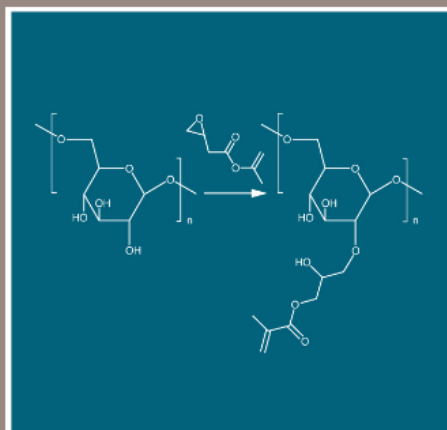




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Biomedical hydrogels

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Biomedical hydrogels

Biochemistry, manufacture and
medical applications

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Hydrogel swelling behavior and its biomedical applications

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Abstract: The ability of hydrogels to respond to relatively small changes in stimuli with relatively large changes in volume allows a wide variety of applications. This chapter addresses hydrogels with regard to the chemical identity of hydrophilic polymers and copolymers, polymer synthesis, the degree of crosslinking and hydrogel porosity, and bulk geometry of hydrogels in the form of matrix, membrane and erodible systems. The relationships between these features and hydrogel swelling behavior upon stimulation are also described. Finally, various exploitations of hydrogel swelling behavior in developing highly sensitive, real-time biosensors are discussed.

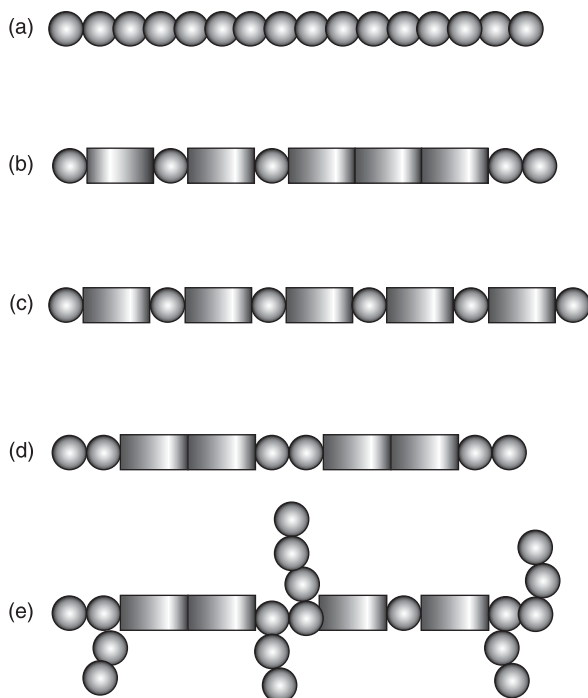
Key words: hydrogels, superporous hydrogels (SPHs), swelling, environment-sensitive.

1.1 Basics of hydrogels

Hydrogels gained increased attention from the scientific community in the latter half of the 20th century (Brannon-Peppas and Peppas, 1991). The ability of hydrogels to respond to relatively small changes in external stimuli with relatively large changes in bulk volume enables direct detection of a variety of stimuli (Gemeinhart *et al.*, 2000; Lee and Park, 1996; Peppas *et al.*, 2000; Roy and Gupta, 2003). The chemical makeup, synthesis, crosslinking, and geometry of hydrogels are briefly described.

1.1.1 Chemical identity of hydrophilic polymers and copolymers

Hydrogels are composed of hydrophilic polymer chains. These chains may consist of repeating monomers (homopolymers) or chemically different monomers (copolymers) (Peppas *et al.*, 2000). As depicted in Fig. 1.1, monomers can be arranged in such a way to make random copolymers, alternating copolymers, block copolymers, or graft copolymers. Additionally, the polymer chains may form more intricate three-dimensional structures, such as five-pointed star polymers, or dendrimers (Jeong *et al.*, 2002). The selection of chemical makeup of a polymer is critical to controlling swelling behavior, since these constituents are responsible for interactions with water and subsequent volume change (Peppas *et al.*, 2000). For example, hydrogels with hydrophobic internal cores would be well suited for delivery of poorly water-soluble drugs (Jeong *et al.*, 2002).



1.1 Chemical diversity of hydrogel polymer chains. (a) homopolymer, (b) random copolymer, (c) alternating copolymer, (d) block copolymer, and (e) graft copolymers. Rectangular and circular units represent chemically different monomers.

1.1.2 Polymer synthesis

Polymer synthesis is tailored according to the need to develop chemically diverse hydrogels for specific applications (Brannon-Peppas and Peppas, 1991; Peppas *et al.*, 2000). Depending on the application, the synthesized polymers may require biocompatibility, mechanical strength, or analyte specificity in addition to sensitivity to stimuli (Brannon-Peppas and Peppas, 1991; Lee and Park, 1996; Peppas *et al.*, 2000; Kim and Park, 2001b; Kim and Park, 2004). Table 1.1 lists monomers used in synthesizing hydrogels for pharmaceutical applications. Polymers are synthesized by various mechanisms, such as radical polymerization, condensation polymerization, graft-copolymerization, photopolymerization, and ring-opening polymerization (Lee and Park, 1996; Kim and Park, 2001b; Lee *et al.*, 2003; Xiao, 2007; Pearton *et al.*, 2008; Xue *et al.*, 2004; Plunkett *et al.*, 2003; Gu *et al.*, 2002). Care must be taken to purify the synthesized hydrogels for pharmaceutical and biomedical applications by removing the residual monomer, initiator, crosslinking agent and other contaminants (Markowitz *et al.*, 1997; Risbud *et al.*, 2000; Peppas *et al.*, 2000).

Table 1.1 Examples of monomers used in pharmaceutical applications

Monomer
Acrylic acid (AA)
Ethylene glycol (EG)
Hydroxyethyl methacrylate (HEMA)
<i>N</i> -isopropyl acrylamide (NIPAAm)
<i>N</i> -vinyl-2-pyrrolidone (NVP)
Poly(ethylene glycol acrylate) (PEGA)

Source: Peppas *et al.* (2000)

1.1.3 Degree of crosslinking and porosity

Hydrogels are crosslinked either physically or chemically to form networks (Peppas *et al.*, 2000; Roy and Gupta, 2003). Physical crosslinking occurs via noncovalent interactions, whereas chemical crosslinking utilizes covalent interactions (Lin *et al.*, 2005). The degree of crosslinking plays a significant role in the integrity and swelling properties of hydrogels, influencing hydrogel structure and swelling capacity (Flory and Rehner, 1943; Brannon-Peppas and Peppas, 1991). The greater the extent of crosslinking, the less flexible a hydrogel is to shrink, swell or change phase in response to stimuli (Peppas *et al.*, 2000). Hydrogel brittleness has been observed at high degrees of crosslinking (Peppas *et al.*, 2000). Physical crosslinks are often used in hydrogel formation due to their ability to reform crosslinks upon removal or presentation of the stimulus (Roy and Gupta, 2003; Lee and Park, 1996; Lee *et al.*, 2004).

Hydrogels have a range of porosities that influence the diffusion coefficients involved in mass transfer during swelling (Peppas *et al.*, 2000; Bezemer *et al.*, 2000). Pore size is dependent on the average molecular weight of polymer chain segments between adjacent crosslinks and acts as a selective barrier with regard to the permeability of substances (Peppas *et al.*, 2000). Specifically, swelling can be decreased by decreasing the average molecular weight of the polymer chain segments between crosslinks (Brannon-Peppas and Peppas, 1991). Pore size can be further controlled by various techniques, such as freeze drying, porosigen method, or gas formation method (Gemeinhart *et al.*, 2000). Therefore, the pores can range from a few nanometers to several micrometers (Kim and Park, 2004).

1.1.4 Bulk geometry of hydrogels

Hydrogels can be molded into various geometries, ranging from microspheres to films, and this makes their application highly versatile (Roy and Gupta, 2003). Hydrogel matrixes can be used as implantable scaffolds, due to their structural

properties and ability to absorb or release bioactive substances (Roy and Gupta, 2003; Pearton *et al.*, 2008; Markowitz *et al.*, 1997; Risbud *et al.*, 2000; Lee *et al.*, 2003; Mauck *et al.*, 2002; Gombotz and Wee, 1998). Table 1.2 lists hydrogels that have been studied for controlling the release of bioactive substances. Due to the relative thickness of a hydrogel matrix (as compared to membranes), the rate of diffusion for drug molecules through the matrix may be impeded (Zhang and Wu, 2002). Conversely, hydrogel membranes are relatively thin and offer increased response rate (swelling or shrinking) to stimuli due to the shorter distance required for diffusion (Zhang and Wu, 2002). Such hydrogels, capable of preventing degradation of labile substances, act as their reservoirs until stimulated (Pearton *et al.*, 2008; Markowitz *et al.*, 1997; Risbud *et al.*, 2000; Mauck *et al.*, 2002; Bezemer *et al.*, 2000). Similarly, erodible hydrogels are of interest in the

Table 1.2 Examples of the use of hydrogels in biomedical applications

Hydrogel composition	Substance released	Stimulus	Ref.
Polyacrylic Carobopol-940 or PLGA-PEG-PLGA	Plasmid DNA (pDNA)	Hydration Temperature	(Pearton <i>et al.</i> , 2008)
Polyacrylamide	Monoclonal antimouse IgG-FITC	Hydration	(Markowitz <i>et al.</i> , 1997)
Poly(chitosan-pyrrolidone)			(Risbud <i>et al.</i> , 2000)
PEG-PLGA-PEG Agarose hydrogel	Plasmid TGF- β 1 Chondrocytes*	Temperature	(Lee <i>et al.</i> , 2003) (Mauck <i>et al.</i> , 2002)
α -tocopheryl methacrylate-co-2-hydroxyethyl methacrylate (VEMA-co-HEMA)	α -tocopherol	pH	(Plasencia <i>et al.</i> , 1999)
Poly(HEMA-co-DMA), GOD	Insulin	Glucose	(Brahim <i>et al.</i> , 2002)
Poly(ethylene glycol)/poly (butylene terephthalate)	Lysozyme	Hydration	(Bezemer <i>et al.</i> , 2000)
Alginate microbeads	Albumin, HRP, Insulin, TGF- α , Hepatocytes		(Gombotz and Wee, 1998; Singh and Burgess, 1989; Igari <i>et al.</i> , 1990; Gray and Dowsett, 1988; Downs <i>et al.</i> , 1992; Miura <i>et al.</i> , 1986)

Note:* seeded in hydrogel

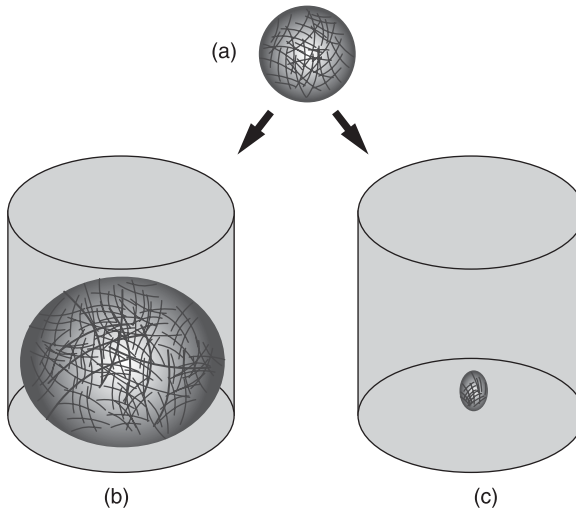
pharmaceutical field as their ability to exhibit zero-order release kinetics has been well established (Peppas *et al.*, 2000; Lee, 1984).

1.2 Swelling of hydrogels: water diffusion into hydrogels

The ability to display a measurable change in volume in response to external stimuli is a fundamental property of hydrogels (Lee and Park, 1996). Some hydrogels exhibit this volume change by swelling (see Fig. 1.2), while others undergo transitions between sol and gel phases (Brannon-Peppas and Peppas, 1991; Gemeinhart *et al.*, 2000; Lee and Park, 1996; Jeong *et al.*, 2002). When hydrogels swell, the glassy phase turns into the rubbery phase (Lee, 1984). The degree of crosslinking influences the area permitted for diffusion across the hydrogel network and, subsequently, the capacity for hydrogels to take up water (Peppas *et al.*, 2000). The water capacity is depicted from the equilibrium swelling ratio shown in Equation 1.1, as the ratio of the mass of a fully swollen hydrogel (in equilibrium with aqueous medium) to the mass of a dehydrated hydrogel (Brannon-Peppas and Peppas, 1991).

$$\text{Swelling ratio} = \frac{M_{\text{hydrated}} - M_{\text{dehydrated}}}{M_{\text{dehydrated}}} \quad [1.1]$$

where M represents hydrogel mass. Interactions between polymers in hydrogels and water are similar in nature to those between non-crosslinked polymers and



1.2 Dehydrated (a), swollen (b), and shrunken (c) hydrogels as the result of small changes in external stimuli, such as pH, temperature and analyte concentration that influence hydrogel hydrophilicity.

water. Hydrogels, consisting of networks of crosslinked hydrophilic polymers, undergo swelling instead of dissolution in water (Peppas *et al.*, 2000). Hydrogels made of polyelectrolytes swell more due to the charge repulsion among polymer chains, and such swelling property is useful in environment-sensitive swelling of hydrogels for controlled drug release (Peppas *et al.*, 2000; Roy and Gupta, 2003).

In the dehydrated state, hydrogels exist in the glassy state (Lee, 1984). The hydrogel may contain a substance of choice incorporated into the polymer network (Lee, 1984). As a glassy hydrogel swells, the inner portion of the hydrogel remains in a glassy phase, while the portion of the hydrogel that swells develops into a rubbery phase, expanding to accommodate water fluxed in (Peppas *et al.*, 2000). Substances in the glassy phase are extremely slow in diffusing through the hydrogel network, while substances located in the outer, rubbery phase are released easily (Peppas *et al.*, 2000). A swelling agent can be included to penetrate the hydrogel more rapidly than the encapsulated substance would normally diffuse, enhancing substance release through the swollen network (Lee, 1984; Peppas *et al.*, 2000).

Hydrogels can have ionic or neutral side groups attached to their backbone chains, and either group will influence water uptake (Peppas *et al.*, 2000). The Flory-Rehner theory aids in describing swelling of neutral hydrogels (Peppas *et al.*, 2000). Briefly, a neutral hydrogel experiences a thermodynamic force of mixing and a contractive force that become balanced once a hydrogel reaches its equilibrium swelling state (Peppas *et al.*, 2000). The theory was modified by Peppas and Merrill to account for hydrogels synthesized in water (Peppas *et al.*, 2000). Anionic and cationic hydrogels have an additional force exerted on their networks due to their ability to form ionic interactions (Peppas *et al.*, 2000). Peppas and Merrill derived relationships between ionic strength, the swollen state hydrogel volume fraction, and the average molecular weight of a polymer chain segment between two adjacent crosslinks, for polyelectrolyte hydrogels (Peppas *et al.*, 2000). Altering the ionic strength of the hydrogel swelling agent (via salting out or salting in reagents) influences the equilibrium swelling volume (Peppas *et al.*, 2000; Jeong *et al.*, 2002; Suzuki and Kumagai, 2003).

Superporous hydrogels (SPHs) are capable of rapid swelling and shrinking via capillary forces (Gemeinhart *et al.*, 2000). Fast swelling occurs as a result of convection of water into the porous hydrogels. Specifically, SPHs have pore sizes to the order of 10–1,000 μm , formed by gas blowing during hydrogel synthesis and gelation (Kim and Park, 2004). Because of the highly porous structure, SPHs often lack the mechanical strength required to be effective biosensors (Kim and Park, 2004). Polyethylenimine (PEI) interpenetrating polymer networks (IPNs) have been incorporated into poly(acrylamide-co-acrylic acid) P(AAm-co-AA) SPHs to improve the compressive strength (Kim and Park, 2004). PEI has a highly branched and ionizable structure capable of interacting

with P(AAm-co-AA) electrostatically (Kim and Park, 2004). Reduced pore size from the resulting interactions with PEI was the trade-off for improved strength (Kim and Park, 2004).

1.3 Stimulus-responsive hydrogels

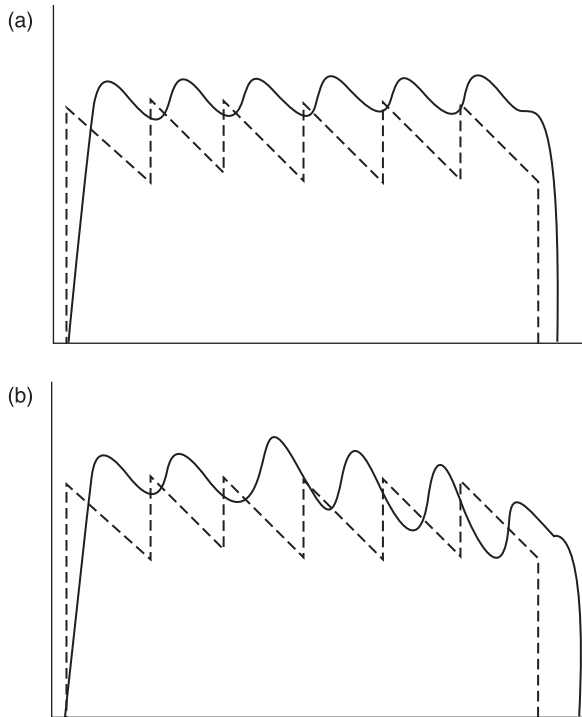
1.3.1 Linear hydrogel responses to external stimulus

Environment-sensitive hydrogels usually respond to external stimuli in a linear fashion after a stimulus reaches a setpoint. Hydrogels that can release insulin as a function of glucose concentration in the environment usually exhibit linear responses (Zhang and Wu, 2002; Kim and Park, 2001b; Obaidat and Park, 1997). Frequently, there is a lag time after the hydrogel is first stimulated until it responds to the stimulus (Kim and Park, 2001b; Obaidat and Park, 1997). If the hydrogel swelling depends on the changes in environment initiated by the components attached to the polymer chains (e.g., immobilized enzymes or other bioactive molecules), the hydrogel response rate is further delayed (Suzuki and Kumagai, 2003).

