volume ratio:

$$Y_T = K \cdot 100/K + V_B/V_T$$

where Y_T is the percentage yield in the top phase, K is the partition coefficient

and V, the volume of the top (T) and bottom (B) phase. For example, a yield of 83% can be obtained at a K value of 5 and a volume ratio of 1, however, if a volume ratio of 2 is used, then a yield of 91% can be achieved. The size and/or volume ratio of the system should enable a sufficient volume of top phase to be removed and thermoseparated, especially for analysis of phase concentration; i.e., for polarimetric readings, a minimum volume of 7 mL is generally required.

5. Some proteins that display low solubilities, i.e., relatively hydrophobic proteins, should first be dissolved in top-phase polymer prior to addition. For example, when adding salts, buffer, or crude extract, the volume ratio may change owing to a shift in the binodal ***(7,8)***.
6. Graduated test tubes are not always accurate and therefore should be further calibrated with water (i.e., 1 g = 1 mL), to ensure a correct reading of the phase volume ratio.
7. Ideally, preparing dilutions should be carried out by volume, however, because of the viscosity of the polymer solutions, pipetting is not always accurate and therefore dilutions are carried out by weight. It should be noted that the latter method does not take into account the density of the phase.
8. Determination of protein concentration can be carried out by measuring the wavelength at 280 nm or using a dye binding method such as Coomassie Brilliant Blue G-250. A blank system should be used to take into account any absorbance of the phase forming components. Coomassie can be prepared following the method of Bradford ***(9)***, i.e., dissolve 100 mg coomassie in 50 mL of 95% ethanol. Add 100 mL of 85% phosphoric acid and make up to 1 L with water and filter (0.2 μm) prior to use. Add 100 μL sample to 1 mL Coomassie and mix. After approx 10 min, measure the absorbances at 595 nm and 465 nm and calculate A_{595}–A_{465}. A standard curve can be prepared within the range of 0–0.1 mg/mL.
9. A similar graduated test tube used for the extraction system can be used for this purpose. Alternatively, in our laboratory, we have found that a graduated glass pipet (e.g., 10 mL), with the bottom end sealed and the top end cut off, is quite useful for this purpose.
10. For heat-sensitive proteins, copolymers with a high PO content can be used, for example, with $EO_{20}PO_{80}$ the thermoseparation can be carried out at 24°C ***(2)*** or alternatively a salt can be added to the system, which causes a decrease in the cloud-point temperature, (i.e., with the addition of 0.2 *M* Na_2SO_4 the cloud-point temperature of $EO_{50}PO_{50}$ can be reduced from 55°C to 35°C ***[1]***).
11. When removing the top phase after thermoseparation, leave a small volume above the interface to ensure that protein is not contaminated with polymer.
12. Owing to the viscosity of the concentrated EO-PO phase, prior to analysis, mix well to ensure a homogeneous mixture; pipetting can be rather arduous and therefore it may be easier to dilute the phase in the same test tube.
13. Except for $EO_{50}PO_{50}$, the concentration of polymers calculated for the protein-free system remains the same in the presence of protein. For 10% $EO_{50}PO_{50}$ in water, we have found an increase of approx 20% for recovery of EO-PO in the presence of 10 mg/mL BSA.

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Extraction of Amphiphilic Proteins Using Detergent-Based Aqueous Two-Phase Systems

Torsten Minuth

1. Introduction

Extraction systems based on nonionic surfactants have been described as an alternative to the standard polymer/polymer or polymer/salt systems. Phase-forming surfactants are, for example, the nonionic polyoxyethylene-type detergents. This kind of aqueous two-phase system (ATPS) is simply induced by a switch in the temperature; on the basis of the temperature-dependent reversible hydration/dehydration of the polar ethylene oxide headgroups. A single isotropic micellar phase separates into two isotropic phases: one of the resulting two aqueous phases, the so-called coazervate phase, is enriched in detergent, whereas the other is depleted ***(1)***. The detergent forms micelles in the detergent-depleted phase and is believed to exist in the form of lamellar stacks in the coazervate phase ***(2)***. Both phases have a high water content. The temperature at which the phase separation occurs is referred to as the cloud-point. The clouding temperature depends on the structure of the polyoxyethylated surfactant. This kind of ATPS is especially suited for the extraction of amphiphilic/hydrophobic biomolecules.

The potential of nonionic detergents to lyse a biomembrane and to solubilize proteins that are membrane-associated without loss of their biological activity is well-documented ***(3–5)***. Polyoxyethylene-type nonionic detergents solubilize membrane proteins in a gentle fashion. During the solubilization, the nonionic detergent replaces most lipid molecules in contact with the hydrophobic domain of the amphiphilic protein and leads to the formation of a soluble protein–detergent mixed micelle ***(3)***. Upon alteration of the conditions of the micellar solution (temperature, pressure, addition of salt or other addi-

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tives), a phase separation is induced. The lamellar structures formed in the miscibility gaps of polyoxyethylated fatty alcohol/water systems are responsible for the selective extraction of amphiphilic/hydrophobic substances ***(6)***.

Figure 1 summarizes the steps involved in the application of such a cloud-point extraction process schematically.

An important advantage of this extraction method is that using only one auxiliary chemical the desired biomolecule can be solubilized and after alteration of the temperature of the micellar solution an ATPS is formed, enriching amphiphilic/hydrophobic proteins in the small volume element of the coazervate phase, whereas hydrophilic proteins are recovered in the detergent-depleted phase.

The potential of ATPS based on nonionic polyoxyethylene detergents for separating integral membrane proteins from peripheral membrane and cytosolic proteins was first demonstrated by Bordier in 1981 ***(7)***. Since then, this kind of ATPS has been successfully utilized to extract selectively a variety of integral membrane proteins from peripheral membrane and cytoplasmic proteins into the coazervate phase for purification and characterization ***(8–14)***. Ramelmeier et al. ***(15)*** and Terstappen et al. ***(16)*** have demonstrated that the cloud-point extraction technique can also be used for selective partition of water-soluble extracellular proteins into the detergent-rich phase.

This chapter deals with general methodologies required in the preparation and application of nonionic surfactant-based ATPS in the small as well as in the pilot scale. More comprehensive reviews have been published by Hinze and Pramauro ***(17)*** and Sanchez-Ferrer et al. ***(18)***.

As a practical example, some of the recent investigations by Kula and coworkers will be presented to illustrate the potential and usefulness of this type of ATPS ***(19–21)***. The potential of such nonionic detergent-based extraction systems to simplify downstream processing of a bacterial integral membrane protein has been shown ***(19,20)***. In this study, the membrane-bound cholesterol oxidase (CHO) from *Nocardia rhodochrous* (NCIB 10554), an enzyme that is well-characterized ***(22)*** and is of commercial interest in the field of clinical diagnosis of human serum cholesterol ***(23,24)***, was extracted successfully. An ATPS of nonionic polyoxyethylene detergent with linear alkyl chains for direct solubilization and extraction of the membrane-bound CHO from unclarified fermentation broth of *N. rhodochrous* has been used. Furthermore, a method for recycling the detergent used for the protein extraction has been developed and the use of conventional techniques as anion-exchange chromatography for further purification of the enzyme is demonstrated. **Figure 2** outlines the optimized extractive purification of cholesterol oxidase from *N. rhodochrous* ***(20,21)***.

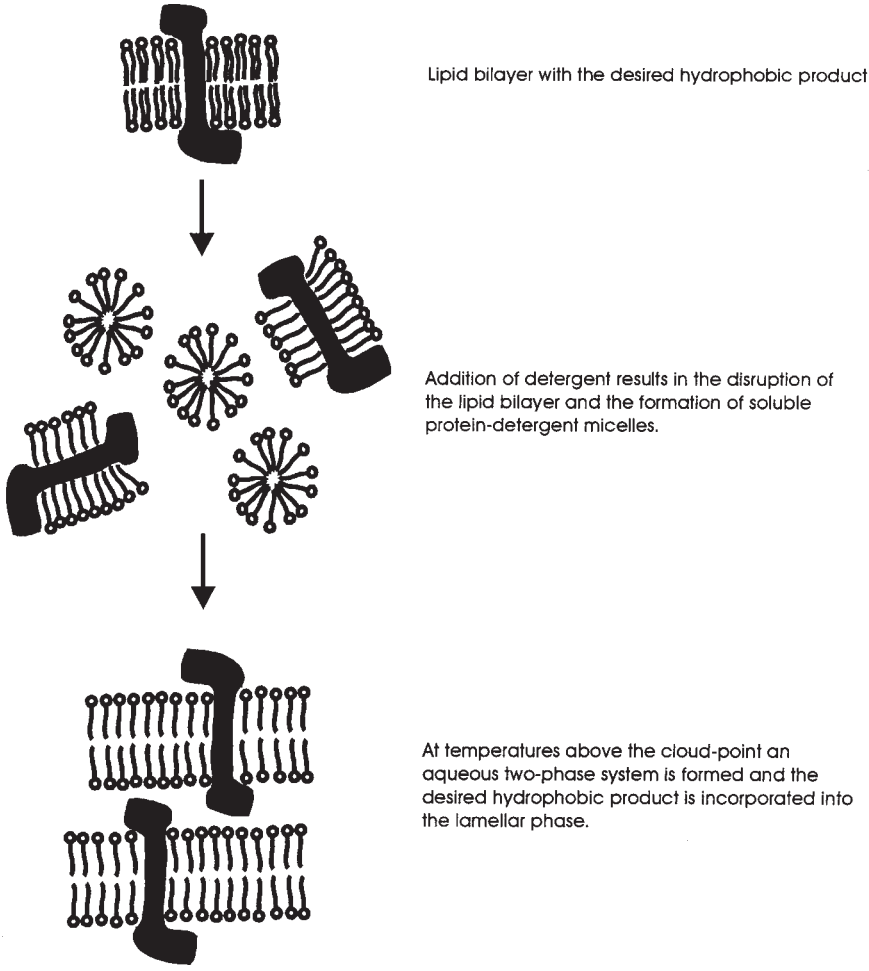

Fig. 1. Schematic representation of the cloud-point extraction technique.

2. Materials

1. Phase forming surfactants (*see* **Note 1**): Polyoxyethylene detergent C12EO5 (pentaethyleneglycol mono n-dodecyl ether), C12EO6 (hexaethyleneglycol mono n-dodecyl ether), C14EO5 (pentaethyleneglycol mono n-tetradecyl ether), and C14EO6 (hexaethyleneglycol mono n-tetradecyl ether) (all these detergents have been kind gifts of Henkel KGaA, Düsseldorf, Germany). These detergents are homogeneous with respect to the linear alkyl chain and exhibit a narrow-range ethylene oxide (NRE-type) distribution with an average of five (C12EO5 and C14EO5) or six (C12EO6 and C14EO6) of these groups. The quaternary surfactant system C12-18EO5 exhibits a narrow-range ethylene oxide distribution with

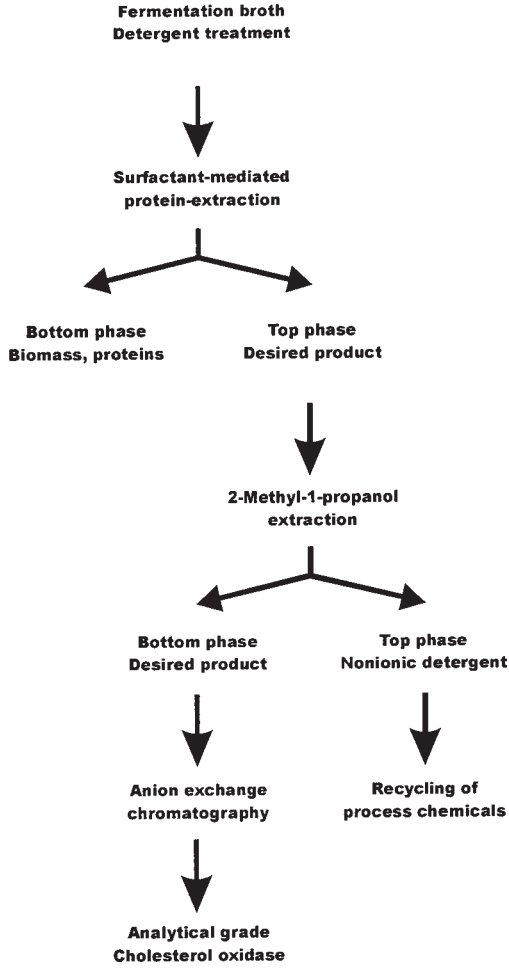

Fig. 2. Flow sheet of cholesterol oxidase purification.

an average of five of these groups and is inhomogeneous with regard to the alkyl chain length, varying from n-dodecyl to n-octadecyl. The relative ratios of the straight-chain fatty alcohols is not known. All detergent preparations are used without further purification.

2. Chromatographic material: diethyl aminoethyl (DEAE) Sepharose Fast Flow (Pharmacia Biotechnology, Freiburg, Germany/Uppsala, Sweden).
3. Graduated centrifuge glass tubes: For partition experiments in the 10 g scale.
4. Tube rotator (Heidolph, Kelheim, Germany): For mixing in the 10 g scale.
5. Bench centrifuge for phase separation; in the 10 g scale.
6. 30 L stainless steel vessel: For experiments in the pilot scale (21 kg).

7. Centrifugal liquid-liquid separator (SA 1-01-175; Westfalia Separator AG, Oelde, Germany) (*see* **Note 2**): For phase separation in the pilot scale.
8. Liquid chromatography system:
   a. Glass column (C10/10, Pharmacia Biotech).
   b. Peristaltic pump (Minipuls, Gilson).
   c. A variable wavelength monitor (Knauer, Berlin, Germany).
   d. A computer for registering and processing the absorbance data.

3. Methods

3.1. Determination of the Cloud-Point and the Phase Ratio (see Note 3)

The phase behavior for each nonionic system under different experimental conditions is simply determined by heating up a clear aqueous solution of 2% (w/w) detergent until clouding of the solution occurs, and subsequent cooling down of this solution until clarification is observed again.

1. Place the detergent samples (5 mL) in a water bath, and maintain the rate of temperature change at about 0.1°C/1 min.
2. The mean value of the observed clouding and clarification temperature is noted. This is defined as the cloud-point of the surfactant.
3. The volumes of the separated phases are measured using graduated glass tubes. Aqueous solutions of 2% (w/w) detergent are placed in a water bath until a constant phase ratio is reached. The incubation temperature must be constant and must be above the prior determined cloud-point.

3.2. Preliminary Screening Experiments at the 10 g Scale for Determining Suitable Partition Conditions (see Note 4)

1. Concentrated detergent solutions are added in graduated centrifuge tubes to final detergent concentrations of 1.0, 2.0, 4.0, and 5.0% (w/w), respectively, in 10 g systems.
2. 6 mL of the protein samples (0.2 to 1.0 mg/mL) are overlaid on the detergent solution.
3. Bring the system to the final total weight of 10 g by the addition of water.
4. After mixing the protein–detergent solutions for 15–30 min using a tube rotator, the samples are incubated for maximal 2 h at a constant temperature of 3–5°C above the cloud-point of the corresponding pure detergent solution (2%, w/w).
5. The phase separation can be achieved rapidly by centrifugation of the mixture for 10–15 min at 400*g* at 3–5°C above the cloud-point of the chosen system.
6. Phase volumes are noted after separation. If the phase volumes are constant after repeated centrifugation, a real phase system has been formed.
7. Samples from the top and the bottom phase are taken with a pipet or a Hamilton microsyringe and are analyzed for the concentration of the desired product (*see* **Note 5**). The detergent-rich phase is in general quite viscous and must be diluted prior to analysis.

8. Calculate the partition coefficient, K, which is defined as

$$K = C_T/C_B$$

where C_T and C_B are the concentrations of the desired product in the top and the bottom phase. The extraction yield of the desired protein is calculated on the basis of the concentrations of the product in both phases and the phase volumes of the detergent-rich and the detergent-depleted phase (*see* **Note 6**).

3.3. Whole Broth Extraction

1. Solubilization experiments are carried out with cell-containing cultivation medium of different cell concentrations (0.5, 1.0, 2.0, 5.0, and 10.0% [w/w]). The different cell concentrations are achieved by harvesting and resuspension of the cells in the clarified culture medium.
2. The concentrated detergent solution is added to 10-mL samples of cell suspension to concentrations of 1.0, 2.0, 4.0, 5.0, 8.0, and 10.0% (w/w), in tapered graduated tubes.
3. The cell-detergent solutions are incubated for maximal 2 h in a tube rotator. The temperature must be constantly 4/5°C below the cloud-point of the corresponding pure detergent system.
4. After solubilization of the desired product, phase separation is carried out by subsequent centrifugation at a constant temperature above the cloud-point for 10–15 min (*see* **Note 7**).

3.4. Formation of a Surfactant-Based ATPS at the Pilot Scale

1. The two-phase systems are made up by adding the undiluted surfactant to the aqueous suspension or the unclarified fermentation broth under continuous stirring using a propeller in the vessel.
2. After 45 min mixing at room temperature the dispersion is heated above the cloud-point and stirred at a constant temperature for 15–30 min.
3. Mixing in the reservoir is continued during the separation procedure.

3.5. Phase Separation under Gravity

Separation under gravity can be carried out on any scale.

1. The thoroughly mixed dispersion is transferred to a graduated vessel.
2. Separation is conducted at a temperature close to the cloud-point of the system employed. In general, the separation time needed to obtain sufficiently pure phases and a constant phase volume ratio is rather long. This is owing to the small density differences between the two liquid phases, the high viscosity of the detergent-rich phase, and the low interfacial tensions of these detergent-based extraction systems.

3.6. Continuous Phase Separation in a DiskStack Centrifuge

1. The dispersion is fed to a disk stack centrifuge (SA 1-01.175, Westfalia) using a peristaltic pump (VERDER 2004). With regard to the small density differences

between top and bottom phase, use is made of discs with rising channels, positioned at the outside of the discs.
2. The two clarified liquids are continuously discharged with two centripetal pumps.
3. Solids are discharged discontinuously by activating a hydraulic intermittent desludging mechanism.

3.7. Determination of Phase Purity after Separation

1. 10 mL samples of both the top and bottom phase are placed in a water bath for 1 h at a constant temperature above the cloud-point of the system employed.
2. The samples are centrifuged in a thermostatted bench-scale centrifuge.
3. The purity of the coazervate phase is calculated after determination of the volume ratio of the detergent-rich phase to the total sample volume. A pure coazervate phase is defined at a volume ratio of 1.
4. The purity of the detergent-depleted phase is calculated after determination of the volume ratio of the detergent-depleted phase to the total sample volume. A pure detergent-depleted phase is defined accordingly at a volume ratio of 1.

3.8. Detergent Recycling (see Note 8)

1. Solvent extraction of the coazervate phase is carried out in a stirred glass vessel at 4°C. To 20 mL of coazervate phase, add 12 mL of precooled (4°C) 2-methyl-1-propanol using a pump at a flow rate of 15 mL/h.
2. After solvent addition stirring is continued for 10 min.
3. Phase separation is achieved by gravity settling.
4. The detergent is extracted in the organic top phase and the desired product is recovered in the detergent-depleted aqueous bottom phase.
5. This aqueous solution can be used as a feed stock solution for the final purification by chromatographic methods (*see* **Subheading 3.9.**).
6. The polyoxyethylene detergents can be separated from the organic solvent simply by distillation at reduced pressure (40 mbar) and 65°C (*see* **Note 9**).

3.9. Fine Purification of Cholesterol Oxidase by Subsequent Anion Exchange Chromatography (see Note 10)

1. The chromatographic purification is carried out by frontal chromatography. The aqueous phase of the second extraction system is applied onto DEAE-Sepharose Fast Flow equilibrated with 10 m*M* potassium phosphate, pH 7.0, containing 10% (v/v) 2-Propanol.
2. The sorbent is washed with the equilibration puffer.
3. The desired enzyme is desorbed with 50 m*M* potassium phosphate, pH 7.0, 50 m*M* sodium chloride, and 0.02% (w/v) of the nonionic detergent decaethyleneglycol mono n-lauryl ether (C12EO10).
4. Standard protocols according to the instructions of the manufacturer can be used for column regeneration and washing.

4. Notes

1. To date, Triton X-114 surfactant systems are widely used for protein extraction, because this surfactant is commercially available, possesses a cloud-point below

30°C and effectively solubilizes membrane-bound proteins without destroying the biological, active conformation ***(3,25)***. Nevertheless, we prefer nonionic polyoxyethylated surfactants like C12EO5 to generate detergent-based ATPSs. There are three arguments to use polyoxyethylene glycol monoethers instead of *t*-octylphenoxy polyoxyethylene ethers (e.g., Triton X-114).

a. The coazervate phase of a C12EO5 based extraction system is less dense than the detergent-depleted phase and forms the top phase. In contrast, in Triton X-114 based ATPS the detergent-rich phase forms the bottom phase. The density difference is small and in the presence of salt isopycnic situations may arise, making separation impossible ***(15)***.
b. The *t*-octylphenoxy polyoxyethylene ether detergents exhibit a specific ultraviolet (UV)-absorption at 280 nm, and thus drastically impair process control of the following protein purification steps.
c. The polyoxyethylene glycol monoethers are more easily degraded in biological wastewater treatment than the *t*-octylphenoxy polyoxyethylene ether detergents. The rate of biodegradation of the two nonionic polyoxyethylene surfactants appears to be highly dependent on the structure of the hydrophobic component. Degradation of the unbranched alkyl structure appears to occur more readily than that of the branched alkyl and the aromatic ring structure of Triton X-114 ***(26,27)***.

With regard to economical aspects of the cloud-point extraction technique, mixed surfactant systems as phase-forming agents are quite interesting. But, systems with a broad ethylene oxide distribution are not suitable for protein extraction, because these systems exhibit no definite cloud-point temperature. The phase behavior of mixed surfactant systems with a narrow range ethylene-oxide distribution is generally suitable for protein extraction. Using the quaternary surfactant system C12-18EO5, and the same solubilization and extraction conditions as described for the C12EO5 system, the extractable amount of CHO was similar to the C12EO5 system ***(20,21)***.

2. Some technical data of the disk stack centrifuge from Westfalia type S 1-01-175 are listed in **Table 1**.
3. The temperature at which clouding occurs depends on the structure of the nonionic surfactant. For a homologous series of polyoxyethylated nonionic surfactants, the cloud-point increases with decreasing length of the hydrocarbon chain and increasing length of the oxyethylene chain (**Ref. *28*** gives an extensive list of cloud-point temperatures for aqueous solutions of several nonionic surfactants). Furthermore, the miscibility gaps of polyoxyethylated fatty alcohol/water systems can be influenced by several additives, like salts and alcohols ***(15,29,30)***. The phase ratio depends on the separation temperature. The volume of the coazervate phase decreases with increasing temperature.
4. At present, the optimum detergent and the optimum ratio of detergent-to-membrane ratio must be determined empirically. Independent of individual detergent characteristics, a general rule emerges to obtain maximal exchange of lipid for detergent surrounding the proteins: to ensure an effective solubilization process

Table 1
Technical Data of the Westfalia Disk Stack Centrifuge

Parameter	SA 1-01-175
Maximum centrifugal force, *g*	5700
Total volume, mL	600
Number of discs in the stack, N	35
Angle of discs in the stack, ϕ, degree	38
Disc radius (max r1), cm	5.1
Disc radius (min r2), cm	2.6
Number of centripetal pumps	2
Area equivalent (Ae), m^2	1207.6

directly from the fermentation broth, a higher ratio of detergent-to-membrane than described for complete membrane disintegration of partially purified membrane fractions must be used.

5. Protein contents in the presence of high detergent concentrations can be determined according to Neuhoff et al. ***(31)*** or, alternatively, using the commercially available detergent compatible protein assay from Bio-Rad according to the instructions of the manufacturer. The reaction is similar to the Lowry assay ***(32)***.
6. The relation between theoretical product yield in the top (coazervate) phase Y_T the volume ratio of the phases (V_T/V_B), and the partition coefficient of the product K is given by the following equation:

$$Y_T = (100)/(1 + [V_B/V_T][1/K])\ (\%)$$

In principle, a high volume ratio must be chosen at a low K value. On the other hand, at a K value of 10, a volume ratio of 1 is sufficient to yield 90% of the desired product in the coazervate phase by a single extraction step.

7. An alteration of the clouding temperature has not been observed. In contrast, the velocity of the phase separation is significantly altered. The settling velocity of cell–detergent solutions is too low under gravity for practical purposes (*see* **Subheading 3.5.**), which is attributed to the relatively small density differences as well as the viscosities of the phases. Separations with high reproducibility must use centrifugal forces.
8. We have investigated in detail the potential of liquid–liquid separation for detergent removal. Three criteria were used for the selection of a suitable nonpolar phase:
   a. high extraction efficiency for the alkyl polyoxyethylene monoethers,
   b. the desired product must be stable in the detergent-depleted aqueous phase, and
   c. the boiling temperature of the organic solvent should be low with regard to further processing of the organic detergent-containing phase by distillation. The second point especially should be taken into consideration when optimizing this recycling process for different proteins.
9. The operating conditions for the distillation process are not optimized and the nonionic surfactant solution was used without additional purification for further

protein extraction from whole fermentation broth. With this simple approach, we could use the nonionic surfactant solution for three extraction experiments with nearly constant product yields in the coazervate phase. Also, after the second recycle, the phase behavior of the surfactant solution was identical to that of the original solution. However, the effect of residual materials (e.g., pigments) enriched in the recycled detergent solution on the extraction performance and the quality of the product needs further investigation.

10. After successful solubilization, membrane proteins can be handled like hydrophilic proteins for further purification by chromatographic methods. Two major aspects must be taken into consideration:
    a. To avoid aggregation and precipitation phenomena during the chromatography process all buffers should contain small amounts of detergent or mild organic solvents to enhance protein solubility, and
    b. the chromatographic resolution performance can be reduced in comparison for water-soluble products because of microheterogeneity of the product resulting from the solubilization process.

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28

Use of Charged PEG and Dextran Derivatives for Biomolecule Partitioning

Göte Johansson

1. Introduction

The partitioning of biopolymers and bioparticles between the two phases of polymer–polymer aqueous two-phase systems depends on the kind of electrolytes present in the system. This is a consequence of the ionic charge of the partitioned material and the difference in affinity of cation and anion of the electrolyte for the phases. One of the ions can be given a very high affinity for a phase by attaching the ion to the polymer concentrated in this phase. In the case of polyethylene glycol (PEG)-dextran systems, PEG has been used as the carrier of ion charge ***(1)***. This molecule has two functional groups—the two terminal hydroxyl groups—that can be used for introducing the charged elements. A number of ways to synthesize such derivatives has been published ***(2,3)***. If all PEG in a system carries two ionic groups per molecule, the phase-forming properties of the PEG are reduced and the phase diagram is considerably changed ***(4)***. By introducing relatively high concentration of salt (i.e., 100 m*M* NaCl) in the system, this effect can be eliminated, but then the effect of the modified PEG on the partitioning of biomaterials will also be strongly reduced. To get the optimal steering effect, monosubstituted PEG should be used; only a part of the PEG in the system normally has to be in modified form, and the salt should have an optimal concentration (2–10 m*M*) ***(5,6)***.

A charged PEG derivative makes the partitioning of proteins very pH-dependent, which can be used for adjustment of their partitioning in a most favorable way ***(6–8)***. Also, the partitioning of bioparticles (such as cells) is affected by charged PEG, but in a much more sensitive way ***(9)***. The comparison of the partition, as function of pH, in two systems containing trimethylamino-PEG (TMA-PEG) and PEG-sulfonate (S-PEG), respectively, gives information of

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the net charges and isoelectric points of proteins ***(10)*** as well as of particles ***(11–13)***. Furthermore, the charged PEGs have been used in connection with studies of weak enzyme–enzyme interactions by counter-current distribution using the aqueous two-phase systems ***(14)***.

Proteins, as well as particles, have been partitioned between three phases of PEG-Ficoll-dextran systems where PEG is concentrated in the top phase, Ficoll in the middle phase, and dextran in the bottom phase ***(15,16)***. Both the partitioning between top and middle phase, as well as between middle and bottom phase, are influenced by charged PEG. Beside the PEG derivatives, other heavily charged polymers (polyelectrolytes) may also be added in small amount to effect the partitioning of biomaterials. The partitioning of the polyelectrolytes can be effected by low concentrations of salts in the system and the polyelectrolytes will, in turn, determine the partitioning of biopolymers ***(17)***.

This chapter describes the use of charged PEG and dextran, respectively, for partitioning of proteins and particles. Synthesis of positively charged TMA-PEG and negatively charged S-PEG is also dealt with ***(1)***.

2. Materials

2.1. Synthesis of Bromo-PEG

1. PEG 8000 (Union Carbide), M_r = 8000.
2. Toluene (analytical reagent quality).
3. Thionyl bromide (Fluka, **Use care**; *see* **Note 1**).
4. Triethylamine, anhydrous.
5. Active carbon.

2.2. Synthesis of TMA-PEG

1. Absolute ethanol.
2. Trimethylamine (gas or in alcohol solution, *see* **Note 2**).
3. Nitrogen.

2.3. Synthesis of S-PEG

1. Water-ethanol mixture (5:1 v/v).
2. Sodium sulphite (analytical grade).
3. 5 *M* Phosphoric acid.
4. Toluene.
5. Absolute ethanol.

2.4. Partitioning in Two-Phase Systems with Charged PEG

1. 40% (w/w) PEG 8000 (Union Carbide) solution: Dissolve 40 g of PEG 8000 in 60 g of water at room temperature.
2. 20% (w/w) Dextran T500 (Pharmacia) solution: Layer 21 g of Dextran T500 powder over 79 g of water in a 250-mL Erlenmeyer flask and heat the mixture,

with occasional shaking, to gentle boiling. When cooled down, the exact concentration may be determined by weighing out 4 g portions on an analytical balance (accurate weight = m) in 25 mL volumetric flasks and dilute with water to the mark. The dextran concentration (c_{Dx}) in% w/w is determined polarimetrically (*see* **Chapter 3**).

3. 40% (w/w) TMA-PEG 8000 solution (as prepared in **Subheading 3.2.**).
4. 40% (w/w) S-PEG 8000 solution (as prepared in **Subheading 3.3.**).
5. 50 m*M* sodium phosphate buffer, pH 7.0: The concentration is based on phosphorous. Mix 42 mL of 50 m*M* NaH_2PO_4 and 58 mL of 50 m*M* Na_2HPO_4.
6. 100 m*M* Tris-HCl buffer, pH 6.5: The concentration is based on Tris. Mix 50 mL of 0.2 *M* Tris and 50 mL of 0.2 *M* hydrochloric acid.
7. 2 *M* NaOH.
8. 2 *M* phosphoric acid.
9. 2 *M* Tris.
10. 2 *M* HCl.
11. Coomassie Brilliant Blue G (100 mg/L) in 8.5% phosphoric acid and containing 5% ethanol (Bradford reagent; *see* **Note 3**).

2.5. Use of Highly Charged Dextran to Direct Partitioning of Proteins

1. 40% (w/w) PEG 8000 solution (*see* **Subheading 2.4., item 1**).
2. 20% (w/w) Dextran T500 solution (*see* **Subheading 2.4., item 2**).
3. 3% (w/w) diethyl aminoethyl (DEAE)-dextran (M_r = 500,000) (Pharmacia).
4. 1 *M* potassium thiocyanate (KSCN).
5. 1 *M* KCl.
6. 0.5 *M* K_2SO_4.

3. Methods

3.1. Synthesis of Bromo-PEG

1. Dissolve 25 g of PEG 8000 in 200 mL of warm (50–80°C) toluene in a 500-mL round-bottomed flask.
2. Some solvent (30–40 mL) is distilled off (*see* **Note 4**).
3. Let the PEG solution cool down to 30–40°C. Add 0.9 mL of triethylamine.
4. Add drop-wise under mixing 0.5 g of thionyl bromide in 4 mL toluene (*see* **Note 5**).
5. Heat the mixture to boiling and let it boil gently for 10 min (*see* **Note 6**).
6. Filter the mixture while hot and collect the filtrate.
7. Leave the filtrate overnight at 3–5°C.
8. Collect the precipitate formed (bromo-PEG) by suction filtration and wash the polymer on the filter with 100 mL of cold toluene.
9. Dissolve the precipitate in 250 mL of toluene and allow to crystallize (*see* **Note 7**). If the solution has a dark brown color it may be treated with 2–3 g of active carbon at 40–50°C for 1 h and filtered before crystallization.

3.2. Synthesis of TMA-PEG

1. Dissolve 10 g of bromo-PEG (**Subheading 3.1.**) in 50 mL of absolute ethanol by gentle heating.
2. Add 1 g of trimethylamine dissolved in 10 mL of ice-cold absolute ethanol.
3. Leave the mixture for 1 h at room temperature.
4. Reflux the mixture for 24 h.
5. Remove excess of amine by passing a strong stream of nitrogen gas through the solution.
6. Add absolute ethanol to a total volume of 150 mL and warm if any polymer has precipitated.
7. TMA-PEG crystallizes at 0°C overnight.
8. Collect the polymer by suction filtration at low temperature (0–5°C) and wash with ice-cold absolute ethanol.
9. Recrystallize twice from 150 mL of absolute ethanol.
10. Melt the polymer and remove traces of alcohol by evaporation in vacuum at 100°C in a rotary evaporator.

