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Biofunctionalization of Polymers and their Applications

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Preface

Polymer chemistry has dominated our daily lives through its application in all kinds of materials thanks to the ingenuity of polymer chemists and material scientists. Building on these successes, recent advances in the field of biotechnology have witnessed increased collaborations with polymer chemists and material science engineers resulting in the development of novel biofunctionalized polymers. This has partly been necessitated by the new global policy of the "polluter pays" principle favoring the development of green technologies and/or maximum exploitation of renewable polymers. It is with this hindsight that biofunctionalization of polymers (broadly defined in this book as the use of biological molecules or processes for the development of functional polymers) has emerged as one of the most rapidly expanding field finding applications in medical, textile, construction, electrical fields, etc. The developments and trends in biofunctionalization of polymers have facilitated the selective fusion of biological molecules and/or grafted bioactive molecules onto synthetic polymers, resulting in novel polymers. In many of these fields, enzymatic processes are replacing conventional chemical processes and again enzymes are actively being pursued as parts of the molecular biology toolbox necessary to design tailor-made natural polymers through genetic engineering. The development of biofunctionalized materials is consistent with the principles of green chemistry which is aimed at designing and commercializing processes and products that reduce the generation of pollutants, thereby eradicating health risks.

This volume therefore provides a concise overview of the advances in different fields of biofunctionalization of polymers for varied applications and will therefore inspire professionals in chemistry, biochemistry, biotechnology, engineering, agriculture, pharmacy, materials science, and related fields because it gives a good framework for understanding the complex nature of biofunctionalization of polymers. Although heavily biased toward biological processes, it also summarizes chemical synthesis processes where biological processes are still scarce. This is intended to capture and provoke the imagination of many scientists to explore in their wildest imagination the possibility of using green technologies for the described processes. The book covers the basic fundamentals and the biochemical

processes involved, as well as the technologies themselves within different areas of application and stages of development.

Examples in this book include the production of highly complex functional polymers through genetic engineering (the so-called recombinamers) with potential applications in biomedical and biotechnology. This approach exemplified by elastin-like recombinamers (ELRs), combine sequential and architectural molecular complexity with diverse bioactivities, allows for easy and highly effective design and synthesis of complex polymers with selected and sophisticated properties for cutting-edge applications in biomedicine and nano(bio)technology. Similarly advances in exploitation of polyhydroxyalkanoates (PHA) (a family of biopolyesters synthesized by many types of bacteria with many attractive properties including processability, biodegradability, biocompatibility, and sustainability) are presented explaining their applications in the production of bioplastic packaging, biofuel, medical implants, drug delivery, protein purification, chiral chemicals, and drug development with some products already at industrial scale. To achieve this goal, genetic engineering, pathway modification, or synthetic biology approaches are being actively pursued.

Technologies for the maximum exploitation of natural polymers such as chitin are also being investigated. The polysaccharide chitin is intensively being investigated in the biomedical field for a variety of application among them for the preparation of wound dressing materials and scaffolds for tissue regeneration. Functionalization of these biopolymers for improvement of properties such as solubility, or introduction of active functional groups and blending with other intrinsically bioactive polymers is the subject of intense research. Such modifications would allow going the beyond traditional approaches for treatments of dermal injuries. Recent progress in dermal tissue management is directed toward physiological repair at molecular level. The introduction of a variety of targeted functionalities to chitin/chitosan backbone for dermal tissue repair is an active research area aimed at developing polymers that interact with the injured skin at molecular level, thereby accelerating the healing process. Another area of interest is the exploitation of cyclodextrin-based polyrotaxanes and polypseudorotaxanes that have attracted scientific and industrial interest over the past 18 years for the development of supramolecular biomaterials for drug and gene delivery. Because of their attractive properties such as low toxicity, sliding, dethreading, and easy to modify, cyclodextrin-based supramolecular polymers have attracted an increasing attention as vectors for effective drug and gene carrier. Intense research is aimed at developing temperature-responsive, pH-sensitive, and controllable hydrolyzable polyrotaxane hydrogels as injectable drug delivery system for sustained and controlled drug release.

Another active field covered is the “biomimetics field or enzymatic polymer functionalization”, where biologists and engineers endeavor to produce bioinspired materials through enzymatic modifications as exemplified by the functionalizations of different polymeric matrices using enzymes such as transglutaminases, laccases, peroxidases, lipases, and tyrosinases. This area is fast expanding with current research efforts covering advanced materials processing

for different fields such as electronics, construction, textile, pharmacy, medicine, etc. Tranglutaminases and tyrosinases, for example, are being explored for the modification of scaffolds through the crosslinking of lysine and glutamine residues of proteins to enhance the mechanical properties and the in vivo stability of scaffolds based on natural polymers; in situ synthesis of gelling hydrogels and functionalization of scaffolds with appropriate ligands for cell adhesion and/or growth factors. Lipase-catalyzed polymerization has been employed to synthesize a variety of polymer materials. Grafting of functional molecules onto lignocellulose polymers using laccases and peroxidases is actively being pursued in the areas of textile, particle board manufacture, and surface functionlization of many polymers. Enzymes (cutinases, lipases, and esterases active) have been identified and characterized for the biofunctionalization of poly(ethylene terephthalate) aimed at improving their poor water permeability and reactivity. Surface-modification of PET is also being actively studied to impart antimicrobial properties.

It is the intended wish of these editors to inspire scientists and industrialists from different backgrounds to make a difference by presenting these interesting achievements,and processes under development, and above all demonstrate the convergence of ideas from different fields of science. This contribution will in no doubt accelerate the development of novel processes and biofunctionlaized products. The editors wish to thank all the authors for their excellent contributions and the European Science Foundation for support and organization of the COST868 network in this fast emerging and important field.

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Chitin, Chitosan and Derivatives for Wound Healing and Tissue Engineering

Antonio Francesko and Tzanko Tzanov

Abstract Naturally derived polymers possess a number of properties beneficial to wound healing and tissue engineering. The polysaccharides chitin and chitosan appear to be suitable candidates for the preparation of dressing materials and scaffolds for tissue regeneration due to their unique structural, physico-chemical and functional properties. Functionalization of these biopolymers for improvement of properties such as solubility or introduction of active functions and blending with other intrinsically bioactive polymers has attracted considerable attention in recent years. Such modifications would allow going beyond traditional approaches for treatments of dermal injuries. This chapter is a critical review of the advances in chitin and chitosan functionalization for wound-healing and tissue-engineering applications.

Keywords Blends · Chitin · Chitosan · Fibers · Hydrogels · Tissue engineering · Wound dressing

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1 Introduction

Modern industries necessarily need to exploit renewable resources in a sustainable manner, promoting sustainable, bio-based, environmentally friendly or beneficial technologies in order to keep competitive market positions. The highly demanding market calls for constant improvement in product quality as well as competitive technologies for their generation. In the last decades, naturally derived polymers, due to their intrinsically beneficial properties for a broad spectrum of applications, have been gaining importance as raw materials in many industrial sectors such as food, textile, packaging, medicine and pharmacy. Biodegradable and biocompatible biomaterials that do not cause immune responses in organisms and/or are able to integrate with a particular cell type/tissue are required for medical and pharmaceutical uses. Biopolymers nowadays play crucial roles in applications where the materials are in direct contact with body tissue, e.g., dermal tissue repair. Dermal tissue (skin) repair includes regeneration of the injured skin, and in the most general sense is described as wound-healing and dermal-tissue (skin) engineering.

Wound healing is a dynamic biological process, a body response to the dermal tissue injury, which comprises several overlapping stages: hemostasis, inflammation, migration, proliferation and maturation. Upon injury, a cascade of complex biochemical events, as a normal body response, takes place to repair the damage [132]. When this response is not sufficient, the treatment usually comprises surgical debridement, topical antimicrobial agent application and, most commonly, dressing of the wounds.

Tissue engineering involves the development of temporary, or in some cases permanent, biological substitutes for failing tissues and organs. In particular, skin engineering is based on seeding of cells onto a porous biodegradable polymer scaffold that serves as a starting platform for dermal tissue recovery. A primary factor for skin repair is the availability of suitable biomaterials to support and evoke regeneration of the skin.

The materials used in skin repair should display intrinsic biocompatibility and biodegradability at an ideal rate corresponding to the rate of new tissue formation [74]. Moreover, the biomaterials used in tissue regeneration and their degradation products should not be toxic, immunogenic and carcinogenic.

Polysaccharides and proteins, being natural components of all living structures, are fascinating candidate materials for dressings and scaffold fabrications. Carbohydrate moieties of the polysaccharides interact with integral components of many extracellular matrix glycoproteins and cell adhesion molecules in the skin. Chitin and its deacetylated derivative chitosan, due to their molecular and supramolecular structure, and intrinsic antimicrobial and wound-healing properties, have been identified as suitable bioplatforms that can be further improved by targeted functionalization for skin repair.

This chapter gives an overview of the recent progress in the use of chitin, chitosan and derivatives thereof for wound healing and tissue engineering. The most important biochemical functionalizations, blending with other biopolymers

that are beneficial to tissue repair, and the available wound dressing designs are reviewed herein.

2 Chitin and Chitosan

Chitin, a polysaccharide (aminopolyglucan) composed of β(1–4)-linked *N*-acetyl-D-glucosamine residues, is a structural material in the exoskeletons or the cuticles of many invertebrates and in the cell walls of green algae, some fungi and yeasts. It is one of the most abundant organic materials obtained through biosynthesis. This poly-*N*-acetyl glucosamine exhibits structural similarity to cellulose, differing only in the replacement of C-2 hydroxyl residues by acetamide groups. Depending on the polysaccharide source and isolation conditions, chitin could have a different degree of acetylation. The length of the chitin molecule in nature varies largely. For instance, 5,000–8,000 *N*-acetyl-D-glucosamine residues are found in crab chitin, while the one from yeast contains only up to 100 residues [135]. Chitin is insoluble in water, diluted acid/base solutions and in most of the organic solvents. It is usually dissolved in concentrated acids as well as in hexafluoroisopropanole or hexafluoroacetone.

Chitosan is produced by chitin deacetylation with concentrated alkali solutions at elevated temperatures. The degree of deacetylation and the molecular weight, ranging from 30 kDa to well above 1,000 kDa, of chitosan can be controlled by the temperature of the process. The rigid D-glucosamine structures, characterized by high crystallinity and hydrogen bonding capacity between chitosan macromolecules, define the poor solubility of chitosan in common organic solvents and complete insolubility in aqueous solutions above pH 7. Reducing the molecular weight and lowering the crystallinity by random deacetylation improves its solubility in dilute acids below pH 5 where the free amino groups are protonated. Chitosan displays interesting physicochemical properties, including both its solid-state structure and the dissolved state conformation [106]. In solid state, relatively rigid crystals are formed due to the regularly arranged hydroxyl and amino groups, while in solution hydrogen bonding drives the formation of microfibrils, depending on the chitosan concentration [105]. Furthermore, chitosan is a biopolymer that is easy to develop in various designs, i.e., films, sponges, scaffolds and hydrogels, an essential fact for the preparation of a great diversity of wound dressings and tissue-engineering materials. Regarding targeted functionalization, chitosan bears two types of reactive groups that can be modified—the free amino groups on the deacetylated units and the hydroxyl groups on the C_3 and C_6 carbons on both acetylated and deacetylated units. This allows its functionalization with a variety of (bio)active molecules or the use of crosslinking reagents for blending with other biopolymers.

The unique biological properties of chitin and chitosan, including biocompatibility, biodegradability, non-toxicity, antibacterial and hemostatic properties, assure their biochemical significance in skin repair processes. Both biopolymers have an effect on fibroblast (the cells regenerating the extracellular matrix and therefore

playing a crucial role in wound healing) activation, facilitate the production of cytokines (cell-signaling molecules—peptides, proteins and glycoproteins), cell migration and stimulation of granulation tissue formation. Chitin accelerates macrophage migration and fibroblast proliferation with a particular role in vascularization. It has superior hemostatic activity compared to chitosan [38], an important fact in the early treatment of the injury. Pietramaggiori et al. [110] demonstrated rapid closure of full-thickness cutaneous wounds using poly-*N*-acetyl glucosamine membranes and attributed this effect to the fast growing of new blood vessels (angiogenesis) in the injured tissue. On the other hand, chitosan has an indirect role in wound healing, supporting the regeneration of the extracellular matrix. Chitosan promotes physiologically ordered dermal regeneration during skin reconstruction, regulating the deposition and faster arrangement of thin collagen fibers [129].

The recent discovery of elevated levels of matrix metalloproteinases (MMPs), a group of collagenases with Zn^{2+} in the enzyme active center, in inflammation diseases such as chronic wounds gave new insights for their treatment. The effect of chitin, chitosan and their derivatives on the MMPs' activity has been the subject of a limited number of scientific reports and recently summarized by [101]. It was found that especially chitooligosaccharides (COS) display an inhibitory effect on MMP-2 expression and its transcriptional activity. Both zymogen and active MMP-2 were inhibited with 3–5-kDa COS, and this effect was attributed to the chelating ability of COS on Zn^{2+} [75]. At the same time, partially hydrolyzed chitosans were found to be potent inhibitors of gene and protein expression of MMP-9 according to the same inhibitory mechanism [142]. High molecular weight chitosan (500 kDa) showed elevated binding specificity for MMP-2 and also displayed inhibitory activity by non-competitive inhibition. Atomic force microscopy revealed complex formation between the chitosan and the enzyme [42].

Owing to their biodegradability, both chitin and chitosan have attracted much attention as materials for tissue engineering, and in particular for skin engineering, because the materials should degrade as the new tissue is formed [4]. Tissue engineering generally makes use of biocompatible and biodegradable scaffolds with mechanical properties closely matching those of the target tissue. These materials and their degradation products should be further characterized by non-inflammatory response and non-toxicity. All these properties are met by chitin and chitosan. The cationic nature of chitosan facilitates the preparation of various scaffolds in combination with anionic glycosaminoglycans, important molecules in human dermal tissue, forming polyelectrolyte complexes (PEC). Chitosan-based PEC scaffolds also provide controlled drugs, growth factors and extracellular matrix component release.

3 Enzymatic Modification of Chitin/Chitosan

The enzymatic methods for polymer modification rely on the high specificity of the biocatalysts at mild reaction conditions to achieve targeted functionalizations,

while eliminating a series of complicated protection and deprotection steps typical for the chemical synthetic routes. Enzymatic modifications of natural polymers are less frequently reported than the conventional chemical modifications; however, this trend is progressively changing. Recent studies clearly indicate that the modification of natural polymers with enzymes is an environmentally friendly alternative to the chemical methods using harsh conditions [44].

3.1 Enzymatic Functionalization

The enzymatic tools for functionalization of chitin/chitosan are selected on the basis of the chemical structure of these biopolymers (functional groups and targeted enzyme activities related) and include oxidases, e.g., tyrosinase, peroxidase, laccase and transferase (transglutaminase) types of enzymes. The same enzymatically assisted reactions could be further employed to crosslink the functionalized biomaterials in order to improve their exploitation characteristics, i.e., stability at use.

Grafting of phenolic compounds onto chitosan using tyrosinase (EC 1.14.18.1) to confer water solubility under basic conditions has been reported by Kumar et al. [80]. The tyrosinase converts the phenol moieties into electrophilic *o*-quinones in a wide variety of phenolic substrates, which subsequently undergo non-enzymatic reactions with chitosan amino groups to yield either Schiff bases or Michael-type adducts. Coupling reactions of the phenolic active species result in a complex mixture of mono- and oligomeric products grafted on chitosan. The modified chitosan is soluble in both acidic and basic media. In addition, the tyrosinase-catalyzed modification of chitosan with phenols dramatically alters the rheological and surface properties of the biopolymer. Generally, this reaction would allow for grafting of any phenol group-containing molecule and therefore introduces various functionalities into polysaccharides that contain amino residues. For example, grafting of 3,4-dihydroxyphenethylamine onto chitosan could find application as a water-resistant adhesive [149]. In another study Kumar et al. [81] used various phenolic substrates with different molecular weights to induce enzymatic gelation of chitosan. Interaction between chitosan and polymers, such as poly(4-hydroxystyrene) in the mixture of methanol and water, has also been reported [128].

The increased interest in blending of two or more naturally derived compounds to improve their physicochemical properties resulted in many publications in the field. Chitosan was blended with sericin peptides [6] using tyrosinase. Following the same route, chitosan was combined with proteins such as silk fibroin [123] and gelatin to induce gelation [21]. The so obtained gels showed improved mechanical properties when compared to gels formed by gelatin cooling.

Transglutaminases (EC 2.3.2.13) catalyze the formation of a covalent bond between a primary amino group of lysine and the γ-carboxamide group of protein-bound glutamine that is highly resistant to chemical and enzymatic degradation.

Preparation of chitosan–gelatin gels using transglutaminases has been reported. Although chitosan was not required to obtain strong gels from gelatin (as in the case of tyrosinase), it was observed that its presence leads to faster gelation, and the resulting protein–polysaccharide gels were stronger [21].

A horseradish peroxidase (HRP)-catalyzed gelation system has been studied as a novel and effective route for obtaining hydrogels from various biopolymers. HRP catalyzes the oxidation of donors using H_2O_2, resulting in polyphenols linked at the aromatic ring by C–C and C–O coupling of phenolic moieties. The versatility of the HRP-catalyzed gelation system has been demonstrated by the gelation of biopolymer derivatives bearing phenolic groups such as modified polysaccharides, e.g., hyaluronic acid [82], alginate [118], dextran [70], carboxymethylcellulose [104] and chitosan [119], and proteins such as gelatin [121]. Sakai et al. [120] also reported on simultaneous conjugation and hydrogelation of phenol-bearing polysaccharides and proteins using HRP. The gelation time of the resulting gels decreased with increasing HRP concentration and was controllable from a few seconds to 6 min. The tunable gelation, biodegradability, mechanical properties and cell adhesiveness of the obtained polysaccharide–protein conjugated hydrogels indicate high potential for a wide range of applications, among which are scaffolds for tissue engineering and carriers for drug delivery systems. Recently, HRP was used to crosslink chitosan derivatives prepared by conjugation of chitosan and phenol containing α-hydroxy acids to produce biodegradable injectable hydrogels for cartilage regeneration [69]. In the same study, these hydrogels showed good biocompatibility by in vitro culturing of chondrocytes, and they can be readily degraded by lysozymes.

Despite the increasing number of reports on using biotools for tuning chitin/chitosan properties, their functionalization aiming at wound healing and dermal engineering application has not been reported so far.

3.2 Enzymatic Depolymerization

Chitin and chitosan can be depolymerized by various hydrolases, e.g., lysozyme, pectinase, cellulase, lipase, digestive amylase, papain, chitin deacetylase, and especially chitosanase and chitinase [40, 103, 116]. Generally speaking, the efficacy of chitosan oligomers for wound healing is superior to other forms of chitins and chitosans. Chitins (including *N*-acetyl-D-glucosamine and a mixture of its oligomers up to the pentamer) and chitosans (including D-glucosamine and a mixture of its oligomers up to the hexamer) have been compared using a linear incisional wound model in rats. The wound breaking strength in the cases where chitosan was applied was higher than in the chitin-treated group of wounds. Collagenase activity was also higher in the chitosan group than in the chitin group [95]. Additionally, chitosan oligomers were confirmed to inhibit the growth of deleterious bacteria and to boost immune function [58].

4 Chitin and Chitosan in Wound Healing and Tissue Repair

Chitin and chitosan have been used as materials in wound healing and tissue engineering due to their beneficial skin repair properties. However, despite the fact that these polysaccharides appear to be efficient to a different extent in the tissue repair process, the currently marketed products exploit only the intrinsic properties of the biopolymers without further upgrading them with active functions to react at molecular level with wound pathogens. Numerous reports dealing with chitin/chitosan functionalization at laboratory scale exist and are summarized below.

4.1 Chemically Modified Chitin/Chitosan

Chitin and chitosan modification to improve their biofunctionality (biocompatibility, biodegradability, antibacterial activity and wound-healing promoting) tends to preserve the original physicochemical and biochemical properties of the polymers, while widening their application potential. The major limitation for chitin and chitosan functionalization and application is their poor water solubility. The most important chitin derivates may be classified into two categories, sharing as a common feature the removal of the *N*-acetyl groups and further exposure of the primary amino functions to reactions with: (1) either acyl chlorides or anhydrides forming NHCOR, or (2) modification by reductive amination to $NHCH_2COOH$. The derivatives of chitin include carboxymethyl chitin, hydroxyalkyl chitin (glycol chitin, hydroxyethyl chitin, hydroxypropyl chitin and hydroxybutyl chitin), fluorinated chitin, *N*- and *O*-sulfated chitin, (diethyl amino)ethylchitin, phosphoryl chitin, mercaptochitin, chitin carbamates, di-*O*-butyryl chitin, *O*-acylchitin, tosyl chitin, iodochitin, alkyl deacetylated chitin, triphenylsilyl chitin, 6-oxychitin and 6-deoxychitin, and have been extensively reviewed by Kurita [83]. The use, however, of modified chitin in skin repair application has been sparsely reported. Water-soluble chitin with a controlled degree of deacetylation (DD) and molecular weight for wound-healing acceleration has been prepared through alkaline and ultrasonic treatment [24]. Miyatake et al. [97] reported on phosphated and sulfated chitin with an increased anti-inflammatory effect.

Major chitosan functionalization could be carried out by: (1) substitution, introducing small functional groups to the chitosan backbone, and (2) depolymerization by chemical, physical or enzymatic treatments. Moreover, further chemical modifications of the functionalized chitosans can be performed in order to extend the range of their applications [47].

Introducing small functional groups to the chitosan structure, such as alkyl or carboxymethyl groups (*O*-/*N*-carboxyalkylation), can drastically increase the solubility of chitosan at neutral and alkaline pHs without affecting its cationic character. The conversion of chitosan to a variety of *N*-alkylated derivatives can be achieved by treatment with aldehydes or ketones leading to formation of Schiff

base intermediates, followed by reduction of the imine linkage finally yielding *N*-alkylated chitosan [113]. Most of these reactions proceed smoothly in binary solvent mixtures of aqueous acetic acid and methanol. The *N*-alkylated derivatives can also be prepared by introducing sugar branches at the *N*-amino groups. These reactions involve reductive alkylation of chitosan using sodium cyanoborohydride and diverse reducing sugars such as D-galactose, D-glucose, cellobiose and lactose [87].

N-Carboxyalkylation of chitosan involves the introduction of acidic (anionic) groups onto the polymer backbone. Besides good solubility, carboxymethylation of chitosan promotes the proliferation of normal skin fibroblasts significantly, but inhibits the proliferation of keloid (scar) fibroblasts and reduces the ratio of collagens I/III in keloid fibroblasts by inhibiting the secretion of collagen type I, while not affecting the secretion of collagens I and III in normal skin fibroblasts [20, 22, 64, 154]. Increase of the antimicrobial activity is also observed with carboxymethyl chitosan, which either renders essential transition metal ions unavailable for bacteria by complexation or disturbs the cell membrane binding to the negatively charged bacterial surface [91]. Finally, carboxymethyl chitosan can be used in the development of different protein drug delivery systems as super porous, pH-sensitive, cross-linked hydrogels for wound healing applications [19, 89].

N-Acylation of chitosan is the most typical and extensively studied modification reaction. The process involves a reaction between chitosan and an acid anhydride or acyl halide. The reaction proceeds through an addition/elimination type mechanism, where the amide functionality of the *N*-amino groups is restored. Several *N*-acyl derivatives comprising aliphatic side chains have been prepared. For example, a series of water-soluble *N*-saturated fatty acyl derivatives of chitosan have been synthesized through reactions with propionic, butyric, pentanoic, hexanoic and octanoic anhydride, and the longer chain acid anhydrides decanoic, lauric, myristic, palmitic and stearic anhydride [54]. Hu et al. [57] prepared a series of *N*-acylated chitosans with different degrees of substitution and evaluated them in vitro for antibacterial activity. The results showed that the characteristic intermolecular aggregation of low DD *N*-acetylated chitosans may be responsible for the interaction with bacterial cell. *N*-Acylated chitosan using butanoic, hexanoic and benzoic anhydride under homogeneous conditions in the presence of methanol and the nanoparticles prepared from *N*-acyl chitosan were blood compatible [85].

N-Sulfation of chitosan is another example of introducing anionic charge to the polymer backbone. Various methods involving combinations of sulfating agents and reaction media have been used for the sulfation of chitosan. These include concentrated sulfuric acid, oleum, sulfurtrioxide, sulfurtrioxide/pyridine, sulfurtrioxide/trimethylamine, sulfur trioxide/sulfur dioxide, chlorosulfonic acid–sulfuric acid and the most commonly used chlorosulfonic acid in homogeneous or heterogeneous conditions in DMF, DMF–dichloroacetic acid, tetrahydrofuran, and formic acid at different temperature range or under microwave irradiation [99]. Sulfated chitosans are analogues to the natural blood anticoagulant heparin [31,

49], a highly sulfated glucosaminoglycan used in medicine for treatment of various cardiovascular diseases.

Phosphate groups, due to their ion-exchange properties, are appropriate functionalities for specific binding of biologically active species. Phosphate carriers in solution are cation exchangers of ionically bound cations (e.g., Na^+, Ca^{2+}) with cationic residues of biomolecules. Phosphorylation of porous chitosan matrices can be used to immobilize signaling biomolecules and growth factors [5]. The ionic character of binding together with the mild conditions for immobilization leads to minor conformational changes in the immobilized biomolecule with subsequent high retention of activity. Phosphorylation of chitin and chitosan can be achieved by various chemical methods, reviewed by [65], such as heating of chitin/chitosan with orthophosphoric acid and urea, reaction of chitin/chitosan with phosphorous pentoxide in methanesulphonic acid, and reacting chitosan, phosphorous acid and formaldehyde. The products of these reactions are normally water-soluble phosphorylated chitin/chitosan with a high degree of substitution (DS), especially when phosphorous pentoxide is used.

The derivatization of the primary amino groups of chitosan with coupling reagents bearing thiol functions leads to the formation of thiolated chitosans. Sulfhydryl-bearing agents such as cysteine and thioglycolic acid can be covalently attached via amide bond formation between the carboxylic acid group of the thiolating agent and the primary amino group of chitosan mediated by a water-soluble carbodiimide [13]. Thiolated chitosans display a series of interesting functions such as mucoadhesive [115], permeation-enhancing [39] and in situ gelling properties within a pH range of 5–6 [71]. In addition, thiolated chitosans are biodegradable. Further studies in the direction of its use as novel scaffold material were performed with L-929 mouse fibroblasts seeded onto chitosan–thioglycolic acid sheets. The thiolated chitosan provided a porous scaffold structure for cell anchorage, proliferation, and tridimensional tissue formation [72]. Schmidt [124] patented the preparation of water-insoluble thiolated polysaccharides (e.g., chitin/chitosan) with antioxidant properties against reactive oxygen species (ROS) present in early stages of chronic wounds. The thiolated polysaccharides were also able to complex Fe^{3+}, which is essential for the bio-cycle of some bacteria found in the chronic wound environment.

Quaternary ammonium functionalities could be introduced to chitosan either by direct alkylation of the *N*-amino functional groups or via covalent attachment of quaternary ammonium substituents to the *N*-amino groups. These processes are referred to as quaternization. The simplest synthetic approach to give chitosan quaternary ammonium functionality is via conversion of the *N*-amino groups into *N*-trimethylammonium halide salts. Different degrees of quaternization of amino groups in chitosan can be achieved with methyl iodide in alkaline solution of *N*-methyl pyrrolidinone [25]. The major advantage of these derivatives over the parent chitosan is the permanent positive charge of the macromolecules. Permanent positive charge on the chitosan backbone results in increased antimicrobial activity [9, 67]. Alkylchitosans quaternized by methylation [46] increased their

free radical scavenging activity, making them potentially useful in inflammatory diseases such as chronic wounds [45] characterized by elevated levels of ROS.

Other interesting biomedical applications using biochemically modified chitosan have also been reported. Ishihara and co-workers [63] prepared a new photocrosslinkable chitosan bearing *p*-azidebenzoic acid and lactobionic acid. This derivative could be crosslinked by UV irradiation, resulting in a rubber-like flexible hydrogel. The hydrogel showed strong tissue-adhesion properties, significant induction of wound contraction, and acceleration of wound closure and healing properties, which makes it suitable as a biological adhesive in surgical applications. Ethylene diamine tetraacetic acid (EDTA) grafted onto chitosan increased its antibacterial activity by complexing magnesium that under normal circumstances stabilizes the outer membrane of gram-negative bacteria [12].

Grafting of cyclodextrins (CDs), cyclic oligosaccharides built from six to eight D-glucose units that are formed during the enzymatic degradation of starch and related compounds, onto chitosan may lead to a molecular carrier that possesses the cumulative effects of inclusion, size specificity and transport properties of CDs together with a controlled release ability of the polymeric matrix. The different methods used to graft CD on chitosan and the inclusion ability, sorption and controlled release properties of the products have been reviewed recently by [112]. Chitosan-based systems bearing cyclodextrin cavities have been proposed as a matrix for controlled drug release [78]. Due to the presence of the hydrophobic-cyclodextrin rings, these systems provide slow release of the entrapped hydrophobic drug. El-Tahlawy et al. [34] used a novel technique for preparation of β-CD grafted chitosan of reacting β-CD citrate with chitosan dissolved in formic acid solutions and evaluated these polymers as antimicrobial agents.

4.2 Chitin and Chitosan Fibers

Research on (bio)polymer fiber manufacturing has attracted considerable interest because of advantages such as the high specific surface area and porosity of the fibrous designs. In tissue repair processes, such fibrous assemblies would allow loading with active substances and would provide water and vapor permeability and better contact of the material with the injury.

Several fabrication techniques such as spinning, meltblown, phase separation and self-assembly have been employed to produce fibers suitable for different purposes [153]. Fibers based on chitin and chitosan have been known for a long time, and in the early stages of man-made fiber development much of attention was focused on a chitin as a potential raw material for producing artificial silk. Only after recognition of the natural renewable resources as materials with potential for fiber production were major breakthroughs in the preparation of chitin and chitosan fibers achieved starting in the 1970s. In most studies, chitin and chitosan fibers are obtained by wet-spinning, consisting of dissolving the polymer in an appropriate solvent followed by extrusion of the polymer solution through the

spinneret into a non-solvent to solidify the fibers. The polymer precipitates in the form of filament, which can be washed, drawn and dried to form the fibers [1]. Traditionally, chitin is dissolved in di- or trichloroacetic acid alone or in conjunction with other organic solvents such as formic acid, acetic acid, chloral hydrate, methylene chloride, etc. Development of fibers from chitosan is comparatively easier as it is soluble in dilute acids such as acetic acid. The most commonly used chitosan solvent is aqueous acetic acid (normally 2%). Additionally, 2% acetic acid–methanol (1:1 v/v) aqueous solution is considered to be particularly suitable as a spinning solvent for preparation of chitosan filaments. The mechanical properties of chitin/chitosan fibers produced by wet-spinning depend on both the chemical nature (e.g., degree of deacetylation) and production (spinning) conditions.

The strong interaction through intermolecular hydrogen bonds and electrostatic forces in mixtures of alginate and water-soluble chitin for fiber spinning ensures the miscibility of the biopolymers [37]. The presence of water-soluble chitin in the fibers improved the water-retention properties of the blend compared to pure alginate fiber. However, the wet tensile strength and breaking elongation decreased with the increase of chitin content.

Significant improvement of functional properties has been reported for fibers obtained from blends of chitin with various natural polymers such as cellulose, silk fibroin and glycosaminoglycans [48, 50, 51]. Fibers of chitosan blended with alginate, collagen and gelatin have been evaluated for wound dressing and artificial skin applications [52, 53, 108, 136].

Hirano et al. [53] prepared cotton-like fibers from chitosan and glycosamynoglycans, namely hyaluronic acid, chondroitin sulfate and heparin using wet-spun technique. Glycosaminoglycans present from 5 to 33% in the fibers allowed the development of targeted release strategies in alternative wound-healing approaches, however, at reduced mechanical strength of the composite fiber. Considering the mechanical properties, fibers obtained from chitin/chitosan with a low degree of deacetylation showed dry and wet tensile strength higher than the fibers obtained from more deacetylated polymers. Different drying conditions can affect the fibers arrangement. The major drawback of chitin and chitosan fibers formed in the wet-spinning process is their relatively low tensile strength.

A recent breakthrough in nanofiber technology is the use of electrospinning as a convenient method for preparation of polymer fibrous materials with very fine diameters, enormous surface-to-weight area and superior mechanical properties. Simplified, the electrospinning setup consists of a pipette that doses the polymer solution and two electrodes with the positive one connected to the pipette, while the negative one serves as a collector under a direct current (DC) voltage supply in the kV range. The electrified jet of polymer solution drops from the tip of the pipette and accumulates on the collector in the form of fiber mesh because of rapid solvent evaporation. The morphology of the prepared nanofibers is influenced by both system and process parameters. The modern electrospinning technology is able to generate continuous fibers with diameters in the range of nano- to a few micrometers [30].

Chitin, chitosan and their derivatives show promising properties as starting materials for the preparation of nanofibers for tissue engineering and regeneration. The nanofibers are continuous and potentially allow for manufacturing of 3D nanofibrous scaffolds with high porosity and high spatial interconnectivity [33, 93]. In different studies chitosan nanofibers in combination with other polymers were beneficial to wound-healing and skin-engineering properties, such as antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* [60], cell adhesion [14] and biocompatibility [134]. The generated nanofibers could mimic the structure and function of the natural extracellular matrix (ECM) and could serve in tissue engineering as scaffold materials to restore, maintain or improve the function of human tissues. However, due to the relatively recent development of the electrospinning technique, a limited number of reports could be found on chitosan nanofibers for tissue repair. Available reports address ultrafine electrospun fibers from chitosan derivatives with improved solubility. Skotak et al. [131] grafted L-lactide oligomers onto chitosan via ring opening polymerization. Cytotoxicity tests using fibroblasts on the electrospun L-lactide-modified chitosan fibers showed that the specimen with the highest molar ratio of L-lactide (1:24) appeared to be the most efficient for tissue-engineering purposes. The same modification approach allowed controlling of the biodegradation rate and hydrophilicity of the scaffold material [148].

Chitin and chitosan nanofibers, methods for their preparation as well as their potential biomedical applications, including dressing materials, have been recently reviewed by Pillai et al. [111] and Jayakumar et al. (2008). Nevertheless, most of the reported chitin and chitosan nanofiber developments are still at the laboratory scale level; thus, optimization of the production process and performance of the fibers are necessary before large-scale manufacturing and commercialization.

4.3 Chitosan Hydrogels

Hydrogels are highly absorbent water-insoluble networks of polymer chains with a high degree of flexibility similar to that of the natural tissue. The hydrogels are normally classified according to the nature of network formation as physical and chemical hydrogels. The physical hydrogels are obtained by reversible electrostatic interactions (e.g., PEC) or through secondary interactions (e.g., hydrogen bonds), while the chemical hydrogels are covalently crosslinked. Several other types of hydrogels exist, such as ionically crosslinked or entangled hydrogels as suggested by Berger et al. [10], but their application in skin repair has not been reported.

4.3.1 Physical Hydrogels

In physical hydrogels the cationic chitosan interacts with any polyanionic polymer forming PEC. Complexation based on the opposite charges of the two polymers

requires their ionization, which means that the PEC formation occurs in the vicinity of the pK_a interval of the two polymers. However, precipitation due to strong ionic interaction is considered the main disadvantage for large-scale preparation of PEC. One possibility to avoid precipitation is to weaken the electrostatic interactions, contributing to the counter-ion environment by adding salts, i.e., sodium chloride [122].

The most commonly used polyaniones for preparation of PEC in combination with chitin and chitosan are carboxylic groups bearing polysaccharides such as alginate [73], pectin [151] and xanthan [32], and proteins such as collagen [139] and gelatin. Biodegradable, pH-stable PEC membranes of chitosan and alginate have been generated by Yan et al. [150]. Chitosan and alginate have also been combined into a PEC membrane for treatment of highly exuding wounds and prevention of bacterial infections [114]. In skin repair the most efficient chitosan PEC hydrogels are those with glycosaminoglycans (GAG)—long unbranched polysaccharides with repeating disaccharide units. Anionic in nature, GAGs are found in the skin matrix and are well known to bind and modulate the activity of a number of cytokines and growth factors [61, 126]. Membranes of chitosan combined with heparin (the biological molecule with the highest negative charge density) stimulated the dermal wound healing by slowly releasing heparin into the wound area in human skin, thereby protecting locally produced growth factors [77]. Chitosan-heparin hydrogels were also more efficient in treatment of full-thickness skin defects in rats compared to chitosan alone [84]. However, no protective effect on early extension of burn wounds in rats has been observed [68]. Fucoidan, another sulfated polysaccharide, interacting with the growth factors in skin, in combination with chitosan induced significant wound contraction and healing of dermal burns in a rabbit model [127]. According to Denuziere et al. [29], hyaluronic acid/chitosan PEC hydrogels allow the culture of keratinocytes (the cells producing the skin matrix), leading to wound-healing acceleration in rats without any inflammatory reactions and toxicity to the animals.

Huang et al. [59] developed a chitosan–gelatin PEC scaffold that supported fibroblast viability with a clearly increased cell-spreading area compared to chitosan alone. However, degradation results showed that gelatin-containing chitosan scaffolds had a faster degradation rate and higher loss of material than chitosan over 2 months period.

Ionically crosslinked PEC hydrogels exhibit pH- and to a minor extent ion-sensitive swelling in acidic and basic media. In such systems the swelling and drug release profiles can be modulated by selection of the preparation conditions [11]. The delivery of fibroblast (FGF) and transforming growth factor-β (TGF-β) involved in the wound-healing process could increase the efficiency of these materials, especially in the case of chronic inflammation. The formation of chitosan hydrogels by non-covalent crosslinking is a useful method for preparation of drug delivery devices. Fibroblast growth factors 1 and 2 (FGF-1 and FGF-2) and heparin were incorporated into a high molecular weight chitosan hydrogel formed by UV-initiated crosslinking. The biologically active FGF-1 and FGF-2 together with heparin were released in a controlled manner from the hydrogel upon

biodegradation in full-thickness wounds in mouse [62]. The slow release of growth factors is essentially important in the treatment of chronic wounds. Recently, Park et al. [109] developed a chitosan hydrogel scaffold impregnated with basic fibroblast growth factor (bFGF)-loaded microspheres that were able to accelerate wound closure in chronic ulcers.

In cutaneous wound healing, targeted ablation of smad3 (a mediator in intracellular signaling of TGF-β1) resulted in acceleration of epidermal resurfacing with less scar formation [7, 8]. Antisense oligonucleotides (ASOs) of smad3 offer a new therapeutic approach in which the production of the disease-causing protein is directly inhibited at a genetic level. Thus, addition of ASOs into the wound environment would promote healing. Hong et al. (2008) generated smad3 ASOs impregnated PEC comprising chitosan and sodium alginate. ASOs-PEC, PEC alone, ASOs and gauze dressing have been applied to determine the concentration of TGF-β1 and collagen in tissues in full-thickness excisional wounds, observing the wound contraction and tissue histology. The healing process evaluated by wound closure and histological observation in the ASOs-PEC-treated group was faster than in the other groups with increased collagen contents.

Recently much attention has been focused on the controlled release of antimicrobial peptides. Besides being a physical barrier, the skin contains a chemical barrier consisting of constitutively and inducible produced antimicrobial peptides, which control microbial growth on the skin surface. Skin infections might be caused by defects in this barrier suggesting that the use or the induction of these compounds could be a viable strategy for preventing skin infections [125]. Unlike antibiotics, the antimicrobial peptides do not induce bacterial resistance [133]. Release of antimicrobial peptides from chitosan matrices has not been reported so far, and the current investigations are based on model proteins. Grenha et al. [43] loaded the model protein ovalbumin into chitosan–carrageenan nanoparticles providing controlled release for up to 3 weeks. Previously, chitosan and the seaweed-derived biopolymer carrageenan (bearing sulfate groups and anionic in nature) have been used in different ratios for preparation of nanoparticles not exhibiting cytotoxicity in biological in vitro tests performed using L929 fibroblasts. The developed chitosan–carrageenan nanoparticles have shown promising properties to be used as carriers of therapeutic macromolecules with potential application not only in drug delivery, but also in tissue engineering and regenerative medicine.

Chitosan complex with polyvinyl alcohol (PVA) is the exception in the physical hydrogel formation, since the chitosan–PVA network is stabilized by secondary and non-ionic interactions. In this complex the less hydrophilic chitosan is concentrated on the air-surface side of the formed hydrogel. High concentration of chitosan on the surface favors cell attachment (an important criterion in tissue engineering application) because of electrostatic interactions between the amino groups of chitosan and the cells. Chitosan–PVA complex represents a better medium for the cell and fibroblast culture than the covalently cross-linked hydrogels. However, dissolution and cells release might occur [96]. The effect of polyvinyl alcohol (PVA)/chitosan/fibroin (PCF) sponge on wound healing in rats

has been investigated by Yeo et al. [152]. The polymers formed semi-interpenetrating networks through hydrogen bonding. The water permeability increased by increasing the PVA ratio in the sponges, resulting in improved flexibility, softness and absorption capacity. Histopathological inspection of the wound 12 days later showed increased vascular ingrowth and absence of inflammatory cells. Regeneration of the skin around the wound was faster than that of the controls (chitosan in combination with PVA or fibroin alone). Finally, the PCF sponge was found to promote wound healing by facilitating collagenization.

Hydrogen bonds were also responsible for blending of chitosan and cellulose [147]. The mechanical and dynamic thermomechanical properties of the material appear to be dominated by cellulose. The reduced rate of water vapor transport through the blend appears suitable for wound dressings, providing an optimal moisture environment of the wound by preventing excessive dehydration. The blend demonstrated effective antimicrobial activity against *E. coli* and *S. aureus*.

4.3.2 Chemical Hydrogels

The preparation of chemical hydrogels requires the use of crosslinkers that in the case of chitosan most commonly are dialdehydes, such as glyoxal and glutaraldehyde. Dialdehydes react directly with amino groups in chitosan (and in less extent with hydroxyl moieties) in aqueous media under mild reaction conditions. Their use as crosslinkers, however, was lately avoided due to toxicity concerns [86]. Other reagents that have been used for crosslinking chitosan include tripolyphosphate, ethylene glycol, diglycidyl ether and diisocyanate. Toxicity studies have shown that these synthetic crosslinking reagents are cytotoxic to a certain extent and may impair the biocompatibility of the chitosan delivery system. Recently, the naturally occurring crosslinking agent genipin (isolated from *Genipa Americana* and *Gardenia jasminoides Ellis*) has been used to promote crosslinking reactions between polysaccharides and proteins. The biocompatibility of genipin in humans has not yet been assessed, while in vitro tests and injection in rats did not show cytotoxicity. Based on biochemical data on genipin safety, Muzzarelli [102] concluded that the crosslinked complexes formed with genipin were not cytotoxic for the animal and human cells tested so far. Moura et al. [98] treated chitosan solutions (1.5%) at close to neutral pH with low amounts of genipin (0.05–0.20%) in the presence of glycerol phosphate and found that by changing the genipin concentration in the solution the gelling reaction can be controlled. Crosslinking of chitosan using genipin increased the hydrogel tensile strength, while decreasing the swelling capacity compared to physical hydrogels. The chitosan bacteriostatic activity remained unaffected after crosslinking with genipin [94]. These findings suggest that genipin could be a promising crosslinker of chitosan for fabrication of wound dressings and tissue-engineered scaffolds.

Chitin was used in combination with poly(acrylic acid) to obtain hydrogels for wound-healing application. For hydrogel crosslinking, an esterification reaction in acid medium between chitin hydroxyl groups and carboxyl moieties of acrylic

acid has been employed. Cell culture tests using L929 mouse fibroblasts carried out on chitin-poly(acrylic acid) hydrogels showed good cytocompatibility of the hydrogel with well-adhered and proliferated fibroblast after 14 days of culture [138].

4.4 Blends of Chitin/Chitosan and Other (Bio)Polymers

The combination of chitin and chitosan with other synthetic or natural polymers, in order to provide a wide range of new physicochemical properties, preserving at the same time the biocompatibility and biodegradability of the blends, is currently being investigated extensively.

The hydrophilic and biodegradable c-poly(glutamic acid) has been used to modify chitosan matrices, and the resulting cytocompatible composite biomaterial showed to be suitable for tissue engineering applications [56]. Another potential skin replacement blend has been prepared using chitosan and the cysteine-rich major structural fibrous protein keratin that supported fibroblast attachment and proliferation, demonstrating to be a good substrate for mammalian cell culture [137].

Sponge-like chitosan and alginate (anionic, biocompatible, highly hydrophilic and biodegradable biopolymer) blends prepared by a deep freeze, freeze-dried method was biodegradable in vitro using lysozymes and showed extended drug release over 20 days. An in vivo animal test using SD rat showed that sponge had a better effect than cotton gauze, and addition of curcumin into the sponge enhanced the healing effect [27, 28]. In order to create a moist environment for improved wound healing, a functional dressing sheet composed of alginate, chitin, chitosan and fucoidan has been developed. The fluid absorption in the dressing became constant within 18 h. The wound dressing was characterized as easy to apply and remove, and to have good adherence properties. Full-thickness skin defects on the back of rats and healing-impaired wounds using mitomycin C solution were prepared. The repair of healing-impaired wounds was significantly stimulated using the above dressing material. Histological examination demonstrated significantly advanced granulation of tissue and capillary formation already on day 7 [100].

The homobifunctional crosslinking agent glutaraldehyde was once widely used for (bio)polymers crosslinking. Crosslinked chitosan materials displayed prolonged drug (e.g., antibiotic) release [79], whereas the addition of other polysaccharides such as cornstarch and dextran has been reported to improve the physical strength of films formed [146]. Ma et al. [92] fabricated biocompatible chitosan–collagen scaffolds crosslinked with glutaraldehyde with improved biostability. The potential cytotoxicity of glutaraldehyde is claimed to be compensated by the presence of higher amounts of chitosan in the blend. The scaffold showed potential as a dermal equivalent as it could successfully induce fibroblast infiltration from the surrounding dermal tissue. The incorporation of collagen into chitosan

enhanced the attachment of seeded cells in a scaffold for tissue-engineering application developed by Cuy et al.[26]. Wang et al. [143] generated another sponge-like dressing for diabetic wounds comprising chitosan and collagen loaded with recombinant human acidic FGF. The dressing displayed a uniform and porous ultrastructure, small interval porosity, high resistance to collagenase digestion and extended release of FGF. Successful treatment of diabetic wounds in rats has been achieved within 14 days compared to 18–21 days in control treatment groups, providing rapid tissue collagen generation, high TGF-β1 expression and dermal cell proliferation.

Water-soluble carbodiimides such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) or *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC) have been frequently applied as crosslinking agents for biological materials. Carboxyl residues in biopolymers are activated with carbodiimides usually in the presence of *N*-hydroxysuccinimide (NHS). The activated carboxyl groups then react with nucleophiles such as primary amine groups to form stable zero-length amide bonds [145]. Byproducts of the reaction are easily rinsed away, leaving a stable non-cytotoxic matrix. Accordingly, carbodiimides have become popular in a wide range of tissue engineering-based applications [36]. In one of the earliest studies, Wang et al. [144] covalently coupled chitosan to type I collagen using EDC in morpholinoethane sulfonic acid buffer. The platelet deposition and endothelial cell culture experiments showed that the chitosan/collagen matrices (CCM) had good cell compatibility and excellent blood compatibility, therefore suggesting that the CCM creates an appropriate environment for the regeneration of endothelial cells and are promising biomaterial candidates for tissue-regenerating scaffolds. In another study, sponge scaffolds composed of different ratios of chitosan, collagen and hyaluronic acid crosslinked with EDC showed good hydrophilicity (all specimens could absorb 35- to 40-fold of physiological fluid) maintaining their form and stability. For tensile strength, the optimal ratio of collagen and chitosan was 9:1. The thermal stability of the scaffolds studied by differential scanning calorimetry increased at a higher chitosan ratio. A steady improvement of the biostability of the sponges has also been observed with increasing chitosan concentration. The biocompatibility test showed high proliferation of fibroblasts cultured on the sponges [88].

As mentioned above (see Sect. 4.3.2) by varying the concentration of genipin, the crosslinking degree of chitosan and/or chitosan with another biopolymer can be modulated. In general, genipin concentrations used for gel formulations are very low (about 0.1% w/w of polymer). At higher genipin concentration, the formulations obtained have more defined and dense structure similar to films, sponges and scaffolds. In example, the mixture of chitosan and silk fibroin has been crosslinked with genipin and freeze-dried to obtain chitosan-silk sponges. The reaction velocity in this case was dependent on chitosan concentration in the mixture due to the higher content of amino groups in chitosan readily reacting with genipin compared to the low content of amino groups in silk fibroin. The sponges displayed low cytotoxicity for mouse fibroblast and promoted adhesion, proliferation and matrix production of chondrocyte-like cells [130]. Liu et al. [90]

presented a novel design of an easily stripped bi-layered wound dressing material that consists of a non-woven, soybean protein fabric coated with a genipin-crosslinked chitosan film. The degree of crosslinking and the in vitro degradation rate of the films could be controlled by varying the genipin concentrations. Water contact angle measurements on the genipin-crosslinked chitosan film revealed low hydrophilicity; therefore, the chitosan layer was not entangled with the soybean protein non-woven fabric, the latter being easily stripped away. This new wound dressing material provided an adequate moisture environment, minimizing the risk of wound dehydration, and exhibited good mechanical properties. Finally, the in vivo histological data confirmed the epithelialization and reconstruction of wounds on the back of rats. The composite was easily stripped from the wound surface without damaging the newly generated tissue.

Genipin was also used to blend chitosan and gelatin for tissue engineering [23]. Higher genipin concentration (2.5% w/w of gelatin) negatively affected properties desirable for dressing applications such as wettability, dissolution and swelling degree of the blends. On the other hand mouse fibroblast adhesion and proliferation on substrates increased because gelatin contains an Arg-Gly-Asp (RGD) attachment site, a major recognition sequence for cell adhesion and proliferation [117]. The relative comparison of biological response involving cell proliferation and viability on chitosan–gelatin scaffolds suggested that blending of gelatin and chitosan could improve the cellular efficiency. Studies involving scanning electron and fluorescence microscopy, histological observations and flow cytometry analysis of the constructs implied that the polygonal cells can attach to and penetrate the pores in the scaffold, proliferating well during the 28-day culture period. Furthermore, chitosan–gelatin scaffolds were cytocompatible with buffalo embryonic stem cells [140]. This study disclosed for the first time that the chitosan–gelatin scaffolds were promising candidates for stem-cell-based tissue engineering, considered as the next generation of skin substitutes. Stem cells, as cells being able to renew themselves through mitotic cell division and differentiating into a diverse range of specialized cell types, have been increasingly reported to confer benefits in vivo as agents of angiogenesis and multilineage restoration in the face of soft tissue defects [2, 76]. Evidence suggests that adipose-derived stem cells (ASCs) are permanent residents in tissues and on stand-by for tissue repair as needed [141]. However, with the extensive tissue damage, the local pool of stem cells available for repair is putatively insufficient to fully correct the deficiency and thus therapeutic application of stem cells derived from relatively low-risk surgical interventions is needed [41, 155]. In a recent study chitosan has been combined with silk fibroin to prepare scaffolds serving as a delivery vehicle for human ASCs in a murine soft tissue injury model [3]. Green fluorescent protein (GFP)-labeled ASCs have been seeded on the fibroin–chitosan scaffolds (SFCS) and suture-inlaid to a full-thickness skin defect in mice. The results showed significant enhancement in the extent of wound closure in the ASC–SFCS group compared to SFCS and no-graft controls at postoperative day 8 (90, 75 and 55%, respectively), with a microvessel density at the wound bed significantly higher in the ASC–SFCS group compared to SFCS alone. GFP-positive stem cells

differentiated into epidermal epithelial cells within 4 postoperative weeks. The generated silk fibroin–chitosan scaffold seeded with human ASCs enhanced the wound healing and showed differentiation into fibrovascular, endothelial and epithelial components of the restored tissue. In the prepared scaffold the chitosan mimics the glycosaminoglycan constituents in the extracellular matrix and serves as a substrate for cell adhesion, migration and ultimately tissue incorporation. In yet another example, chitosan has been cross-linked with genipin to obtain an asymmetric structure chitosan membrane further loaded with type I collagen particles (CGC membrane). Cell adhesion tests after 7 days of dynamic culture revealed a flat cell morphology and good spreading on the membrane scaffold. In animal studies, this potential skin substitute seeded with fibroblast and grown for 7 days was more effective for healing wounds than the commercial ones. In vivo histological assessment indicated that covering the wound with CGC membrane resulted in epithelialization and reconstruction [18].

5 Commercially Available Wound Dressing Materials Based on Chitin and Chitosan

Despite the large number of reports, there are only few chitin and chitosan-based medical products at the market. Commercialization of chitin and chitosan (including oligomeric forms) for medical applications has been limited rather to the unmodified forms. Currently several chitin-based wound dressings are being marketed. Chitipack S® and Chitipack P® (Eisai Co.) represent sponge-like chitin from squid and dispersed, swollen chitin supported on poly(ethylene therephthalate), respectively. Chitipack S® sponge is used in the treatment of traumatic wounds and surgical tissue defects in animals, favoring early granulation, while Chitipack P® is used also in the dressing of large skin defects in animals. Marine Polymer Technologies (http://www.syvek.com) is a privately run company that researches, develops and markets technologies based on a proper microalgae-based polymer that accelerates the body's hemostatic processes. The series of products under the name of Syvek-Patch®, based on chitin fibrils, has been clinically tested as a hemostatic agent on 200 low-risk patients [107].

HemCon® from HemCon Medical Technologies Inc. (http://www.hemcon.com) is a hemostatic dressing of freeze-dried chitosan acetate salt for emergency use. A series of studies has been conducted to evaluate HemCon® performance in the prevention of fatal infections [16, 17] in civilian emergency medical services [15] and as antimicrobial dressings on infected burns, all indicating its efficiency. The bandage TraumaStat® has been recently introduced on the market by Ore-Medix (http://www.oremedix.com/home/default.asp) for the same application [35]. Freeze-dried sponge comprising chitosan and collagen, Vulnosorb® from Tesla-Pharma (http://www.tesla-pharma.com), has been commercialized in Europe since 1996.

6 Conclusion

Recent progress in dermal tissue management has been directed toward physiological repair at the molecular level. Chitin and chitosan, with their unique structural, functional, physical and chemical properties, appear as biopolymers of choice for wound-healing and tissue-engineering applications. Biochemical modifications of these biopolymers and their preparation in different designs have been reported extensively, however with limited commercial impact. The introduction of a variety of targeted functionalities to the chitin/chitosan backbone for dermal tissue repair would allow the interaction of these biopolymers that are intrinsically beneficial for wound healing with the injured skin at the molecular level, thereby accelerating the healing process. In addition, the use of chitins and chitosans in the form of nanofibers, hydrogels, films and sponges will provide the ordered regeneration of wounded tissue.

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Biofunctionalization of Polymers and Their Applications

Guo-Qiang Chen

Abstract Polyhydroxyalkanoates (PHAs) are a family of biopolyesters synthesized by many types of bacteria as carbon and energy reserve materials. PHAs combine properties of thermal processibility, biodegradability, biocompatibility and sustainability. They have attracted attention from fermentation, materials and biomedical industries. Recent environmental concerns such as CO_2 emissions and plastic pollution as well as rapid exhaustion of petroleum resources have increased public and industrial interests in these unique materials. In fact, PHA has slowly evolved into an industrial value chain ranging from microbial fermentation, bioplastic packaging, biofuel, medical implants, drug delivery, protein purification, chiral chemicals and drug development. This chapter will discuss microbial PHA production and its applications in various fields.

Keywords Biofuel · Biomaterials · Bioplastics · Biopolyesters · Drug targeting · Fermentation · PHB · Polyhydroxyalkanoates · Production · Protein purification

Contents

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1 Introduction

Production of polyhydroxyalkanoates (PHA) by bacteria is a universal phenomenon; at least 30% of bacteria inhabiting soils were found to produce various PHAs when different carbon sources were supplied as nutrients to the bacteria [27] (Fig. 1). PHAs are composed of diverse monomer structures with over 120 variations depending on the bacterial species, growth conditions and especially on the specificity of the respective PHA synthase PhaC [7, 8] (Fig. 2). Depending on the monomer composition, PHAs can be brittle, flexible or elastic, allowing them to be exploited for various applications [10] (Fig. 3). As thermally processible materials, PHAs can be used as environmentally friendly plastics for packaging purposes (Fig. 4). Their biodegradability and biocompatibility allow them to be used as bioimplant materials for medical applications [5]. More excitingly, methyl esters of PHA monomers are combustible, and they hold promise as biofuels or as fuel additives for gasoline [29] (Fig. 5). Recent results have showed that PHA can be produced in continuous and non-sterile process conditions lasting up to 3 years without microbial contamination, demonstrating that the cost of PHA can be dramatically reduced [11].

Besides the above applications, PHA synthesis in bacteria was found to provide resistance against environmentally adverse conditions for bacteria [12, 30], and this can be exploited for the development of robust industrial bacterial strains for enhanced production of chemicals [3, 14].

Chiral monomers of PHA can also be used for the synthesis of drugs or others used for fine chemicals [5, 6]. For example, one of the most common PHA monomers, 3-hydroxybutyrate (3HB), is a potential memory-enhancing drug when turned into 3HB methyl ester [32] (Fig. 6). Several amphiphilic proteins, namely PhaP or phasin attached to hydrophobic PHA granules, were used to achieve protein purification or specific drug delivery (targeting) [26, 28] (Fig. 7). It appears that PHA has many potential applications waiting to be exploited.

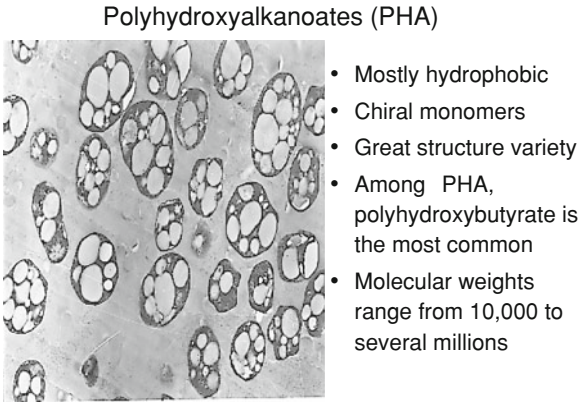

Fig. 1 PHAs are accumulated as granules in bacterial cells

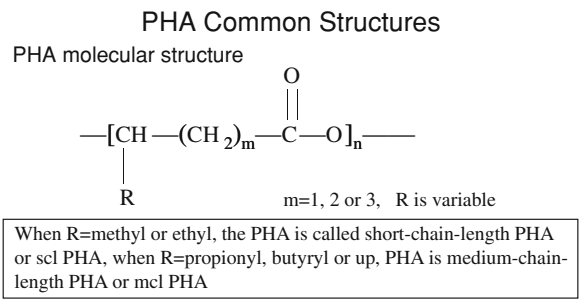

Fig. 2 PHA molecular structures

Fig. 3 Products based on PHA

It is now obvious that PHA's industrial value chain will soon generate revenues [3] (Fig. 8) and that uses of PHA will provide great benefits to our society.

2 Microbial Production of Polyhydroxyalkanoates (PHA)

Because of the applications mentioned above, various attempts have been made to produce PHA of various kinds beginning in the 1970s. British Imperial Chemical Industry (ICI) company was the first to produce PHA copolymers of poly(3-hydroxybutyrate-co-3-hydroxyvalerate), abbreviated as PHBV, at an industrial scale, for making shampoo bottles for the German Wella company. The Austrian Chemie Linz AG began to produce PHBs in the 1980s, and the technology was

Fig. 4 PHA packaging materials

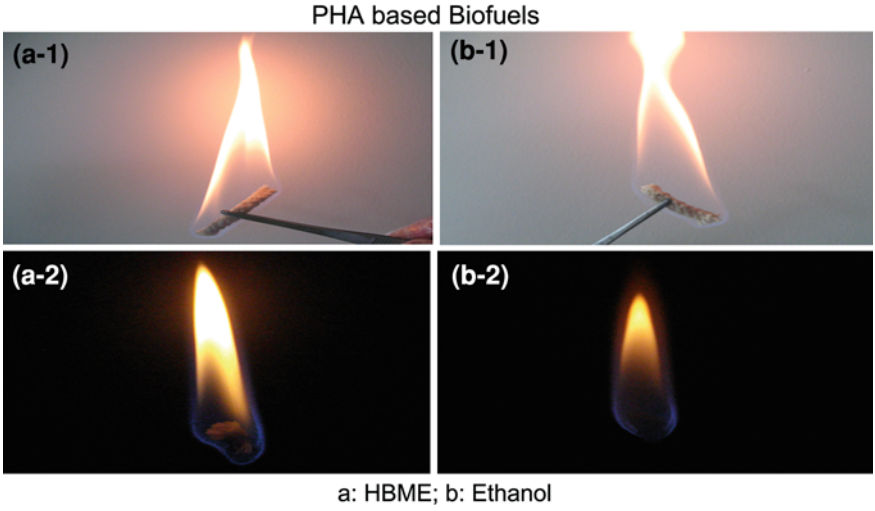

Fig. 5 Biofuels based on PHA

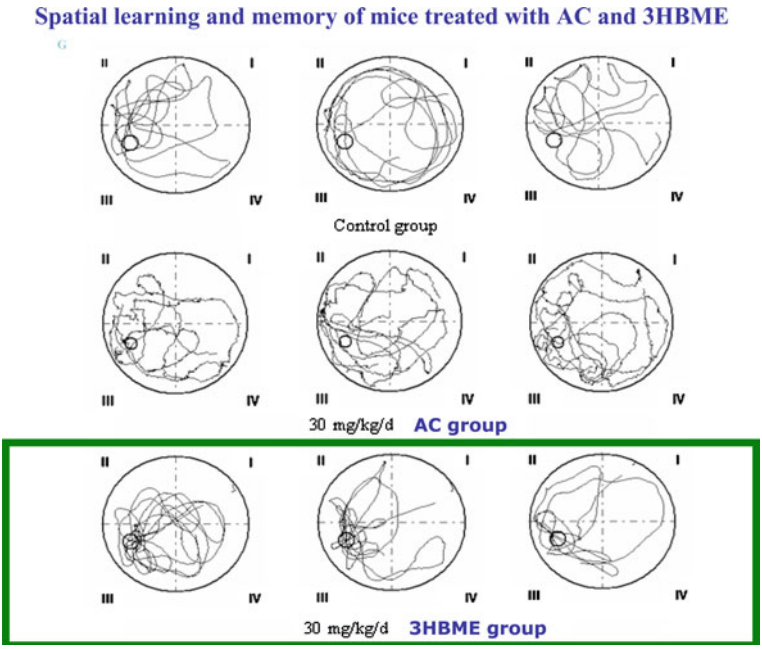

Fig. 6 Methyl ester of PHB monomer (3HBME) can enhance memory in mice. Spatial learning and memory of mice were enhanced after treatment with positive control acetyl-L-carnitine (AC) and 3HBME

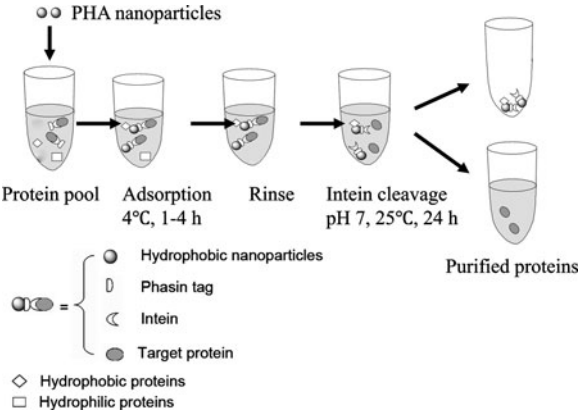

Fig. 7 PHA granule-binding protein PhaP can be used to achieve protein purification

later transferred to Biomers of Germany. The Boston-based company Metabolix was also founded in the 1980s and has been actively developing technologies for processing PHA. A joint venture between Metabolix and the US ADM Company led to a plan to produce 50,000 tons of PHA in 2006. Beginning in the 2000s, the Chinese Ningbo TianAn Company started to produce thousands of tons of PHBV

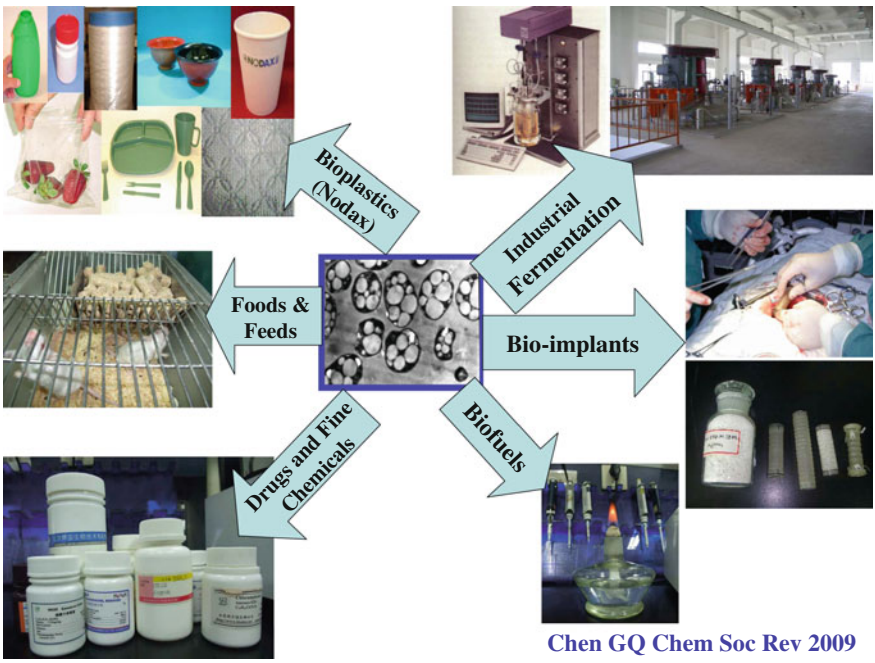

Fig. 8 PHAs are developing into a bio- and material industrial value chain with applications ranging from industrial microbiology, plastics, biofuels, medicines and fine chemicals [3]

for various applications. In early 2000, a collaboration among the Tsinghua University of China, KAIST of Korea, the US company Procter & Gamble (P&G) and the Chinese Jiangmen Biotech Development Center succeeded in producing poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), abbreviated as PHBHHx, at industrial scale, allowing P&G to develop a series of products based on PHBHHx under the trade name Nodax^{TM}. Recently, the Chinese company Green Biosci, based in Tianjin city in northern China, successfully launched a PHA plant in December 2009 with a production capacity of 10,000 tons of PHA (Table 1). These industrialization attempts are associated with a reduction of CO_2 emissions, environmental protection and sustainable development avoiding dependence on petroleum for material manufacturing [3].

2.1 Short-Chain-Length PHA

Short-chain-length PHAs (scl PHA) include PHB, PHBV and copolymers of 3-hydroxybutyrate and 4-hydroxybutyrate (P3HB4HB), copolymers of 3-hydroxypropionate (3HP) and 3HB (PHPHB) as well as copolymers of P3HP4HB.

Table 1 Worldwide PHA production and research companies [3]

Company	Types of PHA	Production scale (t/a)	Period	Applications
ICI, UK	PHBV	300	1980s to 1990s	Packaging
Chemie Linz, Austria	PHB	20–100	1980s	Packaging and drug delivery
BTF, Austria	PHB	20–100	1990s	Packaging and drug delivery
Biomers, Germany	PHB	Unknown	1990s to present	Packaging and drug delivery
BASF, Germany	PHB, PHBV	Pilot scale	1980s to 2005	Blending with Ecoflex
Metabolix, USA	Several PHAs	Unknown	1980s to present	Packaging
Tepha, USA	Several PHAs	PHA medical implants	1990s to present	Medical bio-implants
ADM, USA (with Metabolix)	Several PHAs	50,000	2005 to present	Raw materials
P&G, USA	Several PHAs	Contract		manufacture
1980s to 2005	Packaging			
Monsonto, USA	PHB, PHBV	Plant PHA production	1990s	Raw materials
Meredian, USA	Several PHAs	10,000	2007 to present	Raw materials
Kaneka, Japan (with P&G)	Several PHAs	Unknown	1990s to present	Packaging
Mitsubishi, Japan	PHB	10	1990s	Packaging
Biocycles, Brazil	PHB	100	1990s to present	Raw materials
Bio-On, Italy	PHA (unclear)	10,000	2008 to present	Raw materials
Zhejiang Tian An, China	PHBV	2000	1990s to present	Raw materials
Jiangmen Biotech Ctr, China	PHBHHx	Unknown	1990 s	Raw materials
Ecoman, Shandong, China	PHA (unclear)	3,000	2008 to present	Raw materials
Tianjin Northern Food, China	PHB	Pilot scale	1990s	Raw materials
Shantou Lianyi Biotech, China	Several PHAs	Pilot scale	1990s to 2005	Packaging and medicals
Jiangsu Nan Tian, China	PHB	Pilot scale	1990s to present	Raw materials
Shenzhen O'Bioer, China	Several PHAs	Unknown	2004 to present	Unclear
Tianjin Green Bio-Science (+DSM)	P3HB4HB	10,000	2004 to present	Raw materials and packaging
Shandong Lukang, China	Several PHAs	Pilot Scale	2005 to present	Raw materials and medicals

PHB, PHBV and P3HB4HB have been produced on a large scale (Table 1). Bacterial strains, such as *Ralstonia eutropha* (formerly called *Alcaligenes eutrophus*), *Alcaligenes latus*, *Bacillus* spp. and recombinant *E. coli* containing PHA synthesis gene *phaCAB*, are used as industrial strains for scl PHA production [3]. Compared with other PHAs, scl PHA-producing strains grow fast on simple substrates with high PHA content in microbial cells. In addition, scl PHAs are synthesized by only three key enzymes named PhbA, PhbB and PhbC, allowing convenient genetic manipulations. This explains why scl PHAs are now commercially produced with relatively low cost while others are not. Various products have been developed using scl PHA (Fig. 8).

2.2 Medium-Chain-Length PHA

Medium-chain-length PHAs (mcl PHA) have the most structural diversity (Fig. 9), are soft, elastic and even sticky. They can potentially be used as latex or adhesives. Some of the unsaturated bonds in mcl PHA can be crosslinked by UV radiation or chemical reactions, leading to rubber properties. Most mcl PHAs are produced by *Pseudomonas* spp., including *P. putida*, *P. aeruginosa* and *P. oleovorans*, among many other microorganisms. Until now, mcl PHAs have not been produced at large scale because of the lack of successful commercial application. However, the unique structures of mcl hydroxyalkanoic acids have yet to be exploited for chiral chemicals synthesis. When mcl PHAs are available in large quantities and have low costs, many applications could follow.