A hydrogel-actuated microvalve (HAM) was designed to respond to glucose concentration (Gu *et al.*, 2002). Specifically, the HAM was a phenylboronic acid-based hydrogel, configured in an apparatus to modulate fluid flow, depending on whether the hydrogel was in a swollen or shrunken state (Gu *et al.*, 2002). A swollen hydrogel closed the valve, while a shrunken hydrogel permitted flow (Gu *et al.*, 2002). The HAM device had consistent responses (depicted as a flow rate) to changes in glucose concentration (Gu *et al.*, 2002).

1.3.2 Hysteresis in hydrogel responses to external stimulus

Hydrogel swelling and response rate are further complicated when the stimulus setpoint is modulated as depicted in Fig. 1.3 (Zhang and Wu, 2002; Kim and Park, 2001b; Kataoka *et al.*, 1998; Xiao, 2007; Satish and Shivakumar, 2007). The main challenges in development of environment-sensitive hydrogels are to make the hydrogels able to detect small changes in the stimulus with corresponding response, and, equally importantly, to maintain the hydrogel sensitivity over the entire spectrum of stimulus setpoints as well as during the lifetime of the intended applications. The ingenuity of future hydrogel developments will lie in the ability to predict and reproduce hydrogel swelling response with repetitive stimulation. Although hydrogels are capable of undergoing reversible transitions, repeated cycling between phases does not imply that mass transport through the hydrogel is reproducible on each cycle (Kim and Park, 2001b; Zhang and Wu, 2002; Miyata *et al.*, 2002; Makino *et al.*, 1990; Satish and Shivakumar, 2007). It is yet to be identified how the formation and re-formation of crosslinks during cycling account for altered mass transport in reversible hydrogel systems.



1.3 Modulation of external stimulus and subsequent hydrogel response. (a) theoretical hydrogel response to stimulus with respect to time. Constant hydrogel response rate and level baseline are achieved over time. (b) Hysteresis in hydrogel response to stimulus with respect to time. Hydrogel response rate and baseline level are time-dependent. Dashed grey line represents a change in stimulus setpoint with respect to time. Solid black line represents hydrogel response with respect to time.

Studies utilizing antigen-antibody sensitivity incorporated in a hydrogel network showed reproducible swelling behavior during a limited number of repeated cycles (Miyata *et al.*, 1999; Miyata *et al.*, 2002). Here, swelling resulted from introduction of free antigen to the hydrogel, which competes with the antigen attached to the hydrogel polymer chains (Miyata *et al.*, 1999; Miyata *et al.*, 2002).

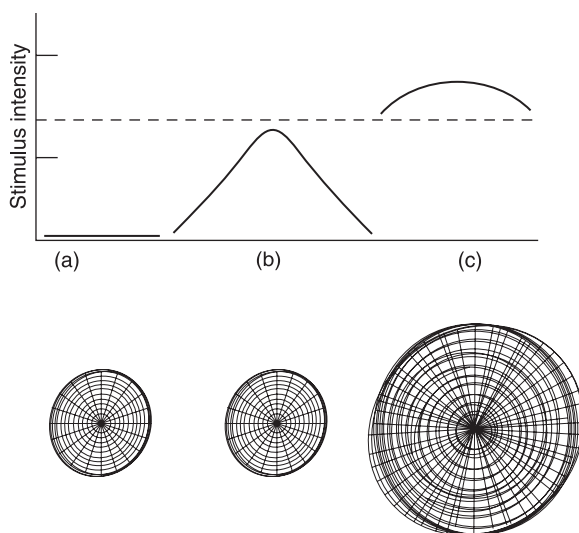
One study employing a disposable hydrogel biosensor showed that hydrogel glucose-sensitivity changed with time (Suzuki and Kumagai, 2003). Cycling through stimulus setpoints has revealed an increase in hydrogel response rate over time in some hydrogel delivery systems (Kim and Park, 2001b; Zhang and Wu, 2002). Specifically, glucose-sensitive hydrogels, such as (poly(allyl glucose-co-3-sulfopropylacrylate) (P(AG-co-SPAK), poly(allyl glucose-co-N-vinyl pyrrolidone) (P(AG-co-VP), and poly(allyl glucose-co-acrylamide) (P(AG-co-AAm), exhibited faster release rates of insulin when exposed to cycles of either 1 or 4 mg/mL glucose solutions (Kim and Park, 2001b). Furthermore, the baseline for

such responses rates drifted significantly upon cycling between predetermined setpoints, confirming that some inherent feature of the hydrogels was altered upon resuming the original setpoint (Kim and Park, 2001b; Kataoka *et al.*, 1998; Xiao, 2007; Miyata *et al.*, 2002; Makino *et al.*, 1990; Satish and Shivakumar, 2007). Other hydrogel systems consisting of PNIPAAm exhibit constant response rates during cycling, yet fail to achieve reproducible swelling behavior over time (Xiao, 2007).

A likely reason for hysteresis in the response behavior is deterioration of the hydrogel components with repeated exposure to stimuli (Kim and Park, 2001b). Measures have been taken to reduce hydrogel degradation by enclosing hydrogels in dialysis tubes or membranes that allow the free flow of water and small solutes (Lee and Park, 1996; Obaidat and Park, 1997). Specifically, the inability of physical crosslinks to completely reform once dissociated may be responsible for the increased release rates observed. As a result of incomplete crosslink formation, pore size may remain enlarged, allowing diffusion of more drug molecules. Additionally, re-formation of some crosslinks may have a time-dependence. The chemical species involved in hydrogel stimulus recognition may also be affected during cycling.

1.3.3 Delayed swelling (threshold-dependent swelling)

Hydrogels can be designed to respond only beyond a certain threshold stimulus intensity but do not respond to stimuli although present below the threshold, as conveyed in Fig. 1.4 (Lee and Park, 1996; Kim and Park, 2001b; Kikuchi and



1.4 Overcoming a stimulus threshold to elicit a hydrogel response. Hydrogel behavior without stimulus (no swelling) (a), below stimulus threshold (no swelling) (b), and above stimulus threshold (swelling) (c). The X-axis represents different degrees of stimulation.

Okano, 2002; Kataoka *et al.*, 1998). An example of the threshold-dependent response is the use of the concanavalin A (Con A) lectin as a crosslink of glucose containing polymers (allyl glucose) (Lee and Park, 1996; Obaidat and Park, 1997; Kim and Park, 2001b). Soluble free glucose is supplied four times the molar quantity of Con A incorporated into the hydrogel structure in order to elicit a sol–gel phase response (Lee and Park, 1996). Free glucose has the ability to competitively bind with Con A, replacing some of the allyl glucose bound to Con A, and facilitating a sol–gel phase transition (Lee and Park, 1996). Reducing hydrogel dimensions and thickness may improve the hydrogel response rate (Obaidat and Park, 1997).

1.4 Examples of environment-sensitive hydrogels

Environment-sensitive hydrogels have the capability to imitate feedback mechanisms often observed in nature (Miyata *et al.*, 2002). For instance, glucose, cholesterol and galactose amperometric hydrogel biosensors have been designed (Brahim *et al.*, 2002). Impressively, it has been suggested that these sensors exhibit quick, linear responses to their respective stimuli (Brahim *et al.*, 2002).

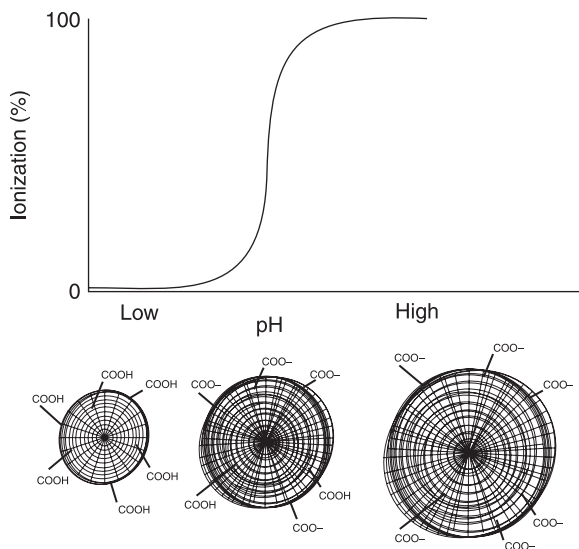
A variety of environment-sensitive hydrogels have been designed to harness the hydrogel swelling potential into a sensory device (see Table 1.3) (Roy and Gupta, 2003). The most common hydrogel systems swell in response to changing pH, temperature, and analyte concentration (Roy and Gupta, 2003).

1.4.1 pH-sensitive hydrogels

Hydrogels made of polyelectrolytes serve as pH-sensitive sensors (Peppas *et al.*, 2000). Depending on solution pH and dissociation constants (pK_a or pK_b) of polymer side groups, the hydrogel becomes ionized and swells as a result of electrostatic repulsion of polymer chains (see Fig. 1.5) (Brannon-Peppas and Peppas, 1991; Peppas *et al.*, 2000). Conversely, as the hydrogel becomes unionized, it shrinks due to reduced electrostatic repulsion (Peppas *et al.*, 2000). For instance, N,O-carboxymethyl chitosan (NOCC) and alginate copolymer hydrogels were synthesized for use as carriers for oral administration of protein drugs (Mi *et al.*, 2005). NOCC behaves as a zwitterion over a range of pH values,

Table 1.3 Examples of ion-sensitive natural hydrogels

Hydrogel composition	Stimulus	Ref.
Alginate	Ca^{2+} and other divalent ions	(Roy and Gupta, 2003; Gombotz and Wee, 1998; Byrom, 1991)
Chitosan	Mg^{2+} or pH	(Roy and Gupta, 2003)
κ -carrageenan	K^+	(Roy and Gupta, 2003)



1.5 Swelling behavior of pH-sensitive (acidic) hydrogel. Acidic groups are unionized (bottom left), partially ionized (bottom middle), or completely ionized (bottom right).

and was crosslinked to alginate covalently using either genipin or glutaraldehyde, or ionically using calcium ions (Mi *et al.*, 2005). The swelling ratios for the three types of crosslinked NOCC/alginate hydrogel systems were observed over acidic and slightly alkaline values at physiologic temperature in an attempt to simulate conditions in the gastrointestinal tract (Mi *et al.*, 2005). Additionally, drug release was simulated from these three hydrogel types using 1% (w/v) bovine serum albumin (BSA) (Mi *et al.*, 2005). It was observed that hydrogels consisting solely of NOCC had relatively high swelling ratios that decreased with the introduction of alginate during synthesis (Mi *et al.*, 2005). Additionally, the lowest swelling ratios for the three crosslinked types of hydrogels all coincided at pH 4, suggesting low electrostatic interactions between NOCC and alginate (Mi *et al.*, 2005).

In yet another pH-sensitive hydrogel system utilizing chitosan as a drug carrier to the colon, sodium tripolyphosphate (Na^+ -TPP) and dextran sulfate (DS) were incorporated into porous hydrogel microspheres (Lin *et al.*, 2005). Specifically, chitosan, chitosan/ Na^+ -TPP and chitosan/ Na^+ -TPP/DS hydrogels were observed for their relative swelling properties and drug release (Lin *et al.*, 2005). Ibuprofen was chosen to depict the ability of the microspheres to adequately encapsulate hydrophobic drugs in both alkaline and acidic media (Lin *et al.*, 2005). In the hydrogel categories listed above, chitosan functioned as the polycation, while ionized Na^+ -TPP and DS functioned as anions (Lin *et al.*, 2005). In all hydrogel categories, swelling ratios increased with increasing pH, where most chitosan amines are deprotonated and negative charges of DS and TPP become dominant

Table 1.4 Examples of pH-sensitive hydrogels

Hydrogel composition	Substance released	Stimulus	Ref.
Poly(acrylamide-co-acrylic acid)/ polyethylenimine interpenetrating network (P(AAm-co-AA)/PEI IPN)		pH	(Kim and Park, 2004)
Poly(acrylamide-co-acrylic acid) (P(AAm-co-AA))		pH	(Gemeinhart <i>et al.</i> , 2000)
Chitosan, chitosan/Na ⁺ -TPP, chitosan/Na ⁺ -TPP/DS	BSA	pH	(Lin <i>et al.</i> , 2005)

(Lin *et al.*, 2005). As the DS constituent of the chitosan-based hydrogels increases, the degree of swelling increases as a result of the hydrophilicity of the sulfate group (Lin *et al.*, 2005). A partial list of pH-sensitive hydrogels is shown in Table 1.4.

1.4.2 Temperature-sensitive and phase-reversible hydrogels

Phase-reversible hydrogels do not swell but rather have the ability to change solubility from a free flowing solution to a gel phase and vice versa (Jeong *et al.*, 2002; Lee and Park, 1996). Sol–gel (reversible phase) hydrogels have been designed to respond to changes in pH, temperature and analyte concentration, in addition to other stimuli in as little as 5–30 minutes (Lee and Park, 1996; Obaidat and Park, 1997). For temperature-sensitive sol–gel hydrogels, the transition between the solution and gel phases occurs at the upper critical solution temperature (UCST) or the lower critical solution temperature (LCST) (Peppas *et al.*, 2000). This temperature can be identified upon inverting a vessel containing the hydrogel, and noting the temperature at which the gel phase begins to flow or the solution phase becomes restricted to flow (Jeong *et al.*, 2002). The falling ball method has also been described for determining the sol–gel transition (Yoshida *et al.*, 1998). Table 1.5 highlights the LCST for commonly synthesized hydrogels. For example,

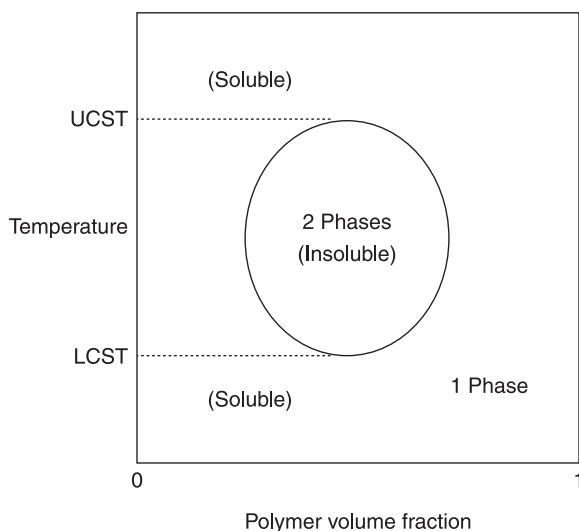
Table 1.5 Examples of LCST values of some hydrogels

Polymer	LCST (°C)
Poly(<i>N</i> -isopropylacrylamide) (NIPAAm)	~ 32
Poly(ethylene glycol) (PEG)	~ 120
Poly(vinyl alcohol) (PVA)	~ 125
Poly(vinyl pyrrolidone) (PVP)	~ 160
Methylcellulose (MC)	~ 80

Source: Jeong *et al.* (2002)

thermosensitive sol–gel hydrogels such as the poly(D,L-lactic-co-glycolic acid)-b-polyethylene glycol-b-poly(D,L-lactic-co-glycolic acid) (PLGA-PEG-PLGA) triblock copolymer forms a gel phase above the polymer's LCST ($\sim 32^{\circ}\text{C}$) (Pearton *et al.*, 2008). A homogeneous solution phase exists below LCST, but a gel is formed or polymers precipitate at temperatures higher than LCST. If the temperature is increased further, the gel phase becomes a sol phase again. Poly(*N*-isopropylacrylamide) (PNIPAAm) has a similar LCST as PLGA-PEG-PLGA (Xiao, 2007). The presence of salting in or salting out reagents influences the observed transition temperature (Jeong *et al.*, 2002; Suzuki and Kumagai, 2003; Kawasaki *et al.*, 1997). Depending on the polymers used in synthesis, either a solution ($T > \text{UCST}$) or gel ($\text{LCST} < T < \text{UCST}$) phase may exist above the critical solution temperature, depicted in Fig. 1.6 (Peppas *et al.*, 2000).

Other hydrogels are capable of undergoing a sol–gel phase transition in the excess of analyte concentration (Lee and Park, 1996; Jeong *et al.*, 2002; Gemeinhart *et al.*, 2000). In such circumstances, the stimulus is capable of inducing detachment of crosslinks, so that the hydrogel flows as a solution. Conversely, gelation occurs as crosslinks re-form among polymer chains. The ease of gel formation increases with increasing molar ratio of the crosslinking agent to backbone polymers (Lee and Park, 1996). Naturally occurring polymers that undergo sol–gel phase transition include chitosan (stimulated by pH), alginate (stimulated by calcium and other divalent ions excluding the magnesium ion), and



1.6 Phase changes of a temperature-sensitive polymer. At temperatures lower than LCST a homogeneous solution exists and a crosslinked hydrogel swells. As temperature increases above LCST, water-soluble polymer precipitates out of solution, and a crosslinked hydrogel shrinks.

κ -carrageenan (stimulated by potassium ions) (Roy and Gupta, 2003; Gombotz and Wee, 1998; Byrom, 1991). Although these polymers are natural, their derivatives may contain impurities that may prove toxic if implanted directly *in vivo* (Gombotz and Wee, 1998). For instance, heavy metals, mitogens and endotoxins may exist in the kelp sources used in producing alginate (Gombotz and Wee, 1998; Smidsrod, 1973).

1.4.3 Glucose-sensitive hydrogels

Interest in glucose-sensitive hydrogels has increased over the last few decades, as the projected number of people (diagnosed and undiagnosed) living with diabetes increases (Boyle *et al.*, 2001; Fagot-Campagna *et al.*, 2000). A decade ago, it was suggested that a third of people living with non-insulin dependent diabetes-mellitus (NIDDM or type II diabetes) did not even realize that they had this disease (Engelgau *et al.*, 1998; Petersen *et al.*, 2003; Anon., 1997; Gavin *et al.*, 1997). In addition to the disease itself, secondary diseases are further exasperated by NIDDM and insulin-dependent diabetes-mellitus (IDDM or type I diabetes) such as retinopathy, nephropathy, neuropathy and macrovascular disease (Control *et al.*, 1993). Additionally it has been suggested that between 1988 and 1994, approximately 71% of adult diabetics in the U.S. had hypertension or prehypertension as well (average blood pressure $\geq 130/85$ mm Hg) (Geiss *et al.*, 2002). Prevention of these diseases and further damage to the body is dependent on the regulation of blood glucose (Control *et al.*, 1993). The focus has frequently been on implantable devices that could sense abnormal glucose levels (generally hyperglycemic) in the body, and respond by delivering the appropriate amount of insulin, which signals glucose uptake into neighboring cells (Lee and Park, 1996; Obaidat and Park, 1997; Kim and Park, 2001b; Zhang and Wu, 2002; Zhang *et al.*, 2007; Shenkman *et al.*, 2007).