3.3. Synthesis of S-PEG

1. Dissolve 10 g of bromo-PEG (**Subheading 3.1.**) in 60 mL of water-ethanol mixture (5:1 v/v) by gentle heating.
2. Dissolve 0.8 g of Na_2SO_3 in the aforementioned solution.
3. Keep the solution in a 60°C water bath for 24 h.
4. Cool to room temperature and adjust pH to 6.5–7.0 with 5 *M* phosphoric acid.
5. Remove solvent by evaporation in vacuum at 100°C.
6. Dissolve the remaining polymer in 200 mL of toluene. Distil off 30–40 mL toluene (+water) (*see* **Note 8**).
7. Filter the remaining hot solution of S-PEG to remove precipitated salts (NaBr and Na_2SO_3).
8. Let the S-PEG crystallize overnight at 3°C.
9. Collect the S-PEG by suction filtration.
10. Remove traces of toluene by evaporation in vacuum at 100°C.
11. Recrystallize twice from 150 mL of absolute ethanol.
12. Melt the polymer and remove traces of alcohol by evaporation in vacuum at 100°C.

3.4. Partitioning in Two-Phase Systems with Charged PEG

3.4.1. Partitioning of Proteins by Variation of pH

The dependence of the partition of enzymes and other proteins on pH can be determined by titrating the systems with diluted base and acid and removing small sample systems after each adjustment. The sample can be any soluble protein or protein mixture. The two-phase system is chosen to be used at room temperature, but it works as well in the cold. It contains 7% (w/w) dextran

T500 and 5% (w/w) total PEG 8000, of which half is in the form of TMA-PEG or S-PEG. The partitioning of target protein (e.g., via its activity in case of enzyme) and of total protein are determined at various pH values to find the best combination of pH and type of charged PEG for optimal purification.

1. Weigh out 6.25 g of 40% (w/w) PEG 8000, 6.25 g of 40% (w/w) TMA-PEG 8000, and 35 g of 20% (w/w) Dextran T500 in a 200-mL beaker (*see* **Note 9**).
2. Add 5 mL of 50 m*M* sodium phosphate buffer together with water and protein solution (sample) to a total system weight of 100 g (*see* **Notes 9** and **10**).
3. Mix the system well and divide it into two approximately equal parts in 100-mL beakers (high model).
4. Keep one of the systems mixed with aid of a magnetic stirrer and measure the pH with a glass electrode.
5. Take out a small sample of mixed system, around 2 mL, and transfer it to a centrifuge tube (*see* **Note 11**).
6. Adjust pH of the mixed systems in intervals of 0.4–0.7 units by addition of 2 *M* NaOH and transfer 2 mL sample to centrifuge tubes after each adjustment (*see* **Notes 10** and **11**).
7. Repeat the procedure with the other system, but reduce the pH stepwise by addition of 2 *M* phosphoric acid (*see* **Note 10**).
8. Centrifuge (if necessary) all the tubes with collected systems to get well-separated phases.
9. Analyze both top and bottom phases for the presence of activities of interest (*see* **Note 12**).
10. Analyze both top and bottom phases for the presence of protein (*see* **Notes 3** and **12**) using the Bradford method at 595 nm.
11. Plot the amount of protein and various activities as function of pH (*see* **Note 13**).

3.4.2. Partitioning of Bioparticles

Particles like cells, viruses, cell organelles, and membrane fragments not only partition between the two phases, but also can be accumulated at the interface. Bioparticles are, in most cases, negatively charged at physiological pH values. Because of their charge, their partitioning within the system can be effected already at very low concentrations of charged PEG. By using charged PEG the mean partitioning can be adjusted to a value suitable for fractionation by countercurrent distribution and, at the same time, increasing the selectivity of the separation. The two-phase system (for room temperature) contains 5.5% (w/w) Dextran T500 and 4.0% (w/w) PEG 8000. When the particles in the original system are concentrated to the bottom phase, their affinity for the interface and the upper phase can be successively increased by addition of TMA-PEG. On the other hand, if the particles are accumulated in the upper phase, the partitioning can be directed to the interface and the lower phase by increasing amount of S-PEG ***(9)***.

1. Mix 0.55 g of 20% dextran, 0.20 g of 40% TMA-PEG, and 1.25 g of water (system A) (*see* **Note 14**).
2. Mix 0.55 g of 20% dextran, 0.20 g of 40% S-PEG and 1.25 g of water (system B) (*see* **Note 14**).
3. Make a blank system by mixing 0.55 g of 20% dextran, 0.20 g of 40% PEG, and 1.25 g of water, and let the phases separate.
4. Mix 11.0 g of 20% dextran, 4.0 g of 40% PEG, the sample in a weak buffer (5–25 m*M*, e.g., phosphate or Tris-HCl), and eventually water to a total system weight of 40 g (system M).
5. Transfer 3 mL of the mixed system (system M) to a test-tube and let the phases separate (10–20 min).
6. Check the partitioning of the particles by visual inspection. If the particles are in the bottom phase and partly at the interface, use TMA-PEG for titration (system A). If they are mainly in the top phase use S-PEG (system B).
7. Mix system M (*see* **item 4**) continuously with a magnetic stirrer, add stepwise either system A or system B in 98 mL (=100 mg) portions and withdraw 3 mL samples after each addition (*see* **Note 15**).
8. Mix each of the 3 mL systems and take out immediately a 400 μL sample that is diluted with 2.20 mL of water (or buffer).
9. Allow the remaining phase systems to settle for 15 min.
10. Take out 400 μL samples of top and bottom phase, respectively, and dilute each with 2.20 mL of water (or buffer).
11. Measure the apparent absorbance at 400 nm using diluted mixed blank system and diluted separated phases of this system, respectively, as blanks.
12. Calculate the percentage of particles recovered in top and bottom phase, respectively. The difference from total is assumed to be collected at the interface (*see* **Note 16**).
13. Plot the percentage in top phase, bottom phase and at the interface as function of the percent of PEG in charged form (*see* **Notes 15** and **17**).

3.5. Use of Highly Charged Dextran to Direct Partitioning of Proteins

1. Weigh out six systems containing 1.25 g of 40% (w/w) PEG 8000 and 3.5 g of 20% (w/w) Dextran T500 with 0.5 mL of 1 *M* KSCN (two systems), 0.5 mL of 1 *M* KCl (two systems) or 0.5 mL of 0.5 M K_2SO_4 (two systems) in 10-mL centrifuge tubes. Add water to a total weight of 7.00 g and mix the systems.
2. Add 2.5 mL of protein sample in 10 m*M* buffer to each system (*see* **Note 18**).
3. For the pair of systems containing the same salt, one system is fed with 500 μL of water, the other one with 500 μL of 3% (w/w) DEAE-Dextran.
4. Equilibrate the systems by careful mixing, and centrifuge them for 5 min at moderate speed, 1000–2000*g*, in a desktop centrifuge.
5. Analyze both top and bottom phases for the presence of activities of interest and/or protein by using the Bradford method (*see* **Notes 3** and **12**).
6. Compare the partitioning with and without DEAE-Dextran.

4. Notes

1. Thionyl bromide may slowly decompose at room temperature forming bromine; the reagent should therefore be kept at –20°C. Open the bottle carefully when it is still cold to avoid overpressure. Protect hands and eyes.
2. Trimethylamine can be purchased in gas tubes, in glass ampoules, or as an alcoholic solution. It may also be generated from its salt (trimethylamine hydrochloride) by addition of solid base, heating the mixture, and collecting the developed gas in ice-cold ethanol.
3. The Bradford reagent ***(18)*** is obtained by dissolving 100 mg of Coomassie Brilliant Blue G in 50 mL of ethanol and adding 100 mL of 85% phosphoric acid. Dilute to 1 L with water and filter after 1 d. Precipitate may form with time but will sediment (do not shake). For protein determination, 250 μL sample (diluted phase) is mixed with 3 mL of reagent and a blank is prepared in the same way from 250 μL phase of a system not containing protein. The absorbance is measured at 595 nm. A calibration curve using bovine serum albumin (BSA) as standard is used for evaluating the protein concentration.
4. Traces of water are removed by azeotropic distillation. The water will otherwise consume part of thionyl bromide by reacting with it.
5. By keeping the reaction mixture under nitrogen atmosphere, the side reactions, which give dark-colored byproducts, will be considerably reduced.
6. Reflux under exclusion of moisture by using a drying tube with blue silica on the top is suggested.
7. The filter cake of crystallized polymer is transferred from the filter to an open bowl and the traces of toluene are allowed to evaporate in the hood. Divide the cake into smaller pieces with aid of a glass rod while drying to help the evaporation process.
8. Traces of water are removed by azeotropic distillation.
9. The same experiment can be carried out with S-PEG instead of TMA-PEG.
10. When S-PEG is used the phosphate buffer is replaced by a Tris-HCl buffer and pH is adjusted upwards with 2 *M* Tris and downwards with 2 *M* HCl. If the protein solution contains enough buffer, no extra buffer has to be added. Instead, this space (5 mL) can be used to introduce more protein solution or it has to be filled with water.
11. The system must be well-mixed and the transfer should be quick enough so that the system is not settled in the pipet. No accurate volume is needed. A centrifuge tube of 5 or 10 mL made of clear plastic or glass may be used.
12. Enzyme activities can be measured by adding known volume of phase to the assay solution. Because of the high viscosities, especially of the bottom phase, the content of the pipet should be washed down in the assay solution by sucking it up in the pipet and emptying it back again. The phase often forms a layer at the bottom of the cuvet and the latter should be mixed intensively to get a homogeneous mixture before measuring. For protein measurements, the phases are diluted 3–10 times before addition to the Bradford reagent ***(18)***.
13. The partitioning of some enzymes in a TMA-PEG containing system is shown in **Fig. 1**.

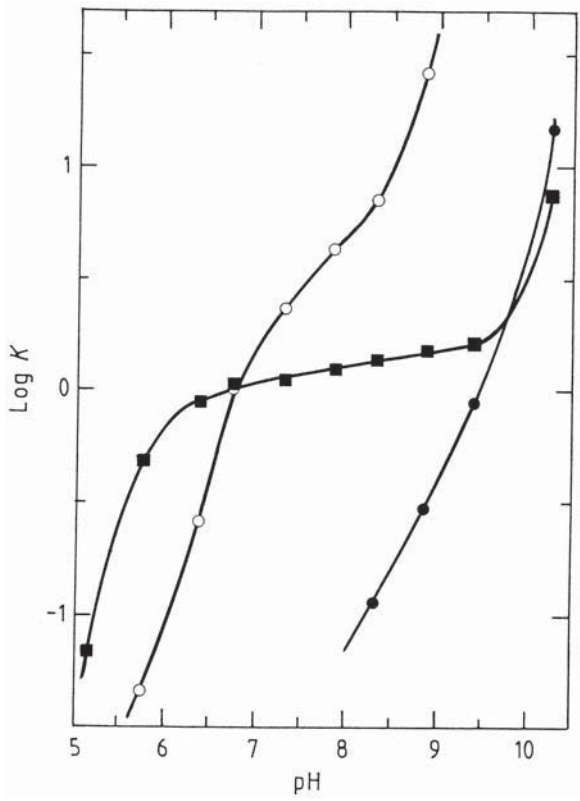

Fig. 1. The dependence of the partitioning of the enzymes fumarase (○), enolase (●), and aspartate-glutamate transferase (■) on pH in a system containing 6.6% (w/w) Dextran T500, 6.6% (w/w) TMA-PEG 8000, and 1.5 m*M* sodium phosphate buffer together with an extract of beef heart (12.5% w/w). Temperature, 22°C. (Adapted with permission from **ref. *5***.)

14. The system may eventually be just one phase depending on the degree of substitution of the PEG. This is owing to the electric repulsion of the charged PEG molecules. Because only a fraction of the PEG in the final systems is replaced, this will not markedly influence the phase formation properties.
15. The charged PEG will be 0.27, 0.56, 0.88, 1.23, 1.62, 2.07, 2.57, 3.17, 3.90, and 4.82% of total PEG, respectively.
16. If a linear relation between the absorbance and the concentration of particles is assumed, the percent of material in the top phase (m_T), in the bottom phase (m_B), and at the interface (m_I) can be calculated from the absorbance of the diluted mixed system (A_{mix}), top phase (A_T), and bottom phase (A_B). The volume ratio, top phase/bottom phase = V_r, has also to be known.

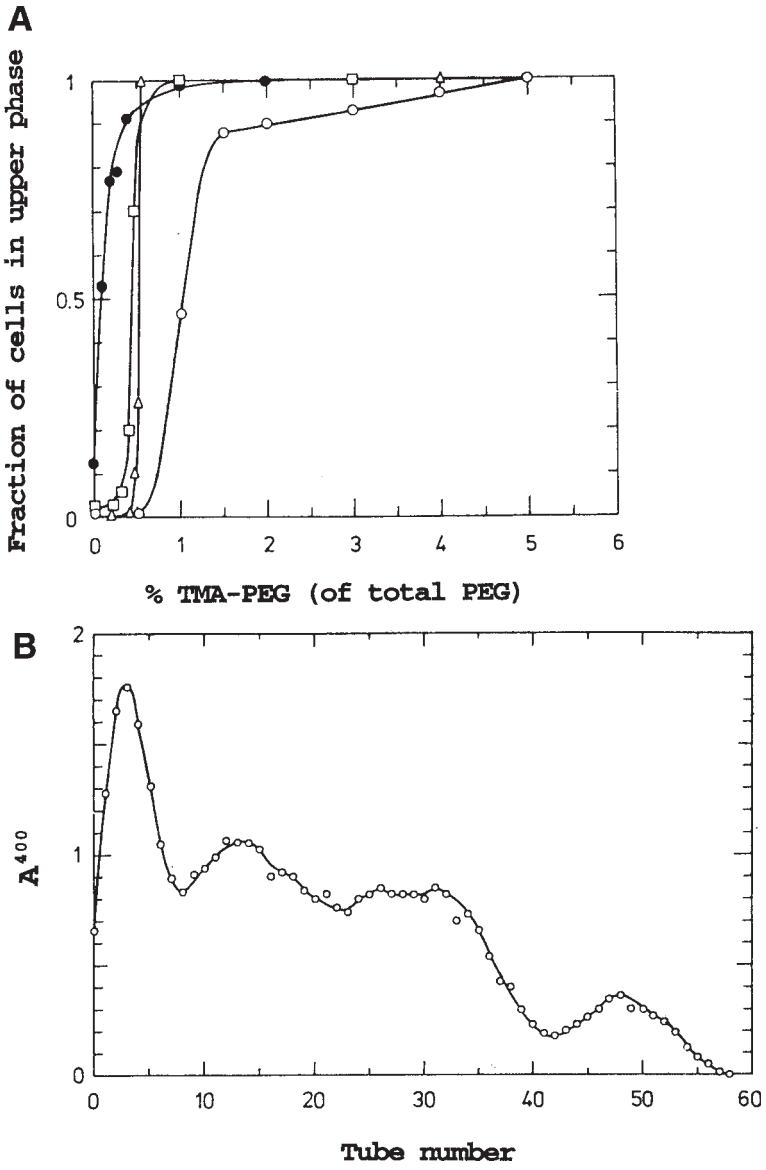

Fig. 2. **(A)** The dependence of the partitioning of *Rhodotorula glutinis* (●), *E. coli* (□), *Sarcina lutea* (Δ), and *Saccharomyces cerevisiae* (○) on the concentration of TMA-PEG in the system expressed as percent of total PEG. System composition: 8.0% (w/w) Dextran T500, 8.0% (w/w) PEG 3400 including TMA-PEG, 5 m*M* potassium phosphate buffer, pH 6.8, and particles (5 g wet weight/L). Temperature, 22°C. **(B)** Countercurrent distribution of *S. cerevisiae* in the same system with 1.1% TMA-PEG (of total PEG). (Adapted from **ref. *9***.)

$m_T = 100 \cdot A_T.V_r/(A_{mix} \cdot [1 + V_r])$; $m_B = 100 \cdot A_B/(A_{mix}.[1 + V_r])$; $m_I = 100\text{-}m_T\text{-}m_B$.

17. The distribution of some micro-organisms in systems with increasing amount of TMA-PEG is shown in **Fig. 2A**. In **Fig. 2B** the heterogeneity of the commercial baker's yeast is demonstrated by counter-current distribution in the system, where 50% of the yeast cells are in the upper phase.
18. The pH value of buffer should be such that the protein(s) have a net negative charge, pH 8.5–10.

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Affinity Partitioning Using Poly(ethylene glycol) with Covalently Coupled Hydrophobic Groups

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1. Introduction

Many biological processes, such as regulation, catalysis, and complex formation, depend on the capacity of biopolymers to interact with apolar groups, i.e., their surface or "effective" hydrophobicity ***(1)***. The determination of surface hydrophobicity is therefore important for understanding the mechanism of interaction of a biopolymer with its ligands. The determination of surface hydrophobicity is also important for designing a method for the purification or the concentration of biopolymers, especially proteins.

This chapter describes the usefulness of affinity partitioning in aqueous two-phase systems containing Dextran and Poly(ethylene glycol) (PEG), where a fraction of the latter is replaced by PEG to which hydrophobic groups have been covalently coupled, for the aforementioned purposes ***(2–4)***. Comparison of affinity partitioning in the presence of PEG carrying a hydrophobic group with that in the presence of PEG carrying the same group but unsaturated or with a terminal-charged (e.g., COO^- or NH_3^+) or polar (e.g., OH) group, permits further characterization of the binding site ***(5)***. This chapter also describes the use of hydrophobic affinity data for the characterization of conformational changes in proteins ***(4,6,7)***.

Shanbhag and Axelsson ***(2)*** have described the theoretical basis of hydrophobic affinity partitioning. In effect, one measures the partition coefficient (K') in the presence and (K) in the absence of the PEG-coupled hydrophobic ligand (PEG-L_H). The respective partition coefficient is given by

$$\log K' = \log K'_0 + \gamma' Z \quad (1)$$

$$\log K = \log K_0 + \gamma Z \quad (2)$$

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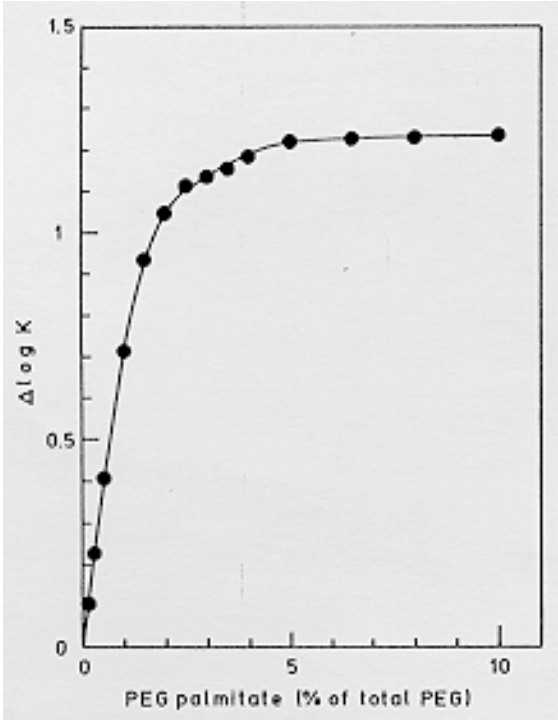

Fig. 1. Increase in Δ log *K* of human serum albumin as a function of the concentration of PEG-$(CH_2)_{15}CH_3$ in the two-phase system at 23°C. The system was composed of 4% Dextran T500, 4% PEG 8000 (including PEG 8000-$(CH_2)_{15}CH_3$), 100 m*M* KCl, 10 m*M* potassium-phosphate buffer, pH 7.1, and 2 mg human serum albumin per g system. (Reprinted from ***ref. 12*** with the kind permission from Elsevier Science, Amsterdam.)

where K'_0 and K_0 are the charge (*Z*)-independent values of the partition coefficients, in the presence and absence of PEG-L_H, respectively, γ' and γ are the corresponding values of the interfacial potential in the two-phase system, and *Z* is the net charge of the partitioned molecule. At a constant pH, *Z* is constant and if γ is not affected by the presence of PEG-L_H i.e., γ' = γ (a plausible assumption), the difference, Δ log *K*, between the partition coefficient in the presence respectively the absence of PEG-L_H , is given by

$$\Delta \log K = \log K'_0 - \log K_0 \quad (3)$$

The difference, Δ log *K*, is a measure of the affinity of the biopolymer for L_H (if Δ log $K > 0$) or repulsion of the biopolymer by L_H (if Δ log $K < 0$). When Δ log $K = 0$ the biopolymer is unaffected by the presence of PEG-L_H in the system. If Δ log *K* is plotted as a function of concentration of PEG-L_H, usually expressed

as a percent of the total PEG in the two-phase system, the plot will resemble a binding curve (as exemplified in **Fig. 1**).

It should be noted, that the partition curve provides only a relative and not an absolute measure of the affinity or the repulsion of a biopolymer for the hydrophobic ligand bound to PEG.

If PEG in PEG-L_H is the dominating part, the latter will be soluble in water in spite of the bound hydrophobic ligand. This is in contrast to the methods depending on the binding of hydrophobic probes, e.g., fluorescence probes, which will have to carry polar or charged groups to be soluble in water. These groups on the probe could affect the binding, which, therefore, might not reflect the true hydrophobicity. Hence, if the aforementioned precautions are taken, hydrophobic affinity partitioning ($\Delta \log K$) should yield a better measure of surface hydrophobicity of a biopolymer, a type of hydrophobic site, and should be useful in comparing different conformational or oligomerization states of the same biopolymer or in comparison between two biopolymers.

Further, the knowledge of the $\Delta \log K$ in a system containing a particular PEG-L_H should facilitate the design of a method for the separation of the biopolymer concerned from a mixture if the $\Delta \log K$ for it is very much different.

The preparative use of hydrophobic affinity partitioning can be exemplified by the specific extraction of serum albumin from human plasma ***(8)***. For a general use of partitioning for preparative purposes, the readers should refer to **refs. *9*** and ***10***.

Examples of the use of hydrophobic affinity partitioning, mostly for characterization of the surface hydrophobicity of proteins are recorded in **Table 1**.

2. Materials

2.1. Synthesis of Fatty Ester of PEG

1. Solvent: Toluene.
2. Derivatives of fatty acids: Chloride or anhydride derivatives of fatty acids to be esterified with PEG, e.g., palmitoyl chloride, (Synthesis grade from Merck, Darmstadt, Germany).
3. PEG: PEG 8000, PEG 3400, or monomethyl PEG (Union Carbide, New York).
4. Catalyst: Triethylamine or dicyclohexyl-carbodiimide (Merck).

2.2. Determination of the Degree of Substitution, DS, of PEG-L_H

1. 0.1 *M* NaOH.
2. 0.1 *M* HCl.
3. PEG-L_H (e.g., as prepared in **Subheading 3.1.**).
4. Iodobromide, NaI and 0.65 *M* Na_2SO_4.

2.3. Determination of Partition Coefficient

1. Stock solution of PEG: Solution of PEG (usually 40%) is prepared by mixing solid PEG, of the required molecular weight, with the necessary amount of dis-

Table 1
Examples of Hydrophobic Affinity Partitioning

Hydrophobic ligand	Protein/s	Measured effect	References
Saturated (C_2–C_{18}) and unsaturated (19:1) acyl groups	Human serum albumin, β-lactoglobulin, CO-hemoglobin, myoglobin, and cytochrome C	Effect of a) chain length of ligand; b) concentration of PEG-L_H on Δ log K	***(2)***
Saturated (C_2–C_{18}) and unsaturated (18:1, 18:2, 18:3) acyl group and benzoate	Serum albumins, lysozyme, ovalbumin, and ribonuclease	Effect of chain length and type of ligand on Δ log K	***(11)***
Saturated (C_2–C_{18}) and unsaturated (18:1, 18:2, 18:3)	Total histone and individual histones of salt on Δ log K	Effect of a) chain length and b) type and concentration	***(3)***
Palmitoyl (C_{16})	β-lactoglobulin	Effect of pH on Δ log K	***(4)***
Palmitoyl (C_{16}) α-lactalbumin	Human serum albumin and with polymer concentration	Variation of Δ log K	***(12)***
Saturated (C_6–C_{18}) acyl group and aromatic groups	Calmodulin on Δ log K	Effect of [Ca^{2+}] and chain length	***(13)***
Saturated (C_6–C_{18}) acyl group	α-lactalbumin	Effect of [Ca^{2+}] and pH on Δ log K	***(14)***
Saturated acyl groups (C_6–C_{18}) without and with terminal COO^- group	Human serum albumin group on Δ log K	Effect of terminal COO^-	***(5)***
Saturated (C_8–C_{18}) acyl group	α_2-macroglobulin and pregnancy zone protein	Dependence of Δ log K on conformation	***(15)***
Palmitoyl (C_{16}) zone protein	α_2-macroglobulin and pregnancy on conformation	Dependence of Δ log K	***(16)***
Palmitoyl (C_{16}) zone protein	α_2-macroglobulin and pregnancy on conformation	Dependence of Δ log K_{acc}	***(17)***

tilled or Milli-Q water by slow stirring with a magnetic stirrer to obtain the required weight fraction of PEG. The correct concentration of PEG in its stock solution is determined by refractometry using solutions with a known concentration of PEG as blanks.

2. Stock solution of PEG- L_H: Solution of PEG-L_H, of the required weight fraction, in water is prepared in the same manner as solution of PEG. The true proportion of PEG-L_H can be calculated from the degree of substitution (DS) of the derivative of PEG.
3. Stock solution of dextran: Dextran powder (Amersham Pharmacia Biotech, Uppsala, Sweden) is added to the water and should be dissolved by slow stirring while simultaneously heating the mixture to boiling. The heating not only speeds up the dissolution of dextran but also helps to partly sterilize the solution. The concentration of dextran in the stock solution is determined by polarimetry using the equation : Dextran $[Dx] = [V \times \alpha]/ 199 \times G$; where $[Dx]$ is the concentration in w/w of dextran, V is the volume in which G is the weight of the Dextran stock solution in water, α is the rotation of the plane-polarized light of 589 nm, and 199 is the specific rotation of Dextran at that wavelength ***(18)***.
4. Stock solutions of salt and buffer: The stock solutions of salt and buffer are prepared in distilled or Milli-Q water so that their molar concentration is 20–50 times their concentration in the two-phase system.
5. Stock solution of the biopolymer to be partitioned: The concentration of the biopolymer in its stock solution will mainly depend on the method available for determination of the concentration of the biopolymer in question, i.e., the more sensitive the method available for the determination of concentration of a given biopolymer is, the lower can its concentration in each of the phases be. Enough biopolymer must be added to the phase system so that its concentration, in the phase with the lower affinity for it, can be measured accurately. The minimum amount of the biopolymer that can be added to the system will not only be determined by its concentration in the start solution, but also the space available in the two-phase system after the other components, i.e., the polymers, the salt and the buffer, have been added.

3. Methods

3.1. Synthesis of Fatty Ester of PEG

Synthesis of PEG-palmitate is described here (*see* **Note 1**).

1. Dissolve 100 g of PEG in 600 mL toluene.
2. Remove moisture by azeotropic distillation by which 100 mL of toluene is removed.
3. Add trimethylamine (5 mL for PEG 3400 or 2.5 mL for PEG 8000).
4. Add palmitoyl chloride (30 mmol for PEG 3400 or 15 mmol for PEG 8000) dropwise under stirring.
5. Reflux the mixture for 30 min.
6. Remove the salt formed by filtration.

7. Add 500 mL toluene to the filtrate and the PEG-palmitate formed is allowed to crystallize at 4°C.
8. The PEG-palmitate is purified by recrystallization 2–3 times from 1 L portions of absolute ethanol.
9. Finally, the traces of ethanol are removed by rotatory evaporation under vacuum.

3.2. Determination of the Degree of Substitution (DS)

$$\text{DS} = 100[\text{wt. bound ligand} /2 (\text{wt. PEG-}L_H - \text{wt. bound ligand})] \times [\text{mol. wt. PEG/mol. wt. bound ligand}]. \quad (4)$$

DS of PEG-L_H denotes per cent of the two terminal hydroxyl groups of PEG substituted by the ligand. Thus, PEG-L_H with DS = 100% means that each PEG molecule carries a ligand molecule at the two termini. The DS should ideally be high, but below 50%. A value of DS higher than 50% could mean that a significant fraction of PEG molecules in PEG-L_H carry a ligand at each end and there is a risk that such a PEG-L_H would bind to two sites on a biopolymer, or crosslink two biopolymer molecules, especially when the biopolymer studied has a strong affinity for the ligand bound to PEG and the pH chosen.

3.2.1. Determination of DS by Saponification of PEG-Esters

1. The PEG-ester is dissolved in 10 mL of 0.1 *M* NaOH or 0.1 *M* HCl (for esters of dibasic acids) and the mixture is incubated at 50°C for 15 h.
2. Dilute the incubated mixture with 40 mL of distilled water and measure the pH.
3. The diluted solution is titrated with 25 m*M* NaOH to the equivalent point for esters with monobasic acids or first to the lower and then to the upper equivalent point for esters with dibasic acids.

3.2.2. Determination of DS when L_H Is an Unsaturated Fatty Acyl Group

1. Dissolve 2 g of PEG-L_H in 15 mL acetic acid.
2. To this solution, add 10 mL of 0.4% solution of iodobromide and incubate the mixture for 30 min in dark at room temperature.
3. Add NaI to the incubated mixture until the concentration of the salt is 2%.
4. The mixture is then rapidly titrated with 0.65 *M* Na_2SO_4. There is a change from colorless to yellow at the end point of titration.
5. Identical titration of PEG, of the same molecular weight as that used in the synthesis of PEG-L_H, recrystallized from absolute ethanol, is performed to serve as the blank.

3.3. Determination of Partition Coefficient

1. Prepare the required two-phase systems by mixing stock solutions of PEG, PEG-L_H, dextran, salt, buffer, and the biopolymer so that the concentration of all the components including that of the total PEG ([PEG] + [PEG-L_H] is identical in all the systems (*see* **Notes 2** and **3**). To obtain an affinity partitioning similar to that depicted in **Fig. 1**, [PEG-L_H] in systems is successively increased with a corresponding decrease in [PEG] (*see* **Note 4**).

2. To obtain a series of extraction curves, e.g., of different conformational states of the same protein, it is preferable to make two-phase mixtures, one with and one without PEG-L_H. The phase mixture should be such that a two-phase system with the highest required concentration of PEG-L_H can be obtained. The phase mixtures are mixed in definite proportions and then the required amount of protein solution is added to obtain the final two-phase systems containing different proportions of PEG-L_H. (*See* **Notes 3** and **5**.)
3. The systems are mixed by about 40 inversions and equilibrated at the temperature at which the partition coefficient is to be measured, if this is different from that at which the components of the systems were mixed.
4. After equilibration, the systems are mixed by inversions as before and the phases separated by centrifugation at around 3000*g* for 5 min or longer at the same temperature. The longer time for centrifugal separation is used if the systems contain large polymers at high concentration or the systems are large, i.e., the distance of separation is large.
5. At this stage, the interface should be examined visually for any signs of precipitate, which would indicate that the biopolymer is not soluble in the system at the concentration or other conditions used.
6. Identical aliquots are then withdrawn from each phase and diluted with water or buffer if necessary. If unequal aliquots are withdrawn from the two phases, one should correct for this inequality before calculating the partition coefficient.
7. Use identically treated aliquot from the corresponding phase from a system not containing the biopolymer as a blank.
8. The aliquots are used for determination of biopolymer concentration (*see* **Note 4**).

4. Notes

1. The synthesis of PEG-esters with fatty or aromatic acids can be achieved by the methods described by Shanbhag and Johansson ***(8)*** or Johansson ***(11)***. General methods for the synthesis of PEG-L_H have been described by Harris ***(19)*** For commercial availability of a particular PEG-L_H please enquire with Shearwater Polymers Inc. (Huntsville, USA).
2. Up to now, the hydrophobic affinity partitioning method has only been applied to the study of proteins. The phase systems used for this purpose have all contained PEG 8000 (Union Carbide) and Dextran T70 or Dextran T500 (Amersham Pharmacia Biotech). The choice of the types of polymers and their concentration depends on the K_0 of the biopolymer desired, which also guide the ionic conditions and the pH chosen. Because the sensitivity of the results is directly proportional to $\Delta \log K$ between systems with and without PEG-L_H, it is best to choose systems in which the biopolymer studied partitions to the lower Dextran-rich phase, i.e., K_0 is very low, in the system not containing PEG-L_H. Two systems with charge-independent protein partitioning are recorded in **Table 2** and they should be preferred when $\Delta \log K$ for a protein is to be measured over a large range of pH values.

Table 2
Systems for Charge-Independent Partitioning

Temp.	Dextran type	Concentration (%)	PEG type	Concentration (%)	Salt type	Concentration (m*M*)
22°C	T70	8	8000	8	K_2SO_4	100
4°C	T500	4	8000	4	Na-acetate	100

As an example, a 2-g phase system containing 8% Dextran T70, 8% PEG 8000 (total), 0.1 *M* K_2SO_4, 10 m*M* sodium phosphate, pH 7.0, 0.2 mg protein, and different fractions of PEG as PEG-L_H is given in **Table 3**.

When the partition curve is obtained for the first time for an unknown biopolymer, the fraction of PEG-L_H is varied in large intervals up to the maximum possible, which is the DS of PEG-L_H. However, if K' reaches a plateau value after only a small fraction of PEG is replaced by PEG-L_H, i.e., when the affinity of the partitioned biopolymer for PEG-L_H is high, it is advisable to change the fraction of PEG-L_H in small steps so as to obtain an accurate extraction curve.

Avoid using PEG-L_H with an unknown DS or old PEG-L_H where the bond between PEG and L_H may be broken, leading to free L_H (whose partition between the phases would be unknown) and also to a change in DS. As consequences of this, wrong conclusions might be drawn from hydrophobic affinity partitioning data as the assumed DS is wrong and the free L_H may also bind to the biopolymer, but with a different affinity than PEG-L_H.