2.3 Short- and Medium-Chain-Length Copolymers of PHA

PHA copolyesters of scl- and mcl monomers combine the toughness of scl PHA and elasticity of mcl PHA, demonstrating the most desirable properties for various

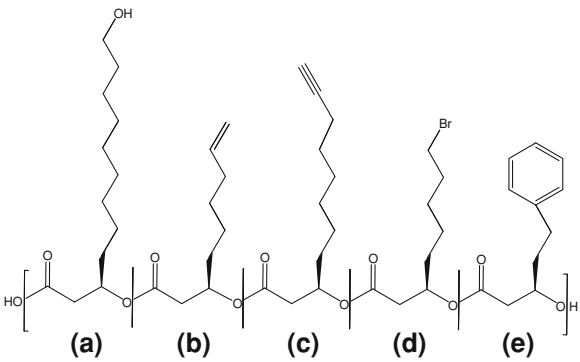

Fig. 9 Medium-chain-length PHA [31]

applications. Among these copolymers, so far, only PHBHHx has been successfully produced and is available in large quantities for research. In PHBHHx, 3-hydroxyhexanoate (3HHx) is responsible for the elasticity while 3-hydroxybutyrate (3HB) for the toughness due to its high crystalinity. The US-based company, Procter & Gamble (P&G), has given these copolymers the trade name Nodax™. P&G has used PHBHHx to develop various applications by including films, foam, coatings, bottles and utensils (Fig. 4). However, due to the high cost of using lauric acid for producing PHBHHx, its large-scale application as a packaging material has not yet been realized. Many attempts have been made to genetically engineer the PHBHHx-producing strain *Aeromonas caviae* and *Aeromonas hydrophila* for enhanced production of PHBHHx with different degrees of success; now PHBHHx can be produced with different compositions and properties [15, 16, 21] (Fig. 10).

2.4 PHA Production by Plants

PHA production in plants, particularly by agricultural crops, is viewed as a promising approach that can reduce costs compared to bacterial fermentation [2]. Synthesis of PHA in crops also fits into a larger concept of using plants as reactors for the renewable and sustainable synthesis of carbon building blocks that are currently almost exclusively provided by the petrochemical industry. If PHA production in plants is indeed achieved, it will be a very successful example of CO_2 reduction or industrial green products.

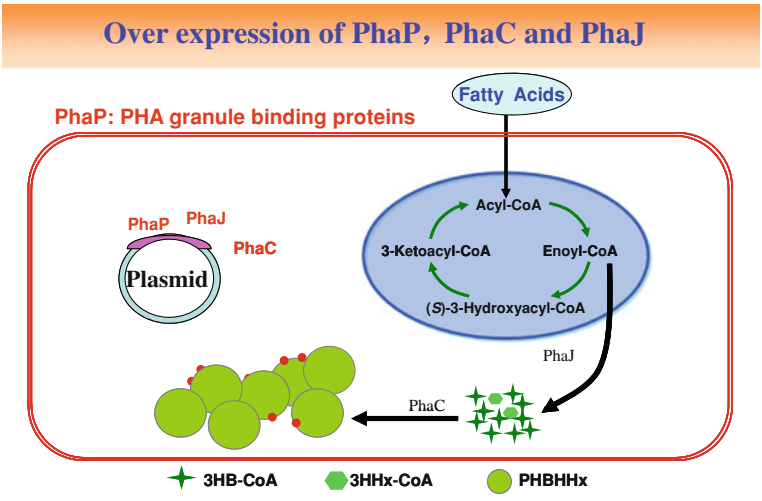

Fig. 10 Metabolic engineering for enhanced PHBHHx production

Synthesis of PHA in plants was first demonstrated in 1992 by the accumulation of PHB in the cytoplasm of cells of *A. thaliana* [19]. Since then, a range of different PHAs has been synthesized, including various co-polymers such as PHBV and mcl PHA, in a variety of plants, including corn, sugarcane and switchgrass [20]. This has been achieved through the modification of various pathways localized in different subcellular compartments, such as the fatty acid and amino acid biosynthetic pathways in the plastid or the fatty acid degradation pathway in the peroxisome. Although the initial driving force behind synthesis of PHA in plants has been for the biotechnological production of biodegradable polymers, PHA synthesis in plants has also been used as a useful tool to study some fundamental aspects of plant metabolism [18].

The success of using transgenic plants as a source of novel material will not only depend on the production levels in plants achieved, but also on whether the polymers can be extracted efficiently, economically and using environmentally friendly methods. Although a number of strategies have been described in the literature for the extraction of PHA, some relying on solvents while others not, further work is required to validate these extraction procedures in the context of large-scale production in crop plants [17].

3 Applications of Polyhydroxyalkanoates (PHA)

Successful application developments depend on the availability of large amounts of PHA, preferably at low cost. Until now, only PHB, PHBV, P3HB4HB and PHBHHx are available in sufficient quantities. Therefore, most applications are centered on these PHAs. PHAs were initially used by Wella AG Germany to make articles such as shampoo bottles and packaging materials. PHAs were also developed as packaging films mainly for use as shopping bags, containers and paper coatings, disposable items such as razors, utensils, diapers, feminine hygiene products, cosmetic containers and cups as well as fibers, medical surgical garments, upholstery, carpet, packaging, compostable bags and lids or tubs for thermoformed articles by P&G, Biomers, Metabolix, Polyone and several other companies [4].

Beginning in 2010, large amounts of PHA will be made available to the polymer processing companies, which will be able to develop more PHA-based products. In addition, high value added applications of PHA have also been continuously developed, as described below [4].

3.1 Bio-Implant Materials

PHB, PHBV, P4HB (short for poly-4-hydroxybutyrate), PHBHHx and poly-3-hydroxoctanoate (PHO), which are available in sufficient quantities, have been

used to develop devices including sutures, suture fasteners, meniscus repair devices, rivets, tacks, staples, screws (including interference screws), bone plates and bone plating systems, surgical mesh, repair patches, slings, cardiovascular patches, orthopedic pins (including bone filling augmentation material), adhesion barriers, stents, guided tissue repair/regeneration devices, articular cartilage repair devices, nerve guides [1, 5, 24], tendon repair devices, atrial septal defect repair devices, pericardial patches, bulking and filling agents, vein valves, bone marrow scaffolds, meniscus regeneration devices, ligament and tendon grafts, ocular cell implants, spinal fusion cages, skin substitutes, dural substitutes, bone graft substitutes, bone dowels, wound dressings and hemostats [5]. Changing PHA compositions allow favorable mechanical properties, biocompatibility and degradation times within desirable time frames under specific physiological conditions.

With successful approval of P4HB as an implant biomaterial by FDA (http://www.tepha.com), more PHA-based biomaterials are expected to go into clinical trials soon. With the diversity of PHA materials, one can expect a diverse family of bio-implant materials with multiple applications.

It was found that the cytotoxicity of oligo PHA decreased with increasing oligo PHA side chain length. From the biocompatibility point of view, medium chain length oligo PHAs have been proposed, e.g., PHBHHx and medium-chain-length PHA Poly(3-hydroxyalkanoates) (mcl PHA), and are preferred for development and application as PHA-based tissue engineering biomaterials due to their low toxicity of their degraded oligo- and monomers [25].

3.2 A Pool for Chiral Hydroxyalkanoic Acids

Chiral R-form hydroxyalkanoic acids are normally difficult to synthesize by chemical means. The occurrence of over 120 different chiral R-forms of PHA monomers reflects the low substrate specificity of PHA synthases, which are the key enzymes of PHA biosynthesis [7, 8, 22]. These can be used as a rich pool of chrial compounds [13]. In addition, the importance of bacterial anabolism and catabolism, which provide the coenzyme A thioesters of the respective monomers as substrates to these PHA synthases, is also important in controlling the structure of hydroxyalkanoic acids [23].

R-3-hydroxybutyrate (3HB) was reported to be used for the synthesis of carbapenem antibiotics [6]. Other 3-hydroxy acids were also reported to be used as chiral building blocks for the synthesis of macrolides such as pyrenophorin, colletodiol, grahamimycin A1 and elaiophylidene [6]. The transformation of 3-hydroxyalkanoates (3HA) to their dioxanone enolates allows the subsequent synthesis of various 3HA derivatives. 3HB dioxanone enolates can be used as intermediates for synthesis of beta-lactones and 2-alkylated 3HB [6].

3HB can also be used to synthesize dendrimers that possess the advantages of biodegradability, monodispersity and large numbers of surface functional moieties, which can be expected to be promising for in vivo drug delivery. Sodium 3HB

was also found to represent many clinical advantages against normal glucose injection when used as a vein injection or taken orally in starvation treatment of adiposis [6].

Learning and memory requires energy-demanding cellular processes and can be enhanced when the brain is supplemented with metabolic substrates. It was found that neuroglia cell metabolic activity was significantly elevated when cultured in the presence of 3HB and its methyl ester derivative. The proliferative effects of these compounds correlated with increased gap junction intercellular communication. It was demonstrated that the receptor for 3HB, namely, PUMA-G, protein upregulated macrophages by IFN-γ, was expressed in brain and upregulated in mice treated with 3-hydroxybutyrate methyl ester (M-3HB). Increased expression of connexin 36 protein and phosphorylated ERK2 (extracellular signal-regulated kinase 2) in brain tissues following M-3HB treatment was also observed. Mice treated with M-3HB performed dramatically better in the Morris water maze than either the negative controls (no treatment) or positive controls (acetyl-L-carnitine treatment). Thus, 3HB and derivatives enhance learning and memory by increasing protein synthesis and GJIC through a signaling pathway requiring PUMA-G [32].

3.3 A Source of Biofuels

When PHB and mcl PHA were, respectively, esterified to become R-3-hydroxybutyrate methyl ester (3HBME) and medium chain length hydroxyalkanoate methyl ester (3HAME) via acid catalyzed hydrolysis [29] (Fig. 11), it was found that 3HBME and 3HAME had combustion heat values of 20 and 30 kJ/g, respectively. Ethanol has a combustion heat of 27 kJ/g, while addition of 10% 3HBME or 3HAME enhanced the combustion heat of ethanol to 30 and 35 kJ/g, respectively. The addition of 3HBME or 3HAME into *n*-propanol and *n*-butanol led to a slight reduction of their combustion heats. Combustion heats of blended fuels 3HBME/diesel or 3HBME/gasoline and of 3HAME/diesel or 3HAME/gasoline were lower than those of the pure diesel or gasoline, although reasonable as a fuel. It was roughly estimated that the production cost of PHA-based biofuel should be around US$ 1,200 per ton when PHA was produced from activated sludge.

3.4 Drug Delivery Carriers

Many proteins located on the surfaces of PHA granules are amphiphilic. For example, PHA granule-binding protein, PhaP, which is produced by PHA-positive bacteria, is available in abundant quantities on the granule surfaces. A receptor-mediated drug-specific delivery system was based on PHA nanoparticles, and PhaP was reported to be successful for achieving specific drug targeting to liver

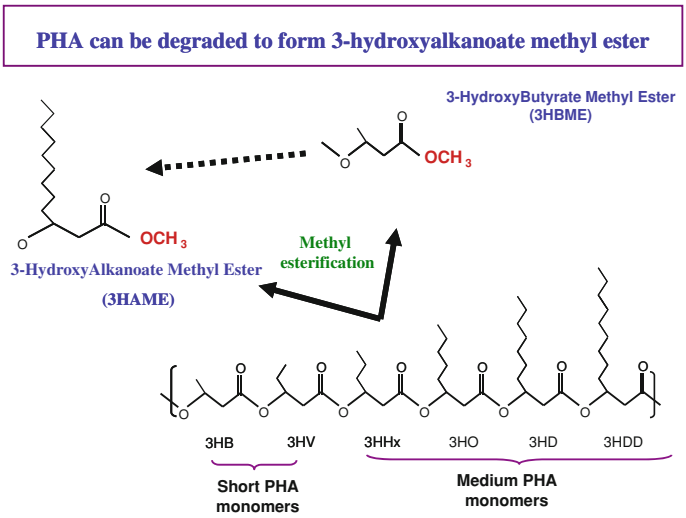

Fig. 11 PHA as a source of biofuel [29]

cancer cells in mice [28]. The receptor-mediated system consists of PHA nanoparticles, PhaP and ligands fused to PhaP (Fig. 12). The PHA nanoparticles were used to package mostly hydrophobic drugs, and PhaP fused with ligands produced by over-expression of their corresponding genes in *Pichia pastoris* or *E. coli* was able to attach to hydrophobic PHA nanoparticles. At the end, the ligands were able to pull the PhaP–PHA nanoprticles to the targeted cells with receptors recognized by the ligands. In our study, the receptor-mediated drug-specific delivery system ligand–PhaP–PHA nanoparticles were taken up by macrophages, hepatocellular carcinoma cell BEL7402 in vitro and hepatocellular carcinoma cells in vivo, respectively, when the ligands were mannosylated human α1-acid glycoprotein (hAGP) and human epidermal growth factor (hEGF), respectively, which were able to bind to receptors of macrophages or hepatocellular carcinoma cells. The system was clearly visible in the targeted cells and organs under fluorescence microscopy when rhodamine B isothiocyanate (RBITC) was used as a delivery model drug due to the specific targeting effect created by specific ligand and receptor binding. The delivery system of hEGF–PhaP–nanoparticles carrying model drug RBITC was found to be endocytosed by the tumor cells in an xenograft tumorous model mouse (Fig. 13). Thus, the ligand–PhaP–PHA-specific drug delivery system was proven effective both in vitro and in vivo [28].

3.5 Protein Purification Platform

Another application for PHA granule-associated protein phasin (PhaP) is protein purification. To do this, a pH-inducible self-cleaving intein and PHA nanoparticles

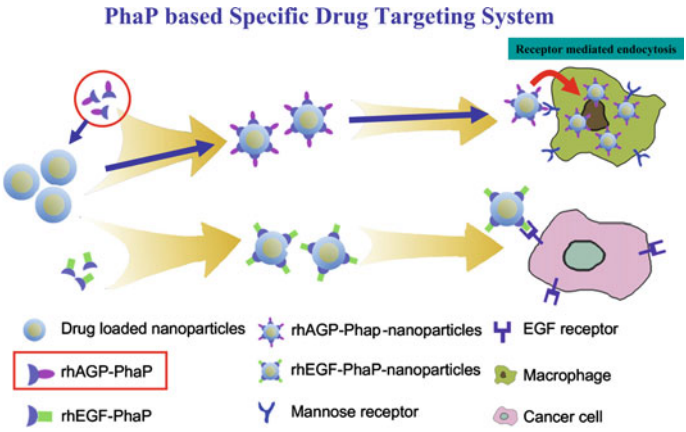

Fig. 12 A receptor-mediated drug-specific delivery system based on PHA nanoparticles and PhaP [28]

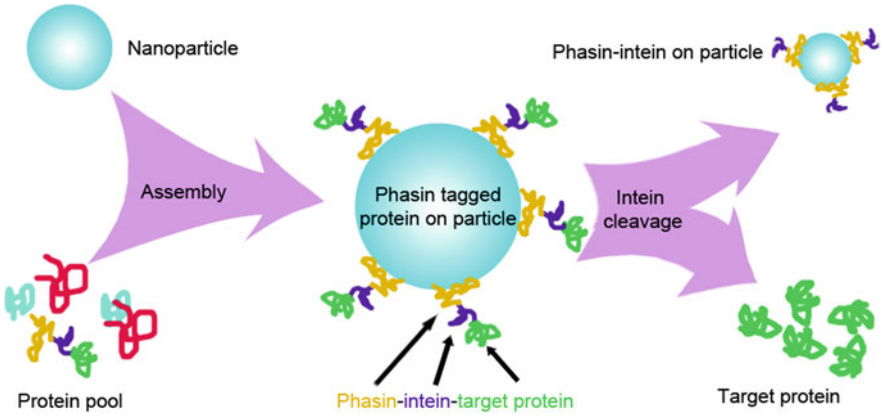

Fig. 13 PHA granule-associated protein phasin (PhaP) can be used for developing the protein purification platform[26]

combined can be used as a protein purification system (Fig. 13). Genes for producing and purifying the target proteins were fused to genes of intein and phasin, and the genes were jointly overexpressed in vivo, such as in *E. coli* cells. The fused proteins containing target protein, intein and phasin produced by the recombinant *E. coli* were released together with all other *E. coli* proteins via a bacterial lysis process. They were then adsorbed in vitro to the surfaces of the hydrophobic polymer nanoparticles incubated with the cell lysates. The nanoparticles attached with the fused proteins were concentrated via centrifugation. Then, the reasonably purified target protein was released by self-cleavage of intein and separated with

nanoparticles by a simple centrifugation process. Using this system, enhanced green fluorescent protein (EGFP), maltose-binding protein (MBP) and β-galactosidase were successfully purified in their active forms with reasonable yields, respectively, demonstrating the effectiveness and reliability of this purification system. This method allows the production and purification of high value-added proteins in a continuous way with low cost [26].

3.6 Feed Additives

There is a growing awareness that antibiotics should be used less in animal husbandry because of the risk of developing antibiotic resistance. However, a decreased use of antibiotics could result in a growth of pathogenic bacteria, leading to an incidence of infections. Short-chain fatty acids (SCFAs) have long been known to exhibit bacteriostatic activity. These compounds also specifically downregulate virulence factor expression and positively influence the gastrointestinal health of the host. As a consequence, there is currently considerable interest in SCFAs as biocontrol agents in animal production. Some evidence has shown that PHA can also be degraded upon passage through the gastrointestinal tract of animals, and consequently adding these compounds to the feed might result in biocontrol applications similar to those described for SCFAs [9].

It might be highly interesting to evaluate PHAs as biocontrol agents in different host-microbe systems, such as, for instance, in *Salmonella* infections in poultry and swine or bacterial infections in crustaceans and fish. In these studies, the chemical composition of the polymer, its physicochemical state (crystalline or amorphous) and the size of the polymer particles will be of specific interest. Given the fact that medium- and long-chain fatty acids are more bactericidal than SCFAs, it could be interesting to study the biocontrol potential of these medium- and long-side-chain PHAs [9].

4 Conclusion

Future development of PHAs should be focused on two fields: lowering the production costs and finding high value-added applications of PHAs. To achieve this goal, genetic engineering, pathway modification or synthetic biology approaches should allow the development of super PHA production strains that are able to grow to high cell density within short periods of time on low cost substrates. A synthetic strain containing minimum genome could help increase the yield of PHA. Simple purification and extraction technology employing controllable lysis of bacteria that have accumulated large PHA granules is an area requiring further investigation that can potentially reduce the cost of centrifugation, filtration and extraction.

Low cost PHA will not only benefit the application of PHA material as bioplastics, but will also promote the application of PHA as biofuel. This is an area full of promise, since low cost PHA could also be obtained from activated sludge and wastewater fermentation.

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Enzymatic Polymer Functionalisation: Advances in Laccase and Peroxidase Derived Lignocellulose Functional Polymers

Gibson S. Nyanhongo, Tukayi Kudanga, Endry Nugroho Prasetyo and Georg M. Guebitz

Abstract Enzymatic polymer functionalisation has entered its most fascinating period with development in this field largely at the basic research level and pilot scale applications. Development of enzymatic processes for the development of lignocellulose-based functional polymers has not been spared, ranging from textile fibres with novel properties (antimicrobials properties, hydrophobic properties, attractive shed colours, etc.) to fibreboards. Enzymatic processes are also being actively pursued aimed at developing functional polymers from lignin (a major by product of the pulp and process).

Keywords Functional polymer · Laccase · Lignocellulose · Peroxidase

Abbreviations

MDF	Manufacturing of medium-density fibreboards
HBT	1-Hydroxybenzotriazole
VA	Violuric acid
NHA	*N*-hydroxyacetanilide
ABTS	2,2′-Azinobis-(3-ethylbenzothiazoline)-6-sulphonate
LiP	Lignin peroxidases
MnP	Manganese peroxidases
AAO	Aryl-alcohol oxidase
SBP	Soya bean peroxidase
HDF	High density fibreboard
PB	Particleboard
PF	Phenol formaldehyde
TMP	Thermomechanical pulps

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Contents

1 Introduction

Since the late 1990s, enzyme technology, as pointed out by Guebitz and Cavaco-Paulo [29], has evolved from the biotransformation of small substrates to biotransformation of complex natural and synthetic polymers. In 2004, economists predicted a 25-fold increase in turnover for polymers processed through biotechnological methods up until 2010, compared with a sevenfold turnover increase for fine chemicals [94]. The current revolution is underpinned by the accrued understanding of enzyme reaction mechanisms, a new global thrust towards green chemistry technologies and advancement in analytical technologies gained in applying enzymes in waste treatment, pulp and paper industry, organic synthesis, diagnostics and food industries. Among the enzymes intensively sought for biotransformation of complex polymers are laccases and heme peroxidases. These enzymes have proved to be versatile biocatalysts with remarkable abilities to oxidise a wide variety of natural and synthetic molecules. The ability of laccases and peroxidases to generate reactive species (phenoxy radicals), thereby enhancing the reactivity of molecules/polymers, forms the basis of their application in polymer chemistry [73, 74]. The generated reactive species provide ideal sites for crosslinking (coupling) of desired functional molecules leading to grafting and consequent formation of new materials or materials with novel properties. The resulting grafting products depend on the position of the radical as the oxidised molecule tries to stabilise itself through resonance [74].

The discovery of mediators (small molecules which when oxidised by peroxidases or laccases form highly reactive species), which have the ability to oxidise high redox potential substrates difficult to be oxidised by the enzyme alone, has further expanded the applications of these enzymes in modifying "inert" polymers. Examples of widely investigated mediators are 1-hydroxybenzotriazole (HBT), violuric acid (VA), *N*-hydroxyacetanilide (NHA) and 2,2′-azinobis-(3-ethylbenzothiazoline)-6-sulphonate (ABTS). Alternatively, active research is aimed at developing natural cost-effective lignin-derived mediators [10].

Although horseradish peroxidase (HRP) has been studied for more than a century and was the first enzyme to be employed for oxidative polymerisation during the 1980s [17, 80], since the mid-1990s laccases have become increasingly pursued for similar applications. The observation of the ability of laccases and peroxidases to crosslink different small molecules has extended their application in surface modification of complex polymers by grafting (coupling) functional molecules (antimicrobials, reactive molecules, hydrophobic enhancing molecules, etc.). Therefore, peroxidase and laccase-mediated grafting of functional molecules is and has become an attractive option to introduce a variety of desired functional groups onto polymers resulting in impressive functionalities, which in many cases cannot be obtained through conventional chemical synthesis. The excitement generated by this new approach is justified given the potential benefits, such as environmentally friendly processes, processes operated at ambient temperatures and consequently low operational cost. Therefore, timeliness of reviewing the progress made in peroxidase/laccase-mediated grafting is justified by the recent advances in the field. This chapter summarises and critically discusses the current status of the application of heme peroxidases and laccases in grafting functional molecules onto lignocellulose polymers with the aim to respond to the increasing interest in this relatively recent but very promising field.

1.1 Heme Peroxidases: Sources and Brief Review of Biochemical and Catalytic Properties

A number of reviews have been dedicated to describing the sources, distribution, biochemical properties and general applications of heme peroxidases and laccases, and therefore are not subject of discussion in this work. Briefly, peroxidases (peroxidase EC 1.11.1.7) are iron(III) protoporphyrin IX containing enzymes, widely distributed in plants, microorganisms and animals. In plants many physiological functions have been assigned to peroxidases including indole-3-acetic acid metabolism, lignification, crosslinking of cell wall polymers, suberin formation and resistance to infection [64]. Lignin peroxidases (LiPs) and manganese peroxidases [EC 1.11.1.13 (MnP)] are widely distributed in fungi. In fungi, LiPs are involved in degrading non-phenolic lignin units that make up to 90% of the lignin polymer, while MnP generates Mn^{3+}, which acts as a diffusible oxidiser attacking both phenolic and non-phenolic lignin units. More recently, versatile

peroxidases have been described as a third type of lignolytic peroxidase that combines the catalytic properties of LiP, MnP and plant/microbial peroxidases in oxidising phenolic compounds [63]. Nevertheless, most of our current understanding of biochemical properties and biotechnological applications of heme peroxidases is based on horseradish peroxidase obtained from *Armoracia rusticana* (P.Gaertn., B.Mey. & Scherb.; Cruciferae), a hardy perennial herb cultivated in temperate regions for the culinary value of its roots [93].

Heme peroxidases catalyse the oxidation of several organic substances (phenols) using hydrogen peroxide or a few of the hydroperoxides. Their catalytic mechanism involves a two-electron oxidation of the heme moiety to an intermediate known as compound I. Successive one electron reductions return the enzyme to its resting state using a second intermediate, compound II, resulting in the production of free radical [93]. In vivo, oxidases such as aryl-alcohol oxidase (AAO) play a crucial role in providing the H_2O_2 required as a cofactor for lignin attack by fungal peroxidases.

1.2 Laccases: Sources and Brief Review of Biochemical and Catalytic Properties

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are multicopper containing enzymes that catalyse the oxidation of a broad range of substrates, such as phenols, polyphenols, anilines, aryl diamines, methoxy-substituted phenols, hydroxyindols, benzenethiols, inorganic/organic metal compounds and many others with the simultaneous reduction of molecular oxygen to water [75, 79]. Laccase catalyses the four-electron reduction of O_2 to water coupled with oxidation of the substrates. Laccases were first extracted from the exudates of a Japanese lacquer tree, *Rhus vernicifera*, by Yoshida in 1883. However, new and novel sources of laccases are continuously been reported, including from insects, bacteria, terrestrial and marine fungi (Ascomycetes, Deuteromycetes, litter-decomposing and ectomycorrhizal fungi, and Basidiomycetes). In insects and bacteria, laccases are associated with the synthesis of pigments, whereas in fungi laccases are involved in the degradation of lignin.

2 Functionalisation of Lignin

Although lignin is the second most abundant polymer on earth constituting 30% of non-fossil organic carbon [7] and a major by-product of the pulp and paper industry, only approximately 2% of lignin is currently used in polymer blends for mortar, construction systems, adhesives, biodegradable plastics, polyurethane copolymers, paints, dispersants in dyes, in pesticides and printed circuit boards [1, 32, 56, 82, 85]. Exploitation scale-up has been hampered by the huge

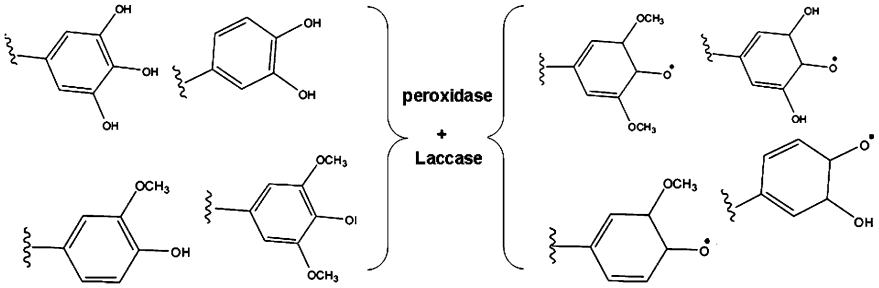

Fig. 1 Radical generated by laccase/peroxidase oxidation of lignin molecules

physicochemical heterogeneity of lignin, which renders it immiscible due to low entropy [18, 72]. Therefore, successful industrial applications of lignins require modifications to enhance its reactivity, reduce its heterogeneity and improve its dispersion properties among other physicochemical characteristics. The ability of oxidoreductases (peroxidases and laccases) to oxidise lignin (Fig. 1) has renewed the hope for potential maximum exploitation of the technical lignins in the synthesis of various functional polymers.

Treatment of lignins or aromatics by laccases/peroxidases in the presence of different redox mediators has been shown to produce different effects as summarised by Sena-Martins et al. [82], Stewart [85], Kobayashia and Higashimura [44]. Several authors have observed a decrease in phenolic and carboxylic groups, and an increase in new C–C, aryl–aryl and aryl–alkyl bonds [28, 65, 78, 84].

2.1 Grafting Reactive Functional Molecules onto Lignin

Laccase and heme peroxidase mediated grafting of small reactive molecules onto lignin in an effort to produce phenolic resins was probably the first demonstration of the potential industrial application of technical lignins. Phenolic resins are widely used in surface coatings, as adhesives, in laminates, molding, friction materials, abrasives, flame retardants, carbon membranes, etc. [19]. Dordick et al. successfully produced phenolic resins by grafting cresol onto lignin using HRP and also provided evidence that acetylated lignins or methylated lignins were not good substrates for producing phenolic resins. Milstein et al. [66] successfully used laccase to graft a variety of compounds onto organosolv-lignin in dioxane-H_2O. Laccase-mediated grafting of guaiacol sulfonate and vanillylamine onto Indulin (kraft lignin) formed a precipitate containing nitrogen, indicating copolymerisation, while the reaction with guaiacol sulfonate did not form a precipitate becaus of the presence of the sulphur groups. [57] produced water soluble kraft lignin through oxidative coupling with water soluble phenols. Liu et al. [52, 53, 55], using a reversed micellar system, succeeded in manipulating the mean molecular weight of a copolymerised polymer of lignin with cresol. The hybrid copolymer showed quite different properties compared to native lignin,

including lower glass transition temperature. Similarly, peroxidase-mediated polymerisation of lignocatechol and lignocresol produced phenolic resins that have potential application in adhesives as bonding agents and in engineering materials [100, 104]. Using soya bean peroxidase (SBP), Ikeda et al. [34] generated polyphenol resins that could replace conventionally polymerised novolac resins without the involvement of formaldehyde. In another interesting development, treatment of brown-rot-fungus-decayed wood with sodium borohydride followed by mixing with polyethylenimine resulted in a formaldehyde-free, strong and water-resistant wood adhesive [51]. The pretreatment of wood with brown-rot fungi enabled preferential degradation of cellulose and hemicellulose in wood, while simultaneously the oxidoreductases oxidised and demethylated lignin [51], resulting in oxidised ortho-quinone structures, a suitable subtsrate for adhesive production. Recently, [71] developed hybrid copolymers from different technical lignins and silane precursors that could well be used as either coating material or adhesives (Fig. 2). In another study, [72] further demonstrated the ability of laccase to modify conjugated carbonyl, biphenyl, phenylcoumarins and stilbene groups of the lignosulphonates in the presence of 1-hydroxybenzotriazole, leading to the formation of new ether and C–C aryl–aryl or aryl–alkyl linkages. This was accompanied by a loss of phenolic hydroxyl and carboxylic groups leading to extensive polymerisation. The modifications were shown to increase the dispersion properties of the lignosulphonates, a positive feature for improving lignin polymer blends. Nevertheless, the use of ABTS as a mediator was repeatedly shown to result in depolymerisation of lignin [8, 31]. This shows the great potential of manipulating the enzymatic treatments either towards polymerisation or depolymerisation depending on the target application.

Enzymatic functionalisation of lignin with acrylamide is also gaining a lot of research interest. The grafting of acrylamide onto lignin has the potential of producing a new class of engineering products, e.g., plastics. Laccase-mediated grafting of acrylamide onto lignin resulted in increased Mw from 14,900 to 27,800 [67]. However, addition of organic peroxides, particularly dioxane peroxide, tetrahydrofuran peroxide and t-butylhydroperoxide to the reaction of laccase with organosolv-lignin and acrylamide significantly increased the yield of the acrylamide/lignin copolymerisation [59–62]. It was postulated that peroxides acted as laccase mediators, producing radicals upon reaction with acrylamide, while phenoxy radicals from lignin are produced by laccase. Mai et al. [59] further successfully demonstrated the chemo-enzymatic grafting of acrylic compounds onto different technical lignosulfonates and noted that the grafting of phenolics was dependent both on their redox potential and on the type of acrylic monomer applied.

2.2 Factors Affecting Industrial Application of Industrial Lignins

An assessment of the reported grafting studies onto lignin described above reveals the significant role played by the redox potential of the enzyme, reaction

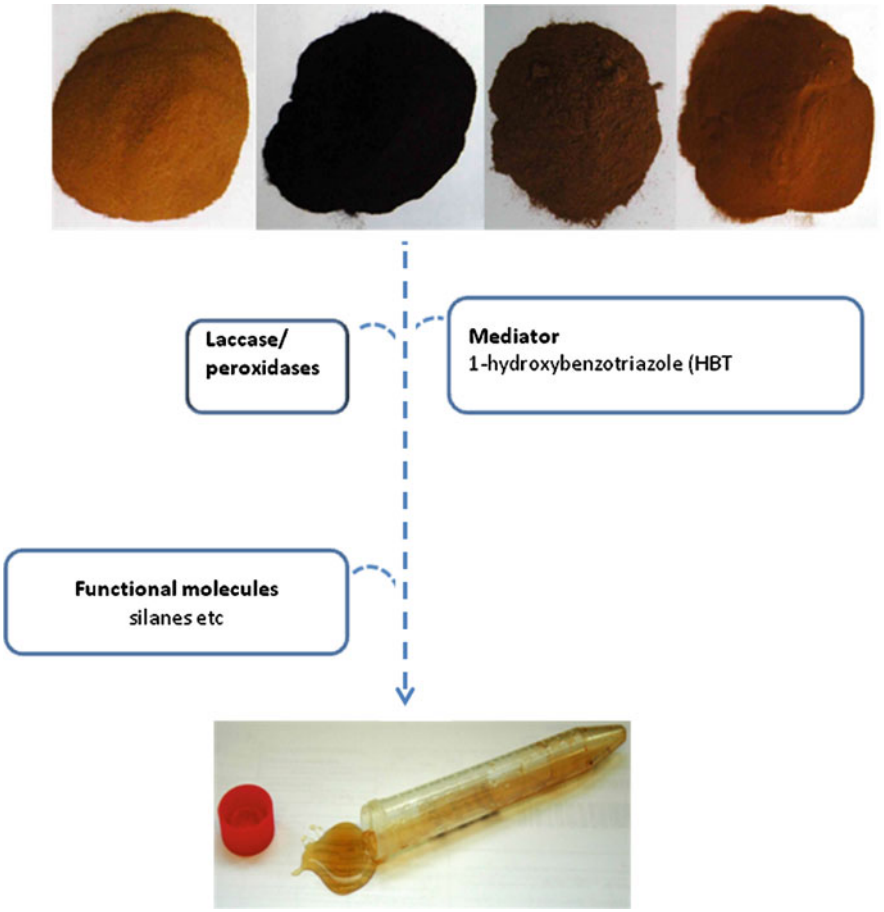

Fig. 2 Functional polymers derived from enzymatic grafting and/or copolymerisation of different silanes (tetraethylorthosilane and aminoslinane)

conditions, nature of the grafting molecule (functional molecule) and nature of the technical lignin (functional groups, side chains, etc.). Regardless of the origins of the lignin, enhancing its reactivity is a prerequisite for incorporation of functional molecules leading to successful production of new polymers. Excellent reviews on oxidative polymerisation and the influence of different substituents on the activity of peroxidases and laccases have been provided [44, 77, 91, 92, 97]. In summary, successful enzymatic functionalisation of lignin depends on:

(1) Its reactivity, which is influenced by the number of hydroxyl groups and available for reaction sites on the aromatic structure.
(2) The nature and position of side groups, e.g., methyl/methoxyl, acetyl groups or other aliphatic side-chains. For example, *p*- and *m*-substituted phenolics have been widely reported as good substrates for oxidative polymerisation [27].

(3) The predominant substituent groups, whether electron-withdrawing groups or electron-donating groups (electron-donating molecules enhance activity of laccases and peroxidases, whereas electron-withdrawing groups have a negative effect).
(4) Type of lignin (condensed or hydrolysable lignin)
(5) Method used to obtain the lignin, e.g., kraft (insoluble in water) and lignosuphonates (soluble in water).

3 Functionalisation of Lignocellulose Materials

3.1 Grafting Natural Antimicrobial Functional Molecules

Plants are increasingly being investigated for their ability to produce many secondary metabolites with antimicrobial activities [2, 70]. Simultaneously, studies investigating the ability of enzymes to mediate grafting of natural antimicrobial compounds, especially those of phenolic nature onto lignocelluloses polymers, are increasing. Lignocellulose materials grafted with antimicrobial phenolics were shown to be effective against *Bacillus subtilis*, *Escherichia coli* and *Klebsiella pneumoniae* [20, 81, 95]. The antimicrobial activities were attributed to the presence of hydroxyl groups and delocalised electrons on their structure [42, 88]. Widsteins et al. [95] also provided mechanistic evidence of the binding of catechin (as an antimicrobial) onto putative lignin monomers (Figs. 3, 4) as well as gallic acid (as a model of flavonoid-based tannins) onto putative lignin monomers (Fig. 5). Catechin was bonded to the phenolic molecules mainly through 5–5 linkages, whereas bonding to monomers with sinapyl units was mainly through 4-*O*-5 linkages.

In contrast, *β*-*O*-4 coupling products were observed in addition to 4-*O*-5 when gallic acid was coupled onto unsaturated phenolic molecules (caffeic acid, ferulic acid and sinapic acid, respectively) (Fig. 5). Preformed lignin oligomers usually couple through 5–5 and 4-*O*-5 linkages [7], whereas *β*-*O*-4 bonds are favoured in vivo, which probably explains the different frequencies of lignin substructures obtained in vitro from those obtained in vivo. Figure 5 also shows the effect of the position of the radical (due to resonance) on the coupling product.

3.2 Grafting Hydrophobic Enhancing Functional Molecules

Another interesting emerging study is enzyme-mediated grafting of hydrophobicity-enhancing molecules. Hydrophobicty enhancement is very important as it reduces the chances of growth of microorganisms that are responsible for biodeterioration as well as providing favourable conditions for the growth of pathogenic microorganisms. Laccase-mediated grafting of laurylgallate onto pulps increased their

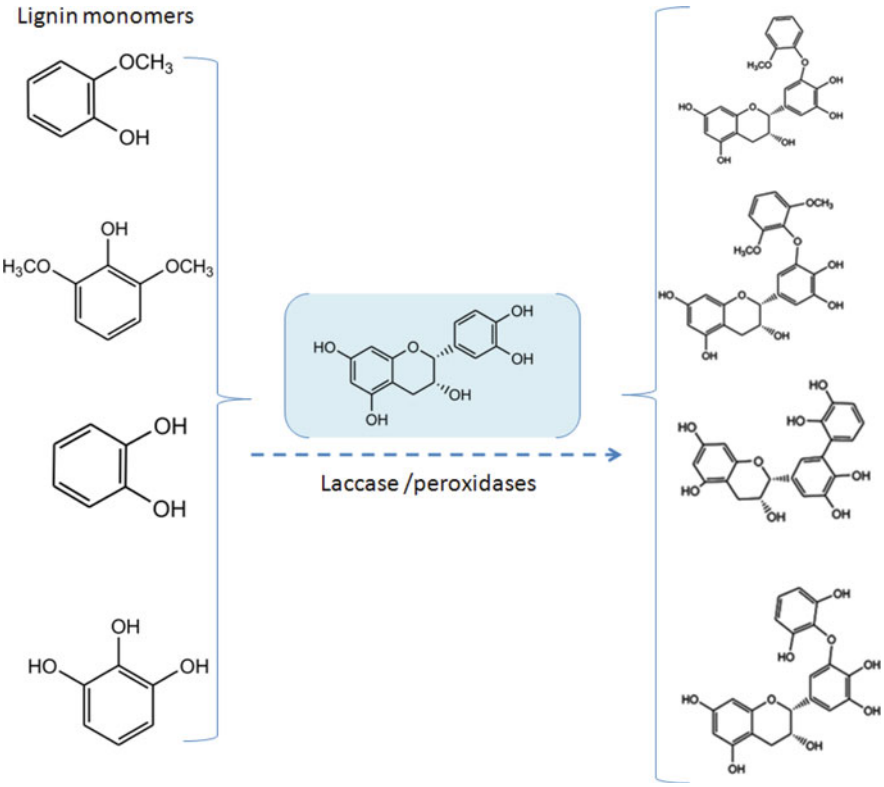

Fig. 3 In vitro grafting of catechin onto different lignin monomers (simple phenols)

hydrophobicity [86]. Recently, laccase-catalysed coupling of fluorophenols and alkylamines increased the hydrophobicity of wood veneers as illustrated in Fig. 6 ([47, 49]). The authors also provided the first in vitro evidence for the possibility of establishing covalent bonds between complex lignin models [syringylglycerol *β*-guaiacylether (G-S-*β*-ether), guaiacylglycerol *β*-guaiacyl (erol) and dibenzodioxocin] and different alkylamines as well as fluorophenols (Fig. 6; [47, 49]). The proposed mechanism showed that the fluorophenols were bonded to sinapyl units via 4-*O*-5 linkages, whereas coupling to guaicyl units occurred through 5–5 linkages (Fig. 6). The alkyl amines were bonded onto simple phenolics and lignin models through the $–NH_2$, thereby establishing a C–O–N and a –C–N bond (Fig. 6).

4 Manufacturing of Wood Composites

The production of high density fibreboard (HDF), medium-density fibreboard (MDF), particleboard (PB) and other wood composite materials uses huge amounts

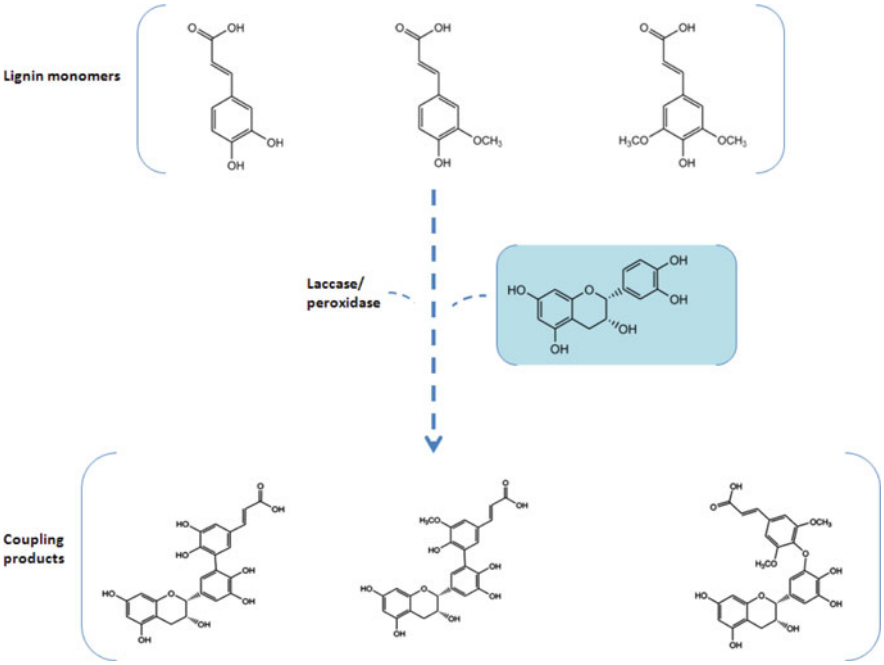

Fig. 4 In vitro grafting of catechin onto different lignin monomers (hydroxycinnamics)

of petrochemical-based adhesives. Nevertheless, recent trends show the great interest in exploring the use of phenols from renewable sources as binding agents or simply promoting auto-adhesion of fibres by increasing the reactivity of wood or fibre component materials mediated by peroxidases and laccases.

4.1 Use of Lignin as a Binder for Particle Boards

Previous attempts to use technical lignins (sulphonate or kraft) to partially substitute phenol in phenol formaldehyde (PF) adhesives have failed mainly because of the problems described elsewhere in this work. However, enzymatic oxidation of lignin is an attractive option to provide a reactive interface for further processing of lignocellulosic materials. Attempts to enhance lignin reactivity by use of enzymes to mediate bonding in wood started in the mid 1960s. Later the use of lignosulphonates oxidised by peroxidase or laccase as binders for the production of particle boards was reported by [68, 69]. They successfully produced particle boards that surpassed the requirements of transverse tensile strength (DIN 52365 test) of 0.35 MPa specified by European standard EN 312-4. Unfortunately, the particle boards swelled in water because of the presence of the sulphonate groups,

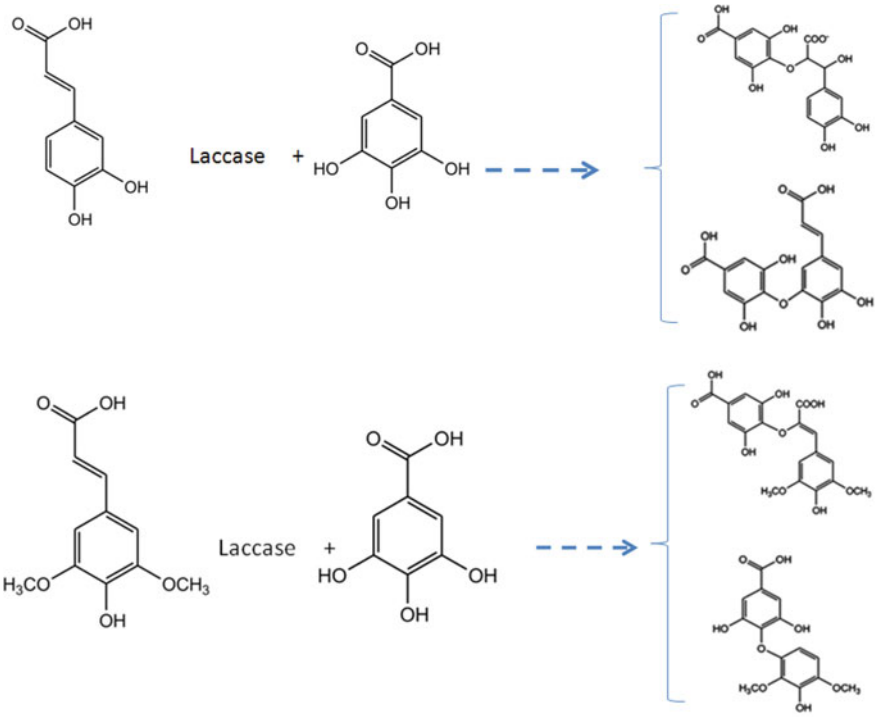

Fig. 5 In vitro grafting of gallic acid onto cafeic and sinapic acid

making them suitable for indoor use only. However, using brown-rotted lignin isolated from decayed wood instead of lignosulphonates was beneficial, leading to a 35% substitution of phenol and boards with strength comparable with that of boards glued by conventional PF resins [35, 36], whereas incoporation of methylene diphenyl diisocyanate reduced swelling and doubled the tensile strength [32, 33].

4.2 Creating a Stable Reactive Surface on Polymers

A number of authors have reported oxidation and activation of wood surfaces using oxidoreductases. However, once formed, these radicals are usually stabilised through resonance, and therefore the reactivity of the oxidised molecule depends on the position of the radical as previously described above. If the radical is shielded by substituent molecules like the methoxyl group, it becomes less accessible for coupling. Therefore, it is not surprising that up to 90% of radicals formed on wood surfaces were quenched within a few hours in laccase-bleached

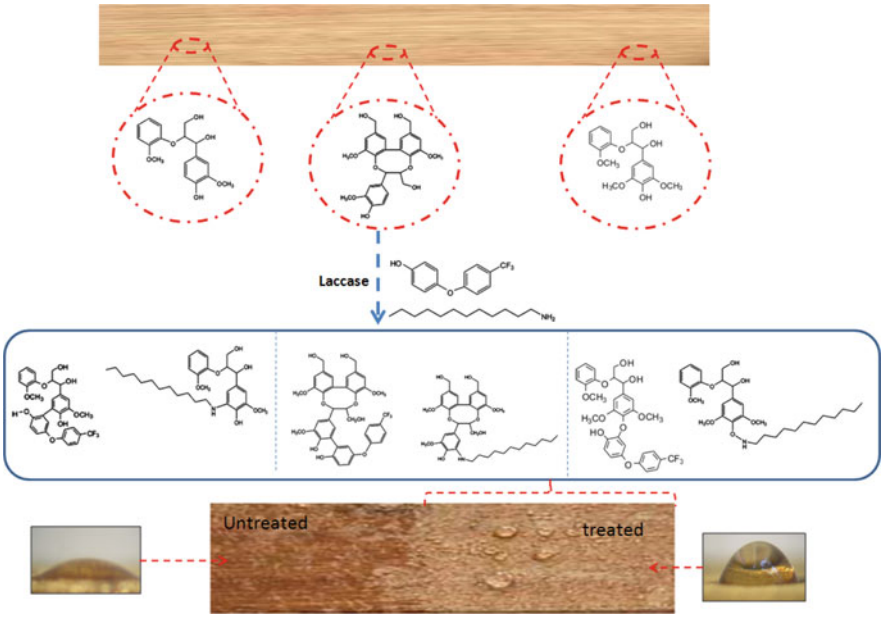

Fig. 6 Grafting of alkylamines and flurophenols onto different complex lignin model compounds and onto wood material. Grafting of alkylamines or flurophenols onto wood resulted in increased hydrophobicity

thermomechanical pulp [28]. To overcome this limitation, scientists have introduced stable reactive molecules that act as anchor groups that could be used for further coupling of functional molecules. Laccase-catalysed functionalisation of spruce wood particles with 4-hydroxy-3-methoxybenzylurea significantly increased the internal bond strength of fibreboards in the subsequent processing steps [22]. Kudanga et al. [48] also grafted various reactive aromatic amines onto lignin resulting in increased coupling of antifungal molecules onto the reactive surface. The authors also provided the mechanistic evidence for the covalent binding of aromatic amines [46], demonstrating that tyramine was bound onto syringylglycerol-guaiacylether via a 4-*O*-5 bond, leaving the $-NH_2$ group free for further attachment of functional molecules. It was also evident from the coupling products obtained with syringylglycerol–guaiacylether that the presence of methoxyl groups block the 5th position, promoting binding at the 4th position (Fig. 7).

In contrast to the binding mechanism proposed for syringylglycerol–guaiacylether, the same authors also noted that regardless of the chemical structure, all the phenolic amines preferred to couple onto dibenzodioxocin through position 5 resulting in C–C (5–5) coupling (Fig. 8). However, in both cases NH_2 group was free for futher grafting reactions.

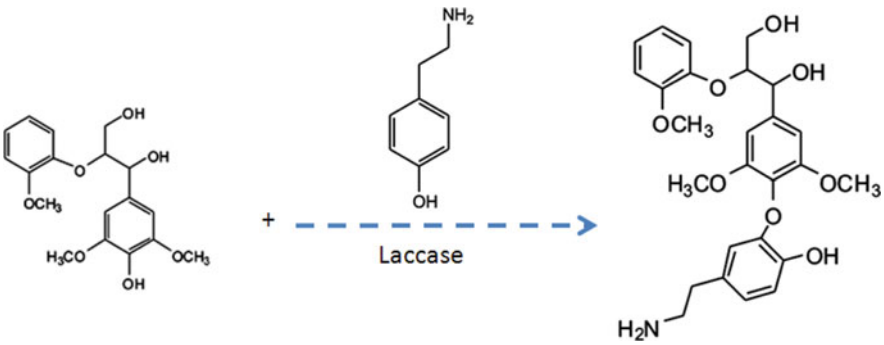

Fig. 7 In vitro grafting of tyramine onto syringylglycerol-guaiacylether

4.3 Manufacturing of MDF

Based on the experience gained through laccase- or peroxidase-mediated polymerisation of phenolic molecules, the ability of peroxidases and laccase to promote auto-adhesion of lignin in medium-density fibreboards (MDF) is widely investigated. Among some of the pioneering work, HRP and laccase treatment of brown-rotted wood produced laminates with relatively good shear strength, although lower than those obtained with adhesives [35, 36]; however, the material swelled in water. In yet another study, energy consumption was reduced by 40% when wood chips were pretreated with *Trametes hirsuta* and *Gloeophyllum trabeum* [89, 90], *Coniophora puteana* and *Fomitopsis pinicola* [45]. Interestingly, the pretreated wood chips bonded without adding any adhesive. Körner et al. [45] also demonstrated that fibres obtained from fermented wood chips produced MDFs with a 3.5-times higher bending strength and a 3-times higher modulus of elasticity than boards pressed from untreated fibres. In other parallel studies, improving on reaction engineering resulted in MDFs with increased internal bond strength and reduced swelling in water [23–26, 37, 38, 89]. Both laccase and peroxidase produced MDF boards fulfiling the European standard CIN DIN 622-5). The use of a whole fermentation culture was shown to produce MDF with twice the strength compared to those obtained by purified *T. versicolor* laccase [50, 89], demonstrating that the phenolic molecules in the whole cultures helped in crosslinking. Recently, Widsten et al. [96] using laccases and the addition of wax to the tannic acid–laccase formulation improved the dimensional stability of the fibreboards.

4.4 Improving Paper Properties

Laccase and peroxidase applications in the pulp and paper industry have been intensively studied for biopulping, biobleaching, and deinking mill process water and effluent treatment. However, recent research has shifted towards

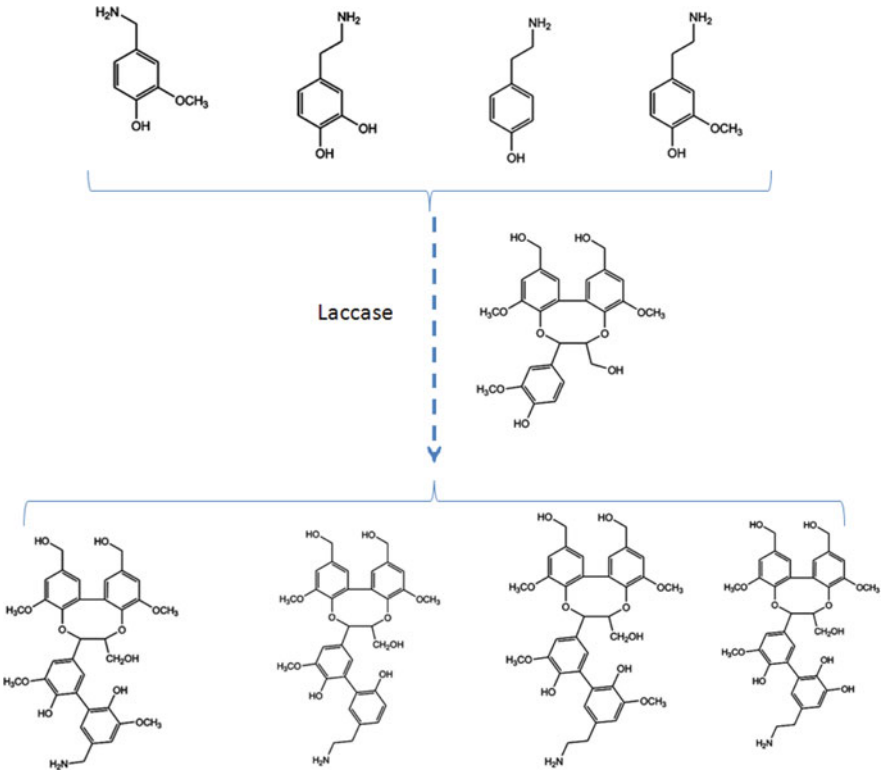

Fig. 8 In vitro grafting of different phenolic amines onto dibenzodioxocin 8a complex lignin model compound

functionalisation of pulps in order to produce novel paper products. The enzyme-assisted grafting of functional compounds to pulps provides novel opportunities to alter the physicochemical properties of the resulting paper products. A recent trend is the development of functional packaging materials with antimicrobial properties aimed at reducing the need to include antimicrobial agents in the food. The main applications of laccases and peroxidases in improving paper properties are summarised in Table 1. Laccase-mediated grafting of phenols possessing antimicrobial activities onto unbleached kraft liner fibres was reported by [21]. The enzymatically treated handsheets were effective in inhibting both gram-positive and gram-negative bacteria as compared to handsheets treated only with monomeric phenol derivatives. The pioneering works of Yamaguchi et al. [101–103] demonstrated the ability of laccase to polymerise various phenolic compounds to form dehydrogenative polymers that were subsequently coupled to thermomechanical pulps (TMP). Incorporation of phenolics (vanillic acid, catechol, mimosa tannin and tannic acid) in the presence of peroxidase or laccase enabled production of paper with increased tensile strength and water-resistant properties attributed to the coupling of free phenolic groups on the fibre surface with the added

Table 1 Laccase/peroxidase mediated grafting of functional molecules onto paper

Functional molecules	Product	References
Vanillic acid, catechol, mimosa tannin and tannic acid onto TMP	Paper with improved tensile strength and water resistance	[101–103]
Kraft lignin + spruce sulfite pulp	Pandsheets with higher tear and wet strength	[33]
Beech TMP fibres + ferulic acid-arabinoxylan dimer	Paper with higher wet strength and decrease in bulk thickness	[23–26]
Acrylamide + lignin	Fibril-like particulate	[59, 60]
Unbleached high-yield pulp	Increase in wet strength of kraft paper prepared from unbleached high-yield pulp	[58]
Lignin-rich extractives + high-yield kraft pulp	Improved the paper wet strength	[58]
Low Mw phenolics + high-kappa pulps	Improving strength properties of paper made from high-kappa pulps	[11–15]
Cationic dye phenol celestine blue onto kraft paper	Increased the strength of high-kappa kraft paper	[15]
Antimicrobial phenols onto unbleached kraft liner fibres	Handsheet papers were effective in inhibting both gram-positive and gram-negative bacteria	[21]
Low-molecular-weight ultra-filtered lignin fraction + kraft pulp	Kraft liner pulp	[21]
Amino acids onto high-lignin content pulps to	Improve their physical properties in paper products	[97]
Methyl syringate + unbleached kraft pulp	Wet tensile strength increase	[54]

dehydrogenative polymers. Huttermann et al. [33] combined laccase-pretreated kraft lignin with spruce sulfite pulp resulting in handsheets with higher tear strength and higher wet strength than untreated controls. In another attempt, Felby et al. [24, 25] observed an increase in the wet strength and decrease in bulk thickness after treatment of beech TMP fibres with laccase and a ferulic acid-arabinoxylan dimer. They noted an increase in dry tensile strength from 8.1 to 47.0 N m/g and attributed it to the coupling of ferulic acid to the pulp fibre, leading to enhanced fibre bonding. Using a low dose of laccase/HBT, Wong et al. [98, 99] improved the strength of paper made from high-yield kraft pulp by 5–10%. Further, [58] reported that addition of lignin-rich extractives to laccase-treated high-yield kraft pulp improved the wet strength of the paper. The potential of laccase-assisted grafting of low Mw phenolics was also extensively studied by Chandra et al. [11–15] for improving strength properties of paper made from high-kappa pulps. The observed improvements in paper tensile and burst strength were attributed to the capacity of carboxyl groups to promote hydrogen bonding and to the crosslinking of phenoxy radicals in the paper sheet. Surface-grafting of the cationic dye phenol celestine blue also increased the strength of high-kappa kraft paper [15]. Recently, [97] investigated the possibility of grafting various

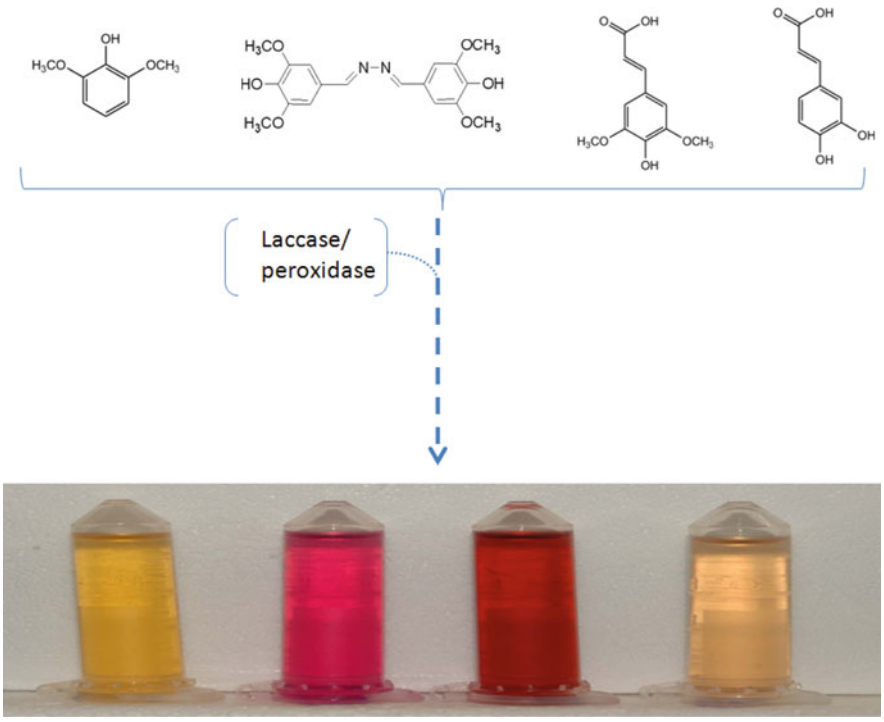

Fig. 9 Beautiful colours obtained after laccase/peroxidase oxidation of different lignin monomers (2,6-dimethoxyphenol, syringaldazine, sinapic acid, cafeic acid, respectively)

amino acids onto high-lignin content pulps. Laccase-histidine treated pulps showed an increase in strength properties of the resulting paper. The addition of methyl syringate to unbleached kraft pulp in the presence of laccase resulted in doubling of its wet tensile strength [54]. [21] reported that treatment of kraft liner pulp with laccase in the presence of a low-molecular-weight ultra-filtered lignin fraction leads to a twofold increase in the wet strength of the produced kraft liner pulp handsheets. Another emerging application of laccases and peroxidases is the improvement of the antimicrobial properties of pulps and paper.

4.5 Improving Aesthetic Look of Lignocelluloses Polymers

In situ enzymatic synthesis of dyes during the dyeing process could provide an efficient way of coupling dyes onto textile fibres under mild conditions. Laccase and peroxidase have been repeatedly shown to oxidise colourless aromatic compounds such as phenolics, aminophenols and diamines to coloured compounds. Figure 9 shows some of the beautiful colours obtained from laccase oxidation of colourless phenolic compounds.

Perhaps the first and most notable attractive activity of peroxidases, which even led to their discovery, was the observation by Planche [76], of their ability to catalyse the formation of a beautiful intensive blue colour when a tincture of gaiac resin came into contact with fresh horseradish root, long before the enzyme was known. With respect to laccases, the more than 6,000-year-old Chinese lacquer-work demonstrates the use of this enzyme in producing colourful artworks [32, 43]. However, it was not until recently that this principle was applied to colour textile fibres. Building on this experience, there has been a sudden increase in using natural phenolics for dyeing purposes as evidenced by several recent studies [3–6, 9, 30, 39–41, 83, 87]. Schroeder et al. [81] successfully coated flax fibres with different phenols in the presence of laccase, which also conferred antimicrobial properties to the flaxs. Laccase-mediated dyeing of cotton has also been attempted by many research groups [87]. Recently, using the anchor group concept (creating the reactive surface approach), tysolated cotton cellulose was dyed by coupling catechol, resulting in coloured product covalently fixed on the fabric [16]. Similarly, amine-functionalised cellulose was coated in situ with enzymatically synthesised poly(catechol) in the presence of *Trametes villosa* laccase [40, 41]. The authors also demonstrated the molecular mechanisms of the coupling where the polycatechol covalently binds to cellulose through the $–NH_2$ group and showed that the flavonoids naturally present on unbleached cotton could be perfectly used as anchor groups.

5 Concluding Remarks and Future Perspectives

Grafting of functional molecules onto lignocellulosic polymers using laccases and peroxidases shows encouraging results. However, many of the various possible applications are still at laboratory scale. Further reaction engineering studies of the reaction conditions would bring about faster industrial implementation. In the case of lignin, intensive efforts have to be directed towards increasing the reactivity with detailed studies of the enzymatic modification in order to gain insight into bottlenecks. The search for novel, more effective redox mediators may enhance reactivity through, e.g., demethylation or modification of other aliphatic side chains. Combining the acquired experience with the intensive, on-going searches for novel sources of peroxidase and laccases using culture-independent methods (metagenomics approaches), and intensive invstigation into cheaper and more effective mediators in a very short time will make industrial products highly likely. This is because developments in bioprocess engineering methods, molecular engineering of these enzymes and other site-directed mutagenesis activities aimed at improving production and the catalytic properties of these enzymes are encouraging.

Acknowledgments This work is a special dedication to Professor Walter Steiner on his retirement, to whom we owe much of our professional careers. Indeed he has been more than a

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Lipases in Polymer Chemistry

Bahar Yeniad, Hemantkumar Naik and Andreas Heise

Abstract Lipases are highly active in the polymerization of a range of monomers. Both ring-opening polymerization of cyclic monomers such as lactones and carbonates as well as polycondensation reactions have been investigated in great detail. Moreover, in combination with other (chemical) polymerization techniques, lipase-catalyzed polymerization has been employed to synthesize a variety of polymer materials. Major advantages of enzymatic catalysts are the often-observed excellent regio-, chemo- and enantioselectivity that allows for the direct preparation of functional materials. In particular, the application of techniques such as Dynamic Kinetic Resolution (DKR) in the lipase-catalyzed polymerization of racemic monomers is a new development in enzymatic polymerization. This paper reviews selected examples of the application of lipases in polymer chemistry covering the synthesis of linear polymers, chemoenzymatic polymerization and applications of enantioselective techniques for the synthesis and modification of polymers.

Keywords Polyesters · Lipase · Polycondensation · Ring opening polymerization · Block copolymers · Chemoenzymatic polymerization · Chiral polymers

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Contents

1 Introduction

Over the last decade in vitro enzymatic catalysis has established itself as an indispensable tool in the synthesis of small molecules both at the academic and industrial level. Examples can be found in the production of pharmaceutical intermediates where biotechnology is generating significant turnover and reducing environmental impact [1]. The success of enzyme catalysis in these reactions is based on the selectivity and efficiency of enzymes by promoting reactions that are not easily accessible by conventional techniques. Examples are the replacement of tedious protection/deportation chemistry (chemo- and regioselectivity) and asymmetric synthesis of chiral compounds (enantioselectivity).

In recent years enzyme catalysis was also successfully applied in polymer synthesis. Notably, three of the six enzyme groups have been reported in enzymatic polymerization in vitro, i.e., oxidoreductases (e.g., polyphenols, polyanilines), transferases (e.g., polysaccharides, polyesters) and hydrolases. Hydrolases including glycosidases, lipases and proteases are enzymes catalyzing a bond-cleavage reaction by hydrolysis. Under synthetic conditions they have been employed as catalysts for the reverse reaction, i.e., the bond-forming reaction, for example, for polyesters. Specifically, lipases appeared in the last decade as outstanding catalysts for polymerization reactions as a result of their broad substrate scope, high activity and excellent stability in a broad range of reaction media. Lipases are powerful catalysts for the preparation of polyesters, polycarbonates, and even polythioesters and polyamides. Moreover, a variety of different polymer architectures such as (block) copolymers, graft copolymers, etc., have been prepared using chemoenzymatic approaches, and most recently stereoselective synthesis and modification of polymers have been investigated. Irrespective of the

type of polymerization, *Candida Antarctica Lipase* B (CALB) dominates the literature of enzymatic polymerization. Specifically in its immobilized form, known as Novozym 435, it is by far the most applied lipase in polymer applications. The reasons are both technical as well as practical since Novozym 435 not only is one of the most active lipases in these reactions, but it is also the easiest to use in the hands of chemists unfamiliar with enzymology due to its robustness, stability and simple removability.

In this paper, we will introduce selected examples illustrating the application of enzymes in the synthesis of different classes of polymers as well as the combination of enzymatic polymerization with conventional polymerization techniques. This is followed by a section on enantioselective applications of lipase catalysis in the polymer field. Several excellent recent review articles on enzymatic polymer synthesis are also recommended [2–8].

2 Examples of Lipase-Catalyzed Polymerizations

2.1 Ring-Opening Polymerization of Lactones

Since the discovery of lipase-catalyzed ring-opening polymerizations (ROP) of lactones in 1993 by two independent groups, i.e., Uyama, Kobayashi and coworkers [9] and the group of Knani [10], ring-opening polymerization of lactones has been by far the most studied enzymatic polymerization. Reaction parameters investigated are, for example, the enzyme origin [11–15], concentration [16], temperature [17, 18], solvent [11, 16, 19, 20] and water content [21, 22]. Enzymatic ROP reactions have also been investigated in alternative solvents such as supercritical media [23–25] and ionic liquids [26, 27]. Mechanistically, lipase reactions are well understood. The active site of CALB, for example, contains the catalytic triad, Ser105-His224-Asp187, where Ser acts as the nucleophile, His as the basic residue and Asp as the acidic residue [28, 29]. The catalytic amino acids, serine and histidine, and histidine and aspartic acid form a hydrogen-bonding network, which is essential for the catalytic activity of a lipase [30]. It is generally accepted that the monomer activation proceeds via the formation of an acyl-enzyme intermediate by reaction of the serine residue with the lactone, rendering the carbonyl group more prone to nucleophilic attack (Fig. 1) [11, 16, 19, 31–33]. Deacylation of the acyl-enzyme intermediate by an appropriate nucleophile such as water produces the corresponding ω-hydroxycarboxylic acid/ester. If the nucleophile is an alcohol or amine, the product contains the corresponding ester or amide as an end-group. This step is often referred to as initiation in analogy to chemical polymerizations. Chain growth or propagation then occurs by deacylation of the acyl-enzyme intermediate by the terminal hydroxyl group of the growing polymer chain to produce a polymer chain that is elongated by one

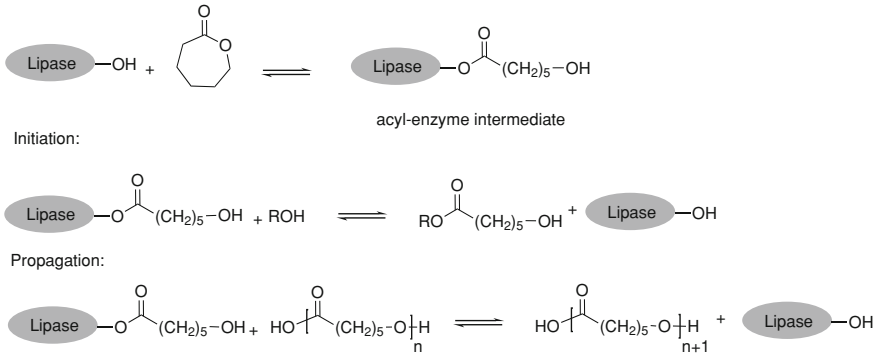


Fig. 1 Proposed mechanism for lipase-catalyzed ROP of lactones [32]

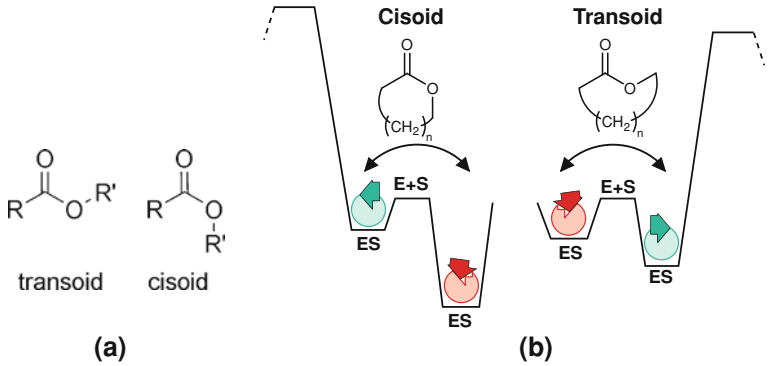


Fig. 2 **a** *Cisoid* and *transoid* conformation of the ester bond. **b** Productive and unproductive binding due to the *cisoid* or *transoid* nature of the ester bond in lactones in the CALB active site [38]. Reproduced by permission of Wiley

monomer unit. Lipase-catalyzed polymerization thus follows a monomer activation mechanism.

Kinetic investigations of lipase-catalyzed ROP of unsubstituted lactones revealed that the polymerizations follow Michaelis-Menten kinetics and that the formation of the acyl-enzyme intermediate is the rate-determining step [11]. In substituted lactones, depending on the size of the substituent, the deacylation step can become rate-determining [34]. The determination of the Michaelis-Menten constant, K_M, and the maximal rate of polymerization, V_{max}, of unsubstituted lactones of varying ring size showed that K_M is relatively independent of the ring size, while no obvious trend could be discerned for V_{max} [35, 36]. Interestingly, lactones possessing a *cisoid* ester bond conformation appeared less reactive than large ring lactones in a *transoid* conformation in CALB-catalyzed reactions (Fig. 2a), an opposite trend compared to the chemical reactivity of lactones where ring strain in the small, and *cisoid* lactone is the driving force for reaction/polymerization [37].