Concanavalin A and allyl glucose hydrogel systems

Glucose-sensitive hydrogel systems have been developed using concanavalin A (Con A), isolated from the *Canavalia ensiformis* jackbean, as a crosslinking agent of glucose-containing polymeric chains (allyl glucose), depicted in Table 1.6 (Kim and Park, 2001a; Kim and Park, 2001b; Lee and Park, 1996). As a tetramer at physiologic pH, the Con A lectin is capable of noncovalently binding four glucose molecules (Lee and Park, 1996; Kim and Park, 2001a; Kim and Park, 2001b). To maintain its structure and binding ability, seven co-ordinated calcium ions and six co-ordinated manganese (II) ions bind with Con A (Hardman *et al.*, 1982). The hydroxyl groups on carbons three through six of the allyl glucose interact with Con A (Kim and Park, 2001a). In one study, the allyl attachment to glucose was made at carbon one (Kim and Park, 2001a). Below the threshold free glucose concentration, four allyl glucose chains are bound to Con A (Kim and

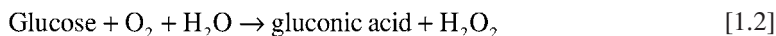
Table 1.6 Examples of glucose-sensitive hydrogels

Hydrogel composition	Substance released	Stimulus	Ref.
1-vinyl-2-pyrrolidinone-allyl glucose (VP-AG), Con A		Glucose	(Lee and Park, 1996)
Poly(hydroxyethyl methacrylate) (PHEMA), Con A	Insulin, Lysozyme	Glucose	(Obaidat and Park, 1997)
Poly(allyl glucose-co-3-sulfopropylacrylate potassium salt) (P(AG-co-SPAK)), Poly(allyl glucose-co-vinyl pyrrolidone) (P(AG-co-VP)), Poly(allyl glucose-co-acrylamide) (P(AG-co-AAm)), Pegylated Con A	Insulin	Glucose	(Kim and Park, 2001b)
Poly(<i>N</i> -isopropylacrylamide-co-methacrylic acid) (PNIPAAm-co-MAA), Glucose oxidase (GOD), Catalase	Insulin	Glucose	(Zhang and Wu, 2002)
Poly(acrylamide-co-3-acrylamidophenylboronic acid) (P(AAm-co-3-AAmPBA))		Glucose, Cis-diols	(Lee <i>et al.</i> , 2004)
Poly(<i>N</i> -isopropylacrylamide) (PNIPAAm) core, Poly(<i>N</i> -isopropylacrylamide-co-phenylboronic acid) (PNIPAAm-co-PBA) shell		Glucose	(Zhang <i>et al.</i> , 2007)
Hydrogel matrix, Glucose binding protein (GBP), Cyan fluorescent protein (CFP), Yellow fluorescent protein (YFP)		Light (400–400 nm), Glucose	(Shenkman <i>et al.</i> , 2007)
Poly(2-hydroxyethyl methacrylate-co- <i>N</i> , <i>N</i> -dimethylaminoethyl methacrylate), Glucose oxidase (GOD), Catalase	Insulin	Glucose	(Satish and Shivakumar, 2007)

Park, 2001b). Specifically, allyl glucose has a higher binding affinity for Con A than free glucose below this threshold free glucose concentration (Kim and Park, 2001b). As the concentration of free glucose is increased to four times the concentration of Con A, free glucose begins to compete with allyl glucose for binding sites on Con A (Kim and Park, 2001a; Kim and Park, 2001b). Excess free glucose concentration induces a sol phase, whereas lower glucose concentration induces a gel phase (Kim and Park, 2001b). A major concern about the use of Con A as a constituent in implantable hydrogels is the immunogenicity associated with the lectin (Kim and Park, 2001a; Kataoka *et al.*, 1998). To reduce immunogenicity and increase stability, poly(ethylene glycol) (PEG) units were grafted to Con A (Kim and Park, 2001a). A ratio of five PEG units to one Con A has displayed an optimum binding affinity of free glucose to Con A (Kim and Park, 2001b).

Coupled glucose oxidase and catalase hydrogel systems

Researchers have looked towards 'natural' ways of implementing blood glucose control by encapsulating glucose oxidase (GOD) isolated from *Aspergillus niger* within a hydrogel network made of pH-sensitive polymers (Zhang and Wu, 2002; Satish and Shivakumar, 2007). Free glucose, presumably from the blood, can be converted to gluconic acid via GOD (Zhang and Wu, 2002; Satish and Shivakumar, 2007).



As the concentration of gluconic acid increases, the pH correspondingly decreases, causing the hydrogel to swell or shrink in response and releasing stored insulin to combat an increasing glucose concentration (Zhang and Wu, 2002; Satish and Shivakumar, 2007). Limitations in the use of immobilized GOD include the need to replenish depleted enzymes, and buildup of hydrogen peroxide as a result of reduction-oxidation reactions (Zhang and Wu, 2002; Satish and Shivakumar, 2007). Hydrogen peroxide, a product of GOD, inhibits GOD function (Zhang and Wu, 2002; Satish and Shivakumar, 2007). To counteract this inhibition, catalase isolated from *Aspergillus niger* has been coupled with GOD in hydrogel systems, to convert hydrogen peroxide back to free oxygen and water (Zhang and Wu, 2002).



It has been reported that by incorporating catalase into the GOD immobilized hydrogel, as much as 50% of the oxygen used in converting glucose to gluconic acid can be recovered (Zhang and Wu, 2002). Additionally, it has been suggested that gluconolactonase may be used to increase the rate that gluconic acid is formed, converting the gluconolactone intermediate to gluconic acid (Suzuki and Kumagai, 2003; Hanazato *et al.*, 1988; Ogawa *et al.*, 2002). See Table 1.6 for a compilation of hydrogel systems utilizing GOD in the presence or lack of catalase.

Glucose binding protein and fluorescent resonance electron transfer (FRET) technology hydrogel systems for glucose sensing

A glucose binding protein (GBP) isolated from *Escherichia coli* was engineered and encapsulated in a hydrogel system (Shenkman *et al.*, 2007). GBP was engineered to depend on two additional proteins, cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), which act as an electron donor and acceptor, respectively (Shenkman *et al.*, 2007). Initially stimulated by blue light (400–400 nm), CFP and YFP form a closed circuit in the absence of free glucose (Shenkman *et al.*, 2007); thus, fluorescent resonance electron transfer (FRET) fluorescence from YFP is observed (Shenkman *et al.*, 2007). In the presence of free glucose, a conformational change takes place in GBP, increasing the distance between CFP

and YFP so that CFP is unable to donate its electron to YFP; therefore, fluorescence from CFP is observed (Shenkman *et al.*, 2007). Using FRET, the concentration of free glucose in the hydrogel environment can be readily quantified (Shenkman *et al.*, 2007).

Boronic acids and free glucose hydrogel systems

A glucose-sensitive hydrogel system made of boronic acid does not rely on lectins, enzymes or other proteins in detecting changes in glucose concentration (Kataoka *et al.*, 1998). For example, phenylboronic acid has been frequently incorporated into hydrogels due to its ability to bind with free glucose (Kataoka *et al.*, 1998). Specifically, polymer chains consisting of 3-acrylamidophenylboronic acid (3-AAmPBA) and poly(*N*-isopropylacrylamide) (PNIPAAm) become ionized in an alkaline environment (Kataoka *et al.*, 1998). Ionization of phenylboronic acid encourages covalent, yet reversible binding with glucose (Kataoka *et al.*, 1998; Lee *et al.*, 2004). In a medium with pH > 9, the phenylboronic acid portions of the hydrogel partially ionize (Kataoka *et al.*, 1998). As the concentration of glucose increases in the alkaline medium (pH 9), equilibrium shifts in favor of the ionized form of phenylboronic acid, which now has the ability to bind with glucose (Kataoka *et al.*, 1998). As a result, increasing aqueous glucose concentration increases phenylboronic acid ionization, which in turn increases the repulsive charges on polymer chains (Kataoka *et al.*, 1998). These charges increase hydrogel hydrophilicity and swelling is observed (Kataoka *et al.*, 1998).

In yet another glucose-sensitive hydrogel involving 3-AAmPBA, an acrylamide hydrogel film containing a hologram has been developed with the ability to detect glucose as well as other cis-diols in the environment (Lee *et al.*, 2004). Hydrogels of this sort would be useful nutrient monitors in bioreactors (Lee *et al.*, 2004). Specifically, in the presence of alkaline medium, 3-AAmPBA groups of the hydrogel bind cis-diols (i.e. deoxyribose, fructose, galactose, lactate, glucose, etc.), resulting in an observed swelling of the hydrogel (Lee *et al.*, 2004). Impinged with white light, the degree of hydrogel swelling shifts the light diffracted from the hydrogel from blue to red, depending on the cis-diol concentration (Lee *et al.*, 2004). Holographic hydrogel response rate was faster for 2 mM lactate than for 2 mM glucose solutions, yielding half-lives of 0.7 min and 10.5 min, respectively (Lee *et al.*, 2004).

1.5 Future trends

For clinical applications, future hydrogel biosensors will have to overcome several obstacles (Brahim *et al.*, 2002). For instance, sensors should be resistant to biofouling if they are to be employed in implantation or if interactions with biological fluids are required (Brahim *et al.*, 2002; Wisniewski and Reichert, 2000). While sufficiently sensitive and responsive to the specific stimuli, the

'smart' biosensors should remain insensitive to commonly observed interferents such as acetaminophen, uric acid, and L-ascorbic acid (Brahim *et al.*, 2002; Suzuki and Kumagai, 2003). Additionally, hydrogel biosensor sensitivity and stability over prolonged time and when not in use will need to be addressed (Brahim *et al.*, 2002). Disposable biosensors may be an alternative for sensors lacking sufficient stability or mechanical strength over time (Suzuki and Kumagai, 2003). Additionally, in order to lower hydrogel detection limits for reasonable use, synthesis of smaller 'microgels' will need to be employed (Plunkett *et al.*, 2003).

If the above challenges are overcome, hydrogel use may even prove convenient in diagnostic testing. It has been suggested that hydrogels, designed to be degraded by enzymes concomitant with specific disease states, can be used to determine the progression of disease states (Miyata *et al.*, 2002; Plunkett *et al.*, 2003). Targeted drug delivery may be enhanced with the use of hydrogels that degrade in the presence of specific enzymes. For instance, hydrogels have been synthesized to degrade with either dextranase or azoreductase, both which are readily available in the colon, for local treatment of diseases present in the colon (Hovgaard and Brondsted, 1995; Yeh *et al.*, 1995). Others have synthesized hydrogels that require multiple stimuli to degrade hydrogel networks (Yamamoto *et al.*, 1996; Kurisawa *et al.*, 1997).

The subject of hydrogels has already allowed the integration of a variety of scientific backgrounds, in understanding both chemical functions of natural and synthetic materials and biological functions of small molecules (Miyata *et al.*, 2002). For now, the ingenuity of future hydrogel developments will lie in the ability to predict and reproduce hydrogel swelling response with repetitive stimulation. For hydrogels to be used as real-time biosensors in the future, we must understand how the formation and re-formation of crosslinks during repetitive stimulation account for altered mass transport in phase reversible hydrogel systems.

1.6 References

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Superabsorbent cellulose-based hydrogels for biomedical applications

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Abstract: Hydrogels are macromolecular networks able to absorb and release water solutions in a reversible manner, in response to specific environmental stimuli. Such stimuli-sensitive behaviour has made hydrogels appealing for the design of ‘smart’ devices that find application in a variety of technological fields. This chapter surveys the design and the manufacture of cellulose-based hydrogels, which are extensively investigated due to the large availability of cellulose in nature, the intrinsic degradability of cellulose and the smart behaviour displayed by some cellulose derivatives. The sorption mechanism of cellulose-based hydrogels is discussed, as a function of the desired application.

Key words: cellulose derivatives, hydrogels, crosslink, swelling, thermodynamics, manufacturing applications.

2.1 Introduction

Hydrogels are macromolecular networks widely used in the biomedical industry for different applications and are capable of absorbing, retaining and releasing water solutions in a reversible way and in response to specific environmental stimuli. Due to this feature this class of material has been widely used in many fields ranging from personal care products (e.g. napkins and diapers) to drug delivery systems and in catalysis and in biosensing. Their application in agriculture has been investigated as they could represent a suitable solution for water storage and controlled release. The manufacture of cellulose-based hydrogels is extensively investigated due to the large availability of cellulose in nature and their intrinsic degradability. Also, the ‘smart’ (stimulus responsive) behaviour displayed by some cellulose derivatives is important.

A considerable drawback for traditional hydrogels, for example crosslinked sodium polyacrylates, is their potential environmental impact. Since environmentally friendly products and processes are of great importance nowadays, much industrial research has focused attention on the synthesis of novel cellulose-based hydrogels. In fact, cellulose is a natural polymer that can be employed as renewable-based polymeric material with biodegradable properties.

It is also well known that the effective swelling of cellulose-based hydrogels requires a chemically crosslinked network that can be obtained following different pathways. Hydrophilic polymers can swell and absorb water without dissolving, provided that chemical or physical crosslinks exist among the macromolecular

chains. The polymer network resulting from the crosslinks swells in the aqueous solvent, until the thermodynamic force of swelling is totally counterbalanced by the elastic, retractive force exerted by the crosslinks. This ‘solid-like solution’ of polymer and water resulting at equilibrium is known as a hydrogel. The amount of water retained by the mesh of the hydrogel network depends on the structure of the polymer network itself and on the environmental conditions, such as the temperature, pH and ionic strength of the water solution in contact with the polymer.

The volume or mass-swelling ratio of the hydrogel is the most important variable to be evaluated for given environmental conditions, as it affects the diffusive, mechanical, optical, acoustic and surface properties of the hydrogel itself. In cases where sharp and/or fast swelling-shrinking transitions happen in response to changes of external stimuli, hydrogels are potentially useful for the development of a variety of smart devices, such as valves, artificial muscles and substrates for controlled drug release. Since the first hydrogels based on poly-(hydroxyethyl-methacrylate) (PHEMA) developed by Otto Wichterle in the 1950s and later patented for use as soft contact lenses, great steps have been taken by researchers towards obtaining novel hydrogels, based on synthetic, natural or hybrid polymers, which possess given swelling properties and/or biocompatibility and bioactivity. Innovative hydrogel products have thus been developed either as water absorbents for specific applications (e.g., personal hygiene products, underwater devices, water reservoirs for dry soils) or as biomedical devices, including soft contact lenses, lubricating surface coatings, phantoms for ultrasound-based imaging, controlled drug release devices, wound healing dressings, cell immobilization islets, three-dimensional cell culture substrates and bioactive scaffolds for regenerative medicine.

2.1.1 Cellulose and cellulose-based hydrogels

Cellulose structure and biodegradability

Cellulose is the most abundant naturally occurring polymer of glucose and it can be found as the main constituent of plants and natural fibres such as cotton and linen. Some bacteria (e.g., *acetobacter xylinum*) are also able to synthesize cellulose (Ross *et al.*, 1991). Microbial or bacterial cellulose (BC) is chemically identical to plant cellulose (PC), although it possesses different macromolecular structure and physical properties (Czaja *et al.*, 2007). In both BC and PC, the glucose units are held together by 1,4- β -glucosidic linkages, which accounts for the high crystallinity of cellulose (usually in the range 40–60% for PC and above 60% for BC) and its insolubility in water and other common solvents.

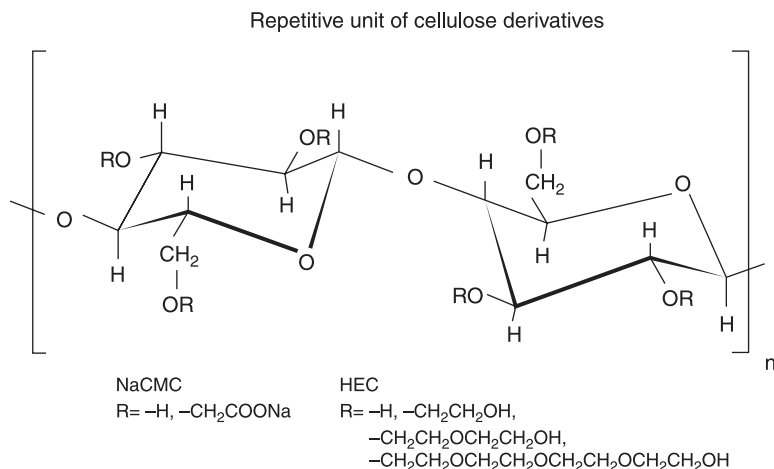
However, BC biosynthesis yields nanosized fibres, which are about two orders of magnitude smaller than PC fibres. BC thus shows a peculiar, ultrafine fibre network with high water holding capacity and superior tensile strength compared to PC. Moreover, BC is totally pure, unlike PC, which is usually associated with other biogenic compounds, such as lignin and pectin. Therefore, while BC is used as

synthesized by bacteria, PC requires further purification and modification. Chemical modification of cellulose, usually involving esterification or etherification of the hydroxyl groups, is performed to produce cellulose derivatives, named cellulotics, which are more easily processable and find large application in the industry. Cellulose and its derivatives are environmentally friendly because they are degraded by several bacteria and fungi, which are able to synthesize cellulose-specific enzymes (i.e. cellulases), present in air, water and soil (Tomsic *et al.*, 2007). The biodegradation of cellulose has been widely investigated, and progressively leads to decreased molecular weight, lower mechanical strength and increased solubility. Moreover, higher biodegradation rates of cellulose are likely yielded by lower degrees of crystallinity and improved water solubility (Miyamoto *et al.*, 1989).