3. In the example given in **Table 4**, a 9-g phase mixture, PM I, without PEG-L_H is prepared by mixing, in required amounts, all the components except the protein solution. A second phase mixture, PM II, is prepared likewise but with 9% of PEG replaced by PEG-L_H. On mixing PM I and PM II in the proportions recorded in **Table 4**, a 0.9-g mixture is obtained, which on addition of 0.1 g protein solution, will yield a 1-g phase system containing the recorded concentrations of PEG-L_H.

   The size of the two-phase system used can be varied depending on the concentration of the stock solutions and sensitivity of the method used for determining the concentration of the biopolymer concerned. Extraction curves have been obtained using only 150–200 μL of phase system ***(20)***.

   Tubes made of a material with an affinity for PEG-L_H for partitioning studies will be a source of serious mistake in the calculation of Δ log *K* at a given [PEG-L_H] owing to adsorption of PEG-L_H to the walls of the tube. When serum albumin was subjected to counter-current distribution (CCD), in systems containing PEG-palmitate, the protein was found in all the chambers of the CCD-plate because the plate was made of acrylate, which adsorbs the PEG-derivative. For partition work in systems containing PEG-L_H, it is advisable to use glass or tubes with a surface with no affinity for PEG-L_H.

4. For the determination of concentration, one can use absorbance at a given wavelength, enzyme activity, enzyme-linked immunosorbent assay (ELISA), or

Table 3
Protocol for Preparation of Two-Phase Systems

Concentration of PEG-LH (%)	Wt. Dextran T70 (20%) (g)	Wt. PEG8000 (40%) (g)	Wt. PEG-LH (DS=40%) (20%) (g)	Wt. K_2SO_4 500 m*M* (g)	Wt. Na-phosphate 100 m*M* pH 7.0 (g)	Vol. protein solution[a] (2 mg mL^{-1}) (mL)
0	0.8	0.400	0	0.4	0.2	0.2
2	0.8	0.384	0.016	0.4	0.2	0.2
5	0.8	0.368	0.032	0.4	0.2	0.2

[a]Here, the protein is dissolved in water. If the protein solution contains salt and buffer, correction must be made for their contribution to the concentration of salt and buffer in the system and the system is made up to the required weight (2 g above) by addition of salt, buffer, and water. For the blank, the protein solution is replaced by an identical amount of the solvent used for making the protein solution.

Table 4
Protocol for Preparation of Two-Phase Systems from Two-Phase Mixtures (PM)

Concentration of PEG-L_H (%)	0	1	2	4	6	8
Weight of PM I (g)	0.9	0.8	0.7	0.5	0.3	0.1
Weight of PM II (g)	–	0.1	0.2	0.4	0.6	0.8

radioactivity of ^{3}H,^{14}C, ^{125}I, ^{32}S-labeled biopolymer or other type of activity to determine the concentration of the biopolymer in the two phases, and to calculate the partition coefficient from the data. The measurement of radioactivity should not be affected by the presence of the synthetic polymers. The sensitivity of measurement of radioactivity can be increased by proper dilution of the phases and the use of appropriate scintillation cocktail, whenever necessary. However, the specific activity of ^{125}I-labeled proteins is observed not to be affected by the presence of synthetic polymers. When protein concentration in the phases is measured by absorbance or ELISA, it has been found necessary to dilute the phases at least five times. Even though the sensitivity of measurement of radioactivity is very high, the partition coefficient should be determined by alternative methods, e.g., the measurement of absorbance or ELISA in order to confirm that the affinity of the biopolymer for PEG-L_H is not affected by labeling. This is also true for other forms of labeling e.g., with fluorescence probes. The determination of protein concentration by the method of Bradford ***(21)*** or the reagent bicinchoninic acid (BCA) (Pierce, USA) yield very high blank values (*see* Chapter 22, **Note 6** for modification of the method).

5. The data can primarily be used to decide:
   a. whether a biopolymer has surface hydrophobicity or not;
   b. the specificity of the hydrophobic interaction; and
   c. if changes in conformation or oligomerization of the biopolymer are accompanied by changes in surface hydrophobicity.

   From the data collected for a number of proteins (see **Fig. 2** and **Table 1**) it can be concluded that:
   a. the partitioning of some, but not all, proteins is affected when PEG-L_H is added to a system ***(2,8)***;

Fig. 2. Effect of the chain length of the fatty acyl group bound to PEG on hydrophobic affinity partitioning, i.e., Δ log *K* at 23°C. **(A)**, human serum albumin (■), β-lactoglobulin (○), carboxyhemoglobin (+), and cytochrome C (Δ). The system contained 7% Dextran T500, 4% PEG 8000, 10% of which was PEG 8000-L_H, 100 m*M* K_2SO_4, and 2 m*M* potassium-phosphate buffer, pH 7.1. **(B)**, Partition of total histone in systems containing PEG-bound fatty-acyl chain. The system was composed of 6% Dextran T70, 6% PEG 8000, 20% of which was PEG 8000-L_H, 100 m*M* potassium fluoride, 2 m*M* potassium phosphate buffer, pH 7.1, and 2 mg total histone per g

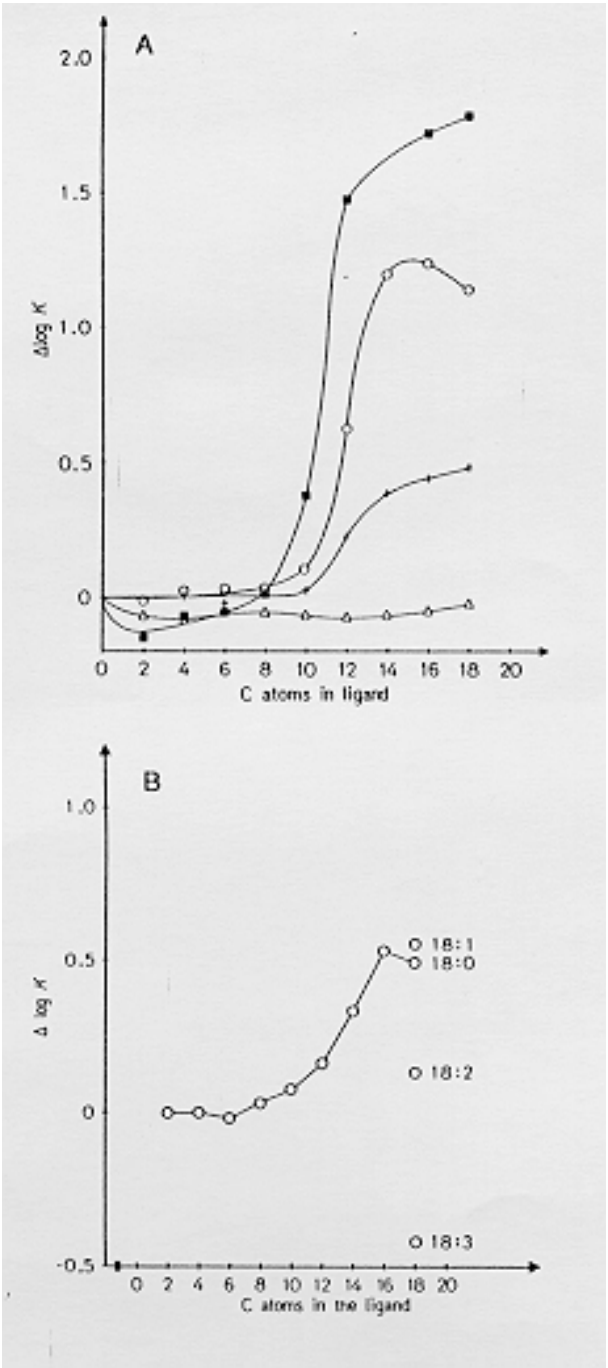

Fig. 2. *(continued)* system. The number of double bonds in ligands containing 18 carbons are indicated in the figure. [**(A)** Reprinted from **ref. *2*** and **(B)** from **ref. *3*** with the kind permission from Elsevier Science, Amsterdam.]

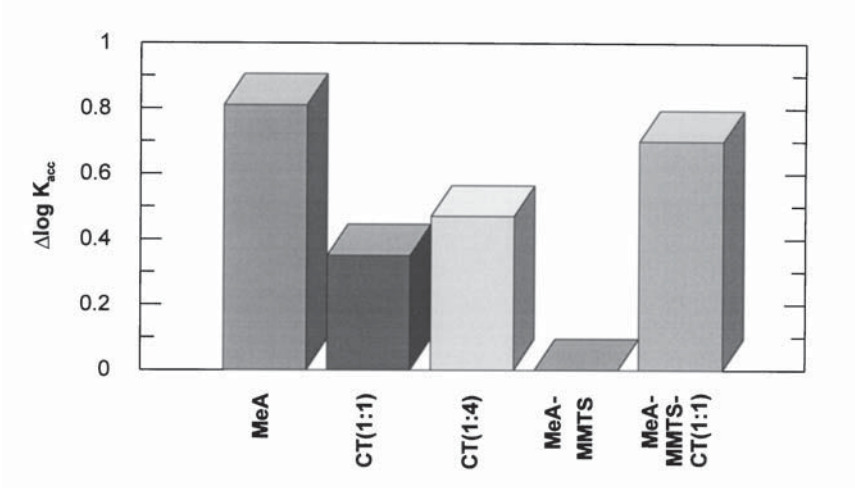

Fig. 3. Δ log K_{acc} as a measure of the conformational changes in α_2M upon modification with methylamine (MeA) or chymotrypsin in a molar ratio of 1:1 (CT 1:1) or 1:4 (CT 1:4) to α_2M, methylamine plus methyl-methanethiosulfonate (MeA + MMTS) which was followed by treatment with chymotrypsin in a molar ratio of 1:1 to α_2M. (Adapted from **ref. *6***, with the kind permission of the author.)

b. for proteins that exhibit affinity for PEG-coupled fatty acyl groups (PEG-L_H) Δlog K > 0 when L_H is longer than at least eight C-atoms ***(2,3,9,15)***;
c. Δ log K for a given protein in the presence of a particular PEG-L_H increases with the concentration of the latter in the system and usually reaches a plateau value at high concentration of PEG-L_H ***(2,9,15,16)***,
d. the method can yield information about the hydrophobic site on the surface of a biopolymer (e.g., the increased affinity of human serum albumin for PEG with $(CH_2)_{15}COOH$ as compared to $(CH_2)_{15}CH_3$ described in **ref. *5***),
e. changes in conformation and oligomerization can be traced when they are accompanied by changes in surface hydrophobicity and therefore Δ log K ***(6,17)***. In this case, a new term Δ log K_{acc} given by

$$\Delta \log K_{acc} = \Delta \log K_{max\ (modified)} - \Delta \log K_{max\ (unmodified)} \quad (5)$$

has been defined by Jensen et al. ***(17)*** where Δ log $K_{max\ (modified)}$ and Δ log $K_{max\ (unmodified)}$ stand for the maximum value of Δ log K (the plateau value) for a protein with a change in conformation or the oligomerization and the unchanged form, respectively, of the same protein. The subscript "acc" stands for the abbreviation of "apparent conformational change." An example of the usefulness of Δ log K_{acc} is given in **Fig. 3**.

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Dye-Ligand Affinity Partitioning of Proteins

Gerhard Kopperschläger and Jürgen Kirchberger

1. Introduction

1.1. Principle of Affinity Partitioning and Theoretical Considerations

Affinity partitioning combines the capability of biological macromolecules to partition in aqueous two-phase systems with the principle of biorecognition. As a rule, a biospecific ligand is bound to one of the phase-forming polymers causing partitioning of the ligand in favor of the phase enriched in that polymer. Partitioning of target protein in an aqueous two-phase system containing the ligand is governed by the formation of ligand–protein complexes, and is favored into the phase containing the ligand–polymer. As a result, a drastic change in the partition coefficients of these proteins can be observed.

Therefore, the general equation describing the partition of a protein ***(1)*** has to be completed by the term $\ln K_{lig}$.

$$\ln K_p = \ln K_{el} + \ln K_{conf} + \ln K_{hphob} + \ln K_{hphil} + \ln K_{lig} + \ldots \quad (1)$$

If the term $\ln K_{lig}$ becomes dominant, the partition of a protein is determined by the ligand–polymer concentration. Under optimum conditions, the protein of choice may be transferred completely from one phase into the other.

In order to quantify affinity partitioning, a general equation has been developed ***(2)***

$$K_C = K_P \cdot \{[1 + (C_L/K_D)_T]^N\}/\{[1 + (C_L/K_D)_B]^N\} \quad (2)$$

where K_C and K_P are the partition coefficients of the ligand-protein complex and the free protein, respectively, $(K_d)_T$ and $(K_d)_B$ are the dissociation constants of the complex in the top (T) and the bottom-phase (B), respectively, $(C_L)_T$ and $(C_L)_B$ are the equilibrium concentrations of the ligand-polymer in the top (T)

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and bottom (B) phase, respectively, and N is the number of ligand-binding sites. Under condition of ligand saturation in both phases **Eq. 3** results:

$$K_C = K_P \cdot (K_L)^N \cdot \{[(K_d)_B]/[(K_d)_T]\}^N \quad (3)$$

where K_L is the partition coefficient of the free ligand–polymer. If the dissociation constants of the ligand molecules in the two phases are equal and independent of each other, **Eq. 3** simplifies according to ***(3)***

$$K_C = K_P \cdot (K_L)^N$$

or in logarithmic form:

$$\Delta\log K_{\max} = \log (K_C)/(K_P) = N \cdot \log K_L \quad (4)$$

However, this approach also assumes equal binding constants for all the binding sites, which is often not the case ***(4)***. For these reasons, it is generally not possible to calculate the number of binding sites for a certain ligand by estimating $\Delta\log K_{max}$ and the partition coefficient of the free ligand according to **Eq. 4**.

Several ligands used in affinity partitioning are biospecific, such as nucleotides, inhibitors, hormones, hormone receptors, antibodies, and antigens. Alternatively, pseudobiospecific ligands such as reactive dyes, metal chelating groups, and hydrophobic and hydrophilic ligands have often been used (**Table 1**).

1.2. Applying Affinity Partitioning in Preparative and Analytical Scale

Affinity partitioning has been employed for single or multistep protein extraction of proteins in aqueous two-phase systems from crude cell material. Usually, the ligand is coupled to polyethylene glycol (PEG) because of the ease of steering the partition of proteins into the opposite phase, composed of dextran or other polymers. The extent of extraction may be quantified by determining the partition coefficient (K) of the target protein in presence and absence of the ligand. The theoretical yield (Y) of a single-step extraction for the top (V_T) and bottom (V_B) phase may be calculated by the following equations:

$$Y_T\,(\%) = 100/[1 + [V_B/(V_T \cdot K)] \qquad Y_B\,(\%) = 100/[(V_T \cdot K)/V_B] \quad (5)$$

Both the K-values in absence and presence of the ligand–polymer and the volume ratio of two phases (V_T/V_B) are determining factors for the net mass transfer of a protein from one phase into the other. The main advantages of affinity partitioning of proteins in preparative scale are:

1. Ease of scaling up the results of analytical approaches into preparative scale;
2. High ligand concentration per volume of polymer solution, providing sufficient concentration of the bound target protein in one phase (more than 5 g protein could be extracted in a system of 1 kg containing 8% [~10 mM] dye-PEG, related to the total PEG) ***(16)***;

Table 1
Affinity Partitioning of Proteins

Affinity ligand	Coupling polymer	Target protein	Reference
NAD^+ NADH	PEG	Dehydrogenases	***(5)***
ATP	PEG	Nucleotide-dependent enzymes	***(6)***
Protein A	PEG	Antibody-labeled cells	***(7)***
Antibodies	PEG	Surface antigens of diverse cells	***(8)***
Receptor-Ligands	PEG	Opiate receptors	***(9)***
Lecithin	PEG	Colipase	***(10)***
Estradiol	PEG	3-Oxosteroid isomerase	***(11)***
p-Aminobenzamidine	PEG	Trypsin	***(12)***
Reactive dyes	PEG, Dextran, UCON	Nucleotide-dependent enzymes	***(13)***
Metal chelates	PEG	Metal ion-binding proteins	***(14)***
Fatty acids	PEG	Plasma proteins	***(15)***

3. Fast adjustment of the equilibrium; and
4. The potential for continuous processing.

The recommended concentration of dye-PEG for extraction of proteins is 4–8%.

In addition, affinity partitioning of proteins has also been elaborated as a highly sensitive method for investigating ligand–protein interactions. The effect of affinity partitioning, expressed as $\Delta\log K$ (i.e., the difference of the partition coefficients of a protein in presence and absence of the ligand presented in logarithmic scale), when plotted vs the concentration of ligand–polymer, follows a saturation function in most cases, from which $\Delta\log K_{max}$ (maximum extraction power) and the half saturation point $0.5 \times \Delta\log K_{max}$ may be calculated. Although $\Delta\log K_{max}$ is somehow related to the number of bindings sites (*see* **Eq. 4**), $0.5 \times \Delta\log K_{max}$ is valid as a relative measure for the affinity of a ligand to the protein.

So far, affinity partitioning is suited for extracting proteins from crude cell material, for screening ligands, for estimating binding forces, and for analyzing competing effectors ***(17)***.

In order to increase binding capacity of the target protein to the ligand, the removal of some metabolites present in the cell extract is recommended. This may be achieved by fractionated precipitation of the proteins by PEG or salts.

1.3. Coupling of Ligands to Phase-Forming Polymers

In principle, all phase-forming polymers are capable of carrying covalently linked affinity ligands. The nucleophilic coupling procedure should be performed

under mild chemical conditions in order to avoid thermic and chemical breakdown of the respective polymer in the course of reaction. Preferentially, PEG has been used as carrier because of its availability in diverse fractions of different molecular weights, its low price, and its simple stoichiometry to bind maximally two ligands per molecule. For a single derivatization of the polymer, monoalkylated PEG can be used. Double-substituted PEG may induce protein precipitation by cross-linking with ligand-binding proteins. In addition, very high viscosity or even gel formation by self-interaction of the dye-ligands is often observed at higher concentration of double-substituted dye-PEG.

In order to increase the reactivity of PEG, the hydroxyl groups may be substituted by more reactive electrophiles, such as bromides or chlorides. Sulfonate esters (tosylate and mesylate) are somewhat more reactive than halides. Reactive epoxide derivatives have also been synthesized by the reaction of PEG with epichlorohydrin. For the attachment of PEG to free amino groups (especially of proteins), cyanuric chloride, carbonyl diimidazole, and the succinimidyl derivatives have been synthesized. In some cases, a spacer was introduced between the polymer and the ligand. For example, N^6-(2-aminoethyl)-NADH-PEG has been prepared by the reaction of carboxylated PEG with N(1)-(2-aminoethyl)-NAD^+ in the presence of carbodiimide, followed by the reduction of the product by sodium dithionite ***(5)***. Adenosine triphosphate (ATP)-PEG was prepared by the modification of PEG to the adiponyldihydrazo-form, which easily reacts with periodate-oxidized ATP ***(6)***.

In principle, the substitution of dextran and other polyalcohols may be performed similarly. However, in comparison with PEG, there are only a few reports dealing with this matrix as ligand carrier. Procedures elaborated for the activation are: glycol oxidation, activation by cyanogen halide, oxirane, carbodiimide, triazine, divinylsulfone, and others ***(13,18)***.

1.4. Reactive Dyes as Pseudo-Biospecific Ligands for Affinity Partitioning of Proteins

The use of reactive dyes as ligands for affinity partitioning has been elaborated for enrichment of enzymes and other proteins, for differentiation of isoenzymes, and for separation of membranes and cells ***(17,19)***. The advantages of pseudo-biospecific dyes, i.e., large variety of different specificities, low costs, ease of coupling and high chemical and biological stability, makes these compounds useful as ligands also for other affinity separation techniques ***(20,21)***. The synthesis of so called "biomimetic" dyes which are designed to fit more precisely to the target protein has made progress in many applications of protein purification ***(22)***.

Reactive dyes of the mono- and dichlorotriazinyl and the vinylsulfonic-acid type may be easily coupled directly under alkaline conditions by substitution to several functional residues of the polymers such as PEG,

dextran, ficoll, polyvinylalcohol, and hydroxypropyl starch ***(13)***. Under optimum conditions (reaction time, temperature, ionic strength), a yield of 10–30% monosubstituted PEG is obtained ***(16)***. Higher yields are achieved by coupling reactive dyes to amino-substituted PEG ***(23)***. If desired, a spacer such as alkylenediamine links the PEG and the dye by reaction of the halide-PEG with alkylenediamine followed by coupling of the reactive dye. Deactivated or nonreactive dyes may be coupled via their amino or hydroxyl groups of the chomophoric part to the respective activated polymer ***(18)***.

This chapter describes the coupling of reactive dye to PEG by two different procedures, and the application of the dye-polymer conjugates for affinity partitioning. The latter is illustrated by examples of partitioning of alkaline phosphatase isoenzymes and phosphofructokinase, respectively.

2. Materials

2.1. Preparation of Dye-Ligand PEG

2.1.1. Direct Coupling Method

1. Polyethylene glycol 6000 (Serva, Heidelberg, Germany).
2. Cibacron Blue F3G-A (Ciba-Geigy, Basel, Switzerland) (*see* **Note 1**).
3. Procion Yellow H-E3G (ICI Organics Division, Blackley, UK) (*see* **Note 1**).
4. LiOH, analytical reagent (AR) grade.
2. NaH_2PO_4, AR grade.
3. KCl, AR grade.
8. Na_2SO_4 (anhydrous), AR grade.
9. Chloroform, AR grade.
9. Butan-1-ol, AR grade.
10. Propan-2-ol, AR grade.
11. Ethyl acetate, AR grade.
12. DEAE-Cellulose (Whatman DE-52, Maidstone, England).
13. Silica plates G60 (Merck, Darmstadt, Germany).
14. Filter paper 595 (Schleicher & Schüll, Dassel, Germany).

2.1.2. Amino-PEG Method

1. PEG 6000 (Serva).
2. Reactive dyes (mono-, dichlorotriazine dyes; Sigma [Deisenhofen, Germany] or ICI).
3. Thionyl chloride (Merck, Darmstadt, Mannheim, Germany).
4. Nitrogen (Merck).
5. Ammonia (25%, w/w).
6. KCl; AR grade.
7. Sephadex G-50 (Pharmacia Biotech Europe, Freiburg, Germany).
8. Glass autoclave 1.51 (Schott, Jena, Germany).

2.2. Preparation of Aqueous Two-Phase Systems

1. 30% (w/w) Dextran T70, 20% (w/w) Dextran T500 (Amersham Pharmacia Biotech, Uppsala, Sweden). The exact concentration has to be determined (*see* Chapter 3).
2. 20% or 40% (w/w) PEG 6000 (Serva).
3. NaH_2PO_4, Na_2HPO_4, AR grade.

2.3. Affinity Partitioning of Human Alkaline Phosphatase

1. 30% (w/w) Dextran T70 stock solution; stable at 4°C for 2 wk (*see* Chapter 3 for preparation).
2. 20% (w/w) PEG 6000 stock solution; stable at 4°C for 4 wk.
3. 20% (w/w) Procion Yellow H-E3G-PEG 6000 (containing 0.05–2.5% dye-PEG related to the total PEG) stock solution; stable at 4°C for 4 wk (*see* **Note 2**).
4. 0.2 *M* Tris-HCl-buffer, pH 7.5, containing 40 m*M* $MgCl_2$.
5. 0.2 *M* potassium phosphate buffer, pH 7.5.
6. $MgCl_2$.
7. Isoenzymes of human alkaline phosphatase (adult liver, intestine, and placenta) (*see* **Note 3**).

2.4. Affinity Partitioning of Phosphofructokinase

1. Soluble extract of baker's yeast (*see* **Note 4**).
2. 20% (w/w) Dextran T500, stable at 4°C for 2 wk.
3. 40% (w/w) Polyethylene glycol 6000, stable at 4°C for 4 wk.
4. 40% (w/w) Cibacron Blue F3G-A-PEG 6000 (containing 5% dye-PEG related to the total PEG), stable at 4°C for 4 wk (*see* **Note 2**).
5. Buffer A: 25 m*M* sodium phosphate, pH 7.1, 0.5 m*M* ethylenediaminetetraacetic acid (EDTA), 5 m*M* 2-mercaptoethanol and 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF) (Serva).
6. 0.5 *M* Sodium phosphate buffer, pH 7.1.
7. 0.5 *M* PMSF, dissolved in dioxane.
8. 2-Mercaptoethanol (Serva).
9. Mixture of solid KH_2PO_4 / K_2HPO_4 (molar ratio 1:1.2).

3. Methods

3.1. Synthesis of Dye-PEG

3.1.1. Direct Coupling Method (28)

1. Dissolve 20 g of PEG 6000 (M_r 6000–7500) in 30 mL of water and heat the solution in a water bath to 85°C.
2. Add 0.2 g of LiOH and 0.6 g of the respective reactive dye and stir the reaction mixture for 15 min.
3. Add again 0.6 g of LiOH and maintain the mixture under stirring for 40 min at 85°C, followed by the addition of 1.5 g of the dye.

4. Stir again for 60 min at 85°C, and add 1.0 g of the dye.
5. Allow the mixture to react at 25°C overnight.
6. Dilute the mixture with 250 mL of water and add solid NaH_2PO_4 until a pH of 7.0 to 7.5 is reached.
7. Add solid KCl to give a final concentration of 1.5 *M*.
8. Extract the reaction mixture five times with 100 mL of chloroform each; dry the pooled chloroform with anhydrous Na_2SO_4 and filter it through a filter paper.
9. Remove the solvent by distillation, and dissolve the sticky polymer in 300 mL of water.
10. Add DEAE-cellulose (Whatman DE-52, about 30 g wet weight) equilibrated at about pH 7.0 and stir the mixture until nearly all colored material is bound.
11. Wash the ion-exchanger several times with water on a Buchner funnel in order to remove unmodified polymer.
12. Desorb the dye-polymer from the DEAE-cellulose by slowly passing a 2-*M* KCl solution through the ion-exchanger bed.
13. Extract the dye-polymer from the KCl-containing solution with chloroform as described above and dry the chloroform.
14. Remove the solvent by evaporation in a rotary evaporator and store the dye- PEG at room temperature under conditions preventing the pickup of moisture (*see* **Note 5**).
15. Check the purity of the dye-PEG by thin-layer chromatography on silica plates 60 with butan-1-ol/propan-2-ol/ethylacetate/water (20:35:10:35) as eluant. Under these conditions the dye- polymer does not migrate in contrast to the free dye which is moving with the eluant. If the dye-PEG is still seen to be contaminated with the uncoupled dye, the chloroform extraction has to be repeated.

3.1.2. Amino-PEG Method ***(23)***

1. Liquefy PEG overnight in an incubator at 70°C, and then make it water-free by rotation in a rotary evaporator at the same temperature.
2. Add a molar excess (50%) of thionyl chloride. Pass dry nitrogen through the vessel during the reaction (*see* **Note 6**).
3. Allow the mixture to react for 8 h at 70°C and then remove the remaining thionyl chloride by evaporation, likewise at 70°C.
4. Dissolve PEG-chloride in a glass autoclave 1.51 (Schott, Jena, Germany) with a large excess of ammonia (25% w/w) such that the vessel is filled to about 75% of volume.
5. Allow the mixture to react at 110°C for 30 h under pressure. Remove excess of ammonia in a rotary evaporator at 70°C.
6. Mix the reactive dye with the amino-PEG in a molar ratio of 2:1 in aqueous solution at concentration as high as possible.
7. Stir the reaction mixture at pH 11.0 and 60°C for 24 h.
8. Pass the reaction mixture over a Sephadex G-50 column using 0.01% potassium chloride as eluant in order to separate the unbound dye from the product.
9. Remove the remaining salt by dialysis.

3.2. Preparation of an Aqueous Two-Phase System

An example of preparation of a two-phase system with a PEG modified ligand is described here.

1. Prepare 10 g of an aqueous two-phase system containing 5% PEG 6000 (including 5% Cibacron-Blue F3G-A-PEG 6000), 7.5% Dextran T500, 50 m*M* sodium phosphate-buffer, pH 7.0. For this, weigh 3.788 g of Dextran T500 (19.8%), 1.250 g PEG 6000 (40%, containing 5% Cibacron Blue F3G-A-PEG, related to the total PEG), 1.000 g of 0.5 *M* sodium phosphate-buffer, pH 7.0 (*see* **Note 7**), and add 3.962 g of water or sample volume to give a total of 10 g.
2. Mix the phases carefully after attaining a constant temperature by inverting the tubes 10–20 times or using a Vortex mixer for 20 s.
3. Separate the two phases within 15 min by gravity or by a quick centrifugation at low speed (2000*g*) for 3–5 min (*see* **Note 8**).

3.3. Affinity Partitioning of Human Alkaline Phosphatase Isoenzymes (27)

3.3.1. Determination of the Partition Coefficient (K), the $\Delta log\ K_{max}$ and the Relative Affinity ($0.5 \times \Delta log\ K_{max}$)

1. Prepare 2 g of aqueous two-phase systems containing 9.75% Dextran T70, 6.5% PEG 6000 (with and without various amounts of Procion Yellow H-E3G-PEG, related to the total PEG), 10 m*M* Tris-HCl buffer, pH 7.5, 2 m*M* $MgCl_2$, and 5 units of the respective alkaline phosphatase isoenzyme and equilibrate the systems at 25°C.
2. Separate the phases by low-speed centrifugation, withdraw 50 µL of sample from each phase (*see* **Note 9**), dilute at least fivefold and determine the enzyme activity (*see* **Note 10**).
3. Calculate the partition coefficients in systems without and with dye-PEG and compare (**Fig. 1** and *see* **Note 11**).
4. Determine Δlog K as a function of different concentrations of dye-PEG (0.05, 0.1, 0.2, 0.5, 1.0, 2.0, and 2.5%, respectively, of the total amount of PEG) in the system.
5. Estimate $\Delta \log K_{max}$ and $0.5 \times \Delta \log K_{max}$ by plotting of $1/\Delta \log K$ vs the reciprocal concentration of dye-ligand PEG (**Table 2** and *see* **Note 12**).

3.3.2. Competitive Effect of Phosphate

1. Prepare 2 g of systems containing 9.75% Dextran T70, 6.5% PEG 6000, (containing 2.5% Procion Yellow H-E3G-PEG, related to the total PEG), 10 m*M* Tris/HCl, pH 7.5, 2 m*M* $MgCl_2$, and 0.5 m*M* and 5 m*M* potassium phosphate.
2. Add 5 units of the respective isoenzyme, mix and let the systems settle at 25°C.
3. Take off 50 µL of sample from both phases (*see* **Note 9**), dilute and determine the enzyme activity (*see* **Note 10**).
4. Calculate the residue Δlog *K* as described (**Table 2** and *see* **Note 12**).

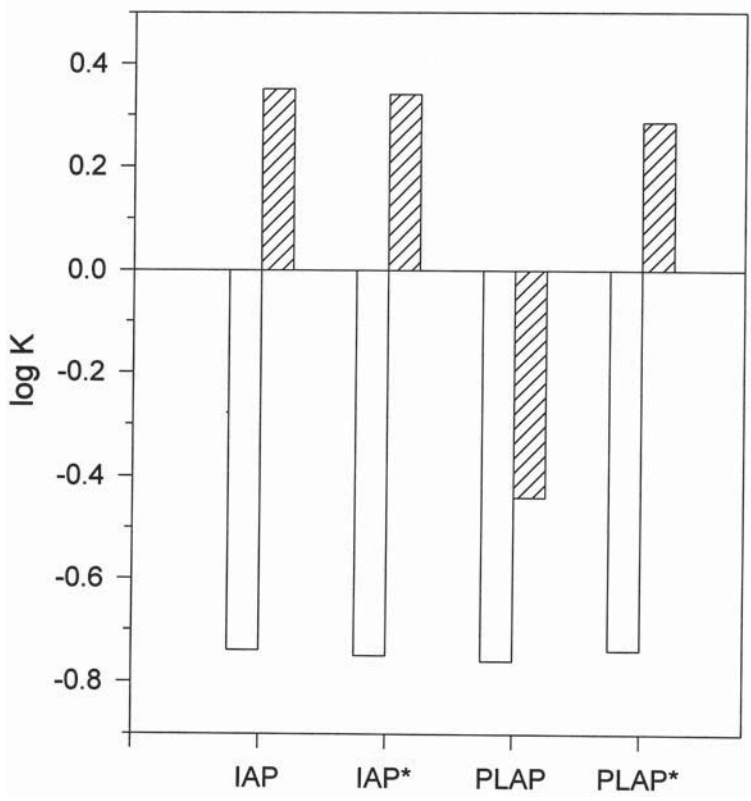

Fig. 1. Dye-ligand affinity partitioning of alkaline phosphatase from human placenta (PLAP) and intestine (IAP). The systems (2 g), containing 9.75% Dextran T70, 6.5% PEG 6000 (partially replaced by 2.5% Procion Yellow H-E3G-PEG), 10 m*M* Tris-HCl-buffer, pH 7.5, 2 m*M* $MgCl_2$, and 5 units of the respective isoenzyme, were equilibrated at 25°C; □, without Procion Yellow H-E3G-PEG; ▨, with 2.5% Procion Yellow H-E3G-PEG; ◆, neuraminidase treated form of the isoenzyme *(27)*.

3.4. Purification of Phosphofructokinase from Baker's Yeast by Affinity Partitioning (30)

1. Add to 120 mL of the soluble fraction of the yeast extract (*see* **Note 4**), 105 g of Dextran T500 solution (19%), 33.4 g of PEG 6000 solution (40%), 6.7 mL of 0.5 *M* sodium phosphate-buffer, pH 7.1, 65 µL of 0.5 mM PMSF solution and 100 µL of 2-mercaptoethanol, and cool the system down to 4°C (*see* **Note 13**).
2. Allow the components to equilibrate by gently mixing for 3 min and separate phases by centrifugation at 2000*g*.
3. Discard the upper phase and wash the lower phase by addition of a fresh upper phase obtained from a phase system containing 7.5% Dextran T500 and 5% PEG 6000 and the same buffer composition as in the first extraction (*see* **Note 14**).