Docking studies by Veld et al. suggest that for the *cisoid* lactones, competitive inhibition by wrongly bound substrate is responsible for the low experimentally observed reactivity (Fig. 2b) [38]. This finding indicates that in ROP of lactones to polyesters where a monomer activated mechanism is operational, the polyester product can be more reactive in the activation step than the lactone substrate. This has important implications for the ability to control the polydispersity, the molecular weight and the end-group fidelity in lipase-catalyzed ROP.

Control over the molecular weight has been achieved reasonably well in enzymatic ROP. In the absence of water, high molecular weight polyesters are typically obtained [39–41], while increasing the water content (which acts as an initiator) leads to a higher number of polymer chains and thus lower molecular weight polyesters [20, 33]. Also the use of initiators containing an alcohol allows the preparation of polymers of predictable molecular weight [22, 42–44]. However, the polydispersities—typically between 1.5 and 2.0—suggest a limited control during the polymerization. The fact that a lipase can activate every ester bond, and preferably accepts *transoid* ester bonds as substrates, implies that transacylation reactions will always compete with monomer activation during the polymerization. In other words, competing transacylation reactions and chain transfer cannot be avoided during lipase-catalyzed polymerization reactions. In the end, a PDI of 2.0 is expected as a result of the statistically randomized chain length distribution.

An important factor in the synthesis of functional polymer is end-group fidelity. Many applications require a high degree of polymer end-functionalization, and the ability to control end-groups in enzymatic ROP has thus been intensively investigated. Predominantly the addition of functional alcohols as nucleophiles (initiators) was applied to achieve incorporation of functional groups into the growing polymer chain. A systematic study by de Geus et al. [22] on the initiating efficiency of different alcohol initiators in the CALB-catalyzed ROP of ε-caprolactone (CL) showed that cyclic species, water-initiated PCL chains and PCL chains with the desired initiator attached were formed. Depending on the concentration of the monomer and water activity of the reaction, the relative amount of the three products varied. The maximum degree of PCL functionalization was 95%. A careful evaluation of the polymerization of CL in the presence of different initiators by a combination of LCCC, SEC and Maldi-TOF-MS revealed that water, even when stringent drying conditions were applied, dominated the initial initiation process but, depending on the exact reaction conditions, the initiator was incorporated into the polymer structure to a high degree as a result of transacylation reactions [45]. Interestingly, recent work by Hult and coworkers hypothesized a water tunnel in CALB that provides the active site with substrate water [46].

Careful selection of the reaction conditions and working under thermodynamic control i.e., performing reactions under equilibrium condition allow high end-group fidelity in a lipase-catalyzed ROP. For example, vinyl(meth)acrylates were found to be suitable end-cappers during the polymerization reactions [47, 48]. Using hydroxyl compounds like hydroxyethyl (meth)acrylate (HEA or HEMA) as

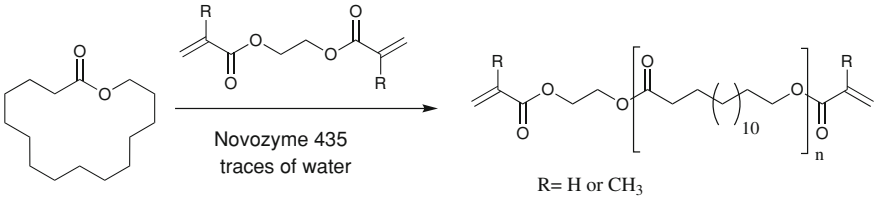


Fig. 3 Well-defined, fully dimethacrylated polyester by lipase-catalyzed polymerization [51]

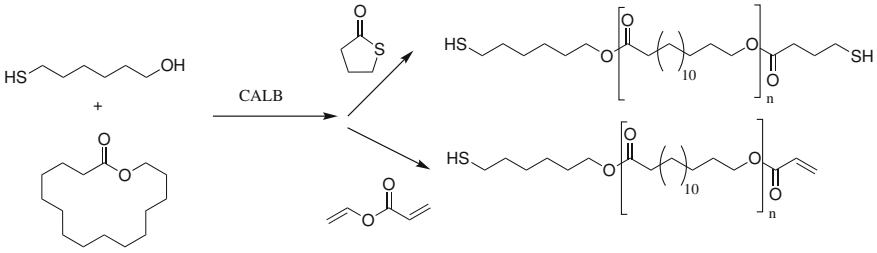


Fig. 4 Polymerization of PDL initiated by 6-mercapto-1-hexanol [54]

an initiator, di(meth)acrylate macromonomers were accessible in a one-pot enzymatic procedure [49–52]. Takwa et al. found that a complex mixture of polymer end groups is obtained in these reactions owing to the transesterification process in which the ester groups in HEMA and HEA took part [49, 50]. Although the initiators with cleavable ester groups are thus of limited use to synthesize well-defined (meth)acrylated polymers, it was shown that it is possible to prepare well-defined, fully di(meth)acrylated polymers when combined with end-capping using vinyl methacrylate. Equally efficient is the use of ethyleneglycol di(meth)acrylate in these reactions (Fig. 3) [51]. The same authors also introduced a simple solvent-free enzymatic single-step route for the synthesis of α, ω-functionalized macromonomers with a high fraction of thiol-thiol or thiol-acrylate end groups, which can be used to give cross-linked thin film when exposed to UV light [53]. Initiation with mercapto-ethanol or 6-mercapto-1-hexanol in lipase-catalyzed ROP of CL and (pentadecalactone) PDL, respectively, resulted in a polymer functionalization of 95% (Fig. 4). The alcohol was shown to act as the initiating specie while the thiol was unreactive in the initiation step [54]. The latter is a result of the high chemoselectivity of lipases for the alcohol group over the thiol group in the deacylation step. In situ end-capping of the polymers with thiobutyrolactone or 11-mercaptoundecanoic acid resulted in high yields of dithiol end-capped polyesters with a high degree of functionalization [51, 54, 55]. In addition, glycidol initiation in combination with a divinyl ester initiator also resulted in epoxide end-capped polyesters in a one-pot approach that can be cured into biodegradable coatings [53, 56].

The discovery of the fact that the reaction kinetics and the achievable molecular weights in lipase-catalyzed ROP increase with the lactone ring size has led to an

increasing interest in the polymerization of macrolactones that can be derived from natural sources. The polymerization of macrolactones by lipase catalysis occurs with higher reaction rates and higher conversions in contrast to traditional chemical methods in which generally a slow polymerization rate and low molecular weight products are observed [35, 36]. Kobayashi and co-workers were the first to introduce the enzyme-catalyzed polymerization of macrolactones, ω-undecanolide (UDL), ω-dodecanolide (DDL) and ω-pentadecanolide (PDL) (12-, 13- and 16-membered lactones) [57, 58]. Poly(pentadecalactone) (PPDL) with molecular weights up to 150 kDA [40, 59] employing Novozyme 435 and up to 200 kDA in miniemulsion employing *Pseudomonas Cepacia* lipase [60] was reported. PPDL is of potential technical interest since it resembles the properties of polyethylene (PE); PPDL is a semi-crystalline polymer with a melting point around 100 °C and a glass transition temperature of −27 °C [41, 61, 62]. The crystallization behavior, the crystal structure of PPDL and the mechanical properties reveal large similarities with polyethylene (PE) [62, 63]. The first application-related PPDL reports were on end-functionalized low molecular weight PPDL for coating applications [64] and high molecular weight PPDL spun into fibers revealing a tensile strength up to 0.74 GPa (Fig. 5) [40].

van der Meulen et al. investigated the Novozyme 435-catalayzed ring opening polymerization of unsaturated analogues of pentadecalactone and hexadecalactone, ambrettolide and globalide, respectively, and obtained high molecular weight products (> 24 kg/mol) (Fig. 6), which are fully biocompatible [39]. It is noteworthy that these two unsaturated polyesters allow for the manipulation of the polymer properties in the solid state by post modification, for example, cross-linking. Another approach to cross-linked polymers from macrolactones is to

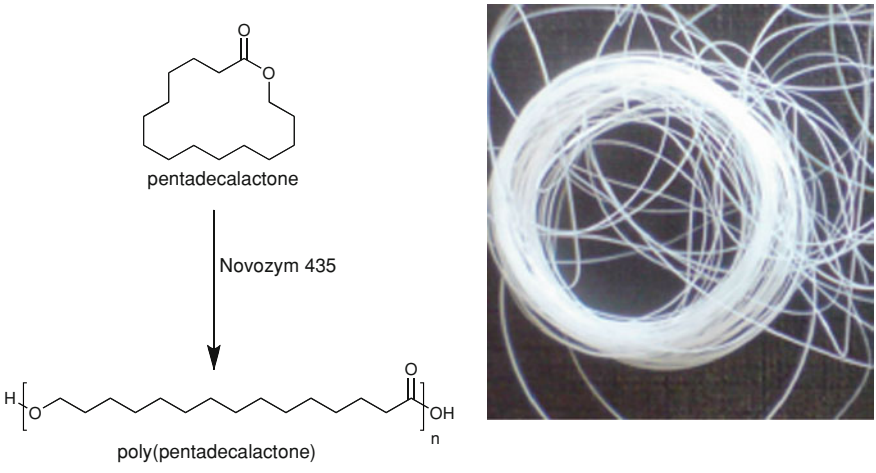


Fig. 5 Enzymatic ROP of pentadecalactone (*left*) and fibers spun from poly(pentadacalactone) (*right*) [40]. Reproduced by permission of The Royal Society of Chemistry (RSC)

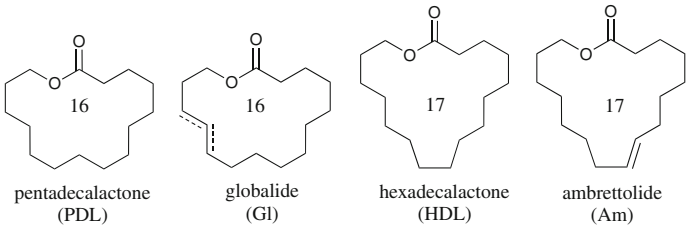


Fig. 6 Structures of PDL, GI, HDL and Am used in lipase-catalyzed ring-opening polymerization [39]

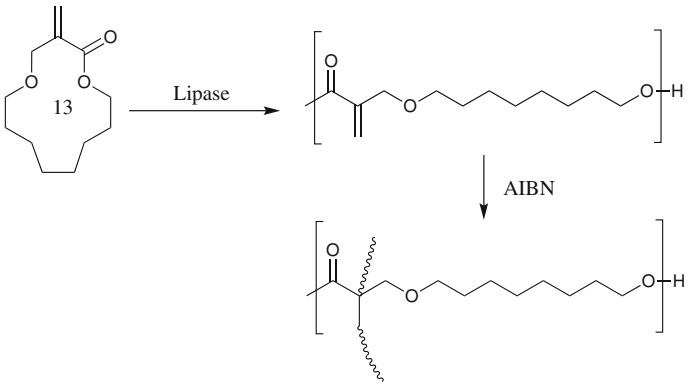


Fig. 7 Enzymatic polymerization of α-methylenemacrolides and subsequent vinyl polymerization [66]

enzymatically polymerize α-methylenemacrolides to polyesters and then free radically polymerize the methylene groups in the main chain (Fig. 7) [65, 66].

2.2 Ring-Opening Polymerization of Carbonates

Polycarbonates have attracted attention in recent years because of their potential use in biomedical applications based on their biodegradability, biocompatibility, low toxicity and good mechanical properties [67]. These polymers can be prepared by the ROP of cyclic carbonate monomers by anionic, cationic, and coordination catalysts. However, lipase-catalyzed polymerization seems to be a feasible alternative to prepare polycarbonates as chemical methods often suffer from partial elimination of carbon dioxide (resulting in ether linkages), require extremely pure monomers and anhydrous conditions.

Trimethylcarbonate (TMC) was the first cyclic carbonate to be polymerized by ring-opening polymerization by various lipases, particularly CALB [68, 69] and

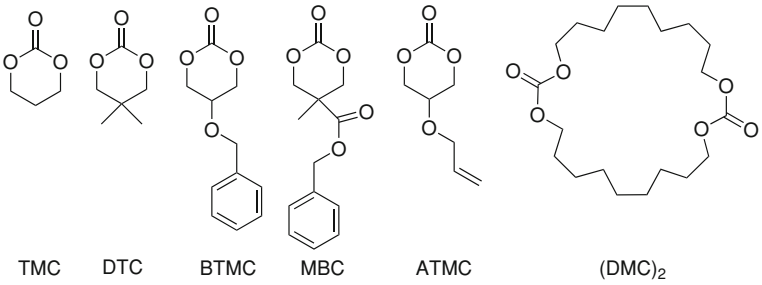


Fig. 8 Examples of cyclic carbonates employed in lipase-catalyzed ring-opening polymerization

porcine pancreatic lipase (PPL) [70]. Recently many efforts have been made to synthesize polycarbonates with different pendant functional groups. A variety of functional groups were introduced into aliphatic polycarbonates to tune the polymer properties such as hydophilicity, permeability, bioresorption and mechanical properties [71]. Functional cyclic carbonates having pendant hydroxyl [72], carboxyl groups [73, 74], amino groups [75], benzyl [76] or ally ether [77] have been investigated by different research groups. In a recent paper, a 26-membered macrocyclic carbonate, namely cyclobis(decamethylene carbonate) $(DCM)_2$, was also successfully polymerized enzymatically employing CALB. In this study, a much lower reaction activity of large-sized $(DCM)_2$ relative to TMC was observed as opposed to the results obtained with different ring-size lactones [78].

An alternative way to tune the polymer properties and insert desired functionalities is copolymerization with different monomers. Cyclic carbonates have been copolymerized with various other cyclic monomers, such as lactones or lactides. For example, TMC was copolymerized with 5-methyl-5-benzyloxycarbonyl-1,3-dioxan-2-one (MBC) using lipase from *Pseudomonas flourescens* (PF) resulting in a highly amorphous random copolymers (Fig. 8) [74]. In another study, 5-benzyloxy-trimethylene carbonate (BTMC) was copolymerized with 5,5-dimethyl-trimethylene carbonate (DTC) using an immobilized lipase on silica particles [79]. In the copolymerization of TMC with a lactone, ω-pentadecalactone (PDL), employing Novozyme 435, highly crystalline TMC-PDL copolymers were obtained, and as opposed to chemical catalysts, enzyme catalyst (Novozyme 435) polymerized PDL more rapidly than TMC [80].

2.3 Lipase-Catalyzed Polycondensation

A condensation reaction leads to the formation of covalent bonds under removal of small molecules (condensation by-product). The most commonly known example is the formation of an ester bond from an alcohol and a carboxylic acid derivative under release of water, alcohol or other molecules. In a polycondensation typically

diol and diester (AA-BB type) derivatives or hydroxy acids (AB type) react to form linear polyesters. Polycondensations are equilibrium reactions requiring removal of the formed condensation product to shift the equilibrium to high conversion. Usually these reactions are acid or metal catalyzed and in some cases conducted on large industrial scale. Initially the replacement of the conventional catalyst by lipases was investigated because of the milder reaction conditions and potentially advantageous energy balance. The use of lipases (mostly Novozym 435) has been described in great detail by a number of groups [81–94], and pioneering work by Baxenden Chemicals Ltd. showed the feasibility of performing this process on a large scale [95].

However, while aliphatic polyester can be readily produced by lipase catalysis, the generally poor substrate acceptance of aromatic acid derivatives like terephthalates generally limits the synthesis of commercially important polyesters like poly(ethylene terephthalate) (PET). Recently, another benefit of lipase catalysis in polyester synthesis, namely their high regioselectivity, have received increased attention. It allows multifunctional monomers to be directly polymerized in high selectivity while avoiding the necessity of protective group chemistry. Polymers with pendant hydroxy or carboxylic acid functionalities can be readily synthesized by this approach and are discussed as biomedical and biodegradable materials. Chemical polymerization under these conditions would result in polymer networks because of the non-specific condensation of all functional groups. For example, the copolymerization of sorbitol, adipic acid and octanediol using Novozym 435 in bulk afforded water-soluble polymers with M_n of 10.6 kDa and a PDI of 1.6 [96]. The reaction occurred predominantly at the primary alcohol groups with a regioselectivity of 95% (Fig. 9a). Thermal analysis showed that these copolymers are semi-crystalline and have a low melting point. Similar polymerizations with

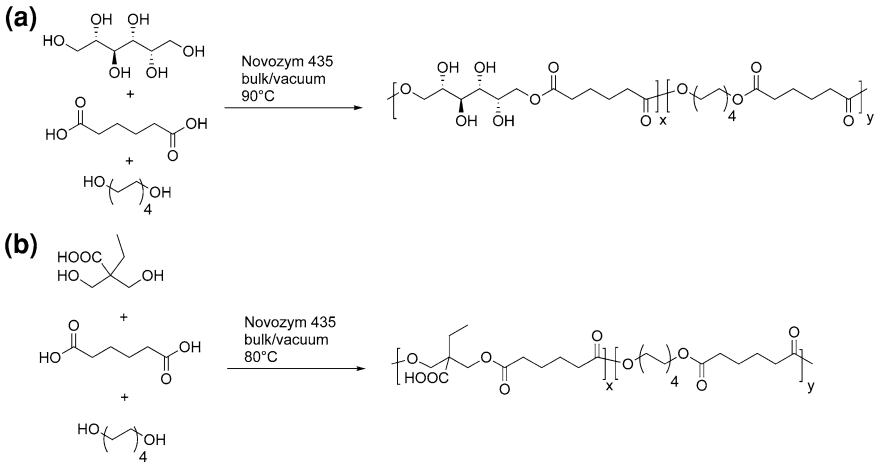


Fig. 9 Different approaches to the direct formation of hydroxy- [96] or carboxylic acid [97] functional polyesters using Novozym 435

glycerol or bis(hydroxymethyl)butyric acid (BHB) resulted in terpolymers with free hydroxy or carboxylic acid groups, respectively (Fig. 9b) [54, 97].

The polymerization of isopropyl aleuritate with Novozyme 435 afforded linear polymers (M_n = 5.6 kDa, PDI = 3.2) [98]. In this case, the regioselectivity was close to 100%, a result of the sterically hindered secondary alcohols. The thermal properties of the polymers could be tuned by copolymerization with CL, and the degree of branching remained close to 0, showing that these secondary alcohols are unreactive. Subsequent reaction of the secondary alcohols with hexylisocyanate showed that these polyesters can be readily post-modified by suitable reagents.

3 Chemoenzymatic Polymerizations

While lipases were successfully applied for the synthesis of a large variety of polymers by ring-opening polymerization and polycondensation, a general shortcoming of lipase-catalyzed polymerizations is the lack of control over the molecular structure. This prohibits the synthesis of complex molecular architectures like block and graft copolymers. This can be overcome by the combination of lipase catalysis with other polymerization techniques similar to multi-catalytic (enzymatic) cascade approaches in pharmaceutical synthesis [99]. Chemoenzymatic polymerizations have thus the potential to further increase macromolecular complexity and design materials with interesting properties.

Enzymatic polymerization has been combined with various chemical polymerizations for the synthesis of block copolymers. Several examples for the enzymatic initiation from hydroxy-terminated macroinitiators, for example, polybutadiene [100] and PEG [101–104], are known but in the majority of the reports the enzymatic polymerization was conducted first. Controlled polymerization techniques have been widely applied for the subsequent macroinitiation, which generally results in well-defined block copolymers. Atom Transfer Radical Polymerization (ATRP) is one of the most widely used techniques in this synthetic strategy in which the end-functionalization of the enzymatic block with an initiator moiety capable of selectively initiating the subsequent polymerization is required. A very efficient way to achieve this is to use a dual initiator. These initiators have a functional group capable of initiating the enzymatic polymerization (hydroxy) and a group for the chemical polymerization. In this process, controlling the end-group fidelity is very critical to obtain high yields of block copolymers. In the initial report, the combination of Novozym 435-catalyzed ROP and ATRP using the dual initiator yielded a block copolymer of styrene, and ε-caprolacone (CL) was described [105]. This concept was further investigated by several groups with respect to the reaction conditions, the initiator structure and variations of monomers [22, 43, 45, 106–114]. For example, de Geus et al. investigated the possibility of conducting both polymerizations as a one-pot cascade reaction in the presence of the dual initiator, the ATRP metal catalyst, CL and methacrylates [106].

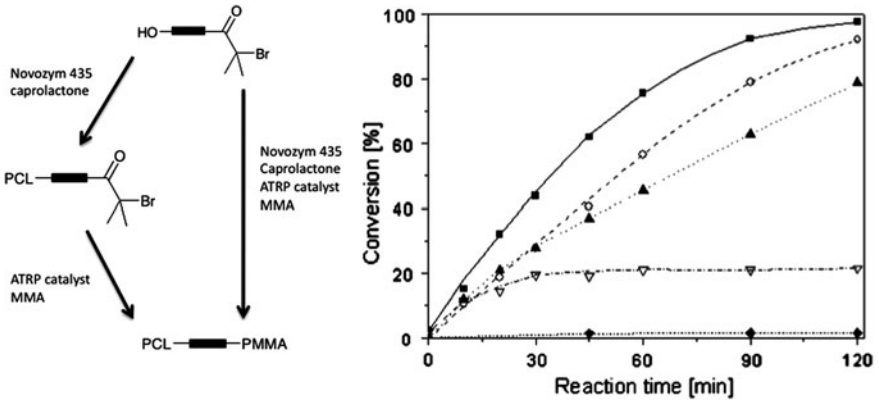


Fig. 10 *Left*: Strategy for consecutive chemoenzymatic and simultaneous one-pot block copolymer synthesis combining enzymatic ROP and ATRP using a dual initiator. *Right*: Influence of ATRP-catalyst system on the conversion of CL in the enzymatic ROP of MMA at 60°C using ATRP-3 as initiator: *filled squares* reaction in absence of ATRP-catalyst; *open circles* CuBr/PMDETA (1:1:1-ratio with respect to initiator); *filled triangles* CuBr/dNbpy (1:2.1:1-ratio with respect to initiator); *inverted triangles* CuBr (1:1-ratio with respect to initiator); *filled diamonds* $CuBr_2$ (1:1-ratio to initiator). CL conversion was determined with ^{1}H-NMR [106]. Reproduced by permission of Wiley

The study revealed that ATRP catalysts like nickel have a strong inhibiting effect on the enzyme, while typical ATRP copper catalysts were tolerated by the enzyme (Fig. 10). By taking advantage of the simultaneous nickel lipase inhibition, the nickel-catalyzed ATRP and the stereoselectivity of the enzyme, chiral block copolymers were obtained by this method from 4-methyl caprolactone (MCL) by Peters et al. [43]. The polymerization of racemic MCL showed good enantioselectivity and produced a chiral macroinitiator with ATRP end group by selectively polymerizing only the (*R*)-MCL. Macroinitiation was then started by adding the nickel catalyst and MMA to the reaction mixture, which simultaneously inhibited the enzyme and activated the ATRP process. Chiral poly(MMA-b-(*R*)MCL) was successfully obtained in this synthesis. Howdle et al. reported that a one-pot, simultaneous synthesis of block copolymers by enzymatic ROP and ATRP is possible in the green solvent supercritical CO_2 ($scCO_2$) [115]. The authors could show that the CL acts as a $scCO_2$ co-solvent was crucial to allow the radical polymerization to remain homogeneous. The unique ability of $scCO_2$ to solubilize highly fluorinated species was also utilized for the synthesis of novel copolymers consisting of a semifluorinated block of poly(1*H*, 1*H*, 2*H*, 2*H*-perfluorooctyl metharylate) (PFOMA) and PCL [116]. These and other enzymatic polymerization procedures in $scCO_2$ have recently been reviewed [117].

A series of interesting block copolymer architectures has also been prepared by Zhang et al. In one of the papers, the synthesis of H-shaped triblock copolymers was demonstrated from enzymatically obtained PCL diol after end-functionalization with a difunctional ATRP initiator [118]. This allowed growing two

polystyrene chains from each end of the telechelic PCL. When methanol instead of glycol was used as the initiator in the initial enzymatic CL polymerization, a PCL with one hydroxyl end group was obtained. Functionalization of this end group with the difunctional ATRP initiator and subsequent ATRP of styrene and glycidyl methacrylate (GMA) resulted in Y-shaped polymers (Fig. 11) [113, 114].

However, all synthetic approaches involving ATRP rely on a metal catalyst. Full metal-free and thus greener approaches to block copolymers were realized by the combination of lipase ROP with nitroxide-mediated living free radical polymerization [44]. With this system it was also possible to successfully perform a one-pot chemoenzymatic cascade polymerization from a mixture containing a dual initiator, CL and styrene (Fig. 12). Moreover, it was shown that this approach is compatible with the stereoselective polymerization of 4-methylcaprolactone for the synthesis of chiral block copolymers. A metal-free synthesis of block copolymers using a radical chain transfer agent as a dual initiator in enzymatic ROP to yield poly(CL-*b*-styrene) was also reported recently [119].

Recently, the first example of the metal-free combination of enzymatic and chemical carbene-catalyzed ROP for the formation of block copolymers (Fig. 12) was described [120]. The approach took advantage of the fact that CALB has a high catalytic activity for lactones but not for lactides, while it is the other way

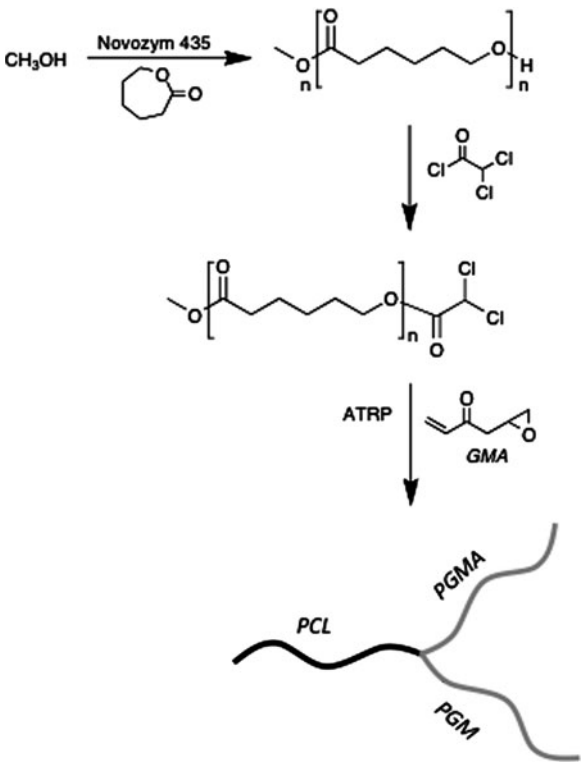


Fig. 11 Chemoenzymatic route to the Y-shape block copolymers [113]

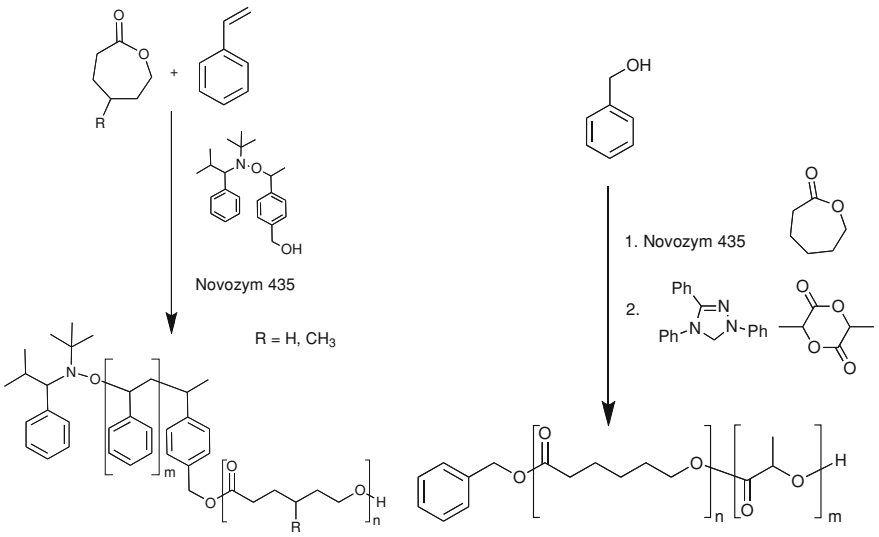


Fig. 12 *Left*: One-Pot enzymatic ring opening and living free radical cascade polymerization [44]. *Right*: Block copolymers by combination of enzymatic ROP and carbene-catalyzed ROP [120]

around for carbenes. In the applied synthetic strategy, the enzymatic polymerization of CL was conducted first. Addition of lactide and carbene to the reaction mixture led to the macroinitiation directly from the hydroxyl end-group of PCL allowing the synthesis of PCL/PLLA block copolymers.

The modification of functional polymer backbones was also investigated as a green route to graft copolymers. An important contribution was made by Hans et al. using well-defined polyglycidols as promising biomedical materials [121, 122]. In an initial paper, the authors compared the chemical and enzymatic ROP of CL from linear and star-shaped polyglycidol obtained from anionic ROP. It was found that the zinc-catalyzed ROP of CL from the multifunctional polyglycidol resulted in a quantitative initiation efficiency, while with enzymatic ROP the PCL was only grafted to 15–20% of the hydroxyl groups. The difference in the initiation efficiency is due to the different polymerization mechanisms. While the chemical ROP is an end-group activated and controlled, the enzymatic ROP is monomer activated. In the latter case the steric constrains in the nucleophilic attack of the hydroxyl groups on the enzyme-activated monomer prevents the reactions of all hydroxyl groups. This was also confirmed by a study of Duxbury et al. on the enzymatic ROP from polystyrene containing 10% of 4-vinylbenzyl alcohol [123]. While quantitative enzymatic modification of the hydroxyl groups with the small vinyl acetate was observed, the grafting of PCL produced only a 50–60% grafting efficiency. Moreover, the results suggested that the grafting action is a combination of grafting and grafting by transesterification of PCL. Similar results were reported for the lipase-catalyzed polymer grafting in scCO2

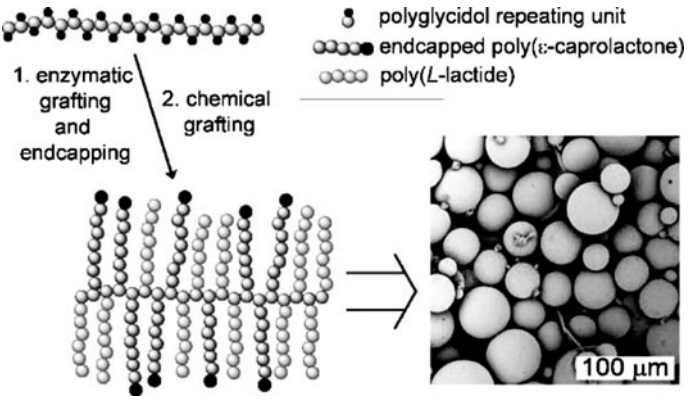


Fig. 13 Chemoenzymatic synthesis of heterografted polymers comprising PCL and PLA grafts on a polyglycidol backbone. After acrylation these polymers can be formulated into cross-linked microspheres [126]. Reproduced by permission of Wiley

from a poly(HEMA-co-MMA) random copolymer by Villarroya et al. About 33% of the hydroxy groups participated in the grafting reaction. The grafting efficiency was significantly improved when highly randomized poly(MMA-co-HEMA) from starved-feed polymerization was used (80%) and when a PEG spacer was introduced between the polymer backbone and the hydroxyl group (100%) [124].

The incomplete enzymatic grafting from polyalcohols opens opportunities to synthesize unique structures. For example, heterografted molecular bottle brushes were synthesized starting from PCL-grafted polyglycidol [125]. The grafting yield in this step was about 50%. Selective acetylation of the hydroxyl groups at the PCL graft ends was achieved via enzymatic reaction with vinyl acetate without acetylation of the remaining hydroxyl groups at the polyglycidol backbone. The remaining hydroxy groups on the polyglycidol backbone were used to initiate chemically catalyzed ROP of lactide to produce a heterografted polymer comprising PCL and PLA grafts. When the end-groups of the PCL were capped with acrylates, the resulting materials could be formulated into UV cross-linked microspheres (Fig. 13) [126].

4 Enantioselectivity in Lipase-Catalyzed Polymer Reactions

Many naturally occurring polymers, such as proteins, DNA and cellulose, are optically active, and some of them show characteristic functionalities such as molecular recognition ability and catalytic activity, owing to their specific chiral structure as represented by genes and proteins. These considerations have motivated considerable interest in the synthesis and application of optically active polymers [127–129]. Introducing chirality into polymers has distinctive advantages over the use of non-chiral or atactic polymers since it adds a higher level of

complexity allowing for the formation of hierarchically organized materials. This may have benefits in high-end applications such as nanostructured materials, biomaterials and electronic materials.

In polymer chemistry, one of the most challenging tasks is to efficiently synthesize optically active synthetic polymers. The extraordinary enantioselectivity of lipases offers new perspectives towards these materials, and it is therefore not surprising that some research efforts have focused on the use of lipases to synthesize chiral polymers from racemic monomers. Methodologies like kinetic resolution and even chemoenzymatic dynamic kinetic resolution (DKR) have already been exploited on the industrial scale to afford chiral intermediates for the pharmaceutical and agrochemical industry. Recently, these methodologies have been successfully applied in the synthesis of chiral polymers.

4.1 Enantioselectivity of Lipases

The enantioselectivity of hydrolases towards secondary alcohols and amines are well documented in the literature [130–132]. Although the overall structure and serine triad conformations are similar in all lipases, their substrate specificities and degree of stereoselectivity differ widely [133]. In general, lipases display a strong *R*-selectivity towards secondary alcohols. In the case of CALB, for example, an *E ratio* of 10^6 has been reported in the kinetic resolution of (*rac*)-1-phenylethanol [134]. Substrate mapping of lipases has been carried out for secondary alcohols, and it was shown that the stereoselectivity is higher if there is one small and one large substituent on the chiral carbon rather than two of approximately equal size. This is because the lipases normally consist of two binding pockets for the substrates, a large acyl binding hydrophobic pocket and a medium hydrophilic binding pocket for the secondary alcohol moiety. In these stereospecific pockets, the smaller substituent of the secondary alcohol can be placed during the reaction, while the larger substituent resides in the larger cavity (Fig. 14). The spatial orientation of these cavities implies that *R*-secondary alcohols are highly preferred; the docking of an *S*-secondary alcohol leads to significant steric hindrance [133, 135]. Moreover, the absence of the hydrogen bond between the catalytic histidine residue of the triad and the oxygen of the alcohol moiety of *S*-enantiomer in the enzyme substrate complex, which is normally present in case of *R*-enantiomer

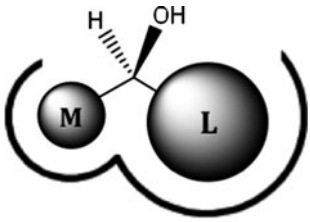


Fig. 14 The conformation of the large (L) and medium (M) substituent and the hydroxyl group of the fast reacting enantiomer of secondary alcohols as predicted by Kazlauskas' rule

complex with the enzyme, favors high selectivity towards the *R*-enantiomer [136]. Although the preference toward one of the enantiomers of the secondary alcohol is similar for most of the lipases, the space available (pocket size) for the two substituents differs among enzyme species and some kind of flexibility has been observed in this region of the protein.

4.2 Enantioselective Polycondensation

A lipase-catalyzed high enantioselective oligomerization was first reported by Klibanov and co-workers in 1987 from a racemic mixture of bis(2-chloroethyl) (±)-2,5-dibromoadipate and a chiral 1,6-hexanediol using lipases from *Aspergillus niger* and *Chromobacterium* sp. to obtain oligomers with optical density $[\alpha]^{25}$ of +4.5 to +4.3 and +1.3 to +1.5, respectively (*S*-enantioselective) [137]. Moving the asymmetric carbon far from the reaction site as in the case of bis(2,2,2-trichloroethyl) (±)-3-methyladipate reduced the enantioselectivity. However, oligomerization between a racemic mixture of diol (e.g., 2, 4-pentanediol) and an achiral diester, bis(2-chloroethyl) adipate using porcine pancreatic lipase inversed the enantioselectivity, giving high *R*-enantiorich oligomer after 14 days in toluene with optical activity $[\alpha]^{25}$ of −7.9. Later in 1989, Wallace and Morrow reported a lipase-catalyzed high enantioselective polymerization between a racemic bis(2,2,2-trichloroethyl) *trans*-3,4-epoxyadipate and 1,4-butanediol in anhydrous diethyl ether using porcine pancreas lipase (PPL) catalyst wherein they activated the acyl donor using 2,2,2-trichloroethyl to improve the polymerization [90]. After 5 days, with a diester to the diol feed molar ratio of 2:1, an enantioenriched polyester with M_W of 7.900 kDa and an optical purity of 95% was formed. Knani and co-workers synthesized optically active oligomers with a degree of polymerization (DP) <6 from racemic ε-hydroxyesters using porcine pancreas lipase (PPL) catalyst in hexane [10]. The size of the substituent at the ε-position influences both the reaction rate and enantioselectivity of the enzyme in the order Me < Et < Ph; the bulkier the substituent, the slower the reaction and higher the enantioselectivity [138]. In another report, *Candida Rugosa* lipase catalyzed the self-condensation of 10-hydroxyundecanoic acid in anhydrous hexane to an optically active oligoester with a high enantiomeric excess (*ee* ∼ 60%) in (*S*-) enantiomer, and the residual monomer was recovered with 33% *ee* favoring (*R*-) enantiomer [139].

Very recently, methodologies of DKR were applied in polycondensations (Fig. 15). The extension of DKR to polymer chemistry is not trivial in practice since side reactions that are relatively unimportant in DKR (e.g., dehydrogenation and hydrolysis) have a major impact on the rate of polymerization and attainable chain lengths since the stoichiometry of the reactants is an important issue. As a result, the reaction conditions and catalyst combinations used in a typical DKR process will not a priori lead to chiral polymers from racemic or achiral monomers with good molecular weight and high *ee* (>95%).

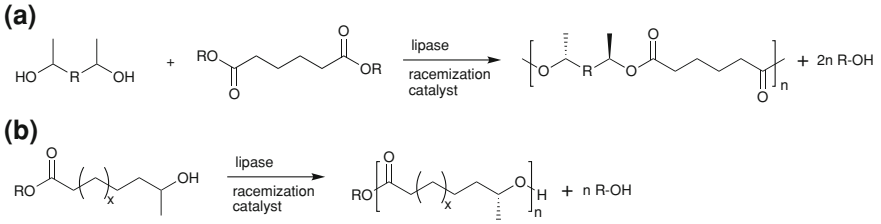


Fig. 15 Variants of the extension of DKR to polymercondensations: Route A uses AA and BB monomers to prepare chiral polymers from racemi/diasteromeric diols. Route B converts an enantiomer mixture of AB monomers to homochiral polymers

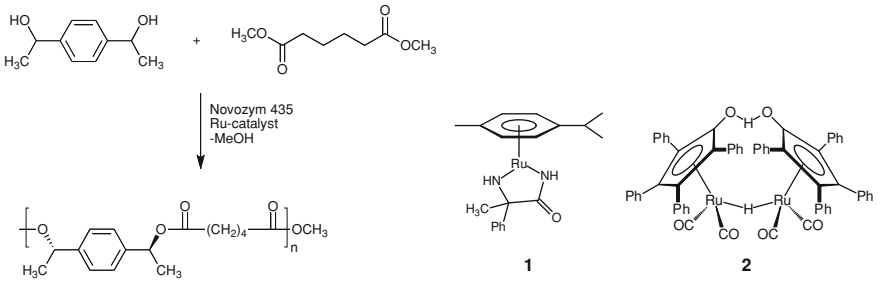


Fig. 16 Dynamic kinetic resolution polymerization of a 1,4 diol and dimethyl adipate, and structures of Noyori (*1*) and Shvo (*2*) type racemization catalyst [140]

Hilker and coworkers reported the first successful DKR polycondensation from a stereoisomeric mixture of the secondary diol α,α′-dimethyl-1,4-benzene-dimethanol and dimethyl adipate (Fig. 16) [140]. The applied catalytic system consisted of a Ru-Noyori type racemization catalyst **1** and Novozym 435. Due to the enantioselectivity of the lipase CA, only the hydroxyl groups at the (*R*) center of the diol reacted to form the ester bond under liberation of methanol. In situ racemization from the (*S*) to the (*R*) configuration by the Ru-Noyori catalysis allowed the polymerization to proceed to a hydroxyl group conversion of 92% after 70 h. Eventually all polymers were end-capped with (*S*) stereocenters (chain stoppers). The molecular weights of the polymer were moderate ($M_W = 3.4$ kDa), and Novozym 435 had to be added every few hours to compensate for the activity loss of the lipase. This suggests that Ru-catalyst **1** and Novozym 435 are not fully compatible. van As and coworkers reported an improved catalytic system by changing the racemization catalyst to Shvo's catalyst **2** (Fig. 16) and by adding a hydrogen donor 2,4-dimethyl-3-pentanol (DMP) to suppress dehydrogenation reactions [141]. Although Shvo's catalyst **2** is a significantly slower racemization catalyst than **1**, it does not require the addition of K_2CO_3, which appeared to cause the lipase deactivation. For the 1,4-diol this resulted in polymers with *ee* of 94% and M_p of 8.3 kDa within 170 h. The 1,3-isomer α,α′-dimethyl-1,3-benzenedimethanol (1,3-diol) showed

slightly better results under similar conditions and had a higher solubility in toluene. After an optimization study, chiral polymers were obtained with molecular weights up to 15 kDa, enantiomeric excess (*ee*) up to 99% and 1–3% ketone functionalities in $\sim$120 h.

Kanca applied DKR to aliphatic AB monomers (e.g., methyl 6-hydroxyheptanoate, methyl 7-hydroxyoctanoate, methyl 8-hydroxynonanoate and methyl 13-hydroxytetradecanoate) encompassing a secondary hydroxy group and an ester moiety with high enantioselectivities in a Novozym 435-catalyzed transesterification reaction (Fig. 15b) [142]. The enantiomeric ratio (*E* ratio), a measure for the enantioselectivity of an enzyme for a particular substrate, was high for all monomers studied (*E* ratio $>$ 200). An additional advantage of the use of AB monomers over AA-BB monomers is the lack of sensitivity on stoichiometric issues. The polymerization of the monomers employing Novozym 435 and Ru-catalyst **2** was slow (RT = 170 h), but did result in chiral polymers of good molecular weigth and *ee*.

4.3 Enantioselective Ring-Opening Polymerization

Adding a substituent to a lactone introduces a chiral center in the lactone ring. Systematic studies of ROP of substituted caprolactones revealed that all lactone monomers except 6-methyl-caprolactone (6-MeCL) could be polymerized with (R)-selectivity [43, 143]. This was extended to the synthesis of chiral block copolymers [43, 44] and chiral polymer particles [144] when combined with chemical polymerization methods. Investigation of the Novozym 435-catalyzed polymerization of 6-MeCL revealed *S*-selectivity in the initial ring-opening step. As Novozym 435 is highly *R*-selective, it cannot accept *S*-configured nucleophilic *sec*-alcohol in the propagation step, which prevents polymerization of 6-MeCL. However, by combining the Novozym 435-catalyzed ROP of racemic 6-MeCL and the Ru-catalyzed (Noyori type Ru-Catalyst, **1**) racemization of the propagating secondary alcohol in one-pot, optically pure oligoesters with up to a maximum of five repeating monomer units were obtained (Fig. 17) [145]. A dramatic improvement was achieved when Shvo's Ru-catalyst **2** (Fig. 16) was employed in combination with 2,4-dimethyl-3-pentanol to suppress dehydrogenation reactions with full conversion of the monomer, a promising *ee* of 86% and M_W of 8.2 kDa [146]. Based on the dynamic kinetic resolution (DKR) method, this method was termed iterative tandem catalysis (ITC) since chain propagation can only be achieved by the combination of two fundamentally different catalytic systems.

Novozym 435-catalyzed ROP of ω-methylated six lactones (4-, 6-, 8-, 9- and 13-membered ring) was studied for elucidation of the relationships between enantioselectivity and lactone structure [147]. The rate of polymerization was much affected by the ring size as suggested by k_{cat} values, and the enantioselectivity was switched from (*S*)-selective for small (4-, 6-, and 7-membered) lactones to (*R*)-selective for large (8-, 9-, and 13-membered) lactones. The selectivity switch

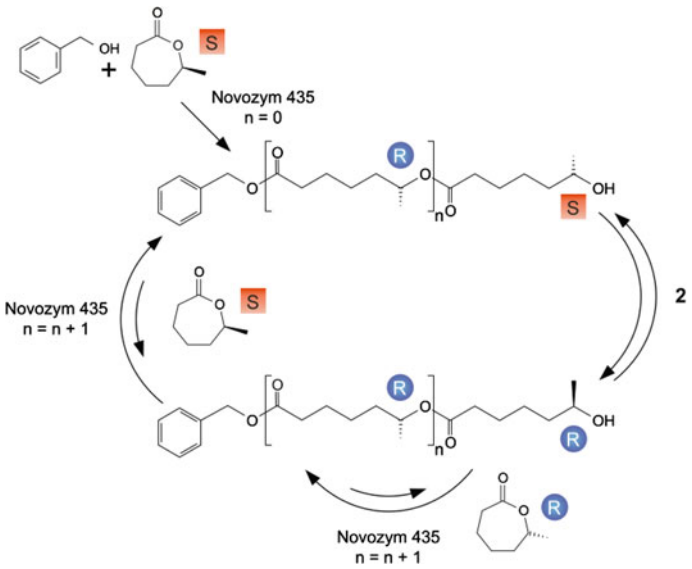


Fig. 17 Polymerization of 6-MeCL by iterative tandem catalysis [146]. Reproduced by permission of The Royal Society of Chemistry (RSC)

was further supported by the molecular modeling studies on the free energy difference between the lactone structure and the active site cavity of the lipase. This is attributed to the fact that the lactone takes *transoid* and *cisoid* conformations; virtually small lactones for *cisoid* and large lactones for *transoid*. ROP of the small *cisoid* lactones was (*S*)-selective (3-MePL and 6-MeCL) or aselective (5-MeVL), whereas that of the larger *transoid* lactones was (*R*)-selective with very high enantioselectivity. A selectivity switch was also observed in the copolymerization from (*S*)- for 3-MePL (4-membered) to (*R*)-selectivity for 5-MeVL (6-membered) [148], 4-alkyl-substituted ε-CL [34] and methyl-substituted ε-CL [43].

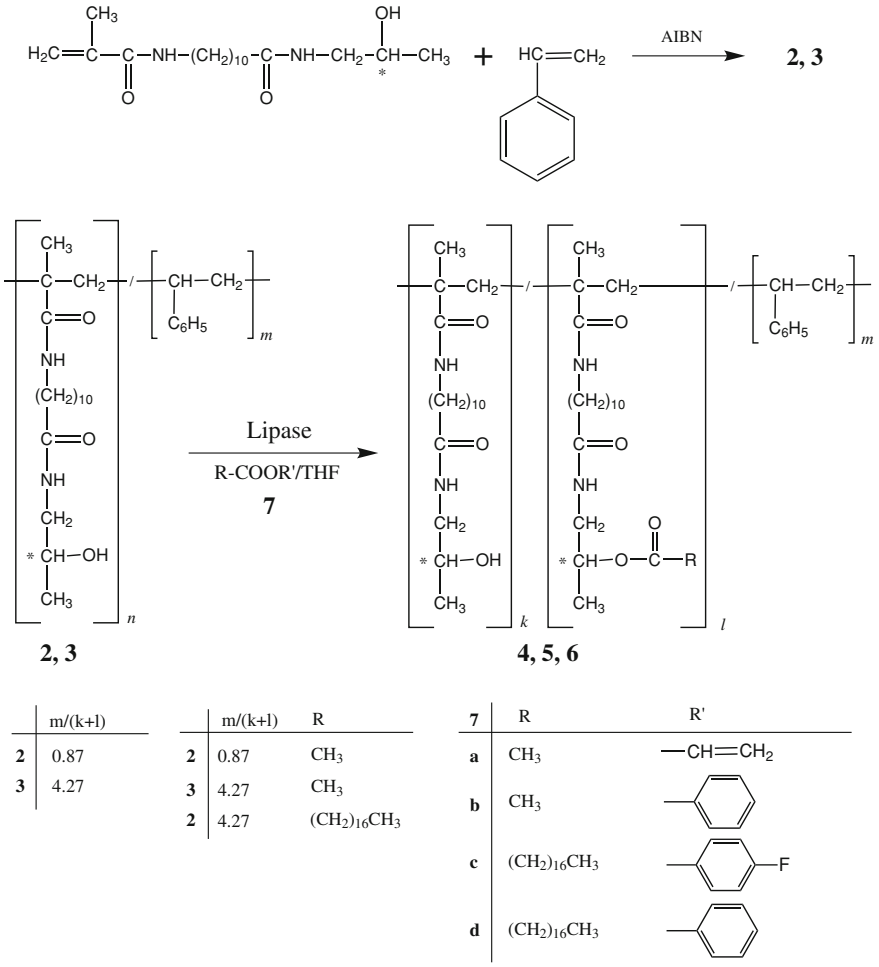
Zhou et al. combined the simultaneous dynamic kinetic resolution (DKR) of a secondary alcohol initiator with lipase-catalyzed ROP of ε-CL. (*R*,*S*)-1-Phenylethanol (PhE) was used as a model secondary alcohol and incorporated into PCL under DKR conditions using lipase CA and a Ru catalyst. A total of 75% of the PhE was incorporated as (*R*)-PhE-PCL with over 99% *ee* in 23 h at 75°C in toluene [149].

4.4 Enantioselective Polymer Modification

A lipase-catalyzed acylation of comb-like methacrylate polymers was induced through OH groups in the side chains using the activated esters, vinyl acetate, phenyl acetate, 4-fluorophenyl acetate, and phenyl stearate, as acylating agents. The OH groups in the side chains of the methacrylate/styrene copolymer were

acylated in THF at ambient temperature for 6 days (Fig. 18) [150]. Typically, the reaction of the copolymer ($n = 19\%$) with phenyl acetate catalyzed by lipase PF (*Pseudomonas fluorescens*) gave the acetylated copolymer in 40% conversions ($m = 7.6\%$) after 7 days. Due to the enantioselectivity of the acylation, the product showed an optical rotation of $[\alpha]_D^{20}$ of $-1.20°$.

Duxbury and coworkers recently developed a new chiral enzyme-responsive polymer based on enantioselective polymer modification [151]. With the aid of two alcohol dehydrogenases that show opposite enantioselectivities in the reduction of ketones (ADH–LB and ADH-T), the two enantiomers of *p*-vinylphenylethanol were obtained in excellent yield and *ee*. Copolymers of these monomers with styrene using free radical polymerization afforded random block copolymers



	m/(k+l)
2	0.87
3	4.27

	m/(k+l)	R
2	0.87	CH_3
3	4.27	CH_3
2	4.27	$(CH_2)_{16}CH_3$

7	R	R'
a	CH_3	$-CH=CH_2$
b	CH_3	
c	$(CH_2)_{16}CH_3$	F
d	$(CH_2)_{16}CH_3$	

Fig. 18 Selective modification of side chain secondary alcohol by lipase-catalyzed acylation [150]

with compositions ranging from 100% *R* to 100% *S* alcohol in the side chains. From differential scanning calorimetry (DSC) analysis, a similar glass transition temperature T_g of approximately 115°C was found for all polymers, irrespective of their chiral composition, suggesting that the enantiomeric composition has no effect on the thermal properties of the polymer. The optical rotation of the polymers increased linearly from −20 to +20° in accordance with their chiral composition. Novozyme 435 was then used to catalyze the esterification of the alcohol groups on the polymer backbone with vinyl acetate in toluene (Fig. 19). Novozym 435 was perfectly (*R*)-selective for the acetylation of phenylethanol with vinyl acetate. When a backbone containing 100% *S*-secondary alcohol groups was used for the enzymatic grafting of vinyl acetate, no reaction was observed over a period of 24 h (Fig. 6B). In contrast, when a backbone containing 100% *R*-secondary alcohol groups was used, the enzymatic esterification of vinyl acetate occurred from 75% of the alcohol groups within 24 h. This means that the encoded chiral information stored in the polymer was accurately read-out by an enantioselective enzymatic reaction.

In another study, Padovani showed that CALB-catalyzed modification of pendant ester groups of a polystyrene derivative provided a clear-cut regioselective transesterification reaction [152]. Of the pendant two ester groups available on the polymer backbone, only the ester group distant from the polymer backbone was involved in the lipase-catalyzed esterification reaction. The results suggested that formation of the acyl-enzyme intermediate is sterically not possible for the ester

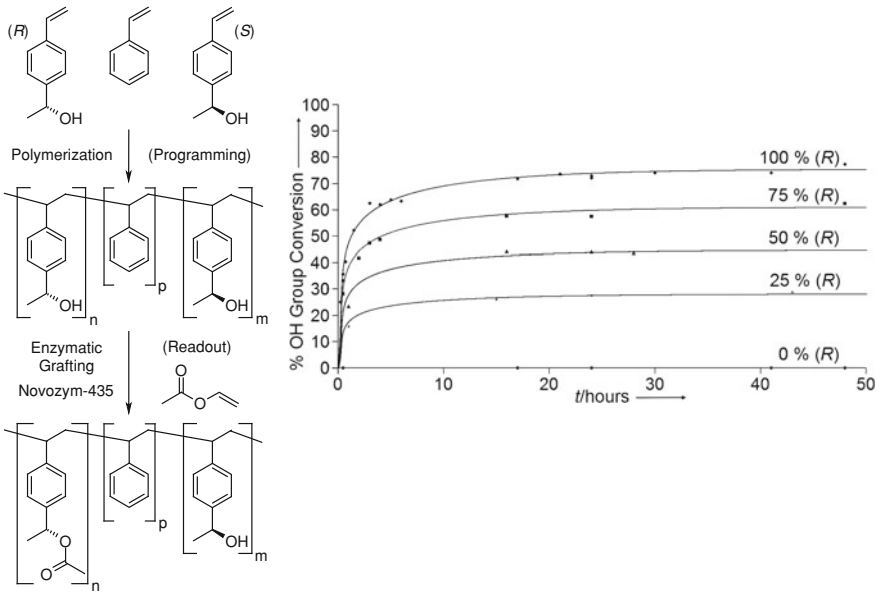


Fig. 19 Stereoselective modification of pendent secondary alcohol catalyzed by Novozym 435 [151]. Reproduced by permission of Wiley

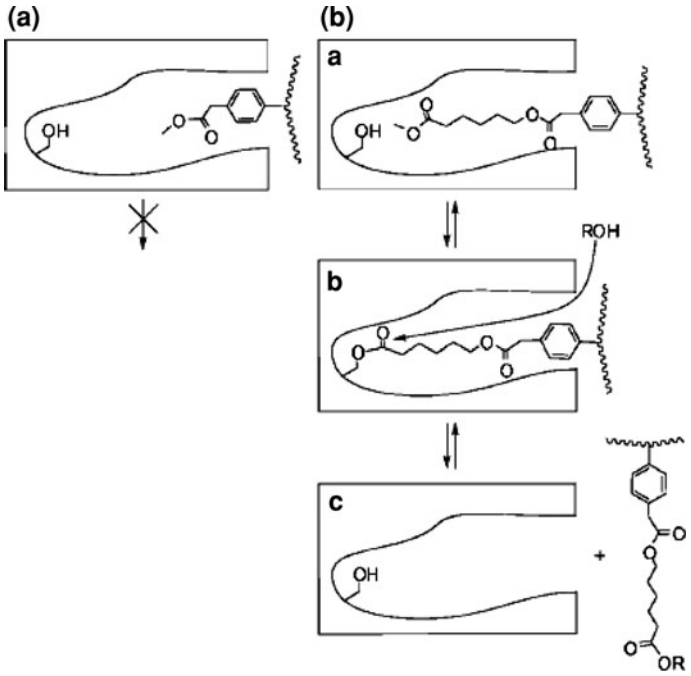


Fig. 20 Schematic illustration of regioselective polymer modification: **a** Pendant group is too short to form the acyl enzyme intermediate in the active site (illustrated by the serine OH group). **b** When the pendant group is longer the acyl-enzyme intermediate is formed (**a**), and the transesterification occurred (**b**) to give the product (**c**) [152]. Reproduced by permission of the American Chemical Society (ACS)

group in the short pendant (A) or the ester group near the backbone, but only for the distant group (B) (Fig. 20). This type of enzymatic functionalization of spacer-dependent regioselectivity has not been observed in the chemical reactions. Combined with enantioselective conversion of (rac)-1-phenylethanol in the transesterification reaction, a maximum of conversion of 47.9% of the R-alcohol was obtained in a regioselective fashion.

5 Outlook

Lipase-catalyzed polymerizations have developed into a promising and versatile technique in the synthetic toolbox of polymer chemists. The increased understanding of the scope as well as the drawbacks of this technique has enabled the synthesis of a large variety of homo- and copolymers. Specific unique attributes of the lipases are already applied to develop materials and processes difficult to achieve chemically. Examples are the synthesis of multifunctional polymers, the

selective modification of polymer end-groups using enzymatic chemoselectivity, the synthesis of fluorinated block copolymers in a fully green process in $scCO_2$ and the synthesis of optically active materials from racemic mixtures. It can be expected that this trend will continue and we will see more examples in which the specific strength of lipases (selectivity) leads to unique materials. As a result, a combined multidisciplinary effort at the interface of biocatalysis, polymer chemistry and organic catalysis may lead to a paradigm shift in polymer chemistry and allow a higher level of structural complexity in macromolecules, reminiscent to those found in nature.

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Enzymes for the Biofunctionalization of Poly(Ethylene Terephthalate)

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Abstract The functionalization of synthetic polymers such as poly(ethylene terephthalate) to improve their hydrophilicity can be achieved biocatalytically using hydrolytic enzymes. A number of cutinases, lipases, and esterases active on polyethylene terephthalate have been identified and characterized. Enzymes from *Fusarium solani*, *Thermomyces insolens*, *T. lanuginosus*, *Aspergillus oryzae*, *Pseudomonas mendocina*, and *Thermobifida fusca* have been studied in detail. Thermostable biocatalysts hydrolyzing poly(ethylene terephthalate) are promising candidates for the further optimization of suitable biofunctionalization processes for textile finishing, technical, and biomedical applications.

Keywords Biofunctionalization · Cutinase · Enzymes · Esterase · Lipase · Poly(ethylene terephthalate) · Polyester · Thermostability

Abbreviations

AFM	Atomic force microscopy
BEB	Ethylene glycol dibenzyl ester
BHET	*bis*(2-hydroxyethyl terephthalate)
BHPT	*bis*(3-hydroxypropyl terephthalate)
CTR	Cyclo-*tris*–ethylene terephthalate, cyclic PET trimer
DET	Diethyl terephthalate
DMT	Dimethyl terephthalate
DP	Diethyl *p*-phthalate
DSC	Differential scanning calorimetry
EBT	1,2-Ethylene-*bis*-terephthalate
EMT	1,2-Ethylene-*mono*-terephthalate-*mono*(2-hydroxyethyl terephthalate)
ESCA	Electron spectroscopy for chemical analysis
FTIR	Fourier transform infrared spectroscopy
HPLC	High performance liquid chromatography
MEG	Monoethylene glycol

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MHET	*mono*(2-hydroxyethyl) terephthalate
MHPT	*mono*(3-hydroxypropyl) terephthalate
PBT	1,2-Propylene-*bis*-terephthalate
PEG	Polyethylene glycol
PET	Poly(ethylene terephthalate)
PET dimer	*bis*-(*p*-methylbenzoic acid)-ethylene glycol ester
PET trimer	*bis*(benzoyloxyethyl) terephthalate
PTT	Poly(trimethylene terephthalate)
PCL	Poly(ε-caprolactone)
SEM	Scanning electron microscopy
Triton X-100	Octyl phenoxy polyethoxy ethanol
T_g	Glass transition temperature
T_d	Thermal denaturation temperature
T_m	Melting temperature
TLC	Thin layer chromatography
TPA	Terephthalic acid
XPS	X-ray photoelectron spectroscopy

Contents

1 Introduction

Poly(ethylene terephthalate) (PET) is a synthetic polyester composed of terephthalic acid (TPA) and ethylene glycol. This thermoplastic polymer is widely used to produce fibers, films, and resins. Most of the PET produced worldwide is needed for the production of textile fibers, while another large part constitutes PET resins for beverage containers and food packaging. PET films are utilized for example in electronics and packaging industries.

PET fibers have excellent mechanical strength properties, high chemical resistance, and low permeability to gases. PET fabrics are wrinkle resistant and show low abrasion and shrinking. While PET filament yarns are employed in clothing, and technical and medical textiles, staple PET fibers find applications in knitted and woven textiles for clothing and furnishings. Due to its competitive price and versatile performance characteristics in comparison to competing materials, PET is taking a significant market share of both synthetic and natural fibers. The global polyester fiber demand is expected to grow at 6% per year, resulting in worldwide production of 36 million tons in 2011 [17].

Synthetic polymers, including PET, are highly hydrophobic. By hydrophilization of the surface of PET materials, their properties and performance can be improved. The undesirable features of PET fabrics, such as their very poor water permeability and wettability, low moisture regain, the build-up of entangled fibers on the surface of fabric subjected to mild abrasion during wash and wear, static charge, and oil stain retention, can be improved by a controlled surface hydrolysis process [2, 5, 44, 45, 101]. Increased surface hydrophilicity also results in better dye uptake in PET fabrics [49, 51, 107]. For other applications, surface-modified PET can be coated with bioactive compounds to create biocompatible or antimicrobial surfaces to be used in textile finishing, medical devices, and tissue engineering [34].

Several surface modification methods for synthetic polymers have been described, for example, the use of chemical finishers based on carboxyl-containing polymers [100]. Alkaline and acid hydrolysis treatments are unspecific and result in strength and weight losses [97, 108]. Ionized gas treatment of PET materials using plasma has also been investigated to introduce hydrophilic groups at the surface of the polymer [80]. However, the application of this method is limited because it is complicated to use, and it can be difficult to control the extent of the material modification [16].

Alternatively, surface hydrolysis of PET can be achieved by treatment with enzymes that introduce polar groups to the polymer surface. A number of hydrolytic enzymes, such as lipases, cutinases, and esterases, have shown potential for surface functionalization of PET [36, 99]. The biocatalytic method can be performed under mild reaction conditions avoiding the use of large amounts of chemicals and energy for the finishing and dyeing processes. The enzymatic modifications are specific and can be limited to the polymer surface. Consequently, the bulk properties and mechanical stability of the polymer are not compromised, and material savings and products of better quality or with new functionalities can be obtained.

2 Analysis of Enzymatic Effects on PET Surfaces

The partial enzymatic hydrolysis of ester bonds on the surface of PET films or fibers resulting in changed surface properties has been quantified using a number of analytical methods (Table 1). Surface hydrophilicity can be assessed by contact

Table 1 PET hydrolases and analysis of their effects on PET

Enzyme	Source	Type of substrate	Analytical method	Effect on PET	Reference
Cutinase	*Fusarium solani*	Fabric	UV and fluorescence spectroscopy, dye assay, water absorption tests	Hydrolysis, increased hydrophilicity, improved dye uptake	[3, 6, 38, 82, 84, 85, 98]
		Film	Titration, HPLC, gravimetric analysis, SEM, ESCA	Hydrolysis, degradation, weight loss, changes in surface morphology	[91, 102]
		PET trimer	HPLC	Hydrolysis	[27, 38]
	Pseudomonas mendocina	Fabric, fibers	Contact angle, water absorption tests, HPLC, UV spectroscopy, dye assays, gravimetric analysis, SEM	Increased hydrophilicity, weight loss, improved dye uptake, changes in surface morphology	[10, 25, 44, 45, 49, 107]
		DET	UV spectroscopy	Hydrolysis	[25, 49]
		Film	Titration, HPLC, gravimetric analysis, SEM	Hydrolysis, changes in surface morphology, weight loss	[91]
	Thermobifida fusca	Fabric, fibers	HPLC, UV-spectroscopy, dye assay, water absorption tests, gravimetric analysis; SEM, AFM, XPS	Hydrolysis, increased hydrophilicity, improved dye uptake, weight loss	[2, 3, 11, 26, 27, 31, 38]
		Film (PET; PTT)	Gravimetric analysis, contact angle measurement	Weight loss, increased hydrophilicity	[26, 27, 76]
		CTR, BETEB, BHPT, cyclic PTT dimer, BHET, PET trimer	HPLC	Hydrolysis	[19, 26, 27, 38]

(continued)

Table 1 (continued)

Enzyme	Source	Type of substrate	Analytical method	Effect on PET	Reference
Lipase	*Thermomyces insolens*	Fabric, fibers	HPLC, dye assay, water absorption tests, gravimetric analysis	Hydrolysis, increased hydrophilicity, improved dye uptake, weight loss	[1, 62, 72, 101]
		DET, BEB, PET trimer, CTR	HPLC	Hydrolysis	[1, 5, 32, 40, 48, 90, 101]
		Film	HPLC, titration, turbidity measurement, gravimetric analysis, SEM	Hydrolysis, changes in surface morphology, weight loss	[1, 91]
	Porcine (hog) pancreas	Fabric	Contact angle measurement, water absorption tests, SEM, dye assay	Increased hydrophilicity, improved dye uptake, changes in surface morphology	[44, 51, 52]
	Burkholderia cepacia	Fabric, PET trimer	HPLC, water absorption assays	Hydrolysis, increased hydrophilicity	[38, 51]
	Candida antarctica	Fabric, fibers BEB, DET	HPLC, titration, SEM, contact angle measurement, water absorption tests	Hydrolysis, increased hydrophilicity, changes in surface morphology	[5] [50] [51, 60]
	Thermomyces lanuginosus	Fabric, fibers, film (PET; PTT)	HPLC, fluorescence spectroscopy, water absorption tests, dye assay, XPS	Hydrolysis, increased hydrophilicity	[4, 11, 26, 27]
		BHPT, BHET, PET trimer	HPLC	Hydrolysis	[26, 27]
	Aspergillus sp.	Fabric, PET trimer	HPLC, water absorption assays	Hydrolysis, increased hydrophilicity	[33, 38]

(continued)

Table 1 (continued)

Enzyme	Source	Type of substrate	Analytical method	Effect on PET	Reference
	Aspergillus oryzae	Fabric, DP	UV spectroscopy, contact angle measurement, dye assay, gravimetric analysis, water absorption assays	Hydrolysis, increased hydrophilicity, improved dye uptake, weight loss	[103]
	Bacillus subtilis, Bacillus licheniformis	PET dimer, PET nanoparticles, dialkylphthalates	Gas chromatography/mass spectrometry, TLC	Hydrolysis	[74, 87]
Esterase	*Cladosporium cladosporioides*	Film, fibrs	Determination of tensile strength, viscoelasticity, extension behavior, viscosity	Hydrolysis, change of physical properties	[93]
	Thermobifida fusca	CTR, PET nanoparticles	HPLC	Hydrolysis	[8, 86]
	Melancarpus albomyces	Fabric	Contact angle measurement, dye assay, water absorption assays	Increased hydrophilicity, improved dye uptake	[59]
	Porcine liver	Fibers, DET	HPLC, SEM	Hydrolysis, changes in fiber surface morphology	[109, 110]
	Penicillium citrinum	Fabric, PET trimer	HPLC, TLC, water absorption assays	Hydrolysis, increased hydrophilicity	[61]
Protease	Papain	Fabric	Titration, water absorption assays	Hydrolysis, increased hydrophilicity	[53]

angle determinations measuring the spread of a drop of water on the PET surface [23, 38, 41, 42, 60, 107].

Changes in the water transport rate of treated PET fabrics have been measured by a vertical wicking test in which the rising height of water in a strip of fabric is determined [3, 33, 77]. Measurements of the dissipation of a drop of water on fabric [11, 38, 62], liquid retention capacity (the ratio of the amount of liquid to the dry fabric quantity), and moisture regain (the amount of water a dry fiber absorbs from the air at a defined relative humidity) [43, 44, 51, 60] also aim to determine changes in the water absorption behavior of treated PET fabrics. Incomplete removal of enzymes adsorbed to the PET surface can, however, easily lead to incorrect results obtained using these methods [11, 23, 102].

Dye assays can be employed in a more direct approach to quantify hydroxyl and carboxyl groups on the PET polymer surface introduced by enzymatic treatments [34]. Improved dye uptake of enzyme-treated fabrics shown by an increase in the color shade of samples dyed with cationic or reactive dyes determined by reflectance measurements indicated a partial enzymatic hydrolysis of PET surfaces [2, 11, 27, 84, 107]. Likewise, the formation of free carboxyl groups formed by enzymatic treatment of PET films can be analyzed following esterification using a fluorescent alkyl bromide [23].

Among spectroscopic methods, X-ray photoelectron spectroscopy (XPS), also known as electron spectroscopy for chemical analysis (ESCA), has been used for the analysis of enzyme-treated PET substrates [11, 27, 31, 102]. With XPS, the elemental composition of the top 10 nm of the PET surface is measured, and the presence of specific functional groups can be identified. XPS analysis of PET treated with a lipase from *Thermomyces lanuginosus* and a cutinase from *Thermobifida fusca* indicated increased amounts of hydroxyl and free acid groups, while the amount of carbon–carbon bonds decreased [11, 27]. XPS has been shown to be very sensitive to contamination by residual enzyme protein strongly adsorbed to the polymer surface [31, 102].

Fourier transform infrared spectroscopy (FTIR) is another method that can determine chemical changes on enzyme-treated PET surfaces in the micron range of the sample [34]. In FTIR, by subjecting the specimen to a modulated infrared beam, molecules absorb infrared energy at characteristic frequencies. The transmittance and reflectance of the infrared rays at different frequencies result in a spectral pattern providing information about the chemical bonding and molecular structure of the analyzed polymer. Using attenuated total reflectance FTIR with improved surface sensitivity, significant spectral changes in the amorphous and crystalline PET domains in enzyme-treated PET films have been reported [23]. The results indicated increased crystallinity of the PET film surface following partial enzymatic hydrolysis.

Surface topography of enzyme-treated PET has been studied using scanning electron microscopy (SEM) and environmental scanning electron microscopy (ESEM), in which a focused electron beam is scanned across a rectangular area of the sample that then emits secondary electrons used to generate a three-dimensional surface image. SEM analysis has revealed cracks, scaling, and increased roughness

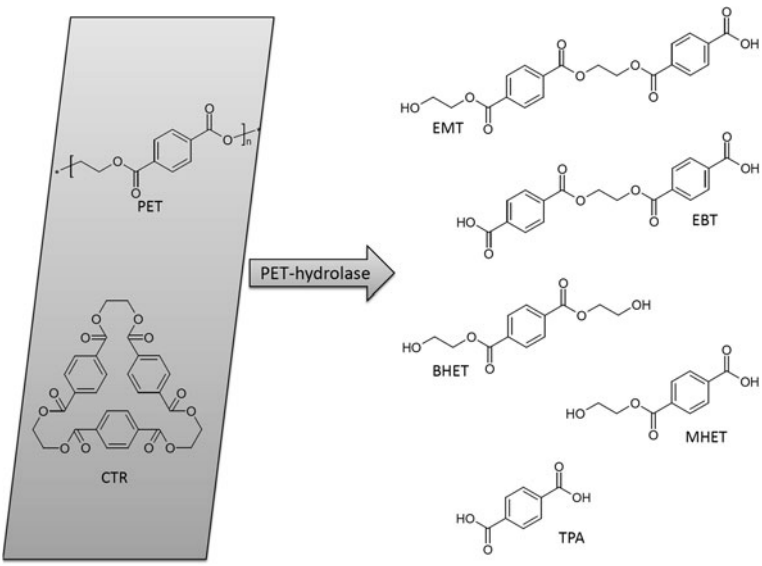

Fig. 1 Chemical structures of PET, cyclic PET trimer (CTR) and enzymatically released hydrolysis products. Terephthalic acid (TPA), *mono*(2-hydroxyethyl) terephthalate (MHET), *bis*(2-hydroxyethyl terephthalate) (BHET), 1,2-ethylene-*mono*-terephthalate-*mono*(2-hydroxyethyl terephthalate) (EMT), and 1,2-ethylene-*bis*-terephthalate (EBT)

of the surface of PET fibers following treatment with lipases from *Candida antarctica* and porcine pancreas, and with porcine liver esterase [52, 60, 109, 110]. Similar results were obtained with PET hydrolases from *Pseudomonas mendocina* and *Thermomyces insolens* [49, 107]. SEM analysis of low-crystallinity PET film treated with the enzyme from *T. insolens* showed that with increasing incubation time the surface roughness of the film increased, and after 96 h of incubation, holes formed that penetrated the surface [91]. In contrast, no changes in surface morphology were detected by ESEM after treatment of PET and poly(trimethylene terephthalate) (PTT) fibers with a cutinase from *F. fusca* [11, 26].