The excellent biocompatibility of cellulose, cellulotics and cellulase-mediated degradation (Entcheva *et al.*, 2004, Martson *et al.*, 1999) has prompted the wide use of cellulose-based devices in biomedical applications. With regard to *in vivo* applications, it is worth reminding that cellulose is a biodurable material. Indeed, resorption of cellulose in animal and human tissues does not occur, since cells are not able to synthesize cellulases. Such a consideration points out the fundamental distinction between biodegradability and bioresorbability: the former refers to the ability of the material to be degraded by microorganisms, whereas the latter indicates the ability of the material to be digested or metabolized when implanted *in vivo*. In a pioneering long-term study by Martson *et al.* (Chen and Sun, 2000), a cellulose sponge implant seems to undergo a slow degradation in the rat subcutaneous tissue. However, the time length of the study (i.e., 60 weeks), together with the poor observed degradation and the lack of any knowledge about the possible mechanism of *in vivo* resorption, suggests that cellulose-based implants should be considered as biodurable. Nevertheless, the chemical modification and/or crosslinking of water-soluble cellulotics with bioresorbable moieties can yield resorbable cellulose-based devices (Sannino *et al.*, 2004, Sannino *et al.*, 2005, Ito *et al.*, 2007).

Water-soluble cellulose derivatives

Most water-soluble cellulose derivatives are obtained via etherification of cellulose, which involves the reaction of the hydroxyl groups of cellulose with organic species, such as methyl and ethyl units. The degree of substitution, defined as the average number of etherified hydroxyl groups in a glucose unit, can be controlled to a certain extent in order to obtain cellulose derivatives with given solubility and viscosity in water solutions. Cellulose-based hydrogels, either reversible or stable, can be formed by covalently crosslinking aqueous solutions of cellulose ethers, such as methylcellulose (MC), hydroxypropyl methylcellulose (HPMC), ethyl cellulose (EC), hydroxyethyl cellulose (HEC) and sodium carboxymethylcellulose (NaCMC), which are among the most widely used cellulose derivatives. The structure of such derivatives is shown in Fig. 2.1. It is



2.1 Structure of cellulose derivatives.

worth highlighting that all these polymers find wide application as thickeners and/or emulsifying agents in the food, pharmaceutical and cosmetics industries, due to their non-toxicity and low cost.

Among the above mentioned cellulose ethers, only NaCMC is a polyelectrolyte, and thus a 'smart' cellulose derivative which shows sensitivity to pH and ionic strength variations. Indeed the presence of NaCMC in a cellulose-based hydrogel provides the hydrogel itself with electrostatic charges anchored to the network, which have a double effect on the swelling capability. On one side, the electrostatic repulsion established between charges of the same sign forces the polymer chains to a more elongated state than that found in a neutral network, thus increasing the swelling. On the other, the counterions that are present in the gel to ensure macroscopic electrical neutrality induce more water to enter the network, due to a Donnan type effect (Flory, 1953). The Donnan contribution to the osmotic pressure is dependent on the different concentration of mobile counterions between the gel and the external solution, thus making the gel sensitive to variations of pH or ionic strength. The polyelectrolyte nature of NaCMC makes it ideal for the development of superabsorbent hydrogels with smart behaviour (Esposito *et al.*, 1996, Sannino *et al.*, 2000)

2.2 Cellulose-based hydrogels and crosslinking strategies

Cellulose-based hydrogels can be obtained via either physical or chemical stabilization of aqueous solutions of cellulose. Additional natural and/or synthetic polymers might be combined with cellulose to obtain composite hydrogels with specific properties (Chen and Fan, 2008, Chang *et al.*, 2008).

Physical, thermoreversible gels are usually prepared from water solutions of methylcellulose and/or hydroxypropyl methylcellulose (in a concentration of 1–10% by weight) (Sarkar, 1979). The gelation mechanism involves hydrophobic associations among the macromolecules possessing the methoxy group.

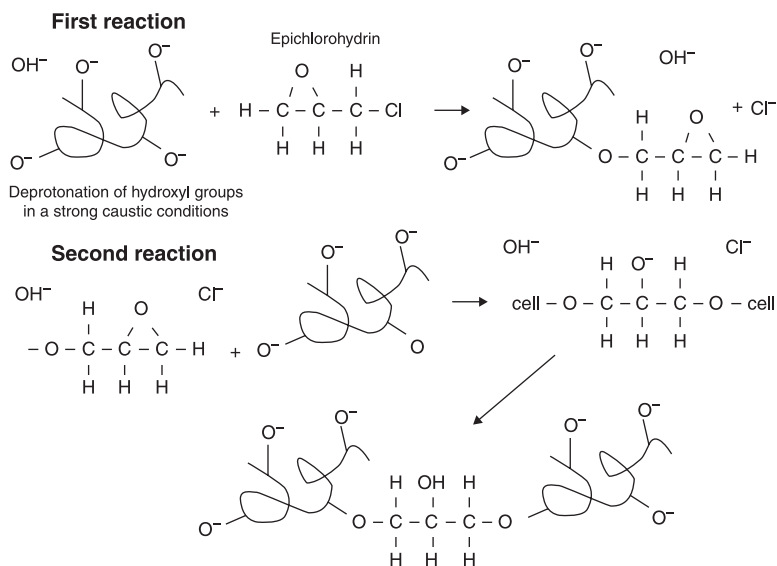
At low temperatures, polymer chains in solution are hydrated and simply entangled with one another. As temperature increases, macromolecules gradually lose their water of hydration, until polymer–polymer hydrophobic associations take place, thus forming the hydrogel network. The sol–gel transition temperature depends on the degree of substitution of the cellulose ethers as well as on the addition of salts. A higher degree of substitution of the cellulose derivatives provides them a more hydrophobic character, thus lowering the transition temperature at which hydrophobic associations take place.

A similar effect is obtained by adding salts to the polymer solution, since salts reduce the hydration level of macromolecules by recalling the presence of water molecules around themselves. Both the degree of substitution and the salt concentration can be properly adjusted to obtain specific formulations gelling at 37°C. Thus they are potentially useful for biomedical applications (Tate *et al.*, 2001, Chen *et al.*, 2006, Stabenfeldt *et al.*, 2006). Liquid formulations, whether mixed with therapeutic agents or not, are envisaged to be injected *in vivo* and their crosslinking reaction triggered by the physiological environment. However, physically crosslinked hydrogels are reversible (Te Nijenhuis, 2007) and might flow under given conditions (e.g., mechanical loading) or they might degrade in an uncontrollable manner. Due to such drawbacks, physical hydrogels based on MC and HPMC are not recommended for use *in vivo*. *In vitro*, MC hydrogels have been recently proposed as novel cell sheet harvest systems (Vinatier *et al.*, 2005).

As opposed to physical hydrogels, which show flow properties, stable and stiff networks of cellulose can be prepared by inducing the formation of covalent, irreversible crosslinks among the cellulose chains. Either chemical agents or physical treatments (i.e., high-energy radiation) can be used to form stable cellulose-based networks. The degree of crosslinking, defined as the number of crosslinking sites per unit volume of the polymer network, affects the diffusive, mechanical and degradation properties of the hydrogel and can be controlled to a certain extent during the synthesis. Specific chemical modifications of the cellulose backbone might be performed before crosslinking, in order to obtain stable hydrogels with given properties. For instance, silylated HPMC has been developed which crosslinks through condensation reactions upon a decrease of the pH in water solutions. Such hydrogels show potential for the *in vivo* delivery of chondrocytes in cartilage tissue engineering (Vinatier *et al.*, 2007, Ogushi *et al.*, 2007). As a further example, tyramine-modified sodium carboxymethyl cellulose (NaCMC) has been synthesized to obtain enzymatically gellable formulations for cell delivery (Wang and Chen, 2005). Photocrosslinking of water solutions of cellulose derivatives is achievable following proper functionalization of cellulose.

Depending on the cellulose derivatives used, a number of crosslinking agents and catalysts can be employed to form hydrogels. Epichlorohydrin, aldehydes and aldehyde-based reagents, urea derivatives, carbodiimides and multifunctional carboxylic acids are the most widely used crosslinkers for cellulose. As example the reaction mechanism of epichlorohydrin with cellulose is shown in Fig. 2.2. However, some reagents, such as aldehydes, are highly toxic in their unreacted state. Although unreacted chemicals are usually eliminated after crosslinking through extensive washing in distilled water, as a rule toxic crosslinkers should be avoided, in order to preserve the biocompatibility of the final hydrogel, as well as to ensure an environmentally sustainable production process. The crosslinking reactions among the cellulose chains activated by chemical agents might take place in water solution, organic solvents or even in the dry state (e.g., polycarboxylic acids can crosslink cellulose macromolecules via condensation reactions which are favoured at high temperature and in the absence of water (Coma *et al.*, 2003; Xie *et al.*, 2006; Demitri *et al.*, 2008). Novel superabsorbent cellulose-based hydrogels crosslinked with citric acid have recently been reported, which combine good swelling properties with biodegradability and absolute safety of the production process (Charlesby, 1955).

In light of environmental and health safety concerns, radiation crosslinking of polymers, based on gamma radiation or electron beams, has been receiving increasing attention in recent years as it does not involve additional chemical reagents, is easily controllable and, in cases of biomedical applications, allows the simultaneous



2.2 Reaction mechanism between epichlorohydrin and cellulose derivatives.

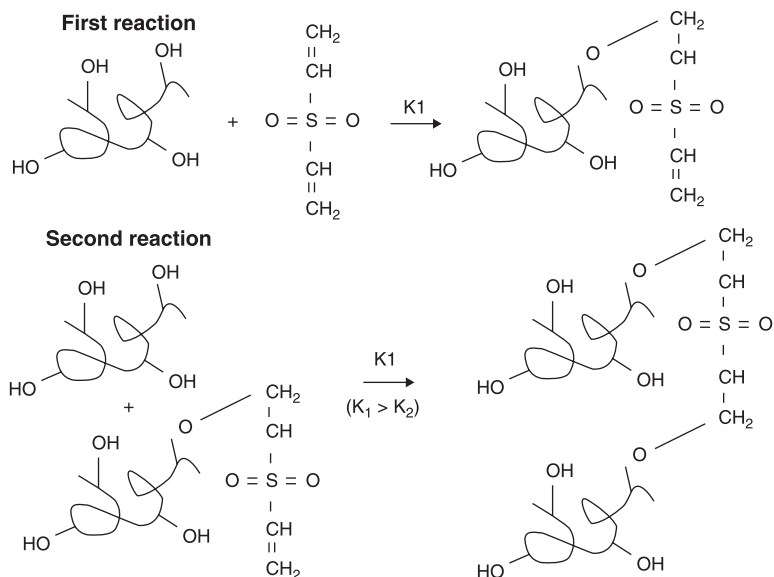
sterilization of the product. High-energy radiation usually leads to chain scission of the polymer and this has also been shown for cellulose (Wach *et al.*, 2003). However, several cellulose derivatives can be crosslinked (i.e., crosslinking prevails over degradation) under relatively mild radiation, both in aqueous solutions and solid form (Pekel *et al.*, 2004, Liu *et al.*, 2005). The crosslinking reaction is affected by the irradiation dose as well as by the cellulose concentration in solution.

In cases where biodegradability of a hydrogel is required or recommended, cellulose derivatives are appealing hydrogel precursor materials, due to their low cost, the large availability and biocompatibility of cellulose, and the responsiveness of some cellulose derivatives to variations of external stimuli. This section deals with some of the possible applications of cellulose-based hydrogels, which range from the traditional use of hydrogels as water absorbents to more innovative biomedical applications.

2.2.1 Crosslinking by means of divinylsulphone (DVS)

Biodegradable cellulose-based hydrogels have been chemically crosslinked by the use of the small difunctional molecule of divinylsulphone (DVS) to create intermolecular covalent bonds among polymer chains (Anbergen and Opperman, 1990, Esposito *et al.*, 1996). What is noticeable is the fact that the swelling properties of these materials are comparable with those displayed by acrylate based products (Lionetto *et al.*, 2005). The DVS molecule presents two carbon–carbon (C=C) double bonds that can be opened and linked to the OH[−] groups of the cellulose molecules. The polymerization is thus characterized by a first addition of DVS carbon–carbon double bond to the cellulose chain and a second addition. Only the latter leads to a crosslinked network. It should be noted that, although the reactive sites of the cellulose chain (OH[−]) are the same both for the first and the second addition, the two reaction steps have different rates, and seem to be independent of the amount of cross linker.

Nevertheless, with increasing reaction time, the ultrasonic velocity increases slightly faster in the solution with a lower DVS content, which reaches the plateau value before the other one. This can be explained by accounting for the different reactivity between the first and the second addition reaction of the DVS carbon–carbon double bonds to the cellulose molecule. The DVS molecule contains two alkene bonds that can be linked to the OH[−] groups of the cellulose molecules via a Michael reaction. The polymerization is thus characterized by a first addition of a cellulose OH group to a DVS alkene followed by a second addition. The second reaction leads to a cross-linked network. It should be noted that, although the reactive sites of the cellulose chain (OH[−]) are the same both for the first and the second addition, the two reaction steps have different rates. The first addition to the alkene (rate constant K_1 in Fig. 2.3) occurs between a compound of a very low molecular weight (DVS) and a side group (OH) of a polymer chain (cellulose). The second addition (rate constant K_2 in Fig. 2.3), instead, always occurs between

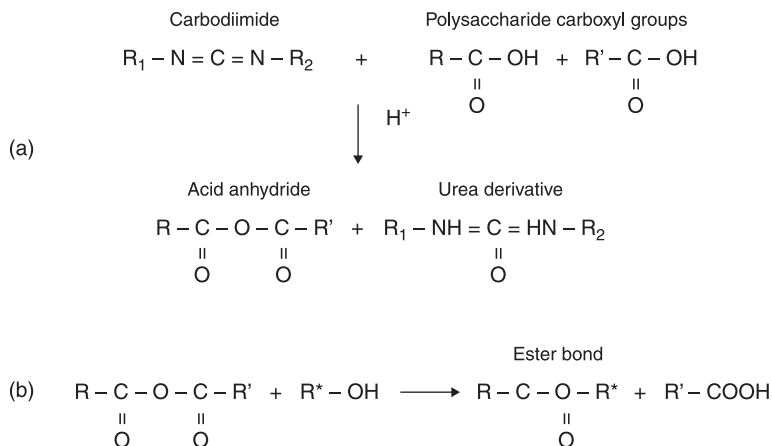


2.3 Reaction mechanisms of the first and second addition of the DVS carbon-carbon double bonds to the cellulose chain.

a cellulose OH group and an alkene bond, but the latter is now the pendant group of a macromolecule and hence $K_1 > K_2$. Therefore, it may be assumed that at higher DVS concentrations, the first addition competes with the second for a longer time, reducing also the number of OH groups that are accessible on cellulose to the crosslinking reaction. In this case, the second addition leading to crosslinking is slightly delayed and, consequently, the growth of viscoelastic properties, monitored by the ultrasonic technique, is observed later compared to the hydrogel obtained with a lower DVS concentration. Nevertheless, once the first addition is completed and the second is in progress, a larger amount of DVS produces a network with a higher crosslinking density and, consequently, improved elastic properties (Lionetto *et al.*, 2005).

2.2.2 Crosslinking by means of water soluble carbodiimide (WSC)

Water soluble carbodiimide (WSC) has been widely investigated as a crosslinking agent because of its ability to induce crosslinking of HA and other polysaccharides (Nakajima and Ikada, 1995, Tomihata and Ikada, 1997). As reported by Tomihata and Ikada (1997), WSC does not chemically bind to polysaccharide molecules, but seems to mediate ester bonds formation between carboxyl and hydroxyl groups belonging to different polysaccharide molecules. WSC can be found as a by-product of the reaction, in the form of a urea derivative, which displays a very



2.4 Reaction scheme of WSC with polysaccharide molecules:
 (a) WSC-mediated intramolecular or intermolecular formation of an acid anhydride between two carboxyl groups, with a non-toxic urea derivative as by-product; (b) anhydride-mediated formation of an ester bond between two polysaccharide molecules.

low degree of cytotoxicity (Tomihata and Ikada, 1997, Choi *et al.*, 1999, Park *et al.*, 2002, Xu *et al.*, 2003). For this reason, WSC can be considered as a non-toxic crosslinking agent. The reaction scheme of WSC with polysaccharide molecules is reported in Fig. 2.4 and can be summarized as follows: first the presence of WSC induces the intramolecular or intermolecular formation of an acid anhydride between two carboxyl groups, changing the WSC itself into a urea derivative; this anhydride is then responsible for the reaction with a hydroxyl group, to yield an ester bond between two polysaccharide molecules. However, because of the high instability of the acid anhydride in water solutions at room temperature, the reaction cannot take place if any hydroxyl groups do not quickly encounter the acid anhydride (Tomihata and Ikada, 1997). Therefore, the crosslinking reaction is markedly affected by the chemical composition of the starting polymer solution (Nakajima and Ikada, 1995, Tomihata and Ikada, 1997). Moreover, the initial reaction of WSC with carboxyl groups is dependent on pH, the optimal pH ranging from 3.5 to 4.5, as reported in the literature (Tomihata and Ikada, 1997).

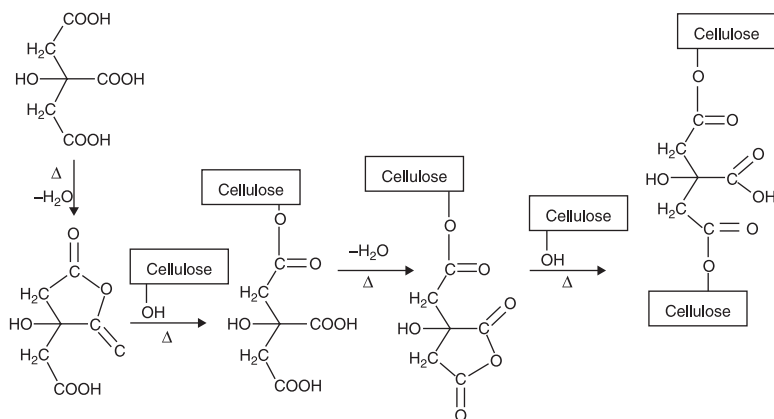
In our study we assume that cellulose derivatives can be crosslinked by WSC through the same reaction scheme reported for HA alone (Nakajima and Ikada, 1995). In fact in acid water solution the CH_2COOH^- anions provided by the CMCNa react with the HC^+ ions, leading to carboxyl groups formation and subsequently to the crosslinking reaction. However, we found that, for a WSC concentration of 5 wt%, hydrogel formation does occur when polymer concentration in the starting solution is at least 3 wt% (unreported data), confirming

that the chemical composition of the starting solution is fundamental to achieve a good crosslinking (Nakajima and Ikada, 1995, Tomihata and Ikada, 1997).