Table 2
Affinity Partitioning of Human Alkaline Phosphatase Isoenzymes

Isoenzymes of human alkaline phosphatase	Parameter		Residual Δ log K (%)[b] at phosphate concentration of	
	Δlog K_{max}	0.5×Δlog K_{max}[a]	0.5 m*M*	5.0 m*M*
intestine	1.08	0.15	93	34
intestine◆	1.10	0.15	92	31
placenta	0.94	1.32	40	0
placenta◆	0.98	0.15	90	25

◆Neuraminidase-treated form of the isoenzyme (*see* **Fig. 1**).
[a]Concentration of dye-PEG (in % of total PEG) (*see* **Fig. 2**).
[b]The Δlog K obtained in the absence of phosphate was taken as 100%.

4. Mix the system again and separate as previously described.
5. Extract phosphofructokinase by mixing the lower phase with 135 mL of a new upper phase containing 8.5% PEG 6000, in which 5% of the total PEG is replaced by Cibacron Blue F3G-A-PEG 6000, and buffer A.
6. Wash the upper blue phase once with 100 mL of the fresh lower phase not containing the dye-ligand (*see* **Note 14**).
7. Separate the upper phase, warm up to room temperature and dissolve 15 g of solid KH_2PO_4 and K_2HPO_4 (molar ratio 1:1.2) in it.
8. Separate the colorless salt phase (approx. 90 mL) from the sticky dye-PEG phase which appears after a couple of minutes (*see* **Note 15**).
9. Perform ion-exchange chromatography and gel filtration to improve the purity of the enzyme (*see* **Note 16**).

4. Notes

1. Reactive dyes are composed of a chromophore and a reactive part, which are linked by auxochromic groups in a variety of chemical combinations. According to the chemical nature of the chromophore, reactive dyes are divided in four main groups:
   a. anthraquinone dyes like Cibacron Blue F3G-A,
   b. azo dyes like Procion Red H-E3B and Procion Yellow H-E3G,
   c. metal complex dyes like Procion Brown MX-5BR, and
   d. phthalocyanine dyes like Procion Green H-4G (**Fig. 2**).

   Although a large variety of compounds have been synthesized, the Cibacron® dyes (Ciba-Geigy) and Procion® dyes (ICI) containing mono- or dichlorosubstituted triazine rings as reacting part and the Remazol® dyes (Hoechst, Frankfurt, Germany), which contain sulphatoethylsulphone groups, have found most application.
2. Stock solutions of dye-PEG are made in the same way as PEG. The molar concentration of the dye-ligand is determined by measuring the absorbance of the dye-PEG solution at pH 7.0 at a wavelength defined by the respective absorbance

coefficient. Various molar absorbance coefficients of dyes are given in the literature ***(24–26)***.

3. Adult human intestinal and liver alkaline phosphatase are prepared from fresh tissues, obtained from autopsies, by homogenization, extraction with n-butanol, acetone precipitation, DEAE-ion-exchange chromatography and immunosorption. For the isoenzyme from placenta, the same scheme is used, but a preparative isoelectric focusing and a gel-permeation chromatography is applied additionally. Specific activity of the purified isoenzymes are: h-PLAP = 533 U/mg, h-IAP = 1400 U/mg and h-LAP = 4055 U/mg. The enzyme treatment with neuraminidase digestion is carried out by incubation of the respective isoenzyme for 12 h at 4°C and pH 6.9. The liberated sialic acid and the neuraminidase are removed by HPLC on a Superose 12-column (Pharmacia) ***(27)***.
4. To prepare the cell-free yeast extract, suspend 1 kg of fresh baker's yeast in 1 L of ice cold 25 m*M* sodium phosphate-buffer, pH 7.1, 0.5 m*M* EDTA, 5 m*M* 2-mercaptoethanol and 0.5 m*M* PMSF (buffer A) and disrupt the cells at 4°C by sonication of the suspension with an ultrasonic disintegrator (Type 250, Schöller & Co.), by shaking the suspension in a Vibrogen Vi4 Cell Mill (E. Bühler, Tübingen, Germany), or by passing through a French press (SLM Instruments, Inc. Urbana, IL). For smaller suspension volumes, shaking with glass beads on a vortex mixer or a Minibead-beater (Biospec Products, Bartlesville, USA) is of advantage. Intermediate cooling steps are necessary during mechanical disruption to reduce heat denaturation.

   Fractional precipitation with PEG: Add solid PEG 6000 (4%, w/w) to the cell homogenate over a period of 15 min with vigorous stirring. Then centrifuge the slurry at 6000*g*, and add again PEG 6000 up to 10%, (w/w) to the supernatant to precipitate the enzyme. After centrifugation at 6000*g* dissolve the pellet in 100 mL of buffer A. The enzyme obtained by fractional precipitation with PEG increases the binding to the dye-PEG, because competing nucleotides present in the yeast extract are removed.
5. Using nonactivated PEG and monochlorotriazinyl dyes, the average yield is 10%, related to the mass of PEG.
6. At all events, water contamination has to be avoided!
7. The buffer should be added in high concentration (almost 10-fold) in order to save volume for the protein sample.
8. The systems without addition of the respective protein may be prepared several days before use, but should be stored at 4°C.
9. Because of the high viscosity of PEG and particularly of dextran solutions, it is difficult to measure exact volumes into the assay cuvet. To minimize errors, the holes of the pipet tips should be enlarged a little bit to facilitate the filling. After carefully cleaning the outer part of the tip, the viscous solution is transferred quantitatively into the assay cuvet by repeatedly rinsing the tip with the assay solution. At least 50 µL of sample volume should be taken from both phases in order to minimize volume inaccuracies. The samples are appropriately diluted for the enzyme assay, if necessary.

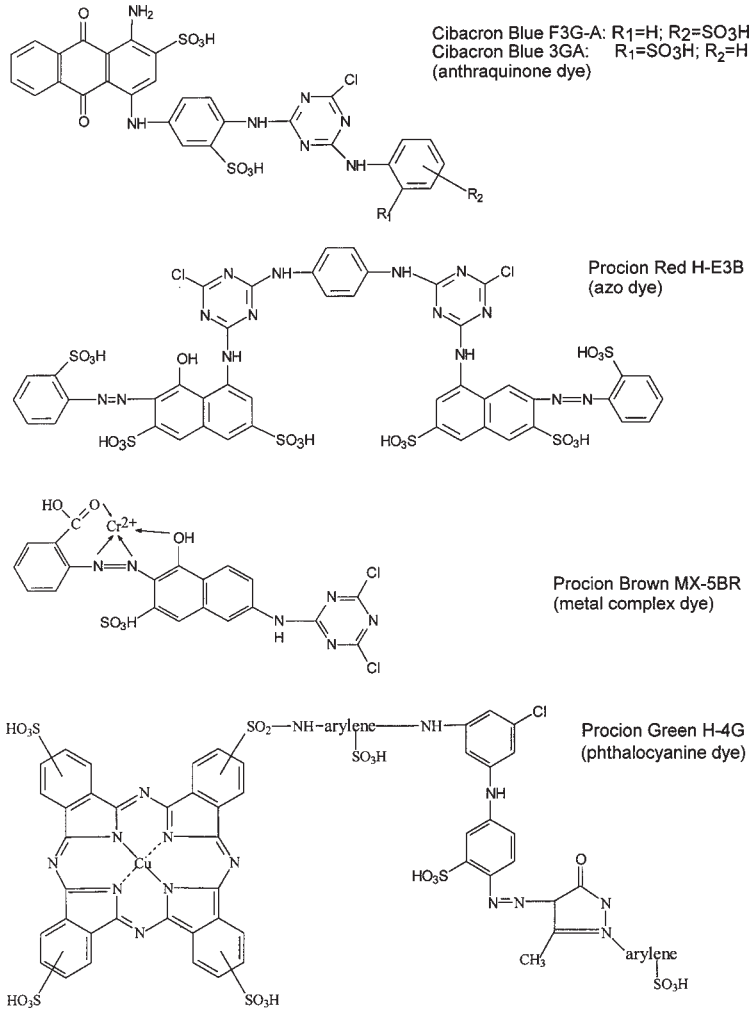

Fig. 2. Structures of some reactive dyes.

10. The activity of alkaline phosphatase is determined by measuring the rate of hydrolysis of *p*-nitrophenylphosphate at 405 nm and 25°C. The samples are preincubated for 15 min in the test buffer (1 *M* diethanolamine, pH 9.8). The enzyme activity is calculated by using the molar absorbance coefficient of 4-nitrophenol as $\varepsilon_{405\ nm} = 18.5 \cdot 10^3 \cdot L \cdot mol^{-1} \cdot cm^{-1}$ ***(29)***.
11. The recovery of the enzyme after affinity partitioning may be checked by determining the enzyme activity in both phases and multiplying the activity/mL with the volume of the respective phase. As a rule, aqueous two-phase systems containing dye-polymer do not affect proteins irreversibly. The presence of the dye

Table 3
Purification of Phosphofructokinase from 1 kg (wet weight) Baker's Yeast by Affinity Partitioning

Purification step	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor (*n*-fold)	Total proteolytic activity (U)
Homogenate	1370	5400	13170	0.4	100	1	13370[a] 3690
PEG precipitation	120	4810	1836	2.6	89	6.4	670
Affinity partition	**120**	**3610**	**153**	**23.6**	**67**	**58**	**34**
DEAE-cellulose batch	40	2520	63	40	47	98	15
Gelfiltration	4	1625	28	58	30	142	1.7

[a]Proteolytic activity in absence of phenylmethylsulfonyl fluoride (PMSF).

may cause a reversible inhibition in some cases, but the increase of substrate or cofactor concentration in the assay buffer and the dilution of the sample abolishes this interference. Most of the polymers used are known to protect proteins. Therefore, affinity partitioning can be performed preferably at room temperature.

12. The difference in partitioning behaviour of the placenta and the intestine isoenzyme of human alkaline phosphatase in the presence of Procion Yellow H-E3G-PEG is only caused by the differences in the carbohydrate moiety (sialic acid content. In contrast to the intestinal form, the placenta isoenzyme contains oligosaccharide residues, which are modified after treatment with neuraminidase. Therefore, the *K*-value is changed only in the case of the placenta enzyme (**Fig. 1**).
13. All partitioning steps are carried out in centrifuge beakers with sealing cups. Phase separation is easily performed by carefully suctioning off the top phase. Upper and lower phase used for the washing steps are prepared from separate blank aqueous two-phase systems.
14. Washing of the lower phase with a fresh upper phase (**Subheading 3.4., step 3**) and the upper blue phase with a fresh lower phase (**Subheading 3.4., step 6**) may improve the purification of the enzyme significantly.
15. Recycling of dye-PEG/PEG mixtures may be performed by chloroform extraction followed by evaporation of the solvent. Separation of the dye-PEG from PEG may be carried out as described in **Subheading 3.1.1.** However, the repeated use of the crude PEG/dye-PEG mixture after forming the PEG/salt phase is recommended for preparative application. In that case, the ionic strength has to be lowered by dialysis or ultrafiltration. Particularly for large scale processes, recycling of PEG may reduce the total process costs ***(31,32)***.
16. Dialyze the salt phase of the PEG/salt system in the cold against buffer A and adsorb the enzyme on 10 g of (wet weight) activated DEAE-cellulose. After washing, desorb the enzyme by treating the matrix with 25 mL of buffer A containing 200 m*M* KCl and 1 m*M* fructose 6-phosphate. Collect the enzyme containing fractions and precipitate the enzyme with ammonium sulphate (80% saturation at 4°C).

    Next, dialyze the dissolved enzyme against buffer A containing 0.5 *M* ammonium sulphate and 1 m*M* fructose 6-phosphate and pass the sample through a Sepharose CL -6B column. Collect the enzyme containing fractions and concentrate by precipitation with ammonium sulphate. A typical purification protocol is presented in **Table 3**.

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Metal Affinity Protein Partitioning

Roberto Z. Guzmán and Javier E. García

1. Introduction

The partitioning in aqueous two-phase systems (ATPS) has become an established and well-known method for the separation and purification of biological materials ***(1–3)***. In order to increase the selectivity and effectiveness of the separation, a ligand that partitions favorably into one of the phases and with affinity for the material of interest is introduced into the system. Thus, once binding occurs, the desired material preferentially distributes into the polymer-ligand rich phase. In general, the affinity ligand is attached covalently to one of the phase forming polymers, which ensures its primary distribution to one phase of the system. In affinity partitioning, ligands attached to poly(ethylene glycol) (PEG) have been widely described and used extensively in the separation and purification of proteins and cells, derivatives include PEG-linked dyes (***4***; Chapter 30), PEG-linked antibodies ***(5)*** and PEG-linked long-chained fatty acids (***6***; Chapter 29). Similar derivatives for dextran (Dx) and procedures to prepare new affinity ligands are described by Harris ***(7)*** and Harris and Yalpani ***(8)***.

Metal affinity partitioning (MAP), in a similar fashion, has been developed as an alternative approach to increase the selectivity of separation by incorporating chelated transition metal ions covalently bound to PEG as affinity ligands ***(9–11)***. In this method the affinity interaction depends on the chelated metal ions with particular accessible amino acids (metal-coordinating ligands) on the protein surface.

This partitioning method evolved from the concept of immobilized metal ion affinity chromatography (IMAC), a technique first introduced by Porath ***(12)***, who used immobilized metal ions chelated to insoluble chromatographic matrices to specifically fractionate serum proteins. Resembling the IMAC separation scheme, metal ion chelators have been attached to water soluble poly-

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mers such as PEG and used as specific ligands for separation of proteins ***(9–11)*** and cells ***(13–15)***. Typical two-phase systems for protein separations include aqueous solutions of PEG and dextran or PEG and a salt such as sodium sulfate or sodium carbonate. When a metal-chelated-PEG is added to the system containing the protein mixture, proteins with affinity for the chelated metal interact and partition preferentially in the PEG-rich phase. In these systems, the partition coefficients *(K)* of proteins which contain surface accessible amino acids, particularly histidine residues, can significantly increase with the addition of a relatively small amount (1–2%) of the metal-chelated polymer derivative.

Some of the chelating-PEG derivatives used in protein partitioning include iminodiacetic acid (IDA)-PEG, the L- and D-isomers of aspartic acid (Asp)-PEG, the L- and D-isomers of methionine (Met)-PEG ***(16)***, as well as derivatives of tris-carboxymethylated ethylxene diamine (TED), and carboxymethylated (tris[2-aminoethyl]amine) (Cm-TREN) ***(17)***. The binding properties of these chelating-PEG ligands are based on the chelating effect that some of their individual atoms display by acting as electron donors. Oxygen and nitrogen atoms in these molecules form coordination bonds with the metal ion in solution producing a metal chelate. Chelates are much more stable than a metal complex due to the loss in free energy when a ring is formed. The IDA ligand is tridentate and can form a double five-membered ring chelate with hexacoordinate metal ions, while TED (tris-carboxymethylated ethylene diamine) is pentadentate and capable of forming four five-membered rings. These ligands work better with transition metal ions, which have several possible coordination sites and act as electron acceptors in the presence of this type of derivatives.

The most commonly used chelating-PEG derivative by far has been IDA-PEG complexed with first row transition metal ions such as Cu(II), Co(II), Zn(II) and Ni(II), particularly with copper. Other metal ions for example, Fe(III) which have affinity for phosphoryl groups, have been studied in the partitioning of phosphorylated proteins in PEG-Dx two-phase systems ***(10)***. A significant observation in MAP is the fact that, unlike many affinity interactions which are disrupted in high concentrations of salts, metal ion coordination in aqueous solutions is promoted by phase-forming salts. As a consequence, the partitioning of proteins with Cu(II)IDA-PEG appears to be more effective in PEG-salt compared with PEG-Dx systems ***(18,19)***. Some of the most typical PEG-salt systems are prepared using sodium, magnesium and ammonium sulfate, potassium phosphate and sodium carbonate salts.

Metal affinity protein partitioning has become a very useful and versatile method for protein fractionation and characterization. However, certain requirements have to be considered in order to take full advantage of this affinity technique. First, an appropriate chemistry must be developed for the synthesis

of soluble chelating-polymers. Second, one has to consider the relevance of the protein surface topography on their recognition by metal chelates. It is now well established that accessible histidine residues serve as predominant metal binding sites. Even in the case of a protein from different species (e.g., isoenzymes) binding to the chelating-derivatives is a function of their primary structure with respect to the content and distribution of histidine residues ***(19)***. In addition, the microenvironment of the electron donors on the surface protein may result in a variable mechanism of protein recognition by the metal ligand ***(12)***. By choosing an appropriate chelating-polymer and metal ion in a given aqueous two-phase system, a high partition coefficient *(K)* for the protein of interest or degree of purification can be achieved. The methodology described here is focused on the metal affinity protein partitioning in PEG-salt systems.

2. Materials

1. PEG for phase formation. Poly(ethylene glycol) 8000 (OH-PEG-OH) (from Sigma Chemical, St. Louis, MO).
2. PEG for synthesis of chelating derivatives (*see* **Note 1**). Methoxy poly(ethylene glycol) 5000 (M-PEG-OH) (Sigma).
3. Salts for phase formation and buffers. Anhydrous sodium sulfate; sodium and potassium phosphate monobasic and dibasic; sodium acetate (J.T. Baker Chemicals, Phillipsburg, NJ).
4. Buffer solutions. 0.1 *M* sodium phosphate buffer, pH 8.0; 50 m*M* sodium acetate buffer, pH 4.0.
5. Proteins. Model proteins used to characterize two-phase partitioning systems with effective chelating-polymers can be purchased from Sigma. Typically, to prepare standard proteins solutions, dissolve 1 mg of protein in 1 g of 0.1 *M* sodium phosphate buffer at various pH values (e.g., pH 8.0). Examples of proteins used in affinity metal partitioning are listed in **Table 1**.
6. Reagents for synthesis of chelating derivatives (IDA-PEG). All reagents should be of analytical grade. Thionyl chloride, bromoacetic acid, ammonium hydroxide, ethylene diamine, iminodiacetic acid, absolute ethanol (Aldrich, Milwaukee, WI). Prepare ammonia solution of 25%.
7. Metal ions. Sulfate salts of Cu(II), Ni(II), Co(II), and Zn(II) and Fe(III) from $FeCl_3$ (Sigma).

3. Methods

3.1. Preparation of Chelating-PEG Derivatives, IDA-PEG, and TED-PEG

1. Preparation of poly(ethylene glycol) chloride (PEG-Cl). Melt 60 g of methoxy (M)-PEG-OH 5000 (*see* **Note 1**) at 65°C. The residual water contained in the MPEG can be removed under vacuum. Add 3 mL of distilled thionyl chloride and rotate the sample for 6 h at 65°C under a nitrogen atmosphere to exclude mois-

Table 1
Suitable Proteins for Characterization of Metal Affinity Partitioning

Protein	Surface histidines
Human hemoglobin	24
Bovine hemoglobin	20
Whale myoglobin	5
Horse myoglobin	4

ture (*see* **Note 2**). After removing the excess of thionyl chloride by evaporation using a rotary evaporator, dissolve the residue in 3 L of absolute ethanol and place the mixture at 4°C in a refrigerator. Under these conditions the PEG-Cl is much less soluble in ethanol and will simply precipitate (crystallize) out of the solution. After filtration to separate the solid (PEG-Cl) from the liquid, place the PEG-Cl to dry in an oven at low temperature. The yield is usually high, from 90 to 95%.

2. Preparation of PEG-amino derivative. Dissolve 50 g of dried PEG-chloride in 150 mL of water. Add 150 mL of ammonia solution (25%). Place the solution in a sealed plastic tube and leave it reacting for 100 h at 55°C in an oven. After evaporation of the solvent and residual ammonia using a rotary evaporator (usually the temperature needed to remove the solvent and reactants is 40–55°C), the amino-PEG derivative is obtained in high yield.
3. Carboxymethylation, preparation of derivative PEG-IDA (*see* **Note 3**). Dissolve 15 g of the amino-PEG in 100 mL of water. Carboxymethylation is carried out by adding 15 g of bromoacetic acid and adjusting the pH to 8.5 (*see* **Note 4**). Allow the reaction to proceed for 12 h at room temperature. Then, add 100 mL of water and extract the PEG-IDA three times with 300 mL of chloroform. The chloroform in the PEG-rich chloroform phase can be removed by distillation or evaporation using a rotary evaporator (usually the temperature needed to remove it completely is 30–40°C). The characterization of IDA-PEG is usually done by determining the nitrogen content by elemental analysis. The yield is usually found between 75–85%.
4. Preparation of tris-carboxymethylated ethylene diamine-poly(ethylene glycol) PEG-TED. The synthesis of the derivative M-PEG-TED or PEG-TED is practically similar to the above procedure, the compound M-PEG-Cl is reacted with ethylene diamine (NH_2-CH_2-CH_2-NH_2) instead of ammonium hydroxide for amination. The group PEG-NH-CH_2-CH_2-NH_2 is similarly carboxylated with bromoacetic acid to give the final compound PEG-TED (*see* **Note 5**).
5. Preparation of metal-chelator-PEG. Metal loading of the ligand is performed by dissolving 6 g of the ligand in 30 mL of 50 m*M* sodium acetate buffer, pH 4.0, containing the desired metal ion as sulfate salt in 15–20 molar excess over the ligand (*see* **Note 4**). Stir the solution for 1 h and extract the metal chelator five

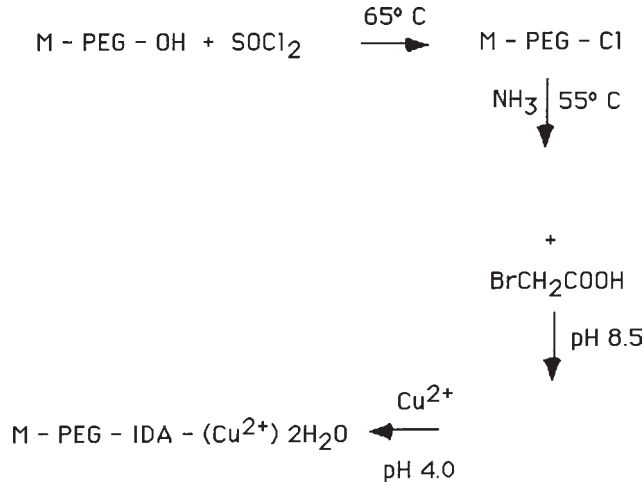

Fig. 1. Schematic presentation of preparation of Cu(II)-IDA-PEG. *See* **Subheading 3.1.** for details on the procedure.

times with chloroform. Remove the chloroform by evaporation. The metal content in the aqueous solution (metal not bound to chelator) is usually determined by atomic absorption using appropriate standards. The general scheme of the reaction steps to prepare Cu(II)-IDA-PEG(5000) is presented in **Fig. 1** (*see* **Note 3**).

3.2. Preparation of Affinity Two-Phase Systems

1. Preparation of PEG-salt two-phase systems. The two-phase systems are prepared on a weight basis from stock solutions of PEG 8000 (OH-PEG-OH) (40%, w/w), and sodium sulfate (16%, w/w). Two-phase systems (4.0 g total weight) are made by combining 1.4 g of the PEG stock solution, 2.0 g of the salt solution, and 0.6 g of a 0.5 mg/mL mixture of protein solution in the desired buffer. For affinity partitioning using PEG-chelate-metal derivatives, replace a portion of the PEG 8000 with the metal chelator as needed (e.g., usually 1–3% w/w of total PEG is replaced for example, by the chelator Cu[II]-IDA-PEG[5000]). The final compositions of the polymer and the salt in this case are 14% PEG and 8% sodium sulfate (*see* **Notes 6** and **7**).
2. PEG-Dx systems. A two-phase systems can be prepared on a weight basis from stock solutions of PEG 5000 or PEG 8000. A two-phase system (4.0 g total weight) consisting of PEG 5000 (40%, w/w), and Dx T500 (13.5%, w/w) is made by combining 0.70 g of the PEG stock solution, 1.30 g of the Dx solution, and 2.0 g of 1 mg/mL protein solution in the desired buffer. For the metal containing systems, replace a portion of the PEG 5000 with the metal chelator (e.g., Cu(II)-IDA-PEG(5000)) as needed, as in the PEG-salt case. The final compositions of both polymers in this case are 7.0% PEG and 4.4% Dx (*see* **Notes 6** and **7**).

3. Once the systems have been prepared, invert them 50 times at room temperature and allow them to reach equilibrium for 15–30 min (*see* **Note 8**). Use a syringe with a large thin needle (in order to avoid contamination as much as possible) to withdraw samples from top and bottom phases, mix them with a known volume of distilled water and analyze them for protein content (*see* **Note 9**).

3.3. Determination of Protein Partition Coefficients

All protein partitioning experiments in these systems are usually carried out at room temperature. The partition coefficient (K_o) for each protein is calculated as the ratio of the concentration in the top and bottom phase (*see* **Note 9**). If measured by light scattering (at 409 or 280 nm), K_o is defined as the ratio of the absorbance of the protein in the top phase to the absorbance of the protein in the bottom phase. The effect of the metal-chelator-PEG in the partition of any protein is expressed as Δlog K which is given by $\Delta \log K = [\ln K_{aff} - \ln K_o]$, where K_{aff} and K_o are the partition coefficient of the species in the presence and absence of the metal-chelator PEG in the system, respectively (*see* **Note 10**).

3.4. Recovery of Proteins Under Study

After the partition experiment, separate the phase containing the complex protein-metal-chelate-PEG (top phase) from the bottom phase. The protein of interest is unbound from the complex by changes in pH or by adding competing ligands or chemical agents (such as EDTA) to dissociate the protein-metal chelate complex (*see* **Note 11**). Once dissociation occurs, to the polymer phase containing the desired protein add high concentrations of salt, enough to form a new two-phase system with the residual PEG phase (e.g., to form a system consisting of PEG (40%, w/w), and salt (16%, w/w). The salt could be the same salt used in the initial two-phase formation (sodium sulfate) or another one (e.g., potassium phosphate), always mantaining a low pH (<5.0). Under these conditions, owing to the absence of affinity binding groups for the protein in the system and to the high ionic strength, the protein of interest will partition preferentially to the salt rich phase.

4. Notes

1. Monomethoxypoly(ethylene glycol) 5000 is one of the few PEG's of this type available commercially. To avoid protein precipitation during extraction, it is necessary to use metal affinity ligands that are essentially monofunctional (M-PEG-OH) or by derivatizing bifunctional PEG (OH-PEG-OH) with a very small amount of IDA.
2. Thionyl chloride and chloroform are highly toxic chemicals. It is necessary to handle these compounds with care under a hood.
3. Alternatively, PEG-IDA can be synthesized by reacting 5 g of the derivative PEG-Cl with 2.1 g of iminodiacetic acid in 50 mL of water and adding 2 g of potassium

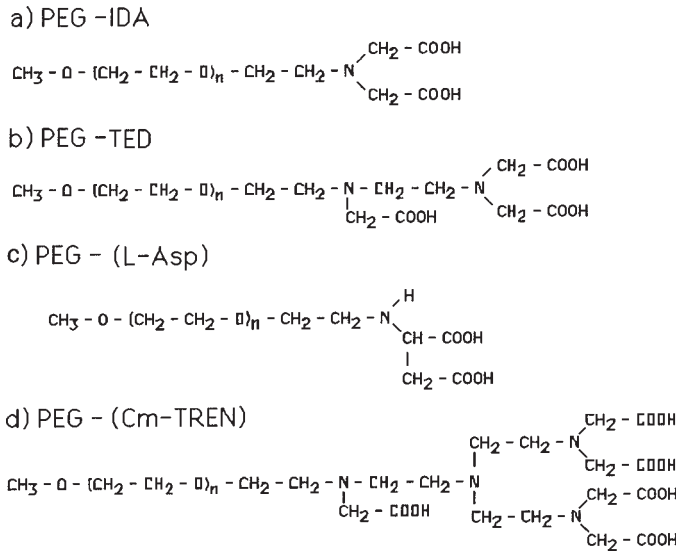

Fig. 2. Structure of various chelating-PEG ligands.

carbonate. The solution is refluxed for 48 h. Then 5–8 g of sodium sulfate are added to the hot reaction mixture, which after cooling down separates into two phases. The PEG phase (top phase) is retained and diluted to 50–100 mL. The PEG solution is dialyzed against 1% sodium bicarbonate, and finally extensively against water for 48–60 h. After lyophilization the final product PEG-IDA is obtained ***(9)***.

4. Check that carboxymethylation and metal loading reactions are carried out at the proper pH at all times so as to maintain the reactivity of the reactants.
5. **Figure 2** shows schematically the structure of several chelating-PEG ligands used in affinity partitioning of proteins.
6. Affinity chelating ligands that are required in small final concentrations can be prepared at high enough concentrated stocks, and added in small aliquots (1–3% of the total PEG phase volume) to the complete phase system, with little effect on the phase composition. In this way a series of systems with constant polymer concentration and different ligand concentrations can be easily prepared. On the other hand, if large quantities of the chelating-PEG ligand are needed, the derivative is substituted on a weight basis for PEG during the preparation of the specific phase system ***(20)***.
7. The effectiveness of partitioning in aqueous phase systems as a separation technique lies in its versatility and its sensitivity to a wide range of features of the partitioned material. Therefore, appropriate phase systems must be chosen. The selection of a useful phase system involves some experimentation. It is important to observe all the operating conditions during the formation of the two-phase systems. Detailed description of phase diagrams with different polymer–polymer

Table 2
Metal Affinity Partitioning of Proteins Using PEG-IDA-Cu(II) in PEG-Salt and PEG-Dextran Aqueous Two-Phase Systems[a]

Protein	PEG-salt ln *(K/Ko)*	PEG-dextran ln *(K/Ko)*	Surface histidines
Horse myoglobin	5.29	0.6	4
Whale myoglobin	7.29	0.7	5
Bovine hemoglobin	7.92	3.3	20
Human hemoglobin	7.67	3.6	24

[a]In the absence *(Ko)* and presence *(K)* of PEG-IDA-Cu(II) derivatives. Composition of PEG-salt two-phase systems: 8.0% sodium sulfate, 14% PEG (8000) (with and without substitution of 1.4% PEG(5000)-IDA-Cu(II)), and 10 m*M* sodium phosphate, pH 7.8 ***(17)***. Composition of PEG-dextran two-phase systems: 4.4% Dextran T500, 7% PEG 8000 (with and without substitution of 1% PEG(8000)-IDA-Cu(II)), 0.1 *M* NaCl, and 10 m*M* sodium phosphate, pH 8.0 ***(9)***.

and polymer–salt systems are given in the literature ***(1,21)***. In metal-affinity separations, the change in protein partitioning coefficients due to the chelating ligand increases with increasing polymer–chelate concentration to a saturation value. Some of the most relevant parameters to consider include pH, temperature, polymer base molecular weight and concentration, etc. In PEG-salt systems it has been observed particularly that the higher the molecular weight of the polymer, the lower its concentration required for phase separation. The procedures described here for the formation of PEG-Salt and PEG-Dx two-phase systems correspond to commonly used two-phase systems.

8. To speed up phase separation, the systems can be centrifuged at 1200–1600*g* during 5–15 min to ensure that phase equilibrium has been reached.
9. Several analytical techniques for protein analysis can be applied to quantify the protein content in each phase. Protein concentrations should be calculated whenever possible, spectrophotometrically. Alternatively protein concentrations can be determined by the biuret method, which is unaffected by the presence of PEG. When using other analytical methods because the polymers may affect certain chemical assays it is convenient to measure the effect of the phases and ligands on the intended assay. In other cases K will be calculated as the ratio of the concentration or radioactivity or any biological activity, e.g., enzyme activity or immuno-assay (ELISA) of a species in the upper phase and the lower phase.
10. Partitioning of histidine rich proteins with PEG-IDA-Cu(II) can be greatly enhanced in PEG-salt compared to PEG-Dx systems. In order to assess the effect of the chelating-PEG (say IDA-PEG [5000]) on the partitioning of the selected proteins, different amounts (e.g., 1%, 2%, etc.) of the polymer forming phase (say PEG [8000]) is substituted with the chelating-polymer. Control partition experiments are carried out using simply the unmodified polymer PEG (5000) instead of the chelating-polymer. Comparison of partitioning results for several model proteins are shown in **Table 2**.

11. At pH values above 5.0, the binding of proteins to Cu(II)-IDA-PEG is strengthened in the presence of salts. At lower pH values salts seem to suppress the binding. Interactions between the chelate metal ion and histidine-containing proteins can be reversed by Lewis acids (H^+), which compete for binding to the protein, alternatively the protein can be displaced by a Lewis base (imidazole, N-acetylhistidine), which competes for coordination to the chelated metal ion. In other cases a strong chelating agent such as EDTA is used to break up the ternary protein-metal ion-IDA complex.

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Recovery of Proteins and Phase Components

Göte Johansson

1. Introduction

Two-phase extraction of protein mixtures leads to one or several protein fractions, which also contain mainly one of the phase-forming polymers. To separate proteins from the phase polymer, a number of methods can be used ***(1)***. If proteins and polymer differ strongly in their respective molecular weights (10 times or more), they can be separated by gel chromatography or ultrafiltration ***(2,3)***. A more common way is to use ion-exchange chromatography ***(1)***. Other methods are those based on electrophoresis ***(4)*** and two-phase techniques ***(1,4,5)***.