In high-resolution atomic force microscopy (AFM), a cantilever with a sharp tip is used to scan the sample surface. Deflection is measured using a laser beam reflected to a photodetector to generate a surface map of the specimen. Analysis of *T. fusca* cutinase-treated PET fibers by AFM has indicated an increase in surface roughness of the fibers [31].

Enzymatically released aromatic PET degradation products can be monitored by UV detection at 240–255 nm, following separation by reversed-phase high performance liquid chromatography (HPLC). Soluble hydrolysis products of PET films, fibers, and cyclic PET trimers (CTR) that have been identified include: TPA, *mono*(2-hydroxyethyl) terephthalate (MHET), *bis*(2-hydroxyethyl terephthalate) (BHET), 1,2-ethylene-*mono*-terephthalate-*mono*(2-hydroxyethyl terephthalate) (EMT), and 1,2-ethylene-*bis*-terephthalate (EBT) [8, 33, 38, 40, 90, 102] (Fig. 1).

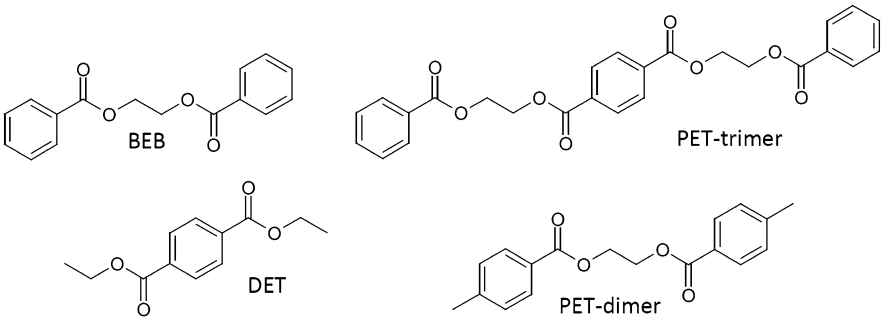

Fig. 2 Chemical structures of the PET model compounds ethylene glycol dibenzyl ester (BEB), *bis*(benzoyloxyethyl) terephthalate (PET trimer), diethyl terephthalate (DET), and *bis*-(*p*-methylbenzoic acid)-ethylene glycol ester (PET dimer)

While TPA released by enzymatic hydrolysis of PET has been directly monitored at 240 nm [2, 107], the sensitivity of the method can be increased by fluorimetric determination following conversion of TPA to hydroxy-terephthalic acid [82, 84]. Alternatively, hydrolysis of water-insoluble PET oligomers and nanoparticles to soluble products has also been monitored by turbidity measurements [1]. Nanoparticles can be prepared by dissolving PET in 1,1,1,3,3,3-hexafluor-2-propanol and dropping the solution in water, which creates a suspension of particles ranging from 50 to 100 nm [87, 104].

Furthermore, the release of TPA from PET substrates and the hydrolysis of alkylphthalates has been analyzed by thin layer chromatography (TLC) [33, 61, 87].

Hydrolase activity on PET substrates has also been determined by titration to measure NaOH consumption during the hydrolysis reaction [37, 52, 53]. A pH-stat technique that maintains a constant pH as acid is released has been used to determine the kinetic parameters of hydrolase-catalyzed PET ester cleavage [37, 91].

Oligomeric PET model compounds have been widely used to study enzymatic hydrolysis of PET, since their degradation is faster and easier to analyze compared to a polymeric substrate. Diethyl terephthalate (DET), diethyl *p*-phthalate (DP), *bis*(benzoyloxyethyl) terephthalate (PET trimer), ethylene glycol dibenzyl ester (BEB), and *bis*-(*p*-methylbenzoic acid)-ethylene glycol ester (PET dimer) have been employed (Fig. 2). Their degradation by PET hydrolases from *Fusarium solani* [27, 38], *T. insolens* [1, 5, 48, 101], *P. mendocina* [25, 49], *T. fusca* [26, 27, 38], *Burkholderia cepacia* [26, 27, 38], *Aspergillus oryzae* [103], *Bacillus* spp. [87], and porcine liver esterase [109, 110] has been reported, and the corresponding hydrolysis products have been partially characterized (Table 1).

3 Biocatalytic Surface Modification of PET

While many natural and synthetic aliphatic polyesters are susceptible to enzymatic hydrolysis, aromatic polyesters such as PET have been previously considered

resistant to enzymatic attack [55, 75]. The degradability generally decreases as the amount of aromatic moieties in the polyester increases [105]. In spite of the recalcitrance of PET, a number of mostly fungal and bacterial enzymes that can hydrolyze PET substrates have been identified (Table 1).

Changes in the physical properties of PET fibers and films following treatment with an esterase from *Cladosporium cladosporioides* was already reported in 1983 [93]. Treatment of PET fabrics with a cutinase from *F. solani* f. sp. *pisi* resulted in the release of aromatic compounds, increased hydrophilicity, and improved dye uptake of the fabrics [3, 6, 38, 82, 84, 85, 98]. The cutinase from *P. mendocina* improved the wetting and water absorption properties of PET and sulfonated PET fabrics [44, 45]. In further studies, partial hydrolysis, increased hydrophilicity, and improved dye uptake of PET and PTT fabrics have been confirmed [25, 107].

Similar effects have been obtained with a cutinase from *T. fusca.* Treated PET and PTT fabrics showed increased hydrophilicity, and improved dye uptake and water absorption properties [2, 3, 11, 26, 27, 31, 38]. A esterase from *Penicillium citrinum* hydrolyzed the surface of PET, resulting in increased hydrophilicity. The PET trimer was hydrolyzed to TPA, BHET, MHET, 2-hydroxyethyl-benzoate, and benzoic acid [61].

With lipases, improved dyeing behavior, water absorption, and oil stain resistance of PET fabrics have been claimed following treatment with enzymes from *T. insolens* [1, 62, 72, 101] and *T. lanuginosus* [4, 11, 26]. Treatment of PET fabrics with lipases from *Aspergillus* spp., *Beauveria brongniartii*, *C. antarctica*, *C. cylindracea*, *Rhizomucor miehei*, *Burkholderia cepacia*, *Pseudomonas fluorescens*, *Rhizopus* spp., and porcine (hog) pancreas lipase resulted in increased hydrophilicity, moisture regain, and water absorption values [4, 33, 38, 44, 51, 52, 60, 103].

While a lipase from *C. antarctica* has been reported to hydrolyze polyester fabric and PET model compounds [5, 50, 51, 60], no hydrolysis products could be detected after treatment of PET films with this enzyme [102].

A carboxylesterase (EC 3.1.1.1) from *T. fusca* [8, 86] and a steryl esterase (EC 3.1.1.13) from *Melanocarpus albomyces* [59] have also shown activity with PET oligomers and fabrics. The enzyme from *M. albomyces* with high specificity for fatty acid esters of sterols increased the hydrophilicity of PET fabrics. The highly hydrophobic serine hydrolase from *T. fusca* with a catalytic triad composed of serine, glutamic acid, and histidine hydrolyzed CTR and PET nanoparticles. The esterase showed high specificity towards short and middle chain-length fatty acyl esters of *p*-nitrophenol. In addition, *p*-nitrobenzyl esterases from *Bacillus subtilis* and *B. licheniformis* that hydrolyzed short chain dialkylphthalates and PET nanoparticles have been reported [74, 87].

Recently, the hydrolysis and increase in the hydrophilicity of PET fabrics treated with papain, a cysteine protease (EC 3.4.22.2), have also been reported, further indicating that the ability to hydrolyze PET cannot be allocated to a single family of hydrolytic enzymes [53].

The biofunctionalization of PET fabrics using enzymes can improve the pilling behavior of treated polyester fabrics. PET hydrolases with high activity and stability could therefore be employed in the textile and detergent industries to

improve the anti-pilling quality of PET textiles. Pilling is the formation of small, fuzzy balls on the surface of PET fabrics resulting in an unsightly worn appearance of the textile. Pilling could be reduced by treatment with PET hydrolases from *P. mendocina* and *T. insolens* [5, 25, 48, 49, 101, 107]. Two enzyme variants from *T. insolens* obtained by site-directed mutagenesis have been used for an effective reduction of PET fabric pilling in treatments at 70°C without the addition of the surfactant Triton X-100 (octyl phenoxy polyethoxy ethanol) [48].

Depending on the processing and thermal treatment, PET can occur in a low-crystalline amorphous form and in a semi-crystalline form. It has been shown that the extent of polyester hydrolysis depends on the degree of its crystallinity and chain orientation [83, 106]. In the amorphous regions, the polymer chains are less densely packed and are more susceptible to hydrolytic attack compared to the crystalline regions. The differences in mobility of the polymer chains in the amorphous and crystalline regions of PET therefore contribute to a large extent to its enzymatic degradability. The observed hydrolytic activities of PET hydrolases from *T. insolens*, *P. mendocina*, *F. solani*, and *T. fusca* were considerably lower with semi-crystalline or biaxially oriented PET films with crystallinity values of about 35–40% as substrate compared to low-crystallinity PET films with about 4–7% crystallinity [11, 91, 102, 107]. Similarly, it has been demonstrated that amorphous PET in the form of nanoparticles is readily degraded by a cutinase from *T. fusca* [104]. This cutinase also released 50% more degradation products from amorphous PET fibers compared to semi-crystalline fibers [11]. In agreement with these results, crystallinity determination following treatment of PET film with PET hydrolases has revealed a relative increase in crystallinity due to the preferential hydrolysis of the amorphous regions of the polymer [23, 91].

The enzymatic degradation rate of polyester has been correlated with the temperature difference between the melting temperature of the polymer and the hydrolysis temperature. The polymer chain can be considered to be more mobile and accessible to enzymatic attack close to the glass transition temperature (T_g) of amorphous PET or the melting temperature (T_m) of semi-crystalline PET [69, 70]. Therefore, increased enzymatic hydrolysis rates of PET can be expected by performing the reaction at temperatures near the T_g of the amorphous polyester [69, 105]. For example, with increasing reaction temperatures from 30 to 80°C, the hydrolysis rate of low-crystallinity PET film with a T_g at about 75°C by the PET hydrolase from *Thermomyces insolens* also increased. The most substantial increase in activity was observed at temperatures above 65°C. Similarly, hydrolysis with the less thermostable PET hydrolases from *F. solani*, *P. mendocina*, and *T. fusca* performed between 40 and 55°C resulted in much lower hydrolysis rates compared to those obtained with the *T. insolens* enzyme at 70°C. These results indicate that the better performance of the *T. insolens* PET hydrolase can be accredited to its higher thermal stability enabling hydrolysis reaction temperatures close to the T_g of the amorphous PET film [91].

Although the desired extent of enzymatic hydrolysis of PET should be limited to the material surface for the purpose of biofunctionalization of the materials, considerable weight losses can be obtained following prolonged treatment with

PET hydrolases [25, 48, 49, 101, 103]. With low-crystallinity PET film, a 50% weight loss after 3 weeks of incubation with a cutinase from *T. fusca* [76] and a 97% weight loss after 96 h of incubation with a lipase from *T. insolens* [91] have been reported. These results indicate the potential of using PET hydrolases for the complete degradation and recycling of PET [77, 91].

PET hydrolases strongly adsorb to the hydrophobic surfaces of polyester substrates, which influences the extent of enzymatic hydrolysis of PET films and fibers [54, 102]. This adsorption involves electrostatic as well as hydrophobic interactions. Depending on the protein concentration, it can result in enzyme inactivation due to irreversible conformational changes and blocking of the surface, which prohibits further hydrolysis. Protein concentration has been found to have a stronger influence on the adsorption characteristics on PET surfaces than the adsorption time [39]. For example, it has been observed that the hydrolysis of amorphous PET films incubated with cutinases from *F. solani* and *P. mendocina* reaches a plateau after several hours of incubation. However, soluble enzymes in the media were still active, suggesting that the enzymes adsorbed on the surface became inactivated during incubation and prohibited access of active soluble enzyme, which resulted in no further degradation [91].

Agitation of the reaction mixture is important in heterogeneous reactions involving insoluble substrates. Cutinase activity on PET fabrics could be improved by a low level of mechanical agitation during enzyme treatment [85]. In addition, it has been shown that the enzymatic hydrolysis of CTR could be strongly increased by agitation of the substrate dispersion resulting in a decreased particle size and a better enzyme delivery to the CTR particle surface [40]. The hydrolysis rate of CTR was found to depend strongly on the particle size and exposed surface area of the substrate. Analysis of the reaction kinetics of the hydrolysis by the lipase from *T. insolens* showed that the hydrolysis reaction followed zero order kinetics when a CTR dispersion was used as the substrate with a small surface area. When CTR was precipitated from dilute dioxane solutions, the surface area of the smaller particles obtained was much larger, and first order rate constants were obtained [32].

The hydrolytic activity of PET hydrolases and the resulting increase in surface hydrophilicity of PET by the addition of non-ionic surfactants such as Triton X-100 have been evaluated. The observed effects appeared to depend on the type of PET hydrolase used. Treatment of PET fabrics with the lipase from *T. insolens* in combination with Triton X-100 resulted in a strong increase in the hydrophilicity of the PET material [62]. Likewise, hydrolysis of the PET trimer model compound with the lipase from *T. lanuginosus* was strongly increased in the presence of Triton X-100, which can be explained by the interfacial activation of lipases involved in the hydrolysis of PET substrates [27]. However, Triton X-100 did not increase the moisture regain of PET fabrics treated with a lipase from *C. antarctica* [60]. The hydrolytic activity of the PET hydrolases from *T. fusca* and *F. solani* were also not improved in the presence of Triton X-100 as can be expected from the lack of a lid structure and interfacial activation in cutinases [19, 27]. A considerable increase in the hydrolysis of PET fabrics and film by the

T. lanuginosus lipase and the *T. fusca* cutinase was found after addition of the plasticizer *N*,*N*-diethylphenyl acetamide, which reduced the glass transition temperature and increased the chain mobility of PET [27].

4 Properties of PET Hydrolases

The best characterized PET hydrolases are the fungal enzymes from *F. solani*, *T. insolens*, *T. lanuginosus*, and *A. oryzae*, and the bacterial cutinases from *T. fusca* and *P. mendocina* (Table 2).

Cutinases (EC 3.1.1.74) are able to degrade cutin, an aliphatic polyester covering the surface of plants, and the polyester domains of suberin, which forms a protective layer in plant periderms [29, 57]; for a review, see [24]. Cutin is composed of mainly C_{16} to C_{18} mono-, di-, and tri-hydroxy and epoxy fatty acids [58]. Cutinases enabling the penetration of the protective cutin layer of plants play an important role in the infection process by phytopathogenic microorganisms. The enzymes have properties of both carboxylesterases and lipases, since they show lipolytic activity with insoluble triglycerides at the lipidic interface and also hydrolyze short chain acylglycerols and *p*-nitrophenyl esters with short and medium acyl chain lengths in solution [89]. The cutinase from *T. fusca* shows high activity between 65 and 70°C and can be employed at higher reaction temperature conditions strongly favoring PET polymer hydrolysis.

Lipases (EC 3.1.1.3) belong to the second group of PET hydrolases. Lipases, in general, catalyze the hydrolysis of long chain water-insoluble triglycerides [7, 15]. Their activity is greatly increased at the water-lipid interface, a phenomenon described as interfacial activation [35]. The active site of lipases is buried in a short polypeptide chain (lid) [21, 46]. Upon adsorption at the lipid-water interface, the movement of the lid exposes the active site and increases the hydrophobicity of the surface of the enzyme around the catalytic site [12]. In contrast, the catalytic site of cutinases is not covered by a lid, but is directly exposed to the solvent,

Table 2 Comparison of PET hydrolases from *F. solani* [13, 14, 91], *T. insolens* [91, 92], *P. mendocina* [10, 91], and *T. fusca* [18, 56]

	F. solani	*H. insolens*	*P. mendocina*	*T. fusca*
Number of amino acids	197	194	272	261
Molecular mass (kDa)	22	20–21	30	28
Isoelectric point	7.2	8	nd	6.43
pH optimum	6.5–8.5	8.5–9.5	9.0–9.5	6.5–8.0
Temperature optimum (°C)	50	75–80	50	60–70
Catalytic triad	Ser120	Ser140	Ser126	Ser132
	Asp175	Asp195	Asp176	Asp176
	His188	His208	His206	His210
GXSXG motif	G-Y-S-Q-G	G-Y-S-Q-G	G-H–S-Q-G	G-H–S-M-G

nd not determined

which explains the observed lack of interfacial activation typically found in lipases [71].

4.1 Fusarium solani *Cutinase*

The properties of the cutinase from the filamentous fungus *F. solani* f. sp. *pisi* have been reported previously [88, 89]; for a review, see [13]. The three-dimensional structure of the cutinase has been solved at 1.6 and 1.0 Å resolution [47, 64, 65, 71]. The enzyme consists of 197 amino acids with a molecular mass of 22 kDa. It contains four invariant cysteines forming two disulfide bridges. The structure of the enzyme consists of a reduced α/β hydrolase fold with a five-stranded central β-sheet and five α helices (Fig. 3). Together with other PET hydrolases, it belongs to the α/β hydrolase fold superfamily [79]. The catalytic triad of the serine esterases is composed of Ser120, Asp175, and His188. The enzyme hydrolyzes a wide range of esters, including short and long chain triglycerides [67]. In addition to cutin, it hydrolyzes short chain esters of *p*-nitrophenol. The highest activity was

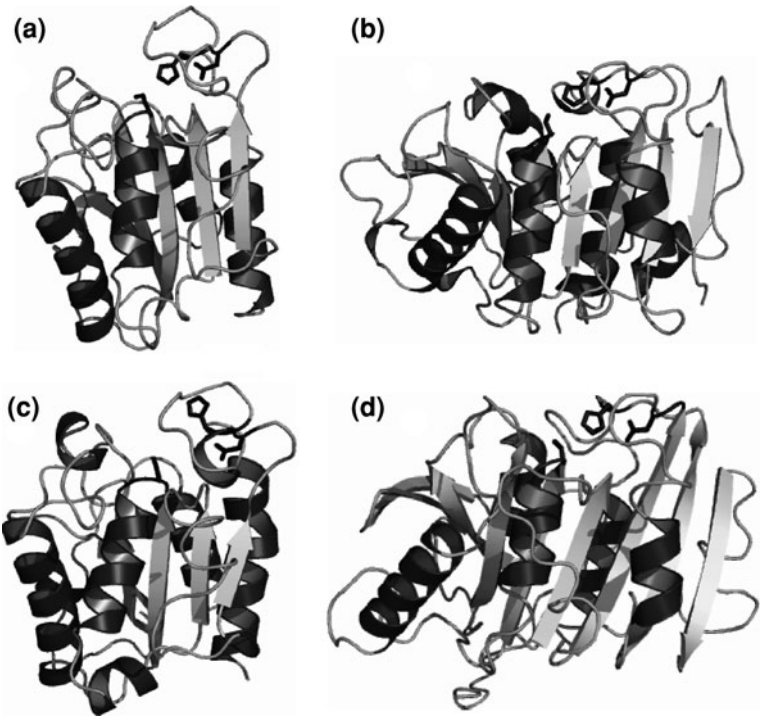

Fig. 3 Ribbon diagram of the modeled structures of PET hydrolases from **a***F. solani* [64, 65]; **b***T. fusca* [18, 66]; **c***T. insolens* [92]; **d***P. mendocina* [10]

observed with *p*-nitrophenyl acetate [89]. The enzyme is also able to degrade the polyester polycaprolactone (PCL) [78]. The maximum activity for the hydrolysis of low-crystallinity PET film was determined at 50°C. The PET hydrolase is not stable at higher temperatures, with a loss of 100% of its activity at 60°C. It is active between pH 6.5 and 8.5, but shows almost no activity at pH 9 [91]. The drastic loss of activity in alkaline conditions has been noted previously [73]. Among soluble products of the hydrolysis of low-crystallinity PET film with 4.1% crystallinity and PET granules with 13.7% crystallinity, TPA, MHET, and BHET were identified [102]. However, only TPA and ethylene glycol were detected as hydrolysis products after incubation of PET film with 7% crystallinity with the enzyme at 40°C for 96 h, indicating that a complete conversion of PET to its monomeric building blocks can be achieved by the cutinase. In contrast, the enzyme released only small amounts of hydrolysis products from a highly crystalline biaxially oriented PET film [91]. The cutinase also hydrolyzed the PET trimer model compound to TPA, BHET, MHET, 2-hydroxyethyl-benzoate, and benzoic acid [27, 38]. The enzyme completely further hydrolyzed 2-hydroxyethyl-benzoate after 24 h of incubation. BHET was hydrolyzed first to MHET, and in a second step TPA was released [27].

Site-directed mutagenesis experiments of *F. solani* cutinase to improve the specific activity towards PET substrates resulted in the construction of several variants obtained by substitution of amino acids at the catalytic site with smaller residues [6]. Simulation studies indicated an enhanced fit and free energy stabilization of the tetrahedral intermediate of the model substrate 1,2-ethanodiol dibenzoate and an increased activity with PET fibers of the L182A cutinase variant when compared to the wild-type enzyme.

4.2 Pseudomonas mendocina *Cutinase*

The cutinase from the phyllospheric fluorescent *P. mendocina* (formerly *putida*) was isolated in 1987 [95]. Besides cutin, this enzyme also catalyzes the hydrolysis of mono- and triglycerides and of *p*-nitrophenyl esters of C_4 to C_{16} fatty acids with decreasing activity with the longer chain esters. However, tripalmitoyl and trioleyl glycerol were not hydrolyzed. The 30 kDa serine esterase displays a broad pH activity range between 8.0 and 10.5 with cutin as the substrate [96]. Structure analysis of the enzyme showed an α/β-hydrolase tertiary fold consisting of a nine-stranded core β-sheet and seven α helices (Fig. 3). Its catalytic triad is composed of Ser126, Asp176, and His206 [9].

Hydrolysis of the PET model compound DET by the cutinase has been reported [49]. Maximum activity for the hydrolysis of low-crystallinity PET film was determined at 50°C, and 100% of the activity was lost at 60°C. Enzymatic activity increased from pH 7.0 to 9.5, indicating high stability under alkaline conditions. Incubation of low-crystallinity PET film with the enzyme at 50°C for 96 h resulted in 5% weight loss. Only TPA and ethylene glycol were detected as hydrolysis

products [91]. By site-directed mutagenesis, several variants were produced with improved stability and hydrolytic activity on PET substrates compared to the wild-type cutinase [10].

4.3 Thermobifida fusca *Cutinase*

The thermophilic actinomycete *T. fusca* produces cutinolytic activity when grown in media containing cutin or suberin [28]. The cutinase preparation showed a half-life of more than 1 h at 70°C and a pH optimum of 11.0. An enzyme preparation obtained from *T. fusca* KW3 grown in media containing PET fibers or suberin released hydrolysis products from PET fibers, poly(butylene terephthalate) fibers, and recycled PET granulate at 50 and 70°C [2].

Thermobifida fusca produces two homologous cutinolytic isoenzymes with 92–93% identity and a molecular mass of about 29 kDa. Both enzymes consist of 261 amino acids [18–20]. Homology modeling suggested a α/β-hydrolase tertiary fold flanked on both sides by α helices and the absence of a lid structure (Fig. 3). The catalytic triad of these serine esterases consists of Ser132, Asp176, and His210. The two cutinases showed similar catalytic properties and were able to hydrolyze cutin as well as insoluble triglycerides and soluble esters at an optimum temperature of 60°C and a pH optimum at 8.0. Their activity was higher with the C_4 triglyceride tributyrin than the C_{18} triglyceride triolein, indicating a preference for short chain fatty acid esters. The enzymes retained 50% of their activity after incubation for 40 h at this temperature. One of the cutinases with a molecular mass of 28.2 kDa obtained from *T. fusca* DSM 43793 efficiently hydrolyzed amorphous PET films [27, 76]. In contrast, the second cutinase showed very low PET-hydrolyzing activity with CTR as the substrate, indicating differences in the substrate-binding site despite the high sequence similarity found [19]. The enzyme hydrolyzed triglycerides, preferentially those with short and medium chain-length fatty acids. Besides cutin, the enzyme showed activity with dibutylterephtalate, PCL, several aliphatic polymers, and an aliphatic–aromatic co-polyester, but did not degrade poly(butylene terephthalate) and poly(β-hydroxy butyrate). It showed the highest activity with the aliphatic–aromatic co-polyester between 65 and 70°C and a pH maximum between pH 6.0 and 7.0. The enzyme lost about 85% of its activity within 30 min of incubation at 70°C [56, 69]. The cutinase also hydrolyzed the PET trimer model compound [27, 38] and BHPT [26] in a similar way as the cutinase from *F. solani*. BHPT was first hydrolyzed to MHPT, and after complete conversion of BHPT to MHPT it was further hydrolyzed to TPA. Likewise, cyclic PTT dimers and films were hydrolyzed to produce MHPT and subsequently TPA without the formation of BHPT. The PET trimer was hydrolyzed to TPA, BHET, MHET, 2-hydroxyethyl-benzoate, and benzoic acid. The enzyme further degraded 2-hydroxyethyl-benzoate completely after 24 h of incubation. As in the case of BHPT, BHET was hydrolyzed first to MHET, and in a second step TPA was released [27].

4.4 Thermomyces insolens *Lipase*

Thermomyces insolens (formerly *Humicola insolens)* is a thermophilic filamentous fungus. It produces a lipase with a molecular mass of 20–21 kDa. It shows activity at 50°C between pH 7.0 to 9.5, and a maximum at 80°C and pH 8.5 [91]. Analysis of the structure of the enzyme has revealed that it has high homology with the cutinase from *F. solani* [5] (Fig. 3). The active site of the enzyme is composed of Ser140, Asp195, and His208 [92]. It hydrolyzed the PET model compounds DET, BEB, and PET trimer [1, 5, 48]. MHET was detected as the major hydrolysis product of CTR with only trace amounts of TPA and BHET formed [90]. Complete degradation of CTR by the enzyme was achieved at pH 8 at 60°C [40]. TPA and MHET were the predominant products found, and BHET was detected in trace amounts. The ratio of TPA/MHET was found to depend on the enzyme concentration used. Higher concentrations of the enzyme resulted in more complete hydrolysis of MHET and a larger amount of TPA detected as hydrolysis product. The PET hydrolase has also been shown to be able to completely degrade low-crystallinity PET film to TPA and ethylene glycol [91].

By random and site-directed mutagenesis, variants of the enzyme have been prepared with improved thermal stability. While the wild-type lipase showed a thermal denaturation temperature (T_d) of 61°C, several of the variants obtained with one or more amino acid substitutions in the vicinity of the N-terminal amino acid had a T_d of 64–66°C. One of the variants showed improved CTR hydrolysis at pH 9 and 75°C, and its temperature optimum was determined at 65°C [1]. The thermostability of the enzyme could be further improved by site-specific mutagenesis using synthetic nucleotides [81], and variants with a T_d of 71–72°C were obtained [101]. The optimized PET hydrolase could be employed for PET fabric treatments performed at 75°C, resulting in a de-pilling effect and improved water absorption.

Using low-crystallinity (7% crystallinity) and biaxially oriented PET films (35% crystallinity), the *T. insolens* lipase showed maximum hydrolysis activity on low-crystallinity PET film between 70 and 80°C [91]. With a T_g value of 75°C, the increased chain mobility of the low-crystallinity film at this reaction temperature strongly facilitated its hydrolysis by the thermostable *T. insolens* PET hydrolase. The low-crystallinity PET film was almost completely hydrolyzed after 96 h at 70°C, while tenfold lower activity was detected with the semi-crystalline PET film. Degradation of the low-crystallinity PET film followed a heterogenous kinetic model considering substrate-limited conditions with a limited surface area available to the enzyme [94].

4.5 Thermomyces lanuginosus *Lipase*

The lipase from the thermophilic filamentous fungus *T. lanuginosus* (formerly *Humicola lanuginosus*) consists of 269 amino acids and has a molecular mass of 31.7 kDa [30]. It contains a α/β-hydrolase tertiary fold consisting of a nine-

stranded core β-sheet and seven α helices. The structure of the enzyme has been solved [22]. It contains a highly mobile α-helical loop forming a lid structure covering the active site and a catalytic triad composed of Ser-His-Asp.

Besides PET and PTT, the enzyme also degrades other polymers such as PCL and poly(bisphenol-A carbonate) [30]. The lipase hydrolyzed BHPT to MHPT, but TPA could not be detected as a degradation product, indicating that the lipase was unable to hydrolyze MHPT. Cyclic PTT dimers and films were also not hydrolyzed [26]. The lipase did not release TPA from the model compound BHET, but the PET trimer was hydrolyzed to TPA, BHET, MHET, 2-hydroxyethyl-benzoate, and benzoic acid. In contrast to cutinases, 2-hydroxyethyl-benzoate was not further hydrolyzed to benzoic acid [27].

4.6 Aspergillus oryzae *Lipase*

The filamentous fungus *A. oryzae* produced by induction with BHET a lipase that hydrolyzes DP and improves the hydrophilicity of PET fabrics [103]. The lipase from *A. oryzae* is also able to degrade synthetic plastics such as poly(butylene succinate), poly(butylene succinate-co-adipate), and PCL [68]. The highest catalytic efficiency with *p*-nitrophenyl ester substrates was found with *p*-nitrophenylvalerate and *p*-nitrophenylbutyrate. The enzyme fully retained its activity at incubation temperatures of up to 40°C for 1 h. Crystallographic analysis of the structure of the lipase indicated a monomeric protein consisting of an α/β hydrolase fold with a central β-sheet of five parallel strands surrounded by ten α-helices. Ser126, Asp181, and His194 residues form the catalytic triad of the enzyme [63]. Comparison with the cutinase from *F. solani* revealed the presence of an additional stabilizing disulfide bond and a catalytic triad in a topologically favored position with a longer and deeper groove (Fig. 4). These differences may contribute to the observed preference for longer chain substrates, higher hydrolytic activity with polymeric substrates, and superior thermal stability of the *A. oryzae* lipase.

5 Conclusions and Perspectives

The biofunctionalization of PET with the aim to increase hydrophilicity of the hydrophobic polyester surface has wide-ranging applications from textile finishing to technical and medical fields. Among the biocatalysts with PET-hydrolyzing activity that have been reported so far, several have shown promising results for the biofunctionalization of PET as an alternative to presently used chemical or physical methods. However, an enzymatic treatment of PET materials requires highly active biocatalysts and short treatment times to become industrially viable. Analysis of the efficiency of PET hydrolysis performed with biocatalysts at

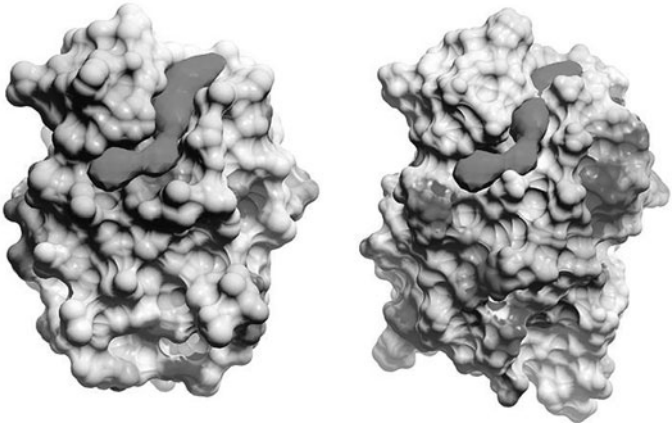

Fig. 4 Electrostatic surface rendering of *A. oryzae* lipase (*left*) and *F. solani* cutinase (*right*). The *dark shaded area* illustrating the groove on the surface proximal to the active site of the *A. oryzae* lipase cannot fit in the narrow groove on the *F. solani* cutinase [63]

different reaction temperatures has clearly shown the requirement of employing thermostable enzymes at elevated temperatures to achieve substantial enzymatic activity with PET materials. While improvements of the thermostability and reactivity of PET hydrolases achieved by protein engineering have already been reported, continuing progress towards the stabilization of the PET-hydrolase structure and optimal design of the active site for PET polymer chain hydrolysis will be required for further industrial development of the biocatalytic functionalization of PET.

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Biology of Human Hair: Know Your Hair to Control It

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Abstract Hair can be engineered at different levels—its structure and surface—through modification of its constituent molecules, in particular proteins, but also the hair follicle (HF) can be genetically altered, in particular with the advent of siRNA-based applications. General aspects of hair biology are reviewed, as well as the most recent contributions to understanding hair pigmentation and the regulation of hair development. Focus will also be placed on the techniques developed specifically for delivering compounds of varying chemical nature to the HF, indicating methods for genetic/biochemical modulation of HF components for the treatment of hair diseases. Finally, hair fiber structure and chemical characteristics will be discussed as targets for keratin surface functionalization.

Keywords Follicular morphogenesis · Hair follicle · Hair life cycle · Keratin

Contents

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1 Structure and Morphology of Human Hair

Human hair is mainly composed of fibrous α-keratin proteins. Hair fibres are not continuous in their full length, but rather result from compact groups of cells within the fibre follicle, from which three further basic morphological components of hair structure originate: the multicellular cuticle sheath, the fibrous cortex and the medulla [1, 2].

At the follicular level, a single layer of cells gives rise to the cuticle, a protective layer covering the core of the fibres. It is mainly composed of *β*-keratins and displays a scaled structure, possessing between seven and ten superimposed layers with the cuticle edges pointing toward the tip of the fibre [3]. The outer surface of the cultricle's scale cells is coated by a thin membrane called the epicuticle, which covers the cysteine-rich exocuticle, a constituent that contains most of the cysteine residues present in the scales [4]. Finally, there is the endocuticle, which is located at the interface of the cortex and is mainly composed of the remaining cell organelles. Endocuticle consists of proteins that, unlike those found in other parts of the hair fibre, have very low sulphur content; thus, it is poor in cysteine, which causes the endocuticle of the scales to swell considerably more in water than the cysteine-rich exocuticle. This might explain the pronounced projection of the scales and the tendency for wool felting in the presence of water [5].

The cuticle tightly encircles the cortex that forms the most voluminous part and the heart of the hair fibre. The cortex is made up of cortical cells, which comprise the macrofibrils, long filaments oriented parallel to the axis of the fibre. Each macrofibril consists of intermediate filaments (IF), known also as microfibrils, and the matrix [6, 7]. It has been established that the molecules that aggregate to form the IFs in keratin fibres are type I and type II keratin chains, arranged parallel to one another and in the axial register. After the formation of the α-helices, it is believed that the two types of chains associate to form a dimer, which then aggregates with another dimer to form a tetramer. Finally, the formation of a pseudo-hexagonal structure (the IFs structure) occurs by the association of seven or eight tetramers. Type I chains are net acidic, with p*I* values in the range of 4.5–5.5, while type II chains are neutral-basic with p*I* around 6.5–7.5 [8–11]. As a consequence, the IFs are low in cystine ($\sim$6%), whereas the matrix contains up to 20% of total amino acid residues [12–14].

The matrix proteins that surround the IFs through intermolecular disulfide bonds act as a disulfide crosslinker holding the cortical superstructure together and conferring high mechanical strength, inertness and rigidity to keratin fibres. High sulphur proteins, ultra-high sulphur proteins and high glycine-tyrosine proteins are present in matrix proteins (*γ*-keratins), depending on their cysteine, tyrosine and glycine content [1, 4, 15, 16].

Apart from albinos, all normal humans have melanin hair pigmentation, whatever the colour. Dispersed throughout the structure of the cortex in granular form are the melanin pigment particles. The number, chemical characteristics and distribution pattern of these cells determine the colour of the hair [2]. The actual

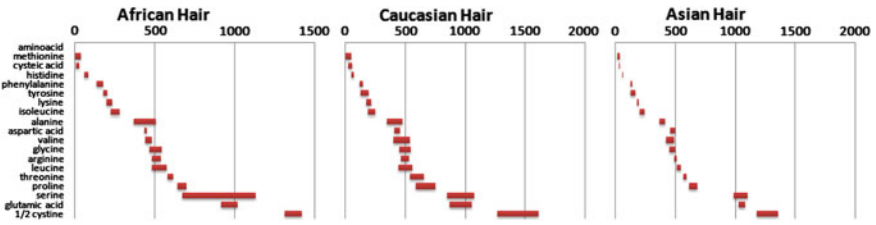

Fig. 1 Amino acid content of human hair of diverse ethnic origins (μM/g) (adapted from [1, 2, 18, 19])

shade of colour in each individual depends not only on which melanin is present, but also its quantity and the site, number and shape of pigment granules in the hair cortex (Cx) [17].

Vacuolated cells may also be present along the axis of coarser α-keratin fibres, forming the medulla. These cells generally constitute only a small percentage of the mass of hair and are believed to contribute negligibly to the mechanical properties of human hair fibres. Physically, the medulla forms the empty space of the fibre [4, 7].

Like all polymeric structures, keratin fibres consist of long, tightly bound molecular chains held together in many different ways from covalent bonds to weaker interactions such as hydrogen bonds, Coloumbic interactions, van der Walls interactions and, when water is present, hydrophobic bonds. Hair reactivity is complex and depends not only on the presence of reactive groups in the fibre, but also on their availability. The latter is significantly affected by fibre morphology and molecular structure [2]. Hair is mostly proteinaceous in nature, while structural lipids and other materials represent only a minor fraction of its constituents.

Human hair is usually categorised ethnically into three major distinct groups: Asian, Caucasian and African. Looking from the perspective of biological variability, environmental effects and diversity of fibre texture, it is remarkable how uniformly the amino acid makeup of protein components is across ethnic groups. The amino acid makeup of the protein components was reviewed by Wolfram and is depicted in Fig. 1 [1, 2, 18, 19].

2 Biology of Human Hair

Hair is an important feature of mammalians, where hair shafts fulfill a number of different functions such as thermoregulation, collection of sensory information, protection against environmental trauma, social communication and camouflage. Each of us displays an estimated total number of 5 million hair follicles (HF), of which 80,000–150,000 are located on the scalp [20].

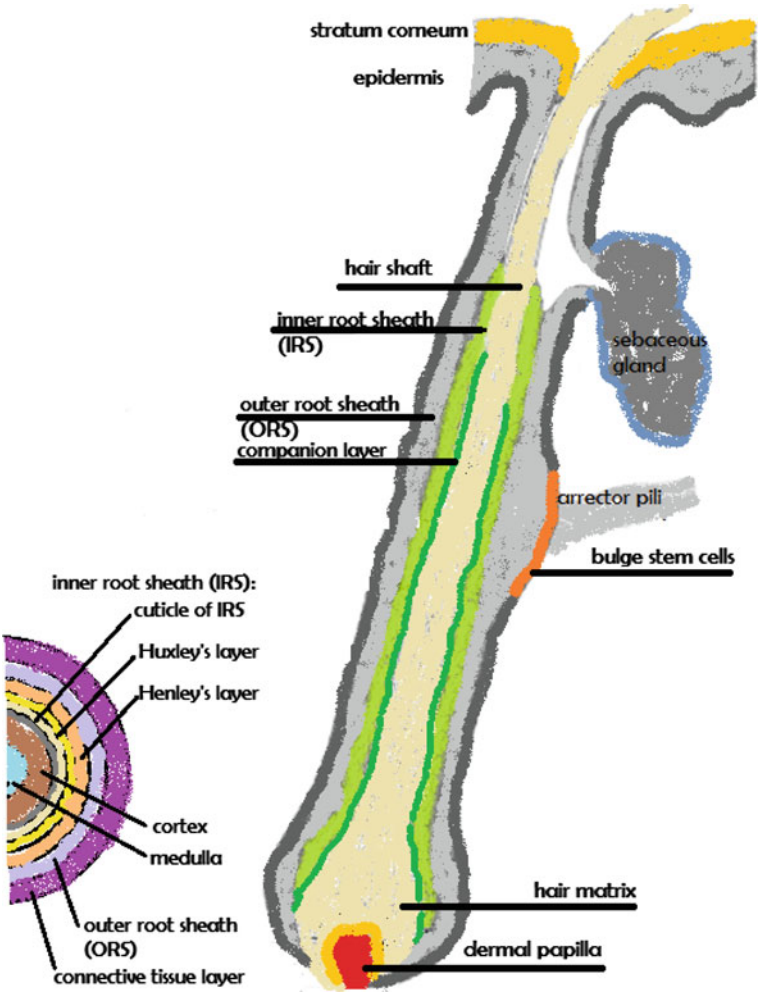

Fig. 2 The human hair follicle: structure, main functional areas and concentrical layers, which constitute the typical hair

The HF (Fig. 2) is one of the most complex mini-organs of the human body with the capacity to reconstitute itself. During postnatal life, HFs show patterns of cyclic activity with periods of active growth and hair production (anagen), apoptosis-driven involution (catagen) and relative resting (telogen) [21]. These cyclic changes involve rapid remodeling of both epithelial and dermal components and suggest the presence of intrinsic stem cells. Stem cells isolated from the bulge area possess high proliferative potential in vitro [22] and the capacity to repopulate HFs, sebaceous glands and epidermis in vivo [23–27]. These transformations are

regulated by variations in the local milieu, based on changes in expression and/or activity of many cytokines, hormones, enzymes, neurotransmitters and their cognate receptors as well as of transcription factors that have become recognised as key mediators of HF cycling.

2.1 Hair Follicle Anatomy

The HF results of interactions between epithelial, mesenchymal and neuroectodermal cell populations as well as transient migratory cells.

The epithelium is divided into an upper permanent region, distal to the arrector pili muscle and an inferior region that includes the hair bulb (Fig. 2) [28]. Each HF is composed of nine distinctive epidermal layers: hair matrix (Mx), medulla (M), Cx, hair cuticle (Ce), cuticle of the inner root sheath (Ci), Huxley's layer (Hx), Henle's layer (He), companion layer (Cp) and outer root sheath (ORS), arranged concentrically from core to periphery, as well as two dermal tissues: dermal papilla (DP) and dermal sheath. Among these tissues, only the medulla is optional, given that some hairs have no medulla, whereas in others it is relatively large. The Cx and Ce constitute the major part of the hair shaft that penetrates the skin. Both Cx and Ce tissues undergo heavy keratinization to form the solid hair shaft. The three concentric layers located externally to the shaft constitute the inner root sheath (IRS), which is thought to support the growth and differentiation of the shaft. The innermost layer of the IRS, called the Ci, consists of thin overlapping scales facing the Ce. The Hx layer is the last layer to undergo keratinization. This layer may help other keratinized cells in terms of nutritional and informational support. Importantly, Hx is known to contribute to relieving the distortion caused by uneven keratinization of the shaft, which occurs in curly hair, for example. On the other hand, the He layer keratinizes at a very early phase of hair growth so these keratinized cells are visible at a positionally low level of the HF. He layer provides mechanical support to the most delicate part of the HF in the early stages of its development.

Located within the hair bulb is a population of cells with the highest proliferation rate in the human body: the keratinocytes of the Mx. These can differentiate into trichocytes or cells of the IRS. The hair bulb in the anagen phase functions as a hair shaft producer and provides the hair shaft's trichocytes with characteristic melanin granules. The ORS, Mx and hair shaft derive from the epithelial bulge stem cells [24, 25, 27].

Mesenchymal stem cells in the tissue sheath serve as a reservoir for new DP cells. The DP determines the size of the anagen hair bulb, the duration of anagen and hair shaft diameter [20, 29, 30]. In adult hair, DP maintains the vascular system that provides the nutritional support and hormonal regulation required for hair growth [31].

2.2 Hair Follicle Morphogenesis

Follicle morphogenesis regularly occurs only during embryonic development, so each mammal is born with a fixed number of follicles that normally does not increase afterwards, although folliculoneogenesis can take place during wound healing [32–34].

2.2.1 Hair Follicle Life Cycle

The hair growth cycle describes the changing histological morphology of the shaft and of the follicle over time. Starting with anagen (rapid growth and hair shaft elongation), the follicle and its shaft progress through catagen (involution and apoptosis-driven regression), telogen (resting) and finally exogen (shedding) [20, 21, 35].

In *anagen*, the growth phase of the hair cycle, hair undergoes morphological and molecular events similar to fetal HF morphogenesis [36, 37]. Many key molecular regulators of hair biology not only activate morphogenesis, but also regulate anagen induction and duration [38, 39]. In this phase, epithelial bulge stem cells differentiate into the various cell types that will reconstitute the entire hair shaft [40, 41]. Hair shaft synthesis and melanin production in melanocytes (melanogenesis) [42], only take place in anagen. Pigmentation begins after the initiation of shaft formation and ends before this process is terminated, which causes the shaft to have an unpigmented tip and root. By the 7th week of gestation, melanocytes are already present in the human epidermis where they remain until hair morphogenesis starts, approximately 2 weeks later [43]. During HF morphogenesis, a few melanoblasts leave the epidermis and distribute randomly in the forming HF and in some sebaceous glands [44, 45]. Once the hair fiber is completely formed, melanocytes concentrate near the basal lamina surrounding the DP [44]. Anagen ends with a tightly regulated involution of the HF, which is accompanied with apoptosis and terminal differentiation of cells, a period designated as catagen [46].

The first sign of *catagen* is the cessation of melanin production in the hair bulb and apoptosis of follicular melanocytes, derived from melanocytic stem cells of the secondary hair germ [47]. Programmed cell death of these stem cells seems to play an important role in hair graying [48–50]. In contrast to what happens in the ORS and the Mx, no apoptosis occurs in the DP due to the presence of antiapoptotic protein BCL-2 [37, 46]. During catagen, the DP condenses, moves upward beneath the HF bulge and halts its activity.

After regression, the HF enters *telogen*, a phase of relative quiescence regarding proliferation and biochemical activity. The follicle remains in this stage until it is reactivated by intra- and extrafollicular signals [51].

Once the growth cycle is complete and the phase of telogen reaches the end, the hair shaft will be shed. This process, *exogen* or teloptosis, is independent of a

possible new HF cycle [52]. In fact, it is most common in mammals that a new hair shaft regrows before the resting shaft sheds, assuring the animal is never completely naked. Apart from normal development, in pathologies like trichostasis, where multiple shafts are formed and retained within the same HF, it also supports the thesis that follicle growth and shedding are independent events. Exogen ends when the shaft is released.

Recently, Rebora and Guarrera used the term kenogen to describe the interval in which the HF remains empty after the telogen hair has been shed and before a new anagen hair emerges. During kenogen the HF remains completely empty and possibly inactive. Frequency and duration of kenogen are greater in men and women with androgenic alopecia [53].

All body hairs undergo a similar life cycle, although its extent, the duration of its phases and the length of individual shafts vary between different body areas and between individuals, depending on genetic programming, genre, age and health status.

2.3 Molecular Control of Hair Follicle Development and Cycling

Epithelial-mesenchymal interactions are crucial in HF development and cycling. During normal embryonic development, interactions between epidermal keratinocytes and DP fibroblasts lead to the formation of the hair shaft. The most important factors regulating both developing and anagen follicles include the lymphoid-enhancer factor 1 (Lef1), Notch signaling pathway, Sonic hedgehog (Shh), bone morphogenetic proteins (BMPs), neurotrophins and several members of the Wnt family of proteins.

Bone morphogenetic proteins are expressed during embryonic development and postnatal life of nearly all mammalian organisms and play important roles in the regulation of both cell proliferation and differentiation [54]. BMP signaling inhibits induction of follicle development [35], and the neutralization of BMP-2 and BMP-4 activity by noggin stimulates the initiation of HF development [55, 56]. However, the differentiation of keratinocytes into mature and cuticle cells is severely impaired if levels of BMPs decrease as these proteins restrict the proliferating population to the bulb region [57]. Recent studies reinforce the important role that the BMP signaling network plays in the development and homeostasis of HF structure through the activation and/or maintenance of stem cell populations [58, 59].

The Wnt signaling pathway has been implicated in multiple cellular events including the regulation of cell proliferation, cell fate, polarity, differentiation and pattern formation [60, 61]. Wnts are divided at least into three groups according to their signal transduction pathways: the canonical pathway in which β-catenin stabilization occurs, the planar cell polarity (PCP) pathway and the Wnt/Ca^{2+}

pathway [62]. Canonical Wnt/β-catenin signaling plays an important role in HF induction and fate [63–69], and expression of several Wnts, Wnt ligands and inhibitors is specifically elevated in developing and postnatal HFs [61, 70–72]. On the other hand, forced activation of β-catenin signaling promotes HF fate in both embryonic and postnatal skin [67, 73–75]. Recent studies also suggest that another pathway, the ectodysplasin signaling pathway, is critical for the induction and maintenance of placode [76, 77].

In a surprising report, Ito and collaborators revealed that HF can form de novo in adult mice after wounding [33], contrary to the belief that follicle neogenesis only occurred during embryonic development. This phenomenon is completely dependent on Wnt signaling, and overexpressing Wnt actually increases the number of HF formed. Interestingly, the cells forming the *new HF* originate in the re-epithelialized interfollicular epidermis and not follicle stem cells.

In vertebrates, Shh is a member of the hedgehog (Hh) family of secreted signaling molecules that play a crucial role in both embryogenesis and organogenesis [78, 79]. Shh is required for HF morphogenesis during embryogenesis and for regulating follicular growth and cycling in the adult. HF formation may start without Shh, but mature HFs fail to develop because hair germs cannot elongate into the dermis to form a hair peg and the DP does not mature [80–82]. In postnatal skin, Shh is relevant for hair cycling, its expression being up-regulated in early anagen. In line with this, treatment with Shh-blocking antibodies is known to cause reversible alopecia, as HF cannot cycle and arrest in telogen [83]. On the other hand, exogenous Hh stimulates the transition from resting (telogen) to growth (anagen) stage [84] and, in some settings, anticipates the start of anagen and promotes hair regrowth in the skin of mice with chemotherapy-induced alopecia [85, 86]. These studies indicate a potential application of Hh modulation in control of hair density.

The Notch signaling pathway is also important for determining cell fate in HF development [87]. Notch signaling acts by blocking cell differentiation, which maintains the competence of undifferentiated cells [88, 89] in both the developing tooth and the HF to respond to inductive signals that determine their developmental fate [87, 90]. Based on expression studies in the HF, Notch may be a competence factor, as its pattern of expression in the HF appears to be restricted to cells that have left the proliferative pool but have yet to terminally differentiate [91, 92].

Although Notch activation is not required for HF formation in the embryo, blocking Notch signaling in postnatal epidermis results in conversion of HF into interfollicular epidermal cysts. In the absence of Notch signaling, the hair shaft still forms and contains appropriately positioned cells expressing markers for each fate; however, because IRS cells fail to adhere to each other, the follicular architecture cannot be sustained, which leads to the transformation of these aberrant HF into epidermal cysts [92–95]. Very recently, Demehri and Kopan [96] proposed that Notch acting on bi-potential bulge stem cells, or their uncommitted migratory descendents, plays an inhibitory role in preventing bulge stem cells from differentiating into epidermal cells, thus ensuring the follicular fate.

It was also reported that Notch signaling, via its target gene *Hes1*, is essential for the maintenance of melanoblasts and adult melanocyte stem cells [97, 98]. Moreover, mice with a conditional deletion of RBP-J_K (Notch transcription mediator) display, soon after birth, impaired hair pigmentation and subsequent progression of hair graying. Specifically, Notch1 and Notch2 targeted deletion in melanocytes led to inactivation of the *RBP-J_K* gene in a dose-dependent manner, and three intact alleles of *Notch1* and *Notch2* are required for preventing precocious hair graying [98]. Notch 3 and 4 are not implicated in this phenotype [99, 100].

Hormones, particularly androgens, are among the most important, although non-essential, modulators of hair growth. Androgens have paradoxically different effects on human HF depending on their body site. Androgens can stimulate hair growth on the face (beard), axilla, pubis and chest, where they are necessary for conversion of vellus hair to terminal hair, while they simultaneously can inhibit HF on parts of the scalp, converting terminal hair to vellus hair, causing balding in genetically susceptible individuals (for reviews, see [101, 102]).

Androgens, except for pubic and axillary follicles, require the intracellular enzyme 5α-reductase to metabolize testosterone to its more potent metabolite 5α-dihydrotestosterone (DHT). DHT then interacts with intracellular androgen receptors in the HF cells to modulate target gene transcription. The exact mechanism is not fully established, but Randal proposed the DP as the primary target [103, 104]. In this model, androgens act directly on DP cells where they bind to androgen receptors and then initiate altered gene expression of regulatory factors modulating indirectly the period of time when hair is growing, size and activity of the DP, keratinocytes and melanocytes. The factors produced by these populations may be soluble paracrine factors and/or extracellular matrix factors, including insulin-like growth factor-1 (IGF-1) in growth stimulation [105], stem cell factor in altered pigmentation [102] and transforming growth factor-β [106, 107] and dickkopf 1 [108] in inhibition. The opposite effects of androgens on beard and scalp follicles probably reflect differences in androgen receptor content, which is higher in beard DP than in occipital hair DP [109] and/or 5α-reductase activity, which is higher in beard than in scalp [110]. Recent observations suggest that the dermal sheath can also respond to androgens without the DP acting as an intermediary [111].

Several other hormones are implicated in the regulation of the hair growth cycle, including melatonin, prolactin, melanocyte-stimulating hormone (MSH), thyroid hormones and oestrogens. Prolactin is implicated in hair growth regulation in both mice and human [112–114]. Similarly, melatonin has been reported to promote hair growth in animals and in humans, where topical application of melatonin induced hair, probably through induction of anagen [115].

Human hair is deeply affected by the level of unbound thyroid hormones that have been described to prolong the duration of anagen, up-regulate the proliferation of Mx keratinocytes and also stimulate intrafollicular melanin synthesis [101, 116, 117]. On the other hand, several studies reveal that a significant percentage of patients presenting hair loss were diagnosed as suffering from hypothyroidism [118, 119].

Estrogens, namely 17β-estradiol, act mainly as hair growth inhibitors. Topical application to mice skin of 17β-estradiol inhibits hair growth and accelerates catagen, while antiestrogens promote early anagen [120, 121]. A similar inhibitory effect on hair growth was observed in humans [122, 123]. Nevertheless, the exact role of estrogens in the regulation of the HF life cycle is still under debate.

Very recently, mammalian circadian clock genes such as Clock and Bmal1 were reported to be involved in the regulation of HF cycling [124], suggesting that modulation of hair growth may also be achieved by modulating circadian genes through control of the cell cycle.

Taken together, these studies suggest that the described genes and signaling pathways regulating HF morphogenesis and cycling can be reactivated and modulated in adult skin, creating new strategies for hair regeneration.

3 Strategies for Cosmetic and Clinical Purposes

3.1 Treatments of Hair Growth Disorders: Room for Improvement

The most common types of hair growth disorders are caused by aberrant HF cycling. One such disorder is androgenetic alopecia, characterized by a shortening of the anagen phase and a prolongation of telogen, combined with miniaturization of HF [125]. Hirsutism, on the other hand, is defined as the presence of excess terminal hairs in females in an adult male typical pattern [126]. To control the amount of hair produced for clinical purposes, the main strategy is to alter anagen duration either by shortening it in cases of hirsutism or by increasing it to correct alopecia.

The first-generation drugs used for hair growth control—topical minoxidil solution and oral finasteride—are the only FDA-approved drugs for this application. Minoxidil prolongs the anagen phase, and promotes growth and enlargement of follicles in telogen phase [30, 127]. The effects of minoxidil on human HF are nevertheless very contradictory. Some studies describe that, at low concentrations, minoxidil stimulates anagen in human follicles [128–130], while it can cause growth inhibition at high concentrations [128]. Other authors reported no effect at all [131].

Nevertheless, it is assumed that treatment with minoxidil lengthens and thickens the small vellus hairs and decreases shedding. Minoxidil is a potassium channel opener that causes vasorelaxation [132] and stimulates cutaneous blood flow to the scalp [133]. Minoxidil sulfate, a metabolite of minoxidil, is a potent vasodilator. Uptake and conversion of minoxidil to minoxidil sulfate occurs within the HF, suggesting a direct action on the follicle [134]. The most probable site of action of minoxidil is the DP [135], and the mechanism of action has been linked to its effects on the Kir6.1/SUR2B potassium channel expressed by the derma papilla [136–138].

Finasteride inhibits 5α-reductase type II and decreases both serum and cutaneous dihydrotestosterone concentrations, thus inhibiting androgen-dependent miniaturization of HF [139]. The DP is probably also the target of finasteride. This drug is not effective in treating androgenetic alopecia in women, but it can be beneficial for women with hirsutism. Finasteride must be nevertheless used very cautiously because of its potential feminizing effects on male fetuses [104] and even in adult male patients where some cases of gynecomastia have been reported [140].

Latanoprost has also been found to reverse alopecia and induce increased growth of the eyelashes [141]. A beneficial role in reversing androgenetic alopecia has not, however, been found, and detailed studies regarding whether latanoprost or another prostaglandin analog could be clinically used are still lacking.

Additional current pharmacological therapies for hair disorders include a range of antiandrogens that block the intracellular androgen receptors [142]. The mechanisms by which all these compounds trigger hair cycle changes are not clear and justify further studies.

In recent years, the molecular characterization and isolation of living bulge stem cells along with the studies that revealed their high proliferative capacity and multipotency opened up new directions for their utilization in cutaneous (hair and skin) regenerative medicine [24, 25, 143, 144]. This new approach suggests the control of hair characteristics from the living interior of the fiber. For this purpose, profound knowledge of genes and signaling pathways involved in hair disorders will permit specific modulation using, for example, RNA interference. miRNAs, which play a critical role in skin morphogenesis, were reported to be involved in the regulation of HFs development and cycling, but neither their expression nor their roles have been characterized yet [145]. This elucidation is crucial to develop new efficient treatments for hair disorders that do not cause hair damage or skin injuries, possibly using RNA interference for highly specific modulation of genes involved in these processes.

As proof of principle, we have achieved effective siRNA delivery and efficient modulation of a ubiquitous, highly expressed KRT1 gene in the epidermis of DBA/2J mice with a topical treatment (Araujo et al., submitted data).

Interestingly, topical application of cationic nanoliposomes loaded with specific siRNA has been tested as an effective approach for control of cutaneous melanomas [146], and siRNAs conjugated to cationized gelatin showed a positive effect on symptoms of alopecia in C3H/HeJ mice [147]. A promising line of research has been developed by Kerner and colleagues that proposes modulating androgen receptor expression with RNAi for hair and skin therapy [148, 149]. Nevertheless, this is still a very incipient area that promises to bring new and highly targeted strategies for skin and hair diseases.

HF as well as sweat glands are ideal targets for drug delivery and may represent an alternative to the intercellular route of skin permeation. HF, in contrast to the stratum corneum, represent an efficient long-term reservoir (up to 10 days) for topically applied substances, as their depletion occurs only through the slow processes of sebum production and hair growth [150–152]. The molecules that

penetrate HF can also access the tissues surrounding the follicle and reach the blood circulation through the dense network of blood capillaries, thereby avoiding the stratum corneum barrier. For example, it was shown that when caffeine was included in a shampoo formulation, it was detectable in blood just 5 min after application [153]. Therefore, HF may serve not only as a major entry point, but also as a reservoir for dermally applied substances. In addition, HF also contained multiple target structures for innovative therapeutic approaches. These include specific cell populations in and around the HF, such as immune cells, stem cells and melanocytes, sebaceous glands and perifollicular blood vessels [154–156].

The sebaceous glands represent an important therapeutic site for follicular targeting since they are implicated in the aetiology of acne and androgenetic alopecia [157], as well as in other sebaceous gland dysfunctions [158]. Hueber et al. [157] suggested that the sebaceous glands specifically promoted the penetration of hydrocortisone and testosterone into the skin. Evidence suggests that topically applied compounds entrapped in liposomes accumulated not only in the HF, but also in the sebaceous glands [159]. While the presence of lipophilic sebum may favor follicular uptake of lipophilic molecules, sebum production may, however, moderate drug transport, especially for hydrophilic drugs, functioning as a physical and a chemical barrier for drug penetration [160].

More efficient drug delivery vehicles are therefore being sought. Among the newly emerging concepts, drug delivery systems based on nano- and microparticles, which efficiently penetrate via the follicular route, are highly promising approaches. Nanospheres of different chemical natures are being tested for their capacity to facilitate the transport of substances to deeper layers of the skin, with obvious potential in topical delivery applications.

Another attractive targeting area within the HF is the bulge region. This region, including Mx cells, controls hair growth and pigmentation [161] and is responsible for follicle reconstitution due to the presence of stem cells with a high proliferative capacity and multipotency. The bulge region, in the ORS, has been described as the reservoir for keratinocyte stem cells in both humans and rodents [24, 26, 143, 162–164]. These cells are the target for gene delivery to facilitate long-term gene correction of congenital hair disorders or genetic skin disorders. Targeting of HF stem cells offers unique therapeutic options for genetic hair and skin therapy and regenerative medicine.

3.2 Modifications of Hair Fiber Surface and Structure

Hair's chemical and biological properties make it an ideal material for undergoing an infinite number of changes and treatments, from cosmetic to clinical applications.

Healthy and beautiful hair is desired, and the need for products that improve the look and feel of the hair surface has created a huge industry for hair care. Products such as shampoos and conditioners, along with damaging processes such as

chemical dyeing and permanent wave or relaxing treatments, alter many hair properties, which results in damage to the hair fibre [165–167]. In recent years, many innovations have taken place, and new approaches for hair treatments have been reported in the literature to overcome this problem.

Unaltered human hair (virgin hair) has an isoelectric point near 3.67 [168]. Hence, during a normal hair washing procedure performed at neutral pH (pH > p*I*), the surface of hair acquires negative charges. For this reason, most conditioning shampoos possess cationic polymers in order to counteract these negative charges, thereby improving hair texture and feel. Electrostatic interactions are believed to play a crucial role in the adsorption mechanism of such compounds [168]. However, these products are a small part of the polymer-based cosmetic products. Polymer-containing compositions represent the second-most common ingredient in cosmetic and personal care products. A diverse range of polymers is applied in this segment as film formers, fixatives, rheology modifiers, associative thickners, emulsifiers, stimuli-responsive agents, foam stabilizers and destabilizers, beneficial skin feel agents and antimicrobials [169].

Studies on protein-based formulations to treat hair fibres have also been widely explored. Several patents disclose compositions capable of restoring hair health by providing excellent finishing effects. Applications of proteins such as a water-soluble compound derived from a vegetable protein derivate [170], non-naturally occurring keratin proteins [171], a mixture of a hydrolyzed protein and an amino acid with an aliphatic side chain [172], and other hydrolyzed proteins [173, 174] are also examples within this category.

Several studies have been published regarding how to improve the structure of the keratin hair shaft mainly for cosmetic purposes. These studies take into account the keratinous structure of hair fibres and explore the potential benefit effects of amino acids and peptides in hair care applications [175–177]. Silva et al. [178] described the importance of peptide structure in hair penetration using conventional fluorescent microscopy. We have been investigating the protein disulfide isomerase (PDI) for the functionalization of keratinaceous surfaces with cysteine-containing compounds (CCC) (Fernandes et al., submitted data). PDI is a multifunctional enzyme that catalyses formation and isomerization of disulfide bonds (disulfide shuffling) in a wide range of substrates. In vivo, PDI promotes the correct formation of disulfide bonds in proteins (oxidative folding), leading them to the native state. The CCCs were shown to penetrate inside the hair shaft and attach to the cortex without damaging hair. This approach can represent a promising strategy for the development of new hair care formulations with the ability to dye and restore the integrity of damaged hair (Fernandes et al., submitted data).

PDI has also been used for treatment of wool [179] and hair fibres [180]. King and Brockway [179] showed that PDI was able to restore part of the original properties on aged or harshly treated wool. The same enzyme was used by Brockway [180] to perform a curling, waving or straightening treatment safely under mild condition.

A method to gently and permanently relax or straighten hair was also attained by Presti [181] using a protease, kerA. This enzyme was found to cleave inter-

peptide bonds, allowing the hair fiber to be relaxed or straightened with less damage to the fiber than would have occurred using traditional or existing straightening methods [181].

Humidity is an important factor when considering hair beauty and styling. Air humidity affects hair form and structure at the level of hydrogen bonds. A humidity increase of 30–70% will augment by twofold the water content of hair, thereby increasing its volume by more than 20%. This influx of water eventually causes the hair fibres to swell, which results in friction between fibres and an additional increase in volume and frizz, changing hair appearance [182]. To overcome this issue, scientists from Massachusetts Institute of Technology (MIT) developed a technology that reduces hair frizz using a polyfluoroester, a molecule smaller than the traditional ones used for frizz control. Because of its chemical nature, the formulation adheres tightly to the hair, promoting long-lasting resistance to moisture. Because of its low surface energy, this technology repels most other materials like water and oils. As an additional benefit, the low refractive index of the coating produces a unique, long-lasting shine and pop in the colour of the hair [183].

Today, people in ever-greater numbers alter their hair colour and appearance. However, the aggressiveness of the available techniques poses a big drawback to hair colouring. Commonly used hair dyeing compositions are driven by a mechanism of diffusion of small molecules into the hair fiber [184]. There are three different types of hair colouring agents: permanent, semi-permanent and temporary colourants. The permanent hair dyes are constituted of small dye precursors, able to penetrate into hair, which develop the colour within the hair shaft in the presence of high alkalinity and oxidative conditions. These dyes provide the best colouring results, but cause significant hair damage. The semi-permanent and temporary dyes are molecules too large to diffuse into the hair, therefore acting on the exterior of the fiber at the cuticle. This process does not harm the fiber because of the absence of alkaline oxidative conditions, but fails in terms of colour durability [166]. For this reason, the development of a colouring agent that provides the durability of the permanent hair dyes without the use of oxidizing agents that damage hair is highly desirable.

It is generally accepted that penetration of chemicals into hair occurs through intercellular diffusion, i.e., by adsorption onto the keratin substrate. Faucher and Goddard [185] have shown that the amount of polymer adsorbed on the hair surface increased with decreasing molecular weight. Similarly, low molecular weight compounds might also penetrate the hair shaft, at the cortex level, since the diffusion process is greatly facilitated when hair is exposed to water. Low molecular weight compounds are, however, only retained while the hair is dry because further contact with water opens the cuticle scales, facilitating their escaping. Recent solutions to this problem rely on the use of hair-binding peptides coupled to dyes or pigments that are able to penetrate into the hair shaft, although they lack the required durability for long lasting colour effects. Huang et al. [186] have tested a hair-binding peptide coupled to carbon black and the use of chemically functionalized carbon nanotubes that provided an enhanced interaction with

the hair, resulting in a more durable hair colouring effect. Nevertheless, more durable hair colourants are still needed.

For this reason, there have been attempts to enhance the binding of the cosmetic agent to hair. Richardson et al. [187] describe the covalent attachment of cosmetic agents to hair using transglutaminases. These enzymes promote the crosslinking of the cosmetic agent's amine to the glutamine residues in hair. Similarly, Green et al. [188] describe the use of the enzyme lysine oxidase to covalently attach cosmetic agents to hair.

Despite all the research for cosmetic applications, conventional products are still being used, with small improvements. Formulation requirements imply very strict criteria for dye/product selection. The remaining problem is achieving perfect compatibility in all respects between the dyes/products and the various other constituents of the products.

4 Final Remarks

Hirsutism, graying, alopecia and other disorders of human HFs have dramatic effects on the appearance, socio-cultural status and self-esteem of the affected individuals. The most current treatments available are very aggressive to hair and potentially to human health. In order to solve these problems, new and more efficient approaches are required.

Much progress has been made in understanding the genetic basis and molecular pathways activated during HF embryogenesis, cycling, disorders and the ageing process. HFs are a rich source of stem cells. The recent advances in the isolation, gene expression profile and propagation of stem cells could lead to the modulation of these cells with therapeutic purposes in the treatment of alopecia, wounds, burns, carcinogenesis and even ageing.

The potential specific targeting of different HF-associated cells, namely DP and immune cells, by bioactive compounds, along with effective follicular delivery systems will allow the development of home-based procedures for the maintenance and treatment of hair and scalp disorders.

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Recombinamers: Combining Molecular Complexity with Diverse Bioactivities for Advanced Biomedical and Biotechnological Applications

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Abstract The rapid development of polymer science has led to literally thousands of different monomers and an almost endless number of possibilities arising from their combination. The most promising strategy to date has been to consider natural products as macromolecules that provide the best option for obtaining functional materials. Proteins, with their high levels of complexity and functionality, are one of the best examples of this approach. In addition, the development of genetic engineering has permitted the design and highly controlled synthesis of proteinaceous materials with complex and advanced functionalities. Elastin-like recombinamers (ELRs) are presented herein as an example of an extraordinary convergence of different properties that is not found in any other synthetic polymer system. These materials are highly biocompatible, stimuli-responsive, show unusual self-assembly properties, and can incorporate bioactive domains and other functionalities along the polypeptide chain. These attributes are an important factor in the development of biomedical and biotechnological applications such as tissue engineering, drug delivery, purification of recombinant proteins, biosensors or stimuli-responsive surfaces.

Keywords Biomaterials · Elastin-like recombinamers · Macromolecules · Proteins

Contents

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1 Introduction

Polymer science has developed rapidly and has had a profound impact on society in the last few decades. The availability of thousands of different monomers and the almost limitless possibilities arising from their different combinations have allowed polymer scientists to produce numerous novel materials with desired applications, ranging from very simple materials for use as bulk commodities to highly sophisticated ones with specific biomedical, engineering or nano(bio)technological uses.

More recently, and somewhat paradoxically, the search for improved functionality to meet current needs has meant that long-since-abandoned natural products are being reconsidered as an alternative to more modern and successful synthetic materials. To be more exact, rather than reconsidering the natural products by themselves, this novel strategy involves the introduction of concepts borrowed from nature into future synthetic materials and systems. Indeed, relatively novel concepts in materials science, such as hierarchical organization, mesoscale self-assembly or stimuli-responsiveness, are common to many natural macromolecules such as proteins, nucleic acids or polysaccharides (or combinations of them). Indeed, the slow but relentless process of natural selection has produced materials that show a level of functionality significantly more exquisite than that reached by synthetic materials, with proteins being perhaps one of the best examples of this.

Natural proteins are usually large and very complex linear polymers that contain diverse specific functional groups that generate and promote self-assembly and function. Nature has designed proteins with specific practical functionality, such as an ability to self-assemble, specific recognition, energy interconversion or monodispersity, and enhances these attributes in protein-based biomaterials. Our basic understanding of amino acid-based peptides and proteins forms the foundation for the current development of peptide-based biomaterials. As such, peptides and high molecular weight polypeptides are emerging as a new class of biomaterials due to their unique chemical, physical and biological properties.