2.2.3 Esterification crosslinking

Citric acid (CA) is widely used in the food and drug industry and is an excellent candidate as a crosslinking agent. CA is widespread in nature (lemon juice contains approximately 5% of CA) and is prepared commercially by fungal fermentation of glucose. CA and its salts, with a good affinity for metal ions, are used in a wide variety of applications: in soft drinks and effervescent salts, as an antioxidant in food, as a sequestering agent for metal ions, as a cleaning and polishing agent for metals, as a mordant in dyeing. Moreover, CA and its salts have fundamental biological functions. For example, CA is involved as intermediate in the ‘Krebs cycle’ in all living cells, also known as ‘citric acid cycle’, for the production of usable energy (Glusker, 1980). Recently, CA was used as crosslinking agent in various cellulose derivative systems (Glusker, 1980, Wang and Chen, 2005, Coma *et al.*, 2003, Xie *et al.*, 2006, Yang and Wang, 1998) and different mechanisms have been proposed in the literature to explain the crosslinking reaction of cellulose polymers with CA.

Xie *et al.* (2006), for example, studied the optimum conditions for corn starch and CA reaction to produce resistant starch and studied the thermal stability of citrate starch products. The authors reported that when CA is heated it will dehydrate to yield the cyclic anhydride that reacts with starch; successively another cyclic anhydride function can be achieved into CA structure through the other two non-reacted carboxylic groups, allowing then the attachment of another hydroxylic starch group (Fig. 2.5).



2.5 Crosslinking reaction mechanism of citric acid with cellulose.

Coma *et al.* (2003) carried out the crosslinking of hydroxyl propyl methyl cellulose with CA, simply heating the reagents and determining the rate of crosslinking. According to Zhou *et al.* (1995) the two main stages of the reaction of polyfunctional carboxylic acids with cellulose are firstly due to the attachment of the polyfunctional carboxylic acids via esterification with a cellulosic hydroxyl group and its further reaction – via esterification – with another cellulosic hydroxyl group producing a crosslink between cellulose chains. This mechanism is based on an anhydride intermediate formation. Attachment of the carboxylic acid moiety to cellulose's hydroxyl group via esterification reaction of the first cyclic anhydride would expose a new carboxylic acid unit in citric acid, which has the proper chemical connectivity to form a new intramolecular anhydride moiety with the adjacent carboxylic acid unit. Further reaction with a cellulose hydroxyl of another chain can then lead to crosslinking.

2.2.4 Effects of the introduction of molecular spacers into the network

The hydrogel equilibrium swelling capacity depends on both internal parameters, related to the macromolecular network, and external, related to the environment contacting the material. In particular, for a polyelectrolyte network, characterized by fixed charges on the macromolecular backbone, there are four polymer properties affecting polymer swelling: (1) the polymer chain hydrophilicity, which promotes polymer–solvent mixing and thus promotes material swelling when in contact with water and water solutions; (2) the presence of fixed ionic charges, which induces a ‘Donnan-type’ effect, an osmotic effect associated with the concentration of ionic charges in the hydrogel and induces more water to penetrate the hydrogel to dilute this higher charge concentration; (3) the electrostatic repulsion between the charges of the same sign present on the polymer backbone, which tends to expand the macromolecular network and thus promotes polymer swelling; and (4) the elastic response of the crosslinks, which is entropic in nature, stabilizes the polymer chains in the hydrogel network and counteracts polymer swelling.

The Donnan effect (also known as the Gibbs-Donnan effect) is related to the behaviour of free charged particles in the presence of a semipermeable membrane separating two different solutions. Being the membrane semipermeable, only some charged species can pass through the membrane in order to reach the equilibrium between the two solutions. A typical Donnan-type mechanism takes place when a three-dimensional polyelectrolyte network is placed in contact with a water solution, since electrical charges are tethered on the polymer backbone, which thus acts as a semipermeable membrane. The equilibrium of the whole system (composed by the swelling solution and the polymer network itself) is thus attainable only if a passage of water is established, going from the external solution to the polymer network, thus diluting the concentration of the charges

inside the network. Due to the same Donnan-type swelling mechanism, when a polyelectrolyte hydrogel is swollen in distilled water, it exhibits a higher swelling capacity if compared to that of the same material swollen in a solution with a certain ionic strength (e.g. in the presence of NaCl).

The second and third properties mentioned above, promoting polymer swelling, are strictly related to the use of a polyelectrolyte cellulose-based system. These contributions are not affected by the presence of spacer molecules in the range of low concentrations. The fourth property, limiting polymer swelling (i.e., the elastic response of the crosslinks), depends on an effective degree of crosslinking of the polymer network. The degree of crosslinking can be evaluated as a function of the average molecular weight between two adjacent crosslinks. In the case of a perfect network with no dangling ends, loops and entanglements, which could be obtained by joining pairs of segments of linear chains through chemical crosslinks, the concentration of elastically effective chain elements corresponds to the concentration of all chemically crosslinked polymer segments.

According to this definition the moles of polymer segments engaged by crosslinks are the moles of crosslinks per unit volume of the network. Clearly, the higher the length of the crosslinker, the higher the average distance between two adjacent joined sites. Thus, the spacer plays the multiple role of (a) increasing the macromolecular network expanding properties; (b) increasing the average distance between two adjacent crosslinking sites, thus reducing the effective crosslinking density of the polymer network; and (c) decreasing the number of crosslinker molecules active for the crosslinking reaction, starting from the same initial crosslinker concentration. As already mentioned, the industrial field of interest in which these materials find wider application is represented by personal-care products. Thus, a set of sorption measurements was carried out in synthetic urine, in which the high ionic strength of the solution negatively affected the hydrogel sorption properties (Sannino *et al.*, 2003).

2.3 Hydrogel properties and thermodynamics

2.3.1 Hydrogel swelling ratio and sorption thermodynamics

The swelling capacity of hydrogels has been studied by different authors. In the previous chapter we saw that the presence of fixed charges, typical of polyelectrolyte gels, determines a significant swelling of the polymer in water. This behaviour can be addressed to a Donnan equilibrium established between gel and the external solution, whose ionic strength strongly affects the degree of swelling.

The hydrogel capability to absorb and retain water is probably the most important parameter to be evaluated. The sorption mechanism in hydrogels depends on the variations of the solvent in which the material is soaking. For

example, the small variations of certain environmental stimuli (e.g., pH, ionic strength, solvent composition) can considerably affect the hydrogel swelling capacity (Tanaka, 1985). The hydrogel swelling capacity (i.e., swelling ratio) can be evaluated by means of the measure of the mass of solvent absorbed by the network following the equation:

$$Q_m = \frac{W_s - W_d}{W_d} = \frac{M_1}{M_2} \quad [2.1]$$

where Q_m is the mass swelling ratio, W_s and W_d are the weights of the network in the swollen and dry state respectively and M_1 and M_2 indicate the masses of the solvent (i.e., water) and the polymer respectively.

The swelling ratio can also be evaluated by means of determining the volume of solvent absorbed by the network:

$$Q = \frac{V_s}{V_d} = \frac{V_1 - V_2}{V_2} = 1 + Q_m \frac{\rho_2}{\rho_1} \quad [2.2]$$

where Q is the volume swelling ratio, V_s and V_d are the volume of the swollen and dry state respectively, V_1 and V_2 are their volumes and ρ_1 and ρ_2 their densities. The polymer volume fraction in the swollen state can easily be determined as:

$$V_{2s} = \frac{1}{Q} \quad [2.3]$$

2.3.2 Sorption mechanism

The hydrogel state can be considered as a solution composed of water and polymer. This particular mixture shows an elastic rather than a viscous behaviour because the presence of the crosslinks—hydrophilic nature of the polymer promotes the adsorption of water. The polymer-solvent interaction can be described by the thermodynamic theory of polymer solutions. Flory (1953) has shown that the free energy change associated with the mixing process between the solvent and the polymer network can be calculated as follows:

$$\Delta G_{mix} = KT \left[n_1 \ln(1 - V_{2s}) + \chi_{1,2} n_1 V_{2s} \right] \quad [2.4]$$

where K is the Boltzmann constant, T the absolute temperature, n_1 the number of solvent molecules and $\chi_{1,2}$ the Flory-Huggins polymer-solvent interaction parameter. This last parameter has positive or negative values for endothermic or exothermic mixing, respectively. In the case of a complete miscibility of the polymer in the solvent over the entire composition range, $\chi_{1,2}$ is less than 0.5. The exact value of this parameter can be calculated according the following equation:

$$\chi_{1,2} = \chi_a + \chi_b V_{2s} + \chi_c V_{2s}^2 + \dots \quad [2.5]$$

where χ_a , χ_b , etc. are function of temperature. In addition it is evident that this interaction parameter is also a function of the polymer concentration. Due to these characteristics, this parameter plays an important role in the design of thermosensitive hydrogels.

In cases in which a hydrogel is required to respond quickly to certain environmental stimuli, the swelling rates are of particular importance. Usually the diffusion process in hydrogels is slow due to the nanometric dimension of its polymer mesh (Tanaka, 1985). Since the time required for diffusion is proportional to the square of the characteristic length of the gel, the swelling rate can be typically enhanced by reducing the size of the hydrogel block in powder or granular form. An excellent alternative to this method consists of producing porous hydrogels, with interconnected micro and macro pores. Porous hydrogels typically display higher water sorption compared to non-porous ones, as long as their pores are small enough to retain the liquid phase by means of capillarity effects (Esposito *et al.*, 1996, Sannino *et al.*, 2004, Sannino *et al.*, 2006). The swelling rate results are also enhanced due to the higher surface area per unit volume of porous hydrogel.

Sorption in solutions at different ionic strengths (constant pH)

A swollen hydrogel is able to change its volume as a result of the changing composition of the swelling solution. The fixed charges linked to the polymer backbone play an important role in driving this action. The equilibrium solution uptake always diminishes for higher values of the ionic strength. However, it can be observed that the polyelectrolyte hydrogels display a higher sensitivity to ionic strength variations. In fact, the osmotic pressure related to the Donnan effect is proportional to the difference in concentration of charges between those contained in the gel and those in the external solution. Being a polyelectrolyte, the CMCNa provides an overall higher ion concentration in the gel, and thus a higher Donnan-type effect is expected. The hydrogel crosslinked in the presence of the polyelectrolyte CMCNa displays a higher sorption capacity if compared with the samples crosslinked only with non-polyelectrolyte polymer (e.g. HEC). Obviously increasing the ionic strength of the external solution decreases the difference between the concentration of ion species in the gel and in the external solution and, as a result, the water uptake decreases. This behaviour can be ascribed to the neutralization of the fixed charges linked to the polymer backbone by the 'free' charges active in the external solution. This neutralization reduces the total active charge of the polymer network and thus reduces both the electrostatic repulsion of the polymer chain and the Donnan-type sorption mechanism. On the other hand, this effect can be explained as a reduction of the chemical potential of the water in the external solution with a resulting reduction of its capability to penetrate the polymer network.

Sorption in solutions at different pH (constant ion strength)

In order to evaluate the sorption mechanism of cellulose-based hydrogels in the presence of solutions at different pH it is useful to observe that the dissociation of the carboxylic group fixed on the cellulose chains are strongly affected by the pH of the external solution. A reduction in the number of dissociated carboxylic acid groups in the polymer network is evident at low pH. This mechanism reduces the swelling of the material in accordance with the reduction of the polyelectrolyte property of the network. On the other hand, when the pH of the swelling solution increases, there is a growth of the number of dissociated carboxylic group with a consequent increment in swelling. It is important to note that at low pH the majority of the carboxylic acid groups are in a non-dissociated state, and the hydrogel seems to be composed by only non-polyelectrolyte chains.

2.3.3 Degree of crosslinking and evaluation techniques

The degree of crosslinking (DC) of a polymeric network can be defined as the density of junctions joining the chains into a permanent structure:

$$DC = \frac{2\nu}{fV} \quad [2.6]$$

where ν is the number of units engaged in crosslinks ($2\nu/f$ is the number of crosslinks) and V is the volume of the polymer network after crosslinking (i.e., the relaxed volume). If we define N_0 as the total number of structural units composing the network, then ν is given by:

$$\nu = N_0 \frac{M_0}{M_c} = \frac{V}{\nu M_c} \quad [2.7]$$

where M_0 is the number average molecular weight of the structural unit, M_c is the number average molecular weight between two consecutive crosslinks, and ν is the specific volume of the polymer.

Defining N the crosslinking density as the moles of crosslinked chains per unit volume of network, the total number of crosslinked units can be expressed by a function of the crosslinking density as follows:

$$p_x = \frac{\nu}{V} = \frac{1}{\nu M_c} \quad [2.8]$$

Defining N as the number of primary linear polymer molecules forming the network, then:

$$N = N_0 \frac{M_0}{M_n} = \frac{V}{\nu M_n} \quad [2.9]$$

where M_n is the number average molecular weight of the primary molecules.

In an ideal and perfect network no terminal chains are present, i.e. the network is infinite, and all chains are elastically effective, or active in deformation ($v = v_e$). In opposition a real network is characterized to have $2N$ terminal chains:

$$v_e = v - 2N \quad [2.10]$$

By combining these last three equations we are able to calculate the value of the elastically effective crosslink density:

$$p_{se} = \frac{v_e}{V} = \frac{1}{vM_c} \left(1 - \frac{2M_c}{M_n} \right) = \frac{1}{v} \left(\frac{1}{M_c} - \frac{2}{M_n} \right) \quad [2.11]$$

The value of the crosslink density coming by the equation (2.11) overestimates the real density, since it is based on the assumption that terminal chains are the only imperfection of the network.

Evaluation of the degree of crosslinking through swelling measurements

The Flory-Rehner thermodynamic theory states that, when a hydrogel is allowed to swell, it is subjected to two forces: the force of mixing and the entropic response of the network. The force of mixing, which contributes positively to the swelling, is related to the hydrophilic nature of the polymer.

The latter, which contributes negatively to the swelling, opposes the deformation of the network to a more elongated state and is directly related to the degree of crosslinking. Considering the balance in the free energy associated to the mixing of the pure solvent and the pure polymer network, we can write:

$$\Delta G_{tot} = \Delta G_{mix} + \Delta G_{el} \quad [2.12]$$

where ΔG_{mix} is the variation in free energy due to the mixing of the solvent molecules with the polymer chains, and ΔG_{el} is the variation associated with the elastic force established within the network upon swelling. The above equation can be differentiated with respect to the number of solvent molecules; at constant temperature and pressure, we obtain the following equivalent equation:

$$\Delta \mu_{tot} = \mu_1 - \mu_{1,0} = \Delta \mu_{mix} + \Delta \mu_{el} \quad [2.13]$$

Here, μ_1 represents the chemical potential of the pure solvent within the gel and $\mu_{1,0}$ is the chemical potential of the pure solvent. When the equilibrium point is reached the chemical potential of the solvent inside and outside the polymer network must be equal. This means that at equilibrium the elastic and mixing contributions to the chemical potential will balance. When the dry network is placed in contact with the solvent, the osmotic pressure driving the solvent inside the gel is maximum, since the polymer concentration in the gel is high. When the solvent molecules start to penetrate the network, the polymer concentration in the gel decreases and the elastic retraction force of the polymer chains increases. At the equilibrium, the

chemical potential of the solvent inside the gel will be equal to that of the solvent outside the gel, i.e. $\Delta\mu_{tot} = 0$. The theoretical expression for $\Delta\mu_{mix}$ and $\Delta\mu_{el}$ can be calculated by means of both the classical theory of rubber elasticity and the thermodynamic theory of polymer solutions. In cases where the crosslinking mechanism occurs in the presence of solvent, the equilibrium condition for neutral gels leads to the following expression for (Peppas and Merrill, 1977):

$$\frac{1}{M_c} = \frac{2}{M_n} - \frac{\left(\frac{v}{V_1}\right) \left[\ln(1 - V_{2,s}) + \chi_{1,2} + V_{2,s}^2 \right]}{V_{2,r} \left[\left(\frac{V_{2,s}}{V_{2,r}} \right)^{1/3} - 2 \left(\frac{V_{2,s}}{V_{2,r}} \right) \right]} \quad [2.14]$$

where all the parameters were explained in the previous section. This equation is valid for loosely crosslinked networks, where the number of repeating units within each chain is large enough so that the chains can be represented by a Gaussian distribution. In addition the expression for $\Delta\mu_{el}$ is derived on the assumption of moderate degree of swelling (i.e., the polymer chains are extended less than half their fully stretched length). For highly crosslinked networks and for non-affine deformations, more complex theories have been developed by different authors (Peppas *et al.*, 1985, James and Guth, 1953, Hermans, 1962).

Evaluation of the degree of crosslinking through mechanical measurements

Assuming that the deformation of the chains is affine and that the volume of the polymer does not change upon uniaxial deformation, a relationship between the uniaxial stress and the uniaxial deformation of the polymer network can be derived (Flory, 1953):

$$\sigma = RT\rho_{s,e} \left(\alpha - \frac{1}{\alpha^2} \right) = G \left(\alpha - \frac{1}{\alpha^2} \right) \quad [2.15]$$

where σ is the applied stress, R is the universal gas constant, T is the absolute temperature, $\alpha = L/L_i$ is the deformation ratio (L and L_i are respectively the actual thickness of the deformed sample and the initial thickness of the samples) and G is the shear modulus of the polymer network.

In the presence of isotropic swelling of the network in a solvent (Flory, 1953), and if the crosslinking reaction occurs in the presence of a solvent (Peppas and Merrill, 1977), the shear modulus can be expressed with the following equation:

$$G = RT\rho_{s,e} V_{2,s}^{1/3} V_{2,r}^{-1/3} = RT \frac{v_e}{V_0} V_{2,s}^{1/3} V_{2,r}^{2/3} \quad [2.16]$$

where V_0 is the volume of the dry polymer network. Consequently the modulus G , and the corresponding effective crosslinking density, can be measured by means of uniaxial compression or elongation (Sannino *et al.*, 2005).