When the two-phase systems are applied in large scale, or when valuable ligand-polymers are used, the recovery of phase polymers is of economical interest. For this purpose, polymer-salt two-phase separation is used ***(5)***, as well as systems based on thermoseparating polymers ***(6)***.

This chapter describes the different means that have so far been used for the separation of proteins and the phase polymer. The choice of the method that is made depends on the system being studied. Recovery of the phase components, for recycling, from a polyethylene glycol (PEG)-salt system, which is commonly used for protein extractions on large scale, is also described.

2. Materials

2.1. Ion Exchanger for Protein Recovery

1. Diethyl aminoethyl (DEAE)-Cellulose, e.g., DE52 from Whatman, Maidstone, England (*see* **Note 1**).
2. 1 *M* ammonia.
3. 50 m*M* Tris-HCl buffer, pH 9.0: 500 mL of 0.1 *M* Tris base is mixed with 50 mL of 0.1 *M* HCl, and 450 mL of water giving a pH of 9.0–9.2. Check pH with pH meter or indicator paper.

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4. 5 m*M* Tris-HCl buffer, pH 9.0.
5. 0.5 *M* sodium phosphate buffer, pH 6.0: Mix 90 mL of 0.5 *M* NaH_2PO_4 with 10 mL of 0.5 *M* Na_2HPO_4.

2.2. Phase Partitioning with Salt for Protein and Polymer Recovery

50% sodium phosphate mixture: Mix 20 g of NaH_2PO_4, 30 g of $Na_2HPO_4 \cdot H_2O$, and 50 g of water. Heat the mixture while stirring until a clear solution is obtained. Allow the liquid to attain room temperature without external cooling, which may cause crystallization.

2.3. Phase Partitioning with DEAE Dextran for Protein Recovery

1. Solid NaCl, ground to a fine powder.
2. 1 *M* ammonia.
3. (DEAE)-Dextran, average molecular weight 500,000 (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.4. Phase Partitioning with High PEG Concentration

1. Solid KSCN.
2. 1 *M* ammonia.
3. 20% (w/w) Dextran T500: Layer 4.2 g of Dextran powder (Amersham Pharmacia Biotech) over 15.8 g of water in a 50-mL Erlenmeyer flask and heat the mixture under occasional shaking to gentle boiling. Replace any water lost by evaporation.
4. Solid PEG 8000 (Union Carbide, NY), finely ground.

2.5. Thermoseparating Polymers

1. 20% (w/w) Ucon 50-HB-5100 (Union Carbide).
2. Solid Na_2SO_4.
3. Solid NaCl.

2.6. Recovery of PEG and Salt

1. PEG 8000.
2. Solid KH_2PO_4.
3. Solid K_2HPO_4.

3. Methods

3.1. Ion Exchanger for Protein Recovery

The protein-containing phase is diluted with water (2–5 times), and the pH is adjusted to a value that gives the protein a distinct negative (or eventually positive) net charge. The diluted phase is filtered through a bed of ion-exchangers that will adsorb the protein, but not the nonionic polymer. After

careful washing of the bed with buffer, the protein can be eluted by addition of salt solution of high concentration and/or by changing pH. If the phase contains valuable polymers, like ligand derivatives, the eluted polymer fraction can be recovered and dialyzed to remove salts and other low molecular-weight substances followed by concentration using evaporation.

1. Protein containing phase, 10 mL top or bottom phase, from a PEG-Dextran system is diluted with 50 mLwater (*see* **Note 2**).
2. Adjust pH, with 1 *M* ammonia, to a value of 9.0–9.5 (*see* **Note 3**).
3. Wash around 10 g of DEAE cellulose with 50 m*M* Tris-HCl, pH 9.0, by decanting until the washing liquid has the same pH (*see* **Note 4**).
4. Pack the DEAE-cellulose in a column with a diameter of 1.5–2.5 cm (*see* **Note 5**).
5. Wash the column with 100 mL of 5 m*M* Tris-HCl, pH 9.0.
6. Add the diluted phase to the column and let it pass through.
7. Wash the column with 25 mL of 5 m*M* Tris-HCl, pH 9.0.
8. Elute the proteins with 20 mL of 0.5 *M* sodium phosphate buffer, pH 6.0 (*see* **Note 6**).

3.2. Phase Partitioning with Salt for Protein and Polymer Recovery

This method is used with top phases from PEG-Dextran systems. The recovered top phase is supplied with highly concentrated phosphate solution and a salt-PEG system is formed. Because of the high concentration of PEG in the resulting new top phase the proteins are strongly recovered in the lower salt phase. The high salt concentration also breaks the interactions between a number of affinity ligands (which are used in PEG-bound form) and extracted proteins. The PEG and eventually ligand–PEG are obtained in highly concentrated form in the upper phase. It can often be used several times without further purification.

1. Take 10 g of protein-containing upper phase from a PEG-Dextran system with the composition 8% (w/w) PEG 8000, 10% (w/w) Dextran T500 (*see* **Note 7**).
2. Add 4.2 g of 50% sodium phosphate mixture (*see* **Note 8**).
3. Equilibrate the system by inverting the closed tube about 10 times.
4. Wait for 10 min to let the phases settle or centrifuge at low speed for 1 min.
5. Collect the main part of upper PEG-containing phase (*see* **Note 9**).
6. Collect the main part of the protein-containing salt phase by using a Pasteur pipet.

3.3. Phase Partitioning with DEAE Dextran for Protein Recovery

The protein in a PEG-containing top phase can be recovered by addition of a small amount of the positively charged DEAE-Dextran, which forms a small bottom phase to which negative proteins have high affinity. The salt content has to be adjusted to allow the phase formation.

1. Take 10 mL of protein-containing top phase from a system with the approximate concentration of 6% PEG 8000, 8% (w/w) Dextran T500. Dissolve 60 mg of NaCl in the top phase (*see* **Note 10**).
2. Adjust pH to 9.0–9.5 with 1 *M* NH_3 (*see* **Note 11**).
3. Add 50 mg of solid DEAE-Dextran (*see* **Note 12**).
4. Mix the system well and centrifuge it for 10 min at 2000–3000*g*.
5. Remove the upper PEG-rich phase.
6. The proteins are largely recovered in the small (0.1–0.5 mL) bottom-phase.
7. To remove the bottom phase polymers, dilute the bottom phase 5–10 times, and apply the method described in **Subheading 3.1.** using DEAE-cellulose.

3.4. Phase Partitioning with High PEG Concentration

Proteins can be recovered in good yield from a PEG-rich top phase by addition of a low amount of Dextran and then by increasing the concentration of PEG to a high value (16–20%). The proteins will be excluded from the large top phase because of the high PEG concentration. The excluding effect can be further enhanced by addition of a salt, KSCN, or $KClO_4$, which directs proteins towards the lower phase at high pH values. The PEG in the resulting top phase can be recovered by evaporating the water and dissolving the polymer in toluene and removing any precipitate by filtration followed by evaporation of the solvent. A better choice, however, is to use a thermoseparating ethylene oxide/propylene oxide copolymer instead of PEG (*see* Chap. 26).

1. 100 mL of protein-containing liquid, e.g., top phase from an extraction, is supplied with 1.0 g solid KSCN.
2. pH is adjusted to 8.0 by addition of 1 *M* ammonia.
3. 0.40 g of 20% Dextran T500 is added.
4. 22 g of solid PEG is added in small portions under vigorous mixing by using a magnetic stirrer. The mixing is continued until all the PEG has dissolved (*see* **Note 13**).
5. Centrifuge the obtained two-phase system for at least 10 min at 400–800g_{max}.
6. The proteins should now, to large extent, be recovered in the small bottom phase, 250–300 μL.

3.5. Thermoseparating Polymers

The solubility of copolymers of ethylene oxide and propylene oxide in water decreases strongly at increasing temperatures. The effect can be strengthened by addition of suitable salts to the solution. By using such polymers instead of PEG in the two-phase systems the polymer of the recovered top phase can be excluded from the water by increasing the temperature to form its own phase containing around 40–50% (w/w) of polymer. Proteins, present in the top phase, will remain in the water solution.

1. Make a partition of protein in a system made of 4.5 g of 20% Dextran T500, 3.25 g of 20% Ucon 50-HB-5100, and 2.55 g of protein solution.
2. Mix well to equilibrate the system.
3. Centrifuge the system for 5 min in a desk top centrifuge at moderate speed.
4. Recover the top phase, add 0.08 g of NaCl and 0.20 g Na_2SO_4, and mix until the salts have dissolved.
5. Place the top phase in a water bath at 40°C for 15 min.
6. Withdraw the formed upper water-rich phase, which contains the extracted proteins, with a Pasteur pipet leaving the dense Ucon-containing phase.

3.6. Recovery of PEG and Salt

Aqueous two-phase systems composed of PEG and a salt such as phosphate have been applied in large scale. Therefore, the recovery of the phase components can be of economical interest. Possible ways to recover the salt and PEG from bottom and top phase, respectively, of a PEG-phosphate system are presented below. The phosphate is recovered from the bottom phase by crystallization at low temperature. The top phase is desalted by dialysis or ultrafiltration and the resulting PEG solution is then, if necessary, concentrated by evaporation.

1. Prepare a two-phase system by dissolving 20 g of PEG 8000, 18 g of K_2HPO_4, and 10 g of KH_2PO_4 in 152 g of water in a 250-mL Erlenmeyer flask. Warm the mixture if necessary to dissolve the two salts.
2. Transfer the system to a separatory funnel. Equilibrate the system by mixing at room temperature for 1 min and let the phases settle for 10 min.
3. Separate the two phases (*see* **Note 14**).
4. Place the upper phase in a dialysis bag with a capacity of at least 150 mL. Put the bag in 5 L of water which is slowly stirred. After 4 h change to fresh water and continue the dialysis for another 4 h (*see* **Note 15**).
5. Concentrate the PEG solution by letting the dialysis bag hang free in a strong air steam, e.g., in a well-ventilated fume hood, for 2 h or until the volume does not decrease any longer.
6. Determine the PEG concentration of the remaining solution by weighing out 2–3 g in a 50-mL glass beaker of known weight and keeping it in a thermostat oven at 110°C. Remove the beaker after 2 h and allow to attain room temperature. Weigh the beaker again and calculate the mass of PEG as well as its concentration in the solution.
7. Place the bottom phase on ice; the phosphates form a crystal cake. Remove the liquid by decantation or filtration.
8. Purify the phosphate mixture by addition of water in the same quantity as the mother liquid removed under **item 7**. Heat the mixture under stirring until dissolved and filter if turbid. Cool again to 0°C and recover the salt crystals formed.

4. Notes

1. Commercial DEAE-cellulose has to be washed with strong base followed by acid before equilibration with buffer. Ten grams of DEAE-cellulose is suspended in 200 mL of 0.5 *M* NaOH and allowed to sediment. The overlaying liquid is removed and the DEAE-cellulose is suspended in 200 mL of 0.5 *M* HCl. After sedimentation the ion exchanger is separated from the liquid and washed, in the same way, with three 200 mL portions of distilled water.
2. The dilution is done to reduce the viscosity of the phases to give reasonable flow rate for the liquid through the ion exchanger but also to dilute the salt normally present in the phase system. If the salt concentration after dilution is still above 5 m*M,* it is advisable to dialyse the diluted phase against 5 m*M* Tris-HCl, pH 9.0.
3. The pH value is raised to give a negative net charge to the proteins. If proteins with high isoelectric points are present even higher pH should be used or, alternatively, a negatively charged ion exchanger, e.g., CM-cellulose, can be applied in combination with buffers with pH values of 4–5. The choice of pH and type of ion exchanger depends on the stability of the protein.
4. The DEAE-cellulose, after washing (*see* **Note 1**), is equilibrated by suspension in 200 mL of 50 m*M* Tris-HCl buffer, pH 9.5. The liquid is decanted after sedimentation of the ion exchanger and the process is repeated until the washing liquid has the same pH as the added buffer.
5. The column should not be more than 1–3 cm high to assure an acceptable flow rate. Do not use pump.
6. The proteins will largely move with the front between the two buffers. By taking out small fractions, e.g., 3 mL, and checking for protein content the volume of the protein eluted can be minimized.
7. A top phase from a system after affinity partitioning of an enzyme can be used (*see* Chap. 30). The composition of the phase system is not critical as long as the molecular weight of PEG is 8000 or higher.
8. The solution should be stored at room temperature. It has to be heated up again if it starts to crystallize. The phase separation takes place at room temperature, but is even more effective at higher temperatures.
9. The upper phase has high viscosity and a pipet with wide opening is suggested to be used for the recovery.
10. The addition of salt is necessary to get a phase system between DEAE-Dextran and PEG. If the salt content is 0.1 *M* or more in the top phase, the addition of NaCl can be omitted.
11. The pH adjustment is done to give the proteins net negative charge.
12. The DEAE-Dextran is readily soluble in the system by mixing for a few minutes.
13. If the original protein solution contains some PEG (top phase) the amount of added solid PEG can be reduced correspondingly.
14. The volume of the upper phase is around half of that of the lower phase.
15. The volume of the liquid in the dialysis bag increases with a factor of 1.5–2. Consequently the PEG concentration is reduced from 30% to 15–20%.

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Combination of Extraction with Adsorption for Protein Purification

Gabriel Raya-Tonetti and Nora I. Perotti

1. Introduction

Affinity chromatography is the most powerful technique for protein purification in terms of specificity. It has the potential of achieving a high degree of purification in one step, but necessitates the use of primary clarification and concentration steps.

Biospecific interactions have also been used in aqueous two-phase systems in order to impart selectivity to partitioning of proteins (Chapters 30 and 31). Polymers modified with affinity ligands have been used to immobilize the ligand in one phase. Affinity partitioning has mostly been performed using polyethylene glycol (PEG) modified with affinity ligands ***(1)***, but Dextran has also been used in a few instances as a ligand carrier ***(2)***. Preparation of the affinity conjugate free from the unmodified-ligand and -polymer is, however, more cumbersome as compared to that in affinity chromatography since all the components are in a soluble form.

This chapter describes the means to integrate adsorption to an affinity sorbent with aqueous two-phase partitioning ***(3–6)***. Affinity adsorption to the chromatographic particles is performed in the presence of two-phase forming components. After phase separation, the particles occupy the interface from where they can be harvested and treated for elution of the bound protein. The advantages of this technique, besides allowing one to perform affinity adsorption without prior clarification of the homogenate, are easier recovery and recycling of affinity ligand, and possibility of elution in a batch or a chromatographic mode.

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As an example of such an integrated technique, affinity purification of alcohol dehydrogenase (ADH) and glucose-6-phosphate dehydrogenase (G6PDH) simultaneously from unclarified yeast homogenate will be described. The textile dye, Cibacron Blue is used as the ligand for which the dehydrogenases exhibit affinity. This method is applicable to other protein-ligand pairs, as long as the binding constants are sufficiently high (10^5 or more), and the ligand is biologically and chemically stable.

2. Materials

2.1. Preparation of Yeast Homogenate

1. Baker's yeast (*see* **Note 1**).
2. 0.25 *M* Disodium phosphate.

2.2. Coupling of Dye to Support

1. Sepharose CL4B is available from Amersham Pharmacia Biotech, Uppsala, Sweden.
2. Cibacron Blue F3GA is available from Sigma Chemical (St. Louis, MO).
3. Sodium carbonate.
4. 1 *M* NaCl solution.
5. 50 m*M* Citric acid-sodium citrate buffer, pH 3.0.
6. 50 m*M* Tris-HCl, pH 9.0.
7. 50 m*M* Sodium phosphate buffer, pH 6.9.

2.3. Integration of Adsorption and Extraction

1. Dye bound Sepharose CL4B as prepared in **Subheading 3.2.**
2. PEG (mol wt 8000) (Sigma).
3. Reppal-PES 200 (Carbamyl AB, Kristianstad, Sweden).
4. Washing buffer: 50 m*M* Sodium phosphate buffer, pH 6.4, containing 10 m*M* $MgCl_2$.
5. 50 m*M* Sodium phosphate buffer, pH 6.4, containing 10 m*M* $MgCl_2$ and 5 m*M* β-NAD^+.
6. 50 m*M* Sodium phosphate buffer, pH 6.4, containing 10 m*M* $MgCl_2$ and 10 m*M* β-$NADP^+$.
7. 50 m*M* Tris-HCl buffer, pH 8.6, supplemented with 1 *M* NaCl.

3. Methods

3.1. Preparation of Yeast Homogenate

1. Forty-four grams of commercial baker's yeast (dry weight 30%) is inoculated in a 1 L medium in a fermenter of 2 L total volume (*see* **Note 1**).
2. Growth under anaerobic conditions is allowed for 16 h at 30°C.
3. Cells are harvested by centrifugation at 16,000*g* for 5 min at 4°C. The yeast cream with 80% of moisture content may be stored at –20°C.

4. Mix 20 g (wet weight) of yeast cells with 50 mL of 0.25 *M* disodium phosphate solution and incubate the suspension at 37°C for 2.5 h (unpublished data) for disruption of the cells.

3.2. Coupling of Dye to Support

1. Fifty mL of distilled water is mixed with 1.5 g of Cibacron Blue F3GA and 75 g of Sepharose CL4B.
2. After about 10 min at room temperature, add 3.0 g of disodium carbonate (final pH 10.8) and incubate the mixture with shaking for 48 h at 45°C (*see* **Notes 2** and **3**).
3. The dyed support is then washed sequentially on sintered glass funnel (porosity 3) with NaCl (1 *M*, 500 mL), citrate buffer (50 m*M* pH 3.0, 500 mL) and Tris-HCl buffer (50 m*M* pH 9.0, 500 mL).
4. The Sepharose-Cibacron Blue is stored in 50 m*M* sodium phosphate buffer, pH 6.9, at 4°C.

3.3. Integration of Adsorption and Extraction

3.3.1. Adsorption Step

1. Drain the buffer from Sepharose CL4B-Cibacron Blue with the help of a sintered glass funnel. Suction dry.
2. In a capped tube, weigh out 0.5 g of PEG 8000, 1.4 g of Reppal-PES 200, 1 g of Sepharose CL4B-Cibacron Blue, 6.1 g of 50 m*M* sodium phosphate buffer pH 6.4 containing 10 m*M* $MgCl_2$ (washing buffer) and finally 1 g of unclarified yeast homogenate (*see* **Note 4**).
3. Equilibrate the mixture on a rocking table for 5 min.
4. Allow to stand at room temperature for 1 h in a separatory funnel for phases to separate. PEG-rich phase (3 mL) forms the top phase, and the Reppal-rich phase (3.6 mL) is the bottom phase, while the adsorbent particles are located in the interface as the third phase (2.2 mL).

3.3.2. Elution Step

1. The aqueous phases, top and bottom are removed from the separating funnel, first the bottom phase is taken out and then the top phase is pippeted out.
2. The remaining solid phase with the affinity complex (Sepharose-dye-enzyme) is transferred into a chromatographic column (0.9 × 5 cm) giving 3.5 cm of packed bed (*see* **Note 5**).
3. Wash with 10 column volumes of the washing buffer, 50 m*M* sodium phosphate, pH 6.4, with 10 m*M* $MgCl_2$, at a flow rate chosen according to the column area (superficial velocity of 0.5 cm/min) (*see* **Note 6**).
4. Elute the ADH using 2 column volumes of the washing buffer further supplemented with 5 m*M* β-NAD^+ (*see* **Note 7**).
5. Wash the column again with 2 column volumes of the washing buffer.
6. Subsequently, elute G6PDH with 2 column volumes of washing buffer containing 10 m*M* β-$NADP^+$ (*see* **Note 7**).

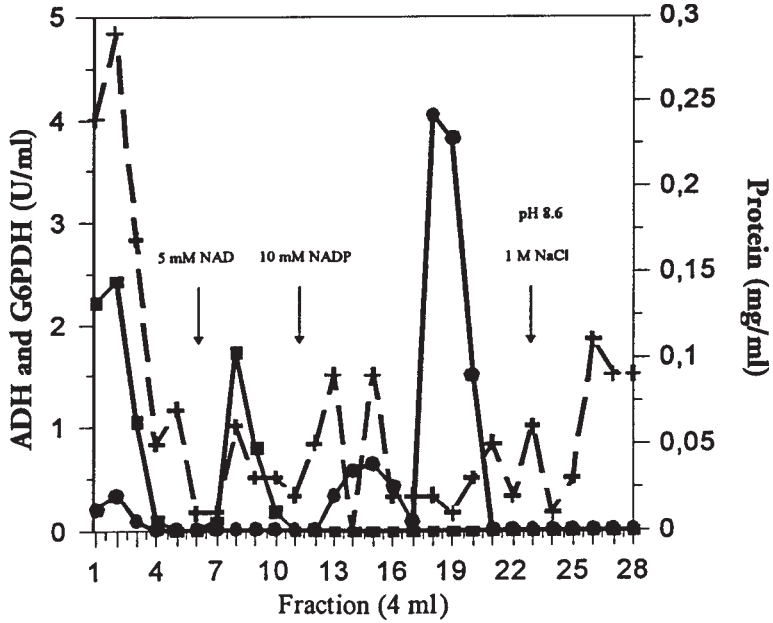

Fig. 1. Elution profile of ADH (■), G6PDH (●), and protein (✚) from Sepharose-Cibacron blue after adsorption in a three-phase system of 5% PEG, 10% adsorbent, and 14% Reppal. The experimental details are described in **Subheading 3**. The chromatographic column was rinsed with 10 mL of washing buffer at a flow rate of 0.3 mL/min. ADH and G6PDH were eluted using 5 mL each of the washing buffer supplemented with 5 m*M* β-NAD^+ and β-$NADP^+$, respectively. In between the elutions, the column was rinsed with 5 mL of washing buffer. Finally, the column was washed with 20 mL of 50 m*M* Tris-HCl buffer, pH 8.6, containing 1 *M* NaCl. Fractions of 4 mL each were collected throughout the experiment.

7. Wash with 2 column volumes of washing buffer and then with 5 column volumes of 50 m*M* Tris-HCl buffer, pH 8.6, containing 1 *M* NaCl to desorb the other proteins bound to the matrix.
8. ADH and G6PDH activities as well as protein content may be determined in the fractions collected during the chromatographic elution and also in the top and bottom polymer-phases (*see* **Notes 8–10**). **Figure 1** and **Table 1** summarize the results obtained from such an experiment.

4. Notes

1. In order to increase the ADH content of the yeast cells, an alcoholic fermentation should be carried out in a culture medium containing (in g/L): sucrose 200, ammonium sulfate 4, and potassium phosphate 0.5.
2. Coupling of the dye to Sepharose can alternatively be performed in a shorter time at higher temperature. The gel is suspended in equal amount of water, heated to

Table 1
Simultaneous Affinity Purification of Alcohol Dehydrogenase and Glucose-6-Phosphate Dehydrogenase from Baker's Yeast Homogenate by Integration of Adsorption and Extraction

	ADH				G6PDH			
Sample[a]	Units	Specific activity	Recovery (%)	Purification factor	Units	Specific activity	Recovery (%)	Purification factor
Cell homogenate	45.5	4.0	100	1.0	54.2	4.8	100	1.0
Top phase	4.0	2.4	—	—	1.6	1.0	—	—
Bottom phase	8.5	6.7	—	—	2.02	1.6	—	—
Eluate	10.3	24.5	22.9	6.1	31.3	324.3	57.8	67.6

[a]The experimental details are stated in the text.

60°C; Cibacron Blue solution (1 g/30 mL water for 200 mL gel suspension) is added slowly and the mixture stirred for 30 min at 60°C. Subsequently 15 g NaCl is added to the above amount and incubation continued for 1 h at the same temperature. Thereafter, the temperature is increased to 80°C, 1.5 g sodium carbonate is added and after 2 h at the new temperature, the reaction mixture is cooled, filtered and washed.

3. It is possible to bind all kinds of textile dyes to Sepharose CL4B, but it is important to take into account the binding conditions (temperature, pH, etc.) suggested by dye manufacturers. Even other affinity ligands could be used to bind onto support. The binding and elution conditions of the desired protein should be determined when these ligands are used for purification. Mainly, the influences of pH and ionic environment should be tested and studied during binding step. Also, binding capacity for desired protein should be determined using frontal analysis in order to find the respective breakthrough curves. Biological and chemical stability of a ligand are important parameters, so as to withstand the action of proteases present in the crude feedstock and also the cleaning conditions prior to recycling of the affinity adsorbent.
4. The separation technique may be designed with other combination of polymers as well. The concentrations of polymers should be optimised in individual cases.
5. Due to viscosity of the phases, care must be taken to separate them. All the affinity adsorbent should be transferred to the chromatography column. If some portion of Reppal-rich and/or PEG-rich phases remains with the solid support, the polymers will be removed during the first chromatography washing.
6. It is important to keep a superficial velocity of 0.5 cm/min during the affinity chromatography mode. The best resolution of protein purification and the necessary residence time in order to obtain the desired protein elution with low amount of eluent are obtained when this superficial velocity is used. Also the resin is not compressed under this flow-rate condition.
7. The process suggests the use of affinity specific elution for ADH and G6PDH, where β-NAD and β-NADP concentrations respectively were previously optimized. It is also possible to define ionic strength conditions, as well as pH change for performing nonspecific elution of the bound enzymes, but often nonspecific elution leads to low recoveries and low purification factors due to lower specificity. The integrated technique shows potential for the separation of enzymes like dehydrogenases and kinases. If the adsorption conditions are evaluated and specific elution conditions are used for each enzyme, the results can be interesting and promising. Recycling of the affinity adsorbent can easily be carried out without loss in binding capacity.
8. The ADH activity is measured according to Worthington Enzyme Manual *(7)* by oxidation of ethanol to acetic acid with β-NAD^+ as coenzyme. The reaction mixture in cuvet is made up by mixing 0.75 mL of 32 m*M* sodium pyrophosphate buffer, pH 8.8, 0.5 mL of 5 m*M* β-NAD^+ and 0.25 mL of 2.0 *M* ethanol. The reaction is started by addition of 0.1 mL of appropriately diluted enzyme solution. The generation of β-NADH is followed by measuring the initial rate of

increase in absorbance at 340 nm. One unit of the ADH activity is defined as the amount of enzyme which catalyses the generation of 1 mmol of β-NADH/min under the assay conditions specified.

9. Glucose-6-phosphate dehydrogenase activity is measured according to Worthington Enzyme Manual *(7)* by oxidation of D-glucose-6-phosphate to D-glucono-D-lactone-6-phosphate with β-$NADP^+$ as coenzyme. To a reaction mixture consisting of 1.30 mL of 55 m*M* Tris-HCl buffer, pH 8.8, containing 3.3 m*M* $MgCl_2$, 0.05 mL of 100 m*M* D-glucose-6-Phosphate and 0.05 mL of 6 m*M* β-$NADP^+$ in a cuvet, add 0.1 mL of enzyme solution. Follow the generation of β-NADPH by measuring the initial rate of increase in absorbance at 340 nm. One unit of the G6PDH activity is defined as the amount of enzyme which catalyses the generation of 1 mmol of β-NADPH/min under the assay conditions specified.
10. The protein content of the samples may be determined by Bradford method. The dye reagent is prepared by mixing equal volumes of Coomassie blue G-250 solution (prepared by dissolving 600 mg of the dye in 500 mL of distilled water) and 6% perchloric acid solution, taking care not to produce flocs ***(8)***. This reagent is light-sensitive.

    Add 500 μL of the dye reagent to 500 μL of sample taking into account the appropriate protein range for this method (0–50 μg/mL). BSA solution (0–50 μg/mL) is used as standard for the protein calibration curve. Absorbance is measured at 595 nm in glass or polystyrene cuvettes at room temperature after 15 min. After some hours of incubation, macroscopic precipitates can be observed. The presence of precipitates is not deleterious to the assay as long as they are resuspended before measuring the absorbance. Because of the colloidal nature of the reagent, gentle mixing of the reagent prior to use and minimal handling of the assay samples are necessary for reproducible results.

    The dye tends to stick to the surface of the cuvet, and the absorbance of the sample changes with pipeting. If using a single cuvet, rinse it with the reagent before adjusting the blank to zero in the spectrophotometer and pipet each sample gently. The cuvet may be rinsed with ethanol between samples. Cuvets may be cleaned by rinsing with concentrated detergent followed by ethanol and water.

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Integration of Extraction with Affinity Precipitation

Masamichi Kamihira, Rajni Hatti-Kaul, and Bo Mattiasson

1. Introduction

Conventional aqueous two-phase extraction by spontaneous partitioning has often problems with low specificity. The introduction of an affinity ligand into one of the phases has been attempted to enhance the specificity of protein partitioning(***1–7***; Chapters 29–31). However, this procedure still has some limitations in the effective removal of impurities as well as recovery and reuse of the ligand. During the past decade, another potentially scalable technique, affinity precipitation, has been studied for the isolation of proteins ***(8–10)***. The affinity ligand coupled to a reversibly soluble-insoluble polymer is used to specifically bind the protein in a complex mixture. The precipitation step which can be accomplished by a change in any environmental parameter such as pH, temperature, salinity, and so forth, facilitates the separation of the bound ligand and affinity complex from the unbound components. Although cells and cell debris should be removed before use, recovery and reuse of the ligand are relatively easy.

We have proposed a new separation procedure using aqueous two-phase separation method integrated with affinity precipitation to compensate for the problems in the two respective methods. A reversibly soluble-insoluble polymer, which partitions into the upper phase, is used as the ligand carrier in a two-phase system. A crude-cell homogenate containing the target protein is mixed with such a phase system. After equilibration and phase separation, the affinity conjugate is precipitated from the upper phase, followed by the elution of the bound protein. This chapter describes the use of an enteric-coating polymer, Eudragit S100 as a ligand carrier ***(11)***. The soluble-insoluble form of the polymer can be controlled by changing pH. Eudragit is specifically partitioned to the polyethylene glycol (PEG)-rich upper phase in the aqueous two-phase

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systems. Therefore, by the use of Eudragit as a ligand carrier in aqueous two-phase system, one can expect to extract target protein specifically to the upper phase, and the target protein–ligand–polymer complex can be recovered as precipitate after changing pH. **Figure 1** illustrates the purification scheme in this method. As a model separation system, this method was applied for the purification of recombinant protein A using human Immunoglobulin G (lgG) coupled to Eudragit. The method should be applicable to other protein–ligand pairs, but may require prior optimization in each case ***(12)***.

2. Materials

2.1. Immobilization of IgG to Eudragit

1. Eudragit S-100 (Röhm Pharma GmbH, Darmstadt, Germany): a copolymer of methacrylate and methylmethacrylate (*see* **Note 1**).
2. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Dojin Co., Osaka, Japan or Sigma, St Louis, MO).
3. Phosphate buffered saline (PBS) solution: 10 m*M* phosphate buffer, pH 7.4, containing 0.14 *M* NaCl.
4. Human IgG (Sigma) (*see* **Note 2**).
5. 0.45 g/mL Ethanolamine solution, pH 8.5.
6. 2 *M* Acetic acid solution.
7. 10 m*M* acetate buffer, pH 4.5.
8. 1 *M* Potassium phosphate buffer, pH 7.0.
9. 2 *M* NaOH solution or 1 *M* HCl solution: required for adjustment of pH.
10. Sodium azide.

2.2. pH Response Test of Eudragit

1. 1% (w/v) Eudragit S100 solution (*see* **Note 1**).
2. 1% (w/v) IgG-bound Eudragit solution (prepared as in **Subheading 3.1.**).
3. 2 *M* acetic acid solution.

2.3. Evaluation of Adsorption Capacity

1. 1% (w/v) IgG-bound Eudragit solution (prepared as in **Subheading 3.1.**).
2. Pure protein A (Sigma).
3. 20% (w/v) Polyethylene glycol 8000 (PEG 8000) (Sigma).
4. PBS solution.

2.4. Preparation of Protein A Crude Extract

1. *Escherichia coli* N4830-1 harboring a plasmid pRIT2T ***(13)*** (Amersham Pharmacia Biotech, Uppsala, Sweden).
2. L-Broth medium: 10 g polypepton, 5 g yeast extract, 5 g NaCl, 10 g glucose, and 100 mg ampicillin/L, pH 7.0.
3. PBS solution.

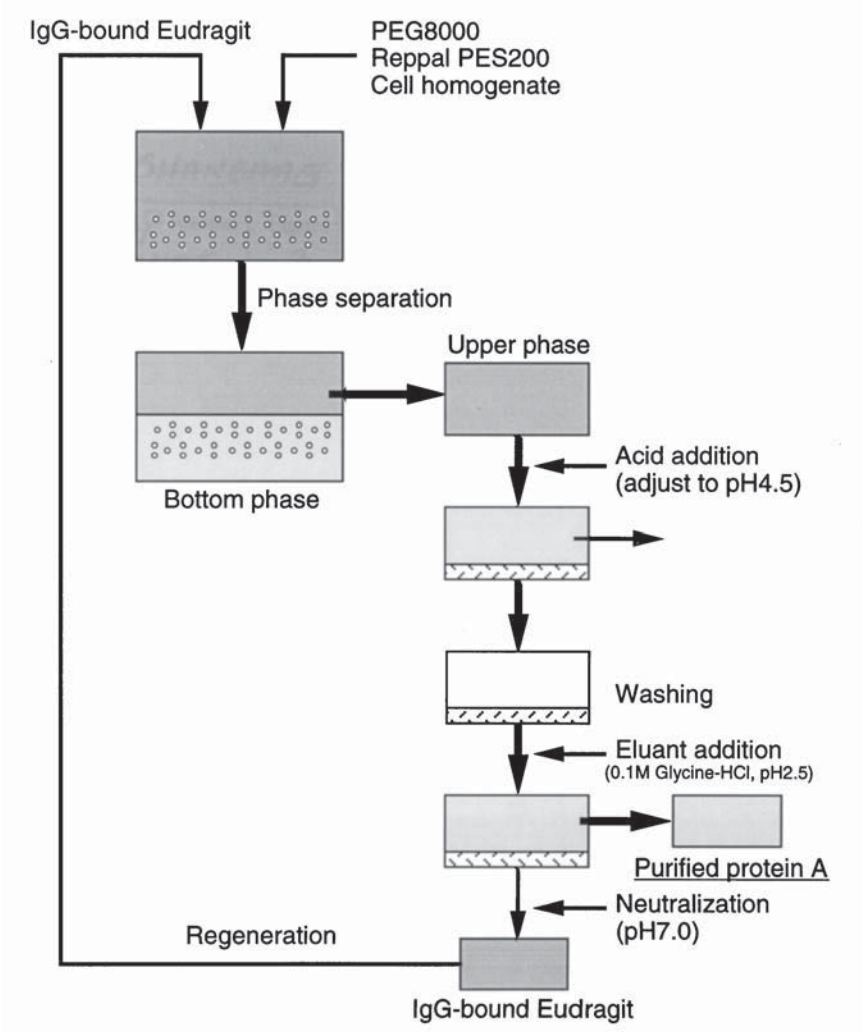

Fig. 1. Purification scheme of aqueous two-phase extraction integrated with affinity precipitation.