Prototypical examples of engineering peptide-based biomaterials include polyamino acids, elastin-like polypeptides, silk-like proteins, coiled-coil domains,

tropoelastin-like peptides, leucine zipper-based peptides, peptide-based amphiphiles, β-sheet forming ionic oligopeptides and β-hairpin peptides. This explosion of new peptide-based materials is founded on parallel developments in both our understanding of protein function and the maturation of recombinant DNA technologies.

The use of recombinant DNA technologies to obtain recombinant protein-based polymers, more commonly known as "recombinamers," has been proposed recently [1]. These polymers, with their total lack of randomness in the macromolecular sequence, permit the required functionalities to be designed in the final material in a highly precise manner. Indeed, this high precision, which is emerging as the new "holy grail" in the field of synthetic polymers, is inherent to such recombinamers. The success of engineering recombinamers in materials applications will, however, depend on our ability to effectively design specific physical and chemical properties. Thus, although the design of highly functional polymers inevitably means the design of complex macromolecules and the close control of their synthesis, both of which are inherent to recombinamers, the real challenge lies in achieving a conventional, chemical polymer synthesis as the increased complexity of the final molecule unavoidably results in an almost exponential increase in the time and cost of the synthesis and therefore to a greater or lesser degree of success.

This review is devoted to a series of highly functional, and hence highly complex, molecules, namely the recombinamers. The genetic engineering of recombinamers enables the easy and highly effective design and synthesis of complex and highly functional molecules with selected and sophisticated properties, such as the incorporation of selected exogenous bioactivities in order to develop materials for cutting-edge applications in biomedicine and nano(bio)technology [2]. Furthermore, genetic engineering can recreate simple or complex properties that are present in natural proteins in these recombinamers. Additionally, as it is possible to construct any coding gene required, base by base, on the basis of original designs without being restricted to the gene fragments present in living organisms, materials and systems exhibiting functions of particular technological interest that are not displayed in living organisms can also be designed and produced [3]. As an example of these approaches, we show here how elastin-like recombinamers (ELRs), which combine sequential and architectural molecular complexity with diverse bioactivities, play an important role in the synthesis of advanced materials, with particular emphasis on advanced biomedical and nano(bio)technological applications.

2 The Example of Elastin-Like Recombinamers (ELRs)

"Elastin-like recombinamer" is a new and more suitable definition that has replaced the former term "elastin-like polymer" (ELP) [1]. It is an explicit term that can be specifically associated with this new type of macromolecule and that is

sufficiently informative to clearly describe the main characteristics of this emerging class of materials. Thus, the expression "recombinamer" clearly indicates the oligomeric nature of these compounds and their production as recombinant proteins. In addition, recombinamer closely resembles the term polymer, thereby suggesting the macromolecular nature of these materials but without requiring that they involve a continuous repetition of small and simple monomers. Recombinamer also prevents the reader from automatically identifying these molecules with natural proteins, or some modification thereof, but rather suggests a molecule whose composition is defined strictly by engineering design. This term will therefore be used throughout this manuscript.

2.1 Properties of ELRs

ELRs are a promising model of biocompatible protein-based polymers. The basic structure of ELRs involves a repeating sequence based on the recurring sequences found in the mammalian elastic protein elastin [4]. As far as their properties are concerned, some of their main characteristics are derived from those of the natural protein. Elastin is an extracellular matrix protein that is present in all vertebrate connective tissue. Its functions include the provision of elasticity and resilience to tissues, such as large elastic blood vessels (aorta), elastic ligaments, lung and skin, which are subjected to repetitive and reversible deformation [5, 6].

The importance of ELRs resides in the fact that these polymers show a versatile and broad range of interesting properties above and beyond their simple mechanical performance that are not easily found together in other materials, including stimuli-responsive behavior or the ability to self-assemble. These properties arise due to a molecular transition of the polymer chain in the presence of water at temperatures above a certain level. This transition, known as the "Inverse Temperature Transition" (ITT) [7, 8], has become the key issue in the development of peptide-based polymers for use as molecular machines and materials.

ELRs show an additional property—an incredibly high biocompatibility—which is highly relevant for the use of these polymers in advanced biomedical applications such as tissue engineering and controlled drug delivery. Indeed, the complete series of ASTM generic biological tests for materials and devices in contact with tissues and tissue fluids and blood demonstrate their unmatched biocompatibility [9]. Perhaps somewhat surprisingly, it appears that the immune system simply ignores these polymers as it cannot distinguish them from natural elastin. In a more detailed interpretation of the basis for the extraordinary biocompatibility of these materials, Urry and co-workers have suggested that the β-spiral, the common structural feature of all ELRs, strongly contributes to preventing the identification of these foreign proteins by the immune system [10]. In addition, their degradation products—natural amino acids—are non-toxic [11–14].

The polymer poly(VPGVG) is considered to be a model for all ELRs [8]. Indeed, most ELRs are based on the pentapeptide VPGVG (or its permutations), with amino-acid side-chains, other than glycine, comprising simple aliphatic and mainly hydrophobic chains, without further functionalization. A wide variety of ELRs based on the ELP model, with the general formula VPGXG, where X is any natural or modified amino acid other than L-proline, has been (bio)synthesized.

Initially, the monomers, oligomers and higher polymers of this repeat, and modifications thereof, were synthesized chemically. More recently, however, with the increased development of molecular biology, these and more complex ELRs have been produced using genetic-engineering techniques [15, 16].

2.2 ELR Production Using Engineering Techniques

The use of genetic-engineering techniques to produce these polymers as recombinant proteins has several advantages over their synthetic counterparts. For example, as they are composed of amino acids, the genetically encoded design of these recombinant polypeptides permits precise control over ELR sequence and chain length [14]. This technique also permits new polymers with unique physical, mechanical and/or biological features, such as variations of folding, chain interactions within the synthetic protein structure, temperature- or pH-responsiveness, etc., to be obtained [17].

The design of an appropriate strategy at the nucleotide level is critical for the efficient synthesis of the protein encoding sequence and to produce a uniform protein product with an optimal quality and yield. The biosynthesis of any artificial protein generally includes: (1) the construction of a synthetic gene that encodes the protein of interest in a plasmid with close transcription control; (2) the cloning of a recombinant gene with the necessary transcriptional regulatory elements into competent cells; (3) the screening of plasmids containing the desired clones and verification of their DNA sequence; (4) transformation of the chosen plasmids into expression-competent host cells; (5) the growth of appropriate volumes of host cells and induction of protein expression; (6) purification of the protein of interest from cell lysates [18].

Molecular biology techniques are typically employed to self-ligate monomer DNA fragments in an oligomerization process that relies on restriction-enzyme-based approaches when designing genes that encode repetitive recombinamers. In this case, the monomeric fragments must be oligomerized in a “head-to-tail” orientation and can either be seamless in sequence or can contain intervening linkers between the desired repeats. Approaches to oligomerization can be classified as: (1) iterative, where a DNA segment is oligomerized in a series of single, uniform steps, each of which extends the oligomer by one unit length of the monomer gene; (2) random method or “concatemerization,” where an uncontrolled number of monomer DNA segments are oligomerized in a single step to create a population of oligomerized clones with different lengths. This method

creates a library of genes of different lengths that encode oligomeric polypeptides with the same repeat sequences but sacrifices precise control of the oligomerization process; and finally, (3) recursive directional ligation (RDL), an alternative method in which DNA segments with two different restriction sites flanking the insert are joined in sequential steps, with the length of the ligated segments growing geometrically in each step. This approach is suitable for the synthesis of repetitive polypeptides with a specific and predetermined chain length as it seamlessly joins the two monomeric inserts and also eliminates the restriction sites placed at either end of the dimerized gene (Fig. 1) [7, 19].

2.3 The Stimuli-Responsive Behavior and Self-Assembly Properties of ELRs

The responsive behavior of ELRs has been defined as their ability to respond to external stimuli. This property is based on a molecular transition of the polymer chain in the presence of water at a temperature above a certain level, known as the "Inverse Temperature Transition" (ITT). This transition, which shares most of the properties of the lower critical solution temperature (LCST), although it also differs in some respects, particularly as regards the ordered state of the folded state, is clearly relevant for the application of new peptide-based polymers as molecular devices and biomaterials. Below a specific transition temperature (T_t), the free polymer chains remain as disordered, random coils [20] that are fully hydrated in aqueous solution, mainly by hydrophobic hydration. This hydration is characterized by ordered, clathrate-like water structures somewhat similar to those described for crystalline gas hydrates [21, 22], although somewhat more heterogeneous and of varying perfection and stability [23], surrounding the apolar

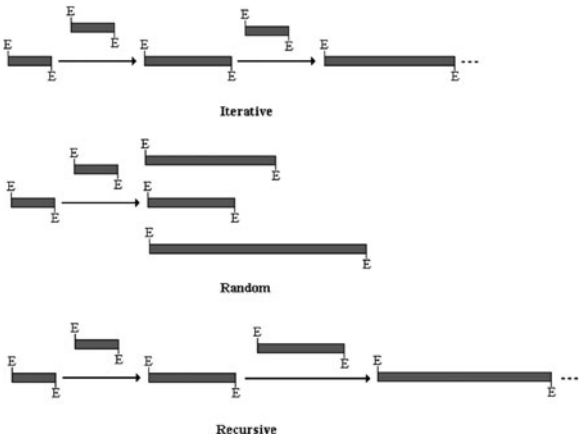

Fig. 1 Schematic showing three approaches to DNA oligomerization: iterative, random and recursive. Reproduced with permission [19]

moieties of the polymer. However, above T_t, the chains fold hydrophobically and assemble to form a phase-separated state containing 63% water and 37% polymer by weight [24]. In this folded state, the polymer chains adopt a dynamic, regular, non-random structure, known as a β-spiral, which contains type II β-turns as the main secondary feature and is stabilized by intra-spiral, inter-turn and inter-spiral hydrophobic contacts [20]. The ITT process is completely reversible, and the polymer returns to its initial state when the temperature drops below T_t [25]. During the initial stages of polymer dehydration, hydrophobic association of the β-spirals results in their taking on a fibrillar form. According to Urry's model, this process begins with the formation of filaments composed of three-stranded, dynamic polypeptide β-spirals that grow to lengths of several hundred nanometers before settling into a visible phase-separated state (Fig. 2) [20, 24].

The specific T_t can be measured by different techniques, such as turbidity analysis or calorimetric methods (Fig. 2). The first method is characterized by a turbidity profile showing a sharp step, with T_t taken to be the temperature that shows a 50% change in the relative turbidity shape. In contrast, differential scanning calorimetry (DSC) measurements are always characterized by a broad peak covering 20 °C or more. In this case, T_t can be considered as either the onset or the peak temperature. The T_t values obtained by these methods usually differ because of the influence of several factors [26].

The magnitude of T_t also depends on several factors. For example, any intrinsic modification to the composition of the polymer chain as a result of changing the sequence or distribution of the different domains, or extrinsic factors such as salt additions, that modifies the tendency of water molecules to take part in hydrophilic vs. hydrophobic hydration will alter the clathrate structure and modify the T_t. This effect is a result of both the mean polarity of the polymer chain and the molecular architecture and distribution of the different amino acid domains in the ELRs [27]. T_t can also be affected by the polymer concentration and by other factors, such as changes in pH or the oxidation state of a side chain or functional group, the

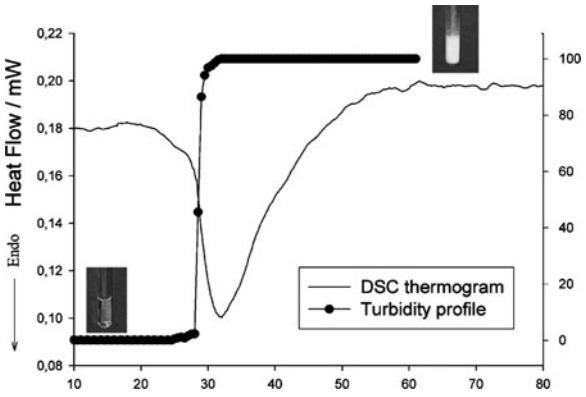

Fig. 2 Turbidity profile as a function of temperature for poly(VPGVG) (5 mg/l) in water. The two photographs were taken below (5 °C) and above the T_t (40 °C). A DSC thermogram of a 50 mg/l aqueous solution of the same polymer is also shown (heating rate: 5 °C/min). Reproduced with permission [8]

addition of extrinsic factors such as salts and organic solutes, the presence of other ions and molecules or pressure changes [28, 29].

As far as self-assembly is concerned, many synthetic strategies have been developed to obtain advanced devices in an attempt to mimic the efficiency of biology in nature. These techniques are usually based on the concepts of self-organization and self-assembly, both of which are widely found in nature, to arrange hierarchically ordered systems in such a way that they can be exploited in synthetic devices. Proteins are useful building blocks for the formation of ordered nanostructures via self-assembly due to their well-understood folding, stability, and protein-protein interactions [30]. Natural elastin undergoes a self-aggregation process in its natural environment that leads to the formation of nanofibers [10]. However, genetic engineering and the tailored molecular design of ELRs allow this possibility to be extended to other topologies and nanostructures. For example, Reguera et al. [31] have shown that a pH-responsive ELR is able to form polymer sheets containing self-assembled nanopores. Other similar examples involving the directed self-assembly of ELRs can be found in the literature [32, 33].

3 Biomedical and Biotechnological Applications

3.1 Tissue Engineering

The general aim of tissue engineering is to design temporary functional scaffolds that mimic essential elements of the structure and functional state of a tissue. Tissues are composed of two major components, namely the cells and the extracellular matrix (ECM) that the cells construct. Each tissue has a particular set of physical and chemical functions that allow it to fulfill its role of sustaining the organ or organism of which it forms a part [13]. The design of functional biomaterials that mimic cellular behavior is a major challenge in the fields of tissue engineering and materials science, with efforts to develop such materials principally involving the design of scaffolds and hydrogels to mimic dynamic interactions between the cells and the extracellular matrix in vivo [34, 35]. In this respect, the incorporation of extracellular adhesion ligands and growth factors into engineered materials has proven effective in directing cellular responses in many applications [36–38].

The proteins found in the ECM (fibronectin, collagen, elastin, etc.) contain a huge number of bioactive peptides in their sequences that are of crucial importance for controlling cellular behavior [39]. In addition to adhesion proteins, the ECM also sequesters and presents a number of morphoregulatory molecules, including growth factors, which control cell division, differentiation, and multicellular morphogenesis processes.

These characteristics of the ECM govern the design of biomaterials for use in tissue engineering and regenerative medicine, with the overall goal of mimicking

key features such as the presentation of adhesion molecules and the sequestration, display and release of growth factors [40]. However, cells should also be able to undergo the normal enzymatic processes that result in the remodeling and eventual replacement of these matrices, principally through matrix metalloproteinases (MMPs), as occurs in the natural ECM. These enzymes, which are only expressed and secreted into the ECM when it needs to be remodeled, act on specific sequences that are only present in ECM proteins, thus meaning that they cannot damage other proteins in their vicinity. It is also known that some fragments of these hydrolyzed ECM proteins, once released, show strong bioactivity, including the promotion of cell differentiation, spreading and regeneration. Finally, growing tissue is delicately controlled by a well-balanced symphony of growth factors and other bioactive substances secreted by the cells.

Biological materials such as ELRs are useful for tissue engineering because they are soluble in aqueous solutions, have good in vivo biocompatibility, a controllable degradation rate in vivo, and break down into natural amino acids that can be metabolized by the body to produce non-toxic degradation products [14]. Besides this proven compatibility and their ability to be easily genetically functionalized to enhance their interactions with cells and provide an optimal platform for cellular activities and tissue functions, they are excellent candidates for use in regenerative and reparative medicine due to their smart and self-assembling characteristics [41]. They are also highly versatile since these properties can be tuned and extended in many different ways simply by changing their amino acid sequence, thereby opening up new possibilities for the formation of biomimetic advanced bio- and nanomaterials that can actively participate in the formation of functional tissue. For instance, biomaterial matrices that mimic the key characteristics of the extracellular matrix, including the presentation of adhesion sites and growth factors in the context of a viscoelastic hydrogel, are currently being developed [40].

The first candidates for tissue-engineering applications were simple polymers of the type poly(VPGVG) (V = L-valine, P = L-proline, G = glycine) and their crosslinked matrices. In principle, these polymers do not appear to be appropriate for tissue-engineering purposes due to their anti-fouling properties [9], which limit cell adhesion. This absolute lack of cell adherence is not, however, a drawback; rather, it is highly desirable since it results in a starting material with the appropriate mechanical properties and biocompatibility that lacks unspecific bioactivities that would promote unspecific cell adhesion. These simple molecules were subsequently enriched with short peptides having specific bioactivities. Due to the polypeptide nature of ELRs, these sequences were easily inserted into the polymer sequence even though, at that time, chemical synthesis was still the only option for obtaining these polymers.

The first active peptides inserted into the polymer chain were the well-known, general-purpose cell-adhesion peptides RGD (R = L-arginine, D = L-aspartic acid) and REDV (E = L-glutamic acid), which are specific for endothelial cells. The incorporation of these active peptides as cell-adhesion ligands resulted in a high capacity to promote cell attachment that was not shown by the simple

poly(VPGVG). Both bioactive peptides showed similar cell-attachment behaviors to that of human fibronectin [42]. Once genetic engineering became the production method of choice, the molecular design started to increase in complexity. This complex and complementary spatial distribution of functions boosted efficiency and selectivity and meant that the cell-attachment domains increased in size as more amino acids were placed around the central active REDV or RGD domains as a way of obtaining a more active cell-binding size. For instance, Panicht et al. have shown that by using the longer CS5 region of human fibronectin, which is an eicosapeptide with an REDV sequence in its central part, the cell adhesion achieved was more effective than with short REDV inserts [43].

The complexity of these still relatively simple ELRs was subsequently increased by adding different functionalities such as crosslinking domains [44–48]. Numerous examples based on more complex designs, including various bioactivities [49, 50] and other functionalities [51–54], have since been synthesized in an effort to mimic the complex composition and function of the natural ECM. Thus, the addition of different functionalities such as crosslinking domains results in more uniform substrates, which are usually based on lysine residues, incorporated in the elastin-based repeat unit (VPGXG) [46, 48, 55].

ELRs are able to form stable viscoelastic hydrogels that mimic the characteristics of the ECM. These ELR hydrogels can be produced by photoinitiation [56], irradiation [57, 58], amine reactivity [59] or enzymatic crosslinking by tissue transglutaminase [49] to maintain their interesting properties as stimuli-responsive substrates. Furthermore, they are a new class of soft materials, which, in response to a small change in temperature, light or other environmental stimulus, swell to several times their original volume or shrink to the same degree [60, 61]. These materials have proved extremely useful in biomedical and pharmaceutical applications because of their high water content and rubbery nature, which are similar to that of natural tissue.

The physical properties of chemically crosslinked hydrogels can be modulated by varying the ELR's concentration, molecular mass and lysine content. This ability to prepare "tunable" hydrogels allows these ELRs to be used in a wide range of applications. Although there are many examples of ELRs hydrogels crosslinked in organic solvents, the application of in situ crosslinking in aqueous solution is limited by factors such as the toxicity of the reagents and by products and slow gelation kinetics. Lim et al. [62] have reported that the chemical gelation of ELRs under physiological conditions results in a biocompatible and injectable biomaterial for support tissue regeneration. Likewise, Tirrell's group has developed this idea further and has demonstrated that chemically crosslinked hydrogels from ELRs containing fibronectin cell-binding domains can successfully be used to support the growth and spreading of endothelial cells [43–45, 47]. The tunable, elastin-like mechanical properties of ELR hydrogels and their ability to mediate cellular adhesion and proliferation make them suitable candidates for use in small-diameter vascular grafts [41].

Highly porous hydrogels formed from recombinant elastin-like-recombinamers chemically crosslinked with hexamethylene diisocyanate have been obtained by a

salt leaching/gas foaming technique for use in 3D cell culture [59]. The pore size, physical properties (porosity and swelling ratio) and mechanical properties of these gels are influenced by the salt/polymer weight ratio during the crosslinking reaction. Their thermal behavior [43–45, 47] was also studied in terms of the physical properties, and it was found that the collapse due to the phase transition above T_t decreased the mean pore size by about 30% (Fig. 3). This technique should provide a simple approach to the fabrication of advanced scaffolds with tunable biological (Fig. 4) and physical applications.

Our group has bioproduced the ELR polymer $[(VPGIG)_2\text{-}VPGKG\text{-}(VPGIG)_2\text{-}(EEIQIGHIPREDVDYHLPY)\text{-}(VPGIG)_2\text{-}VPGKG\text{-}(VPGIG)_2\text{-}(VGVAPG)_3]_n$ ($n = 10$; MW = 80,925 Da) (Fig. 5) [63], whose sequence is a good example of the high complexity that can be attained for ELRs with a structure designed to perform different functionalities. The monomer unit contains four different functional domains in order to achieve an adequate balance between mechanical and bioactive responses. Thus, the $(VPGIG)_n$ sequence confers excellent mechanical properties, extreme biocompatibility and a stimuli-responsive nature, whereas the second building block (VPGKG), which is a modification of the first with a lysine instead of isoleucine, means that the lysine-amino groups can be used for crosslinking and other chemical modifications while retaining the properties of the ELRs. The third group contains the (REDV) peptide sequence found within the alternatively spliced CS5 fibronectin domain, which is specifically recognized by the integrin $\alpha_1\beta_4$ [64]. This integrin is present in a few cell lines, and its specificity for REDV tetrapeptide has been confirmed in endothelial cells, which selectively bind to REDV-coated surfaces [65]. Finally, the polymer possesses another functional block, in this case a recurring hexapeptide derived from the human elastin exon 24-encoded product $(VGVAPG)_3$. This sequence was introduced to drive enzymatic hydrolysis of the synthetic scaffold by the same physiological pathways as natural elastin during ECM remodeling as it is a target for certain

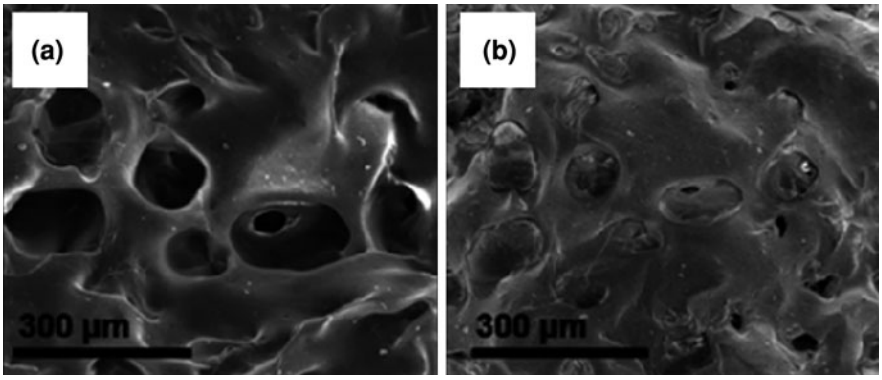

Fig. 3 ESEM micrograph showing the change in pore size with temperature in swollen hydrogels: **a** 4 °C, **b** 37 °C. The mean pore size decreases by about 30% due to the phase transition above T_t. Reproduced with permission [59]

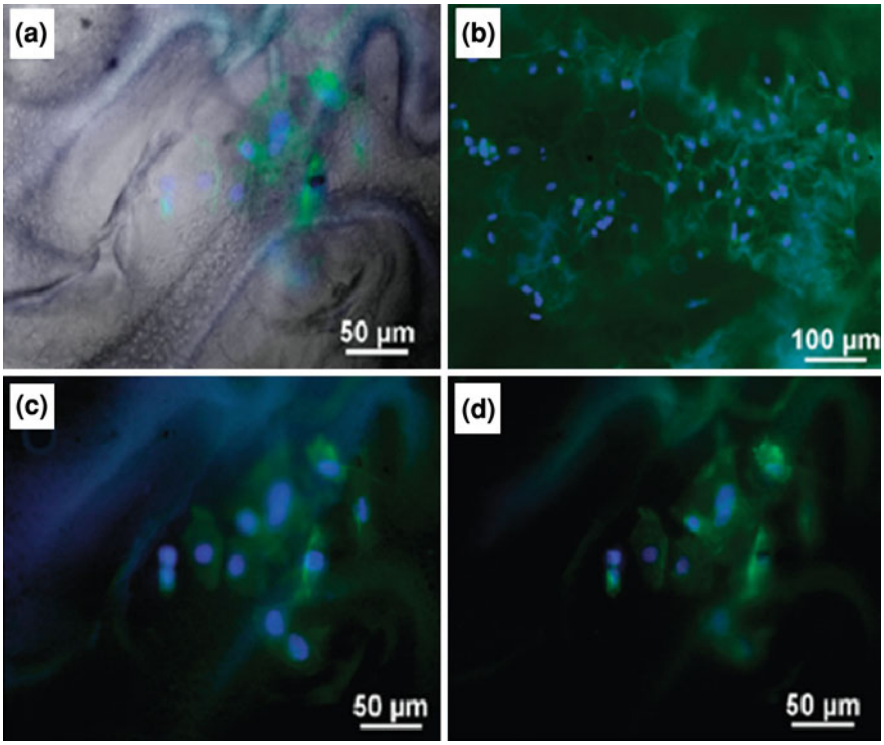

Fig. 4 Microscopy images and SEM micrographs of cells seeded in macroporous ELP hydrogels after incubation for 48 h. **a** Phase-contrast and fluorescence microscopy; **b–d** fluorescence microscopy with Phalloidin Alexa Fluor488 and DAPI staining. Reproduced with permission [59]

Fig. 5 Scheme showing the peptide domains and amino acid sequence of the ELR polymer bioproduced by Girotti et al. Reproduced with permission [59]

proteolytic enzyme elastases [66]. REDV-ELR biopolymer biodegradation was tested with a specific protease, namely human leukocyte elastase I. Thus, when recombinant ELRs were incubated under optimal enzymatic conditions, the elastase was found to quickly and completely digest the REDV biopolymer, whereas no significant degradation of the control biopolymer (lacking the target hexapeptide) was observed even when extending the experimental time (unpublished data). Similar results were obtained upon enzymatic treatment of a chemically crosslinked hydrogel based on the same ELR.

The use of enzymes to create a crosslinked polymer scaffold for tissue engineering is conceptually attractive as it is a bio-inspired method that mimics the natural crosslinking of proteins in vivo to create functional tissue. This ELR-containing REDV cell-adhesion sequence (Fig. 5) has also been used to prepare hybrid scaffolds [49]. Thus, the ELR was introduced as an elastic element in collagen-based scaffolds with a view to providing increased proteolytic sensitivity and increased functionality to the scaffolds by carrying a specific sequence for microbial transglutaminase crosslinking, endothelial cell adhesion and drug delivery. These scaffolds showed that crosslinking ELR-collagen affects the physicochemical properties of the scaffolds such as its porosity, presence of crosslinking, thermal behavior and mechanical strength. These enzymatic crosslinked ELR collagen scaffolds were tested in vitro as substrates to study cell viability with different cell lines. An increasing ELR fraction in the scaffold was found to have an antifouling effect in fibroblasts, whereas endothelial cells displayed normal behavior and proliferation in the hybrid scaffolds. This differential colonization of the scaffolds with a specific cell type makes these scaffolds an attractive platform for biomedical applications, as varying the proportion of both materials should allow us to design the optimal requirements for future applications, such as vascular-tissue or skin-wound healing.

The REDV-containing polymer described above has also been used as a substrate to culture cells from the ocular surface. Epithelial cell adhesion and proliferation were investigated on this ELR, which mimics the functional characteristics of extracellular matrices. Corneal wound healing requires cell adhesion and proliferation, both of which are mediated by binding of epithelial membrane-bound integrins to substrate ligands such as fibronectin. A significant improvement in the adhesion and proliferation of the conjunctival epithelial cell line on the ELR-coated surface containing REDV peptides was observed. This enhancement could be due to the interaction between epithelial integrins and the REDV peptides in the ELR matrix. ELR-coated surfaces containing REDV peptides also supported the normal phenotype and functions of epithelial cells in vitro, thus confirming that this recombinant polymer resembling the ocular surface extracellular matrix is a suitable substrate for sustaining epithelial cell attachment and growth. This type of polymer may be suitable for tissue engineering to restore vision by reconstructing the ocular surface. One of the potential applications we envision for this kind of protein-based polymer is the preparation of scaffolds to be used for ocular surface tissue engineering [50].

Other examples of ELRs demonstrate the complexity attainable for this class of biomaterials. Thus, for injuries to the central nervous system (CNS), it is important to design a family of biomaterials with independently tunable mechanical properties, degradation rates and cell attachment. An ELR that supports neural regeneration through a combination of cell-adhesion and cell-induced degradation and remodeling has been designed for this purpose. This engineered protein includes cell-adhesion sequences to enable neural attachment as well as sequences sensitive to cleavage by urokinase plasminogen activator (uPA), a protease secreted locally at the tips of growing neurites, to enable highly localized and

tunable degradation. These ELRs were chemically crosslinked into highly swollen hydrogels with controllable mechanical properties. In addition, it was found that increasing the density of RGD peptides present in the protein substrates led to increased cell adhesion and more extensive neurite growth. These engineered proteins offer the ability to independently tailor the mechanics, degradation properties and adhesivity of the scaffold for the regeneration of CNS tissue [67].

It is also possible to make use of the thermosensitive behavior of ELRs to form hydrogels. Thus, raising the temperature of a concentrated solution of an ELR above its T_t results in aggregation of the ELR's chains and the formation of an insoluble coacervate phase. Reverse thermosensitive polymers are very promising base materials for "in situ generated implants" due to their ability to form low viscosity physiological solutions at room temperature which gel at higher temperature. This property opens up numerous possibilities, although the two principal areas of research involve hydrophobic materials, which acquire desired mechanical properties, or water-based systems for the controlled release of hydrophilic macromolecules. The structural complexity of ELRs with specific mechanical, chemical and biological properties allows us to design specific features that make it possible to acquire some or all of these properties. The self-assembly behavior of ELRs, for example, has been triggered by the addition of different main peptide blocks in the structure, with the hydrophilic block providing conformationally elastic properties [68] and the hydrophobic blocks forming physical crosslinks through hydrophobic aggregation [1].

In a recent example, ELR tri-block copolymers with different hydrophobic architectures were found to form gels with a complex shear modulus ranging from 4.5 to 10.5 kPa, which can be varied by changing the hydrophobicity of the inner block [69]. Although the modification of ELRs allows some control of their mechanical properties, the physically crosslinked ELR hydrogels lack the strength required for some tissue-engineering applications. When greater mechanical integrity is desired, the elastic modulus can be enhanced by including additional chemical crosslinking sites between the ELR chains [68, 70], thus making them excellent candidates for biomedical applications. Sallach et al. have also developed a recombinant elastin-mimetic triblock copolymer in the absence of either chemical or ionic crosslinking that shows minimal inflammatory response and robust in vivo stability for periods exceeding one year. This example demonstrates the high and extraordinary biocompatibility of ELRs. These triblock copolymers could also be used as structural components of artificial organs and engineered living tissues, as carriers for controlled drug release or as biocompatible surface coatings [71].

The combination of ELRs with chitosan has also led to the development of hydrogels with applications in bone-tissue engineering, which requires the use of temporary scaffolds in order to regenerate bone and improve its healing rate. These hydrogels can be injected into the body as a liquid that gels in situ, thus allowing the material and cells to be implanted using a minimally invasive procedure. In this work, the ELR was incorporated into two different hydrogel formulations. Physically crosslinked chitosan/β-glycerophosphate (Cht-β/GP) thermosensitive

hydrogels were prepared initially and their gelation temperature determined to be around 35 °C. Incorporation of the ELR into the (Cht-β/GP) formulation seems to drastically improve the mechanical properties of this gel under physiological conditions. Chemically crosslinked chitosan hydrogels were then prepared with the ELR acting as a coat on the preformed hydrogel due to electrostatic interactions. An ELR containing an osteoconductive sequence was found to improve the bioactive properties of chitosan-based systems, thereby enhancing the possibility of their use as biomaterials for orthopedic and tissue-engineering applications. In addition, the incorporation of an ELR into both hydrogels has been shown to provide these gels with some ability to induce precipitation of calcium phosphate when the materials are soaked in simulated body fluid for 7 days. These biomaterials may therefore find a use in minimally invasive orthopedic reconstructive applications or in bone-tissue engineering [54].

3.2 Biosurface Engineering

3.2.1 Functionalized Surfaces

One of the most active research areas in the field of materials science concerns the control and modification of surfaces and interfaces, also known as surface engineering [63, 72]. This is indeed an important tool in the design and control of molecular mechanisms for protein adsorption and material-cell interactions for different and specific biological and biotechnological applications. Thus, the surface can be functionalized with fouling/anti-fouling properties, specific groups to promote cell material interactions, smart behavior (stimuli responsive or environmentally sensitive) or with micro- or nano-patterns.

Surfaces modified with stimuli-responsive biopolymers change their physical and chemical properties in response to changes in the environment or under external stimuli. This advantage has been exploited in some smart surfaces covered with a chemically synthesized polymer that exhibits a lower critical solution temperature, especially poly(*n*-isopropylacrylamide) and its derivatives. A change in the environment causes such a surface to undergo a significant change in its bioactive properties, for instance, protein adsorption/desorption or cell or bacterial attachment/detachment [73–76]. For instance, Ozturk et al. have recently prepared micropatterned poly(*n*-isopropylacrylamide) films, which were subsequently modified by adsorption of a thermoresponsive and cell-adhesive (RGD-containing) ELR to improve cell attachment and cell-material communication. These authors studied the thermal responsiveness upon application of mechanical stress to cells under in vitro conditions in order to induce bone formation and showed that the ELR is crucial for maintaining the cell attached to the surface during dynamic culturing [77]. The major disadvantage of this system is the non-degradability of pNIPAM, although it should be possible to resolve this problem by replacing

pNIPAM with an ELR containing biodegradable sequences, such as that described by Girotti et al. (Fig. 5) [63].

ELRs have some additional advantages that make them excellent candidates for the development of smart surfaces, especially the fact that they can be obtained by recombinant technologies, thus enabling a close control of their sequence, length and stereochemistry. This allows, among others, a nanometric degree control of the position where functionality is located along the polypeptide chain and leads to a tremendous potential for the self-assembling and other advanced functionalities displayed by these systems.

Chilkoti's group has also created what they refer to as the "thermodynamically reversible addressing of proteins" (TRAP) [72, 78], where an ELR is covalently micropatterned onto a glass surface against an inert background. The ELR-patterned substrate is then incubated with a solution containing the fusion protein thioredoxin-ELR, which is captured from the solution onto the immobilized ELR by hydrophobic interactions under the influence of an external stimulus such as an increase in NaCl concentration. This TRAP technology enables the reversible, spatial-temporal modulation of protein binding at the solid-liquid interface and can be applied in different systems for bioanalytical applications. For example, the chemical and genetic remodeling of proteins with ligand-binding properties can be used to synthesize various protein-based microsensors for detecting single biomolecules. The construction and characterization of fluorophore-labeled glutamine binding proteins and derivatives coupled to the independently designed hydrophobic polypeptide (APGVGV), which can be adhered onto solid surfaces via hydrophobic interactions, have also been described [79]. Along similar lines, a novel protein for controlling cellular functions has been constructed by combining functional units of various proteins. Thus, the RGD sequence, epidermal growth factor (EGF) and a hydrophobic sequence were combined and incorporated into one molecule. This protein was found to have both cell-adhesive and growth-factor activity, while its hydrophobic sequence contributed to assembly of the RGD and retention of its activity on a solid-phase surface. This fusion protein could therefore prove to be of use for wound healing and tissue regeneration [80].

A novel temperature-responsive culture surface, consisting of a cell sheet harvested from a culture dish, that exploits the stimuli-responsive characteristics of ELRs has been developed. This technique involves changing the temperature once the cells have become confluent in order to reverse the coaggregation. The cells are then detached from the culture dish as a single sheet using a polyvinylidene difluoride (PVDF) membrane and subsequently transferred to new surfaces, thereby suggesting the potential of this approach for the fabrication of multilayer cell sheets [12]. Hyun et al. have also taken advantage of the rapid response of a smart material surface created by adsorption of ELRs to external stimuli for use in cell-based biochips. The smart transition of ELR micropatterns between a hydrophilic and hydrophilic surface glass at T_t enabled these authors to control the reversible cell adhesion by way of the incubation temperature [81].

Another technique commonly used in the field of ELRs is the layer-by-layer deposition of alternating ELR-polyelectrolytes, which is a simple technique for

generating bioactive surfaces [82]. These ultra-thin nanoscale coatings promote cell adhesion and proliferation, and the results described to date show that the thickness and mechanical integrity of the multilayer assembly modulates the cell response. Costa el al. [53], for example, have developed thermoresponsive thin coatings by electrostatic self-assembly (ESA). Thus, a smart thin coating consisting of ELRs containing the cell attachment sequence RGD was fabricated by simple deposition of the ELR dissolved in an aqueous-based solution onto chitosan substrates. It was subsequently shown that the thermoresponsive behavior of these coatings can be exploited for tunable cell adhesion and controlled protein adsorption and that the RGD sequence enhanced cell adhesion in comparison with the original chitosan monolayers or glass substrate. This strategy has shown that the proposed systems have remarkable smart and biomimetic properties that could make them suitable for tissue engineering, delivery of bioactive agents and nanoscale surface tailoring.

The chemical functionalization of ELRs has also been used in biomimetic surface-modification strategies to obtain metallic (commercial pure titanium, Cp Ti) dental implants with osteostimulative capabilities. These surface modifications may provide implants with a rapid rate of new bone growth and osseocoalescence, in other words, direct chemical contact with the surrounding tissue. A simple process using silane chemistry has been shown to be specific, rapid and reliable for the covalent immobilization of biomolecules on the Cp Ti surface to obtain mechanically and thermochemically stable ELR-containing coatings. In particular, this ELR contains an RGDS peptide, an acidic peptide sequence derived from statherin, which is present in saliva and has a high affinity for calcium phosphate and therefore plays a leading role in the remineralization of hard tooth tissue. Two different biomimetic strategies that combine topographical modification, inorganic treatments and/or biofunctionalization were successfully developed to improve the bioactive integrative properties of Cp Ti implants [83].

3.2.2 Nano- and Microtopographical Surfaces

In the last few years, peptides and proteins have been deposited with complete spatial control on specific regions of a surface using lithographic and patterning techniques. The ability to obtain nano- and micrometer-sized patterns of biological macromolecules is of great importance for several applications, including biological assays, miniaturized biosensors and biomedical diagnostics.

ELRs have been employed in the design and development of regenerable biosensors and microfluidic bioanalytical devices, as reported by Chilkoti et al. [84]. Thus, nanostructured surfaces that are able to capture and release proteins using the self-assembly properties of ELRs have been obtained by combining ELRs and dip-pen nanolithography.

Recently, the simple method of replica molding has been adapted to obtain 3D microstructured thermoresponsive hydrogels [59]. Replica molding is a fast, flexible and straightforward micropatterning technique that can be carried out

routinely and consists of only a few steps: dispensing of the polymer on the mold, crosslinking and release of the replica ELR hydrogel. In this study, hydrogels with micropatterns such as lines or pillars (Fig. 6) with different dimensions and spacings were obtained by taking advantage of the thermally responsive behavior of these polymers. The dimensions of the microfeatures with micropatterned lines were tested by varying the water temperature, and a 30–35% decrease in dimensions was observed for both patterns at a temperature above the transition temperature of the hydrogels (20 °C). This thermoresponsive behavior does not modify the topography and can be used to change the dimensions of the micropatterned features during cell culture. Furthermore, these systems permit a controlled topography to be added as a further factor when studying cell behavior and cell-surface interactions, thereby improving the extraordinary properties of ELR hydrogels, particularly their bioactivity, biocompatibility and the "tunability" of their mechanical properties [85].

The ability to generate micro/nanoscale fibers from synthetic and natural polymers has been improved by using a simple fabrication technology known as electrospinning [86, 87]. This process has been widely used in the field of organic polymer science and is now being used as a novel tool for fabricating biopolymer scaffolds [88–90]. The electrospinning process involves applying a high voltage to create an electrically charged jet of the polymer solution, which dries to leave a polymer nanofiber mesh. The fibers produced by this process usually have diameters ranging from a few micrometers to less than a hundred nanometers. Their structural properties depend on processing parameters such as polymer concentration and viscosity, flow rate and applied voltage, among others [91]. The ability to vary the fiber size in the nanometer range opens up the possibility of mimicking the size scale of fibrous proteins found in the natural extracellular matrix. Indeed, fibers made from different proteins such as fibrinogen, gelatin,

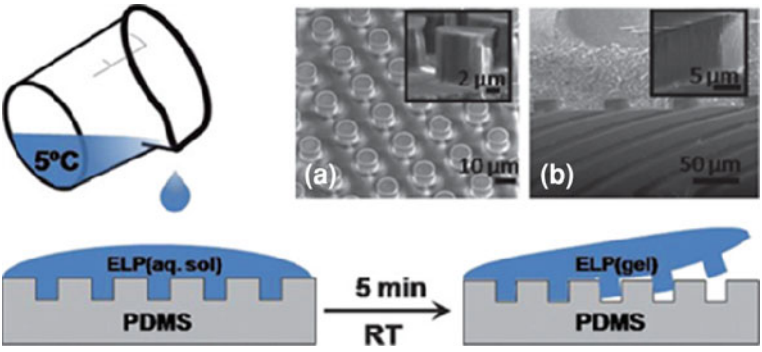

Fig. 6 Steps utilized to carry out replica molding dispensing of the polymer on the mold, crosslinking and release of the replica ELR hydrogel with micropatterns such as lines or pillars. **a**, **b** ESEM micrographs. **a** Cross section of grooves ($h = 15$ mm, $w = 20$ mm, $s = 50$ mm). **b** Magnified surface and cross-section views of pillars ($h = 9$ mm, $w = s = 10$ mm). Reproduced with permission [85]

collagen–elastin mixtures or silk-like proteins have been obtained, with the first elastin-mimetic mixed protein fibers being produced from an ELR. Thus, electrospinning techniques were employed to produce fibers in a form that mimics the diameter of native elastin fibers utilizing an 81-kDa recombinant protein based upon the repeating sequence of elastin $(VPGVG)_4(VPGKG)$. Fibers with diameters varying between 200 and 3,000 nm and three different morphologies (beaded fibers, thin filaments and broad ribbon-like structures) were obtained. The creation of these fibers from carefully chosen protein polymer types and network architectures may lead to the engineering of improved human tissue constructs with enhanced clinical performance [92].

Recently, and continuing with the theme of nanofiber architecture formation, core-shell nanostructures of CdSe nanoparticles with a shell of ELRs have been used as building blocks to fabricate functional one-dimensional (1D) nanostructures. The ELR controls the size and size distribution of the CdSe nanoparticles in an aqueous medium and simultaneously directs the self-assembly of core-shell building blocks into fibril architectures. The ELR also stabilizes the CdSe nanoparticles and controls their nucleation, growth and spatial distribution. The self-assembly of core-shell building blocks into nanofibers was found to be strongly dependent on the pH of the medium. Moreover, the original material is cytotoxic in the absence of ELR, whereas the cytotoxicity results for the ELR-CdSe nanostructures show that this material is a novel and promising class of non-toxic nanofibrous materials that can cross the cell membrane barriers. These types of nanofibers could find future applications in fields such as medical nanotechnology and the diagnosis and treatment of various diseases [93].

3.3 Drug Delivery

Controlled drug delivery can be an important factor when traditional oral or injectable drug formulations cannot be used. A wide range of materials have been employed to control the release of drugs and other active agents, although polymeric systems are the most common because of their desirable physical properties [94].

ELRs display several properties that can be useful for drug delivery purposes, including precise control over size and sequence, smart behavior, self-assembly, biocompatibility, etc. The genetic engineering of ELRs permits the incorporation of targeting peptides, such as cell-penetrating domains [95–97] or receptor ligands, and the introduction of reactive sites for chemical conjugation drugs [98] or fluorescent probes [99] into the ELR sequences. ELRs can also be designed to self-assemble in response to an extrinsic temperature stimulus for applications such as soluble macromolecular carriers that can be used for the systemic delivery of antitumor agents or the local delivery of therapeutics directly to the tumor [14]. The effectiveness of a pharmaceutical treatment, as well as the severity of any side effects, is largely dependent on the precision with which the drug can be targeted

to the site that requires treatment. This is a particularly significant issue in chemotherapy, where the cytotoxic agents used inevitably cause damage to non-target tissue.

The first ELR-based drug delivery systems were reported by Urry. These systems were quite simple devices in which γ-radiated crosslinked poly(VPGVG) hydrogels of different shapes were loaded with a model water-soluble drug (Biebrich Scarlet) [89], which was then released by diffusion. In this design, just the biocompatibility and the lack of pernicious compounds during the bioresorption of the device were exploited. Additionally, the introduction of various chemical functions, such as glutamic acid moieties, along the polymer chain led to enhanced performance, with the free carboxyl groups being used for hydrolysis-controlled crosslinking. The emergence of charged carboxylates on the polymer chain after crosslinker hydrolysis helped to release efficiency by the concurrent swelling of those polymer chains and sections having charged side chains [100].

In a different example, the ability of some ELRs, especially those based on the (VPAVG) pentapeptide, to form stable, drug-loaded, nano- and microparticles has facilitated the development of injectable systems for controlled release [101]. The drug is entrapped in these nano- and microparticles when the particles self-assemble as the temperature is raised above T_t and is then released in a sustained manner over a period of around 30 days. The production of self-assembled nanoparticles (Fig. 7) for the combined release of bone morphogenetic protein-2 (BMP-2) and bone morphogenetic protein-14 (BMP-14) by exploiting the inverse temperature transition of this poly(VPAVG) has been reported. These BMPs could be encapsulated into the elastin-like particles in a highly efficient manner and delivered in a sustained way over a period of 14 days. This approach has significant potential for future bone tissue engineering applications [102]. Slow diffusion of the drug is considered to be the main mechanism of drug delivery for this simple model, although more complex polymer designs are possible.

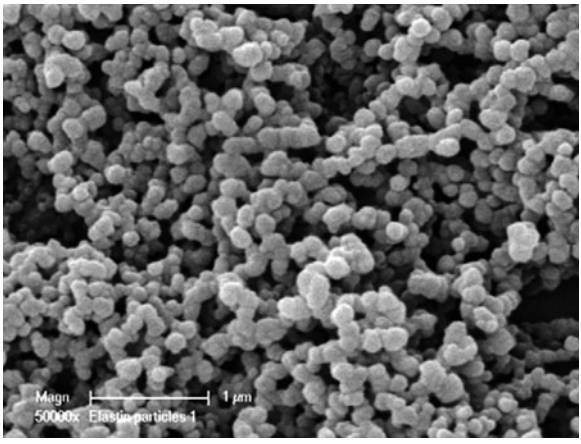

Fig. 7 SEM micrographs of unloaded elastin poly(VPAVG) nanoparticles created by self-assembly. Magnification ×50,000. *Scale bar* 1 µm. Reproduced with permission [102]

Recent DSC studies aimed at determining the influence of NaCl and urea on the ITT of this poly(VPAVG) showed that the T_t can be varied by varying the NaCl and urea concentrations. Thus, while the addition of NaCl causes a decrease in the T_t, with a subsequent increase in the ΔH, the addition of urea disrupts the bulk water structure, thereby decreasing the hydrophobic effect and causing an increase in the T_t. The polymer showed a hysteresis behavior, with thermal absorption/release of components depending on the salt concentration of the polymer solution. In addition, the polymer self-assembles into spherical particles with a size distribution ranging from 700 nm to 2 μm above the T_t [103].

Some polymer designs contain more complexity in their amino acid sequence. One such example concerns ELRs that contain glutamic acid, where the γ-carboxyl groups are maintained in close vicinity in a highly hydrophobic environment by the precise nanometric positioning of phenylalanine (Phe) residues in the polymer sequence in accordance with the β-spiral structure of the folded state [10, 104]. Thus, once the polymer folds into the β-spiral structure, these Phe residues surround the free carboxyl groups to create strong competition between the two mutually exclusive forms of hydration. At neutral or basic pH (including physiological pH), and in the presence of the appropriate oppositely charged drug, this polymer forms strong insoluble aggregates, with the drug being released slowly from its coupling on the outer surface of the aggregate [3].

The most recent breakthroughs in this field point towards exploiting the very characteristics of ELRs themselves and the most powerful way in which they can be produced: genetic engineering. Indeed, the ability to control their aggregation both in vivo and in vitro opens the way to a series of advanced medical applications, such as hyperthermia-induced localized extravascular drug accumulation, drug depot formation, and the design of novel materials suitable for tissue repair or engineering. For example, ELRs specially designed for targetted intra-cellular drug delivery have been produced by taking advantage of the soluble-insoluble transition of ELRs to target a solid tumor by local hyperthermia. Furthermore, the very latest version of these ELRs includes an additional pH-responsiveness to mimic the membrane-disruptive properties of viruses and toxins to provide effective intracellular drug delivery [7, 105]. Besides the efficient dosage of antitumoral drugs, this drug-delivery system could serve as an alternative to fusogenic peptides in gene-therapy formulations and to enhance the intracellular delivery of protein therapeutics that function in the cytoplasm [70, 106, 107].

ELR are widely used as macromolecular carriers of cancer drugs as their biocompatibility, genetically encoded synthesis and stimuli responsiveness provide highly tunable properties that can be rationally optimized for a specific drug. Thus, it is possible to synthesize ELRs whose T_t lies between physiological temperature (37 °C) and the elevated temperature attainable in clinical hyperthermia (42 °C) of solid tumors [108–110]. This mild hyperthermia-assisted approach to enhancing the accumulation of thermally responsive polymer carried in tumors following systemic delivery has been demonstrated by Dreher et al., who investigated the distribution of ELRs in tumors by tagging them with fluorophores. These authors showed that when the tumor heating disappears, the ELR aggregates also

disappear, thereby demonstrating the reversibility of temperature-triggered ELR aggregation in vivo [99].

Continuing with this idea, but following a different strategy that involves enhancing the complexity of ELRs, a simple ELR fusion form of the cell-penetrating Tat peptide (derived from the HIV-1 Tat protein) has been developed. This Tat-ELR was found to inhibit the adhesion, spreading, invasion and migration of ovarian cancer cells in cell culture. Furthermore, it has also been confirmed that this ELR fusion form has anti-metastatic potential in an experimental ovarian cancer metastasis model in vivo. These results suggest a novel role for the ELR T_t as a therapeutic intervention in cancer metastasis. The next step in complexity was achieved by adding the L12 peptide, which is derived from bovine lactoferrin and has been shown to possess anticancer properties in multiples cell lines, to this ELR fusion [111]. L12 is a potent cell-death activator that induces necrosis and apoptosis. By conjugating the lytic peptide L12 to an ELR-based delivery system, the authors of this study were able to overcome several obstacles that commonly thwart drug development, including poor pharmacokinetics and systemic toxicity. The thermally responsive Tat-ELR-L12 was found to be soluble in aqueous solution at 37 °C but to aggregate at 41 °C, thus making it ideal for targeting solid tumors with focused hyperthermia. Indeed, Tat-ELR-L12 showed cytotoxic activity against cancer cells in vitro and has the potential to provide an effective vehicle for the thermal targeting of solid tumors. These conjugates are therefore promising candidates for future in vivo studies because of their degree of selectivity in inducing cancer cell inhibition under hyperthermal conditions [112].

Another technique, namely electrospraying, has also been used to generate nano-scale bioresponsive peptide-based particles of defined morphology that can encapsulate drugs, such as in the preparation of ELR particles that contain the chemotherapy agent doxorubicin [105]. The molecular weight of the ELR, the solution concentration and the spraying solution were found to have a significant influence on the morphology, particle diameter and polydispersity of the resulting electrosprayed ELR particles, with higher flow rates and lower spraying voltages resulting in the formation of particles with tail structures and fibers along with spherical particles. Electrospraying is therefore a versatile, efficient and flexible technique for generating spherical ELR particles that have potential for drug delivery [113].

Polymer vesicles and micelles have recently received an increasing amount of attention as potential carriers for hydrophobic drugs or genes. These self-assembling nanostructures are made from a very attractive and promising class of amphiphilic copolymer structures with a proven ability to mimic some of the basic properties of proteins due to their recombinant synthesis, which allows the amino acid sequence of the ELRs to be fine-tuned, thereby determining specific biological functions. ELR-based block copolymers can be tailor-made to form smart, self-assembling, protein-like micellar systems with controlled structure and function. Chilkoti et al., for instance, have focused on the potential therapeutic application of heat-triggered, reversible ELR-containing micelle formation [104].

Diblock ELRs with a unimer-micelle transition temperature that is between body temperature and that approved for clinical hyperthermia are of particular interest for drug delivery as these diblocks form micelles in regions of the body that are externally heated to 42 °C, thus increasing the affinity of the ELR targeting vehicle in the presence of a thermal stimulus [14]. Similarly, Dreher et al. have utilized ELRs in a linear diblock architecture with an N-terminal peptide ligand to form multivalent spherical micelles when heated slightly above body temperature. The critical micelle temperature is controlled by the length of the hydrophobic block, and the size of the micelle is controlled by both the total ELR length and hydrophilic-to-hydrophobic ratio. This study also identified a subset of elastin-like block co-recombinamers bearing terminal peptide ligands that are capable of forming multivalent spherical micelles with multiple copies of the ligand on their corona in the clinically relevant temperature range 37–42 °C, which can be used to target cancer cells. These species could be useful for drug targeting by thermally triggered multivalency [114].

A large number of examples involving the self-assembly of nanostructures such as micelles or vesicles for use as elastin-like block co-recombinamer-based nanocarriers have been reported. Most of these studies involve diblocks, although there are also some examples based on triblocks. Sallach et al. [115], for example, have used recombinant DNA methods to prepare a triblock co-recombinamer containing a central hydrophilic block and two hydrophobic end-blocks derived from ELR-mimetic peptide sequences. Dilute solutions were found to form monodisperse micelles below the copolymer T_t, whereas an abrupt increase in micelle internal density, with a reduction in micelle size, was observed above this temperature due to a reversible change in micelle compactness triggered by a helix-to-sheet protein-folding transition [116]. Moreover, this group has shown that these triblock co-recombinamers, which can be produced in the absence of either chemical or ionic crosslinking, show minimal inflammatory response and robust in vivo stability for periods exceeding one year. They could therefore be used as carriers for controlled drug release or as biocompatible surface coatings [71].

As a last example, the “one-pot” synthesis of pH- and temperature-sensitive gold clusters mediated by an ELR has been reported recently by our group. Thus, the reduction of auric acid in the presence of an ELR, in this case, ELRGlu15, which contains equally spaced Glu residues and is Cys-terminated, gave the biohybrid Au-Glu15. The gold-biopolymer interface in Au-Glu15 was found to be stabilized by covalent sulfur-gold bonds from the cystein end. Au-Glu15 contained nanosized gold crystals with diameters ranging from 2 to 11 nm. Furthermore, it promoted the formation of 2D linear arrangements of gold clusters, with interparticle distances in the range from 10 to 40 nm, in areas of low particle density. In addition, Au-Glu15 displayed spectroscopic properties (UV-Vis absorption) that could be modulated by varying the pH and temperature of the environment as a result of reversible aggregation-expansion of gold particles. These smart gold-ELR hybrids tuned to physiological conditions could be interesting for the development of biosensors and drug delivery applications [117].

3.4 Photoresponsive ELRs

These ELRs and their fusions are capable of responding to alternative stimuli, in this case light. Strzegowski was the first to demonstrate modulation of the ELR transition in response to light upon incorporation of an azobenzene chromophore into a random ELR copolymer [118]. The cis-trans isomerization of the chromophore by UV irradiation triggered the transition of the ELR within a small temperature range close to 40 °C [14]. The azobenzene is incorporated directly into the poly(VPGVG) and the copolymer is composed of the pentapeptide (VPGVG) and the pentapeptide (VPGXG), where X is L-*p*-(phenylazo)phenylalanine.

In the same manner, the azobenzene group also suffers a photo-induced cis-trans isomerization. Thus, dark adaptation or irradiation with visible light at a wavelength of around 420 nm induces the presence of the most nonpolar trans isomer, whereas UV irradiation (at around 348 nm) causes the appearance of high quantities of the cis isomer, which is slightly more polar than the trans isomer and leads to resolubilization of the ELR chain. Although the polarity change is not high, due to the sensitivity and efficiency of ELRs it is sufficient to obtain functional polymers [29, 119].

In another example, a different chromophore, namely a spiropyran derivative, was attached above the free γ-carboxyl group of a Glu-containing ELR. Spiropyran compounds undergo photoreactions than can be driven by natural cycles of sunlight-darkness without the need for an additional UV source, although UV irradiation causes the same effect as darkness but at a higher rate [120]. These studies demonstrated the enormous potential of ELRs to serve as photoresponsive molecules as only one spiropyran conjugated to 10 pentamers was sufficient to elicit this behavior. These systems are, however, limited by their small range of working polarity change and the need for a temperature of around 14 °C [14]. It is possible to amplify the range over which the ELR phase-transition can be phototriggered without increasing the number of sensitive moieties if this moiety has a state in which it is able to interact with a different compound and the resulting interaction further increases the polarity difference between the two states. α-Cyclodextrins (α-CDs), for example, have been shown to form inclusion compounds with the trans isomer of the azobenzene chromophore, but not with the cis isomer due to steric hindrance [121]. The change in polarity between the dark-adapted sample (trans isomer buried inside the α-CD) and the UV UV-irradiated one (cis isomer unable to form inclusion compounds) led to an increase in the temperature range over which the phase transition could be isothermally triggered. The authors of this study also checked that it was possible to reverse the photosensitivity of the ELRs with α-CD and found that α-CD promoted a tunable offset, gain and inversion of the photoresponse of the polymer. The photoresponsiveness of the polymer could therefore be shifted to room or body temperature, and with a wider range of working temperatures, thus meaning that the need for precise temperature control can be avoided in most conceivable applications such as photooperated molecular machines to macroscopic devices (photoresponsive hydrogels, membranes, etc.)

with nano- and microdevices (phototransducer particles, photo-operated pumps, etc.). This α-CD-related mechanism could be exploited in other smart ELRs that respond to stimuli of a different nature and also adds a further possibility for control since the ability of some modified CDs to form inclusion compounds can be controlled by different stimuli [119, 122, 123].

In the last year, and following a similar approach, photoresponsive gold-ELR hybrids have been prepared in order to obtain multifunctional chromophore-metal nanocomposites that operate in aqueous media for the development of multi-stimuli-sensitive detectors for biosensing applications (bio-inspired applications) and the manufacture of photosensitive smart surfaces, the development of photofunctional membranes or the photocontrol of cell adhesion/proliferation. In order to decouple chromophore photoisomeration with plasmon waves over the surface of the gold cluster, β-CD-capped photoresponsive gold-ELR hybrids were synthesized in one step by addition of thiolated β-CD to a solution of gold-azoGlu15. This procedure resulted in a 65% higher photoisomeration efficiency with respect to the mother hybrid. The optical properties of these hybrids could be tuned by varying the pH and temperature of the environment. Such multifunctional chromophore metal nanocomposites that operate in aqueous media could allow the development of smart photoresponsive host-guest platforms for the manufacture of novel sol-gel systems. Alternatively, the formation of alkenethiol SAMs over the cluster surface of the mother Gold-AzoGlu15 decouples the photoisomerization of azobenzene groups from the bulk phase absorption. There is amplification of the light absorption temperature and pH of the environment. The formation of mixed monolayers over the gold cluster surface with azoglu15 and β-CD or alkene can be correlated to an increase in the azobenzene free volume and a decrease in the steric hindrance between photo-isomers. The optical absorption can therefore be modulated by varying the temperature and pH of the environment. This gold-ELR hybrid could be of interest for the design of multi-functional chromophore-metal nanocomposites that operate in aqueous media for the development of multi-stimuli sensitive detectors with biosensing applications [124].

3.5 Fusion Protein Purification

The demand for recombinant proteins for biomedical and industrial applications is expanding rapidly. However, although many fusion tags have been developed to facilitate the purification of recombinant proteins using affinity chromatography, this technique requires specialized equipment and is difficult to scale-up. Due to these shortcomings, chromatography is not always an efficient and economical method; therefore, the development of new, simpler and broadly applicable purification methods to circumvent these problems is desirable. One such approach takes advantage of the properties of ELRs.

ELR-based protein purification relies on the fact that the thermosensitive properties and smart behavior of ELRs are retained when they are expressed as

recombinant fusions with proteins [125, 126]. ELRs are therefore able to act as environmentally responsive tags for the non-chromatographic purification of recombinant proteins. This ability allows protein purification via inverse transition cycling (ITC), which involves the sequential and repeated aggregation, centrifugation and resolubilization of the fusion protein [125, 126]. In a similar way to other kinds of tags, ELRs can be removed from the target protein by cleavage at a specific protein-recognition site engineered between the ELR tag and the target protein [126, 127]. Moreover, ELR tags have a further advantage in that they can easily be separated from their target protein by a simple additional ITC step. Purification of recombinant proteins via ITC is attracting increasing attention thanks to its numerous advantages, such as the low cost of the purification process, simplicity and robustness. Indeed, both *E. coli* cultures and plant-expression systems have been tested as ELR fusion protein factories [128].

A generic protocol has been developed to purify fusion proteins by ITC. For general implementation, the ELR [V5A2G3-90] (an ELR consisting of 90 pentapeptides, with valine, alanine and glycine amino acids present as guest residues in a ratio of 5:2:3) has been found to be useful for a range of target proteins [125, 126]. Nevertheless, design optimization of the fusion protein and the selection of ITC conditions in order to maximize the yield is advisable [127, 129–131]. Thus, several parameters, including the length of the ELR, guest residue composition, target protein features or fusion order, should be taken into account to achieve the successful purification of a specific protein. It has been reported that the yield of the fusion protein decreases significantly as the ELR chain length increases [125]; therefore, different ELR tags should be tested empirically to determine which tag length is the most suitable to purify a given target protein. Indeed, reducing the size of the ELR tags results in both an increase of the yield and a considerably more complex transition behavior [127]. Moreover, the transition temperature depends on the size of the ELR; therefore, smaller tags require the incorporation of a great fraction of hydrophobic guest residues to keep the transition temperature low enough, since high temperatures during the ITC protocol [129] may denature the target protein [126, 127].

Aliphatic guest residues make the ELR tags only modestly sensitive to changes in salt concentration. The incorporation of ionizable residues into the ELR sequence enhances the sensitivity of the ELR to the salt; therefore, the amount of salt required to induce the inverse phase transition is reduced. The effect that charged amino acids have on T_t must, however, be balanced with the introduction of hydrophobic residues, such as phenylalanine or valine. To date, the shortest ELR that has been used to purify an ELR fusion protein is ELR [KV2F-8] (an ELR consisting of eight pentapeptides with lysine, valine and phenylalanine as the guest residues in a 1:2:1 ratio), with a Mw of roughly 4.3 kDa [129].

As stated above, another parameter to be considered is related to target protein features. The molecular proximity between the ELR and the target protein has important implications since the features of this target protein can modify the physicochemical properties of the environment around the ELR, and therefore its T_t. In other words, fusion of a protein to a tag ELR results in a variation in the T_t of

that ELR relative to the value for the free ELR (ΔT_t parameter). For example, it has been reported that an electrostatic interaction between a cationic ELR containing lysines and thioredonine (Trx, which has a formal charge of nearly –5 at pH 7) results in neutralization of the charged moieties in the ELR, thereby lowering the T_t of the fused ELR and resulting in a negative ΔT_t [129]. On the other hand, Trx-ELR[V2A2G3-90] fusion proteins show a positive fusion ΔT_t due to the low fraction hydrophobic surface area of Trx. Target proteins with a high hydrophobic surface area therefore help to decrease T_t when fused to an aliphatic ELR [130], whereas charged target proteins cause a decrease in T_t when fused to ELRs that contain an ionic guest residue as electrostatic interactions lead to charge neutralization [129]. Bearing this in mind, designing the ELR tag in such a way that its interactions with the tagged protein aid the formation of large aggregates during purification would be desirable to improve recovery and purification efficiency.

The position of the target protein relative to the ELR tag in ELR fusion proteins is another important variable for controlling the expression level and specific activity of the ELR fusion proteins. Protein expression is a complex process; therefore, it is strongly recommended to express both variants of the fusion protein if possible. Nevertheless, it has been reported that placing the ELR at the C-terminal of the target protein results in a higher expression level for the ELR fusion proteins BFP (blue fluorescent protein), CAT (chloramphenicol acetyltransferase) and Trx than that obtained with N-terminal target proteins. The position of the fusion protein also has a significant impact on its specific activity. Four proteins have been studied to determine the role of ELR position on their activity. Protein-ELR constructs whose tagged protein is BFP, CAT or Trx have a higher specific activity than their respective ELR N-terminal fused proteins, whereas the tagged IL1Ra (interleukin-1 receptor antagonist) protein in the N-terminal has higher activity than its respective IL1Ra-ELR fusion constructs [131].

Further modifications of the ELR-base protein purification approach have been made in order to circumvent some problems related to protein purification when the protein is expressed at ultra-low levels. One of the multiple factors that influence thermosensitive behavior is polymer concentration. Some proteins, and their respective fusion proteins, have the drawback of being expressed at low levels, which have repercussions on inverse transition cycling efficiency. To overcome this problem, the addition of free ELR to the soluble lysate containing the fusion protein has been proposed [126, 132, 133]. Free ELR acts as a co-aggregant that leads not only to a decrease in T_t due to the increase in ELR concentration but also to an easier recovery of aggregates thanks to their large size. This ITC variant focused on the addition of excess ELR has allowed the purification of ultra-low concentration ELR fusion constructs, with several such examples having been reported [126, 132, 133].

Another novel approach, based on a combination of the simple environmental trigger experienced by ELR and the specificity and affinity of binding proteins, has been proposed to improve antibody purification. The main advantage of this new platform (known as ELR-mediated affinity capture, EMAC) is its universality as it

does not require the construction of a fusion protein for each individual antibody of interest. The EMAC approach involves the construction of a fusion protein in which the ELR is joined to an antibody-binding domain, such as proteins G, A or L. Selective affinity interaction between the antibody and the antibody-binding domain allows recovery of the antibody after triggering the transition of the ELR. The final step involves incubation with an elution buffer in order to separate the fusion construct from the antibody [134].

4 Outlook

ELRs are a new class of polymers with numerous advantages and superior properties with respect to any other polymer family. Indeed, they show interesting and unconventional features when compared to conventional materials. In this review we have attempted to highlight the enormous potential of genetic engineering for producing highly complex functional polymers with huge potential for a wide range of applications in many different fields. Moreover, these recombinamers have a monodiperse character and are obtained with a precise sequence and molecular weight, which makes them highly valuable as biomaterials. Furthermore, the possibility to tailor them with essentially unlimited complexity, their extreme biocompatibility, and smart and self-assembling characteristics, and their programmed biodegradation make the ELRs an efficient material for the most diverse biomedical and biotechnological applications in fields as varied as tissue engineering, biosurface engineering, drug delivery, protein purification and biosensors.

Although this review has described a large number of examples of ELRs with different functionalities and nano(bio)technological applications, these polymers are still far from reaching their full potential. It is therefore reasonable to expect that the near future will bring new recombinamers with widely diverse functionalities that will establish a new standard in polymer function and, perhaps, a clear quantitative and qualitative leap that will contrast with the current slow but nevertheless continual development of polymer-based innovation, along with encouraging applications.

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Biomimetic Materials for Medical Application Through Enzymatic Modification

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Abstract Living organisms synthesize functional materials, based on proteins and polysaccharides, using enzyme-catalyzed reactions. According to the biomimetic approach, biomaterial matrices for tissue engineering are designed to be able to mimic the properties and the functions of the extracellular matrix (ECM). In this chapter, the most significant research efforts dedicated to the study and the preparation of biomimetic materials through enzymatic modifications were reviewed. The functionalizations of different polymeric matrices obtained through the catalytic activity of two enzymes (Transglutaminase, TGase and Tyrosinase, TYRase) were discussed. Specifically, the biomimetic applications of TGase and TYRase to confer appropriate biomimetic properties to the biomaterials, such as the possibility to obtain in situ gelling hydrogels and the incorporation of bioactive molecules (growth factors) and cell-binding peptides into the scaffolds, were reviewed.

Keywords Biomimetic · Tissue engineering · Transglutaminase · Tyrosinase

Abbreviations

bFGF	Basic fibroblast growth factors
BMMP	Biomimetic materials processing
DOPA	3,4-Dihydroxyphenylalanine
ECM	Extracellular matrix
E_{deoxy}	Deoxytyrosinase
EGF	Epidermal growth factor
E_{met}	Metyrosinase
E_{oxy}	Oxytyrosinase
ESP	Enzyme-sensitive peptides
FN	Fibronectin
K4	Kringle4 domain

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KDP	Lysine donor peptide
LN	Laminin
MMPs	Metalloproteinases
mTGase	Microbial transglutaminase
PDMS	Poly(dimethylsiloxane)
PEG	Polyethylene glycol
PTH	Parathyroid hormone
QAP	Glutamine acceptor peptide
TGase	Transglutaminase
TYRase	Tyrosinase
VEGF	Vascular endothelial growth factor
VN	Vitronectin

Contents

1 Introduction

Biomimetics is a relatively new field, which literally means mimicking of biology. It is a branch of science in which biologists and engineers jointly endeavor to produce bioinspired materials that can be used for medical applications. Biomimetic material processing (BMMP) is defined as the design and synthesis of new functional materials by refining knowledge and understanding of related biological products, structures, functions and processes. Hence, BMMP is not a simple imitation of the processes where biological materials are involved, but it consists in advanced materials processing for different fields, such as bionics, electronics, photonics, mechanics and medicine [103].

The biomimetic approach plays a very important role in tissue engineering for the design of biomaterial scaffolds, providing structural, mechanical and logistic templates to cell attachment and tissue formation [50]. The aim of this approach is to mimic the key features of the extracellular matrix (ECM), which in natural tissues provides structural support for cell attachment, proliferation and differentiation [45]. The ECM is custom designed and manufactured by the resident cells

of each tissue and organ, and it is in a state of dynamic equilibrium with its surrounding microenvironment [49]. The ECM also provides a supportive medium or conduit for blood vessels, nerves and lymphatics, and for the diffusion of nutrients from the blood to the surrounding cells [49].

The properties of ECM molecules make them ideal for cell and tissue engineering. ECM-derived molecules can be used to coat implants, modify surfaces, direct cell growth and differentiation, and to engineer cell phenotype and behavior [87]. Their multifunctional nature makes them ideal for promotion of cell-specific adhesion via integrins and other surface receptors [6]. They serve as efficient co-receptors for sequestration and delivery of growth factors, establishing morphogenic gradients recognized by cellular receptors during development, wound healing and tissue repair [95].

According to the biomimetic approach, recent research efforts in tissue engineering focused on the incorporation of biologically active motifs derived from ECM proteins into matrices to integrate essential molecular elements of the ECM into the biomaterial and to promote a desired biological response. The choice of biologically active motifs incorporated into engineered matrices depends on the ultimate end use of the matrix [87]. The biomimetic approach can be achieved using two different strategies: (i) the incorporation of soluble bioactive molecules, such as growth factors and plasmid DNA, into appropriate biomaterial carriers [83, 109] and (ii) the incorporation of cell-binding peptides into biomaterials via chemical or physical modification.

The second approach includes native long chain of ECM proteins as well as short peptide sequences derived from intact ECM proteins that are able to interact specifically with cell receptors and to confer appropriate functions to the biomaterials [102]. The most studied class of cell-adhesive molecules comprises ECM-soluble serum proteins, including fibronectin (FN), vitronectin (VN), collagen families, thrombospondin, osteopontin, fibrinogen, von Willebrand factor and laminin (LN) [87].