2.4 Applications

In this section some of the possible applications of cellulose-based hydrogels – which range from the traditional use of these hydrogels as water absorbents to more innovative biomedical applications – are shown.

2.4.1 Body water retainers

Due to the intrinsic biocompatibility of cellulose, together with the biocompatibility and the versatile properties displayed by hydrogels in biomedical applications, cellulose-based hydrogels are appealing materials for a number of applications *in vivo*. For example, hydrogels hold promise as devices for the removal of excess water from the body in the treatment of some pathological conditions, such as renal failure and diuretic-resistant oedemas. The hydrogel in powder form is envisaged to be administered orally and absorb water in its passage through the intestine, where the pH is about 6–7, without previously swelling in the acid environment of the stomach. The hydrogel is then expelled as faeces, thus performing its function without interfering with body functions. As sensitivity to pH is required, polyelectrolyte cellulose hydrogels, based on NaCMC and HEC, have been investigated for such an application (Sannino *et al.*, 2000, Sannino *et al.*, 2003, Esposito *et al.*, 2005). The hydrogels showed good swelling properties at neutral pH and low swelling ratios at acid pHs. Moreover, the water sorption capability could be modulated and maximized by adjusting the ratio NaCMC/HEC and the amount of crosslinker used (DVS) (Sannino *et al.*, 2000, Sannino *et al.*, 2003), and by adding molecular spacers to the polymer network, e.g. polyethylene glycol (PEG) (Esposito *et al.*, 2005). In spite of the use of DVS as a crosslinker, the hydrogel formulations tested showed good biocompatibility in both *in vitro* and *in vivo* experiments. The use of hydrogels in combination with diuretic therapies might be useful in substituting some drugs and in using an intestinal pathway, instead of the systemic one, to remove water from the body (Sannino *et al.*, 2003)

2.4.2 Stomach bulking agents

The latest World Health Organization projections indicate that at least one in three of the world's adult population is overweight and almost one in 10 is obese. Additionally there are over 20 million children under age five who are overweight. Obesity and overweight represent the second highest cause of death after smoking, and are major risk factors for several chronic diseases such as type 2 diabetes; cardiovascular disease; sleep apnoea; hypertension; stroke; and certain forms of cancer. Moreover, being overweight or obese often has a dramatic impact on psychological well-being, reducing the overall quality of life (Schachter *et al.*, 2002, Sowers, 2003, James, 2004). The treatment of overweight and obesity usually consists of a supervised diet, often combined with adequate physical

exercise. In the most serious cases, surgical procedures that essentially involve gastric restriction, or particular drug treatments, may be required (Schachter *et al.*, 2002).

However, in recent years a number of dietary supplements and meal replacements have been developed and sold as over-the-counter slimming aids. Dietary supplements are claimed to act either by binding fats and so reducing fat absorption, as reported for chitosan-based products, or by directly reducing the appetite. Examples of the latter are different natural fibres and herbal products, that seem to absorb liquids and swell inside the stomach, thus giving a sense of fullness (Pittler and Ernst, 2004, Saper *et al.*, 2004). This approach, based on the use of natural fillers or bulking agents, is very interesting for its great potential of decreasing the amount of food intake by reducing the available space in the stomach, without the need of complex surgical interventions. However, there is no clear evidence of the effectiveness of currently available bulking agents in promoting weight loss, neither in the short term nor in the long term, whereas their adverse effects, usually including gastrointestinal symptoms, have been well documented (Pittler and Ernst, 2004, Saper *et al.*, 2004). Moreover, it should be taken into account that some fillers may be harmful, causing obstructions in the intestines, stomach or oesophagus, as reported for guar gum (Lewis 1992). Therefore, the development of novel bulking agents, effective in promoting weight loss, is needed.

In this respect, superabsorbent hydrogels are of particular interest since not only can their swelling capacity be properly designed by controlling their chemical composition and physical microstructure, but they can also be modulated by changing the environmental conditions (e.g., pH, ionic strength, temperature). The essential concept is that a xerogel-based pill is administered orally before each meal, and that the xerogel powder swells, once in the stomach. In such a way the space available for food intake is reduced giving a feeling of fullness. The swollen hydrogel is then eliminated from the body as faeces. In this perspective, the hydrogel is envisaged to pass through the gastrointestinal tract, being supposed to encounter the different pH environments of the stomach and the intestine. Along with superporous acrylate-based hydrogels, which swell very rapidly in aqueous solutions (Chen *et al.*, 1999), novel cellulose-based hydrogels, obtained by crosslinking aqueous mixtures of NaCMC and HEC, have been shown to be suitable for the production of dietary bulking agents (Sannino *et al.*, 2005, Sannino *et al.*, 2006). Indeed such hydrogels possess a high biocompatibility with respect to intestinal tissues and a high, pH-sensitive water retention capacity (Sannino *et al.*, 2006). Although the polyanionic nature of the NaCMC network provides higher swelling capabilities at neutral pHs rather than at acid ones, the swelling ratio obtained at acid pHs might still be significant for use of the hydrogel as stomach filler. In particular, cellulose-based hydrogels obtained from non-toxic crosslinking agents are particularly useful for this kind of application (Sannino *et al.*, 2005, Demitri *et al.*, 2008, Sannino *et al.*, 2006).

2.4.3 Wound dressings

Inflammation, autolytic debridement, granulation tissue formation and re-epithelialization are the processes which normally occur during wound healing. Appropriate wound dressings are designed to promote healing while protecting the wound from infection. This is particularly important in cases of chronic wounds (e.g., ulcers), which fail to heal properly. Since a moist environment encourages rapid healing (Winter, 1962), hydrogels are optimal candidates for the development of wound dressings, either as sheets or in amorphous form. Amorphous hydrogels are usually physically crosslinked, thus their viscosity decreases as they absorb physiological fluids. Such gels may be packaged in tubes or in foil packets, and in the latter case the gels are reinforced with a gauze or a polymeric mesh to allow easy removal and prevent gel liquefaction. Hydrogels should be designed to maintain the right moisture balance in the wound bed by hydrating the wound surface and/or absorbing the wound exudates. They also provide non-adherent dressings which can be easily removed without any damage to the wound bed. Hydrogel transparency is a further advantage in this application, as wound healing can be easily monitored. Various types of hydrogel dressings have been patented and are commercially available, based on synthetic or natural polymers, or a combination of them. The most advanced hydrogel dressings include antimicrobial agents in their formulation. Moreover, radiation crosslinking is being increasingly explored in this field, to obtain sterile and crosslinked hydrogel films in a single-step process.

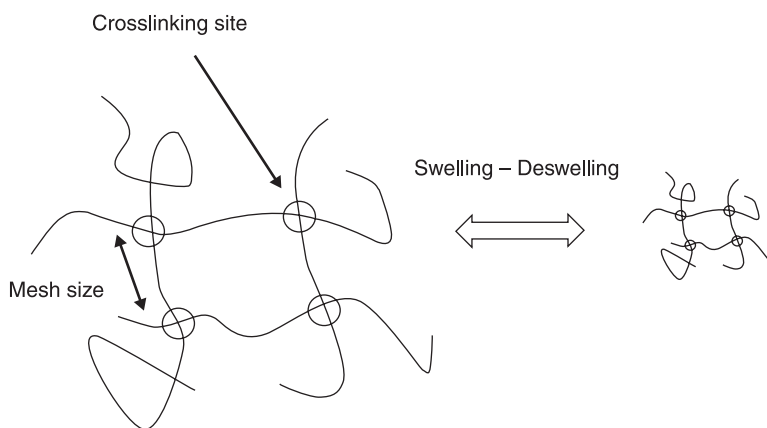
Due to its purity and high water retention capacity BC has been largely investigated for wound healing and a series of BC-based wound dressings are currently marketed (Czaja *et al.*, 2007). To the best of our knowledge gel-forming cellulose derivatives such as NaCMC are included in the formulation of some commercially available hydrogel dressings, usually in combination with propylene glycol, which works as a humectant and a preservative. It is worth noting that the products developed so far are usually indicated for the treatment of specific wounds and often require the use of secondary dressings. Therefore, a number of investigations currently deal with the development of novel wound dressings with improved performance, and cellulose-based hydrogels appear to be promising candidates. Preliminary, unpublished results by the present authors show that cellulose-based hydrogels crosslinked with hyaluronic acid (Sannino *et al.*, 2005) induce a good proliferation of keratinocytes, following a scratch wound model in *in vitro* culture.

2.4.4 Devices for controlled drug delivery

Cellulose ethers have long been used in the pharmaceutical industry as excipients in many drug device formulations (Baumgartner *et al.*, 2002). Their use in solid tablets allows a swelling-driven release of the drug as physiological fluids come

into contact with the tablet itself. The cellulose ether on the tablet surface (e.g., HPMC) starts to swell, forming chain entanglements and a physical hydrogel. As swelling proceeds from the swollen surface to the glassy core of the tablet the drug progressively dissolves in water and diffuses out from the polymer network. The rate of drug release depends on the water content of the swollen hydrogel, as well as on its network parameters, i.e., degree of crosslinking and mesh size (Peppas, 1997, Lin and Metters, 2006). Depending on the structure of the particular cellulose ether used, chain dissolution may take place along with swelling due to the physical nature of the hydrogel network; thus drug release results from the complex combination of swelling, diffusion and erosion mechanisms.

More sophisticated hydrogel-based devices other than swelling tablets have been developed for controlled drug delivery. The most recent advances aim not only at the sustained release of a bioactive molecule over a long period, ranging from hours to weeks, but also at a space-controlled delivery, directly at the site of interest. The need to encapsulate bioactive molecules into a hydrogel matrix or other delivery devices (e.g., microspheres) is also related to the short half-life displayed by many biomolecules *in vivo*. When using hydrogels to modulate the drug release, the loading of the drug is performed either after crosslinking or simultaneously during network formation (Drury and Mooney, 2003). Moreover, the bioactive molecule can be covalently or physically linked to the polymer network, to further tune the release rate. Smart hydrogels are particularly useful to control the time- and space-release profile of the drug because swelling–shrinking transitions, which modify the mesh size of the hydrogel network, occur upon changes of physiologically relevant variables, such as pH, temperature and ionic strength (Peppas, 1997) (Fig. 2.6).



2.6 Schematic structure of a tetrafunctional polymer network upon swelling–shrinking transitions. What changes is the mesh size of the network, which determines the free space available for diffusion and thus regulates the diffusion of molecules (e.g., drugs) through the network itself.

Controlled release through oral drug delivery is usually based on the strong pH variations encountered when transitioning from the stomach to the intestine. Cellulose-based polyelectrolyte hydrogels (e.g., hydrogels containing NaCMC) are particularly suitable for this application. For instance, anionic hydrogels based on carboxymethyl cellulose have been investigated recently for colon-targeted drug delivery (El-Hag Ali *et al.*, 2008).

The most recent advances in controlled release through a hydrogel matrix deal with the delivery of proteins, growth factors and genes to specific sites, the need for which has been prompted by tissue engineering strategies. While hydrogel formulations for oral and transdermal delivery can be non-degradable, the direct delivery of drugs or proteins to different body sites requires biodegradation of the hydrogel, in order to avoid foreign body reactions and further surgical removal. Injectable hydrogel formulations are particularly promising and currently under investigation. The crosslinking reaction has to be performed under mild conditions in order not to denature the loaded molecule. The microenvironment resulting from degradation of the polymer should also be mild. With particular regard to cellulose-based hydrogels, injectable formulations based on HPMC have been developed to deliver both biomolecules and exogenous cells *in vivo* (Vinatier *et al.*, 2005, Vinatier *et al.*, 2007, Trojani *et al.* 2005).

2.5 Conclusions

Due to their biocompatibility and biodegradability cellulose-based hydrogels are important in a number of industrial uses, as well as in biomedical applications. The current trend in the design of cellulose hydrogels is related to the use of non-toxic crosslinking agents or crosslinking treatments, to further improve the safety of both the final product and the manufacturing process. The smart behaviour of some cellulose derivatives (e.g., NaCMC) in response to physiologically relevant variables (i.e., pH, ionic strength) makes the resulting hydrogels particularly useful for *in vivo* applications. In spite of the non-bioresorbability of cellulose, the possibility to functionalize cellulose-based hydrogels with bioactive and biodegradable extracellular matrix domains suggests that, in the near future, such hydrogels might be ideal platforms for the design of scaffolding biomaterials in the field of tissue engineering and regenerative medicine.

2.6 References

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Synthesis of hydrogels for biomedical applications: control of structure and properties

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Abstract: The synthesis of hydrogels is covered assuming little knowledge of the field. The chapter concentrates on general methods for producing natural and synthetic covalently cross-linked homogeneous and multi-component hydrogels including networks composed of hydrophilic polymers modified with hydrophobic polymers. The dominant synthetic technique in this area is radical polymerization and this aspect is covered in some depth. The available techniques for synthesis are illustrated by examples from the literature, with special emphasis on contemporary sources. Finally, the methods for preparing functional hydrogels are covered. This aspect is split into methods that involve copolymerization with functional monomers and techniques for post-polymerization modification.

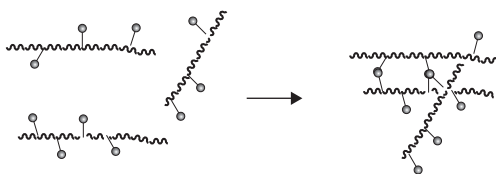
Key words: hydrogel, synthesis, conetwork, interpenetrating network, functional hydrogel, cross-linking.

3.1 Introduction

In common with the synthesis of other polymer networks we can generally place most of the available routes to hydrogels into three categories: 1) cross-linking of high molecular weight polymers; 2) copolymerization with multi-functional monomers and 3) coupling of the end groups of telechelic oligomers. These three routes are shown schematically in Fig. 3.1.

Cross-linking of preformed polymers requires the production of either polymers containing reactive functionality or the generation of reactive functionality on the polymer via a post-polymerization reaction. On the other hand the copolymerization route only requires the availability of often easily synthesised multi-functional monomers. Perhaps the most technically challenging route is the coupling of the ends of telechelic oligomers and only a few examples have been reported in the academic literature. The latter are moderate molecular weight polymers with functionality at the chain ends. This process can be difficult because its success requires the availability of telechelic oligomers with very high degrees of functionality (circa 99+% of all end groups) and extremely efficient coupling chemistry. Low degrees of functionality or coupling mechanisms that do not progress to very high conversion generate highly branched polymers that do not gel, or often materials containing low gel fractions are formed. However, these coupling strategies can potentially provide model networks with well-defined segment structure because it is possible to fully characterise the precursor

Cross-linking of polymer chains



Copolymerization with multi-functional monomers



Coupling of telechelic oligomers



3.1 Schematic diagram illustrating the general routes to cross-linked hydrogels.

telechelic oligomers before coupling. Perhaps the most successful coupling strategy is the use of the Michael addition of thiols ended-oligomers to oligomers with vinyl sulphone end groups (Lutolf and Hubbell 2003; Morpurgo *et al.* 2003; Silviya *et al.* 2010). The choice of a synthesis route is very much dependent on the available chemistry with the required efficiency and compatibility with the target application. In this respect there is a continuous need to develop and improve network-forming chemistry, which can often lead to very significant improvements in materials' performance. However, there are also important processing issues to consider. These issues are focused on the rheology of the precursor's reaction mixtures. Since all devices prepared from covalently cross-linked hydrogels must be produced *in situ* in the form dictated by the application, the rheology of the precursor reaction mixtures can be critical to the choice of processing technique. The cross-linking of high molecular weight polymers requires either melt processing techniques such as extrusion or injection moulding or solution casting techniques. On the other hand, the monomer mixtures or telechelic oligomers are much lower viscosity fluids, which allows for solventless and low temperature processing procedures.

In the following sections the techniques of cross-linking high molecular weight polymers and polymerization with multi-functional monomers will be described and exemplified, and finally some newer variants that are hybrids of these general methods will be discussed.

3.2 Cross-linking of high molecular weight polymers

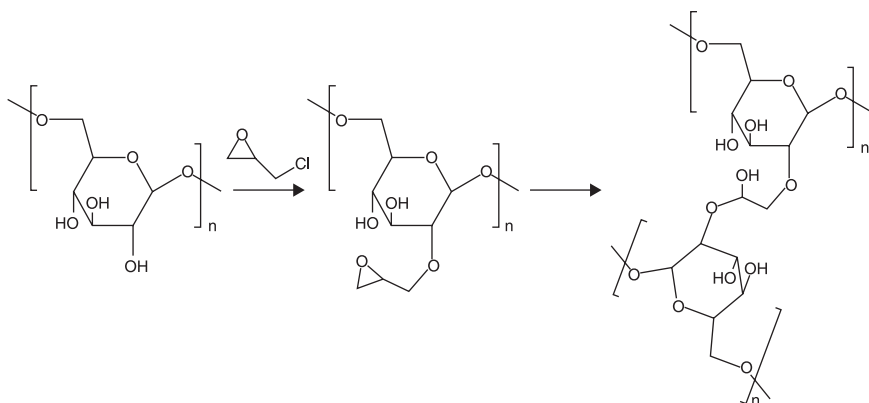
In this route polymers are produced with chain pendant functionality that either self-reacts or which reacts with antagonistic functionality on another polymer chain or small multi-functional molecule. Perhaps the most useful functionalities are hydroxyl and amine.

Hydroxyl functionality is best added using hydroxyl-functional (or protected hydroxyl) monomers or comonomers. Hydroxyl-functional materials based on poly(vinyl alcohol) are by far the most studied and commercially relevant. Poly(vinyl alcohol) is a commodity polymer with a very high degree of hydroxyl functionality and several routes to cross-linking of these functionalities have been used. Epichlorohydrin reacts (Bo 2003) via reaction of the pendant hydroxyls with both the alkyl chloride and epoxide functionalities. Another method involves the formation of phosphate esters as the cross-linking unit using sodium trimetaphosphate as the cross-linking agent (Chaouat *et al.* 2008). The strategy that has received the most attention involves the formation of acetals by reaction of 1,3 diol units (dimer units) with difunctional aldehydes such as glutaraldehyde (Wang and Hsieh 2010, Tang *et al.* 2010). An interesting extension of this idea uses glutaraldehyde to cross-link both PVA and chitosan to produce co-cross-linked blends (Costa-Júnior *et al.* 2009).