2.5. Purification of Protein A Using IgG-Bound Eudragit in Aqueous Two-Phase System

1. PEG 8000.
2. Hydroxypropyl starch (Reppal PES-200) ***(14)***, (Carbamyl, Kristianstad, Sweden) (*see* **Note 3**).
3. Culture broth containing crude protein A.
4. 1% (w/v) IgG-bound Eudragit solution.

5. 0.4 *M* and 0.2 *M* Potassium phosphate solution, pH 7.0.
6. PBS solution.
7. 2 *M* Acetic acid solution.
8. 10 m*M* Acetate buffer, pH 4.5.
9. 0.1 *M* Glycine-HCl buffer, pH 2.5.
10. 0.5 *M* NaOH solution or 1 *M* HCl solution: required for adjustment of pH.

3. Methods

3.1. Immobilization of IgG to Eudragit

1. Dissolve Eudragit S100 to 1% (w/v) in PBS. During the preparation, adjust the pH of the solution at 7.2 with 2 *M* NaOH.
2. Add EDC to a final concentration of 3 mg/mL and incubate for 5 min at room temperature.
3. Prepare human IgG solution of at least 1–3 mg/mL in PBS. Add a volume of human IgG solution to yield a final weight ratio of 60 mg of human IgG to 1 g of Eudragit.
4. Incubate at room temperature for 3 h. Check the pH during reaction and add HCl or NaOH to maintain pH at 7.0–7.5.
5. Add 0.45 g/mL ethanolamine solution, pH 8.5, to a final concentration of 12–20 mg/mL for blocking of the remaining activated sites of the polymer.
6. Incubate for 1 h at room temperature.
7. Stop the reaction by adding 2 *M* acetic acid solution to adjust the pH at 4.5. Precipitation of IgG-bound polymer results.
8. Separate the IgG-bound polymer from the solution by centrifugation at 3000*g* for 20 min in a bench-top centrifuge.
9. Wash the precipitate at least three times with 10 m*M* acetate buffer (pH 4.5). Centrifuge in between washings.
10. Dissolve the precipitate in potassium phosphate buffer and adjust the pH to 7.0 with NaOH. Adjust the concentration of the polymer and potassium phosphate to yield 1% (w/v) IgG-bound Eudragit, 0.2 *M* potassium phosphate, and 0.05% sodium azide for preservation, and store the solution at 4°C until use.

3.2. pH Response Test of Eudragit

1. Prepare 3 mL of 0.15% (w/v) Eudragit or IgG-bound Eudragit solution in test tubes.
2. Add various amounts (3–150 μL) of 2 *M* acetic acid solution.
3. Mix the solution vigorously and allow to stand for at least 20 min.
4. Measure the pH and the absorbance at 470 nm (to determine polymer concentration) for each solution.
5. Calculate a ratio of the absorbance at each pH to the maximum value of the absorbance at pH less than 3.5 as relative value (*see* **Note 4**).

3.3. Evaluation of Adsorption Capacity (see Note 5)

1. Prepare a mixture of 0.5 mL of 0.5% IgG-bound Eudragit solution and 0.5 mL of 20% PEG 8000 solution.

2. Add 100 μg of pure protein A.
3. Incubate for 2 h at room temperature or overnight at 4°C.
4. Adjust the pH of the mixture to 4.5 by adding 2 *M* acetic acid solution.
5. Centrifuge at 3000*g* for 20 min, and collect the supernatant.
6. Dialyze the supernatant against PBS.
7. Measure the amount of protein A in the supernatant (*see* **Notes 6**, **7**, and **8**).

3.4. Preparation of Protein A Crude Extract

1. Cultivate *E. coli* N4830-1 harboring a plasmid pRIT2T in L-broth medium at 37°C.
2. Check the absorbance at 600 nm of the culture broth. When it reaches approx 2.5, raise the culture temperature to 42°C. Continue the culture further for 4 h.
3. Collect the cells by centrifugation, and store at –20°C until use.
4. When required, resuspend the cells in PBS to 1–10% (w/v).
5. Disintegrate the cells by a sonicator.

3.5. Purification of Protein A Using IgG-Bound Eudragit in Aqueous Two-Phase System

1. Prepare an aqueous two-phase system using PEG 8000, Reppal PES 200, 1% IgG-Eudragit solution, 0.4 *M* potassium phosphate solution, and the *E. coli* cell homogenate to yield a final concentration of 5% (w/v) PEG 8000, 14% (w/v) Reppal PES 200, 0.5% IgG-Eudragit and 0.2 *M* potassium phosphate (*see* **Notes 9** and **10**).
2. Incubate for 1 h at room temperature under occasional stirring.
3. Centrifuge at 4000*g* for 10 min, and separate the upper phase and the bottom phase can be separated.
4. Collect the upper phase to recover the protein A.
5. Adjust the pH of the upper-phase solution to 4.5 by adding 2 *M* acetic acid.
6. Collect the resulting precipitate by centrifugation at 4500*g* for 10 min.
7. Wash the precipitate three times with 0.01 *M* acetate buffer, pH 4.5. Centrifuge in between washings.
8. Elute protein A from IgG-bound Eudragit by adding 2 mL of 0.1 *M* glycine-HCl buffer, pH 2.5.
9. Repeat the elution procedure twice, and neutralize each fraction of eluate immediately with 0.5 *M* NaOH.
10. Wash the precipitate (insoluble form of IgG-Eudragit) with 0.01 *M* acetate buffer, pH 4.5, and resolubilize in 0.2 *M* potassium phosphate buffer, pH 7.0. This solution can be used for the next cycle of purification.

4. Notes

1. Eudragit S100 is a pH-sensitive enteric coating polymer. It has been used for coating of drugs, because the solubility in water can be controlled by pH change, i.e., while it is insoluble in gastric juice, it dissolves in the region of the digestive tract where the pH is neutral to weakly alkaline. Actually, the polymer is soluble in water above pH 6.0, insoluble at less than pH 4.5. Thus, to solubilize the poly-

Table 1
Recovery of Eudragit S100 in the Upper Phase of PEG 8000-Reppal PES 200 Two-Phase System as a Function of its Initial Concentration[a]

Initial Eudragit concentration (wt%)	Eudragit concentration in upper phase[b] (wt%)
0.1	0.20 (100%)
0.25	0.59 (99.9%)
0.5	0.86 (97.7%)
1.0	1.38 (85.0%)

[a]Composition of the phase system: 5 wt% and 14 wt% of PEG 8000 and Reppal PES 200, respectively.
[b]Volume ratio of top to bottom phase: 1:2.

mer in water, the pH of the solution should be adjusted with alkaline solution.

2. A ligand to be immobilized to Eudragit should be selected according to the affinity system. In the case of protein ligands, the immobilization procedure described here can be available with slight modification. pH response test and adsorption capacity should be checked for the prepared ligand-bound Eudragit.
3. In the present study hydroxypropyl starch, Reppal PES 200, was used as hydrophilic bottom-phase polymer instead of Dextran, because the polymer is rather inexpensive and promising for industrial-scale use. **Table 1** shows Eudragit concentrations in the PEG 8000-Reppal PES 200 two-phase system as a function of initial concentration of the polymer. Most of the Eudragit is partitioned to the upper phase, although Eudragit concentration of the bottom phase is increased with the increase in the initial concentration. Eudragit from the upper phase can be recovered almost completely after precipitation. Because Eudragit partitions specifically to upper phase for the other aqueous two-phase systems, such as PEG-Dextran and PEG-potassium phosphate, the systems can be also applied for the purification.
4. Native Eudragit has a soluble-insoluble transitional region between pH 4.5 and 5.5 (**Fig. 2**). The region is shifted slightly upward in pH scale after the immobilization of human IgG. However, the response width is not changed. The pH response curves are independent of immobilized ligand density. Moreover, the response behavior is the same both in the absence and presence of 10% PEG.
5. Adsorption capacity of the ligand-bound Eudragit should be determined prior to the practical application to the aqueous two-phase extraction, because overload of the sample leads to the loss of product and the decrease of the purity.
6. Some proteins are adsorbed nonspecifically to Eudragit. This is a potential problem. Actually, 80% of protein A added was adsorbed nonspecifically to Eudragit. However, the nonspecific adsorption can be reduced very much in the presence of

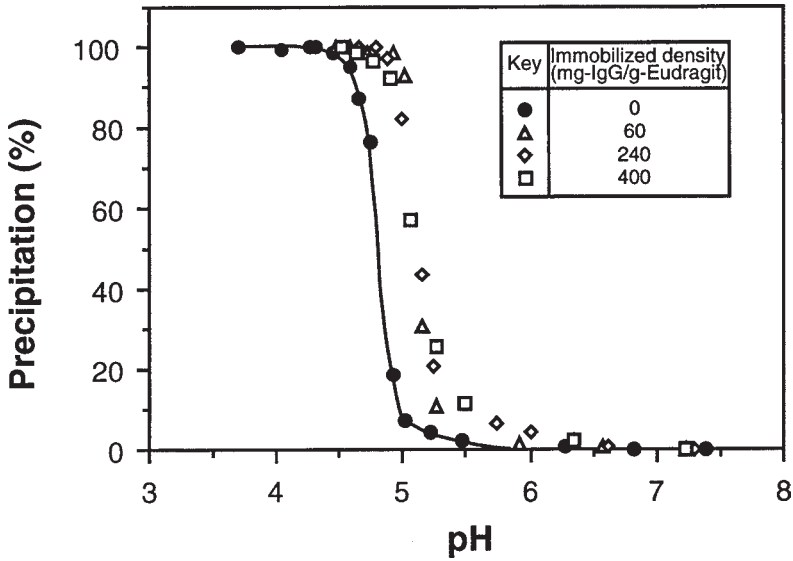

Fig. 2. Precipitation curve of Eudragit S100 and IgG-bound Eudragit in response to pH variation.

PEG. More than 90% of nonspecific binding of protein A was prevented in 10% PEG.

7. The amount of protein A can be measured by enzyme-linked immunosorbent assay (ELISA) and protein assay.
8. For the measurement of adsorption capacity, adsorption of protein A to Eudragit treated only with ethanolamine should also be measured by the same procedure described in **Subheading 3.3.** This amount is regarded as nonspecific adsorption to Eudragit. Adsorption capacity of IgG-Eudragit can be calculated by subtracting the amount of nonspecific adsorption from the amount adsorbed to IgG-bound Eudragit.
9. The partitioning of Eudragit in the aqueous two-phase system is influenced by the initial concentration of Eudragit (**Table 1**), the phase composition (**Table 2**) and salt concentration (**Table 3**).

   In **Table 2**, each composition is almost along the same tie line, and the results indicate the effect of volume ratio between the phases on the Eudragit partitioning. The partition to the bottom phase is increased with the decrease of the top phase volume. Furthermore, the partition to the bottom phase is drastically increased as top phase concentration of Eudragit exceeded 1%.

   The partition to the upper phase is increased by the addition of potassium phosphate (**Table 3**). On the other hand, the addition of NaCl has opposite effect. The difference seems to be attributable to the distribution of the anion in the two-phase system. From the fact that phosphate ion strongly favors the bottom phase and chloride ion is present equally in both the phases ***(15)***, electrostatic repulsion between Eudragit (polyanion in the soluble form) and the salt anion generates the partitioning.

Table 2
Effect of Phase Composition on Partition of Eudragit S100 into Upper Phase of PEG-Reppal System[a]

Composition		Volume ratio (Upper/Bottom)	Eudragit concentration in upper phase (wt%)
PEG 8000 (wt%)	Reppal PES 200 (wt%)		
2	25	0.22	1.81 (65.4%)
5	14	1.2	0.86 (97.7%)
8	6	5.3	0.57 (98.4%)

[a]Initial concentration of Eudragit was 0.5 wt%.

Table 3
Effect of Salt Addition on Partition of Eudragit S100[a]

Salt	Concentration (*M*)	Eudragit concentration (wt%)	
		Upper	Bottom
No addition	—	0.86 (97.7%)	0.025
K phosphate[b]	0.05	0.84 (99.5%)	0.005
	0.2	1.02 (100%)	—[c]
NaCl	0.2	0.67 (76.5%)	0.24
	0.5	0.39 (43.8%)	0.56

[a]Composition of the two-phase system: 5 wt% PEG 8000, 14 wt% Reppal PES 200, and 0.5 wt% Eudragit S100.
[b]K phosphate: equal molar mixture of KH_2PO_4 and K_2HPO_4.
[c]Below detection limit.

10. The cell homogenate can be directly added without elimination of cell debris, because the cell debris is partitioned to the bottom phase after phase separation. However, the purity of the eluate may be higher when the cell debris is removed prior to the extraction.

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Affinity Partitioning Using Magnetic Two-Phase Systems

Masamichi Kamihira

1. Introduction

Aqueous two-phase extraction has appreciable advantages in terms of ease of scaling-up and its applicability to systems containing solids such as cell and cell debris. However, because it is not always possible to achieve an extreme partitioning of a target molecule into either phase, the introduction of an affinity ligand to the system has been examined as a means of enhancing the specificity of partitioning. Covalent binding of a ligand to a phase-constructing polymer (***1–4***; *see also* Chapters 29–31), and the use of ligand-bound chromatographic supports (***5–7***; *see also* Chapter 33) or a pH responsive polymer (***8***; *see also* Chapter 34), that partitions to the upper phase have been employed as ligand-introduction procedures. As described in Chapter 34, the method using a pH responsive polymer can realize easy recovery of the target protein as a precipitate by changing the pH after specific partitioning into upper-phase, and the ligand is also easily recovered and reused after dissociation of the target protein.

The other difficulty in aqueous two-phase extraction is slow-phase separation. A typical aqueous two-phase system can achieve rapid equilibrium with minimum mixing because the surface tension between the phases is relatively low. In contrast, the phase separation is very slow, at least under static condition. Especially in the case of PEG-Dextran and PEG-modified starch systems, the solution is viscous. Therefore, the phase separation is accelarated by centrifugation. Nevertheless, simple and fast phase separation method is still desired. As an approach to this, Wikström and Flygare et al. reported that the introduction of magnetic particles that partition to one of the phases to various aqueous two-phase systems drastically shortened the phase separation time

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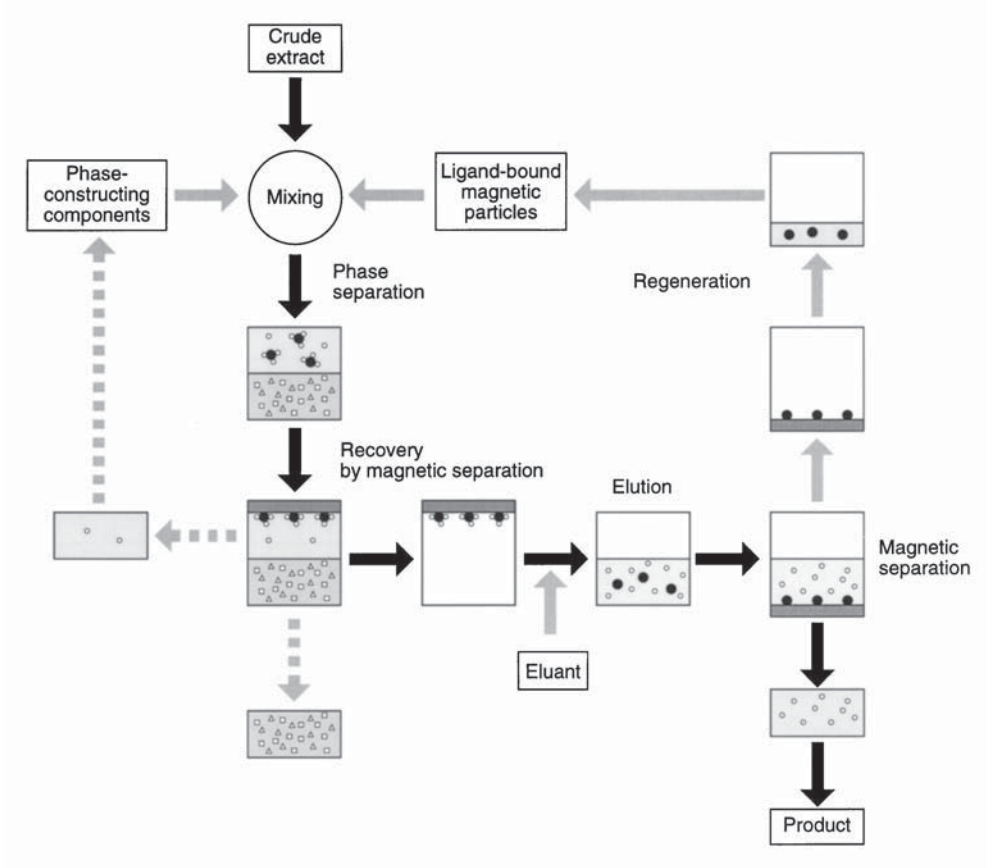

Fig. 1. Flow diagram of separation procedures using magnetic particles as ligand carrier in aqueous two-phase system.

when a magnetic field was applied ***(9,10)***. The aqueous two-phase system was recognized as magnetic aqueous two-phase system. They have also developed the apparatus for magnetic aqueous two-phase systems. In their case, however, magnetic particles were neither modified nor immobilized with an affinity ligand and the particles partitioned to the bottom phase in PEG-based systems.

In this chapter, new magnetic aqueous two-phase system that we developed ***(11)*** will be described. An affinity ligand was immobilized to fine magnetic particles, which partition to the upper phase in aqueous two-phase systems. By introducing ligand-bound magnetic particles to aqueous two-phase systems, not only can selective partitioning and easy recovery be expected, but also the elimination of cell debris and a shortening of the phase-separation time by the application of a magnetic field. A flow dia-

gram of the separation procedures using ligand-bound magnetic particles in aqueous two-phase system is given in **Fig. 1**. As a model separation system, human IgG was immobilized to the magnetic particles and staphyloccocal protein A produced by recombinant *Escherichia coli* was purified with this method.

2. Materials

2.1. Surface Modification of Magnetic Particles

1. Marpomagna FNC-50 (Matsumoto Yushiseiyaku Co., Osaka, Japan): a commercially available magnetic ferro-fluid (magnetite particles 7–15 nm in diameter dispersed in toluene).
2. 3–Aminopropyltriethoxysilane (Tokyo Kasei Kogyo Co., Tokyo, Japan [Also available from Sigma Chemical Co., St. Louis, MO]).
3. Toluene (dehydrated with molecular sieves 4Å).
4. Ethanol.
5. Eudragit S-100 (Röhm Pharma GmbH, Darmstadt, Germany): a copolymer of methacrylate and methylmethacrylate (*see* **Note 1**).
6. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Dojin Co., Osaka, Japan [Also available from Sigma]).
7. 2 *M* NaOH solution or 1 *M* HCl solution: for adjustment of pH.
8. Samarium-cobalt magnet (magnetic field intensity; 3kG).

2.2. Immobilization of Human IgG onto Magnetic Particles

1. Eudragit-modified magnetic particles (as prepared in **Subheading 3.1.**).
2. Human immunoglobulin G (IgG) (Sigma).
3. Phosphate buffered saline solution (PBS): 10 m*M* phosphate buffer, pH 7.4, containing 0.14 *M* NaCl.
4. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide.
5. 2 *M* NaOH solution or 1 *M* HCl solution: for adjustment of pH.

2.3. Evaluation of Adsorption Capacity of IgG-Modified Magnetic Particles

1. IgG-modified magnetic particles (as prepared in **Subheading 3.2.**).
2. Pure protein A (Sigma).
3. PBS solution.

2.4. Evaluation of Paritioning Behaviors of Protein A in Magnetic ATPS

1. IgG-modified magnetic particles (as prepared in **Subheading 3.2.**).
2. Pure protein A.
3. Polyethylene glycol 8000 (PEG 8000; Sigma).
4. Potassium phosphate (an equal molar ratio of K_2HPO_4 and KH_2PO_4).
5. Samarium-cobalt magnet.
6. PBS solution.

2.5. Preparation of Protein A Crude Extract

See Subheading 2.4. in Chapter 34.

2.6. Extraction of Protein A by Affinity Partitioning Using Magnetic Aqueous Two-Phase System

1. Polyethylene glycol 8000.
2. Potassium phosphate (an equal molar ratio of K_2HPO_4 and KH_2PO_4).
3. Culture broth containing crude protein A.
4. IgG-modified magnetic particles.
5. Samarium-cobalt magnet.
6. PBS solution.
7. 3.5 *M* KSCN solution.

3. Methods

3.1. Surface Modification of Magnetic Particles

1. Prepare a magnetic ferrofluid solution at approx. 10% in dehydrated toluene.
2. Add 3-aminopropyltriethoxysilane to 10% (v/v).
3. Incubate at 60°C for 1 h.
4. Wash the magnetic solution with toluene, ethanol, and water, successively. The magnetic particles are now modified with amino groups and suspended in water (approx 5% w/v).
5. Dissolve Eudragit S100 to 1 wt% in water. During the preparation, adjust the pH of the solution at 7.0 with 2 *M* NaOH (*see* **Note 1**).
6. For 100 mL of Eudragit solution, add 3–5 mL of the magnetic solution.
7. Add EDC to a final concentration of 1.5 mg/mL. Adjust the pH to 7.0.
8. Incubate for 3 h at room temperature. Check the pH during reaction and add HCl or NaOH to maintain pH at 7.0–7.5.
9. Wash the resultant magnetic particles well with water (*see* **Note 2**). Suspend the particles in water at 20–50 mg/mL (*see* **Notes 3** and **4**). They can be stored at room temperature until use.

3.2. Immobilization of IgG onto Magnetic Particles

1. Dilute the Eudragit-modified magnetic particles to 20 mg/mL in water.
2. Add EDC to a final concentration of 3 mg/mL. Adjust the pH to 6.0.
3. Incubate for 5 min at room temperature.
4. Prepare human IgG solution of at least 1–3 mg/mL in PBS. Add equal volume of human IgG solution to yield a final weight ratio of 1 mg of human IgG to 10 mg of the magnetic particles.
5. Incubate at room temperature for 3 h. During reaction, add HCl or NaOH to maintain pH at 7.0–7.5.
6. Stop the reaction by adding sodium acetate to a final concentration of 100 m*M*.
7. Separate the IgG-modified magnetic particles from free IgG by centrifugation or a magnet, and remove the supernatant containing free IgG (*see* **Note 5**).

8. Wash the IgG-modified magnetic particles at least four times with PBS. Sodium azide may be added to the magnetic particle solution at a concentration of 0.05 wt% as a preservative, and the solution can be stored at 4°C until use.

3.3. Evaluation of Adsorption Capacity of IgG-Modified Magnetic Particles

1. Wash and suspend the IgG-modified magnetic particles in PBS (50–100 mg/mL).
2. Transfer 0.1 mL of the IgG-modified magnetic particles solution to a microtube.
3. Add 0.1–0.5 mL of pure protein A solution (at least 0.5 mg/mL in PBS). All the tubes are finally made up to the same volume with PBS
4. Incubate at room temperature for 2 h under gentle stirring.
5. Separate the IgG-modified magnetic particles from unbound protein A by centrifugation.
6. Collect the supernatant, and measure the volume.
7. Wash the precipitate with water, and then dry and weigh.
8. Assay protein A concentration of the supernatant (*see* **Note 6**).
9. Calculate the bound protein A to the IgG-modified magnetic particles by subtracting the protein A amount of the supernatant from the total amount added (*see* **Note 7**).

3.4. Evaluation of Partitioning Behavior of Protein A in Magnetic Aqueous Two-Phase System

1. Prepare magnetic aqueous two-phase system in a test tube by mixing PEG 8000, potassium phosphate, IgG-modified magnetic particles, and protein A crude extract. Typically, 50 or 100 μg of pure protein A and 6 or 10 mg of IgG-modified magnetic particles were added to 1 g of an aqueous two-phase system containing 15 wt% PEG 8000 and 10 wt% potassium phosphate (*see* **Note 8**).
2. Incubate at room temperature for 1 h with occasional stirring.
3. Expose the upper phase in the test tube to a magnetic field by placing a samarium-cobalt magnet across the tube wall to facilitate phase separation.
4. Separate the phases and measure the volumes.
5. Remove the magnetic particles from the upper phase by a magnet or centrifugation.
6. Assay protein A concentration of the phases (*see* **Note 6**).
7. Calculate the partition coefficient and estimate the association constant and adsorption capacity in the aqueous two-phase system (*see* **Note 9–13**).

3.5. Preparation of Protein A Crude Extract

1. Cultivate *E. coli* N4830-1 harboring a plasmid pRIT2T in L-broth medium at 37°C.
2. Check the absorbance at 600 nm of the culture broth. When it reaches approx 2.5, raise the culture temperature to 42°C. Continue the culture further for 4 h (*see* **Note 14**).
3. Collect the cells by centrifugation, and store at –20°C until use.
4. Resuspend the cells in PBS at 1–10% (w/v).

5. Disintegrate the cells by a sonicator.
6. Remove cell debris by centrifugation (*see* **Note 15**).

3.6. Extraction of Protein A by Affinity Partitioning Using Magnetic Aqueous Two-Phase System

1. Prepare magnetic aqueous two-phase system in a test tube by mixing PEG 8000, potassium phosphate, IgG-modified magnetic particles, and protein A crude extract. Typically, 2.5 mg of total protein containing 12% of protein A and 40 mg of IgG-modified magnetic particles are added to 8 g of an aqueous two-phase system containing 15 wt% PEG 8000 and 10 wt% potassium phosphate (*see* **Note 8**).
2. Incubate at room temperature for 1 h with occasional stirring.
3. Expose the upper phase in the test tube to a magnetic field by placing a samarium-cobalt magnet across the tube wall. The magnetic particles (IgG-modified magnetic particles adsorbed protein A) are attracted to the magnet and separated from the solution.
4. Wash the magnetic particles three times with PBS (*see* **Note 2**).
5. Elute the protein A by adding 1 mL of 3.5 *M* KSCN.
6. Dialyze the eluate containing purified protein A against PBS.

4. Notes

1. Eudragit S100 is a pH-sensitive soluble-insoluble enteric coating polymer. The polymer is soluble in water above pH 6.0, insoluble at less than pH 4.5. Thus, to solubilize the polymer in water, the pH of the solution should be adjusted with alkaline solution.
2. When washing the magnetic particles, applying magnetic field by a magnet or centrifugation facilitates the separation of the magnetic particles.
3. To determine percentage of solid mass of the Eudragit-modified magnetic particle solution, predetermined small amount of the solution is taken after suspending well, and then dried and weighed.
4. If Eudragit-modified magnetic particles do not partition to upper phase of aqueous two-phase system, the modification should be retried.
5. The amount of immobilized IgG to the Eudragit-modified magnetic particles can be roughly calculated from the difference between the amount of protein used for the coupling reaction and that remaining in the soluble fraction of the solution after the reaction.
6. Protein A concentration can be measured by enzyme-linked immunosorbent assay (ELISA) and protein assay.
7. Adsorption capacity of the IgG-modified magnetic particles is estimated as (bound pure protein A)/(solid mass of IgG-modified magnetic particles).
8. PEG-Dextran (PEG 8000, 6 wt%; Dextran T-500 [Amersham Pharmacia Biotech], 12 wt%) or PEG-hydroxypropyl starch (PEG 8000, 5 wt%; Reppal PES-200 [Carbamyl AB, Kristianstad, Sweden], 14 wt%) systems can also be used. The aqueous two-phase system should be selected according to the separation system.
9. The apparent partition coefficient of protein A can be measured in a PEG-phosphate system with or without magnetic particles as follows. Add various amounts

Table 1
Effect of Magnetic Particles on Partition Coefficient of Protein A in PEG-Phosphate System

		50 μg		100 μg	
		Partition coefficient (–)[a]	Protein A added to the system	Partition coefficient (–)[a]	Protein A added to the system
Without magnetic particles		0.33	(31%)	0.40	(36%)
With Eudragit-Mag	6 mg	0.27	(26%)	0.32	(29%)
	10 mg	0.36	(31%)	0.43	(33%)
With IgG-Mag[b]	6 mg	3.5	(81%)	1.7	(68%)
	10 mg	11.4	(94%)	4.3	(84%)

[a]Total weight of the system was 1 g. Values in parentheses are the percentage of protein A in upper phase.
[b]The density of immobilized human IgG was 75 mg-IgG/g-magnetite.

of pure protein A and magnetic particles to the two-phase system. After mixing the solution for 2 h and then allowing it to settle until the phase interface becomes clear, measure the volumes of the upper (V_U) and bottom phases (V_B) and the concentrations of protein A in the bottom phase (C_B) and/or the upper phase (C_U). The partition coefficient (P) is calculated from the following equation:

$$P = C_U / C_B \tag{1}$$

with the mass balance equation:

$$M = (C_U \times V_U) + (C_B \times V_B) \tag{2}$$

where M is the total amount of protein A added to the aqueous two-phase system. The percentage of protein A in the upper phase (Y) can be calculated from the following equation:

$$Y = 100 \times (C_U \times V_U) / M \tag{3}$$

10. **Table 1** shows experimental data of the partition coefficient and the percentage of pure protein A that partitioned to the upper phase ***(11)***. Protein A spontaneously partitioned with a partition coefficient of 0.3–0.4 in the PEG-phosphate system without magnetic particles, and more than 70% of protein A added partitioned to the bottom phase. The partition coefficients were almost the same as that of spontaneous partitioning when native Eudragit-modified magnetic particles were added in the two-phase system. On the contrary, the apparent partition coefficient increased to 11.4 by the addition of IgG-modified magnetic particles, because protein A bound to IgG and moved to the upper phase with the IgG-modified magnetic particles.
11. The adsorption equilibrium between IgG-modified magnetic particles and pure protein A in the aqueous two-phase system was estimated from the same experi-

mental data. Protein A in the upper phase is distributed into two fractions; the fraction adsorbed to IgG-modified magnetic particles and the residual fraction in the upper phase, which partitions spontaneously to the upper phase at the concentration of C_R. Therefore, neglecting the volume of IgG-modified magnetic particles and using the weight of IgG-modified magnetic particles (X), Eqs. (1) and (2) can be rewritten as follows:

$$P_W = C_R / C_B \tag{4}$$

$$M = C_R \cdot V_U + C_B \cdot V_B + A \cdot X \tag{5}$$

where P_W is the partition coefficient without ligand, so that the value of the partition coefficient obtained under the same condition as with Eudragit-modified magnetic particles was used, and A is the amount of protein A adsorbed to IgG-modified magnetic particles (unit weight). The adsorption isothermal equation between IgG-modified magnetic particles and protein A may be expressed as:

$$A = A_S \cdot K \cdot C_R / (1 + K \cdot C_R) \tag{6}$$

in which K is the association constant and A_S is the saturated adsorption capacity. Using the Eqs. (4)–(6), the values of K and A_S in the aqueous two-phase system were calculated as $9 \times 10^6\ M^{-1}$ and 10 mg-protein A/g-solid, respectively.

12. Assuming the volume of each phase (V_U, V_B), the spontaneous partition coefficient of protein A (P_W), and the affinity between protein A and IgG-Mag (K, A_S) are independent of changes in the amount of IgG-modified magnetic particles (X) and the total amount of protein A (M), the percentage of protein A in the upper phase (Y) can be calculated as a function of X and M:

$$Y = 100 \cdot \frac{C_R \cdot V_U + A \cdot X}{M}$$

$$= 100 \cdot \left\{ 1 + \frac{(\alpha + A_S \cdot X - M) - \sqrt{(\alpha + A_S \cdot X - M)^2 - 4 \cdot \alpha \cdot M}}{2 \cdot \beta \cdot M} \right\} \tag{7}$$

where $\alpha = (P_W \cdot V_U + V_B) / (K \cdot P_W)$ and $\beta = (P_W \cdot V_U + V_B) / V_B$. The profiles of X vs Y for several M values are illustrated in **Fig. 2** and show good fitting to the experimental data.

13. The partitioning behavior varied depending on the amount of protein A added and the amount of IgG-Mag applied in the PEG-phosphate system. The apparent partition coefficients for the systems containing IgG-modified magnetic particles shown in **Table 1** were derived from spontaneous partitioning of protein A without IgG-modified magnetic particles and the adsorption equilibrium between protein A and IgG-Mag in the upper phase of the two-phase system. Increasing the amount of IgG-Mag resulted in an enhancement of the apparent partition coefficient, while increasing the amount of protein A under a fixed amount of IgG-Mag resulted in its reduction. These tendencies were reasonably explained by Eqs. (4)–(7) assuming that IgG-modified magnetic particles had a limited adsorption capacity.