The desired biological effects produced by ECM proteins can usually be attributed to short peptide sequences or motifs. By immobilizing these motifs on surfaces, it is possible to imitate the behavior elicited by the whole molecule and peptides can be immobilized to surfaces in a controllable manner with a defined conformation [107]. Many factors contribute to the effective presentation of peptide motifs within biomimetic materials, including density of the molecules, the nature of the substrate supporting the biomimetic materials, the distance from the surface and the conformation of the molecules presented on the materials [102].

One of the most commonly used ligands in biomaterials and the most physiologically ubiquitous binding motif is the short peptide sequence arginine-glycine-aspartic acid (RGD) [86]. RGD was identified through competitive binding assays involving the integrin family of cell surface receptors. Studies have shown that RGD-modified interfaces can promote integrin-mediated adhesion and migration [105]. Additional peptides, such as REDV [69] and FHRRIKA from heparin [82], PHSRN [4] from fibronectin, YIGSR and IKVAV from laminin [56, 112], have

been explored when the peptide confers a specific advantage to the substrate or serves as a new design paradigm. Among the most commonly used strategies, the coating of polymeric scaffolds (fibers, meshes, sponges) with solutions of ECM protein(s) through adsorption via non-covalent interactions was largely used in the early studies [9]. While this method demonstrated improvements in cell adhesion, proliferation and secretion of ECM for a variety of cell types, it requires the adsorption of a high protein density, since the protein may denature on the surface or adsorb in a suboptimal orientation, which significantly reduces affinity for given cell types [46]. Alternatively, short, bioactive peptides from ECM proteins and growth factors have been attached via chemical methods. For example, the peptide REDV was immobilized on surfaces instead of heparin to mediate the selective adhesion of endothelial cells over smooth muscle cells, fibroblasts and platelets [33]. Additionally, the combination of the RGD and the heparin-binding motifs from bone sialoprotein (FHRRIKA) synergistically improved cell adhesion and mineralization in osteoblast culture [39], and RGD-modified polymers imparted desirable osteoblast adhesion and mineralization on titanium implants [7]. Numerous experiments have been conducted with surfaces and tissue-engineering matrices covalently modified with ECM-derived peptides, and they have confirmed the bioactivity of these peptides and their utility in many tissue engineering applications [67]. A very commonly employed strategy for peptide immobilization is the reaction of the thiol-terminated peptide with surfaces and polymers that are functionalized with maleimide, thiol and vinyl sulfone groups [66]. The reaction of thiol groups with vinyl sulfone-modified polymers and surfaces has been employed in many recent investigations owing to its high selectivity over reactions with amines and its high reaction efficiency at physiological temperature and near physiological pH [47, 93]. Reactions of amine-terminated side chains and carboxylic acid side chains are also used for certain amino acid sequences in which these side chains are not required for biological activity. A variety of chemical reaction strategies can be used, one of the most common being the carbodiimide-activated coupling of amines with carboxylic acids [48].

The advantage of the latter reactions is that they are generally applicable to a variety of proteins, synthetic polymers and plasma-treated surfaces [82]. The overall surface density of the peptides can also be controlled easily via these chemical modification strategies, which can have a large impact on cell proliferation and differentiation, particularly when coupled with manipulation of the chemical composition of the matrix material [85].

As an alternative to traditional chemical methods employing toxic reagents and solvents and harsh conditions, enzymatic approaches may offer cleaner and safer alternatives for the incorporation of ECM-derived peptide sequences into biomaterials. The enzyme-catalyzed reactions are an interesting approach to prepare biomimetic biomaterials: enzymes are expected to offer alternatives to current chemical approaches, owing to their specificity and selectivity, which allows a more careful control of reaction conditions and final biomaterial structure by targeting selected reactive sites of the substrate [94].

The selectivity of enzymes offers the potential for better controlling macromolecular structures without the need for wasteful protection/deprotection steps [91]. In another biomimetic approach, enzymes can be exploited for the crosslinking of ECM-derived materials using mild conditions with the aim to optimize the scaffold mechanical characteristics to the application.

Particularly, in this chapter, the role of two classes of enzymes, tranglutaminases and tyrosinases, for the production of biomimetic biomaterials will be reviewed. These enzymes are involved in the natural gelling mechanisms of blood clotting (transglutaminase) and mussel glue setting (tyrosinase), which may be mimicked for the realization of in situ gelling hydrogels for medical application.

2 Transglutaminase-Mediated Modification of Biomaterials

2.1 TGases, a Family of Enzymes

Transglutaminases (TGases) are a widely distributed group of enzymes (EC 2.3.2.13) that catalyse the post-translational modification of proteins by the formation of isopeptide bonds. The term transglutaminase (TGase) was first introduced by Sarkar and Clarke [90] to describe the transamidating activity observed in guinea-pig liver. Later studies undertaken by Pisano et al. [80] on the stabilization of fibrin monomers during blood clotting demonstrated that transamidation is brought about by enzymes that crosslink proteins through an acyl transfer reaction between the γ-carboxamide group of peptide-bound glutamine and the ε-amino group of peptide-bound lysine, resulting in a ε-(γ-glutamyl)lysine isopeptide bond [80]. As shown in Fig. 1, TGase displays a 'ping pong'-based mechanism, in which the enzyme forms a covalent intermediate between the active-site thiol of its cysteine residue and a glutamine residue in the first protein substrate, with concomitant 'activation' of the glutamine acyl moiety and release of ammonia. This active thioester can undergo through hydrolysis (an unfavorable reaction), releasing glutamic acid in the substrate protein, or an acyl transfer to a primary amine, which can be either a small molecule (like a polyamine) or protein bound (the ε-amino group of a lysine residue). In the transamidation instance, a simple amine-isopeptidyl adduct or a direct glutamyl-lysine protein crosslink is produced, respectively (Fig. 1).

In vitro, the enzyme is able to catalyze crosslinking of whey proteins, soy proteins, wheat proteins, beef myosin, casein and crude actomyosin (which is refined from mechanically deboned meat), leading to their texturization [74, 117]. This capacity of the enzyme has been used in attempts to improve the functional properties of food. Until the end of the 1980s, commercial transglutaminase could only be obtained from animal tissues, most commonly from guinea pig liver.

The rare source and complicated downstream procedure resulted in an extremely high price for the enzyme, which hampered a wide application in food

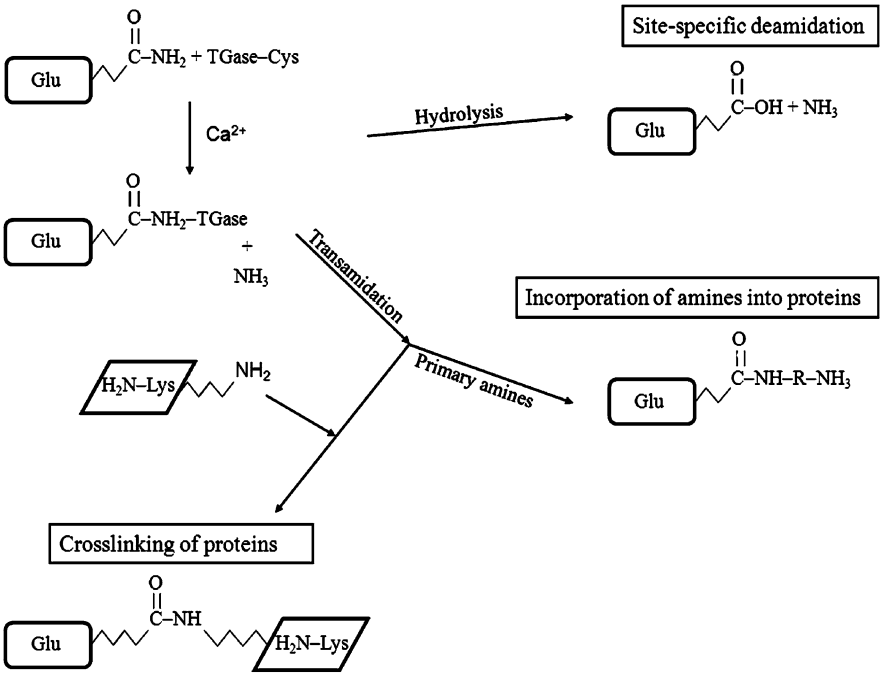

Fig. 1 Transglutaminase-catalyzed reactions

Table 1 TGases characterized at the protein level

TGase	Synonyms	Gene	Chromosome location	Residues (kDa)
Factor XIIIa	Fibrin stabilizing factor	F13A1	6p24–25	732 (83)
Type 1 TGase	Keratinocyte TGase	TGM1	14q11.2	814 (90)
Type 2 TGase	Tissue TGase	TGM2	20q11–12	686 (80)
Type 3 TGase	Epidermal TGase	TGM3	20q11–12	692 (77)
Type 4 TGase	Prostate TGase	TGM4	3q21–22	683 (77)
Type 5 TGase	TGase X	TGM5	15q15.2	719 (81)
Type 6 TGase	TGase Y	TGM6	20q11 15	
Type 7 TGase	TGase Z	TGM7	15q15.2	710 (80)

processing [114]. TGase has also been found in invertebrates [98], vertebrates including amphibians [115], fish [113] and birds [81], plant tissue [106] and microorganisms [3].

In mammals, TGases are present in most tissues and body fluids, and are involved in several biological processes, including blood clotting, wound healing and epidermal keratinization [2]. In mammals, eight distinct TGase isoenzymes have been identified at the genomic level [42]; however, only six have so far been isolated and characterized at the protein level, after purification either from natural sources or as recombinant proteins. As summarized in Table 1, the fully

characterized enzymes include (i) the circulating zymogen Factor XIII, which is converted by a thrombin-dependent proteolysis into the active TGase Factor XIIIa (plasma TGase), involved in stabilization of fibrin clots and in wound healing; (ii) the keratinocyte TGase (type 1 TGase), which exists in membrane-bound and soluble forms, is activated several fold by proteolysis and is involved in the terminal differentiation of keratinocytes; (iii) the ubiquitous tissue TGase (tTGase; type 2 TGase), whose role is still debated; (iv) the epidermal hair follicle TGase (type 3 TGase), which also requires proteolysis to become active and, like type 1, is involved in the terminal differentiation of the keratinocyte; (v) the prostatic secretory TGase (type 4 TGase) [31], essential for fertility in rodents; and (vi) the recently characterized type 5 TGase [14]. All mammalian forms have appreciable structural homology, are the products of different genes arising from duplication, rearrangement and chromosomal shifts [42], and are members of the papain-like superfamily of cysteine proteases [68]. The mammalian TGases are characterized by the common feature of Ca^{2+} dependent catalytic activity [22].

However, more recently, the production of transglutaminase in microorganisms has received increased interest, and an enzyme of microbial origin is now commonly used for food treatment and has been shown to improve food flavor, appearance and texture. Several excellent reviews on the application of microbial transglutaminase in food are available in the literature [26, 54]. A microbial transglutaminase (mTGase) has been isolated from the culture medium of *Streptoverticillium* sp. S-8112 [3], which has been identified as a variant of *Streptoverticillium mobaraense*. This enzyme is the first TGase obtained from a non-mammalian source. Although the physiological role of mTGase is still unknown, this protein is secreted from the cytoplasm membrane as a zymogen and is activated by proteolytic processing [78]. A sequence analysis of mTGase by Edman degradation revealed that the protein consists of 331 amino acids with a molecular mass of 37.9 kDa [57]. In contrast to other members of the TGase family, the mTGase exhibits Ca^{2+} independent activity, but the transamidating activity of this enzyme is relatively evolutionarily conserved, and mTGase has transamidating ability similar to that of the mammalian versions. Moreover, mTGase is considered to be stable over a wide pH range (4–9), and the optimum temperature for enzymatic activity is 55 °C [114].

2.2 Transglutaminase in Biomedical Applications

Although the main applications of transglutaminases remain in the food sector, novel potential applications have emerged during the last decade. These applications cover the areas of biomedical engineering, material science, textiles and leather processing. Below, the potential applications in the medical field are discussed and evaluated, considering the growing interest in the use of TGase enzymes for tissue repair and reconstruction.

Transglutaminase enzymes are expressed in a variety of cell types and have the natural function to crosslink several ECM proteins [10, 51]. Over traditional chemical crosslinking or gelation agents, transglutaminase enzymes have the advantages to be employed under mild conditions (such as temperature and pH) and not requiring chemical initiators.

Different biomimetic approaches of transglutaminase could be used to confer appropriate biological properties to the biomaterials: (i) formation of ε-(γ-glutamyl)lysine isopeptide bonds to crosslink natural polymers, (ii) the possibility of obtaining in situ gelling hydrogels and (iii) the incorporation of bioactive ligands or peptides into the scaffolds through transglutaminase functionalization.

In a biomimetic approach, biologically derived and biologically produced materials containing lysine and glutamine residues, such as collagen, gelatin and fibrin, can be used as substrates for the transglutaminase-catalyzed formation of ε-(γ-glutamyl)lysine isopeptide bonds, leading to material crosslinking or its grafting with functional molecules.

Collagen type I is the most important protein in mammals, having a triple helical structure made of three polypeptide chains containing repeating Gly-X–Y triplets in which the X and Y positions are frequently occupied by proline and 4-hydroxyproline, respectively [15]. Collagen can be readily purified from animal tissues, such as skin and tendon, and from discarded human tissues, such as placenta. Gelatin, which is produced by thermal denaturation or physical and chemical degradation of collagen, is a typical and low-cost cell-adhesive protein material. As in vivo applications of collagen and gelatin gels/scaffolds are limited by the poor mechanical properties, several procedures have been developed to prepare collagen matrices with sufficient mechanical properties to at least partially resist cell-induced contraction. In this context, [15] have compared scaffolds based on native collagen from bovine skin with scaffolds made of collagen treated with either natural transglutaminase from guinea pig liver or mTGase from *S. Mobaraense*. As compared to scaffolds containing native collagen, the ones treated with enzymes of both sources displayed enhanced cell attachment, spreading and proliferation of human osteoblasts and human foreskin dermal fibroblasts [15]. The transglutaminase-treated collagens have also shown a greater resistance to cell-mediated endogenous protease degradation [15]. Similarly, O'Halloran et al. [76] have found that crosslinking collagen from chicken sternal cartilage with mTGase resulted in an increased resistance of the scaffold to degradation. The same group has also demonstrated that an enzymatically crosslinked collagen-hyaluronan material was a potential candidate for developing an injectable cell-seeded hydrogel for nucleus pulposus treatment in degenerated intervertebral discs [76].

Based on the assumption that an enzymatically stabilized collagen scaffold provides a dermal tissue precursor with enhanced wound healing properties, [37] demonstrated that mTGase-crosslinked bovine collagen from calf skin provides an optimal scaffold for cell migration, which prevents wound contraction and stimulates epithelialization (regeneration of the upper skin) and neoangiogenesis (regeneration of the blood vessels) without inducing any significant inflammatory reaction.

For bone tissue regeneration, three-dimensional porous biomimetic hydroxyapatite/collagen composites crosslinked by mTGase were developed [23]. Again, the enzyme was used with the main purpose to increase the mechanical resistance of the organic matrix. The obtained composites supported adhesion, proliferation, viability and differentiation of MG63 osteoblast-like cells and human umbilical vein endothelial cells (Fig. 2).

Currently, the potential of transglutaminase-derived gelatin scaffolds is greater than that for collagen scaffolds, because collagen is often liable to great variation between different batches [62]. Broderick et al. [11] have characterized the mechanical properties of crosslinked gelatin hydrogels confirming their cytocompatibility. Barbetta et al. [8] compared gelatin scaffolds prepared with two different crosslinking procedures: (i) radical polymerization of the methacrylate functionalities previously introduced onto the gelatin chains and (ii) formation of isopeptide bridges among the gelatin chains promoted by the microbial transglutaminase. Enzymatically crosslinked scaffolds were found to have reduced cytotoxicity. Hepatocytes cultured on these scaffolds displayed a more differentiated phenotype, as demonstrated by the expression and correct localization of key adhesion proteins [8]. A protocol was developed for fabricating enzymatically crosslinked gelatin microchannels that included a flexible protein substrate and high cell volume density provided by the micro-molded channels in the gel substrate [77]. The resulting devices were suitable for studying the microenvironment of cultured cells because they closely mimicked their natural in vivo environment with flexible protein gels. The technique is based on the application of photolithography to form molds of poly(dimethylsiloxane) (PDMS), which then can be used several times to act as a mold for a gelatin solution crosslinked with the naturally occurring enzyme transglutaminase via a straightforward process. Cells seeded on these devices can invade the matrix and form three-dimensional structures [77].

Another interesting biomimetic application of transglutaminase is the possibility to obtain in situ gelling hydrogels. In these approaches, polymer crosslinking and gel formation are modeled on crosslinking operations found in biology.

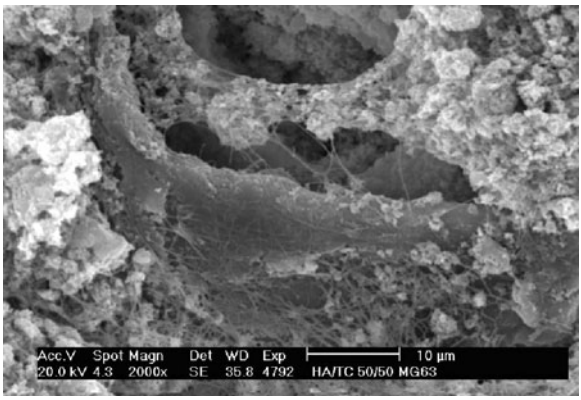

Fig. 2 SEM micrograph of MG63 and human umbilical vein endothelial cells cultured onto hydroxyapatite/collagen/mTGase 50/50 (w/w) for 7 days

The biological models that have probably attracted the most technological interest are (i) the mussel glue that sets under moist/wet conditions [27, 97] and (ii) the transglutaminase-catalyzed reactions that occur during blood coagulation [32]. The first mechanism is activated by tyrosinase enzyme, and it is based on the conversion of phenolic (i.e., 3,4-dihydroxyphenylalanine; DOPA) residues of the adhesive protein into reactive quinone residues that can undergo subsequent interprotein crosslinking reactions. This mechanism will be illustrated in details in the next paragraph.

The second mechanism is based on the reactions of the late stages of blood coagulation, which are illustrated in Fig. 3.

The fibrin matrix is formed by spontaneous polymerization of fibrinogen, a circulating glycoprotein homodimer of a heterotrimer, in the presence of thrombin protease. Thrombin cleaves the so-called fibrinopeptides on fibrinogen that prevent physicochemical self-assembly or polymerization of the molecule. The resulting network is chemically crosslinked by the blood transglutaminase Factor XIIIa, and its complex fibril structure and crosslinked character depend upon the details of its formation. Factor XIIIa transglutaminase catalyzes the formation of covalent crosslinks between lysine and glutamine residues in the α- and γ-chains of fibrin, stabilizing blood clots in vivo [99]. Based on this mechanism, biomimetic approaches for in situ gel formation have considered the use of Factor XIIIa or other tissue transglutaminases [88, 100].

McDermott et al. [72] have been among the first to convert gelatin solutions into hydrogels using transglutaminase as a catalyst. Transglutaminase-mediated

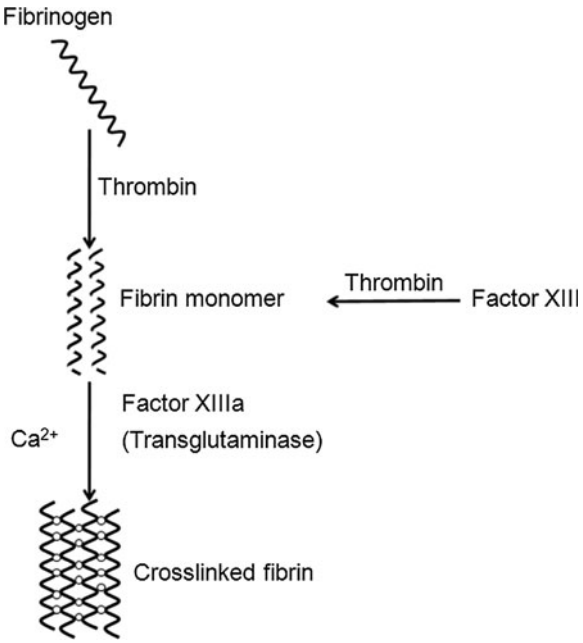

Fig. 3 The mechanisms of fibrin formation in blood coagulation

gelling has been found to be accomplished within minutes depending on the gelatin type and concentration [72]. Gelatin gels can adhere to both moist and wet tissues with a comparable or superior adhesive strength to fibrin-based sealants [17–19]. Liu et al. [64] have found that adhesives produced from mTGase-crosslinked gelatin were able to (i) gel in situ within a relevant timeframe (<5 min), (ii) adhere to tissue in the presence of modest amounts of blood, and (iii) possess appropriate mechanical strength to serve as a hemostatic sealant. According to the same approach, Chen et al. [18] examined the potential of the gelatin-mTGase adhesive for ophthalmic applications and especially for vitrectomy procedures for retinal reattachment.

In addition to collagen and gelatin, fibrin is another typical natural substrate for transglutaminase-catalysed reactions. Fibrin is a specialized protein network that is formed principally in spontaneous tissue repair; it is available from autologous sources and from cryoprecipitated pooled human blood plasma. Although fibrin is not an ECM in the usual sense, as it is not produced by cells in the local environment, the material has the natural role of a provisional matrix for cells to be remodeled and replaced with ECM molecules. Biocompatible hydrogels have been developed using triggered enzymatic crosslinking and using a high concentration of calcium ions [108] or another blood clotting enzyme, thrombin [91], to induce high activity in transglutaminase enzymes. In another approach, the dual activation by calcium and thrombin has been applied to mimic the manner in which Factor XIII is activated in vivo [65] for the crosslinking of the natural Factor XIII substrate fibrin [63] as well as other protein substrates in situ [63, 91]. Strong and biodegradable fibrin-based gels may be produced very quickly (<10 min) by this approach and are used as surgical glues, tissue sealants and drug delivery devices [96]. The in situ crosslinking of the protein substrate by Factor XIII imparts resistance to both mechanical and enzymatic degradation to the material [63]. Moreover, bioactive peptides may be incorporated covalently into fibrin gels, again through exploitation of transglutaminase enzymes, with the aim to tailor the hydrogel properties for specific functions in vivo. For example, incorporation of neuroactive peptides into fibrin gels by Factor XIII has been found to enhance neurite outgrowth and extension into the gel when implanted in vivo [91].

Stimuli-responsive activation of transglutaminase enzymes (e.g., via changes in pH or temperature) has been exploited to achieve in situ enzymatic crosslinking of biomimetic substrates and formation of hydrogels. For instance, stimuli-responsive liposomes entrapping calcium at room temperature and releasing it at 37 °C have been used to trigger the in situ formation of hydrogels. Sanborn et al. [88] conjugated the 20mer peptide CTIGEGQQHHLGGAKQAGDV derived from the Factor XIII crosslinking site of fibrin, having an additional N-terminal cysteine residue to the branches of a four-armed polyethylene glycol (PEG) [88]. Accordingly, a liquid solution containing the peptide-PEG bioconjugate was mixed with calcium-loaded liposomes, Factor XIII and thrombin, and the temperature of the solution raised to 37 °C, causing the release of calcium from the liposomes, thereby activating thrombin and consequently enabling Factor XIII to crosslink the peptide-PEG bioconjugate, resulting in hydrogel formation [88].

The characteristics of ECM guide the design of biomimetic materials, i.e., materials mimicking ECM key features: the presentation of adhesion molecules and the sequestration, display and release of growth factors. Moreover, in a biomimetic scaffold, cells should be able to exert their enzymatic processes to remodel and, in some cases, to replace the matrices, mainly through the action of metalloproteinases (MMPs), as they do with the natural ECM [36]. In this context, cell adhesion peptides, growth factors and peptide sequences targeted by the MMPs may be incorporated into scaffolds to encourage bioactivity and to provide specific biological signals towards cells in order to control or facilitate tissue formation or regeneration. Enzymatic reactions are a promising approach to develop advanced biomimetic materials, as enzymes catalyze chemical reactions under mild conditions, such as body temperature and buffered aqueous solutions at neutral pH, minimizing the risk of peptide/protein denaturation during functionalization. TGases also can be exceptionally selective for their substrates, allowing for sophisticated, biologically inspired material designs without the complication of side reactions and cellular toxicity. The TGases are suitable to functionalize medical devices with the incorporation of necessary biomolecular signals to elicit a desired cellular response. Bioactive ligands such as cell adhesion proteins or peptides have been incorporated into scaffolds to enhance cell adhesion through transglutaminase functionalization. The incorporation of bioactive peptides and proteins has often been achieved by including a Factor XIIIa-reactive peptide domain within the substrate of interest [91, 92].

Ehrbar et al. [32] have recently developed biomimetic hydrogels using transglutaminase Factor XIIIa from Gln- and Lys-containing substrates. A a2-plasmin inhibitor-derived peptide, NQEQVSPL, was used as the Gln acceptor peptide (QAP), while FKGG was used as the Lys donor peptide (KDP) conjugated with an enzyme-sensitive peptide (ESP; GPQGYWGQ). Both QAP and KDP-ESP were attached with a cysteine residue for coupling with multiarm PEG vinyl sulfone (*n*-PEG-VS) through a Michael-type addition, producing *n*-PEG-QAP and *n*-PEG-ESP/KDP, respectively. Factor XIIIa was used for enzymatic crosslinking of *n*-PEG-QAP and *n*-PEG-ESP/KDP to form MMP-sensitive hydrogels. To incorporate cell-adhesive peptides and growth factors, RGD and Vascular endothelial growth factor (VEGF) were attached with QAP to generate RGD-QAP and VEGF-QAP, respectively. Both RGD-QAP and VEGF-QAP were incorporated into the enzyme-sensitive PEG networks by the same enzymatic reaction using Factor XIIIa [32].

Ito et al. [53] incorporated FN and VN peptides into gelatin matrices using mTGase and investigated whether the biomimetic material enhanced the growth of cells.

The motifs of human laminin-332 α3 chain (PPFLMLLKGSTR) modified by addition of transglutaminase substrate (EAQQIVM) and the lysine (KKKKG) residues were successfully conjugated to type I collagen using mTGase by Damodaran [25]. The thering of the two peptides (PPFLMLLKGSTREQQQIVM and PPFLMLLKGSTRKKKKG) using mTGAse enhanced cell attachment, spreading and actin cytoskeleton organization [25].

Chiono et al. [21] successfully functionalized melt-extruded guides based on polycaprolactone and gelatin with poly(L-lysine) by mTGase catalysis. A blend between poly(ε-caprolactone) and uncrosslinked gelatin was produced for the first time and was then melt-extruded into tube-shaped scaffolds. Finally, poly(L-lysine) grafted on the gelatin domains exposed on the inner tube surface using transglutaminase catalysis was synthesized with the aim of conferring guide-specific signaling for nerve cell attachment, proliferation and migration. Confocal microscopy was successfully used to study the accessibility of mTGase towards gelatin substrates using suitable model lysine-rich peptides (FITC-labeled KKKKGY) [21]. Similarly, electrospun gelatin fiber mats were produced from water/acetic acid solutions (10% w/v concentration applied voltage 50 kV; distance needle-collector: 10 cm; collector: wire netting) and then crosslinked by dehydrothermal treatment (Fig. 4). Finally, the gelatin fibers were functionalized with poly(L-lysine) in the presence of transglutaminase and produced a porous fibrous matrix with potential for peripheral nerve repair [24].

Recently, using the same approach of exploiting enzymatic methods (through Factor XIIIa activation), fibrin matrices were functionalized non-covalently with basic fibroblast growth factors (bFGF) fused to a fibrin-binding sequence [116]. To endow bFGF with specific-binding ability, the Kringle4 domain (K4) is fused with the N-terminal end of the human bFGF [116]. For preparing scaffolds able to induce local bone regeneration, an appropriate inactive prodrug-peptide ($TGpIPTH_{1–34}$) that contains an active fragment of human parathyroid hormone was used to functionalize fibrin matrices mediated by Factor XIIIa catalysis [5]. $TGpIPTH_{1–34}$ has been covalently incorporated to release in a manner that depends on local cell-induced proteolysis. The aim of this study was to demonstrate that the $TGpIPTH_{1–34}$ release active $PTH_{1–34}$ only after plasmin-induced proteolytic cleavage [5].

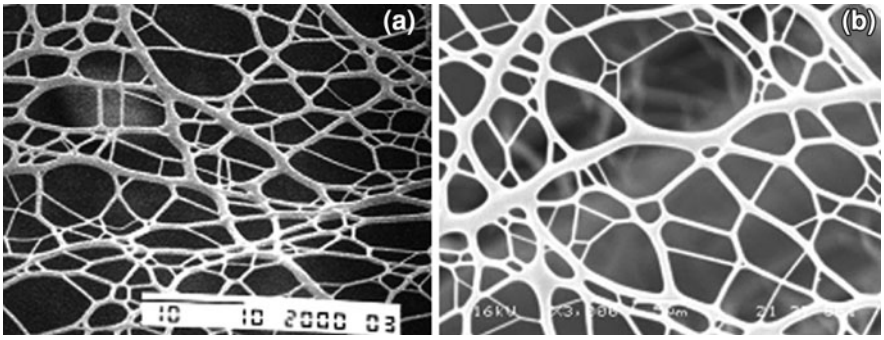

Fig. 4 Electrospun gelatin fiber mats at different magnifications (10%, w/v, concentration in water/acetic acid; applied voltage 50 kV; distance needle-collector: 10 cm; collector: wire netting)

3 Tyrosinase-Mediated Modification of Biomaterials

3.1 Classification and Properties of Tyrosinase

Tyrosinase (E.C. 1.14.18.1, monophenol monooxygenase) is a copper monooxygenases enzyme that catalyzes two different oxygen-dependent reactions, namely: the oxidations of both monophenols (cresolase or monophenolase activity) and *o*-diphenols (catecholase or diphenolase activity) into reactive *o*-quinones [28, 89].

The term tyrosinase refers to its typical substrate, tyrosine. Both tyrosinase activities appear to have broad substrate specificities, although the enzyme has a higher affinity for the L-isomers of the substrates than for the corresponding D-isomers [28]. Tyrosinase is widely distributed throughout the phylogenetic tree, from bacteria to mammals [34], as shown in Table 2, and even presents different characteristics in different organs of the same organisms, such as in roots and leaves of higher plants [13]. The first biochemical investigations were carried out in 1895 on the mushroom *Russula nigricans*, which—once cut—turns red and then black on exposure to air. The best-characterized tyrosinases are derived from *Streptomyces glaucescens*, the fungi *Neurospora crassa* and *Agaricus bisporus*. In fungi and vertebrates, tyrosinase catalyzes the initial step in the formation of the pigment melanin from tyrosine. In plants, the physiological substrates are a variety of phenolics. Tyrosinase oxidizes them in the browning pathway observed when tissues are injured. The enzyme extracted from the champignon mushroom *A. bisporus* is highly homologous with the mammalian ones, and this renders it well suited as a model for studies on melanogenesis. In fact, almost all studies on tyrosinase inhibition conducted so far have used mushroom tyrosinase because the enzyme is

Table 2 Properties of various tyrosinases

Source	Number of subunits	Molecular weight of subunit (kDa)	Spectroscopy of oxy form	
			Absorption (nm)	CD (nm)
Streptomyces glaucescens (eubacteria)	1	30.9	345 640	345 470 575 740
Neurospora crassa (fungi)	1	46	345 425 600	345 520 600 750
Agaricus bisporus (fungi; mushroom)	2	13.4 43	345 600	353
Beta vulgaris (plant; spinach-beet)	1	40	345	–
Human melanocyte (animal)	1	66.7	–	–

commercially available. The notable feature observed in tyrosinases from different sources is that the central copper-binding domain is conserved, which contains strictly conserved amino acid residues, including three histidines [38].

One tyrosinase molecule can contain two copper atoms, and each atom of the binuclear copper cluster is ligated to three histidines. In the formation of melanin pigments, three types of tyrosinase (*oxy*-, *met*- and *deoxy*-tyrosinase) with different binuclear copper structures of the active site are involved. The oxygenated form (oxytyrosinase, E_{oxy}) consists of two tetragonal copper (II) atoms, each co-ordinated by two strong equatorial and one weaker axial N_{His} ligand. The exogenous oxygen molecule is bound as peroxide and bridges the two copper centers. Metyrosinase (E_{met}), similar to the *oxy* form, contains two tetragonal copper (II) ions coupled through an endogenous bridge, although hydroxide exogenous ligands other than peroxide are bound to the copper site. Deoxytyrosinase (E_{deoxy}) contains two copper (I) ions with a co-ordination arrangement similar to that of the *met* form, but without the hydroxide bridge. The resting form of tyrosinase, i.e., the enzyme as obtained after purification, is found to be a mixture of 85% *met* and 15% *oxy* forms [38, 70].

The above considerations have led to the molecular mechanism for the monophenolase and diphenolase activities of tyrosinase (Fig. 5). In the monophenolase cycle, the monophenol can react only with the *oxy* form and be oxidized to the *o*-quinone, resulting in a *deoxy* form ready for further dioxygen binding. Oxytyrosinase is, then, regenerated after the binding of molecular oxygen to deoxytyrosinase. If only *o*-diphenol is present (the diphenolase cycle), both the *oxy* and *met* forms react with *o*-diphenol, oxidizing it to the *o*-quinone. *o*-Diphenol binds to the *oxy* form and is oxidized to *o*-quinone, yielding the met form of the enzyme [73].

In most situations, a diphenol is necessary as the reducing agent to obtain the *deoxy* form, the only one capable of reacting with molecular oxygen and continuing in the catalytic action. For this reason, the monophenolase activity presents a characteristic lag time that exists until a sufficient amount of catechol (needed to reduce the *met* form to the *deoxy* one) is produced by the small amount of the *oxy* form generally present in the resting enzyme preparations. The length of the lag time depends on several factors: the enzyme source; the concentration of monophenol (the lag period being longer when monophenol concentration is increased); the enzyme concentration (with the lag period diminishing, but never totally disappearing, when the enzyme concentration is increased); and finally, the presence of catalytic amounts of *o*-diphenol or transition metal ions, which completely abolish the lag period [60, 73].

3.2 Tyrosinase in Biomedical Applications

Tyrosinase-catalyzed oxidation is a well-known mechanism in nature, responsible for different natural processes, including the setting of mussel water-resistant

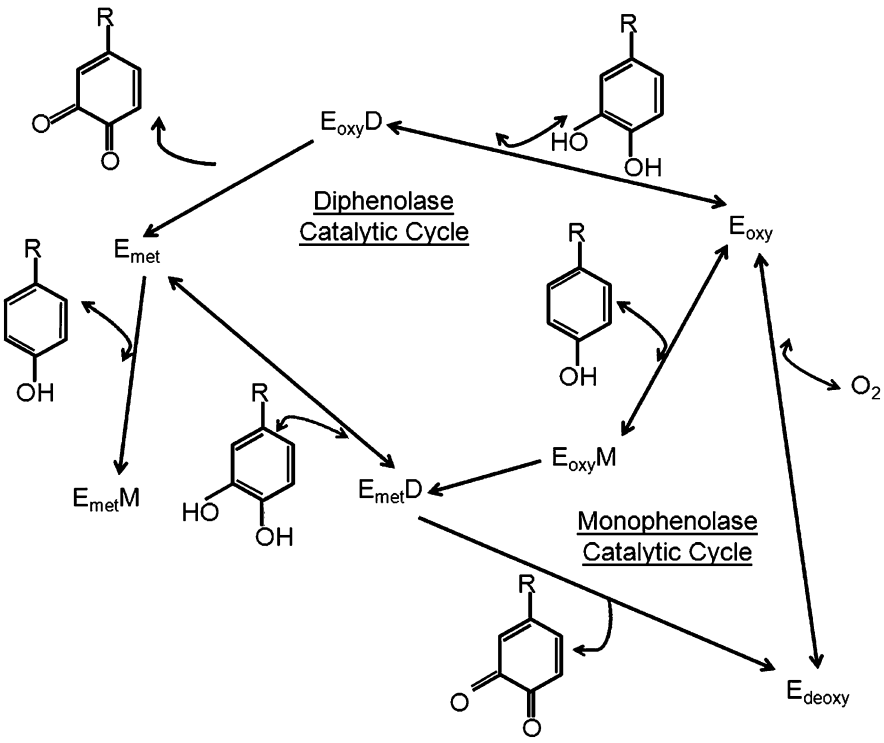

Fig. 5 Catalytic cycles of the two different oxygen-dependent reactions that occur consequently: the oxidations of both monophenols (cresolase or monophenolase activity) and *o*-diphenols (catecholase or diphenolase activity). E_{oxy}, E_{met} and E_{deoxy} are the three types of tyrosinase. $E_{oxy}D$, $E_{oxy}M$ and $E_{met}D$ are E_{oxy}–diphenol, E_{oxy}–monophenol and E_{met}–monophenol complexes

adhesive (by a three-dimensional gel network that gives cohesive strength to the mussel) [101] and the hardening of insect shells during the sclerotization process [43] by the tyrosinase-generated *o*-quinones.

Different biomimetic approaches exploiting enzymatic methods using tyrosinase have been used to prepare protein-polysaccharides conjugates and to create hydrogels from biopolymers for tissue engineering application.

The biochemical approach for preparing protein (or peptide)-polysaccharide bioconjugates via the tyrosinase-catalyzed oxidation-grafting reaction has been investigated for medical, textile and biotechnological applications. Branched copolymers of proteins and polysaccharides, organized to form networks with distinct viscoelastic properties, are very common in nature. Examples include the peptidoglycans of bacterial cell walls, the proteoglycans of the connective tissue and the mucin-type glycoproteins. These complex conjugates are expected to confer appropriate functions and important mechanical properties to the materials.

Different proteins containing tyrosine residues have been analyzed and linked to the polysaccharides (mainly chitosan) such as gelatin [16], cytochrome *c* [20],

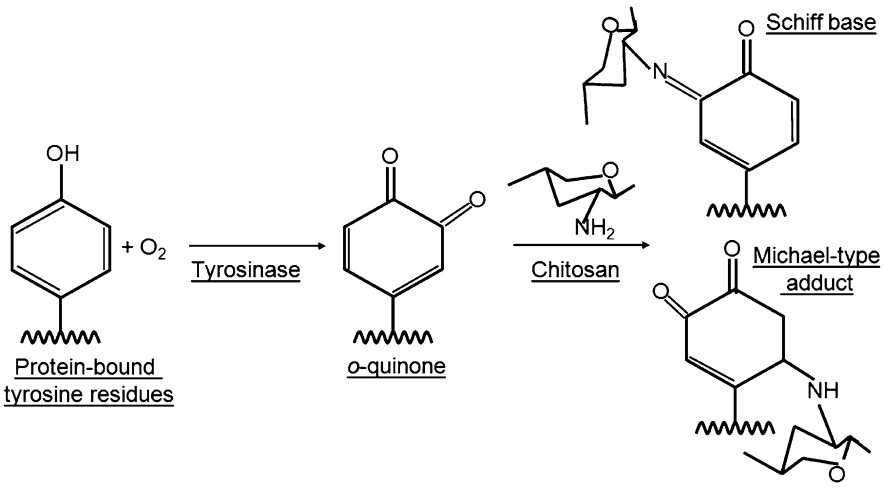

Fig. 6 Tyrosinase-catalyzed reactions for the conjugation of a tyrosine-containing peptide to chitosan

organophosphorous hydrolase [20], green fluorescent protein [17, 19], casein [1] and horseradish peroxidases [59]. As reported by Jee et al. [55], the tyrosinase-catalyzed oxidation and crosslinking of tyrosine-containing peptides take place when the tyrosine residue is located in the N-terminus or in the middle of the peptide.

Gelatin and chitosan represent ideal materials for tissue engineering applications, because they are biodegradable and contain structural groups similar to natural extracellular components [44]. Precisely, gelatin is a partial derivative of collagen that promotes cell adhesion [71]. Gelatin contains a lower number of tyrosyl residues (0.4% of the total residues) with respect to its collagen precursor due to the possible cleavage of the telopeptide region in some of the protein chains during the preparation.

Gelatin has been blended with chitosan to improve the biological activity since (i) it contains RGD-like sequences [52] that promote cell adhesion and migration, and (ii) it may form a polyelectrolyte complex with chitosan. Gelatin-chitosan scaffolds have been prepared in a crosslinked and uncrosslinked form and tested for the regeneration of various tissues including skin [17, 19], cartilage [104] and bone [40].

The mechanism of protein-chitosan conjugation has been investigated and proposed according to a reaction scheme recently developed for the silk-fibroin chitosan polymer system [35].

As shown in Fig. 6, the reaction mechanism of tyrosinase with protein-bound tyrosine residues involves in the first step the hydroxylation of 3,4-dihydroxy-phenylalanine, followed by oxidation to the corresponding *o*-quinone.

In the second step, the *o*-quinones are active species that can condense with each other or react with nucleophiles, such as the amine and sulphydryl groups of

protein-bound amino acid residues, or the amino groups of chitosan. Although the mechanism of the non-enzymatic reaction step is not well known, the hypothesis is that quinones can undergo at least two different types of reaction with NH_2 groups, i.e., via Schiff bases and/or Michael-type adducts [17, 19]. Furthermore, oxidized tyrosyl residues may undergo reactions with lysyl residues of gelatin, but the reaction with chitosan amino groups is favored because of the lower chitosan pK_a (6.3) [84] than for lysyl residues (pK_a 10) in proteins. Chen et al. [17, 19] have demonstrated that tyrosinase can oxidize tyrosyl residues of gelatin, providing chemical and physical evidence for gelatin-chitosan conjugation.

Tyrosinase-mediated chitosan modification is based on the activation of a phenolic substrate into a reactive *o*-quinone, and this in situ activation may offer safety advantages over modification methods that require the use of reactive reagents. Another potential advantage is that the tyrosinase-catalyzed chitosan modification may have generic utility because these enzymes are robust, only require molecular oxygen as a co-substrate, catalyze reactions under mild conditions and have a broad substrate range for phenols [75, 111].

A biomimetic approach exploiting enzymatic methods using tyrosinase to create hydrogels from biopolymers has been reported by Chen [17, 19]. Hydrogels are providing new opportunities for a variety of medical applications. Examples include the use of hydrogels as skin substitutes [58], adhesives [79], matrices for drug delivery [30] and scaffolds for tissue engineering [62]. In many of these applications hydrogel in situ formation is pursued. A system which is able to be injected as a solution and undergoes gelation only after injection requires minimally invasive methods for its implantation [33]. Furthermore, in situ gel formation would lead to filling up of all the available space [41]. Obviously, the main requirement for such gel forming systems is their biocompatibility and lack of toxicity.

Yamada et al. [110] explored in situ generation of gelatin/chitosan hydrogels that can be used as emergency burn dressings. Specifically, they discovered that gelatin and chitosan can be applied directly onto the burn, and gelling can be initiated by the addition of tyrosinase. For this application, the gel needs to form rapidly and to achieve sufficient mechanical strength to be retained as a bandage. This burn dressing offers an approach to protect the wound due to the hemostatic, antimicrobial activities of chitosan as well as the ability to gelatinize promoting cell attachment. Finally, to prevent further tissue damage, loss of strength and dissolution of the gel is recommended during the healing process [110].

Moreover, tyrosinase-catalyzed activity is not limited only to protein substrates. Burke et al. have focused their work on the modification of biocompatible polymers such as poly(ethylene glycol) with DOPA in an effort to impart adhesive qualities to the polymers for biomedical application. Although PEG itself is not adhesive, it represents a candidate building block for a synthetic tissue adhesive because of its high water solubility, low immunogenicity and toxicity, and availability of end groups easily modifiable with amino acids and peptides [61]. Burke et al. [12] synthesized several linear and branched PEG molecules with end groups modified by DOPA residues and have characterized their oxidation-induced

crosslinking through tyrosinase to form robust hydrogels. Under optimal conditions, aqueous solutions of these polymers formed rigid hydrogels within 30s of mixing with tyrosinase oxidizing reagent [12].

Recently, a tyrosinase-catalyzed oxidation-grafting reaction has been used by [29] for the conjugation of de novo designed coil peptides, called Kcoil and Ecoil, that heterodimerize in a highly specific and stable fashion to adopt a coiled-coil structure, characterized by the repetition (five times in each peptide) of K–V-S-A-L-K-E and E-V–S-A-L-E-K heptads, respectively. Demolliens et al. [29] studied the conjugation of the Ecoil/Kcoil system with chitosan to obtain a scaffold or particles for subsequent immobilization of proteins tagged with the Ecoil peptide. Precisely, the system Kcoil/chitosan has been found to be able to recruit the Ecoil-tagged epidermal growth factor (EGF) via coiled-coil interactions. To achieve this purpose, the same enzymatic protocol described above was used, in which tyrosinase-catalyzed oxidation of a tyrosine-containing Kcoil peptide was followed by its condensation onto chitosan in a non-enzymatic step. A limitation of the approach was the limited stability of the coiled-coil complex [29].

4 Conclusion

This chapter reviews the research efforts dedicated to the modification of biomaterials with bioactive molecules through enzyme-catalyzed reactions, with the aim to design biomimetic scaffolds for tissue engineering or other biomedical applications. The reactions catalyzed by two different classes of enzymes—transglutaminase and tyrosinase—for the biomimetic modification of scaffolds were illustrated. The enzymatic method is an interesting alternative to other physical and chemical approaches commonly used for the preparation of biomimetic materials due to the fact that enzymes catalyze chemical reactions under mild conditions (such as body temperature and buffered aqueous solutions at neutral pH), minimizing the risks of peptide/protein denaturation. Tranglutaminases are a family of calcium-dependent enzymes which catalyze an acyl transferase reaction between the γ-carboxamide group of peptide-bound glutamine and various primary amines; it is expressed in a variety of cell types and have the natural function to crosslink ECM proteins. The potential applications of TGase enzymes for tissue repair and reconstruction were extensively described. The TGase-catalyzed reactions may be performed to confer biomimetic properties to biomaterials through different approaches, such as (i) the crosslinking between lysine and glutamine residues of proteins to enhance the mechanical properties and the in vivo stability of scaffolds based on natural polymers; (ii) the obtainment of in situ gelling hydrogels mimicking the same crosslinking approaches existing in biology and (iii) the functionalization of scaffolds with appropriate ligands for cell adhesion and/or growth factors. The potential of tyrosinase (an enzyme that catalyses the oxidation of phenols including tyrosine) to mediate the coupling of bioactive molecules was also described in this work. Tyrosinase activity is not

limited to low molecular weight substrates, but it can also oxidize the phenolic moieties of polypeptides and proteins. For this reason, the mechanism for preparing protein (or peptide)–polysaccharide bioconjugates was discussed. These complex conjugates are able to confer the material appropriate functions and important mechanical properties. For instance, gelatin/chitosan bioconjugates are suitable materials for applications as skin substitutes, adhesives, matrices for drug delivery and scaffolds/hydrogels for tissue engineering.

As reported in this chapter, along with the biocompatibility of the starting materials and mild processing conditions of enzymatic reactions, the biomimetic approach through transglutaminase and tyrosinase activity is a novel and promising strategy for the engineering of a variety of functional, biomimetic materials with properties that are especially well suited for tissue engineering and other medical applications.

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Supramolecular Polymers Based on Cyclodextrins for Drug and Gene Delivery

Jia Jing Li, Feng Zhao and Jun Li

Abstract Supramolecular polymers based on cyclodextrins (CDs) have inspired interesting and rapid developments as novel biomaterials in a broad range of drug and gene delivery applications, due to their low cytotoxicity, controllable size, and unique architecture. This review will summarize the potential applications of polyrotaxanes in the field of drug delivery and gene delivery. Generally, cyclodextrin-based biodegradable polypseudorotaxane hydrogels could be used as a promising injectable drug delivery system for sustained and controlled drug release. Temperature-responsive, pH-sensitive, and controllable hydrolyzable polyrotaxane hydrogels have attracted much attention because of their controllable properties, and the self-assembly micelles formed by amphiphilic copolymer threaded with CDs could be used as a carrier for controlled and sustained drug release. Polyrotaxanes with drug or ligand conjugated CDs threaded on a polymer chain with a biodegradable end group could be useful for controlled and multivalent targeted delivery. In the field of gene delivery, cationic polyrotaxanes consisting of multiple OEI-grafted CDs threaded on a block copolymer chain are attractive non-viral gene carries due to the strong DNA-binding ability, low cytotoxicity, and high gene delivery capability. Furthermore, cytocleavable end-caps were introduced in the polyrotaxane systems in order to ensure efficient endosomal escape for intracellular trafficking of DNA. The development of the supramolecular approach

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using CD-containing polyrotaxanes is expected to provide a new paradigm for biomaterials.

Keywords Cyclodextrin · Drug delivery · Gene delivery · Polypseudorotaxane · Polyrotaxane · Supramolecular structure

Abbreviations

CD	Cyclodextrin
PEI	Polyethylenimine
OEI	Oligoethylenimine
PEG or PEO	Poly (ethylene glycol) or poly (ethylene oxide)
P (EO-r-PO)	Poly [(ethylene oxide)-ran-(propylene oxide)]
PEO–PHB–PEO	PEO–poly [(*R*)-3-hydroxybutyrate]–PEO
PCL–PTHF–PCL	Poly (ε-caprolactone)–poly(tetrahydrofuran)–poly (ε-caprolactone)
PNIPAAm	Poly (*N*-isopropylacrylamide)
PEO–PCL	Poly (ethylene oxide)-b-poly (ε-caprolactone)
PEG-PDMAEMA	Poly (ethylene oxide)-block poly ((dimethylamino ethylmethacrylate)
PAMAM	Polyamidoamine
L-Phe	L-phenylalanine
CEE	Carboxyethylester
DMAB	4-(*N*,*N*-dimethylamino) benzoyl
hPEPT1	Human peptide transporter
H–L-PheGlyGly	L-phenylalanlycylglycine
MA	Methacrylate
LMWP	Low molecular weight protamine
LCST	Lower critical solution temperatures
BAEE	*N*-α-benzoyl-arginine ethylesterv
AEC	Aminoethylcarbamoyl
DMAE	Dimethylaminoethyl

Contents

1 Introduction

The structure of polyrotaxanes with an appropriate polymer axle, macrocycle wheels threaded on the axle, and bulky terminal groups capped on the end of the axle to avoid the dethreading of the macrocycle wheels has attracted great interest for several decades [1–4] (Fig. 1b). Cyclodextrins (CDs) are a series of natural cyclic oligosaccharides composed of 6, 7, and 8 D(+)-glucose units linked by α-1,4-linkages, named α-, β-, and γ-CD, respectively (Fig. 1a), which have been approved by the FDA as food additives. The most common pharmaceutical applications of CDs are to improve the physical properties of drug molecules, such as solubility, stability, and bioavailablity [5]. The presence of 2-, 3-, and 6-hydroxyl groups on the CD ring provides opportunities for structural modification to improve their natural properties and to allow versatile applications [6]. The prominent characteristic of polyrotaxanes is the absence of any covalent bond between the axles and wheels, and the necklace-like structure can be divided into pieces with the use of biocleavable axles or end-caps. Since the most remarkable synthesis of polyrotaxanes composed of α-CD rings threaded on a poly(ethylene oxide) (PEO) chain and capped with bulky terminal groups (Fig. 1), overwhelming interest has focused on the study of the supramolecular structure of polyrotaxanes and their biomedical and pharmaceutical applications [1–3, 7–12].

Polymeric hydrogels have been broadly investigated for biomedical applications because of their high water content and generally favorable biocompatibility [13–16]. Hydrogels provide a favorable environment for protein and peptide drugs, and a controlled release can be achieved via adjustment of the crosslinking density of the structure [17]. Chemical supramolecular hydrogels are usually synthesized from polypseudorotaxanes or polyrotaxanes by covalently binding the cyclodextrin rings with different kinds of linkers [18–21] or modification of polymer chains [22]. Chemical hydrogels were often designed to be biodegradable with hydrolyzable threading polymers for potential applications for drug or gene delivery. Physical supramolecular hydrogels are commonly superior to chemical hydrogels on preparation, purification, drug loading, and other properties. Tremendous CD-based physical hydrogels are self-assembled molecules with high molecular

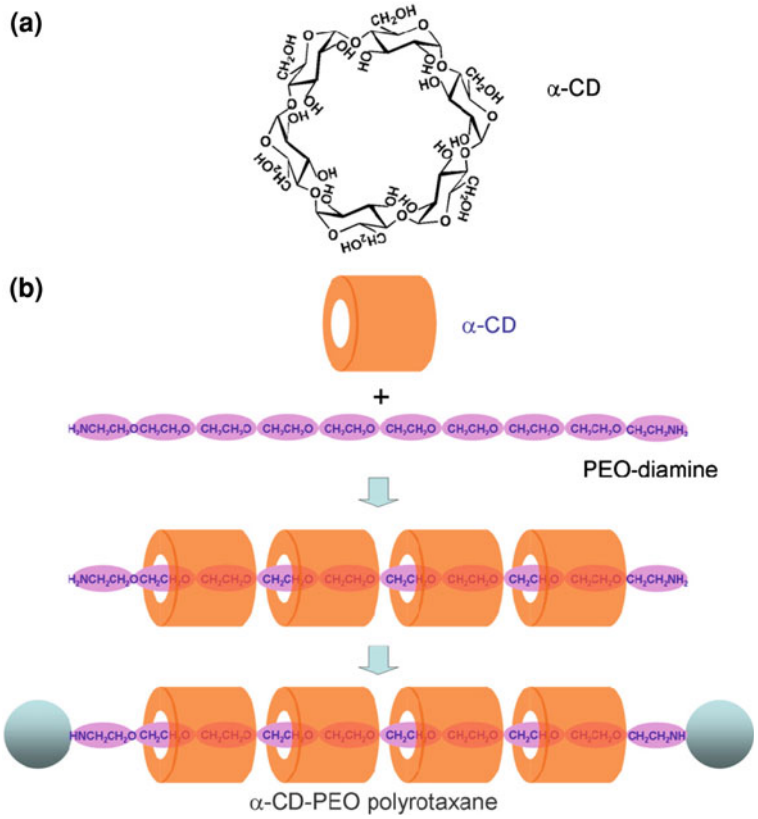

Fig. 1 Demonstration of CD and polyrotaxane: **a** structure of α-CD, **b** synthesis of polyrotaxane from α-CD and PEO-diamine [44]

PEG thanks to their hydrophilic property helping to form hydrogels with the water-insoluble CD-threaded counterparts, and their biodegradability and biocompatibility suitable for in vivo application. Hydrogel formations between block copolymers containing PEO block and cyclodextrin rings were investigated [17, 23–26]. The thermo-reversible and thixotropic properties of physical supramolecular hydrogels were promising as injectable drug delivery systems.

In comparison with viral vectors, cationic polymers such as polyethylenimine (PEI) as a non-viral vector applied for gene delivery showed superior properties of being economic, stable, and easy to modify. However, the application of PEI is limited by its high cytotoxicity and non-biodegradability. The introduction of cyclodextin to construct star-shaped cationic polymers showed lower cytotoxicity and efficient gene transfection [27, 28]. A new class of CD-based polyrotaxanes where multiple PEI-modified cyclodextrin rings are threaded onto a polymer chain and capped by bulky end-caps has been developed. The new supramolecular structure showed efficient DNA-binding capability, and the CD rings can freely move over the polymer chain [29–32]. Yui et al. reported polyrotaxane-based gene

delivery systems with aminoethyl-modified and dimethylaminoethyl-modified CD rings with biocleavable polymer chains [33–35]. The transfection activity was related to the DNA release rate, and high gene transfection efficiency was acheived.

This chapter focuses on the drug delivery and gene delivery applications of polypseudorotaxanes and polyrotaxanes composed of CD rings and various linear polymers. The synthesis of biodegradable hydrogels for potential applications in controlled and sustained drug delivery, drug-conjugated polyrotaxanes for targeting drug delivery, and cationic polyrotaxanes for gene delivery will be discussed.

2 Cyclodextrin-Based Supramolecular Hydrogels or Micelles for Drug Delivery

Since both PEO and CDs are known to be biocompatible and the latter have been approved by the FDA as food and drug formulations, supramolecular inclusion complexes between CDs and PEO or their copolymers as a new class of bioabsorbable systems for controlled drug delivery have been developed. Several attempts have been made to use cyclodextrin-based supramolecular polymers for drug delivery, and an increasing number of polyrotaxane or polypseudorotaxane constructs are appearing in the literature. Based on different methods for introducing drugs or proteins, there have been cyclodextrin-based supramolecular hydrogels or micelle vehicles for sustained drug release and drug-conjugated biodegradable polyrotaxanes for controlled and targeted drug delivery.

Polymeric hydrogels are attractive biomaterials for many biomedical applications, including drug and gene delivery, controlled release devices, and substrates for soft tissue engineering. Due to their high biocompatibility and high water content, they are widely used for controlled release of delicate bioactive agents such as drugs and proteins. In contrast to chemically crosslinked gels, they can be obtained via non-covalent interaction, and the drug loading can be achieved simultaneously in an aqueous environment without impairing the chemical integrity of the drug. In addition, the non-covalent interactions are always reversible, which is an important property for drug delivery systems. Therefore, physical hydrogels formed by synthetic polymers and their potential applications for drug delivery systems have attracted much attention. Recently, studies on inclusion complexes formed between CDs and polymers have led to interesting developments of supramolecular hydrogels with potential application as biomaterials for controlled drug delivery.

2.1 Injectable Drug Delivery Systems Based on α-CD and PEO Homopolymer

Supramolecular hydrogels based on polypseudorotaxanes of CDs and polymers are some of the most attractive physical crosslinking hydrogels that can be applied as

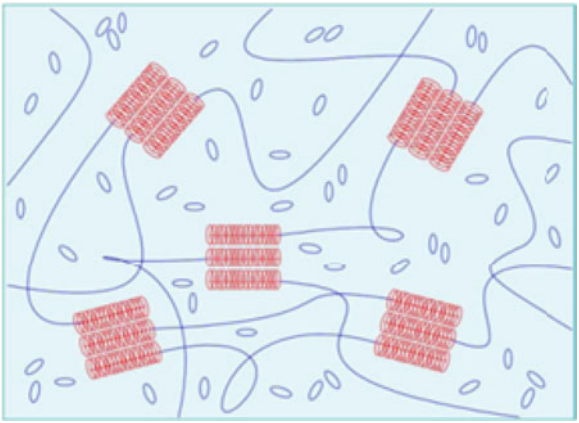

Fig. 2 Supramolecular hydrogel formed by partial inclusion complexation of high molecular weight PEO and α-CD

drug delivery systems. Generally, they are thixotropic and thermo-reversible, which are unique properties for injectable hydrogel drug delivery systems [17, 36, 37]. As a typical example, PEG of high molecular weight was found to form complexes with α-CD in aqueous solution to give gels with a wide range of concentrations [37, 38]. This is an interesting class of hydrogel formation that is based on physical crosslinking induced by supramolecular self-assembling with no chemical crosslinking reagent involved. The X-ray powder diffraction studies indicated that the supramolecular self-assembly was attributed to partial inclusion of PEG chains by α-CD, which consists of the interaction between both complexed α-CD and uncomplexed α-CD (Fig. 2) [38]. The time of gelation was found to decrease with increasing concentration of α-CD or PEG, but increases with increasing PEG molecular weight. Furthermore, the gel-melting temperature measurements showed that the gel-melting temperature increased with an increase in the PEG molecular weight or α-CD concentration, and decreased with an increase in the PEG concentration due to the formation of the longer and shorter α-CD/PEG complex domains, respectively.

According to rheological studies, the α-CD/PEG hydrogels displayed thixotropic and reversible properties. Specifically, the viscosity of the gels was found to be diminished when agitated (Fig. 3a) and was restored within hours without agitation (Fig. 3b). This property renders the hydrogel formulations injectable even through a fine needle [36]. Due to their thixtropic and reversible properties, these unique hydrogels can be applied to injectable and bioabsorbable drug delivery systems. For instance, some bioactive agents, such as drugs, proteins, or plasmid DNAs, can be incorporated into the gel in a syringe, followed by injection into the tissue under pressure because of the thixotropic property. Due to the thermo-reversibility, it will be restored as a hydrogel for controlled drug release (Fig. 4) [1]. Therefore, injectable hydrogels will be much more attractive than implantable hydrogels. The effect of the injection through needles on the viscosity of the hydrogels was further studied. It was found that the viscosity dropped

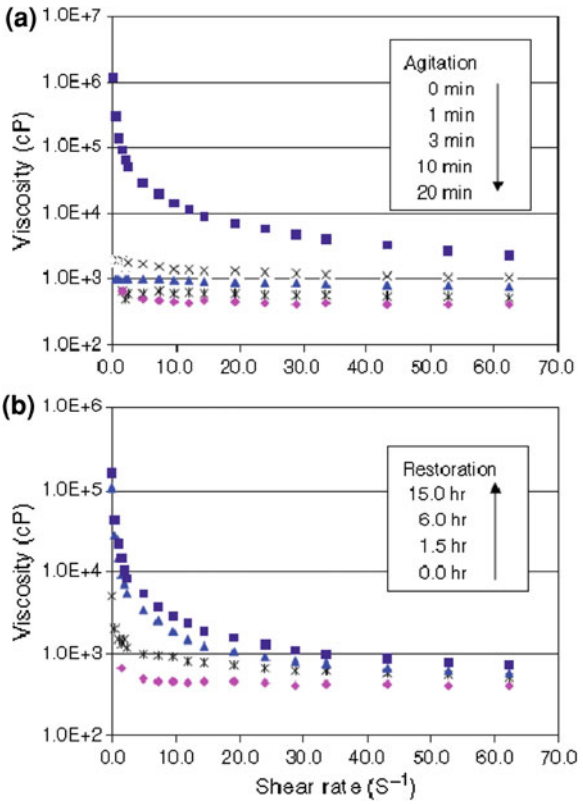

Fig. 3 Changes of the viscosities of Gel-20K-60 as a function of **a** agitation time at a shear rate of 120 s^{-1} and **b** restoration of the viscosities of the gel after 20 min of agitation at a shear rate of 120 s^{-1} [36]

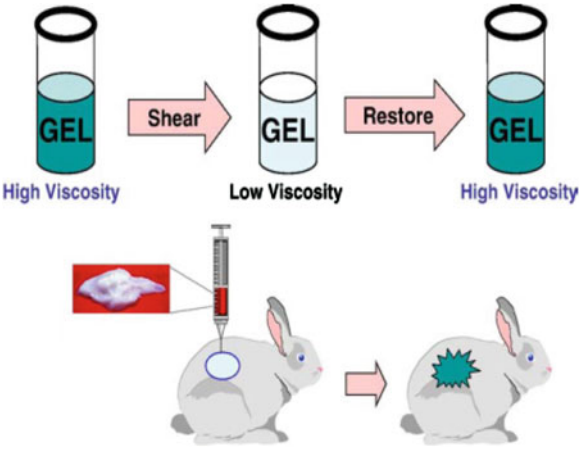

Fig. 4 Injectable drug delivery system based on polypseudorotaxane hydrogels formed between α-CD and PEG [1]

significantly after injection from the syringes because of the higher shear rate caused by the finer needle. Table 1 summarizes the maximum speed at which the hydrogels can be injected through a syringe with different needles. The hydrogels

Table 1 Maximum speeds at which the hydrogels can be injected through needles of different sizes

Needle size	Maximum injection speed of hydrogel (ml/min)			
	Gel-8K-60	Gel-10K-60	Gel-20K-60	Gel-35K-60
18G × 1.5″	[a]	75.1	32.8	8.93
19G × 1.5″	[a]	47.5	15.3	3.15
21G × 1.5″	33.9	13.8	5.72	1.66
22G × 1.5″	22.6	4.35	3.41	0.72
27G × 0.5″	2.61	1.01	0.36	0.09

Terumo 6-ml syringes were used for the experiments
[a] The injection speed is higher than 100 ml/min

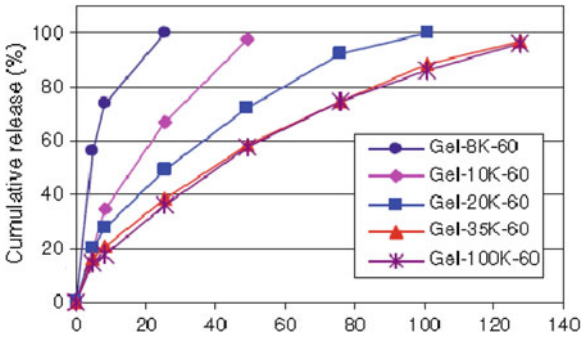

Fig. 5 In vitro release profiles for dextran-FITC (M_W 20,000) released from α-CD–PEO hydrogels formed from PEO of different molecular weights

formed with PEOs of higher molecular weights were more difficult to inject through fine needles [36].

These injectable supramolecular hydrogels are preferred for controlled drug delivery because the components of supramolecular hydrogels are potentially biocompatible, and drugs can be directly encapsulated into the gels at room temperature without any contact with organic solvents. In order to evaluate the in vitro release kinetics of these injectable α-CD-PEO hydrogels, the polypseudorotaxane gels were entrapped by fluorescein isothiocyanate-labeled dextran (dextran-FITC) and characterized in terms of in vitro release [36]. In this research, the release properties of the gel could be fine-tuned by varying the composition, molecular weight, and chemical structure of the polymer. As shown in Fig. 5, the release rate decreases sharply with an increase in the molecular weight of PEO up to 35,000 Da, and the release rate of gel formed with PEO 35,000 (Gel-35K-60) and 100,000 (Gel-100K-60) is quite sustainable. It was presumed that the erosion of hydrogel through de-threading of PEO chains from CD cavities contributed to dextran-FITC release, and the increased sustained release with increasing the PEO molecular weight is a result of the chain entanglement effect of PEO on upon dethreading. However, although Gel-100K-60 showed the most steady release rate with time, it hardly would be used as a drug delivery system because PEO at this large size would be undesirable for in vivo applications due to the difficulty of its clearance from the body.

2.2 Supramolecular Physical Hydrogels Formed by α-CD and PEO Copolymers for Drug Delivery

2.2.1 Supramolecular Hydrogels Formed by α-CD and PEO–PPO–PEO Triblock Copolymer

Although the properties of α-CD–PEO hydrogels are promising, it is challenging to apply this hydrogel for in vivo drug delivery. Firstly, these hydrogels are not suitable for long-term drug release because the physical interaction is weak in aqueous environments because of the hydrophilic property of PEO. In addition, the high molecular weight of PEO is not biodegradable, and it is difficult to eliminate it through the human kidney membrane. Therefore, regarding the first problem of α-CD–PEO hydrogels, a new supramolecular hydrogel self-assembled between α-CD and triblock copolymer PEO–PPO–PEO was studied for long-term and controlled release drugs [23, 25]. The gelation was induced by complex formation between the PEO segments of the PEO–PPO–PEO triblock copolymer and partially threaded α-CDs. It was found that the copolymers containing more than 25 wt% PEO segments can form hydrogels with α-CD at room temperature and low copolymer concentrations [23]. In addition to the injectable and reversible properties, similar to α-CD–PEO hydrogels, the polypseudorotaxane hydrogel formed with α-CD and PEO–PPO–PEO is thermo-sensitive. For example, the same formulation that gelled at room temperature did not form hydrogel at 4°C, where the hydrophobic interactions between PPO segments of PEO–PPO–PEO copolymer are weak. This also confirmed that the driving force for the gelation of PEO–PPO–PEO copolymers and α-CD solutions is a combination of the inclusion complexation between α-CD and PEO blocks and micelization of the middle PPO block.

Furthermore, unlike the thermo-responsive hydrogels formed only with PEO–PPO–PEO triblock copolymers [39, 40], the addition of α-CD largely reduced the concentration of the copolymer needed for gel formation. It may be because the partial formation of polypseudorotaxane between the EO blocks of EO–PO–EO with α-CD largely changed the hydrophobicity of the copolymer and significantly lowered its gelation concentration [25]. These aid effects of α-CD were further evidenced by the temperature-polymer concentration phase diagrams of PEO–PPO–PEO aqueous solutions in the absence and presence of α-CD with different α-CD concentrations (Fig. 6). The region where the pure $EO_{13}PO_{30}EO_{13}$ can form a hydrogel is very small (α-CD 0%), while the concentration must be very high. Increasing α-CD concentration increases the gelation regions, while the lowest concentration at which the copolymer can form a hydrogel becomes much lower with higher α-CD concentrations. This phenomenon proved the hypothesis that the extra inter-molecular hydrophobic interaction of the middle blocks can further strengthen the hydrogel network, resulting in the formation of more stable hydrogels for long-term controlled drugs release. The in vitro controlled release properties were studied using BSA-FITC as a model protein drug release from the

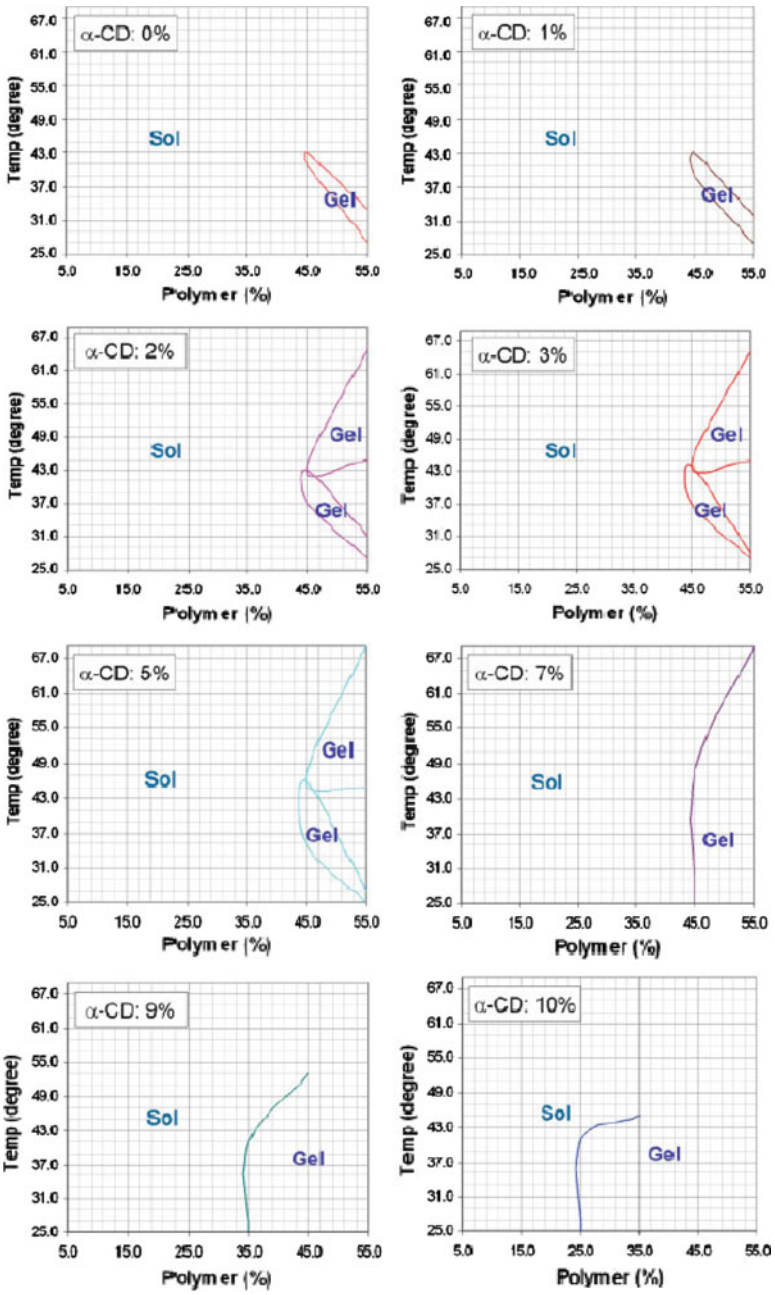

Fig. 6 The temperature-polymer concentration phase diagrams of PEO–PPO–PEO copolymer $EO_{13}PO_{30}EO_{13}$ aqueous solutions in the absence and presence of α-CD with different α-CD concentrations

hydrogel. It was found that the hydrogel with copolymer $EO_{10}PO_{44}EO_{10}$ can achieve sustained release for more than 1 week with linear release kinetics, while others are too unstable for long-term sustained delivery [1]. Pluronics have also been threaded on hydroxypropyl-β-CD. It showed enhanced solubility of the drug and a temperature-dependent sustained release [41].