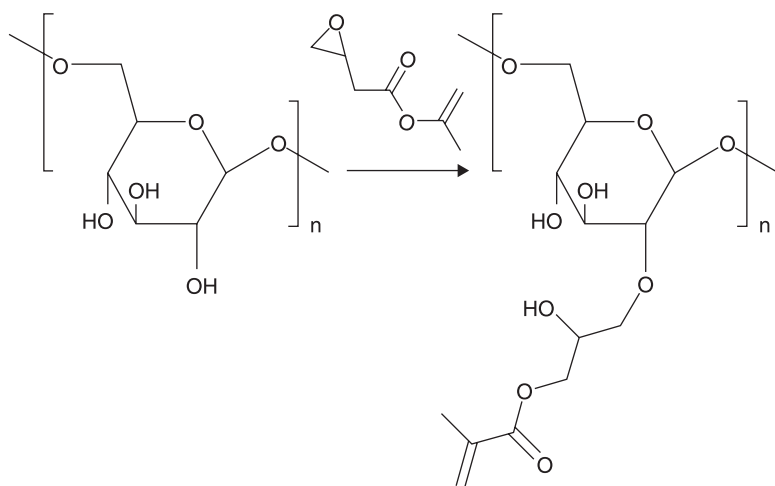
Direct irradiation with electron beams is an established method of cross-linking coatings formed from pre-formed polymers (Ikada *et al.* 1977). Polyacrylic acid is a useful pH responsive medical polymer that can be cross-linked via the coupling of polymer radicals formed by irradiation with an electron beam (Sheikh *et al.* 2010). A similar process has also been used to prepare poly(N-vinyl pyrrolidinone)/poly(acrylic acid) hydrogel blend (Kadubowski *et al.* 2010). Electron-beam cross-linking of preformed hydrophilic coatings also provides an opportunity to produce surface patterns (Saaem and Tian 2007).

Natural polymer hydrogels are of growing importance as materials from renewable resources. Of these materials several polysaccharides are available in high abundance from both plant and invertebrate feed stocks. They are all highly hydroxyl-functionalized and several routes to cross-linking are used. For example, dextran can be cross-linked by reaction of the hydroxyls with epichlorohydrin, phosphoryl trichloride or methylene bisacrylamide (Denizli *et al.* 2004). As shown in Fig. 3.2, epichlorohydrin reacts at both the epoxide and alkyl chloride functionalities and phosphoryl trichloride forms phosphate cross-links.

Dextran can also be formed into a hybrid, synthetic/natural hydrogel by reaction of the epoxy group of glycidyl acrylate with the dextran hydroxyls followed by polymerization of the acrylate groups (see Fig. 3.3) (Edman *et al.* 1980). Later work in DMSO solvent showed that use of glycidyl methacrylate produced a methacrylate directly attached to the chain, which has been explained by proposing that transesterification occurs (van Dijk-Wolthuis *et al.* 1997). A similar method involves the functionalization of dextran with hydroxyl ethyl methacrylate via



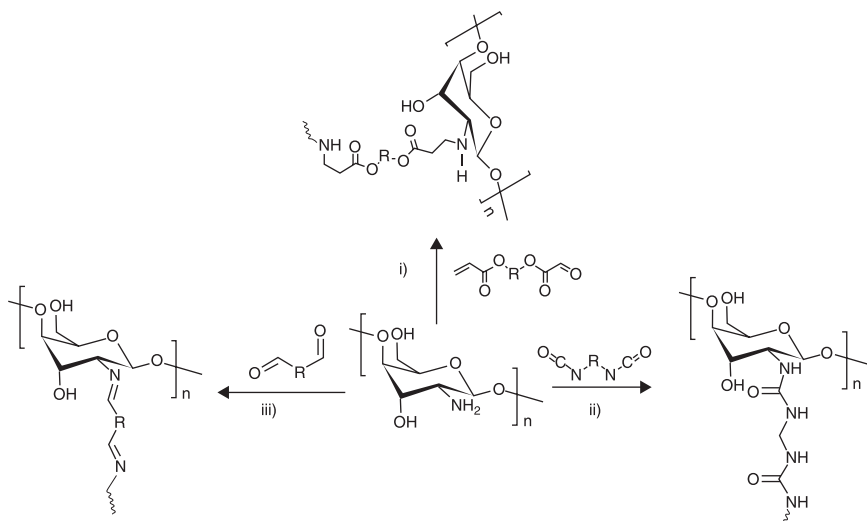
3.2 Cross-linking of dextran by reaction with epichlorohydrin.



3.3 Functionalization of dextran with glycidyl methacrylate prior to cross-linking by copolymerization.

formation of a carbonate link (Kamoun and Menzel 2010). Dextran hydrogels are used as drug delivery systems, particularly for delivery to the colon. Capsules can be formed by reaction with glutaraldehyde, which forms acetal cross-links (Brøndsted *et al.* 1998).

Many examples of the cross-linking of polysaccharides via amino sugars are available. The most important amino glucan, for the synthesis of useful hydrogels, is chitosan. Chitosan is poly(D-glucosamine) and is produced by deacetylation of chitin. As shown in Fig. 3.4, chitosan can be cross-linked in a variety of ways by reaction with aldehyde, alkene or isocyanate multi-functional cross-linking agents.

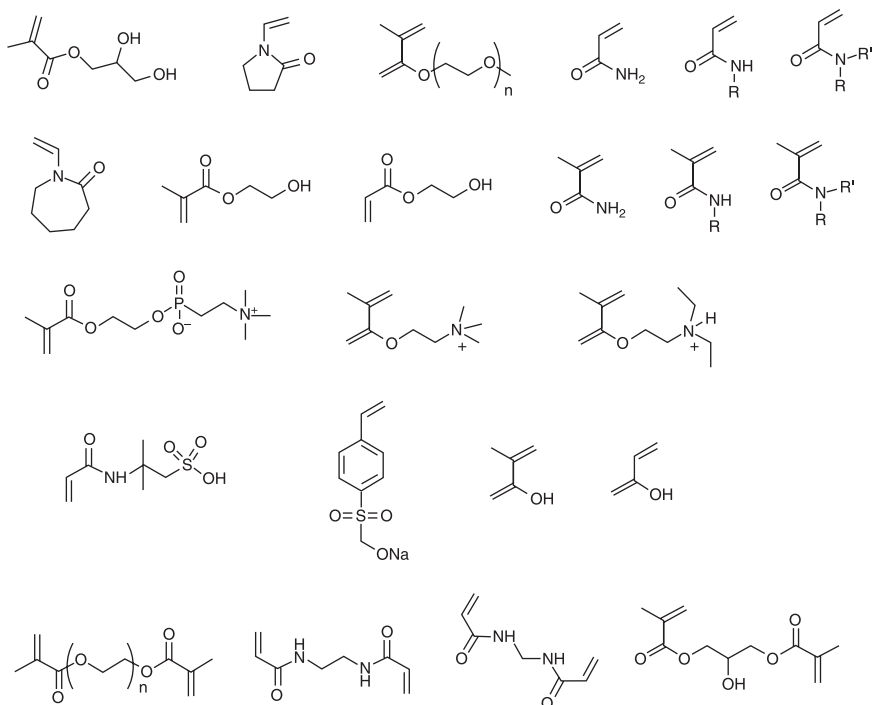


3.4 Cross-linking of chitosan using: i) Michael addition; ii) urea formation with diisocyanate or iii) imine formation with a dialdehyde.

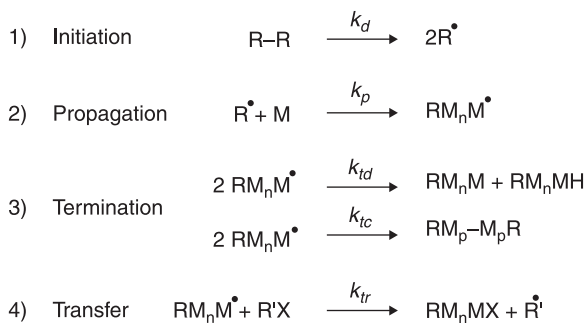
3.3 Copolymerization with multi-functional monomers

Copolymerization with multi-functional monomers leads to network formation (gelation) at relatively low conversion. Gelation generally occurs for n primary chains once $n + 1$ cross-links are formed. By far the most commercially important system is the radical polymerization of water soluble vinyl monomers in the presence of multi-functional alkenes (cross-linkers). However, other systems are known; for example cross-linking of PEG to form castable polyurethane hydrogels. The radical polymerization of multi-functional monomers includes polymerization involving many possible combinations of monomers with cross-linking monomers, shown in Fig. 3.5.

The radical polymerization process is controlled by three processes: initiation, propagation and termination, as shown in Fig. 3.6. Transfer, reaction 4 in Fig. 3.6, can also occur and this process generally leads to lower molecular weights. Significant amounts of transfer can lead to a failure to gel in some systems. Such polymerizations produce soluble highly branched polymers. However, in most systems that produce useful hydrogels transfer processes are avoided by ensuring that transfer rates to monomer, polymer and solvent are very low. The rates of polymerization are described by considering each of these processes and their rates and an analysis of the system shows that the rate of polymerization scales with the monomer concentration and the square root of the initiator concentration.



3.5 Some of the commercially available monomers used to produce hydrogels by radical polymerization.



3.6 The constituent processes of radical polymerization.

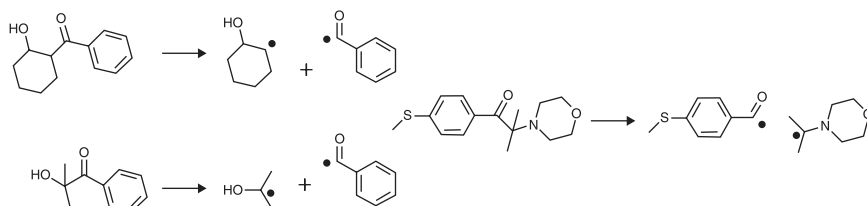
This is an important result that indicates the complexity that can develop when multiple monomers are polymerized together.

Each of these monomers has a k_p associated with homopolymerization and copolymerization is controlled by matrices of crossed k_p s. For the copolymerization of two monomers the ratios of the cross k_p and the homo k_p is known as a reactivity

ratio. For copolymerizations of three, four or more monomers the matrices of reactivity ratios increases in size as the number of monomers increases and analytical prediction becomes more difficult. Also the reactivity ratios refer to instantaneous monomer concentrations but the difference in reactivities of the monomers produces significant changes in the instantaneous concentrations of the monomers as the polymerization progresses. Since these copolymerizations are subject to the reactivity differences described by the reactivity ratios, polymer compositions and sequence distributions are highly dependent on conversion. These conversion dependences lead to broad compositional and sequence distributions at high conversion in conventional radical polymerizations. Since the majority of applications require high conversion of monomer to polymer, these compositional heterogeneities have very significant effects on properties. Thus, hydrogels prepared by conventional radical polymerizations are highly heterogeneous structures at the molecular level but these materials are clearly the most ubiquitous hydrogels available and they have many applications.

Radical polymerization in the bulk or solution can be initiated by thermal production of radicals using peroxy initiators, percarbonate or azo initiators. Thermal polymerization is suitable for thick specimens. On the other hand, UV and other irradiation techniques are suitable for thinner devices and many contact lenses, for example, are prepared in this manner. UV initiated polymerization requires photoinitiators, which in hydrogel synthesis usually produce radicals by homolytic scission of an alkyl-carbonyl bond. Some examples of photoinitiators and their radical generating reactions are shown in Fig. 3.7.

UV polymerization also provides effective sterilization for many devices and this is a major advantage for clinical applications. In medical applications it is also essential to consider toxicity effects. In most cases toxicity is associated with small molecule contaminants, especially monomer, rather than polymer. Only a few, mainly soluble amino polymers, are toxic but in UV polymerized systems it is essential to consider the effective removal of photoinitiators and the by-products of the initiation process. For many applications these potential toxins can be removed by washing, which is facilitated by the swollen nature of a hydrogel. Electron beam and gamma irradiation also can generate radicals directly from



3.7 Typical photoinitiators used for radical polymerization in the production of hydrogels.

monomers and although considerable investment in plant is necessary, photoinitiators are not required. This aspect can offer some advantages if the toxicity of photoinitiators is problematic. In the electron beam process it is generally thought that radicals can be produced by single electron reduction of double bonds to produce radical anions followed by proton abstraction. Many variants of this method are possible and some recent innovative examples include the cross-linking of poly(vinyl alcohol) containing pendant maleic anhydride groups via electron beam initiated polymerization of N-isopropyl acrylamide (Atta *et al.* 2008).

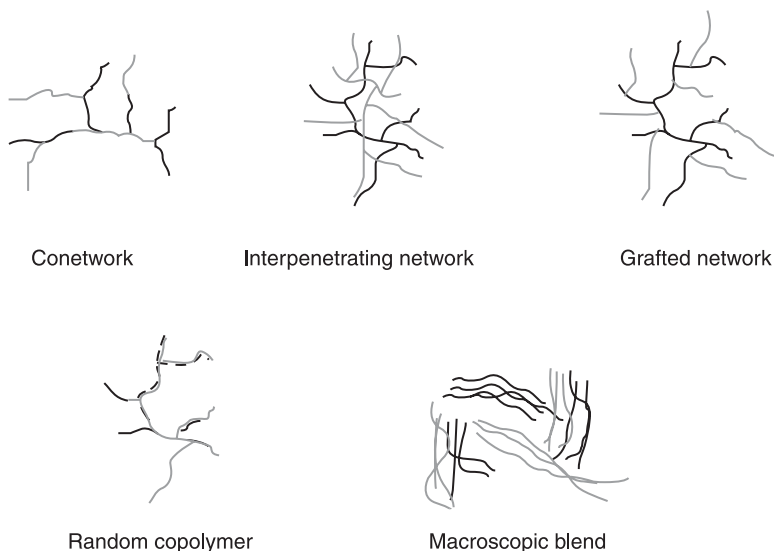
3.4 Multiphase hydrogels

Although fully hydrophilic hydrogels have a large number of applications, there are several areas that require the inclusion of hydrophilic components. For example, fully hydrophilic hydrogels are very poor substrates for cell adhesion but they have other properties that are very useful to applications in regenerative medicine. The inclusion of hydrophobic components that control phase separation can produce dramatic improvements in the performance of hydrogels to support cells. These cell/polymer constructs are at the forefront of major developments in the new clinical and surgical strategies that will transform medicine in this century. Other applications require controlled release of often relatively hydrophobic compounds. In these applications, drug delivery controlled pesticide release, etc., the presence of hydrophobic domains with a non-fouling, hydrophilic matrix can potentially provide control of the rates of delivery. In this type of system most of the properties are controlled by the nano- and micro-phase structures that are derived from the interactions between the phases and the methods (especially the kinetics) of synthesis.

Several systems are possible that can be classed into the following categories: amphiphilic conetworks; interpenetrating networks (IPN); grafted hydrogels; random copolymers and macroscopic blends. Amphiphilic conetworks are characterized as having continuous nanostructure. Materials composed of poly(siloxane) linked to hydrophilic monomer sequences are perhaps the most commercially important new medical material produced in the past 20 years. These conetworks are used in contact lenses with very high oxygen diffusion rates. Conetworks are composed of segments that are covalently attached to each other, as are several other classes of materials, but a key aspect is the co-continuous nature of the morphology. On the other hand, hydrogels with IPN motifs are composed of two intertwining polymer networks. In the case of hydrogel systems the two networks would be either hydrophilic and hydrophobic or hydrophilic and hydrophilic. IPNs have been known for many years but they have recently also become known as double networks. Two varieties are known: full IPNs with both components cross-linked and semi-IPNs in which one component is cross-linked but the second component is not. In a hydrogel application it is vital that in a semi-

IPN, composed of linear and cross-linked polymers, the hydrophilic component is cross-linked: linear hydrophilic components would dissolve and leach out of the device. Grafted hydrogels are either cross-linked hydrophilic polymers or hydrophobic surfaces with a second hydrophilic polymer covalently attached. Random copolymer hydrogels are simply cross-linked copolymers with the monomer sequences distributed statistically. Macroscopic blends feature continuous matrices of a cross-linked component containing a disperse phase of either particles or fibres. Either component could be hydrophilic or hydrophobic but, in a hydrogel blend, at least one component must be hydrophilic. Each of these structures is illustrated in Fig. 3.8.

The synthesis of amphiphilic conetworks requires careful consideration of synthesis (Mespouille *et al.* 2009, Patrickios and Georgiou 2003). In some instances, when the components have reasonable segment compatibility, macromonomers can be copolymerized with low molecular weight monomers of the opposite philicity (Hadjiantoniou and Patrickios 2007, Triftaridou *et al.* 2007, Krasia and Patrickios 2006, Achilleos *et al.* 2007, Karunakaran and Kennedy 2007). However, phase separation during synthesis can produce partitioning of the monomer and macromonomer phases so that the phase domains grow to larger sizes. Both chain growth polymerization, chiefly radical polymerization, and step growth polymerizations are used to form the cross-linked material. This class of materials includes silicone-hydrogel conetworks, which are a new class of biomaterial that have revolutionized the contact lens industry. Phase separation during synthesis can prevent acquisition of the nano co-continuous morphology,



3.8 Multiphase hydrogels: different components in grey or black.

which is important for many applications. In order to alleviate this problem the segments can be linked together before cross-linking or the monomer can be modified then returned to the target functionality after cross-linking. The methods can be summarised as either the use of linear or branched block copolymers with reactive chain ends (Georgiou *et al.* 2010).

Synthesis of IPNs usually involves swelling of a preformed network in another monomer mixture, which is then polymerized. In the context of hydrogels both components can be hydrophilic (Wang *et al.* 2010) or one component can be hydrophobic and the other hydrophilic (Athawale *et al.* 2003).

3.5 Functional hydrogels

Hydrogels are often required to carry either chemical or biochemical functionality. In particular their excellent anti-fouling properties very often make them materials of choice for immobilizing peptides and proteins or medical applications and supporting cells. Two general strategies are available: either functional monomers are used or post-polymerizations reactions are used. Radical polymerization is amenable to the latter process but other polymers produced by other techniques or natural polymers often require post-polymerization strategies.