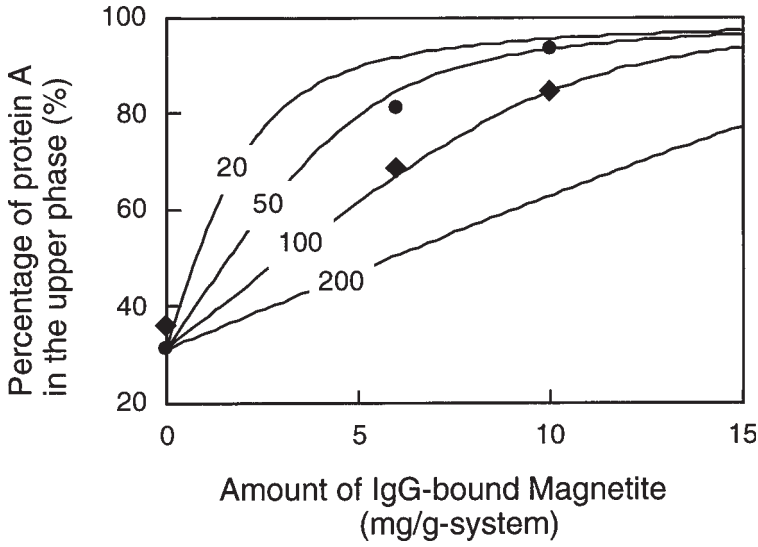

Fig. 2. The profile of percentage of protein A in upper phase as a function of the amount of IgG-Mag applied to the system and total protein A amount added to the system. Lines were calculated from equation *(7)*, and the numbers on these lines are the total protein A amount in μg/g-system. Plots indicate experimental data for 50 (●) and 100 (◆) μg-protein A/g-system.

14. Since the staphylococcal protein A gene exists just downstream of the heat-inducible $P_L P_R$ promoter in the plasmid pRIT2T, the cells are grown first at 37°C after which the temperature is raised to 42°C for the production of protein A.
15. In the preparation of protein A crude extract, the elimination of cell debris can be omitted, although the purity after the extraction may decrease.

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36

Large-Scale Extraction of Proteins

Teresa Cunha and Raquel Aires-Barros

1. Introduction

Purification of proteins using aqueous two-phase systems (ATPS) has been successfully carried out on large scale for more than a decade ***(1–17)***.

One of the major advantages of ATPS is that the scaling up is possible through the use of conventional extraction equipment used for organic-aqueous extraction in chemical industry. The large-scale application of ATPS is mainly limited by the theoretical understanding of phase equilibrium and protein partitioning, the selectivity of these systems and cost of the polymers ***(18–21)***; the possibility to recycle the polymers must also be considered ***(22–24)***.

Large-scale protein recovery using ATPS requires two operations: (i) mixing of the phase components followed by phase dispersal, and (ii) phase separation.

In batch processes, equilibration is usually done in agitated vessels, and in mixer-settler devices. A few minutes of gentle stirring are normally enough to obtain phase and partition equilibrium ***(6)***. The fast approach to equilibrium is owing to the low interfacial tension between the two phases, which enables the formation of very small droplets and thus a large interface for mass transfer with low energy input ***(6)***. Phase separation is performed either by settling under gravitational force for fast-settling systems like PEG-salt systems, or by continuously operating common centrifugal separators ***(6)***.

An alternate scheme is the use of a column-type extractor to improve extraction efficiency using ATPS. The selection of a particular continuous contacting column, depends on the needs of the operation, the properties of the biomolecules, and the type of ATPS involved ***(13)***. Columns can be operated with countercurrent flow of both phases or by keeping one phase stationary. When operating an extraction column with ATPS, the problem is not the generation of sufficient exchange surface between phases as in the case of organic

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solvents, but the avoidance of very small droplet formation, which will decrease the performance and lead to flooding of the column *(2)*.

For the design of commercial-scale columns, data on the following parameters are needed: protein mass-transfer coefficients, dispersed phase holdup, and the extent of mixing in both phases. Usually, these parameters are dependent on the phase velocities, and physical properties, and also on the column geometry *(25)*.

In this chapter we present the equipment and the methodology for aqueous two-phase extrasction of proteins on large scale using mixer-settlers and column contactors. The reader is referred to the literature refs. given in the chapter for details of the specific equipment/procedure.

2. Materials

2.1. Chemicals

1. Phase Components: Most large-scale applications of ATPS are based on polymer-salt systems. Poly(ethylene glycol) (PEG) (Sigma, St. Louis, MO) is the top phase polymer and the salt, namely phosphate, sulfate, or citrate, is the bottom phase component. As an alternative to salt, commercial hydroxypropyl starch (Reppal, Carbamyl AB, Kristianstad, Sweden), or crude dextran (Sigma), can be used as the bottom phase rich polymer (*see* **Notes 1–3**) (*see also* Chapter 3).
2. Salts: In case of polymer–polymer systems, the addition of salts may be needed as buffer components to maintain the pH, or to direct the partitioning of target biomolecule.

2.2. Equipment

The liquid–liquid extraction equipment may be classified, according to the construction and operational characteristics into two groups: stagewise contactors and differential contactors. In the former type of contactors there are discrete number of stages in which two phases are equilibrated and then separated either by gravity (mixer-settler and columns) or by disk stack centrifuges. In the differential contactors the composition of phases changes continuously *(29)*, and two types can be considered: column contactors where separation is achieved by gravity, and centrifugal contactors. The continuous countercurrent flow is maintained owing to the difference in the density of the phases (top and bottom), and either the gravity force (vertical columns) or centrifugal force (centrifugal extractors).

2.2.1. Stagewise Contactors

1. Mixers and Mixer-Settlers: A mixer-settler unit consists of two cylindrical tanks, one provided with a centrally located paddle agitator for mixing the phases and the other for settling the mixed phases (*see* **Notes 4** and **5**). The operation may be

carried out in batch fashion or with continuous flow. For batch operations, the same vessel will serve the purpose for both mixing and settling, whereas for continuous mode, separate vessels are usually used. The mixer-settler units may be arranged in continuous countercurrent mode, either vertically (cascade) or horizontally (batteries) (**Fig. 1A–C**).

2. Mixer-Disk Stack Centrifuges: When the separation time under gravity is too long, commercially available separators can be used to speed up separation. Disk-stack separators are used in a continuous mode for highly efficient separation, and a countercurrent installation of several units can be used to achieve high yields.

   The disk-stack separator consists of a rotating bowl with a double-pump discharge for the light and heavy phases. The liquid mixture is fed through the stationary feed pipe into the rotating bowl. It enters the disk stack through rising channels in the disks and is separated between the narrow disks (**Fig. 2**) ***(31)***.
3. Column Contactors
   a. Perforated Plate Column: Perforated plate (sieve plate) columns are essentially a stack of several small spray columns (usually the diameter of each stage is greater than its height) (**Fig. 3**). Plates separate the stages with large numbers of small holes either drilled or punched on them.
   b. Perforated Rotating Disc Contactor (PRDC): The PRDC consists of a cylindrical vessel containing perforated disks mounted on a central rotating shaft (**Fig. 4**) (*see* **Note 6**).
   c. Kühni Column: This extraction column consists of a cylindrical vessel with a central rotating shaft carrying closed impellers. A perforated plate is located between each stage to control the droplet holdup (**Fig. 5**) (*see* **Note 6**).
   d. York-Scheibel Column: This type of extractor consists of a cylindrical vessel with alternate packed and unpacked sections serving as settling and mixing chambers. A centrally mounted rotating shaft carries a number of equally spaced turbine blade impellers rotating within the unpacked sections (**Fig. 6**). The packed settling chambers are normally filled with York mesh packing having about 90% voids.

2.2.2. Differential Contactors

1. Column Contactors
   a. Spray Column: Spray columns are the simplest of liquid–liquid extractors involving dispersion of one of the liquids. These vertical columns comprise an empty shell where the light and the heavy liquids are introduced and removed as shown schematically in **Fig. 7**. The dispersed phase is distributed with the help of a orifice or a nozzle. If the dispersed phase is the light, it is distributed at the bottom, while the heavy phase is distributed at the top of the column.
   b. Packed Column: Packed columns are essentially a stack of packings arranged regularly or irregularly in vertical towers over a perforated support, as shown in **Fig. 8**. These columns are equipped with suitable liquid distributors and

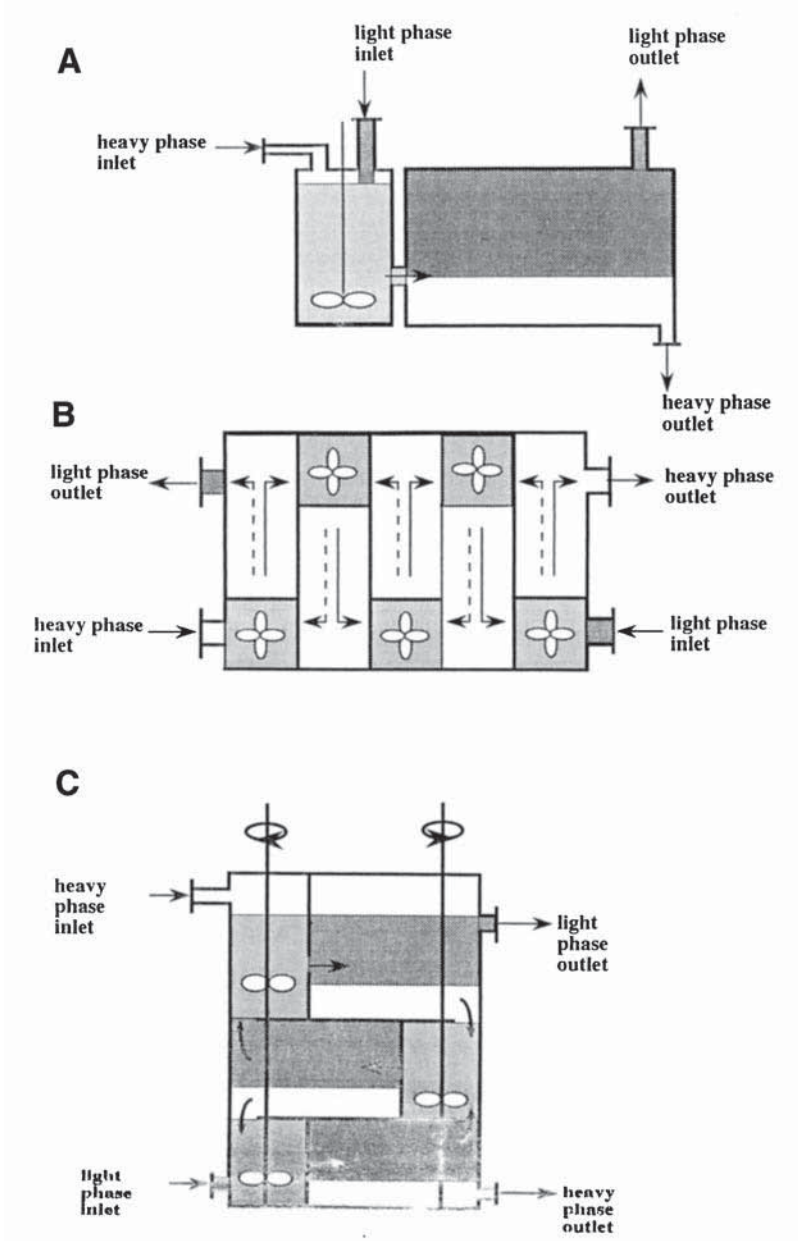

Fig. 1. Schematic representation of **(A)** mixer-settler unit, **(B)** horizontal battery, and **(C)** vertical cascade.

have grids for packing supports. The packings commonly used are Raschig and Lessing rings, and Intalox and Berl saddles, each possessing specific

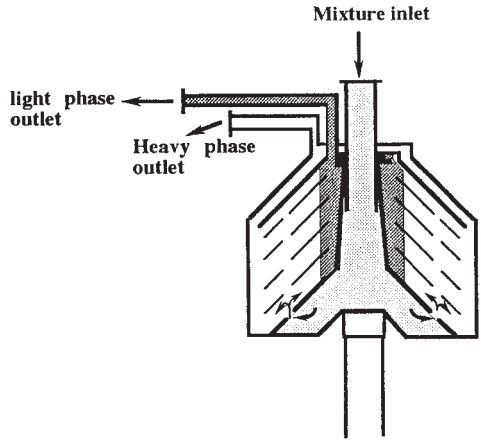

Fig. 2. Schematic representation of a disk stack contactor ***(31)***. Continuous recovery of intracellular products by crosscurrent centrifugal extraction using PEG/salt systems has been used ***(2,5)***.

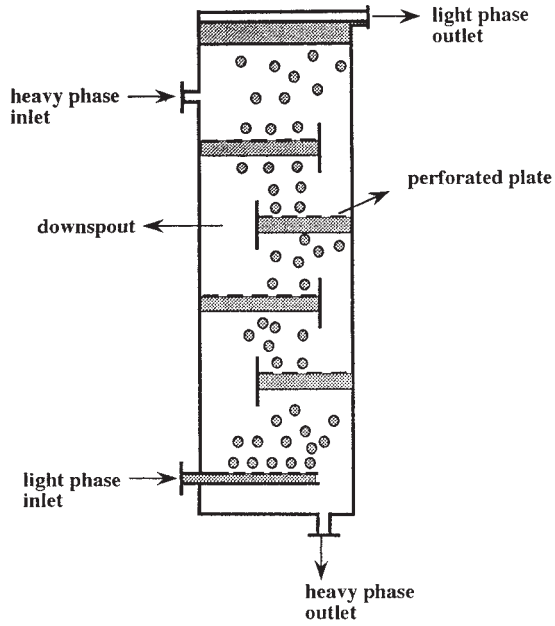

Fig. 3. Schematic representation of a Perforated Plate Column ***(29)***.

advantages with respect to cost, surface availability, interface regeneration, pressure drop, weight, and corrosion resistance ***(30)***.

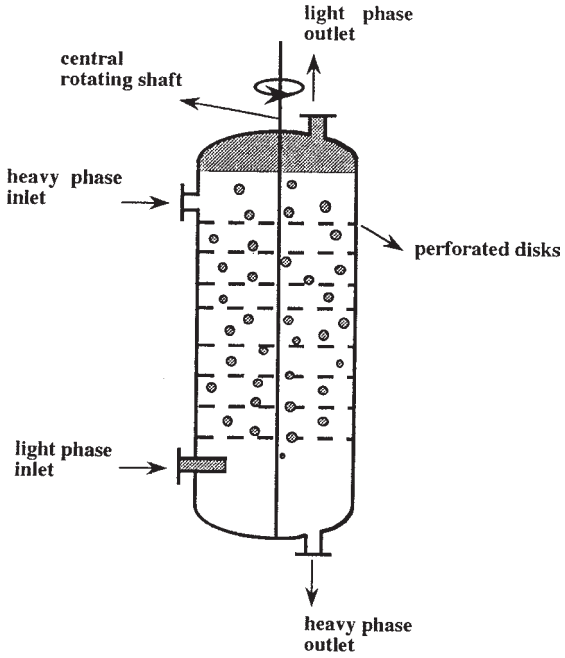

Fig. 4. Schematic representation of PRDC. The PRDC was used to extract a recombinant protein, cytochrome b_5, from disrupted *Escherichia coli* cells with ATPS of PEG/salt ***(38)***.

c. Horizontal Rotary Contactors: The Graesser contactor consists of a horizontal cylinder in which is mounted a rotor disk assembly on a central shaft along the cylindrical axis. The disk comprises several fixed semicircular cups or buckets around the circle diameter. In operation the interface level is kept along the axis of the shaft (*see* **Note 7**).

2. Centrifugal Contactors
   a. Podbielniak extractor: Consists of an internal cylindrical rotor. Typically, concentric cylinders (perforated or not) are wound inside the rotor, rotating about a horizontal shaft. The centrifugal force created makes the heavy liquid flow countercurrently to the light liquid in the direction of the periphery of the spiral, where it is collected out of the machine. Conversely, the light liquid flows to the center and is removed out (**Fig. 9**).
   b. Centrifugal liquid–liquid chromatography: This system operates in continuous mode with a liquid mobile phase and a liquid stationary phase; the latter remains inside the column by the interaction of the special internal geometry of the apparatus and the centrifugal field.

      One type of apparatus design consists of a series of cartridge composed by several fine channels, which are connected in series**,** and are submitted to a centrifugal field. These devices are called *centrifugal partition chromatogra-*

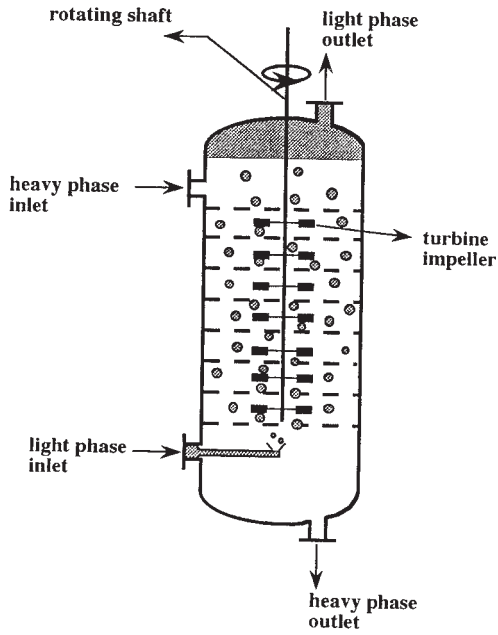

Fig. 5. Schematic representation of a Kühni column ***(39)***. This type of column has been used by Hustedt et al. ***(32)*** for enzyme purification using ATPS.

phy, centrifugal droplet countercurrent chromatography; or *Sanki* from its fabricate name.

A second type of design is denominated *coil planet centrifuge chromatography.* It consists of a column made of teflon tube helical coiled in one or several layers and wrapped around a cylinder. The tube is rotating at a certain angular speed around its own axis. The assembly, column plus cylinder, is also rotating at the same angular velocity and in the same direction around the main axis of the system (synchronous movement). In *cross-axis coil planet centrifuge,* both the rotation axis are positioned in a perpendicular form (**Fig. 10A,B**).

3. Methods

The methods described in the following paragraphs are used for the operation of columns (either stagewise or differential). The operation of the mixer or mixer settler devices, do not differ much from the laboratory test tubes, and is, therefore, not explored (*see* **Note 4**). Numerous processes occur during extraction in columns. Along with mass transfer data, hydrodynamics parameters of hold-up, drop size and velocity, back mixing in the phases, and flooding performance are required for column design. A brief description of the methods used to account for these parameters is also provided.

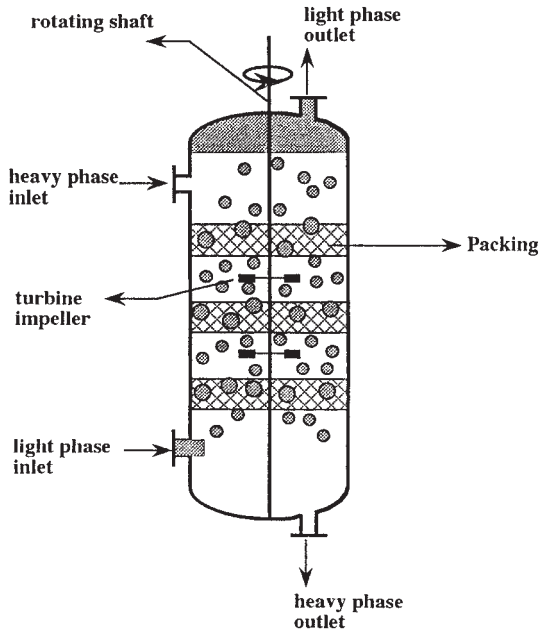

Fig. 6. Schematic representation of a York-Scheibel column ***(29)***. This column has been used for the extraction of proteins using polymer-salt and polymer-polymer systems ***(40,36)***.

1. ATPS preparation: The aqueous two-phase systems are prepared by adding the polymers and salts required according to the desired composition (*see* Chapter 3). The system equilibrium and separation are achieved by mixing and letting it stand for several hours. The time required for separation will depend on the density difference between both phases and on the phases' viscosities. Low-density differences will give rise to high separation times; highly viscous phases will give rise to higher separation times. A centrifuge can be used to enhance the separation. After phase separation, physical properties of both the phases are determined.
2. Determination of physical properties of the phases: The two-phase system is characterized with respect to density and viscosity of the phases, and interfacial tension (*see* **Note 8**).
3. Measurement of partition coefficient of solute: A previous study of the partition is done in a test tube to determine the partition coefficient, *m,* defined as ratio of the solute concentration between the top and bottom phases, respectively.
4. Column operation: The operation of the column is started after separation and characterization of the phases. The apparatus comprises the extraction column (either stagewise or differential) and one or two peristaltic pumps. Before feeding the column with the phases at the desired flow rates, these have to be calibrated due to the different phases' properties. Pump calibration is achieved by

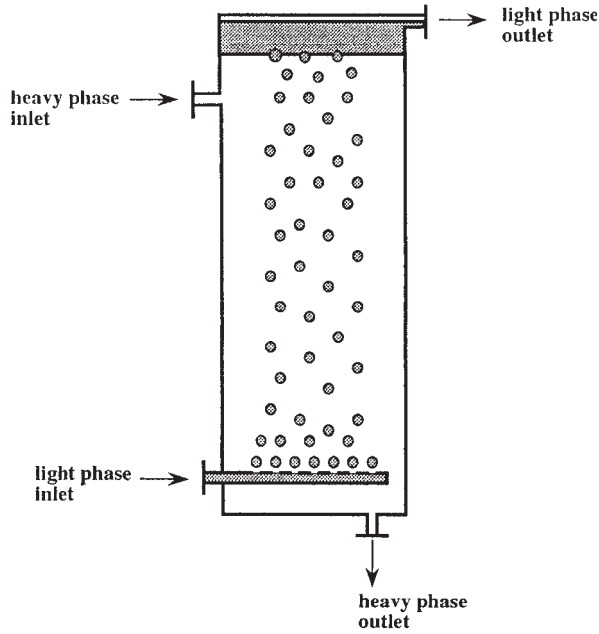

Fig. 7. Schematic representation of a spray column. The spray column is the most studied using ATPS of PEG/Dextran ***(25)***, PEG/salt ***(41,42)***, PEG/maltodextrin ***(43)***, and PEG/Reppal ***(44)***.

fixing different speeds and noting the time required to fill a fixed volume. Pump tubings in different materials and inside diameters are available. The agitation speed is calibrated with a mobile tachometer in columns involving agitation (Kühni, PRDC).

The column is then filled with the two phases, at the desired volume ratio. After that, the phases are fed to the column at a fixed flow rate, and according to the selected operation mode (countercurrent, or keeping one of the phases stationary). In extraction experiments, one of the phases contains already the crude solution having the target solute which is often a protein. Attention should be given to the loading volumes, owing to possible equilibrium deviations and precipitation.

Following start up of the column, the steady-state has to be achieved, defined with respect to the dispersion characteristics. This means that the height of the dispersion stabilizes after this period, which is strongly dependent on the system and operation mode.

For centrifugal liquid–liquid chromatography columns, the apparatus set-up comprises two pumps, the chromatographic column device, an injector, a detector and, eventually, a sample collector. One of the pumps fills up the column with the stationary phase, and the other will pump the mobile phase. For the coil planet

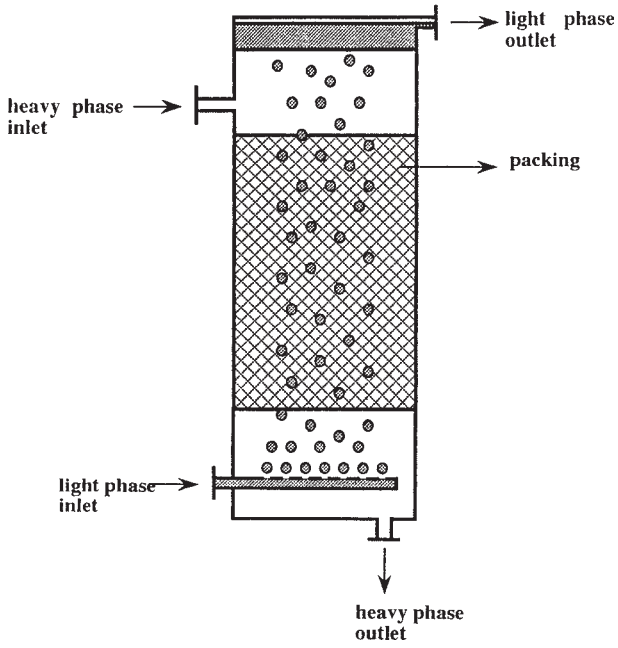

Fig. 8. Schematic representation of a packed column ***(29)***. Extraction of proteins using ATPS in a packed column is reported in ref. ***(13)***.

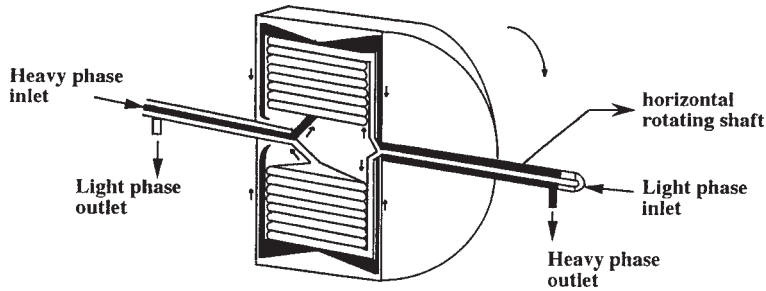

Fig. 9. Schematic representation of Podbielniak extractor. For ATPS application of Podbielniak extractor, *see* **refs. *2*** and ***32***.

centrifuge chromatographic columns, the mobile phase feeding is initiated after starting the rotation of the column. In the Sanki columns, if the light phase is the mobile one it will be pumped in ascending mode and vice versa for the heavy phase. The injector will inject the samples, which may be untreated and include solids, such as fermentation broths.

5. Determination of fractional dispersed hold-up and flooding velocities: The volume of all droplets (dispersed phase) in the contactor at any given time is referred to as the operational holdup of the dispersed phase generally expressed as a frac-

tion of the contactor volume. Fractional dispersed hold-up can be experimentally determined with the displacement method (*see* **Notes 9** and **10**).

6. Determination of drop velocity: The drop velocities of the dispersed phase are measured by following the dynamic disengagement of the heavy phase (*see* **Note 11**).
7. Determination of drop size: Several techniques are available for drop size measurements, such as direct photography, microphotography, light transmission, laser optics, and sampling through a capillary probe (*see* **Note 12**).
8. Axial mixing in the continuous phase: Axial mixing in the continuous moving stream can be evaluated by measurements of residence time distribution (*see* **Note 13**).
9. Mass transfer determination: To determine overall mass transfer coefficients, partition coefficients and inlet and outlet solute concentrations must be monitored (*see* **Note 14**).

4. Notes

1. The cost of the phase components as well as the waste treatment are major factors to be taken into consideration in the process scale-up. PEG and the various salts are regarded as inexpensive, and have almost similar cost per unit weight. Fractionated dextran is prohibitively expensive (~$400/kg) for large scale purposes. Crude dextran (after limited hydrolysis), hydroxypropyl starch (Reppal), maltodextrins, etc., are other less expensive alternatives.

   Phosphate is regarded as one of the more difficult nutrients to be removed efficiently in the wastewater treatment process. Biodegradable and nontoxic polymers, like PEG, dextran, and maltodextrin, are desirable from a pollution point of view. PEG is biodegradable, though by a slow process. Recycling of phase components will, thus, reduce the process cost by decreasing expenditure on chemicals and waste water treatment ***(7,24)***.
2. High viscosity of the phases (e.g., of crude dextran, Reppal, and so forth) and the long time required for its separation is a limitation for the use of some polymers at an industrial scale.
3. For affinity partitioning, application of PEG/salt systems is limited due to high ionic strength, which severely influences the affinity interactions (except in metal ion affinity partitioning) (*see* Chapters 30 and 31).
4. The prime basis of scale-up of batch mixers is often the geometric similarity, particularly equal power per unit volume, although the most desirable practical criterion is equal blending per unit time ***(30)***.
5. The use of static in-line mixers with ATPS has been investigated and used in recovery processes ***(7,22,26–28)***. Agitated vessel-centrifuge or static mixer-centrifuge assemblies are expensive combinations and provide one theoretical stage. In many applications more than one theoretical stage is required to achieve the desired purity.
6. In PRDC and Kühni columns the disks occupy the entire cross-sectional area preventing the backmixing.
7. The Graesser contactor has been tested for separation of PEG/salt systems ***(2)***, and some preliminary work was reported by Hustedt et al. ***(32)***.

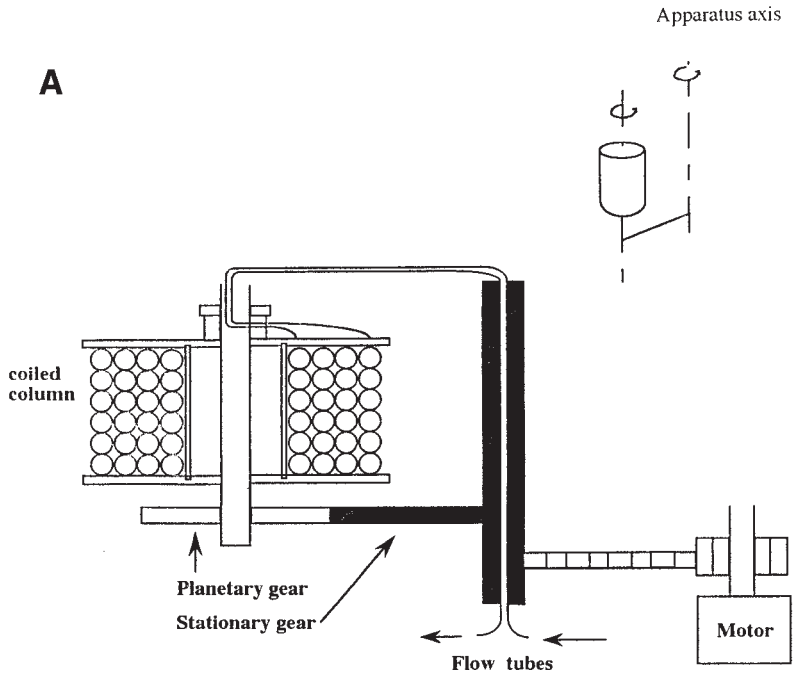

Fig. 10. Schematic representation of a coil planet centrifuge chromatography: **(A)** synchronous and **(B)** crossaxis. See **refs. *45–47*** for application of Centrifugal liquid–liquid chromatography for extraction of proteins in aqueous two-phase systems.

8. Approximate density values can be easily calculated using available empirical equations ***(13)***, or can be measured by using a specific gravity bottle. Viscosity can be measured by using the traditional capillary viscometer.

   Interfacial tension between the phases is very low (0.0001–0.1 dyne/cm) requiring a spinning drop type technique to get accurate values ***(33)***. Approximate values may be determined with a normal staglometer ***(34)***.
9. Once the steady-state is attained, the top (or bottom) interface position is marked. The inlet and outlet streams are then simultaneously closed and the interface position is observed until no further movement takes place. After that, the continuous phase stream is turned on again and the dispersed phase is forced out of the column, being replaced by the continuous phase, until the interface regains its original position. The pump is then stopped instantly and the collected volume of displaced liquid is determined. The volume of the displaced liquid to the actual void volume will be the hold-up under the operated conditions ***(29)***.

   The hold-up can be correlated with the dispersed phase velocity, the physical properties of the phases, and the column design parameters. Different models can be found in the literature. Alternatively, the physical properties can be correlated with the tie line length ***(29)***.

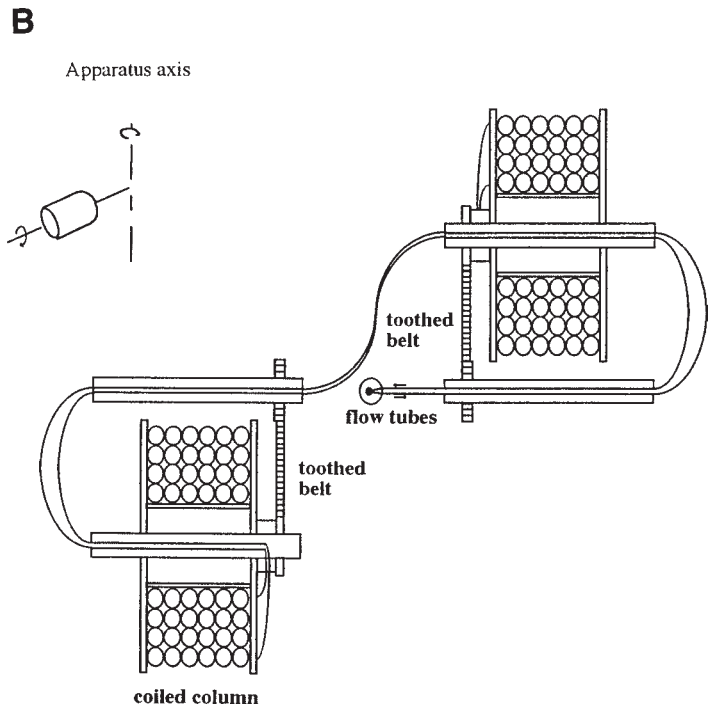

Fig. 10B.

Phase inversion, characterized by interchange between the dispersed and continuous phases, can occur at high hold-up values and when the coalescence rate far exceeds the breakage rate.

As an alternative to the displacement method, electroresistivity, light beam extinction, pressure differential and ultrasonic techniques can be used ***(35)***.