2.2.2 Supramolecular Hydrogels Formed by α-CD and PEO–PHB–PEO Triblock Copolymer

Considering its high molecular weight, PEO is not suitable for filtration through human kidney membranes; therefore, a biodegradable polymer is preferred because of the degradation of material into low molecular weight compounds, which can then be eliminated from the body through natural pathways. PHB, as a poly (β-hydroxyalkanoic acid), is a natural, optically active, and biodegradable biopolyester with high crystallinity and hydrophobicity. It is an attractive polymer for biodegradable drug delivery systems. PEO–PHB–PEO triblock copolymer was found to form block-selective polypseudorotaxanes with α-CD (Fig. 7b) [42, 43]. In comparison with other copolymers, the PHB block in the PEO–PHB–PEO triblock copolymers had a very strong tendency towards self-assembly in aqueous environments. For hydrogel formation, it was found that the cooperation effect of complexation of α-CD with PEO block together with the hydrophobic interaction between the middle PHB blocks results in the formation of a supramolecular hydrogel with a strong macromolecular network [17].

Similar to the hydrogel, formation between PEO–PPO–PEO and α-CD, α-CD also aids the gel formation of PEO–PHB–PEO triblock copolymers at a low copolymer concentration. For example, despite the formation of micelles, 13 wt% of aqueous solution of both polymers did not form hydrogels; however, aqueous solutions containing 13 wt% of either PEO–PHB–PEO copolymers or 9.7 wt% of α-CD formed gels at room temperature. Here the inclusion complexes formed by α-CD and PEO blocks of PEO–PHB–PEO copolymers are thought to aggregate into microcrystals, which act as physical crosslinks and induce formation of a supramolecular polymer network, consequently resulting in the gelation of the solutions. The hydrophobic interaction between the PHB blocks also facilitated the formation of the polymer network. Therefore, the gelation of the aqueous solutions of PEO–PHB–PEO triblock copolymers and α-CD is the result of a cooperation of the inclusion complexation between α-CD and PEO blocks and the micellization of the PHB block of the triblock copolymers. Based on further X-ray diffraction studies, the structures of the supramolecular hydrogel formed by α-CD/PEO–PHB–PEO complex could be illustrated in Fig. 7c. In the complexes, PEO chains penetrated α-CD from both ends and then formed necklace-like inclusion complexes. Among each PEO block (Mn = 5,000), only a length of PEO segments (Mn = 2,000) or less from each end of the copolymers was supposed to be included by α-CD rings based on the previous studies of complex formation between α-CD and PEO–PHB–PEO triblock copolymer with PEO lengths of Mn

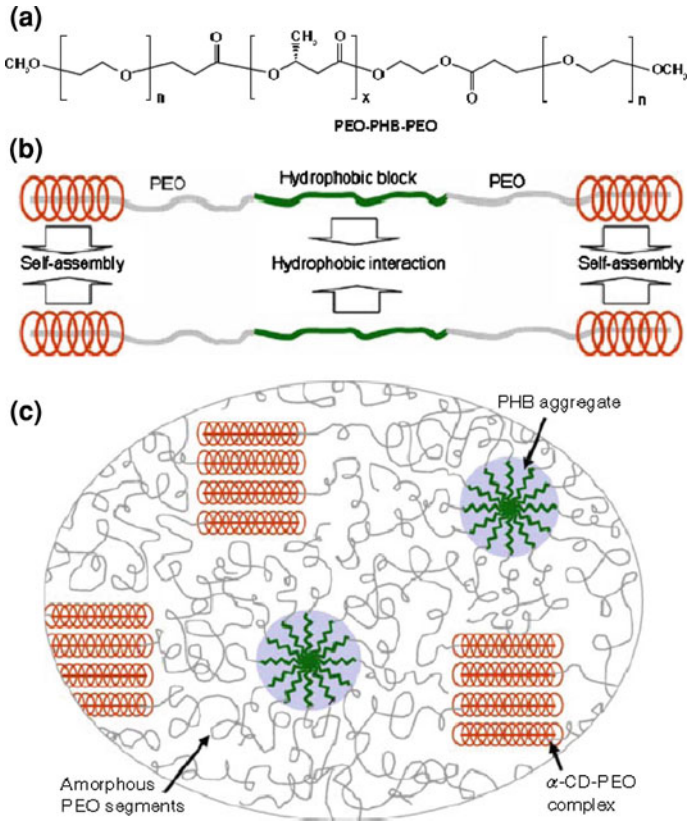

Fig. 7 **a** The stucture of PEO–PHB–PEO triblock copolymer; **b** schematic illustrations of the proposed structures of α-CD–PEO–PHB–PEO inclusion complex; **c** α-CD–PEO–PHB–PEO supramolecular hydrogel

2,000 [42, 43]. The hypothesis is also supported by the number of α-CD rings threaded on a PEO chain and capped by bulky groups at the two ends [44]. In the case of PEO–PHB–PEO block copolymer (PEO Mn = 5,000), the PEO segments covered by α-CD rings should be shorter than 2,000 from each end because of the steric hindrance effect, and the partial inclusion complexation of α-CD with PEO block with hydrophobic interaction of PHB blocks resulted in a strong network and a novel supramolecular hydrogel (Fig. 7c) [17].

In contrast to most physical hydrogels formed simply by copolymers, the in vitro release kinetics studies of the dextran-FITC model drug from this α-CD–PEO–PHB–PEO hydrogel showed that it was suitable for relatively long-term sustained controlled release of macromolecular drugs. Table 2 lists the dextran-FITC encapsulated α-CD–PEO–PHB–PEO hydrogels with different compositions that were used for the in vitro release kinetics studies. The α-CD–PEO (35,000) hydrogel formulation was used as a comparison. Specifically, based on

Table 2 α-CD–PEO–PHB–PEO hydrogels with different compositions that were used for in vitro release kinetics studies (Li et al. [17])

Hydrogel formulation	Polymer used	Gel composition (wt%)			
		α-CD	Polymer	Dextran-FITC	H_2O
Gel-1	PEO (35,000)	9.7	13.3	0.66	76.3
Gel-2	PEO–PHB–PEO (5,000–1,750–5,000)	9.7	13.3	0.66	76.3
Gel-3	PEO–PHB–PEO (5,000–3,140–5,000)	5.4	13.3	0.66	80.6
Gel-4	PEO–PHB–PEO (5,000–3,140–5,000)	7.9	13.3	0.66	78.1
Gel-5	PEO–PHB–PEO (5,000–3,140–5,000)	9.7	13.3	0.66	76.3

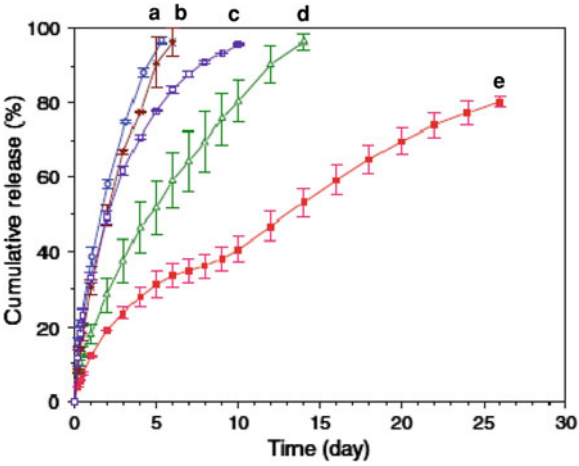

Fig. 8 In vitro release profiles for dextran-FITC released from α-CD–PEO–PHB–PEO hydrogels with different compositions in comparison with α-CD–PEO hydrogel: **a** Gel-1; **b** Gel-2; **c** Gel-3; **d** Gel-4; **e** Gel-5 (Li et al. [17])

Fig. 8, which shows the in vitro release profiles for dextran-FITC released from the supramolecular hydrogels, it can be noted that the α-CD–PEO–PHB–PEO (5,000–3,140–5,000) hydrogels with reasonably high α-CD concentration (9.7 wt%, Gel-5 in Fig. 8) showed an excellent controlled release property, sustaining the release of dextran-FITC for more than 1 month. This is a large improvement when compared with α-CD–PEO homopolymer hydrogel [36]. Futhermore, the hydrogels with lower α-CD concentrations had a much faster release rate, indicating the complexation between α-CD and PEO blocks is important in forming a stable supramolecular gel. Interestingly, α-CD/PEO–PHB–PEO hydrogel with a shorter PHB block only sustained the release for 6 days (Fig. 8, Gel-2), although its α-CD concentration was high (9.7 wt%). It shows that the PHB block length also played a critical role in the stability of the supramolecular hydrogels, which further supported the hypothesis that the cooperation effect of both α-CD complexation with PEO segments and the hydrophobic interaction between PHB blocks result in the formation of hydrogels with strong supramolecular properties. These findings indicate that the properties of the supramolecular hydrogels can be fine-tuned not

only with different lengths of the PHB block, but also with different copolymers, which may open up a wide range of possible drug delivery applications [17].

Like the α-CD–PEO hydrogel, this hydrogel was also found to be thixotropic and reversible. More importantly, PHB is a natural biodegradable biopolyester produced by microorganisms as intracellular carbon and energy storage material [45, 46]. PHB implanted in the animal body was found to undergo hydrolytic degradation and showed good tissue biocompatibility. A copolymer of PHB with PEO should be biodegradable under physiological conditions [47, 48]. Therefore, the biodegradable PEO–PHB–PEO copolymers will have the advantage that the hydrogel will be bioabsorbable and dissociate after drug release, which is more beneficial for the human body. This new supramolecular hydrogel is promising for relatively long-term sustained controlled delivery of macromolecular drugs as an injectable formulation.

2.2.3 Supramolecular Hydrogels Formed by α-CD and PEO–PCL Diblock Copolymer

Similarly, with copolymer PEO–PHB–PEO, another amphiphilic biodegradable copolymer PEO–PCL was also studied in order to form polypseudorotaxane hydrogels with α-CD [26]. In this case, the dethreading of PEO–PCL diblock copolymer chains from α-CD could be assumed to be retarded due to the strong hydrophobic interaction between PCL blocks. The biodegradable hydrogel could be formed by supramolecular self-assembly of the PEO–PCL diblock polymer chains partially included by α-CD molecules in aqueous solution. The same as hydrogel formed by PEO–PPO–PEO/α-CD and PEO–PHB–PEO/α-CD complexes [17, 23], the driving force of the supramolecular structured hydrogel was a combination of the inclusion complexation between the PEO block of PEO–PCL diblock copolymer and α-CD and the hydrophobic interaction between uncovered PCL blocks.

In the research of in vitro release, it was found that when amphiphilic PEO–PCL diblock copolymer was employed instead of pure PEO, the sustained release increased significantly, and almost 20% of the entrapped dextran-FITC was still retained in the hydrogel up to 1 month, even if the molecular weight of the PEO block was only about 5,000. Thus, the requirement of high molecular weight PEO for a long-term delivery system is avoided. This could be attributed to the strong hydrophobic interaction between the uncovered PCL blocks, which was believed to retard the dethreading of PEO–PCL chains from α-CD cavities to release entrapped dextran-FITC. In addition, the drug-carrier properties of supramolecular hydrogels are also influenced by the amount of α-CD in the system. Because the higher α-CD content leads to more α-CD threaded onto PEO–PCL chains, the release rate was accelerated with decreasing α-CD content. For example, the crosslinking density in α-CD (9.7 wt%)/PEO–PCL hydrogel was higher than that in α-CD (8.0 wt%)/PEO–PCL hydrogel, and the α-CD (9.7 wt%)/PEO–PCL hydrogel is more stable and more difficult to dissociate to release entrapped dextran-FITC through dethreading PEO–PCL chains from α-CD cavities [26].

The similar properties of α-CD/PEO–PCL hydrogel as those of α-CD/PEO–PHB–PEO hydrogel indicate that the strategy for fabrication of supramolecular hydrogel through inclusion complexation is also feasible with other types of biodegradable amphiphilic block copolymers besides amphiphilic triblock copolymers, and due to the thixotropic and reversible properties, the new supramolecular hydrogels are promising for minimally invasive therapeutic delivery application with fine-tuned properties [26].

2.3 *Supramolecular Physical Hydrogels Formed by Cyclodextrins with Other Copolymers*

Based on the similar mechanism that α-CD could thread on part of PEO segments in a block or homo polymer to form a supramolecular physical crosslinked structure, an increasing number of polypseudorotaxane-based physical hydrogels are appearing in the literature. For example, by mixing α-CD, PEG, and PAA in an aqueous solution, a competition between host-guest and hydrogen-bonding interactions occurs. The dynamic property of polypseudorotaxane can be strengthened by increasing the α-CD amount, and when the α-CD content reaches 20%, a thermo-responsive hydrogel is formed [49]. Moreover, there were a few reports on hydrogel formation between α-CD and PEO or PPO-grafted polymers. Supramolecular physical gelation can be introduced by a specific host-guest interaction between α-CD and PEO-grafted dextrans [50]. The obtained hydrogels exhibit a unique thermo-reversible gel-sol transition based on supramolecular assembly and dissociation, and the transition temperature was controllable by the polymer concentration or PEG content. Similar hydrogels can be formed between α-CD and PEO-grafted chitosan [51], α-CD and PEO-grafted hyaluronic acid [52], β-CD and PPO-grafted dextran copolymer [53], and α-CD and highly densely PEO grafted polymer brushes (PBIEM-g-P(PEOMA)) [54].

Recently, temperature-responsive or pH-sensitive polyrotaxane hydrogels have attracted much attention concerning drug delivery systems because of their controllable properties and supramolecular functionality. As mentioned above, supramolecular-structured hydrogels prepared on the basis of inclusion complexation between PEG-grafted dextrans and α-CD were found to show thermo-reversible gel-sol transition [50]. In this research, the grafted copolymer inclusion complexes (Mn of PEG = 750) exhibited three kinds of phases: opaque gel, opaque solution, and sol phase. When the supramolecular structures were formed, the solutions became opaque and immobile (opaque gel). As the temperature increased, they were still opaque but mobile (opaque solution). With further increased temperature they became a clear mobile solution (sol phase). On the other hand, they would return to the opaque gel phase again if the temperature decreased below a certain temperature, which represented the gelation temperature ($T_{gelation}$). The properties of the hydrogels, gel melting, and gelation are closely related to supramolecular assembly and dissociation due to a threading-dethreading process of the

α-CD molecules along the PEG grafts. Since the inclusion complexation of a polymer chain into the CDs is entropically unfavorable and prompted by attractive interactions, such as hydrogen bonding and hydrophobic interactions, the temperature increase can induce dissociation of the polymer chains from the CDs with a restoration of its intrinsic entropy from random conformations in solution, and vice versa [55]. Similarly, a PPO-grafted dextran copolymer was used to form thermoreversible hydrogel with β-CD, where the physical crosslinking was induced by the inclusion complex formation between PPO segments and β-CD [53].

Another kind of temperature- and pH-responsive supramolecular hydrogel was synthesized based on the principle of polymer inclusion complex (PIC) formation between PEG-modified chitosans and α-CD [51]. With simple mixing, the resultant supramolecular assembly of the PEG-grafted chitosan polymers and α-CD molecules led to hydrogel formation in aqueous media. The inclusion complex domains played an important role in holding together hydrated chitosan chains as physical junctions. And the gelation property was affected by several factors, including the PEG content in the polymers, the solution concentration, the mixing ratio of host and guest molecules, temperature, pH, etc. Under acidic conditions all the hydrogels exhibited thermo-reversible gel-sol transitions under appropriate conditions of mixing ratio and PEG content in the mixing process. In contrast to the hydrogels formed by a combination of PEG-grafted dextran and α-CD, the gelation temperatures showed similar trends, while the PEG-grafted chitosan/α-CD hydrogel exhibited relatively higher $T_{\text{gel-m}}$. It could be attributed to an enhanced PIC stability arising from the longer chain length of PEG and the higher solution viscosity of chitosan. The hydrogels also showed pH-dependent properties because of the pH-dependent water solubility of chitosan. Such unique properties of this supramolecular hydrogel may extend their potential application in drug delivery systems.

Recently, a water-soluble polyrotaxane composed of multiple methylated α-CD rings threaded on a high molecular weight PEO chain and end-capped by bulky adamant groups was developed [56, 57]. Methylation of the hydroxyl groups on α-CD molecules resulted in a significant improvement of water solubility, and this Me-PR exhibited thermoreversible sol-gel transition in water depending on the degree of methylation. For low degrees of methylation (≤30%), a Me-PR solution was transparent even up to 80°C, while for high degrees of methylation (≥60%), it became opaque with increasing temperature, and gelation took place at high temperature. It was found that a microphase-separated structure was formed, and it formed a network structure. Based on the X-ray diffraction, microdifferential scanning calorimetry, and rheometry, the authors concluded that the crystal-like crosslinks of Me-PR in water originated from the regularly ordered structure of methylated α-CDs along a PEG chain. The temperature dependence of the Me-PR solution is induced not merely by the hydrophobic association among methylated α-CD, but also by the strong crystal-like aggregation as proved by the X-ray diffraction study. Most recently, inclusion complexation between γ-CD and two PEO chains, or the bundling of two PEO chains within the γ-CD cavities, was also used to form a supramolecular polymer network that led to formation of hydrogels [58].

2.4 Supramolecular Chemical Hydrogels Formed by Cyclodextrins with Polymers

Apart from the physical hydrogel, chemical gel crosslinked by biodegradable polyrotaxanes having ester linkages at the terminal of PEG is also reported to be good for drug delivery. These supramolecular networks are also called "sliding gels" [18, 21, 59]. Among all the chemical crosslinked hydrogels, the biodegradable hydrogels were most desirable for drug delivery because the biodegradability avoids the removal of implanted gels when no longer needed. Typically, Yui and coworkers synthesized a series of controlled hydrolyzable polyrotaxane hydrogels for drug delivery and tissue engineering. The supramolecular hydrogel consists of α-CDs and PEG chain capped with bulky end groups (Z-L-Phe) via ester linkages, and the α-CDs were linked with other PEG chains to form hydrophilic PEG networks (Fig. 9a) [60, 61]. The supramolecular crosslinked structure can generate hydrogels capable of swelling between 138 and 1,566% in water. From the results of the gel degradation study, the fastest rates of degradation (∼ 8 h) were observed when the polyrotaxane gels were exposed to basic conditions (pH 8). Slower erosion was achieved by decreasing the polyrotaxane content (i.e., the number of ester end-caps) and increasing the PEG/α-CD ratio, which indicated the number of PEG chains linked with one α-CD molecule (crosslinked density). The higher PEG/α-CD ratio prolonged the time of the hydrogel erosion, indicating the enhanced stability of ester hydrolysis in the hydrogels with a highly water swollen state, which was attributed to a protection of the terminal esters from hydrolysis by CD encapsulation. The prolonged erosion time could also be because of the lower polyrotaxane content; thus, higher PEG crosslink density increased the chance of more PEG being crosslinked to a threaded CD, resulting in enhanced stability. Furthermore, this phenomenon is quite anomalous from the conventional biodegradable hydrogel system: the three-dimensional network of these PEG hydrogels with a highly water swollen state was more stably maintained by using the polyrotaxane as a crosslinker. Thus, the time to reach complete hydrogel erosion was predictable by the

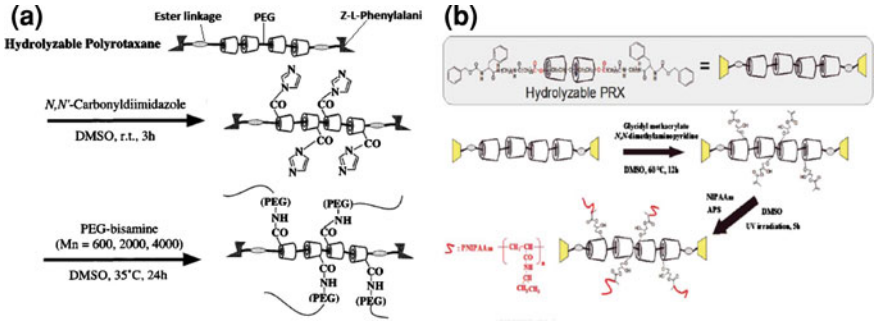

Fig. 9 **a** Preparation of PEG hydrogels crosslinked by the polyrotaxane; **b** preparation of PNIPAAm/MA-introduced PRX hydrogels

polyrotaxane content, and the erosion profile was dominated by the Mn of PEG-bisamine. Such independent control of both the erosion time and the erosion profile of hydrogels is concluded to be of great advantage when considering their use as a drug delivery matrix [62].

Because erosion time is controllable, the hydrogels can be new candidates as long-term stable but actually hydrolyzable hydrogels for scaffolding in tissue engineering. It was suggested that the cell recognizes the surface heterogeneity because of the polyrotaxane structure, and such PEG hydrogels with hydrolyzable polyrotaxane will provide a promising approach for reconstructing damaged tissues. Recently, these PEG/α-CD-based degradable polyrotaxane hydrogels were constructed into porous gels for bone and cartilage regeneration [63]. By seeding chondrocytes onto these highly porous hydrogel scaffolds, it was found that the micrograph showed well-adhered and spread chondrocytes in the pores [64]. These scaffolds were further decorated with carbonate-linked cholesterol, which not only improved the cell proliferation and glycosaminoglycan (GAG) production, but also controlled the degradation rate of the hydrogels [65]. In a later research, many primary amino groups were introduced into the porous polyrotaxane hydrogels, and a significant enhancement of chondocyte attachment was observed [66]. All the results suggested that PEG hydrogel scaffolds crosslinked with hydrolyzable polyrotaxane are a promising candidate for chondrocyte culture and cartilage engineering. In the field of bone regeneration, PEG-crosslinked biodegradable hydrogel was combined with various ratios of hydroxyapatite particles. The compositions containing a 1:1 ratio of polyrotaxane to hydroxyapatite displayed good osteoblast adhesion and appeared to be a good scaffold for bone regeneration.

Another biodegradable hydrogel for temperature-controlled erosion was prepared by crosslinking *N*-isopropylacrylamide (NIPAAm) and methacrylate (MA)-introduced polyrotaxane in which α-CDs were threaded onto a PEG chain capped with bulky end groups via ester linkages (Fig. 9b) [67]. PNIPAAm, as a well-known thermo-responsive polymer, exhibits lower critical solution temperatures (LCSTs) in aqueous solution where its hydration-dehydration change occurs reversibly in response to small temperature changes [68]. It can form an expanded structure below the LCST and form a compact structure above the LCST with dehydratation. In this system, the inclusion of PNIPAAm enables the regulation of the hydrogel degradation times by modulating the inclusion complexation between α-CDs and the ester linkage in polyrotaxanes. In comparison with the supramolecular hydrogel crosslinked by PEG, the complete erosion times of these hydrogels with PNIPAAm crosslinkers were shortened by elevating temperature, while the former one was not changed by elevating temperature. These results suggest that temperature-responsive PNIAAm/polyrotaxane hydrogels contributed to the enhanced degradation at elevated temperatures. It was assumed that temperature-dependent hydration and dehydration of PNIPAAm matrix in the PRX/PNIPAAm hydrogels would alter the inclusion and dissociation of the terminal ester linkages below and above the LCST, respectively. The aggregation of the PNIPAAm matrix above the LCST induces the sliding α-CD molecules from the terminal ester linkages, resulting in exposure of the ester linkage to the alkaline medium to enhance the

erosion. These supramolecular hydrogels could be useful for biomedical applications because of their biocompatibility and thermo-controllable property.

Some other hydrogels have been formed by UV-initiated radical crosslinking of pseudopolyrotaxanes. For example, a novel gelation occurs in water during supramolecular self-assemblies of α-CD being threaded onto amphiphilic LA–PEG–LA copolymer end-capped with methacryloyl groups. The hydrogel was thixotropic and reversible. After UV irradiation with a photoinitiator added in advance, they can be photopolymerized to give rise to chemically crosslinked biodegradable hydrogels with markedly improved mechanical strenghth [22]. The similar mechanism to form polyrotaxane hydrogel can be achieved by inclusion complex formation of α-CD with some other copolymers, such as temperature responsive and biodegradable PCL–PEG–PPG–PEG–PCL copolymer, PCL–PEG–PCL [69] copolymer, and PLA–PEG–PLA [22] copolymer. Furthermore, by dropping the hydrogel with NIPAAm prior to polymerization, it could be made thermo-responsive [70].

2.5 *Supramolecular Structured Micelles Based on Cyclodextrin for Drug Delivery*

Although supramolecular hydrogels or self-aggregates constructed from CDs involving inclusion complexes have been intensively investigated not only for their theoretical merits, but also for the potential drug delivery applications, the self-assembly micelles formed by amphiphilic copolymer threaded with CDs has not been used as a carrier for controlled drug release. Being different from the supramolecular hydrogel matrix for drug delivery, the polyrotaxane micelles could self-assemble the drug into nanometer-scale particles, which is always an attractive factor for effective drug delivery carriers. As a typical example, a kind of novel amphiphilic triblock copolymer containing polyrotaxane as a central block was synthesized via the ATRP of poly (ethylene glycol) methyl ether methacrylate (PEGMA) using 2-bromopropionyl end-capped pluronic F127/β-CD polypseudorotaxand as a macroinitiator at 25°C in aqueous medium (Fig. 10) [71]. Rather than the physically crosslinked hydrogels or the traditional PRs yielded as crystalline precipitates from aqueous solution, these triblock copolymers with a polyrotaxane central block flanked by two hydrophilic brushlike PEGMA oligomers would self-assemble into nano-sized particles with the unique core-shell structure in aqueous medium. In this system, PEGMA oligomers create a dense non-adhesive hydrophilic coating around the PR central block capable of suppressing protein and cell adhesion and extending the residence time of the particles as carriers for controlled drug release.

In order to examine the drug release property of the polyrotaxane micelle system, amphotericin B (AmB) was selected as a model drug to evaluate the potential of the resulting amphiphilic PR-containing triblock copolymers as carriers for the controlled release. AmB is a broad-spectrum chemotherapy and organ antifungal therapy for the treatment of systemic transplantation, but an aliphatic water-insoluble

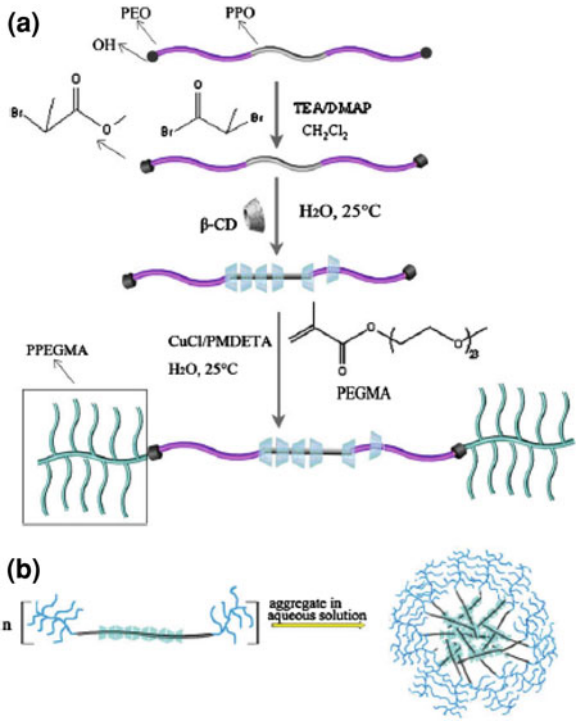

Fig. 10 **a** Synthetic pathway of PR-based triblock copolymer via ATRP of PEGMA in aqueous medium; **b** schematic diagram of self-assembly micelles formed by the PR-based triblock copolymer in aqueous medium

drug [72]. Surprisingly, the nano-sized micelles self-assembled from a selected PR-derived copolymer sample were found to effectively solubilize AmB with high drug-loading content (8.7%) and drug-loading efficiency (87%) as well as a long sustainable release profile. Importantly, there was nearly no initial burst occurring in in vitro release for the drug delivery micelles. By comparison with micelles formed by pluronic PPO–PEO–PPO without CDs, the polyrotaxane-containing amphiphilic micelles released much slower, which indicated that encapsulated AmB may have some interaction with threaded β-CDs, giving longer and sustained release times. Moreover, the entrapment into self-aggregates of amphiphilic PR-derived triblock copolymers can protect AmB molecules from hydrolysis, aggregation, and precipitation by minimizing contact with the bulk aqueous phase. As for the higher stability of polymeric micelles, the AmB formulation based on the PR-derived triblock copolymers holds distinct advantages over those of AmB lipid complex (Abelcet) and conventional liposomal AmB (AmBisome).

Another micellar structure was constructed by using the process of highly threaded pseudopolyrotaxane stacking to drive self-assembly. Poly (ethylene oxide)-block poly (dimethylamino ethylmethacrylate) (PEG-PDMAEMA) polymer is an example. α-CD was able to thread over the PEG segment, but not the PDMA segment of this polymer axle. The threaded part of the polypseudorotaxanes aggregated, leaving the hydrophilic PDMA polymers exposed to solution, thus forming a micellar structure [73].

Recently, suparmolecular aggregates possessing pH-induced reversible micelle-gel transition were synthesized. The polypseudorotaxane consisted of hydrophilic copolymer PEG–b-PLL–HBr copolymer and α-CD [74]. Micellization of this hydrophilic copolymer because of the block-specific threading of α-CD molecules onto the PEG block yielded supramolecular-structured nanoparticles, which underwent pH-inducible gelation in aqueous media. First, at pH 4.5, the PLL block was nonthreaded, while the PEG block of PEG-b–PLL was included inside the cavity of α-CD, inducing the micelle-like aggregation of the hydrophilic block copolymer to nanoparticles around 30 nm. The water-soluble supramolecular nanoparticles possess an insoluble core consisting of inclusion formation α-CD and PEG, and a soluble shell of free PLL. When increasing the pH value of the aqueous solution, the solubility of the PLL shell of the nanoparticles was changed, and above pH 10, PLL became less soluble and aggregated in aqueous solution, leading to gel formation. Furthermore, it became a micelle solution again when changed to acid conditions. The driving force for the formation of the supramolecular hydrogel was believed to be the synergetic effect of selective complexation between PEG block and α-CD, and the pH-inducible hydrophobic interaction between PLL block at pH 10. Such pH switchable and reversible supramolecular hydrogels may find application in biomedical fields, such as controlled drug or protein release.

3 Drug-Conjugated Biodegradable Cyclodextrin-Based Polyrotaxane for Drug Delivery

In addition to a hydrogel matrix for drugs, cyclodextrin-based polyrotaxanes could be widely applied as drug delivery systems with another strategy, which is by conjugating drugs or functional proteins on the CD rings. Polyrotaxane is fascinating and very promising for this kind of drug delivery due to several unique structural characteristics. Firstly, CDs bear many hydroxyl groups that could be easily modified by chemical reactions, allowing the conjugation of bioactive agents on the CD rings. Secondly, self-assembled polyrotaxanes display high mobility of CDs as a result of the CD rotating around the polymer chain. This flexibility is expected to enhance multivalent ligand-receptor interactions and is promising for applications such as targeting drugs, drug-mediated drug delivery, and tissue engineering [9]. Thirdly, CDs can dethread through the polymer chain when the end is capped through biodegradable linkage, which ensures the release of drug into the cell (Fig. 11) [75].

3.1 Targeted Delivery via Multivalent Interactions

In the field of drug delivery, one of the most crucial issues is how to bind the bioactive agents or ligands on receptor sites of proteins on the plasma cell

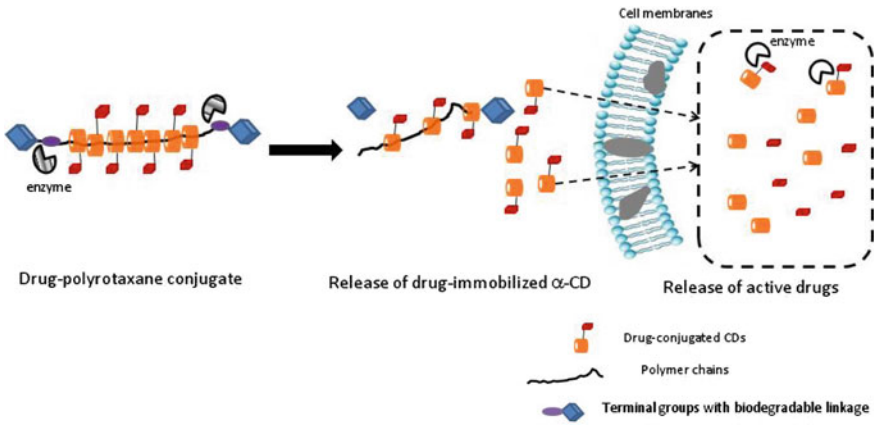

Fig. 11 The schematic illustration of drug-conjugated polyrotaxane and the concept of triggered drug release via enzymatic degradations

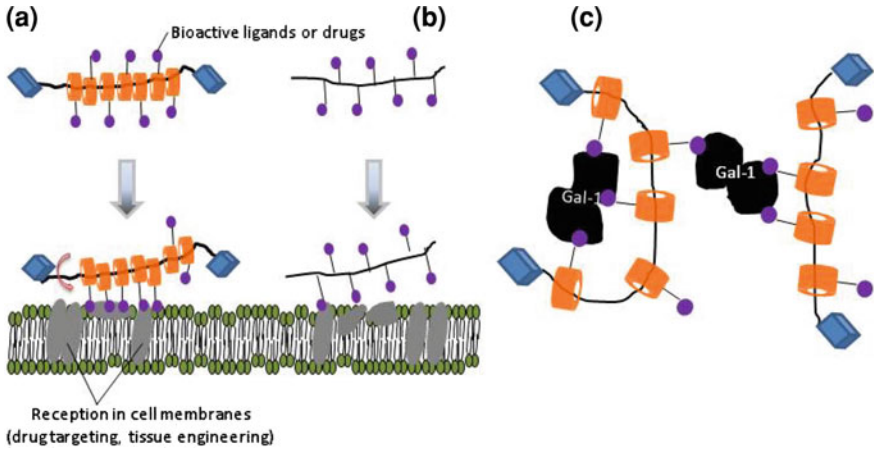

Fig. 12 The effects of mobile CDs in polyrotaxanes on binding receptor proteins in a multivalent manner **a**. Image of ligand-polyrotaxane conjugate, **b** image of ligand-immobilized polymer, and **c** possible binding models for Gal-1 and pseudopolyrotaxnae

membranes effectively and specifically. Over the last decades, the approach of multivalent interaction by using multiple ligands with receptor protein was considered as a possible solution [76]. However, multivalent ligand-immobilized polymers were not satisfactory mainly because of a spatial mismatch between the ligand-polymer and receptor [77] (Fig. 12a). Thus, as mentioned above, cyclodextrin-based polyrotaxanes are advantageous because they spin freely through the polymer chain, which opens the opportunity for multivalent interaction with biological systems (Fig. 12b).

Based on this hypothesis, a self-assembled supramolecular polymer consisting of lactoside-CD-polyviologen-conjugated polypseudorotaxane was investigated for its ability to inhibit galectin-1-mediated T-cell aggulutination [78]. Uniquely, the CDs of the polypseudorotaxane are capable of rotating around the axis of the polymer chain as well as moving back and forth along its backbone to alter the presentation of its ligand (Fig. 12c). Synthetic multivalent ligands for Gal-1 have the potential to act as cancer diagnostics and therapeutics and to shed light on the supramolecular interaction of Gal-1 with its natural and oncogenic carbohydrate ligands. In the agglutination assay, for the purpose of comparing the pseudopolyrotaxane with other multivalent structures, the trivalent lactosides and lactoside-bearing chitosan polymer were synthesized and evaluated in the same agglutination assay. Based on the observation, the trivalent lactosides showed no improvement over native lactose as inhibitors of Gal-1-induced T-cell agglutination. The rigid chitosan polymer bearing lactose epitopes exhibited only a slight enhancement over native lactose in interactions with Gal-1. The improvement may be attributed to the rigidity of the polymer backbone, which could prevent binding to the lectins in the same manner. In contrast to chitosan, the polyviologen backbone of the polypseudorotaxane possesses flexible decamethylene units. The flexibility afforded by the polymer backbone, together with the dynamic property of lactoside ligands, affords a supramolecular structure with the fluidity and adaptability of the biological target of Gal-1. As a result, a ten-fold enhancement of the valency was observed for this lactoside-CD-polyviologen-conjugated polypseudorotaxane. This enhancement is higher than was observed in a hemagglutination assay with β-CD bearing seven lactosides. Therefore, this self-assembled pseudopolyrotaxane, which displayed highly mobile ligands, adds a new dimension to the study of protein-carbohydrate interactions and the exploitation of multivalency for targeting therapeutically relevant lectins. Similar research was also accomplished by Nelson et al. [79]. By modifying lactose on both α-CD and β-CD and threading the CDs onto PTHF and PPG, respectively, they studied the binding capability of these lactose-polyrotaxanes to Gal-1. The methylation of β-CD/PPG analogue improved the water solubility of this polypseudorotaxane, and they showed 6.7-fold improvement over α-CD-lactose for binding to Gal-1.

Similarly, in order to investigate how the multivalent interaction between streptavidin and a biotin-decorated polyrotaxane could serve as a model system for transporter targeting, biotin was conjugated to α-CD, which was treaded in PEO chains and end capped with biodegradable L-phenylalanine (Z-Phe) [40]. These studies were performed to probe the multivalent interactions between a polymeric ligand (i.e., biotin-decorated polyrotaxane) and a model binding protein (i.e., streptavidin). The binding/dissociation kinetics between biotin-polyrotaxane conjugates and streptavidin using the surface plasmon resonance (SPR) technique were performed as a model of multivalent ligand targeting to transporters. The number of biotins conjugated with one polyrotaxane molecule varied from 11 to 78, and it was found that the polytotaxane with the highest density of biotin molecules had the strongest association, suggesting multivalent interaction on the

SPR surface. To quantify the effect of valency, the authors also compared their polyrotaxane bearing 78 biotin molecules to a simple biotin-α-CD conjugate by using a competitive inhibition assay. The results showed that in contrast to biotin-CD conjugate, this biotin-polyrotaxane conjugate model significantly switches monovalent binding to multivalent binding, resulting in much greater inhibitory potency (i.e., 4–5 times greater than that of biotin-α-CD conjugate). This difference could be attributed to the length of the polyrotaxane (theoretically 32 nm) and the fact that it can span multiple binding sites on the 5.5-nm streptavidin tetramer. These model conjugates can be applied for targeting ligand-polymer conjugates to intestinal transporters and temporally inhibiting or altering the transporter properties in conjunction with biodegradable dissociation of the polyrotaxanes [80]. In another study, DMAB-β-CD was firstly threaded onto a biotin-terminated PPG chain and then capped with an avidin-biotin interaction known to have noncovalent bond interactions. During the formation, relatively high temperature and longer reaction time were needed to obtain the polypseudorotaxane. The polypseudorotaxane can be useful to make a polyrotaxane via avidin-biotin molecular recognition using the terminal biotin group [81].

The multivalent interactions of polyrotaxanes with proteins were further exploited by conjugating maltose with α-CD–PEO polyrotaxane and investigating its binding with the plant lectin, concanavalin A (Con A) [82, 83]. A library of maltose-polyrotaxane conjugates (Mal-PRXs) with different maltose loading was prepared by threading maltose conjugated α-CD onto PEG chains capped with bezyloxycarbonyl-L-tyrosine. By using a Con A-induced hemagutination inhibition assay, the inhibitory strength was evaluated. The mobile motion of α-CDs in the polyrotaxane that governed the molecular motion of maltosyl groups could be measured by spin-lattice relaxation time (T_1) and spin-spin relaxation time (T_2) of α-CD C(1), maltosyl C(1), and PEG methylene protons. In the research investigating Con A binding effects (measured by association constant K_a), although the number of maltosyl groups per one Mal-PRX molecule was similar, the K_a of Mal-PRX was much higher (up to 1,000 times) than the monomeric compound (maltose). Mal-PAA, which represented the immobile-multivalent effect, was only one to three powers higher than the maltose. Furthermore, the largest K_a value of Mal-PRX with 38% α-CD threading was well correlated with the T_1 and T_2 values of maltosyl groups and α-CD. The results indicated that rather than the number of maltosyl groups, the mobile motion of maltose-conjugated α-CDs in the Mal-PRXs was the most considerable factor for the high affinity with the Con A binding. On the another hand, different numbers of maltosyl groups were synthesized and compared to evaluate the influence of the local density. It was found that the K_a was increased with the number of maltosyl groups per one α-CD molecule acheiving the highest value of 2.9 maltosyl groups per one α-CD molecule. The relative potency was decreased with a further increase in the density, which was probably due to steric hindrance since not all the maltosyl groups can necessarily bind to the binding sites of Con A. Therefore, the high mobility of the maltose on the polyrotaxane, due to favorable threading levels and high maltose loading, worked together to enhance the Con A binding [83].

This ligand-polyrotaxane-conjugated system can also be applied as an inhibitor that is specifically recognized by transporters without being absorbed to preclude kidney damage. For example, the dipeptide (Val–Lys)-conjugated polyrotaxanes were studied in terms of their inhibitory effect on digested peptide uptake of intestinal human peptide transporter (hPEPT1) on HeLa cells [84]. Specifically, Val–Lys groups were introduced to α-CDs, which were threaded onto a PEO chain capped with bulky polyrotaxane conjugates, and the in vitro efficacy as an inhibitor was evaluated by the uptake of Gly–Sar by HeLa cells, which were transfected to express hPEPT1. The α-CD–Val–Lys showed a slight inhibitory effect on expression of hPEPT1, while α-CD itself did not. Interestingly, the inhibitory effect of the polyrotaxane–Val–Lys conjugates was much greater than α-CD–Val–Lys conjugates, indicating the Val–Lys moieties introduced to the polyrotaxane backbone interacted with multiple hPET1s expressed on HeLa cells. The authors also observed that the higher the Val–Lys loading on the polyrotaxane, the greater the inhibition. By comparison with dipeptide-decorated dextran, their dipeptied-decorated polyrotaxane showed significantly greater inhibitory strength (approximatedly 20% higher). The authors attributed this phenomenon to the mobility of the polyrotaxanes. Owing to their flexibility, the Val–Lys moieties introduced in the polyrotaxane are expected to be well arrayed along the supramolecular backbone, so as to easily face PEPT1 on the membrane. Therefore, the design approach for the conjugation of PEPT1-recognizable oligopeptides with supramolecular-structured polyrotaxanes is expected to be useful for retarding the progression of chronic renal disease, which requires dietary protein restriction and drug delivery [84].

Enzyme inhibition by polyrotaxanes has also been investigated using carboxyethylester-polyrotaxanes to scavenge Ca^{2+} ions, thus inhibiting the protease trypsin. It is a novel calcium-chelating polymer that plays an important role in the field of oral drug delivery. Here, the carboxyethylester (CEE) groups were introduced to all primary hydroxyl groups in α-CDs, which were threaded onto a PEO chain capped with bulky end goups [85, 86]. The solubility of the polyrotaxane is an important issue for drug delivery, and these CEE-polyrotaxanes displayed an excellent solubility in PBS. The solubility increased with pH, indicating ionization-related solubility similar to conventional polyacrylates [85]. In this research, trypsin inhibiton was studied in two steps. First, the Ca^{2+} binding ability was evaluated by comparing with poly (acrylic acid) (PAA) and CEE-conjugated α-CD. PAA had a stronger binding affinity for Ca^{2+} than any CEE-polyrotaxane and CEE-α-CD. The calcium-chelating capacity of CEE-polyrotaxane was similar to or a little bit less than that of PAA, while the maximum binding ability of CEE-α-CD was only 40% of PAA. Secondly, because the trypsin inhibitory mechanism depended not only on the Ca^{2+} chelation, but also on the direct interaction with the enzyme, trypsin inhibition was studied by HPLC analysis of the degradation of the model peptide, *N*-α-benzoyl-arginine ethylesterv (BAEE). PAA, CEE-polyrotaxane, and CEE-α-CD were used for this study. It was found that without the additional calcium chloride, the hydrolyzed BAEE was increased with time in the order of CEE-α-CD $\gg$ CEE-polyrotaxane $>$ PAA, which is inversely proportional to the

capacity of Ca^{2+} chelation. These results suggest that Ca^{2+} chelation by CEE-polyrotaxane and PAA is related to trypsin inhibition.

In a further development, the numbers of α-CD and carboxyl groups in the CEE-polyrotaxanes are also an important factor for the trypsin inhibition [86]. By comparison to polyrotaxanes with various numbers of CEE-modified α-CDs, the authors found that the high number of CEE-α-CDs led to greater calcium-binding affinity and resulted in a greater inhibition of trypsin activity. The CEE-polyrotaxane with the highest number of CEE-α-CDs showed greater inbibition of trypsin activity than PAA. The CEE-polyrotaxane with the smallest number of CEE-α-CDs temporally interacted with trypsin, which was well correlated with the inhibition and recovery of trypsin activity. Therefore, the mechanism and extent of trypsin inhibition can be controllable by the number of CEE-α-CDs.

Although the concept of multivalent targeting is attractive, the cargo capacity of the polyrotaxane is limited from a drug delivery standpoint, especially if all the CDs are ascribed to the targeting task. With regard to this problem, idea of conjugating a multivalent polyrotaxane to a liposome was introduced. This system was able to form stable bilayers and could carry any drug payload desired [87].

3.2 Biodegradable Conjugated Polyrotaxanes for Drug Delivery

Biodegradability is one of the indispensable properties for drug delivery systems. In the last 2 decades, biodegradable polymeric devices for controlled drug release have been attractive because, as effective drug delivery vehicles, they must efficiently carry a bioactive agent and release it locally in high concentration. As compared to biodegradable hydrogel matrices for drug release, drug-conjugated biodegradable polyrotaxanes have the advantages of both controlling the drug release by the degradation rate of terminal group and by the number of CDs threaded onto the biodegradable polymer. The dethreading of CDs modified with appropriate drugs may also enhance drug permeation across biological barriers (Fig. 11). Furthermore, in contrast to the polymer-drug conjugates proposed by Ringsdorf, the biodegradable moieties are only located at the terminals, which avoided the intermolecular and intramolecular association of the polyrotaxanes. Rather than sterically hindering the accessibility of digestive enzymes, the unique structure of polyrotaxane enabled the enzymatic degradation perfectly.

The first example of biodegradable polyrotaxanes was reported in 1995 [88]. The supramolecular assemblies were formed between α-CD and the PEO chain, capping with L-Phe via biodegradable peptide linkages. When the peptide linkages were enzymatically degraded by a protease such as papain, the polyrotaxanes degraded into PEO, α-CD, and L-Phe. In order to investigate the degradation and CD release kinetics of this new drug release system, hydroxyl-propylated (HP) modified polyrotaxanes were synthesized [89] (Fig. 13). The introduction of HP in α-CD improved the solubility of the polyrotaxanes in PBS (pH 7.4). HP-α-CD release from a PEG chain was governed by two processes: the cleavage of terminal

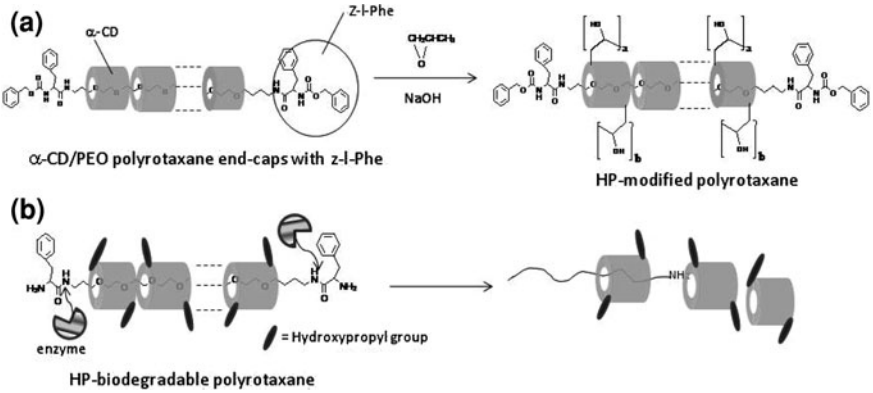

Fig. 13 The biodegradable polyrotaxane systems based on HP-modified α-CD and PEO. **a** The structure of the HP-conjugated polyrotaxane systems; **b** HP-α-CD release by terminal peptide cleavage of HP-α-CD/PEO-Phe system

peptide linkage and the dethreading of HP-α-CDs from a PEG chain. So the HP-polyrotaxanes were characterized by terminal peptide cleavage using papain, and HP-α-CD release was dominated by the degradation of L-Phe moieties in the polyrotaxane. The in vitro release study shows that HP-α-CD can be released when either of two terminal peptides was cleaved, and the effect of degradation could be controlled by several factors. First, the amount and rate of released of HP-α-CDs were governed by the molecular weight of PEG. For example, the release rate of HP-polyrotaxane with lower molecular weight of PEG was much higher than that with higher molecular weight. This was attributed to the rate of terminal peptide cleavage in HP-polyrotaxanes. Second, the steric hindrance to forming enzyme-substrate complexes is also a factor that could affect the degradation rate. As to the formation of an enzyme-substrate complex, the position of biodegradable moieties should be considered for the degradation. Finally, the influence of the adjacent nature of the L-Phe moiety is important. The oxypropyl groups next to the L-Phe moiety can enhance the interaction with papain and increase the HP-α-CD release rate [90]. Thus, the designed polyrotaxanes are feasible as novel drug carriers in which the degradation of the terminal moiety can induce the release of drugs incorporated with a supramolecular structure.

The incorporation of drugs to HP-α-CD in this biodegradable polyrotaxane was accomplished in 1999. Theophylline–polyrotaxane conjugates were synthesized by coupling theophylline with α-CD that threaded onto PEG chain capped with L-Phe [75]. Theophylline-7-acetic acid was activated by coupling with 4-nitrophenol, and the *N*-aminoethyl-theophylline-7-acetoamide was obtained after reacting with ethylenediamine. The derivative was coupled with 4-nitrophenyl chloroformate-activated polyrotaxane to obtain the thephlline–polyrotaxane conjugates. The conjugates formed a specific association under physiological conditions, depending

upon interactions between the theophylline molecules and/or the terminal L-Phe moiety in the conjugates. Upon enzymatic degradation of the terminal groups, theophylline–HP-CD (Theo–HP-CDs) conjugates were completely released via dissociation of the supramolecular structure without steric hindrance. Furthermore, in vitro papain degradation showed that the complete release time of Theo–HP-CDs was shorter than HP-α-CD. This could be attributed to the specific nature of the association between the theophylline and L-Phe moiety, which brings about enhanced degradability of terminal peptide cleavage, resulting in a faster release rate than the HP-α-CD. It was also found that Theo–HP-CD release was faster from polyrotaxane with a smaller number of threaded CDs. It can be presumed that this is because the tightly packed association and the increased number of HP-α-CDs reduced the accessibility of papain to the terminal peptide. The bonds between drug and α-CDs can also hydrolyze and release the active drugs into the cell (Fig. 10). In addition, by utilizing different enzymes (papain and α-chymotrypsin), sustained release of drugs was observed, ranging from 50 to 250 h.

Based on a similar technology, another drug, anti-cancer drug doxorubincin (DOX), was also conjugated to a polyrotaxane polymer via hydrolyzable linkages, and a cell-penetrating low molecular weight protamine (LMWP) peptide was further attached to the chain end in order to facilitate the intracellular uptake of tumor cells [91]. Figure 14 illustrates the structure of DOX–polyrotaxane conjugate. After accumulation of the conjugates at the tumor site, protamine sulfate, a clinical heparin antagonist, allows an effective intracellular uptake of the LMWP-PR-DOX conjugates. Once inside tumor cells, DOX molecules will be released naturally from the PR polymer chain by hydrolysis, yielding a sustained level of DOX within the tumor cells to exert cytotoxic effects. It was demonstrated that the LMWP–PR-DOX conjugates yielded a sustained release of DOX over a period of more than 4 days. The intracellular uptake of these conjugates by A2780 human ovarian cancer cells was evaluated by confocal microscopy and MTT assay. In the intracellular delivery, the PR-DOX conjugates were not taken up by carcinoma cells, while LMWP–PR-DOX was able to internalize into the cells, indicating that the PR-DOX conjugates successfully internalized the cells because of the cell-penetrating activity of LMWP. When heparin was added, this LMWP-induced cell internalization was markedly abolished because of the electrostatic interactions between the positively charged LWMP and negatively charged heparin molecules. In addition, some translocation seemed to occur with the heparin-masked LMWP–PR-DOX conjugates. This is possible because of the competition of cell-surface heparin-sulfate moieties with the administered heparin inhibitor, and the heparin-induced inhibition was nearly completely reversed when protamine was added. This LMWP–PR-DOX conjugate offers great potential for intracellular drug delivery into tumor cells in order to achieve highly effective and safe drug therapy.

The biodegradable HP-α-CDs/PEO polyrotaxane could also be useful in the fabrication of blood-contacting devices based on the intracellular metabolism of platelets and physiochemical interaction of the polyrotaxanes with the plasma membrane. For instance, based on a cellular response study, HP-α-CDs/PEO

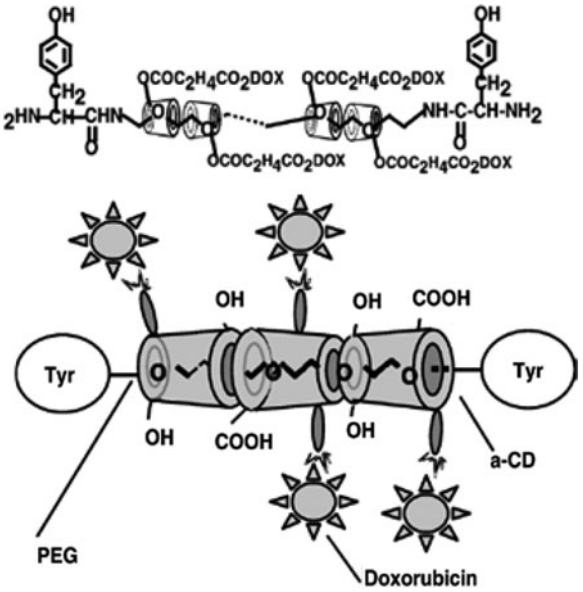

Fig. 14 The structure of DOX–polyrotaxane conjugate

polyrotaxane was found to inhibit cytoplasmic calcium increase in platelets and increase plasma membrane fluidity of red blood cells, and elevate cytoplasmic cyclic-3′, 5′-AMP levels in platelets [92]. With the addition of the polyrotaxane, the inhibition of thrombin-induced cytoplasmic calcium levels in platelets was observed. Further, the polyrotaxanes were found to enhance membrane fluidity in RBC ghosts. These effects of the polyrotaxanes were statistically more significant than those of their constituent molecules, such as a mixture of HP-R-CDs and L-pheterminated PEGs. In a further investigation, the polyrotaxanes with both sulfonyl and carboxyl groups were synthesized and characterized for mimicking the anticoagulant activity of heparin. They were found to exhibit an anticoagulant activity, which was affected by structural factors, such as the lower threading percentage of α-CD, the similar ratio of anionic groups to heparin, and lower molecular weight of PEG. These polyrotaxanes have the potential to be applied in materials for blood-contacting applications [93]. Moreover, by blending with polyurethane films, the sulfonated PRs showed both protein and bacteria repulsion and platelet activation [94].

Another application of these biodegradable HP-α-CDs/PEG polyrotaxanes is to enhance transdermal penetration. A polymer consisting of α-CDs and PEG capped with biodegradable peptide moieties was synthesized, and the interaction with the stratum corneum of hairless rat skin was examined by means of differential scanning calorimetry. According to the study, the polyrotaxane could allow the enhanced permeability of drugs conjugated without penetration into the skin, making it a novel candidates for transdermal drug delivery systems [95].

3.3 Stimuli-Responsive Dethreading Polyrotaxanes for Drug Delivery

Recent studies in biodegradable polyrotaxanes have focused on various stimuli-triggered responses such enzymes, pH, redox, and temperature. Controlling the degradation of polyrotaxanes is a key consideration in site-specific drug release or to trigger the degradation of a scaffold upon successful tissue regeneration. Basically, the biodegradable linkages can be divided into protease-catalyzed peptide bonds, simple hydrolysis based ester bonds, pH-triggered hydrazone bonds, and disulfide bonds for reduction with reductive agents or enzymes. In order to ensure complete CD release from the polyrotaxanes, terminal cleavage should be the first step. After the CDs diffuse into aqueous medium via dethreading, the elimination of hydrogen bonds between CDs is necessary because the hydrogen bonds are the driving force to form polypseudorotaxanes. Considering this problem, Yui et al. [88, 89] eliminated the hydrogen bonds by chemical modification of hydroxyl groups on CDs. As discussed above, after hydroxypropylation, solubility of the polyrotaxane in water was drastically increased, and HP-α-CD was found to be released by the terminal hydrolysis in the presence of papain.

As a typical example of the first kind of biodegradable linkage, polyrotaxane composed of PLLA and α-CD end with Z-L-Phe exhibited papain-triggered hydrolysis (LA-PRX) [96]. In this system, hydrolysis of ester bonds of the PLLA in the polyrotaxane was prevented by the supramolecular structure. However, the hydrolysis of the polyrotaxane could be induced in the presence of protease (papain). The authors investigated the effects of dissociation polyrotaxane structure by enzymatic cleavage of peptide linkages of the bulky end groups on the hydrolysis behavior of LA-PRX. According to the study, in the presence of papain in PBS, drastic decreases in mass were observed for LA-PRX, which reached to about 50% mass loss after 21 days. The release is slower than with PLLA polypseudorotaxane (LA-pPRX) without end capping in the absence of papain. Based on the course of molecular weight remaining in H_2N-PLLA-NH_2 and PLLA chains in LA-PRX and LA-pPRX, it was found that papain has no influence on the degradation behavior of the H_2N-PLLA-NH_2. Such unique hydrolysis behavior, that is, proteinase-triggered degradation of polyester, was achieved by the combination of the supramolecular architecture, biodegradable PLLA, and enzymatically cleavable end groups. Moreover, in order to examine the enzymatic degradation controlling two different peptide end capped polyrotaxanes, L-phenylalanylglycyl-glycine terminated and L-tyrosineglycylglycine-glycine (H–L-PheGlyGly-) terminated polyrotaxane, in which many α-CDs threaded onto PEO, were also synthesized [97, 98]. These polyrotaxanes were shown to be degraded by membrane-bound metalloexopeptidase (aminopeptidase M). In vitro degradation and kinetic studies revealed that the supramolecular structure of the polyrotaxnae enhanced the accessibility toward aminopeptidase M despite the higher molecular weight of the polyrotaxane. This biodegradable polymer provides a new candidate for drug delivery applications with controlled degradation profiles.

Drug release in the human body is a complex area of study. The environment in different parts of the body shows different pHs, ranging from 1 to 8. Therefore, pH could be utilized as stimuli for the triggered release of drugs from polyrotaxane. Recently, the pH-triggered dethreading of polyrotaxane was also demonstrated. α-CD terminated polyrotaxane consisting of β-CD and PEG–PPG–PEG triblock copolymers was biodegradable under acidic conditions [99]. The α-CD is linked to the main polymer backbone by a hydrazone bond and acts as an end-capping group. This polyrotaxane underwent degradation under acidic conditions because of the preferred cleavage of the hydrazone bonds under low pH conditions. Similarly, it was reported that α-CD-PEG polyrotaxanes also exhibited dethreading in low pH environments [100]. Such stimuli-responsive polyrotaxane systems can present a new paradigm of biodegradable materials for biomedical applications.

4 Cyclodextrin Based Polyrotaxanes and Polyseudorotaxanes for Gene Delivery

4.1 Cyclodextrin-containing Cationic Polymers for Gene Delivery

Gene delivery using polycations is believed to be one of the greatest challenges for inventing nonviral gene carrier systems instead of toxic virus-based vector systems [101]. Because the hydroxyl groups in CD rings offer the opportunity for multiple modifications, CDs were explored for oligonucleotide delivery, which enhanced the absorption and resistance to nucleases [102, 103]. The first CD-containing cationic polymer for gene delivery was reported in 1999 [104, 105]. So far, the same group has developed a set of such CD-containing polymers and studied the structural effects of the polymers on gene delivery [27, 28, 106]. Generally, the CD-containing cationic polymers showed lower cytotoxicity and efficient gene transfection. More importantly, the CD-containing polycations can be further modified by inclusion complex formation [107]. Take the pegylation, for example: PEO-adamantane was used for complex formation with β-CD modified polyethylenimine (PEI) by the supramolecular interaction between adamantane and β-CD. It was found to stabilize the polyplex nanoparticles and results in enhanced gene transfection [108, 109].

So far, a number of cationic polymers have been modified by grafting CDs onto the polymers to study gene delivery, such as linear and branched PEIs [110], and polyamidoamine (PAMAM) dentrimers [111, 112]. All the CD-grafted cationic polymers showed reduced cytotoxicity by grafting the CD moieties. Uekama et al. [111–116] systematically studied CD-grafting PAMAM dendrimers with different generations (G2, G3, and G4) and attributed the enhancing gene transfection effect to increasing cellular association and intracellular trafficking of plasmid DNA.

Most recently, a new series of cationic star polymers consisting of α-CD core and oligoethylenimine (OEI) arms was synthesized as a nonviral gene delivery

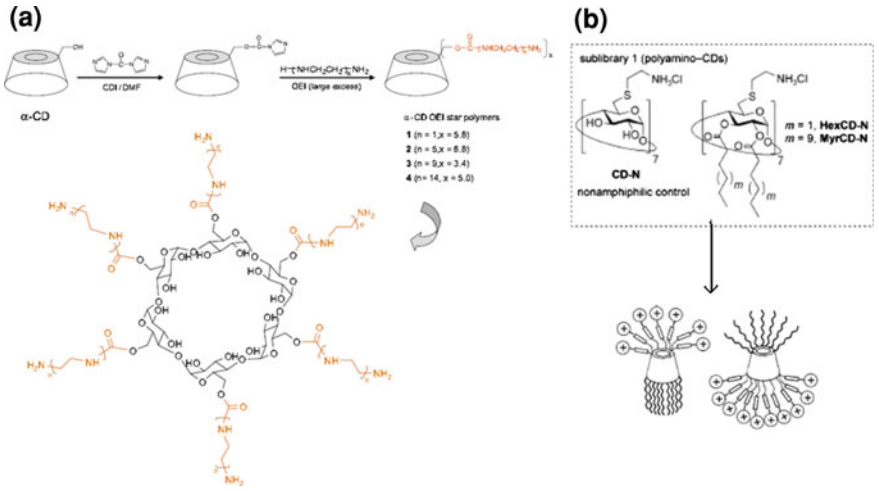

Fig. 15 **a** Synthesis procedures and the structures of α-CD–OEI star polymers. **b** Schematic representation of skirt- (*left*) and jellyfish-type (*right*) polycationic amphiphilic CDs (paCDs)

vector (Fig. 15a) [117]. The molecular structures of the α-CD–OEI star polymers, which contained linear or branched OEI arms with different chain lengths ranging from 1 to 14 ethylenimine units, were characterized by size exclusion chromatography and NMR techniques. The α-CD–OEI star polymers were studied in terms of their DNA-binding capability, formation of nanoparticles with plasmid DNA (pDNA), cytotoxicity, and gene transfection in cultured cells. The α-CD–OEI star polymers were shown to inhibit the migration of pDNA on agarose gel through formation of complexes with pDNA, and the complexes formed nanoparticles with sizes ranging from 100 to 200 nm at N/P ratios of 8 or higher. The star polymers displayed much lower in vitro cytotoxicity than that of branched PEI of molecular weight 25 kDa. The α-CD–OEI star polymers showed excellent gene transfection efficiency in HEK293 and Cos7 cells. Furthermore, the transfection efficiency increased with an increase in the OEI arm length. The star polymers with longer and branched OEI arms showed higher transfection efficiency. The best star polymers for gene delivery showed excellent in vitro transfection efficiency that was comparable to or even higher than that of branched PEIs (25K). These novel α-CD–OEI star polymers with OEI arms of different chain lengths and chain architectures can be promising new nonviral gene delivery vectors with low cytotoxicity and high gene transfection efficiency for future gene therapy applications.

With a similar star structure, a kind of polycationic amphiphilic cyclodextrins was reported (Fig. 15b) [118]. The structure contains both cationic elements and lipophilic tails, which is similar to the lipid structure. The nanoparticle stability and transfection efficiency can be rationally modulated by judicious tailoring of the molecular topology. Importantly, by using polycationic amphiphilic CDs that present a dendritic arrangement of catioinic elements in the BNL-CL2 and COS-7

cell lines, the transfection efficiencies were observed to surpass those of polycationic polymer branched PEI with lower cytotoxicity profiles. The versatility of the synthetic scheme makes it potentially amenable to the selective installation of additional functional elements over the CD core (e.g., for cell targeting, nuclear localization, monitoring cell uptake, and fate). Thus, this system offers further opportunities for gene targeting and delivery.

4.2 Cyclodextrin-containing Cationic Polyrotaxane for Gene Delivery

Another new class of CD-containing gene carriers was developed based on cationic polyrotaxanes where multiple cationic CDs are threaded onto a polymer chain and capped by bulky ends. By taking advantage of the CD's ability to slide and rotate along the polymer axle, this system seems possibly able to generate well-matched DNA complexes and thus to accomplish the gene transfection with a minimum amount of cationic polyrotaxanes. It is similar to the concept of multivalent interaction discussed earlier. Because the CDs are threaded onto a polymer backbone, the hemolytic activity of the CDs would be minimized. Therefore, CD-containing cationic polyrotaxanes are a promising system for non-viral gene delivery.

A desirable model for this system would be CD-containing gene carriers based on cationic polyrotaxanes where multiple cationic CDs are threaded onto a polymer chain and capped by bulky ends. In contrast to the conventional polycations containing long sequences of covalently bonded repeating units, this model developed a novel cationic supramolecule as a new class of gene-delviery vectors. The first example of this kind of model would be the cationic supramolecules composed of multiple OEI-grafted β-CD threaded on a pluronic PEO–PPO–PEO triblock copolymer chain and end capped with 2,4,6-trinitrobenzene sulfonate(TNBS) (Fig. 16) [31]. In this research, OEI-grafted β-CD was selected as the building block because β-CD is larger than α-CD and then could be grafted with more chains of OEI, while an ethylenimine unit has high cationic density. PEO–PPO–PEO triblock copolymer was selected as threading chain, and PPO segments were covered with 13 β-CD rings, whereas PEO segments were free of complexation. This provided some free space for β-CD rings to move along the polymer chain in polyrotaxane, allowing more efficient grafting of OEI to β-CD, because a dense coverage of β-CD on the polymer might be spatially unfavorable to the grafting reaction. In the reaction of grafting OEIs on the CD rings, three OEIs with different lengths of OEI were grafted to the polyrotaxane: ethylenediamine ($k = 1$), pentaethylenehexamine ($k = 5$), and linear OEI with an average molecular weight of 423 ($k = 9$). When a large excess of OEI was used, the maximum number of grafted OEIs per β-CD did not exceed 7, indicating that primary hydroxyl groups in β-CD were modified under this condition.

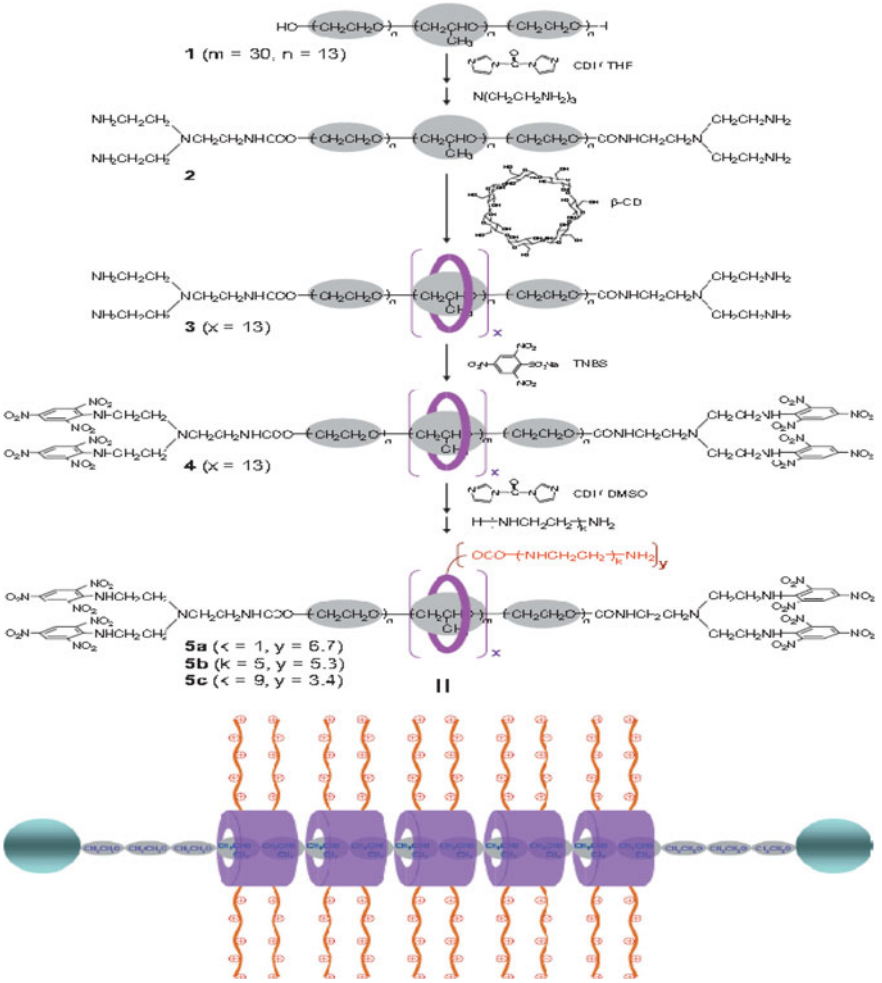

Fig. 16 Synthesis procedures and structures of cationic polyrotaxanes with multiple OEI-grafted β-CD rings [31]. *CDI* 1,1′-carbonyl diimidazole, *THF* tetrahydrofuran

The ability of these cationic polyrotaxanes to condense pDNA into particulate structures was evaluated by agarose gel electrophoresis, particle size analysis, and zeta-potential measurements. All the cationic polyrotaxanes could completely complex the pDNA at N/P ratios of 2 and above, which displayed a similar or slightly better DNA condensation ability than PEI (25K). The particle size of the polyrotaxane/DNA complex decreased with the increase of N/P ratio, and when the N/P ratio reached 6, the particle size remained in the 150–250-nm range. Similarly, the surface net charge of the complexes increased from negative to positive as the N/P ratio increased from 0 to 4 and reached a plateau at N/P ratio of 4 and above. The transfection efficiency of the cationic polyrotaxane was also

assessed using luciferase as a marker gene in HEK293 cells. It was found that the gene transfection was comparable to that of branched PEI (25K), and the efficiency was increased with the increase of OEI chain length (5c > 5b > 5a). Interestingly, the gene transfection efficiency of 5b and 5c in the presence of serum was significantly higher than that in the absence of serum, implying the new cationic supramolecules may have an advantage for in vivo applications [31].