10. Stable operation in countercurrent mode is possible as long as the rate of arrival of droplets in any section does not exceed the coalescence rate at the main interface. Once this value is exceeded, droplet backup will occur at the interface and slowly build back into the column active area, a condition known as flooding ***(30)***.

Flooding occurs owing to high velocity of the continuous phase which will cause the smaller droplets to fall (or rise) more slowly forming a dense region which eventually coalesces, or can be caused by the formation of smaller droplets as it happens when the agitation is too high.

Flooding in the columns occurs for rather low phase velocities compared to the ones commonly observed in oganic-aqueous two-phase systems. Increasing the crosssectional areas can increase the limiting phase velocities.

The limiting flow-rates in the plate and packed columns are lower than those in the spray column. The problem of the low throughputs in these types of columns is decreased in the York-Scheibel column.

11. After the steady-state, the dispersion height is noted and the inflow and outflow streams are simultaneously closed. The interface position is then recorded with respect to time and the velocity is calculated from the variation of interface level with time.

    When the dispersed phase is the light phase, the drop rise velocity can be obtained by the method of Joshi et al. ***(36)***. This method is based on the linear variation of the ratio of the dispersed phase velocity, v_d to the fractional dispersed phase hold-up, E_d, with v_d: $v_d/E_d = C_o\, v_d + C_1$, where C_0 and C_1 are constants.

    The drop rise velocity relative to the continuous phase (slip velocity-v_s) is obtained from the intercept as $v_s = C_1/(1–E_d)$.

12. For the determination of drop size, photography is the most straightforward method when it is possible to take a clear picture. A water pocket is often put on the wall to eliminate the distortion of the droplets image due to the curved surface of the column. Another alternative is to immerse the column in a rectangular tank.

    The microphotography technique enables the determination of bubbles with sizes from 0.1–3 μm and the set-up is the following: camera with motor drive, extension tube, objective lens (planachromatic) with magnification, a protective cup to eliminate the interference of dispersed drops between the objective lens and the object plane; an electronic flash with a fiber-optic light guide, enables lighting of the object plane effectively, and at the same time catches fast moving bubbles ***(37)***.

    To get the drop size distribution, a commercial software package can be used. Some of the observations of drop size with respect to system properties have been as follows:

    a. The lower the interfacial tension, smaller are the drop diameters; the continuous phase viscosity has only a small effect on the drop diameter ***(29)***.
    b. Average drop size and drop velocity increase with an increase in the system composition (increase in the length of the tie-line).
    c. The drop size is smaller when the light phase is dispersed in the heavy phase ***(36)***.
    d. Very fine dispersions are produced by turbo-mixers using high speed impellers, or by the use of orifice plates and jets. Equipment using single nozzles, perforated plates, tower packing, produces discrete droplets of relatively large size that settle quickly ***(29)***.

13. The method used for determination of axial mixing can be either a pulse or step input of tracer soluble in the continuous phase and nontransferable in the dispersed phase. The tracers commonly employed are

    a. soluble dyes;
    b. radioactive tracers; and
    c. electrolyte solutions.

    The tracer can be internal sampling as a function of location at various positions along the column axis in the direction of the bulk flow at any given instant, or the concentration at a specific location such as exit of the column as a function of time.

    High degree of backmixing in the continuous phase can be a serious limitation in the spray column. In the plate and PRDC columns, and in the packed and York-

Scheibel columns the problem of backmixing in the continuous phase is strongly decreased due to the presence of the plates and packings, respectively.

14. Assuming that:
    a. Both phases are immiscible;
    b. The dispersed phase moves in a plug flow manner;
    c. The distribution law holds over the range of polymer concentrations; and
    d. The amount of solute extracted is small;

    the continuous phase solute concentration will change little during the extraction and may be regarded as constant, and a quasi steady-state can be assumed, i.e., the driving force for solute transfer is kept for all column height. Under these operating conditions the equation of the mass balance for solute extraction over a differential height (dh):

$$L_d \, dC_d = K_d a (C_d - mC_c) S \, dh,$$

    simplifies to give the following expression for the overall mass transfer coefficient (K_d, ms^{-1}):

$$K_d a = (L_d/V_d) \ln [(C_{di} - mC_c)/(C_{do} - mC_c)]$$

    where L_d, volumetric flow-rate of dispersed phase, m^3/s; V_d, volume dispersion, m^3; a, interfacial area/U vol, $m^2\ m^{-3}$; m: partition coefficient of solute; S, crosssection of the column, m^2; C, concentration of solute; d, dispersed phase; c, continuous phase; i, inlet; and o, outlet.

    Mass transfer correlations can be determined as previously referred for the holdup. Some hints about mass transfer:

    a. The solute mass transfer between dispersed and continuous phases depends on the dispersion of drops, distribution of drop sizes, the repeated coalescence and redispersion of droplets and hydrodynamic effects. The small drop size of the dispersed phase enables a large interface area, which leads to high-mass transfer. However, very fine sizes of dispersion are not desirable because of consequent higher phase separation times ***(29)***.
    b. Direction of the solute transfer can affect the dispersed phase-transfer coefficient, holdup and flooding velocities owing to the effects it has on the coalescence characteristics of the droplets. Correlations generated in the absence of mass-transfer and then applied to mass-transfer conditions will not give the same accuracy of representation.
    c. If the partition coefficient of the solute, m, is very large, that is, if the solute is nearly insoluble in the bottom phase, the top phase resistance tends to predominate, and vice versa for very low m values.
    d. The diffusivity of the solutes varies inversely with the viscosity. In dispersions, the phase with higher viscosity has higher film thickness or contact times, which leads to lower values of mass-transfer coefficients.
    e. In mass transfer, when the top phase is the dispersed and the bottom phase is the continuous, the overall mass transfer coefficients, K_c and K_d, are given by:

$$1/K_d = 1/k_d + m/k_c \qquad 1/K_c = 1/k_c + 1/mk_d$$

When the top phase is the continuous and the bottom phase is the dispersed, the overall mass transfer coefficients, K_c and K_d, are given by:

$$1/K_d = 1/k_d + 1/mk_c \qquad 1/K_c = 1/k_c + m/k_d$$

The overall mass transfer is dispersed phase controlled if the dispersed phase mass coefficient (k_d) is much lower than the continuous phase mass transfer coefficient (k_c), and if

i) m is low and the top phase is the dispersed $\quad 1/K_d \approx 1/k_d \quad 1/K_c \approx 1/mk_d$
ii) m is high and the bottom phase is the dispersed $\quad 1/K_d \approx 1/k_d \quad 1/K_c \approx m/k_d$

f. The overall mass transfer is continuous phase controlled, if the dispersed phase mass coefficient is much higher than the continuous phase mass transfer coefficient and if

i) m is high and the top phase is the dispersed $\quad 1/K_d \approx m/k_c \quad 1/K_c \approx 1/k_c$
ii) m is low and the bottom phase is the dispersed $\quad 1/K_c \approx 1/mk_c \quad 1/K_c \approx 1/k_c$

g. In the continuous countercurrent extraction columns, axial mixing in either phase lowers the effective coefficient of mass transfer.

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Extractive Bioconversion in Aqueous Two-Phase Systems

Rajni Hatti-Kaul

1. Introduction

Biotechnological processes are characterized, in general, by low productivity and dilute product streams. This is attributed to a variety of reasons:

1. Product inhibition resulting in metabolic control;
2. Toxicity of product—e.g., organic acid, a solvent—to the producing cells; and
3. Instability of the product—e.g., a protein—owing to harsh conditions in the reactor or degradation by proteases.

This leads to high costs in the subsequent isolation and purification of the product. From the point of view of process economy, a high product concentration is desirable. A technological solution for improving the productivity would be to circumvent the biological limitations. This concept is feasible by "extractive bioconversion" or "*in situ* product recovery," in which the product removal step is integrated with that of bioconversion. By this means, the product is continuously removed from its site of production, thus relieving the inhibition/toxicity. It also provides the possibility to run the process as a continuous system, in which the product is recovered directly from the bioreactor and is also protected from degradation. If the product recovery is made selective, it can help to achieve significant purification rapidly and hence facilitate its further upgrading. Different means to accomplish extractive bioconversion include adsorption, membrane filtration, and liquid–liquid extraction ***(1,2)***.

Extractive bioconversion by liquid–liquid extraction is designed in a manner that the biocatalytic reaction takes place in one of the phases and the products are extracted into the other phase. In an ideal situation, this would require extreme partitioning of the biocatalyst, together with the substrate, into the

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phase meant for bioconversion, and that of the product into the extractant phase. Extractive bioconversion using organic solvent as the extracting phase has been attempted. However, a general observation has been that the solvents that are good extractants are also toxic to the producer organism ***(2)***. Furthermore, a high interfacial tension in the water-organic solvent system has a denaturing effect on the biocatalysts. Aqueous two-phase systems (ATPS) circumvent the disadvantages of the water-organic solvent two-phase systems. These systems provide a biocompatible environment; in fact, the polymers constituting the phases may also have a stabilizing influence on the biocatalysts.

The bioconversion process in ATPS is facilitated by the low interfacial tension, which leads to formation of small phase droplets during mixing of the phases, hence minimizing migration distances and promoting mass transfer. The biocatalyst is temporarily immobilized in the phase droplets, and can be regarded as soluble-immobilized systems. In contrast to the insolubilized systems, ATPS seems to be attractive for bioconversions involving macromolecular or particulate substrates because of the lack of steric hindrances. Another distinguishing feature is that it is easier to control a reaction involving a number of enzymes, e.g., it is possible to supplement the system with more of the labile biocatalyst during a continuous reaction.

Several examples of bioconversion in ATPS are known (**Table 1**). These include single-step bioconversions with an isolated enzyme or an intact cell, multistep bioconversions, of both high- and low-molecular weight substrates, involving more than one enzyme and/or living cell, and production of proteins by microbial cells. Bioconversions in ATPS may be performed in a semicontinuous/continuous mode, achieved by integrating the system further with another separation technique e.g., ultrafiltration ***(8,9)***, adsorption ***(10,18)*** for product isolation. **Figure 1** shows the concept of extractive bioconversion in ATPS.

This chapter provides a general description of the procedure adopted for extractive bioconversion in aqueous two-phase systems.

2. Materials

1. Phase forming components (*see* **Note 1**). Often, polymer–polymer systems are used, but polymer–salt systems may also be applicable (*see* **Table 1**) if the biocatalytic reaction is not affected.
2. Biocatalyst, e.g., enzyme(s), nonviable/viable cells (*see* **Note 1**).
3. Buffer/medium components (*see* **Note 1**). For bioconversions not requiring living/growing cells, addition of a suitable buffer would be necessary depending on the optimal conditions required by the particular enzyme(s), whereas fermentations require the addition of nutrients, salts, etc., for growth of the organism.
4. Substrate (*see* **Note 1**).
5. Reactor system (*see* **Notes 1** and **2**).

Table 1
Examples of Extractive Bioconversions in Aqueous Two-Phase Systems

Reaction	Biocatalyst	Two-phase system	Reference
Production of glucose-6-phosphate from glucose	Hexokinase (+ ATP)	PEG/dextran	***(4)***
Deacylation of benzylpenicillin to 6-aminopenicillanic acid	Penicillin acylase	PEG/phosphate	***(5)***
Hydrolysis of casein proteins	α-Chymotrypsin	PEG/dextran	***(6)***
Hydrolysis of cellulose to reducing sugars	endo-β-glucanase + β-glucosidase	PEG/crude dextran	***(7,8)***
Starch hydrolysis to glucose	Amylase + glucoamylase	PEG/crude dextran	***(9)***
Transformation of hydrocortisone to prednisolone	*Arthrobacter simplex*	PEG/dextran	***(10)***
Regeneration of ATP	Chromatophores from *Rhodospirillum rubrum*	PEG/dextran	***(11)***
Fermentation of glucose to ethanol	*Saccharomyces cerevisiae*	PEG/dextran	***(12)***
Fermentation of glucose to acetone-butanol	*Clostridium acetobutylicum*	PEG/dextran	***(13)***
Lactic acid fermentation	*Lactococcus lactis*	Poly(ethyleneimine)/(hydroxyethyl) cellulose	***(14)***
Production of antibiotic, subtilin	*Bacillus subtilis*	PEG/phosphate	***(15)***
Production of toxin	*Clostridium tetani*	PEG/dextran	***(16)***
Production of α-amylase	*Bacillus subtilis*	PEG/dextran	***(17,18)***
Production of cellulase	*Trichoderma reesei*	PEG/pullulan	***(19,20)***
Production of chitinase	*Serratia marcescens*	PEG/dextran	***(21)***
Production of β-galactosidase	*Escherichia coli*	PEG/phosphate	***(22)***
Monoclonal antibody production	Hybridomas	PEG/dextran	***(23)***

3. Methods

1. Determine partitioning of biocatalyst(s), substrate(s), and product(s) in a number of two-phase systems in test tubes. Choose a system showing maximal separation of product from the biocatalyst and the product (*see* **Note 3**).
2. Mix the phase and buffer/medium components with the substrate in a reactor (*see* **Notes 1** and **2**). In case of fermentations, this procedure is done under aseptic conditions. Equilibrate at the desired temperature.

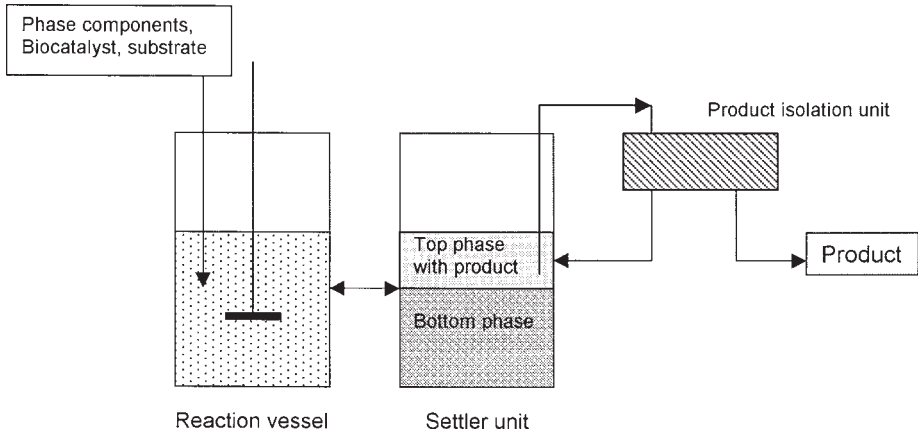

Fig. 1. Schematic presentation of extractive bioconversion in aqueous two-phase system.

3. Add the biocatalyst to initiate the reaction. Mix the phase system continuously and monitor the progress of the reaction (*see* **Note 4**).
4. Pump the phase mixture into the settler unit and allow the phase separation to take place (*see* **Notes 2** and **5**).
5. Drain off the extractant phase and process for the recovery of the product (*see* **Note 6**).
6. To the reaction phase containing the biocatalyst, is added a new extractant phase, or the previous extractant phase (from **step 5**) after removal of the product. More substrate and other required additives, e.g., buffer or medium components are added. Biocatalyst and phase-forming components may also need to be supplemented depending on their loss during each run. Pump the two-phase system back to the reaction vessel for the second batch of bioconversion (*see* **Note 7**).

4. Notes

1. The phase components, buffer or medium substances, substrate, and even the biocatalyst may be prepared as stock solutions, and then mixed in desired concentrations. For bioconversions requiring living cells, sterilization of all the solutions and the reactor is a prerequisite. Solutions may be sterilized by autoclaving or sterile-filtering.
2. The use of batch or mixer-settler type of reactor (*see* Chapter 36) has been mostly reported for extractive bioconversions, however extraction columns (Chapter 36) may also be employed. In the former, phase mixing and separation may take place in one vessel or the phase mixture may be pumped into another vessel for phase separation. The mixing of the phase system is accomplished by magnetic stirrer, turbine-impeller, or static-mixer, depending on the equipment available and also the scale of operation. The settler unit is designed so as to allow fast phase separation.

3. Minimizing the removal of the biocatalyst with the product-containing phase is crucial during extractive bioconversions. Similarily, minimizing the level of the product in the reaction phase is also important. Desirable partitioning of the reaction components in the two-phase systems is not always possible. It is relatively simple to obtain one-sided partitioning of cells, however, cell partitioning may change during fermentation owing to continuously changing ionic composition of the broth ***(14)***. Soluble enzymes show variable partitioning. To obtain favorable partitioning, enzymes may be modified with the polymer constituting the reaction phase ***(6)***. Alternatively, the extractant phase is passed over an ultrafiltration unit for retaining the biocatalysts and the polymers which can then be recycled to the reactor ***(9)***.

   High molecular weight substrates can be easily obtained in one phase, and often the enzymes catalyzing their conversion may also partition favorably by getting adsorbed to the substrate ***(9)***. Low molecular-weight substrates would be equally partitioned between the two phases; this may not affect the reaction efficiency because of high mass transfer rates in ATPS, but may involve loss of the substrate if not completely transformed. Loss of the valuable cofactor is minimized by its modification with the polymer which constitutes the reaction phase ***(4)***.

   In case the product is not preferentially partitioned into the extractant phase, the volume ratio of the two phases may be adjusted to obtain maximal amount into the extractant phase ***(16)***. Even the incorporation of an affinity ligand may help to pull the product into the extractant phase ***(23)***.
4. There are indications that changes in microenvironment of the microbial cells owing to the presence of phase polymers may contribute towards increased product formation in ATPS, e.g., by alterations in metabolism of the microorganisms ***(13)***, or change of membrane integrity ***(21)***.
5. The phase separation under gravity would depend on the viscosity, density difference between the phases, and also the settling area.
6. ATPS is further integrated with another separation technique for recovery of the products from the extractant phase. Small molecular weight products can be separated from the polymers by ultrafiltration which also helps to retain the polymers and biocatalysts (*see* **Note 3**) ***(8,9)***. Combining extraction in ATPS with ultrafiltration also protects the membrane from coming in direct contact with the particulate material (e.g., substrate) in the medium, thus increasing its operational life. Proteins may be separated by passing the phase over a chromatography column ***(18)***.
7. Recycling of cells for extractive fermentations in ATPS may lead to the accumulation of by-products in the reactor which may become inhibitory at high concentrations ***(13)***. Hence a bleed may be necessary to maintain them at a sufficiently low concentration.

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Enzyme Reaction in Polymer–Polymer Organic Solvent Two-Phase Systems

Bo Mattiasson

1. Introduction

Enzymes have been shown to operate successfully under nonphysiological conditions, e.g., in water-poor media consisting of almost pure nonpolar solvents. These new reaction conditions offer interesting possibilities to expand the scope of enzyme technology. An essential issue in this context is the method by which the enzyme is contacted with the solvent phase. A range of solid phase immobilization techniques *(1,2)* are available; use of enzymes in microemulsions *(3,4)*, and chemically modified enzymes *(5–7)* that are solubilized in the organic phase has also been reported.

In an effort to develop a reversible system in which the enzyme is made soluble and then recovered, complex formation between enzyme and a polymer can be performed. By dealing with polymers that are at least partly soluble in water and fully soluble in organic phase, it is possible to first mix the enzyme and the polymer in aqueous solution, to dry the mixture, and then to solubilize the complex in organic solvent. Such complexes are characterized by being held together by noncovalent forces and they will break as soon as water is added to the system.

The basic concepts concerning incompatibility of polymers have been developed for polymers dissolved in organic phase. This makes possible the use of enzyme–polymer complex with another polymer in organic phase to form a two-phase system such that the enzyme–polymer will preferentially be recovered from one phase and the other polymer will mainly constitute the second phase *(8)*. When performing catalytic conversions, one may have a situation where enzyme and substrate/product are partitioned to different phases;

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this will be advantageous for recovering the product from the reactant rich phase without disturbing the enzyme. If, on the other hand, the enzyme and the product partition to the same phase—it is a well-known fact that small molecules like most of the substrate/products studied here will partition fairly evenly between the two phases—one may, by manipulating the volume ratio of the phases, gear the system such that at least 90% of the product will be in the nonenzyme phase.

It should be stressed that the stability of enzyme molecules in general is improved when the water activity is low, and the complex formation between the enzyme molecule and a polymer stabilizes the enzyme even further ***(9,10)***. So far, very little experience is available to judge the potential of polymer-polymer organic two-phase systems, however, they do seem attractive for bioconversions. The advantages are the simplicity in handling, the convenience in mixing and the potential to break the system simply by adding water. The disadvantages still remain to be discovered.

This chapter presents the procedure for the use of enzyme–polymer complex in a two-phase format in organic solvent. Chymotrypsin catalyzed esterification of *N*-acetyl-phenylalanine (Ac-Phe) with ethanol is studied as an example. Additionally, construction of phase diagram of the two-phase system by continuous spectrophotometric measurement of turbidity as a two-phase mixture of polymers is diluted with a solvent is also described.

2. Materials

1. α-Chymotrypsin (E.C. 3.4.21.1) from bovine pancreas with specific activity of 46 U/mg (Sigma, St. Louis, MO).
2. *N*-Acetyl-phenylalanine (Ac-Phe) (Sigma).
3. Polystyrene (PS) with approx mol wt of 150,000 (BDH, Poole, UK).
4. Polymer for complexing with the enzyme (*see* **Note 1**). For example, ethylcellulose (EC) with an ethoxyl content of 48% and viscosity (5% solution in 80/20 toluene/ethanol) of 10 cP; polyvinylbutyral (PVB) with an average mol wt of 36,000 (Aldrich, Steinheim, Germany).

3. Methods

3.1. Phase Diagram

1. Dissolve a pair of polymers in separate volumes of chloroform. For example, 10% (w/w) solutions of EC (or PVB) and PS are prepared.
2. Mix the solutions at concentrations giving a two-phase system. The mixture (5 g) is stirred in a 30-mL beaker by means of a magnetic stirrer and continuously pumped through a flow cuvet in a spectrophotometer and back to the beaker (**Fig. 1**).
3. Dilute the mixture in the beaker with chloroform at a constant flow rate. Record the absorbance (or transmittance) at 600 nm. After a certain time of dilution of the phase system, a dramatic decrease in absorbance (or increase in transmit-

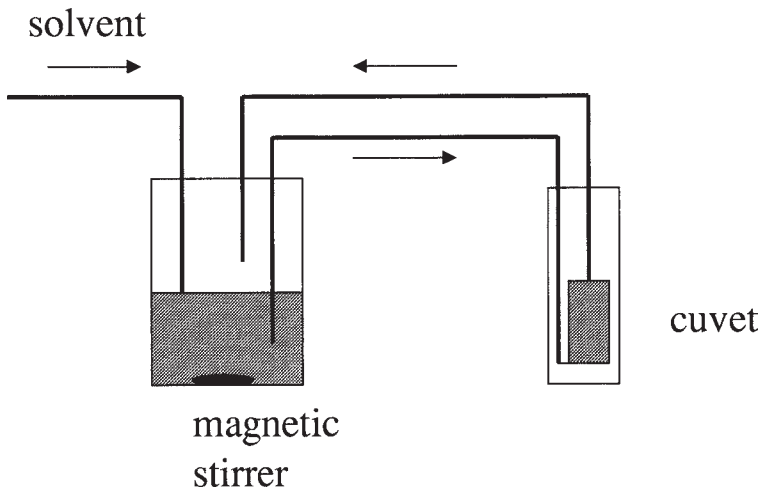

Fig. 1. Experimental set-up for registering phase-transitions in polymer–polymer organic solvent systems. The phase system is placed in a beaker and is stirred using a magnetic stirrer. A stream is continuously pumped via a cuvet placed in a spectrophotometer and back again to the system. Pure solvent is added to the beaker, thereby diluting the total volume. The absorbance is continuously registered in the spectrophotometer.

tance) takes place until a homogeneous solution is formed. Note the initial and final times for phase transition, t_1 and t_2, respectively (**Fig. 2**) (*see* **Note 2**).

4. From the phase transition times, calculate the concentrations of the polymers forming two-phase systems (*see* **Note 3**).
5. Repeat the procedure with a range of two-phase mixtures having varying concentrations of polymers. By measuring a number of systems in this manner, enough points are generated which are used to construct a phase diagram as in **Fig. 3** (*see* **Note 4**).
6. Prepare a mixture of known composition (e.g., 4%/4%, [w/w] of PS/EC) in 8 mL of chloroform and leave to separate into two phases (the two-phase systems could be the same as used in the previous steps). Measure the volume of each phase.
7. Withdraw 1 mL of each phase and mix with 3 mL of acetic acid to precipitate PS. Filter the mixtures on a sintered glass filter and determine the dry weight (after drying at 105°C for 4 h) of precipitated PS. Take 1 mL of aliquot from the filtered solution and evaporate to determine the weight of the other polymer.
8. Construct a tie line by connecting the two points on a phase diagram, each representing the composition of one of the phases.

3.2. Preparation of Modified Chymotrypsin

1. Add 1 g of EC moisturized by ethanol (a few mL ethanol/10 g of EC) to 1 mL of chymotrypsin solution in 100 m*M* sodium phosphate buffer, pH 7.8. Mix vigorously for 2 min.

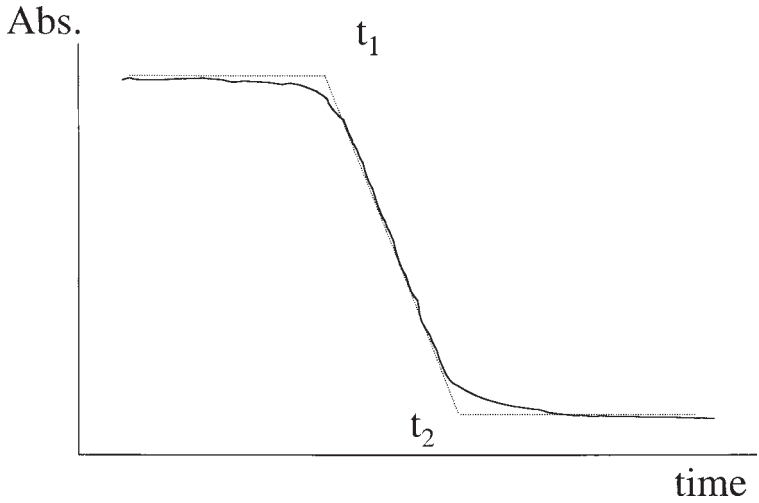

Fig. 2. Typical graph registered by the spectrophotometer upon dilution experiments. The dilution starts when a two-phase is present (represented by high absorbance) and, upon dilution, a transition to homogeneous solution takes place (low absorbance). Because addition of solvent takes place with constant pumping speed, time t_1 and t_2 are defining the degree of dilution of the system.

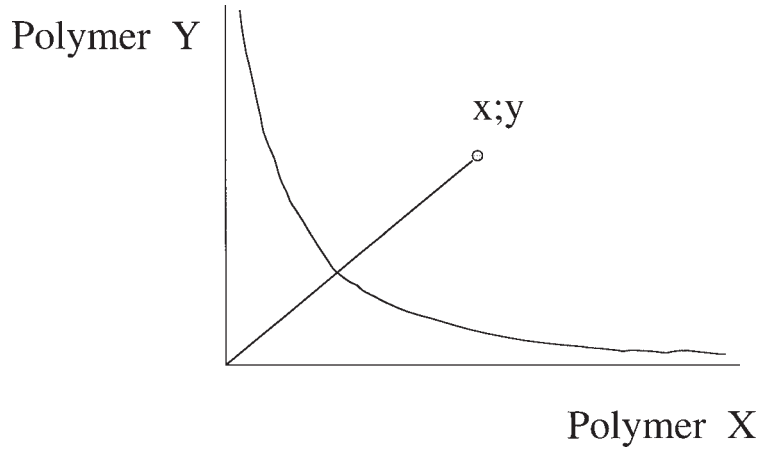

Fig. 3. Phase diagram of a polymer two-phase system. The concentrations of the individual polymers are marked on the axes, and a point in the diagram is then represented by two concentration figures, one for each polymer. The point marked in the diagram has the concentrations (x;y). All systems above the phase line represent two-phase systems, and points below represent homogeneous solutions. When a system of composition (x;y) is diluted with pure solvent, the composition will change according to the line drawn from the point to origin. During this dilution, there is a transition from two-phase to homogeneous system.

2. Freeze the resultant mixture and lyophilize under reduced pressure for 8 h to obtain the EC-modified chymotrypsin (EC-CT) (*see* **Note 5**).

3.3. Enzymatic Reaction in Polymer–Polymer System in Chloroform

1. Add 40 mg of EC-CT preparation, containing 80 mg enzyme/g polymer, to 4 mL of chloroform solution of 9% (w/w) PS (*see* **Note 6**).
2. After about 20 min of mixing, add the reactants, 10 m*M* Ac-Phe and 1 *M* ethanol, and also 0.5% (v/v) water. Shake on a reciprocal shaker at 200 rpm.
3. Once the reaction is complete, separate the phases using centrifugation in a bench top centrifuge for 5 min at 4400 rpm (the speed or the *g*-forces is not critical) (*see* **Note 7**).
4. Samples from each phase are withdrawn and analyzed using high-pressure liquid chromatography (HPLC) for substrate/product content (*see* **Note 6**).
5. To isolate the product, *N*-acetyl-phenyl-alanine-ethylester (Ac-Phe-OEt) from the PS-rich top phase, add methanol (3 mL to 1 mL sample) to precipitate the polymer. Filter the mixture. Recovery of both the product and polymer is more than 90%. The polymer may be recycled.
6. To reuse the enzyme–polymer preparation in the bottom phase, replace the top phase with a fresh solution from a phase system of identical polymer composition (*see* **Note 8**).

4. Notes

1. A critical issue is the selection of polymers. A key feature is that at least one of the polymers is compatible both with the organic solvent and with water. This is needed, otherwise it will be difficult to form the enzyme–polymer complexes. Thus, when choosing the polymer, one should also take into consideration which organic solvent system is to be used. One good way to handle this is through relation of solubility parameters for both polymers and solvents ***(11)***. A measure of interaction between the solvent and polymer can be the difference between their solubility parameters. The solubility parameters are a kind of lumped constant including contributions of dispersion forces, polar forces and hydrogen bonding ***(12–14)***.
2. Alternatively, a solution of EC (1–10%) is recirculated through a flow cuvet, while 10% PS solution is added to the aforementioned solution at a constant flow rate until turbidity appears, i.e., a two-phase system is formed. The time points, t_1 and t_2 correspond to the initial and final points of the increase in absorbance during transition from one phase to two phase ***(8)***.
3. The concentrations of the two polymers forming the two-phase system are calculated from the initial and final times of phase transition, t_1 and t_2, respectively, as follows ***(15)***:

$$C_{i,PS} = (C_{o,PS}\, m_o + t_i\, F)/(m_o + t_i F) \quad (1)$$

$$C_{i,EC} = (C_{o,\,EC}\, m_o + t_i\, F)/(m_o + t_i F) \quad (2)$$

Table 1
Partition of Product (Ac-Phe-OEt) in PVB-PS Two-Phase Systems in Chloroform[a]

PS : PVB (%:%,w/w)	V_t/V_b	K_p[b]	X[c] (%w/w)
5 : 5	0.8	0.80	37
8 : 2	2.5	0.79	67
9 : 1	7.4	0.86	86
9.2 : 0.8	15.8	0.99	94

[a]Samples taken after 24 h of reaction at 98% conversion after centrifugation for 5 min at 4400 rpm to induce phase separation. The reaction was carried out with 10 m*M* Ac-Phe, 1 *M* ethanol, and 0.5% (v/v) water.

[b]Partition coefficient of product (*Kp*) is expressed as C_t/C_b.

[c]Amount of product (*X*) in PS-rich top phase is expressed as percent of the total amount of formed product, i.e., $X = 100 \times (C_t - V_t)/(C_t \cdot V_t + C_b \cdot V_b)$. (Adapted with permission from **ref. *8***.)

where $C_{i,PS}$ and $C_{i,EC}$ are the concentrations (% w/w) of PS and EC, respectively, at point *i*, $C_{o,PS}$ and $C_{o,EC}$ are the initial concentrations (%, w/w) of PS and EC in undiluted two-phase systems; t_i is the time (min) required for transition from a two-phase to a one-phase system, F is the feed-rate (*g*/min) of dilution solvent, m_o is the initial mass (*g*) of the two-phase system.

4. This procedure gives a region on the binodial covering a small concentration range in which the phase transition takes place. In this region, mixtures exist as turbid systems. The variation in the width of this transition region has earlier been ascribed to heterogeneity of the polymer preparations ***(15,16)***. The density of the polymer phases in chloroform is slightly below the density of pure chloroform (1.40–1.46 kg/dm^3).
5. Similar treatment can also been used for other polymers e.g., PVB, poly(methyl methacrylate) (PMMA), poly(ethylene glycol) (PEG), or poly(vinyl methyl ketone) (PVK), when making CT-polymer complexes ***(8)***. Water activity of the enzyme-polymer preparation may be measured using water activity sensor, if necessary.
6. By varying the ratios of PS and EC within the concentration limits required to maintain the formation of two phases, the product and enzyme can be partitioned to different phases. Results obtained from a similar system based on varying ratios of PVB and PS are shown in **Table 1**.
7. The polymer-polymer systems in organic solvent spontaneously separate into two phases under gravity within minutes or several hours depending on the phase composition ***(8)***. Phase separation is facilitated by centrifugation at low *g* forces as for the aqueous two-phase systems.
8. Recycling of the enzyme-polymer complex by replacement of the top phase with another one from an identical two-phase system not containing the enzyme gives product yields above 90% only first two times for a PS-EC system. Upon repeated reuse, the yields drops, probably owing to accumulation of water in the system. Reduction in the amount of water added during every recycle, and using a top phase from a system with polymer-CT complex, provides improvements in yields ***(8)***.

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