In conclusion, this β-CD-OEI/PEO–PPO–PEO system may have the following advantages. First, β-CD is known to selectively thread around the PPO segments [119], providing low spatial hindrance by spare PEO segments and permitting an efficient OEI grafting degree. Second, due to the free mobility of OEI-grafted β-CD along both PPO and PEO segments, it is flexible in order to condense DNA efficiently. Thirdly, because a shorter length of PEI was used, the system showed much less toxicity than PEI (25K) in cell cultures. Finally, there are many "flapping" OEI chains with many primary and secondary amines, which is beneficial for interaction with DNA and cell membranes. As a result, the cationic supramolecular gene delivery vectors showed good DNA-binding ability, low cytotoxicity, and high gene transfection efficiency that is similar to PEI (25K) at the optimized N/P ratio and molecular weight. It even exhibited higher transfection efficiency than dimethylaminoethyl(DMAEC)-α-CD polyrotaxane systems [34].

In a further development, the β-CD-OEI/PEO–PPO–PEO system was reported to display high and sustained gene delivery capability in cancer cells in the presence or absence of serum. The morphology and formation processes of the polyrotaxane/DNA complex were observed by AFM in real time [29]. The real-time AFM confirmed DNA complexation, and the cationic polyrotaxane exhibited much lower cytoxicity and high gene transfection efficiency comparable to or slightly higher than branched PEI in both SKOV-3 and PC3 cancer cells. Of great importance was the observed sustained gene delivery capability in PC3 cells in the presence or absence of serum. Therefore, this β-CD-OEI/PEO–PPO–PEO system has great potential as a novel non-viral gene carrier in clinical cancer gene therapy.

By utilizing a similar system, a few cationic polyrotaxane-based cationic OEI grafted-CDs and chains were synthesized. For example, the cationic supramolecules consisting of OEI-grafted α-CD threaded on PEO, followed by end capping with 2,4-dinitro-1-fluorobenzene (DNFB), were synthesized for gene delivery [120]. After the N/P ratio reached 8, the cationic polyrotanes could efficiently compact pDNA into small nanoparticles with diameters ranging from 100 to 200 nm, and when the N/P ratio increased to 10, the zata potential of the complexes of pDNA with cationic polyrotaxanes was strongly positive and varied within the same range, which resulted in a similar affinity for the cell surface. In the case of cytotoxity and gene transfection, it is worth noting that these cationic polyrotaxanes exhibited less toxicity in both cultured BHK-21 and MES-SA cells than the PEI control (25K). One of the explanations for this phenomenon would be the introduction of CD and copolymer results in the lower density amino groups; the high density of amino groups is always considered an important factor leading to high cytotoxicity of PEI [121]. The gene transfection efficiency was assessed in BHK-21 and MES-SA cells. Generally, the transfection increased with an increase

of the N/P ratio, and under complete serum medium conditions, cationic polyrotaxane displayed comparable or higher transfection efficiency than PEI (25K) at an N/P ratio of 10.

Recently, polyrotaxanes consisting of cationic OEI-grafted α-CD threaded on PEO–PPO random copolymer were synthesized [32]. Unlike the above OEI-α-CD/PEO systems, the OEI-α-CDs were only located selectively on EO segments of the P(EO-r-PO) chain, while PO segments were free of complexation. This increased the mobility of the cationic α-CD rings and the flexibility of the polyrotaxanes, which enhanced the interaction of the cationic α-CD rings with DNA and/or the cellular membrane. Similar to the above β-CD–OEI/PEO–PPO–PEO and OEI-α-CD/PEO systems, the cationic supramolecular polymers showed strong DNA-binding ability, low cytotoxicity, and high gene delivery capability in a variety of cell lines including HEK293, COS7, BHK-21, SKOV-3, and MES-SA. All the systems have great potential as novel non-viral gene carriers in clinical gene therapy.

Rather than grafting cationic OEI on the CD rings, in another study, soluble polyrotaxanes were formed for gene delivery by threading α-CD molecules over PEO and PCL chains of ternary block copolymer PEO–PCL–PEI. The gene transfection efficiency was in the same order of magnitude as PEI (25K), but with much lower toxicity [122].

4.3 Cyclodextrin-Containing Biocleavable Polyrotaxane for Gene Delivery

One of most important difficulties arising in the strategy of polycations as gene carriers is how to ensure the endosomal/lysosomal escape of the polyplex and how to release DNA into the cytoplasm and guarantee that the DNA reaches the nucleus. It is currently considered that endocytosis is a major pathway for DNA entry mediated by DNA/polycation complexes. However, a large fraction of the complexes taken up in the endosome is delivered to the lysosome and consequently degraded. Thus, many efforts have been made to establish strategies for the efficient escape of DNA from the endosome into the cytosol to improve their transfection efficiency. With regard to this issue, the introduction of biodegradable moieties into polycations to dissociate the polyplex has attracted interest recently [123]. Specially, because of its flexibility and multivalent interaction, the cationic polyrotaxane with biodegradable terminal linkage has received much attraction. If the end caps can be cleaved intracellularly, the cationic CDs would be dethreaded and disrupt the endosomal membrane because of cholesterol and/or phospholipid inclusion. At the same time, the CD release can convert the multivalent interactions into monovalent interaction, thereby releasing the DNA for transfection (Fig. 17) [2, 33]. Furthermore, because the hydrophobic cavity of CD is occupied by the polymer chain in the polyrotaxane, the possibility of hemolysis should be negligible in this system.

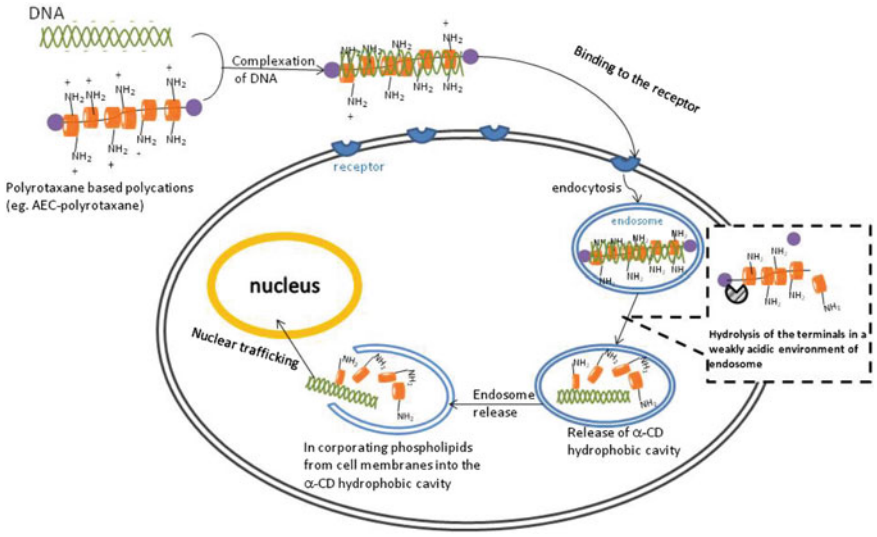

Fig. 17 Strategic image of gene delivery using aminoethylcarbamoyl (AEC)–polyrotaxane

This assumption was first evaluated by Yui's laboratory [33]. Aminoethylcarbamoyl (AEC) groups were introduced to α-CD and were threaded onto a PEG chain capped with Z-L-Phe linking via labile linkage. The numbers of α-CDs and the AEC groups were varied, and the effects on the physiochemical properties of the complexes were investigated. In the steps of DNA complexation, it was interesting to observe that the condensing ability of the AEC-α-CD/PEO system increased with the increasing threading number of CDs in the cationic polyrotaxane. Specifically, the 100, 200, and 300 CDs containing polyrotaxanes completely condensed the DNA at N/P ratios of 5, 0.5, and 0.25. In contrast, changing the number of AEC groups on the individual CD units did not drastically affect the critical N/P values, indicating that the number of AECs is likely to be a minor factor in DNA complexation. The results suggested that the complexation mechanism of the AEC-α-CD/PEO systems was not likely to be neutralization-driven complexation. Since the slope of the DNA condensation intensity curve decreased with increasing the number of α-CDs below the N/P ratio of 1, the authors presumed that the rod-like structure of the polyrotaxane enhanced complexation with DNA in the lower concentration. Furthermore, in contrast to PEI, the gene delivery system showed enhanced and tight complex formation even at lower N/P ratios. And it was found that the introduction of AEC groups with the polyrotaxane backbone enhanced the opportunity of complexation with phosphate groups of plasmid DNA in spite of the lower number of cationic groups than in PEI.

Subsequent to this work, they further designed a cytocleavable polyrotaxane that has a supramolecular structure of DMAEC-α-CDs threading onto a PEG chain capped with benzyloxycarbonyl tyrosine (Z-Tyr) via disulfide (SS) linkage (Fig. 18a) [34, 35]. It was expected that the DMAEC-SS-PRX systems would

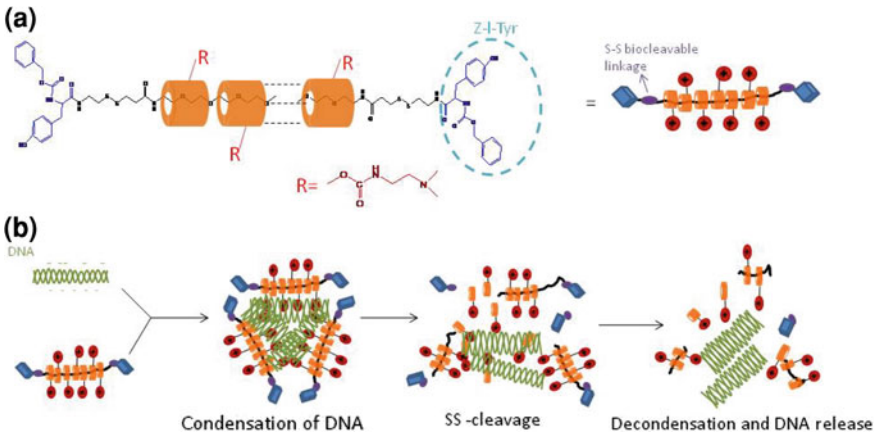

Fig. 18 **a** Chemical structure of biocleavable polyrotaxane, and **b** image of the polyplex formation and terminal cleavage-triggered decondesation of the polyplex

exhibit sufficient cleavage of SS linkages and pDNA release via the dissociation of the non-covalent linkages between α-CDs and PEG, looking like a necklace. As a result, the supramolecular structure could accomplish the rapid endosomal escape and pDNA release, which could be observed by the intracellular trafficking study (Fig. 18b). For this study, two kinds of polyrotaxanes were synthesized: DMAEC-SS-PRX with the respective number of threaded α-CDs and DMAEC groups of 23 and 40, and DMAEC-PRX where the number of α-CD and DMAEC groups was 30 and 40. The complexation of the two polyrotaxanes with pDNA was compared with that of a linear PEI (LPEI 22K). It was found that at an N/P ratio of 0.5, a stable polyplex with a positively charged surface was formed with a diameter of approximately 178–189 nm, while LPEI 22K did not form any tightly packed pDNA and still showed a negative value at the same N/P ratio. It was supposed that the driving force of the pDNA condensation of the DMAE–SS–PRX was not only electrostatic interaction, but also the other factors in the polyrotaxane structure, such as the association of the terminal benzyloxycarbonyl group, rod-like structure, and the mobile motion of α-CDs in the necklace-like structure of DMAE–SS–PRX that enhanced multivalent interaction by preventing spatial mismatching [34].

The in vitro pDNA dissociation effects were investigated in the presence of 10 mM dithiothreitol (DTT). Based on the agarose gel electrophoresis data, pDNA was observed to be released from the DMAE–SS–PRX polyplex in the presence of counter polyanion. However, the polyplex of DMAE–PRX, which has no disulfide linkages, was stable without releasing pDNA under the same conditions. Furthermore, according to the confocal laser scanning microscopy (CLSM) technique, the intracellular trafficking was measured, and the DMAE–SS–PRX polyplex was found to completely escape from the endosome/lysosome 90 min after transfection. This is probably because the proton sponge effect was obtained by the much

more positively charged surface of the DMAE–SS–PRX polyplex and good buffering capacity [34]. Furthermore, it was found that the polyplex with the lowest number of DMAECs exhibited a much faster pDNA release in cytoplasm.

In a further experiment, a variety of DMAEC–SS–PRXs with different numbers of DMAEC groups were synthesized to evaluate the gene transfection efficiency in NIH/3T3 cells [124]. It was found that the transfection activites of DMAE–SS–PRXs are significantly higher than those of nondegradable polyrotaxane, suggesting that the pDNA was released from the DMAE–SS–PRX polyplex in the intracellular milieu. In addition, the DMAE–SS–PRX with low numbers of α-CD and amino groups exhibited a high transfection activity. The authors attributed this effect to the pDNA release from the DMAE–SS–PRX polyplex, which was controlled by the number of α-CD and/or amino groups in the polyrotaxane carrier. Specifically, 29 DMAE-α18–SS–PRXs in which the numbers of α-CD molecules and amino groups were 18 and 29, respectively, exhibited high transfection activity compared with other PRXs. However, although an effective pDNA release was observed, the transfection efficiency of 14 DMAE–α18-SS–PRXs was significantly lower than in other PRXs. This might be due to a premature pDNA release in the cytosol causing the degradation by cytosolic nucleases. Thus, the transfection activity was related to an appropriate timing for DNA release, and high transfection and stability can be achieved by optimizing the numbers of DMAECs [124].

In addition to amino-grafted CD polyrotaxane, LPEI/γCD-based polypseudorotaxanes have also been examined for gene delivery [125]. The γ-CD-based polypseudorotaxanes had improved cellular uptake and lower toxicity. Unlike the cationic polyrotaxane-based polyplex where DNA was complexed at a lower N/P ratio than that of LPEI, these γ-CD-based polypseudorotaxanes required a higher N/P ratio compared to LPEI alone. The transfection efficiency of this polypseudorotaxane was comparable to or greater than that of LPEI, especially at higher N/P ratios.

5 Conclusions and Future Perspectives

Over the past two decades, cyclodextrin-based polyrotaxanes and polypseudorotaxanes have inspired interesting and rapid development of supramolecular biomaterials for drug and gene delivery. Because of their attractive properties, such as low toxicity, sliding, dethreading, and ease to modify, cyclodextrin-based supramolecular polymers have attracted increasingly attention as effective drug and gene carriers. Specifically, cyclodextrin-based biodegradable polypseudorotaxane hydrogels could be used as promising injectable drug delivery systems for sustained and controlled drug release. Temperature-responsive, pH-sensitive, and hydrolyzable polyrotaxane hydrogels have also attracted much attention in the field of drug delivery. The self-assembly micelles formed by amphiphilic copolymers threaded with CDs could be used as carriers for controlled and

sustained drug release. Furthermore, polyrotaxanes with drug/ligand-conjugated CDs threaded on polymer chains with biodegradable end groups could be useful for controlled and multivalent targeted delivery because of the mobility of CDs. And the stimuli-responsive dethreading property of the polyrotaxane is an important factor to ensure effective drug release.

In the field of gene delivery, CD-containing cationic polymers have been exploited as novel polycations as gene carriers. Based on multivalent interaction, a new class of cationic polyrotaxanes consisting of multiple OEI-grafted CDs threaded on a block copolymer chain was developed. They all showed strong DNA-binding ability, low cytotoxicity, and high gene delivery capability, making them fascinating non-viral gene carriers. Furthermore, cytocleavable end-caps were introduced in the polyrotaxane systems in order to ensure efficient endosomal escape for intracellular trafficking of DNA.

Future studies on polyrotaxanes still face many challenges, not only in the design with better functions and performances, but also in the attempt to obtain lower toxicity both in vitro and in vivo for beneficial biomedical applications. Proper and smarter designs are expected for a better controllable supramolecular structure with many different copolymers and CD derivatives, as well as stimuli-responsive end-caps. The possibility of making two- or three-dimensional polyrotaxanes, such as dendritic and hyperbranched, is also very interesting for future research. Furthermore, the safety of the delivery systems is a common concern. Intensive in vitro and in vivo biocompatibility and biodegradability studies are needed for the supramolecular drug and gene delivery systems. The future development of CD-based polyrotaxane would open up new opportunities for designing novel biomaterials for drug and gene delivery systems, and may be developed into novel delivery systems with different properties possibly for a wide range of applications. In addition to drug and gene delivery, some recent research has shown that it also provides great opportunity for application in some other fields, such as fibers, fuel cells, energy transfer, electrochromatography, etc. Therefore, the research on polyrotaxane materials is still a new area, and it is highly promising to study the applications in life sciences and biotechnology.

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Engineering Liposomes and Nanoparticles for Biological Targeting

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Abstract Our ability to engineer nanomaterials for biological and medical applications is continuously increasing, and nanomaterial designs are becoming more and more complex. One very good example of this is the drug delivery field where nanoparticle systems can be used to deliver drugs specifically to diseased tissue. In the early days, the design of the nanoparticles was relatively simple, but today we can surface functionalize and manipulate material properties to target diseased tissue and build highly complex drug release mechanisms into our designs. One of the most promising strategies in drug delivery is to use ligands that target overexpressed or selectively expressed receptors on the surface of diseased cells. To utilize this approach, it is necessary to control the chemistry involved in surface functionalization of nanoparticles and construct highly specific functionalities that can be used as attachment points for a diverse range of targeting ligands such as antibodies, peptides, carbohydrates and vitamins. In this review we provide an overview and a critical evaluation of the many strategies that have been developed for surface functionalization of nanoparticles and furthermore provide an overview of how these methods have been used in drug delivery systems.

Keywords Biological targeting · Drug delivery · Functionalization · Liposome · Nanoparticle

Abbreviations

CAC	Critical aggregation concentration
ConA	Concanavalin A
DCC	*N,N′*-dicyclohexylcarbodiimide
DOPE	1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine

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DOPS	1,2-Dioleoyl-*sn*-glycero-3-phospho-L-serine
DPPE	1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine
DSPC	1,2-Distearoyl-*sn*-glycero-3-phosphocholine
DSPE	1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine
DTT	Dithiothreitol
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELAM	Endothelial-leukocyte adhesion molecule-1
EPR	Enhanced permeation and retention
GPI	Glycophosphatidylinositol
HER2	Human epidermal growth factor receceptor 2
ICAM-1	Intercellular adhesion molecule 1
IgG	Immunoglobulin G
LAMP	Lysosome-associated membrane protein
MAb	Monoclonal antibodies
MESNA	Mercaptoethanesulfonate
mon2C5	Monoclonal antinucleosome antibody 2C5
mon2G4	Monoclonal antimyosin antibody 2G4
NBD	7-Nitro-1,2,3-benzoxadiazole
NHS	*N*-hydroxysuccinimide
PDP	*N*-(3′-(pyridyldithio)propionoyl
PE	Phosphatidylethanolamine
PEG	Poly(ethylene glycol)
PL	Phospholipids
PLA2	Phosholipase A2
*p*NP	*p*-Nitrophenylcarbonyl
QCM	Quartz crystal microbalance
RES	Reticuloendothelial system
SPPS	Solid phase peptide synthesis
TFA	Trifluoroacetic acid
TfR	Transferrin receptor
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide
WGA	Wheat germ agglutinin

Contents

1 Introduction

The pharmaceutical industry has successfully developed numerous drugs for the treatment of cancer, but it remains one of the world's most devastating diseases with more than 10 million new incidences every year [105]. One of the major obstacles to current treatments is inadequate delivery of the therapeutics to the tumor site, which leads to severe side effects [8]. There is therefore increasing demand for delivery systems that transport the drug specifically to the diseased tissue and improve the therapeutic index of the encapsulated drug. Nanoparticle systems have been investigated as drug delivery systems for several decades, and strategies are becoming increasingly complex to fulfill the growing requirement for treating diseases. The nanoparticle drug delivery systems offer new treatment regimes for a large variety of different diseases; however, due to the relatively high costs of many of the utilized materials, they have mainly found applications for treatment of cancer. Of the many classes of nanocarrier systems that are currently under investigation, liposomes remain one of the most successful. Liposomes were first proposed as drug delivery vehicles by Gregoriadis et al. [54] in 1974. Early liposomal formulations suffered from rapid clearance by phagocytic cells of the reticuloendothelial system (RES). This issue was solved by coating the liposomes with polymers, particularly poly(ethylene glycol) (PEG), which suppress protein absorption and opsonization of the liposomes [139]. These *Stealth*® or *sterically stabilized* liposomes were found to accumulate in tumors and inflammatory tissue by passive diffusion because of the leaky vasculature and the lack of an effective lymphatic drainage present in such tissues [82, 87]. This phenomenon is commonly referred to as the enhanced permeation and retention effect (EPR effect). Despite the enhanced accumulation in cancerous or inflammatory tissue as a result of the EPR effect, there is strong motivation for improving the accumulation by

coating the outer liposomal membrane with ligands targeted towards overexpressed or selectively expressed receptors on diseased cells. *Active targeting* with ligands, such as peptides, carbohydrates, glycoproteins, antibodies or fragments thereof, has been utilized to selectively deliver drugs to the desired site of action by increasing the nanocarrier accumulation. Targeted liposomes/nanoparticles are superior compared to drug immunoconjugates since only few targeting ligands are needed to deliver several thousands drug molecules. Furthermore, liposomes are highly biocompatible and can protect encapsulated drugs from premature degradation in the blood stream. However, surface functionalization of liposomes or nanoparticles with targeting ligands are not trivial even though multiple reports have already utilized the targeting strategy. One of the largely overlooked problems when attaching targeting ligands is that reactions that normally work well in solution may proceed very slowly on a surface, and care should be taken to select the chemistry that is suitable for the desired synthetic manipulation. In particular, in many studies there is no evaluation of the success of the functionalization, which is highly problematic as many of the utilized chemistries are far from quantitative.

The aim of this review is to summarize the recent advances in the field of nanoparticle functionalization. The focus is on surface functionalization of liposomes, but the discussed chemistry is equally relevant for other nanoparticle constructs. We will discuss the many new and highly specific conjugation methods that have been developed in recent years to functionalize liposomes and give examples of how different classes of targeting ligands have been attached to liposomes and used to target diseased tissue. We will furthermore briefly discuss the membrane anchors that are employed, which is an overlooked problem in many studies.

2 Surface Functionalization of Liposomes

Three methods are commonly used to functionalize liposomes with targeting ligands. Small targeting ligands, such as vitamins [44, 77, 117], saccharides [25, 33, 122, 149] and small peptides [37, 39], are often covalently attached to a hydrophobic anchor (e.g., a lipid) in organic solvent and purified. The functionalized lipid can thereafter be mixed with natural lipids and hydrated to form liposomes. This approach is only possible when working with smaller ligands and is particularly useful when the ligand comes in relatively large quantities (due to the purification step). A major advantage of the approach is the complete control of the amount of ligands per liposome since the initially added amount can be varied in a controlled manner. However, approximately 50% of the added functionalized lipids will be oriented towards the interior of the liposome, thus, not interacting with the outer environment.

Another way to introduce specific ligands at the outer liposomal membrane is the *post-insertion* approach [62], which is useful for expensive ligands. In this

approach the ligands are typically covalently coupled to preformed lipid–PEG micelles (e.g., DSPE–PEG), which have functionality in the distal end of the PEG that allows coupling to the ligand. Alternatively, synthesized and purified ligand–lipid moieties made by the strategy discussed above can also be used. Succeeding, incubation of the micelles with preformed liposomes allows the DSPE–PEG–ligand conjugates to transfer from the micelles into the outer liposomal membrane in a temperature- and time-dependent manner, if the process is thermodynamically favored. This approach has been used to functionalize liposomes with antibodies [6, 13, 36, 61, 62, 100], peptides [95, 114, 135] and proteins [24]. A major advantage of this approach is that the loading of the liposomes is decoupled from the insertion of the ligands, which allows for optimization of both parameters. Targeting liposomes prepared by the *post-insertion* approach have been shown to have the same in vitro drug leakage rates, cell association profiles and therapeutic efficacies compared to liposomes made by other approaches [6, 61, 95]. However, the amount of ligands inserted into the liposome membrane must be quantified when using this approach.

Functionalization of liposomes with targeting ligands can also be carried out by *post-functionalization*, e.g., performing the conjugation directly on the preformed liposomes with anchors exposing specific functionalities in their respective head groups. This method is primarily used with larger and complex ligands, such as proteins and antibodies or fragments thereof. One should realize that the reactions often do not go to completion, and the degree of functionality should always be quantified. A large number of different anchors, e.g., fatty acids, phospholipids and sterols, have been used. The effect and the properties of these anchors will be discussed in the end of this review.

Ideally, surface coupling reactions should be simple, fast, efficient, reproducible and result in bonds that are non-toxic and non-immunogenic. Furthermore, reaction conditions for surface functionalization should be mild in order to retain the biological activity of the targeting ligands. A wide range of coupling methods has successfully been developed during the last 25 years, resulting in a broad variety of possible methods to functionalize liposomes. Early coupling methods are generally characterized by unspecific surface functionalization resulting in moderate yields, whereas the modern approaches enable site-specific functionalization in high yields. Each coupling reaction used to covalently attach ligands to the liposome surface will be described separately in order to highlight the advantages and disadvantages of the various methods. To limit the scope of this review, only surface functionalization of liposomes is discussed; however, the surface chemistry applies for the majority of other nanoparticle-based drug delivery systems.

2.1 Coupling of Ligands to Amine-Modified Liposomes

One of the earliest developed methods to covalently couple ligands to the liposome surface is based on amine functionalized liposomes. Torchilin et al. [124, 125]

described the use of two homobifunctional crosslinkers (Fig. 1a), glutaraldehyde (**1**) and dimethyl suberimidate (**2**) (Fig. 2), for amine–amine crosslinking. Addition of either **1** or **2** to DPPE-containing liposomes resulted in up to 70% imine or amidine formation, respectively, at the liposome surface. Incubation of these liposomes with rabbit anticanine cardiac myosin antibodies at 4°C in aqueous buffer resulted in 60% conversion [125] without loss of the binding capacity of the antigen.

The major advantage of this surface functionalization approach is the fact that it is based on naturally occurring lipids, which can be used without prior derivatization. However, the use of homobifunctional crosslinkers can result in uncontrollable homopolymerization of ligands or liposomes during the crosslinking reaction, which can lead to liposome aggregation. Furthermore, since multiple amine functionalities are usually present in antibodies, a random attachment can be

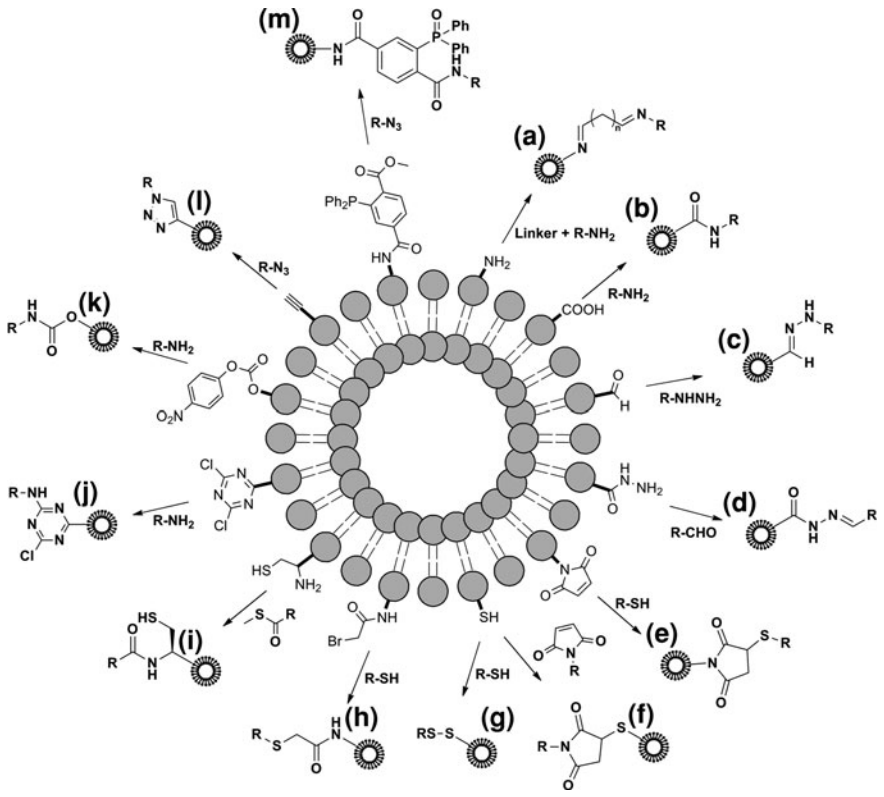

Fig. 1 Schematic illustration of the various coupling methods that have been developed in order to post-functionalize liposomes. **a** Amine functionalization, **b** carboxylic acid functionalization, **c** aldehyde functionalization, **d** hydrazine functionalization, **e** maleimide functionalization, **f** thiol functionalization, **g** thiol functionalization (disulfide bond formation), **h** bromoacetyl functionalization, **i** cysteine functionalization, **j** cyanur functionalization, **k***p*-nitrophenylcarbonyl functionalization, **l** alkyne functionalization, **m** triphosphine functionalization

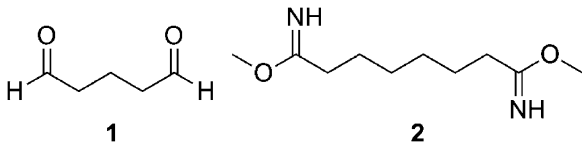

Fig. 2 Chemical structure of glutaraldehyde (**1**) and dimethyl suberimidate (**2**) utilized for amine–amine crosslinking by Torchilin et al. [124, 125]

expected. This may interfere with the binding of the antibody to its receptor and thus alter the binding affinity.

2.2 Coupling of Ligands to Carboxylic Acid-Modified Liposomes

Covalent coupling of ligands to carboxylic acid-modified liposomes (Fig. 1b) is a widely used approach to functionalize liposomes [14, 16, 63, 74, 86, 99, 143]. The method was first introduced by Kung and Redemann [74], who introduced the carboxylic acid functionality by reacting PE-lipids with a wide range of anhydrides in presence of triethylamine. Liposomes, exposing the carboxylic acid functionality, were activated in situ by the addition of water soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). The activated ester was found to react readily with primary amines present on mouse IgG resulting in a stable amide bond. The achieved coupling efficiencies ranged from 1 to 58%, depending on the length of the spacer between the liposome surface and the carboxylic acid functionality.

The effect of the spacer becomes negligible when the ligand is introduced at the PEG terminus, as described by Maruyama et al. [86], who prepared monoclonal IgG 273-34A-modified liposomes by the above-described approach. These immunoliposomes were found to have prolonged circulation times and a higher degree of target cell recognition compared to liposomes exposing antibodies directly on the liposome surface.

The major advantage of using carboxylic acid-modified liposomes for surface modification purposes is that no prior ligand modification is required, thus reducing the risk of denaturation. On the other hand, since multiple amine functionalities are present in antibodies, a random attachment can be expected, which could alter the binding affinity towards the targeted receptor.

2.3 Coupling of Ligands to Aldehyde-Modified Liposomes

Coupling of hydrazino-derivatized ligands to aldehyde functionalized liposomes by hydrazone formation (Fig. 1c) has been described by Bonnet et al. [20], who

introduced the aldehyde functionalized ether lipid di-*O*-hexadecyl-*rac*-glyceraldehyde into the liposomal membrane. Incubation with a hydrazino-derivatized dodecapeptide from the cytoplasmic domain of lysosome-associated membrane protein (LAMP) in aqueous buffer resulted in quantitative conversion within 5 h.

The method described by Bonnet et al. [20] offers an effective approach to conjugate synthetic peptides prepared by solid-phase peptide synthesis (SPPS) to liposomes. The hydrazino functionality is easily introduced to the synthetic peptide on resin by the use of *N*,*N*,*N*-tri(*tert*-butyloxycarbonyl)-hydrazino acetic acid, which is fully compatible with SPPS synthesis [18, 19]. Furthermore, hydrazone formation occurs spontaneously without the need of a catalyst. Thus, this method is one of the most effective for the functionalization of liposomes with targeting ligands when it is possible to introduce a hydrazino group into the ligand. However, this is unfortunately problematic for antibodies and other complex ligands.

2.4 Coupling of Ligands to Hydrazide-Modified Liposomes

A more widely adapted approach, compared to the one described above, is to invert the position of the functional groups, i.e., introducing the aldehyde to the ligand and the hydrazine functionality to the liposome surface (Fig. 1d) [26, 56, 57, 72, 91, 145, 146]. Initially, the hydrazine functionality was introduced by incorporation of lauric acid hydrazide [26] into the liposome membrane (it should be noted that this is a poor anchor). Later, a method to introduce the hydrazine functionality to the distal end of the PEG chain of DSPE–PEG has been described by Zalipsky [145]. Having the hydrazine functionality exposed on the liposomal membranes offers a unique advantage for coupling of antibodies to the liposomal membrane. Mild oxidation of the carbohydrate groups on the constant region of the heavy chain of the immuoglobulin with either galactose oxidase [26] or sodium periodate [26, 56, 57, 72, 91] results in the formation of an aldehyde, which can be chemoselectively attached to the hydrazine-functionalized liposomes through hydrazone bond formation. By utilizing the carbohydrate groups from the Fc region, the antibodies are correctly oriented once attached onto the surface of the liposomes, because only the Fc region is involved in the coupling reaction, leaving the antigen-binding site available for receptor interactions. Comparative studies have indicated that this method results in low coupling efficiencies (17%) [56], yet positive in vitro results have been obtained using this methodology with liposomes targeted towards rat colon carcinoma CC531 cells [72].

2.5 Coupling of Ligands to Maleimide-Modified Liposomes

The most often used approach to functionalize liposomes with targeting ligands is based on the formation of a thioether bond between maleimide-functionalized liposomes and thiol-derivatized ligands [13, 29, 34, 40, 42, 49, 64, 70, 85, 98, 101,

119] by Michael Addition (Fig. 1e). For direct surface functionalization, *N*-(4-(*p*-Maleimidophenyl)butyryl)-phosphatidylethanolamine has been used as the functionalized anchor, whereas DSPE–PEG–maleimide is used for attachment on the distal end of the PEG polymer. Garnier et al. [49] recently used this approach to covalently attach the Annexin-A5 protein, known to target membranes containing negatively charged phospholipids, to DSPE–PEG–maleimide functionalized liposomes. A mutant of the natural Annexin-A5 protein (35 kDa), exposing a cysteine residue at a highly accessible loop on the concave face of the protein, was developed. Addition of this protein to liposomes exposing the maleimide functionality in HBS buffer at pH 6.3 for 4 h resulted in a coupling efficiency of approximately 80%. The Annexin-A5-functionalized liposomes were found to bind to solid supported lipid membranes composed of DOPC/DOPS in a Ca^{2+} depending manner, as monitored by quartz crystal microbalance (QCM). Michael addition of thiolated OX26 MAb Fab′ fragments to DSPE–PEG–maleimide functionalized liposomes has recently been described by Béduneau et al. [13]. Despite optimizing the coupling conditions, the coupling yield was constantly approximately 25%. However, it is noteworthy to mention that quantitative coupling efficiencies have been reported with small thiolated pentameric cRGD peptides using a similar coupling protocol [64].

An interesting study performed by Fleiner et al. [40] concerning the influence of the spacer length between the liposome surface and the reactive maleimido group and its polarity revealed that longer polar spacers resulted in higher coupling efficiencies. Surprisingly, comparison of the reactivity of liposome functionalized with either *m*- or *p*-maleimido benzoic acid esters revealed that the less reactive (less electrophilic) *m*-maleimido benzoic acid ester resulted in a higher coupling efficiency (46 ± 7%) compared to the more electrophilic *p*-maleimido benzoic acid analogue (30 ± 5%). This could be explained by the increasing susceptibility to competing nucleophiles, such as water, of the maleimide group with higher electrophilicity.

Surface conjugation to maleimide-functionalized liposomes is a straightforward and reliable method to attach ligands without prior activation or addition of catalysts to promote the reaction. The reaction proceeds at ambient temperature, close to neutral pH and within a short period of time. Conjugation of smaller ligands often results in quantitative yields, whereas more moderate yields can be expected for larger molecules. Despite the popularity of this conjugation method, maleimide derivatives have been shown to be immunogenic [17, 106].

2.6 Coupling of Ligands to Thiol-Modified Liposomes

Thiol-functionalized liposomes have often been used for the attachment of ligands to the outer liposomal membrane. Normally, the reactive thiol is introduced to the membrane as the disulfide protected derivate *N*-(3′-(pyridyldithio)propionoylamino–PEG–DSPE (DSPE–PEG–PDP), which is activated in situ by reduction with

dithiothreitol (DTT), as described by Allen et al. [5]. Attachment of maleimide-derivatized antibodies (Fig. 1f) was achieved by overnight incubation in yields ranging from 13 to 88%, depending on the liposome composition and amount of maleimide-derivatized antibodies added. Quantitative conjugation yields with maleimide-derivatized My10 antibodies have been reported when the reactive thiol is introduced at the distal end of longer PEG chains than the ones otherwise present in the liposome [92].

Surface conjugation to thiol-modified liposomes can also be achieved by disulfide formation (Fig. 1g). This approach was adopted by Muñoz et al. [96], who introduced the hepatitis A VP3 (101–121) peptide to DSPE–PEG–PDP containing liposomes. Overnight incubation in borate buffer at pH 8 resulted in approximately 50% conjugation yield. A disadvantage of this approach is that free thiols may react among themselves to produce intermolecular disulfide bonds, leading to crosslinking of the reactive ligands or liposomes.

2.7 Coupling of Ligands to Bromoacetyl-Modified Liposomes

Conjugation of cysteine-containing peptides to bromoacetyl-modified liposomes (Fig. 1h) has been described by Frisch et al. [42]. The bromoacetyl functionality was introduced by acylation of DPPE with 2-[2-[2-[(2-bromoacetyl)amino]ethoxy]ethoxy]ethoxy acetic acid (**3**) (Fig. 3) in the presence of *N,N′*-dicyclohexylcarbodiimide (DCC). An octapeptide derivatized from the C-terminal of the histone H_3 peptide was added to liposomes, exposing the bromoacetyl functionality at pH 9.0 resulting in quantitative conversion within 1 h. At lower pH, the reaction was found to be less pronounced. This phenomenon was utilized by Schelté et al. [119], who formulated liposomes exposing both the maleimide- and the bromoacetyl functionality at the outer membrane. This study showed that important kinetic discrimination can be achieved between the maleimide and bromoacetyl functionalities when the reactions with thiols are performed at pH 6.5. Reaction with cysteine-containing peptides was found to be three orders of magnitude faster with the maleimide functionality than with the bromoacetyl derivative, resulting in a high degree of chemoselectivity. These findings enabled the coupling of two different cysteine peptides sequentially. The first coupling was carried out on the maleimide derivative at pH 6.5, followed by a coupling to the bromoacetyl derivative at pH 9.0 under experimental conditions, which were found

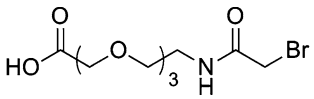

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Fig. 3 Chemical structure of 2-[2-[2-[(2-bromoacetyl)amino]ethoxy]ethoxy]ethoxy acetic acid (**3**) used to introduce the acetyl bromo functionality to DPPE, as described by Frisch et al. [42]

not to alter the integrity of the liposomes. Furthermore, neither the bromoacetyl nor the maleimide functionality was found to react with other nucleophiles such as α- and ε-amino groups or imidazole, which could also be present in peptides.

2.8 Coupling of Ligands to Cysteine-Modified Liposomes

Conjugation of recombinant proteins to the liposome surfaces through native chemical ligation (Fig. 1i) has recently been described by Reulen et al. [109]. Native chemical ligation was first reported by Dawson et al. [32] as a unique method to ligate two unprotected peptide fragments and form an amide bond, thereby facilitating the synthesis of large proteins. Native chemical ligation is a chemoselective reaction, which occurs spontaneously between a thioester and an N-terminal cysteine under aqueous conditions at neutral pH, which makes it ideally suited for liposome conjugation purposes. Reulen et al. [109] introduced the cysteine functionality to the distal end of DSPE–PEG–NH_2 by reacting it with succinimidyl-activated trityl-protected cysteine, followed by deprotection of the trityl protection groups with dilute trifluoroacetic acid (TFA). The C-terminal of the collagen-binding protein domain (CNA35) from the bacterial adhesion protein of *Staphylococcus aureus* was modified with sodium 2-mercaptoethanesulfonate (**4**) (MESNA) (Fig. 4) to form a thioester suitable for native chemical ligation. Cysteine-functionalized liposomes were incubated with MESNA-CNA35 protein in HBS buffer at pH 8 for 48 h in the presence of thiophenol, benzyl mercaptan or MESNA to catalyze the reaction. In the presence of either thiophenol or benzyl mercaptan, approximately 30% conversion was observed, whereas only 10% conversion was achieved with MESNA. However, the poorly water soluble and toxic thiophenol and benzyl mercaptan were found to accumulate in the phospholipid bilayer, making them difficult to remove after ligation. This was not the case with the water-soluble MESNA, which was easily removed by centrifugation. The CNA35-functionalized liposomes were tested in a collagen-binding assay and were found to have a 150-fold increase in affinity compared to the free protein.

Surface conjugation through native chemical ligation is an attractive method to directly conjugate thioester-modified ligands to the liposome surface. The method enables site-specific conjugation, since only a single site in the ligand is available for conjugation. The low coupling yield is an obstacle that needs to be addressed.

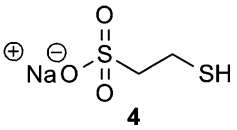

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Fig. 4 Chemical structure of sodium 2-mercaptoethanesulfonate (**4**) (MESNA) used to catalyze the native chemical ligation on the liposome surface, as described by Reulen et al. [109]

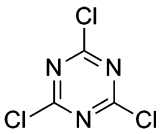

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Fig. 5 Chemical structure of cyanur chloride (**5**) used to introduce the cyanur functionality into liposomes by acylation of DPPE–PEG–OH, as described by Brendas et al. [21]

2.9 Coupling of Ligands to Cyanur-Modified Liposomes

A method for attaching antibodies directly to the PEG terminus of liposomes without prior derivatization has been described by Brendas et al. [21]. Introduction of the cyanur functionality to the PEG terminus of DPPE–PEG–OH was achieved under basic conditions by adding cyanuric chloride (**5**) (Fig. 5). Anti E-selectin monoclonal antibodies were coupled to cyanuric-modified liposomes (Fig. 1j) at pH 8.8 by nucleophilic substitution resulting in immunoliposomes having a high degree of in vitro binding to Chinese hamster ovary cells expressing E-selectins receptors. The nucleophilic substitution between the anti-E-selectin monoclonal antibodies and the cyanur-modified liposomes was found to be very sensitive towards the pH of the buffer. No surface functionalization was observed at neutral pH, whereas a more alkaline environment resulted in hydrolytic degradation of the cyanuric chloride.

This methodology offers a straightforward approach for attaching antibodies to the PEG terminus of liposomes without previous derivatization. However, cyanuric chloride is known to react with a wide range of nucleophilic functionalities, such as alcohols, amines and thiols, which means that a random attachment of the antibodies can be expected. This may interfere with the binding of the antibody to its receptor, thus altering the binding affinity. In addition to this, cyanuric chloride is regarded as a sensory respiratory irritant [113], but has not shown any sign of acute, chronic or genotoxicity [140].

2.10 Coupling of Ligands to p*-Nitrophenylcarbonyl-Modified Liposomes*

An additional method to directly conjugate antibodies to the PEG terminus of liposomes without prior derivatization has been described by Torchilin et al. [128]. The amphiphilic derivate *p*-nitrophenylcarbonyl–PEG–1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (*p*NP–PEG–DOPE), which was obtained in a single step from DOPE and bis(*p*-nitrophenylcarbonyl)-PEG, forms stable and non-toxic carbamate bonds with ligands containing primary amines (Fig. 1k). However, one drawback is the utilization of the bis-functionalized bis(*p*-nitrophenylcarbonyl)-PEG, which may result in dimerization, although this side product is easily

separated from the product. Torchilin et al. demonstrated this approach with several proteins, such as concanavalin A (ConA), wheat germ agglutinin (WGA), avidin, monoclonal antimyosin antibody 2G4 (mon2G4) and monoclonal antinucleosome antibody 2C5 (mon2C5), and observed almost quantitative surface functionalization yields at pH 8.0. Despite the fact that this method does not enable site-specific conjugation, the specific activities of the surface bound proteins were retained after conjugation.

2.11 Coupling of Ligands to Alkyne-Modified Liposomes

One of the more elegant coupling methods to functionalize liposomes is based on the work of Meldal and co-workers [129, 130] and Sharpless and co-workers [111], who reported the use of Cu(I) to catalyze the azide/alkyne Huisgen 1,3-dipolar cycloaddition, commonly referred to as the click reaction. This reaction offers unique flexibility because of the high level of orthogonality to other chemical functionalities and generally proceeds rapidly in high yields. This approach to functionalize liposomes was first described by Hassane et al. [58], who introduced the alkyne functionality to the liposome surface by incorporating the synthetic ether lipid *N*-[2-(2-(2-(2-(2,3-bis(hexadecyloxy)propoxy)ethoxy)ethoxy)-ethoxy)ethyl]hex-5-ynamide (**6**) (Fig. 6) into the liposomal membrane (Fig. 1l). Addition of an azido-modified mannose ligand in the presence of $CuSO_4$ and sodium ascorbate to generate Cu(I) in situ resulted in approximately 25% yield within 24 h. However, by adding the water-soluble Cu(I)-stabilizing ligand bathophenanthroline disulfonic acid [78] to the reaction mixture, complete conversion was observed within 6 h. These reaction conditions did not alter the size of the liposomes or provoke leakage from liposomes loaded with self-quenching concentrations of 5,6-carboxyfluoroscein. Furthermore, the mannose residue was found to be readily accessible to concanavalin A, which upon addition to the liposomes caused instant aggregation.

The click reaction approach has also been adopted by Cavalli et al. [23], who introduced the alkyne functionality by derivatization of DOPE with proliolic acid. Full conversion was in this case achieved within 20 h with a small azido-NBD derivative without the use of bathophenanthroline disulfonic acid.

The Cu(I) catalyzed azide/alkyne Huisgen 1,3-dipolar cycloaddition is a very powerful conjugation reaction for surface modification of liposomes. The

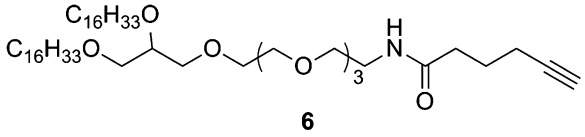

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Fig. 6 Chemical structure of *N*-[2-(2-(2-(2-(2,3-bis(hexadecyloxy)propoxy)ethoxy)ethoxy)-ethoxy)ethyl]hex-5-ynamide (**6**) used to introduce the alkyne functionality into the liposome membrane as described by Hassane et al. [58]

unreactive nature of both alkynes and azides towards the other functionalities present in biomolecules makes site-specific conjugation possible. Furthermore, the formed triazole ring is both thermal and hydrolytically stable, and the reaction can occur efficiently in aqueous media at room temperature. However, the mandatory use of copper catalyst represents a limitation. Copper is considered toxic and complete removal prior to in vitro or in vivo use is necessary. In addition to this, unsaturated phospholipids are known to be oxidized by copper ions in the presence of oxygen [46, 75], which could cause degradation of the liposomal membrane. Alternative methods to promote triazole formation between azides and alkynes, without Cu(I), have been described recently. Strain-promoted [3] or electron-deficient alkynes [79] have been reported to react with azides in absence of Cu(I), but these methods have not yet been applied to the liposome field.

2.12 Coupling of Ligands to Triphosphine-Modified Liposomes

The latest member of the wide range of possible surface conjugation reactions described is based on the Staudinger ligation [118], in which an azide and a triphosphine selectively react to form an amide bond (Fig. 1m). This approach was adopted by Zhang et al. [148], who introduced the triphosphine functionality by acylation of DPPE with 3-diphenylphosphino-4-methoxycarbonylbenzoic acid (**7**) (Fig. 7). Triphosphine-functionalized liposomes were incubated with an unprotected lactosyl derivate carrying an ethyl spacer functionalized with an azide group in PBS buffer, which resulted in 80% surface functionalization within 6 h. The surface conjugation reaction was not found to alter the size or provoke leakage of the liposomes. The surface-conjugated lactose residues were shown to be easily accessible to β-galactose-binding lectin, which, upon addition, caused aggregation of the liposomes.

The methodology described by Zhang et al. [148] offers an efficient and chemoselective conjugation method for liposome surface functionalization. The reaction benefits from being performed under mild conditions without the need of a catalyst. Furthermore, methods to engineer bacteria and yeast enabling them to incorporate azido functionalities into proteins have been developed [53]. This enables direct attachment to the triphosphine-functionalized liposome without prior derivatization of the protein.

3 Targeting Strategies: Active Targeting of Tumor Vasculature and Tumor Cells

Ligand-modified liposomes can be designed to target receptors expressed by cells in the tumor vasculature or on the tumor cells. When targeting tumor cells directly,

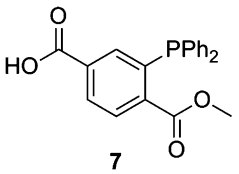

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Fig. 7 Chemical structure of 3-diphenylphosphino-4-methoxycarbonylbenzoic acid (**7**) used to introduce the triphosphine functionality into liposomes by acylation of DPPE as described by Zhang et al. [148]

a number of obstacles have to be overcome as the drug delivery system has to cross the vasculature and travel through the interstitium in the tumor tissue before being able to deliver the drug at the desired site of action. Vascular targeting is generally considered to be advantageous over tumor cell targeting as endothelial cells are genetically stable and the risk of developing drug resistance is minimized [1]. Proliferating endothelial cells in solid tumors share similar characteristics in a variety of tumor types and a number of these can be utilized. In addition, endothelial cells lining the blood vessels in tumors are more accessible for binding by circulating drug delivery systems that are administered intravenously [1]. A strategy that targets both the tumor vasculature and the tumor cells has been envisioned as more effective than targeting the two tissues individually, and Koning et al. have provided proof-of-principle for this hypothesis [73]. These strategies have been investigated using a range of targeting molecules including antibodies, peptides, carbohydrates and vitamin analogs, and an overview of these different targeting moieties is discussed below.

3.1 Immunoliposomes

Antibodies represent one of the most versatile ligands that can be attached to liposomes. However, the antibody functionalization of liposomes is generally challenging, where region specificity and degree of conversion are the main problems, even though a number of good methods exist. One of the main reasons for this is that antibodies can only be attached to liposomes by *post-functionalization*, i.e., the lipsomes are formed prior to attachment, and the chemistry therefore has to work well in aqueous buffer. Early attempts to attach these molecules for the specific targeting of diseased tissue were originally communicated in the 1970s [136] where antibodies were coupled to the surface of non-PEGylated liposomes. Today, it is commonly known that non-PEGylated (or non-polymer) liposomes have little treatment benefit because of fast clearance from the blood stream. Coupling of the targeting ligands to the distal end of PEG is therefore the method-of-choice today to maximize liposome accumulation at the target site [56, 116]. Antibodies can be attached as whole monoclonal antibodies or as a fragment thereof (Fig. 8). Full antibodies have stability advantages over small

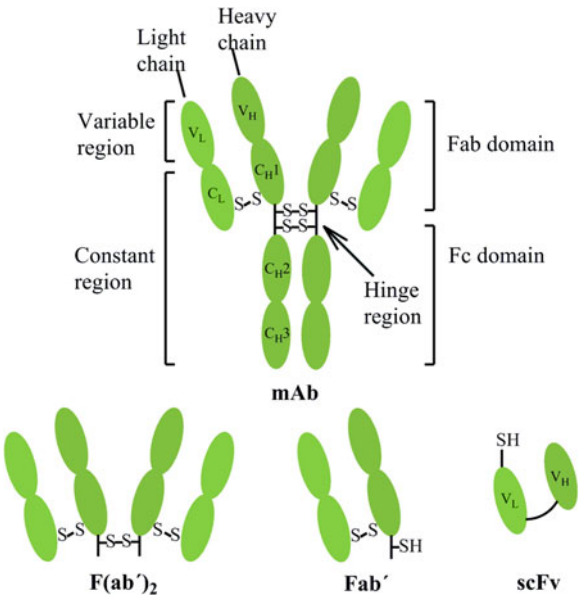

Fig. 8 Schematic representation of various antibody constructs: Monoclonal antibody (mAb); $F(ab')_2$ generated by pepsin digestion of the Fc domain of mAb; Fab′ from reduction of the disulfide bond in the hinge region of $F(ab')_2$; scFv of recombinant V_L and V_H regions linked by a short peptide sequence

fragments such as Fab′ and scFv, but can trigger complement [94] and induce antibody-dependent cell-mediated cytotoxicity [115]. Furthermore, the Fc fragment is known to accelerate clearance of the immunoliposome by uptake of circulating liver and spleen macrophages possibly through opsonization, and thus decreases the circulation time of the liposomes [5]. By attaching fragments of the antibody such as Fab′ or Fv, this undesired clearance can be minimized [12].

The most commonly used antibody-targeting moieties belong to the immunoglobulins of the IgG class [126], which have been coupled to the distal end of PEG using a hydrazido-PEG–DSPE construct [57]. The oligosaccharide moiety of the antibody molecule was oxidized using sodium periodate, creating an aldehyde functionality that reacts with the hydrazido-group on the PEG–DSPE to form a stable hydrazone linkage. Immunoglobulins consist of 82–96% polypeptide and 4–18% carbohydrates [10, 11], and the carbohydrate moieties are mainly situated on the heavy chain in the Fc portion of the antibody. As this region is not involved in the binding to the receptor, a modification in this part should not influence antigen-binding efficacy [52]. However, one potential problem with the oxidation of the carbohydrates is the risk of oxidizing amino acids situated in the antigen-binding Fab′ region. Amino acids most prone to oxidation with sodium periodate are cysteine, methionine, tryptophan, tyrosine and histidine, as well as serine and threonine if they occur as terminal residues [28].

Antibodies against the human epidermal growth factor receptor 2 (HER2) have been widely used in combination with liposomal drug delivery systems. This antigen is frequently overexpressed on various types of cancer cells and is only weakly expressed in normal tissues. One of the first studies of investigating Fab′

and scFv targeting of HER2 was reported by [85]. They utilized PEGylated liposomes that were functionalized with a maleimide functionality in the distal end of PEG. Fab′ was conjugated to the liposomes through a thioether linkage using the free thiol group in the Fab′ hinge region at pH above 7 to deprotonate the thiol. This site is located distant from the antigen-binding site and should not interfere with its function. The thioether bond formed between Fab′ and the maleimide functionalized liposome was stable and not prone to reduction in a reductive environment, e.g., serum [84]. ScFv fragments contain immunoglobulin heavy- and light-chain variables linked by a $[\text{-}(\text{Gly})_4\text{-Ser-}]_3$ motif. At the C-terminal end of the recombinantly produced scFv, a cysteine can be introduced that can be exploited in the covalent attachment to maleimide-activated PEG–DSPE. Two internal disulfide bonds are present in the scFv fragment; however, these have proven to be rather stable to reduction [4]. An analogous approach has been developed with the use of a PDP–PEG–DSPE liposome composition. After reduction with DTT, the liposome was incubated with an anti-HER2 maleimidophenylbutyrate functionalized Fab′ fragment, resulting in the immunoliposome [47]. For targeting the transferrin receptor (TfR), its antibodies or Fab′ fragments have been coupled to maleimide-modified PEG–DSPE liposomes. The TfR expression is elevated in many types of cancerous tissues and correlates with the proliferation rate and aggressiveness of the cancer. Hence, the TfR is a valuable potential target for drug delivery in cancer therapy [13, 141].

For the targeting of endothelial cells in malignant tissues, different forms of adhesion molecules such as endothelial-leukocyte adhesion molecule-1 (ELAM), intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) have been applied. These have been coupled to DOPE-*N*-dodecanoyl lipids by the use of EDC/*N*-hydroxysuccinimide (NHS) chemistry [55]. However, as there are several amine groups in anti-ELAM and anti-ICAM, this strategy leads to a non-specific crosslinking between the liposome and the antibody. This can be avoided by applying the maleimide method described above where the antibody is coupled via a thioether bond to the surface of PEGylated liposomes. However, an additional step is required to introduce a thiol functionality on the cell adhesion molecule. This is readily done using *N*-succinimidyl-*S*-acetylthioacetate [35]. Related to this strategy, immunoliposomes have been prepared using maleimide–DPPE through a reaction with thiolated $F(ab')_2$ fragments of the monoclonal antibody GAH [131]. GAH recognizes surface molecules especially in stomach cancer, and a PEGylated immunoliposome formulation using GAH targeting has been shown to have better efficacy than non-targeting liposomes in Phase I clinical trials [59, 88].

A significant number of monoclonal antibodies have been identified and can be engineered as Fab′ or scFv fragments as well as chimeric or humanized antibodies. Highly effective and reproducible methods of coupling whole antibodies or their fragments to the surface of plain liposomes or PEGylated liposomes exist, and in general immunoliposomes show an enhanced recognition of target cells, as well as a better internalization and intracellular drug delivery over non-targeted liposomes [71, 102].

3.2 Folate-Modified Liposomes

Liposomes functionalized with the vitamin, folic acid, have attracted much attention as the folate receptor is highly overexpressed in a number of cancers, and it can retain its high affinity binding to the folate receptors even after being covalently linked to a variety of macromolecules [144]. The vitamin is internalized to a large extent in proliferating cells as folic acid is essential in the biosynthesis of nucleotides; consequently, cancerous cells overexpress the folate receptor as the fast-dividing malignant cells are in great need of this nutrient. Normally, the folate receptor is expressed at the apical side of epithelial cells and is not accessible to blood-borne drugs. However, when the epithelial cells become malignant, the membrane loses its polarity and the folate receptor can be located at the basal surface of the membrane as well, taking up therapeutics from the plasma [81]. There is no evidence that normal healthy cells are expressing the folate receptor to a significant extent, and healthy tissue should therefore not be affected by the delivery of folate-targeted drugs [103, 138]. The first report of using folate-targeted liposome to transport drugs into tumor cells was published in the 1990s by Lee and Low [76]. They conjugated the NHS-ester of folic acid to the distal end of a NH_2-PEGylated liposomes forming a stable amide bond. Gabizon et al. applied a similar approach simply using a DCC-mediated coupling procedure linking the terminal free γ-carboxylic acid on folate and the free PEG amine functionality [44]. Folate contains both an α- and γ-carboxylic acid moiety, but as the gamma position has a higher reactivity, this is the main coupling product observed [68]. After endocytotic uptake [9] of the liposome complex by the folate receptor-expressing cell, release of the liposome content has been accomplished taking advantage of the endosomal acidic environment, either by introducing pH-sensitive fusogenic peptides to promote uptake into the endosome [134] or adding pH-sensitive lipids to the liposome to increase liposome permeability and accelerate unloading of the content in the acidic endosome [112]. The folate molecule can form dimers, trimers and even higher self-assembling constructs. An increasing level of folate attached to the liposome surface was found not to increase the level of folate–liposome-complex binding to the folate receptor, as this receptor can only bind one folate and will not respond to multiple folates [27, 107]. One of the advantages with folate targeting is that the lipid–PEG–folate conjugates can be synthesized in organic solvent and purified. This allows for a precise control of the amount of targeting ligands that are present in the liposome, and there is no need for *post-functionalization*.

3.3 Saccharides

A large number of mammalian cells express sugar-binding proteins known as lectins. These have been found to be overexpressed on malignant cells and are believed to be involved in metastasis formation [31, 43]; they can thus serve as a

target for drug delivery systems. Liposomes functionalized with carbohydrates are biodegradable, low in toxicity and have a protein-shielding ability that makes them able to minimize liposome clearance from circulation. For instance, monosialoganglioside have been shown to enhance the circulation time similar to that observed for PEGylated liposomes [8, 90]. A number of different carbohydrates have been conjugated to the surface of liposomes. Hassane et al. [58] used click chemistry to couple an unprotected α-D-mannosyl derivative carrying a PEG spacer functionalized terminally with a reactive azide moiety. However, selective protection of the mannose alcohol groups is naturally required during functionalization with the PEG chain to avoid multiple substitutions [58]. Surface functionalization of liposomes using lactose, galactose and a diverse array of polysaccharides as the carbohydrate components have been synthesized using Staudinger ligation [148], thioglycoside-mediated attachment [147] and EDC couplings between the headgroup amine on the lipid, and an activated NHS-ester of the saccharide moiety [123, 142]. The employed methods have both involved *post-functionalization* and complete synthesis of the targeting lipid–ligand conjugate in organic solvent, allowing isolation of the target compound. The latter method is possible with all carbohydrate targeting ligands and should be the method-of-choice.

3.4 Peptides

The use of peptide-targeted liposomes as therapeutics has become highly interesting with the increasing knowledge of specific peptide sequences of proteins involved in cell–cell interactions as well as the improvements in synthesis or expression of synthetic peptides that closely resemble the human ones. For attachment of peptide moieties to the liposome surface, the typically reactive amino acids used are lysine, serine, cysteine, histidine, arginine, aspartic acid, glutamic acid, threonine, tyrosine, the N-terminal amino group and C-terminal carboxylic acid. The primarily used method is to react one of these reactive functionalities with an activated PEG component situated on the surface of the liposome. As lysine is one of the most prevalent amino acids in proteins, this has been a favored linking site; however, upon reaction of a nucleophilic amine group with an electrophilic activated PEG, multiple substitutions are often observed due to the the presence of multiple lysines in the peptide. Amine linkage to PEG has been achieved in multiple ways including the use of PEG dichlorotriazine [2] and PEG tresylate [41]. Many PEG conjugates have furthermore been synthesized using activated carbonate derivatives, such as succinimidyl carbonate, trichlorophenyl carbonate and *p*-nitrophenyl carbonate [93, 132]. Liposomes have been modified with cell-penetrating peptides such as TAT using a *p*-nitrophenyl carbonate-functionalized PEG under slightly basic conditions to form the non-toxic carbamate linkage. TAT and other cell-penetrating peptides such as penetratin and synthetic polyarginines have been conjugated to the surfaces of liposomes to improve liposome uptake by cells [127]. In general, lower reactivity of the

carbonate reagents provides higher selectivity; however, carbonate linkage with several amino acids such as lysine, histidine and tyrosine have been observed [110]. When a highly selective conjugation is required, a PEG–propionaldehyde can be prepared. This functionality will, if pH is controlled, react selectively with the N-terminal α-amine because of the lower p*K*a value of this amine compared to other possible nucleophiles in the sequence [69]. Another possibility is to make the activated ester of PEG carboxylic acids, such as NHS–PEG, which will react with primary amines to form stable amides. This method has been applied for the modification of liposomes with the vasoactive intestinal peptide (VIP) to target VIP receptors in breast cancer [30]. The receptor for this 28-amino acid neuropeptide is found to be overexpressed and homogenously distributed in all parts of the breast tumor and thus serves as a potential tumor-targeting functionality [108]. Other active targeting carriers are based on delivery systems that mimic local bioadhesion. Integrins and in particular the $\alpha_5\beta_1$ integrin have attracted much attention as this and its ligand, fibronectin, are found to be upregulated in blood vessels in tumor biopsies. There is strong evidence that peptides that mimic the cell adhesion domain of fibronectin and contain the peptide sequence RGD (arginine-glycine-aspartic acid) are potent inhibitors of tumor-growth, tumor metastasis and tumor-induced angiogenesis [89]. The RGD sequence has been prepared as a cyclic constrained 5-mer modified with a C-terminal thioacetyl group for linkage to the liposome. Deprotection of the acetyl group and incubation with a maleimide-PEGylated functionalized liposome resulted in a thioester linkage between the peptide and liposome [120]. A similar approach was used for the attachment of the linear ATWLPPR (alanine-threonine-tryptophan-leucine-proline-proline-arginine) sequence, found to have affinity for the vascular endothelial growth factor receptor (VEGF), a receptor overexpressed in the surface of angiogenic endothelial cells [15]. Utilization of cysteine residues is the main approach for site-specific modification due to high specificity and ease of modification of a sequence that lacks a cysteine residue. In addition, few free cysteines are present on the surface of proteins compared to, e.g., lysine. An additional synthetic peptide sequence often applied in blocking cancer cell adhesion is YIGSR (tyrosine-isoleucine-glycine-serine-arginine)—a sequence shown to be important in laminin receptor binding. Conjugation of the peptide moiety to the liposomal surface can be achieved by mild periodate oxidation of the threonine residue in the hexapeptide TYIGSR, leading to a reactive glyoxylyl functionality in the N-terminal end of the peptide followed by attachment to a hydrazide functionalized PEG lipid [146]. The oxidation of carbohydrate residues or N-terminal serine or threonine creates the possibility for site-directed PEGylation using hydrazides. The glyoxylyl functionality formed by the oxidation of N-terminal serine or threonine reacts site-specifically with PEG-hydrazide derivatives [45], and the PEGylated peptide can be purified before use. A similar site-specific N-terminal modification of peptides has been described by Geoghegan and Stroh [50]. In general, the peptides discussed above can be achieved in several ways and can both be inserted by *post-functionalization* and by synthetic procedures in organic solvent. The former is an advantage for larger peptides (>30

amino acids) that are relatively expensive as the purification step utilized in the latter procedure results in loss of compound. However, a synthetic approach that involves purification should always be employed when possible to remove side products and reagents. A number of reports furthermore utilize SPPS of lipopeptide conjugates, and this is a highly efficient method for smaller peptides [66].

4 Membrane Anchors

In relation to liposomes and other self-organized materials, an important consideration when choosing the chemical structure of the targeting conjugate is that membrane affinity for the specific lipid membrane has to be sufficiently high to secure stability during blood circulation and binding to the target receptor. The easiest strategy is of course to choose the same lipid anchor for the targeting conjugate as the majority of the lipids used in the formulation; however, this may not always be possible, and for large hydrophilic targeting conjugates it may not always be sufficient. Depending on the targeting conjugate and the concentration used, the packing parameter of the molecule should also be considered [83]. The flexibility of the functional group as well as the crowding from other molecules on the membrane surface will influence the conformation of the anchored group, e.g., brush-like versus mushroom-like structures of PEG [48]. Two of the most important factors in choosing an anchoring molecule are the critical aggregation concentration (CAC) of the targeting conjugate and how compatible it is with the lipid membrane in terms of membrane thickness and fluidity, e.g., saturated versus unsaturated hydrocarbon chains. It should be noted that using poly-unsaturated hydrocarbon chains might induce a stability challenge, since these are prone to oxidation [133]. A simple example of the importance of fluidity is that rhodamine-labeled DOPE in a DSPC liposome membrane migrates in cell culture to the cell membrane, whereas rhodamine-labeled DSPC remains bound to the liposomes. It is therefore highly important to evaluate the physico-chemical properties of the targeting conjugate and measure the formulation stability with appropriate biophysical methods.

A large variety of lipids has been used to anchor different targeting ligand conjugates in liposomal membranes. Among the most widely used are phospholipids (PL) [137], cholesterol [22], ether lipids [38], acyl chains [60], glycophosphatidylinositol (GPI) [121] and molecular rods [51]. Each anchor type has several subtypes where small modifications can have a significant influence on how well the anchor of the functionalized group associates with the membrane. The choice of anchor depends on the physico-chemical properties of the conjugated group.

Phospholipids are the most widely used anchors since most liposome-based drug delivery systems are composed of commercially available PLs. PE and PE–PEG–NH_2 are the most utilized when synthesizing targeting moieties by

standard chemistry in organic solvent. The linkage chemistry of choice is often activated carbonates, and "click" chemistry is utilized more and more often due to the high level of functional group orthogonality. However, hydrazines should also be considered for reaction with aldehydes as this chemistry works very well and is highly orthogonal to other chemistries. For *post-functionalization*, Michael addition to maleimides with thiols is by far the most often used approach as this chemistry is fully compatible with the aqueous environment. "Click" chemistry can also be considered, but it is more difficult to achieve high coupling efficiency between sterically hindered moieties in comparison to the maleimide chemistries. A special case of PLs is the sphingolipids, which have three major subclasses: (1) ceramides, (2) sphingomyelins and (3) glycosphingolipids, where the latter is mainly used to anchor large proteins. One drawback of using glycerophospholipids as anchors is that enzymes that hydrolyze the lipids giving free fatty acid and lysophospholipids can be overexpressed in diseased tissue, e.g., phospholipase A2 (PLA2) [7, 65]. The enzyme hydrolysis will eventually disrupt the lipid membrane, and the liposome thereby loses its targeting capacity. To circumvent problems with enzymatic hydrolysis or chemical degradation of the ester bonds (which are the weakest chemical bond in the usual phospholipid conjugates), ether lipids can be used. The chemistry of conjugation is the same as for the normal diacyl-glycerophospholipids, but the cost of manufacturing represents a limitation.

Cholesterol and derivatives like thio-cholesterol are also among the frequently used anchors for functional groups on the surface of liposomes. Like PLs, cholesterols are widely used in liposomal drug delivery systems and are also highly present in natural membranes, making cholesterol anchors highly compatible with most membranes. Cholesterol is typically coupled to a functional group via an ester bond, carbamate ester or ether bond using the same type of chemistry as for PLs. Cholesterol will usually serve as a strong anchoring molecule to relatively large targeting conjugates with a low CAC value.

Acyl chains are also commonly used as anchors due to the easy coupling to primary amines and alcohols. The stability of the amide bond makes this approach suitable for solid phase synthesis and usually involves only one acyl chain, but in some cases it involves two or three [80].

The use of only one acyl chain has been seen in multiple studies, but does not generally serve as a sufficient anchor in vivo (or even in vitro) as the water solubility is too high and fast migration to, e.g., cell membranes will be observed. If mono-acylation is used, particular attention should always be given to verifying the anchoring stability.

Molecular rods are a relatively new form of anchors for lipid membranes with functional groups attached to each end. They are designed to fit into membranes spanning the entire bilayer, unlike most other lipid anchors, and can be synthesized with various lengths and desired rigidity needed for the individual target membrane [97]. The principle of lipids spanning the entire membrane is known from archaebacterial membranes, and mimics of the natural occurring lipids have been synthesized [104]. Both molecular rods and tetraether lipids have the advantage of

spanning the entire membrane, making it several orders of magnitude more stable compared to conventional lipids.

5 Future Directions and Conclusion

The chemistry for functionalizing liposomes with targeting ligands is diverse and has been developed over the last 3 decades. Even so, a number of challenges remain as there is a growing need for reactions that are highly regioselective and efficient. "Click" chemistry is a very good example of the type of chemistry that is required as it provides a very high degree of orthogonality to naturally occurring functional groups. However, the reaction suffers from use of copper as catalyst, and it is not a highly efficient reaction on the surface of liposomes. Another important requirement is that the reactions should be relatively cost efficient where the most important factor is that it proceeds in high yield. Lastly, for *post-functionalization*, the reactions should ideally not give any need for successive purification. Good examples of reactions that fulfill this requirement are the Michael addition to maleimides with thiol-containing ligands and hydrazine condensation with aldehydes that do not require purification if all ligands react. Another important step forward will furthermore be to develop chemistries that are easily evaluated for efficiency as it is not always a trivial task to evaluate reaction progression depending on the ligand being used. This can be envisaged in a number of ways, and it is certain that the coming years will provide much better functionalization chemistries giving high coupling efficiencies, high regio-selectivity and methods for fast evaluation of reaction progression. This is not only for use in drug delivery applications, but also to meet the growing needs in the development of new diagnostic tools where nanoparticles are in increasing demand.

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