The synthesis of functional monomers can be time-consuming and special care needs to be taken to ensure that the polymerization does not modify the functionality carried by the monomer. Also, it can be advantageous to delay the activation of functionality until after the polymerization has been carried. This aspect is particularly useful for peptide functional monomers so that orthogonal deprotection of amino acids can be carried out on the hydrogel product rather than during solid phase synthesis (Perlin *et al.* 2008). This has the advantage of providing easier purification of the monomer as well as preventing unwanted side reactions involving reactive side groups. Peptides and proteins can easily be added to carboxyl functional hydrogels by first forming the N-hydroxy succinimide ester. However, glycidyl methacrylate can also provide reactive sites for coupling to amine (lysine) functional entities (Rimmer *et al.* 2007, Johnson *et al.* 2010). A limited number of reactive monomers are available commercially and some of these, such as glycidyl, can provide routes to a wide range of other functionalities. Another important aspect that must be considered in the successful addition of functionality to hydrogels is the solvents that are required for swelling, which provides availability of the polymer sites to reagents, and those required to dissolve reagents and optimize rates. Quite often these requirements can be in conflict and this severely limits the available useful chemistry.

Poly(ethylene glycol) methacrylate copolymer hydrogels produced by radical polymerization can be functionalized by a variety of means that can provide functional polyethylene glycol materials (Zhu 2010). The lack of functionality of the ethylene glycol repeat would otherwise require more exotic chemistry to provide functionality. Recently, strep growth strategies have been used to produce

functional PEGs by utilising the high yield ‘click’ reaction between azides and PEG with alkyne functionality. If the azide molecule carries functionality it can provide both cross-linking and functionalization. Other hydrogels produced by radical polymerization (e.g. poly(N-vinyl pyrrolidinone (NVP), poly(glycerol methacrylate), etc.) also have excellent records in the clinic but they are not as susceptible to oxidation as PEG, *in vivo*. Functionalization of methacrylates is generally simple, since functional monomers, such as peptide methacrylates or glycidyl methacrylate, can easily be incorporated. However, copolymerizations of NVP, which propagate via a non-stabilized radical, require careful attention to copolymerization reactivities. The reactivities of many common commercial monomers dictate that they are only incorporated well into NVP copolymerizations at low feed mole fractions. For example, the use of the difunctional cross-linker ethandiol dimethacrylate produces very large composition drifts during polymerization that often produces weak and friable materials. A better match of the reactivity ratios is achieved by use of allyl carbonate rather than methacrylate polymerizable functionality (Smith *et al.* 2006). Therefore, it follows that functionalization strategies should use monomer functionality that is well matched to the copolymerization with NVP.

3.6 Conclusion

This chapter provides a brief introduction to the methods available for the synthesis of hydrogels for those new to the field. Most of the major techniques have been covered. Many of the aspects covered here are common to general polymer synthesis. However, the requirement for swelling and high conversion in copolymerizations adds complexity. There are many and increasing numbers of applications that require hydrogels. In some of these applications existing systems will be satisfactory but there are many clinical problems where hydrogel solutions will provide major changes in care that could also be more cost-effective than the currently available materials. Clearly, these high impact areas can and should drive continued efforts to provide new hydrogels specifically designed for therapy.

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Processing and fabrication technologies for biomedical hydrogels

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Abstract: This chapter provides a description of selected fabrication or manufacturing processes, technologies and strategies which have the potential to enable emerging biomedical applications. The emphasis is on new technologies which can be used to control microscale physical and chemical characteristics, as well as general functional or mechanical properties. Methods reviewed include processes based on physical crosslinking, photolithography, stereolithography and two photon laser scanning photolithography. The relevance of these techniques to applications in tissue engineering, sensors and diagnostics is highlighted throughout.

Key words: hydrogel, tissue engineering, sensors, bioartificial organs, photolithography, stereolithography, two photon laser scanning lithography.

4.1 Introduction

Recent years have seen significant interest in the development of hydrogels for biomedical applications such as contact lenses (Goda and Ishihara, 2006), tissue engineering scaffolds (Brandl *et al.*, 2007), bioartificial organs (Mironov *et al.*, 2003; Boland *et al.*, 2003), sensors (Richter *et al.*, 2008), drug delivery platforms (Gutiérrez *et al.*, 2007), and wound healing biomaterials (Eisenbud *et al.*, 2003).

As water-swollen three-dimensional networks of polymer chains, many hydrogels exhibit tissue-like elasticity and solute diffusion properties and are showing particular promise in soft tissue engineering research (Peppas *et al.*, 2000, Drury and Mooney, 2003). The advantageous properties have their origins in the polymer chain properties, their interactions with water and other solvents, and the crosslinking or gelation mechanisms used to form the network. High diffusivity properties are necessary for the exchange of cell nutrients and waste products, and allow the development of comparatively thick hydrogel scaffolds before the need for alternative transport networks for growth factors to promote vascularization arises (Ko *et al.*, 2007). For many important biomaterials applications, surface chemistry properties are pertinent, particularly where protein adsorption, or the attachment and guided migration or proliferation of cells, is required.

In many instances, the important functional properties of hydrogel-based products will be dependent not only on the chemistry of the constituent polymers but also on microscale geometric features (Curtis and Wilkinson, 1997, Dalton *et al.*, 2001, Hamilton *et al.*, 2009) and other properties, such as stiffness gradients

(Brandl *et al.*, 2007), which may be controlled or manipulated by the product fabrication process. Processing technologies that offer the opportunity to introduce and control micropores, microchannels or 3D mechanical and biochemical patterning via the fabrication process in order to precisely control these attributes are hence attractive in these applications.

The focus of this chapter will be the description of selected fabrication or manufacturing processes, technologies and strategies which offer control over the surface, microscale and macroscale properties necessary to enable biomedical applications currently under intensive development. There will be an emphasis on new technologies which can be used to tailor physical and chemical properties at the microscale, as well as elasticity and strength at the macroscale, with reference to applications in tissue engineering, sensors and diagnostics. Issues associated with the processing of cell laden hydrogels will also be discussed.

4.2 Applications

4.2.1 Tissue engineering scaffolds and bioartificial organs

The ultimate goal of tissue engineering is the generation of new tissue to replace damaged, missing or diseased tissue. In most approaches, a scaffold with appropriate biomechanical and biochemical properties is used to provide support to the growing tissue and guide the biological processes involved. For soft tissues, several candidate hydrogels have been intensively investigated for scaffold applications. Scaffolds based on hydrogels of PEG (Nicodemus and Bryant, 2008) and PVA (Nuttelman *et al.*, 2002, Schmedlen *et al.*, 2002), for example, have been evaluated with respect to various soft tissue engineering applications, as have hydrogels of natural macromolecules such as chitosan (Fukuda *et al.*, 2006), collagen (Zorlutuna *et al.*, 2009) and gelatin (Broderick *et al.*, 2005). Some recent efforts have employed combinations of synthetic and natural macromolecules (Mathews *et al.*, 2008, Liu *et al.* 2009). Proposed applications for such hydrogels have included corneal implants, vascular tissue and cartilage.

The tissue generation process may be initiated in laboratory bioreactor systems in which appropriate cells are applied to the scaffold (Barron *et al.*, 2003, Martin *et al.*, 2004; Portner *et al.*, 2005). Growth factors, oxygen and cell nutrients can be administered, and appropriate mechanical signals can be applied to the scaffold to stimulate cell proliferation, differentiation, apoptosis and the deposition of extracellular matrix. Alternatively, a cell free scaffold with appropriate bioactivity, often peptide or protein growth factors, may be implanted. This results in a guided influx of cells from the surrounding implant site (Hirano and Mooney, 2004).

The development of new bioartificial organs will involve organization of pockets or compartments of living cells in physical arrangements conducive to providing the correct signaling to reproduce organ function. Adequate transport of oxygen, carbon dioxide and nutrients as well as immunoisolation where required,

must also be ensured. Owing to their tissue-like characteristics, hydrogels are a natural choice of scaffold for such applications and have been widely adopted in such research.

4.2.2 Sensors and diagnostic applications

The potential for the development of hydrogel-based sensors has long been recognized, stemming in many instances from their capacity to undergo significant volume changes in response to small changes in external stimuli such as temperature, light, pH, solvents or particular ions (Richter *et al.*, 2008). In recent years, advances in technologies capable of fabricating hydrogels with features at the micron length scale have given fresh impetus to these possibilities, leading to the development of microdevices operating on these principles (Peppas *et al.*, 2006). Hydrogel inserts can perform combined sensing and actuation functions and have been demonstrated as microactuators operating as autonomous valves within microfluidic channels (Beebe *et al.*, 2000). The utilization of hydrogels for pH sensing applications has recently been reviewed by Richter *et al.* (2008), who summarize mechanisms of optical and mechanical transduction, many of which are also applicable to sensing of other stimuli. They also discuss issues of resolution, sensitivity, range and calibration.

Hydrogels have also recently been receiving attention as 3D physical scaffolds for sensors or diagnostic devices incorporating living cells, particularly for cell types which favour a three-dimensional environment rather than two-dimensional culture (Liu Tsang and Bhatia, 2004, Albrecht *et al.*, 2005, Liu Tsang and Bhatia, 2007). By using these techniques, separately and in combination, a method for producing sensor microarray platforms incorporating live cell assays in three-dimensional hydrated environments was presented.

A common issue across several emerging applications is the need to fabricate hydrogels structures in which the viability of live cells can be supported, and in which the features which interact with cells can be precisely controlled at the micron scale. There are three broad approaches to distributing cells within hydrogel scaffolds (Jen *et al.*, 1996): *seeding* of a scaffold with cells after its fabrication (relying on cell *adhesion* to a surface in or on the scaffold); *microencapsulation* of cells in thin hydrogel capsules; or *macroencapsulation* of cells within a larger structure during a polymerization or gelation process.

4.2.3 Seeding of cells in hydrogels

Seeding relies on the attachment of cells to the substrate, involving binding either on top of gel films or within hydrogel foams. Seeding, or adhesion immobilization, provides a structural template directing cell growth and differentiation. Cell adhesion can be improved by adding immobilized cell-adhesive proteins or oligopeptides, such as fibronectin or laminin. Altering pore size and network

structure can also modify cell adhesion. Cells are generally seeded onto the hydrogel and allowed to attach and migrate. Culture media is usually required to provide nutrients for growth as well as oxygen. Seeding of hydrogels is only effective for development of epithelial layers, such as corneal epithelium or vascular endothelium, since the effective pore size of hydrogels is generally smaller than the average cell size, which makes them more suitable for cell encapsulation procedures.

4.2.4 Microencapsulation of cells

If a form of cell encapsulation is involved, there are significant implications for the selection of the hydrogel processing route. Microencapsulation involves surrounding cells or groups of cells with thin microporous semipermeable membranes. The permeability of the membrane allows diffusion of oxygen, nutrients and cellular products, but prevents the ingress of immune cells responsible for transplant rejection. Photopolymerization has been used to coat hydrogel capsules in order to improve biocompatibility and form the innermost hydrogel layer. This technique has the advantage of the membrane being in direct contact with the encapsulated cells. By minimizing the diffusion distance for oxygen and cell products, therapeutic efficiency can be improved.

4.2.5 Macroencapsulation

Unlike adhesion, matrix entrapment (or macroencapsulation) relies on physical constraint of the cells within the hydrogel network. The three-dimensional porous network is ideal to hold cells in place while allowing transport of nutrients and wastes via the bulk fluid. Hydrogels for matrix entrapment must be formed by very mild polymerization techniques. This precludes the use of harsh solvents, toxic monomers, extensive UV exposure and high temperatures to ensure adequate cell survival.

Macroencapsulation of cells is a suitable option for *in situ* polymerization of injectable cell laden hydrogels, for example, and can also be advantageous in scenarios where guided cell proliferation, migration and differentiation within a scaffold are also desired. Cell encapsulation was first utilized in fields related to tissue engineering as a method to immunoisolate cells, such as pancreatic and hepatic cells (mainly xenogenic or allogenic cells) from the host without compromising their functionality (Canaple *et al.*, 2002). Such treatment schemes can be used for a wide range of endocrine secretion related diseases and also for some neurodegenerative syndromes (Uludag *et al.*, 2000). Where precise control of cell differentiation is needed, encapsulation can be used as a diffusion barrier, thus a rate-controlling element for the diffusion of growth and differentiation factors and moreover as an easier method to distinguish and separate sub-cell populations (Payne *et al.*, 2002).

Encapsulation procedures are inherently difficult, mainly due to the mismatch of the requirements of gelation and the ability of the cells to withstand those conditions. Such limitations were the case for a silk fibroin hydrogel which, under physiological conditions, takes from hours up to days to set; this can be prevented by utilization of low pH high temperature conditions, which are obviously not suitable for cell encapsulation (Wang *et al.*, 2008). However, in cases such as this, gelation can also be facilitated by methods such as ultrasonication. After optimization of gelation for this particular study it was observed that cells survived much better in low concentration hydrogels (4%) than in high concentration hydrogels (8% and 12%). Similar effects of concentration and density of the final gel on cell behaviour have been seen with other encapsulation systems too (Burdick and Anseth, 2002).

Cell proliferation is highly restricted in an encapsulation system due to the absence of the necessary surface area and volume for the dividing cells. Thus the final cell count is reached relatively early during culturing. Further proliferation is then dependent on the hydrogel degradation rate and is further restricted by cells' own secretion of extracellular matrix.

Different encapsulation techniques impose a different set of threats to cell viability. For example, encapsulation via the freeze–thawing cryogelation technique is impeded by the ice crystal formation and rupture of the cells. This can be partly solved by the addition of cryoprotectants such as DMSO, which have been seen to cause a twofold increase in survival rate of the encapsulated cells (Qi *et al.*, 2004). A secondary limitation is the length scales of the hydrogels, since encapsulated cells require a steady diffusion gradient for oxygen and nutrients, and also for the removal of the waste products. This obviously becomes untenable for thicker hydrogels. Diffusion of the nutrients will also be affected by their propensity to be absorbed on the hydrogel network. This would cause sequestering of certain elements in the outer parts of the hydrogel while depriving the inner core. However, hydrogels are still better options for thick products, as evidenced by the reasonable results obtained by the 8 mm thick PEO hydrogels with photoencapsulated chondrocytes (Bryant and Anseth, 2001). Such studies have not, however, established the possible differences between different areas within the hydrogel, due to uneven exposure to the light source (Baroli, 2006). Moreover, the encapsulation process itself would impose abrupt changes to the cellular microenvironment, which would result in phenotypic responses by the cells.

4.3 Gelation

Gelation of the solution containing the cells can be achieved by several methods, such as physical gelation initiated by changes in pH or temperature, photopolymerization or ion addition (e.g. preparation of cell encapsulated alginate microspheres by divalent cation crosslinking). Gelation properties can be controlled by pH, temperature and/or ionic strength. The essential problem for fabricating

hydrogel products with encapsulated cells, however, is the need for processing conditions which are sufficiently benign that they do not affect subsequent cell viability. Thus, optimization of a final hydrogel product for tissue engineering can be severely hindered by the limitations imposed by the presence of the cells.

4.4 Physical crosslinking

4.4.1 Cryogelation

Hydrogel structures can be fabricated using processes that are entirely dependent on physical crosslinking mechanisms. The most ubiquitous example of this is the formation of physically crosslinked polyvinyl alcohol hydrogels by subjecting an aqueous solution to a number of freezing and thawing cycles (Peppas, 1975). The resulting hydrogels (or cryogels) have an open porous morphology and feature both amorphous and crystalline regions. They have been shown to be highly biocompatible and to have high elasticity and relatively high strength. Their unique cryogelation characteristics are attributed to the high propensity for hydrogen bond formation between the hydroxyl groups present in the polyvinyl alcohol chemical structure. Early studies led to a proposed model for this process, involving the formation and growth of ice crystals in polymer poor regions that push chains in polymer rich regions into more intimate contact, resulting in the formation of crystallites (featuring interchain hydrogen bonds) which then act as crosslinking or entanglement points in the hydrogel (Peppas, 1975, Willcox *et al.*, 1999). Transmission electron microscopy has been used to explore the development of morphology in cryogels of various polymer concentrations through freeze–thaw cycles and the ageing process, and propose a process based on a kinetically frustrated crystallization process in the first freeze–thaw cycle (Willcox *et al.*, 1999). In subsequent cycles, the crystallites from the first cycle grow, while new, smaller crystallites also form and create new junctions, albeit with only a slight change to the mesh structure. Recently a low-field NMR spectroscopy study on the structure of polyvinyl alcohol cryogels has been presented. This work confirms the existence of a primary network of crystallites which dictates the network structure as well as the presence of other less perfect crystallites (Valentín *et al.*, 2009).

A method for inducing anisotropy in cryogelation of polyvinyl alcohol hydrogels has also been developed, based on the application of stress during the freezing and thawing processes (Millon *et al.*, 2006, Millon *et al.*, 2007, Hudson *et al.*, 2009). This is significant because most soft tissues are anisotropic in nature, often oriented to withstand functional loads.

4.4.2 Tailored morphologies

As a development of the cryogelation concept, a technique for directional freezing of polyvinyl alcohol solutions has been presented (Gutiérrez *et al.*, 2007). This

results in a hydrogel with a highly oriented structure of parallel microchannels and sheet-like structures, rather than pores. The process (termed ISISA, or ice segregation induced self assembly) involves immersion of the aqueous solution into liquid nitrogen (at -196°C) at a controlled rate. The effect of freezing rates, polymer contents and polymer molecular weights were examined and the channel diameter is shown to decrease with an increase in either the freezing rate or the polymer molecular weight. The capacity of this simple process to deliver a wide range of highly aligned internal architectures has been demonstrated. An interesting outcome of this investigation is the high tensile strengths found (of over 3 MPa in certain cases), with the highest strengths observed for gels with the largest channel diameters and the thickest walls, associated with high molecular weight polymer and slow freezing rates.

A method for modifying the internal architectures of chemically crosslinked gels with a cryogenic treatment has also been presented (Van Blierberghe, 2008), using hydrogels containing modified gelatin (gel-MOD) and/or chondroitin sulphate (CS-MOD). The hydrogels were produced by gelation of solutions of biopolymers, followed by UV photocrosslinking, followed by the cryogenic treatment.

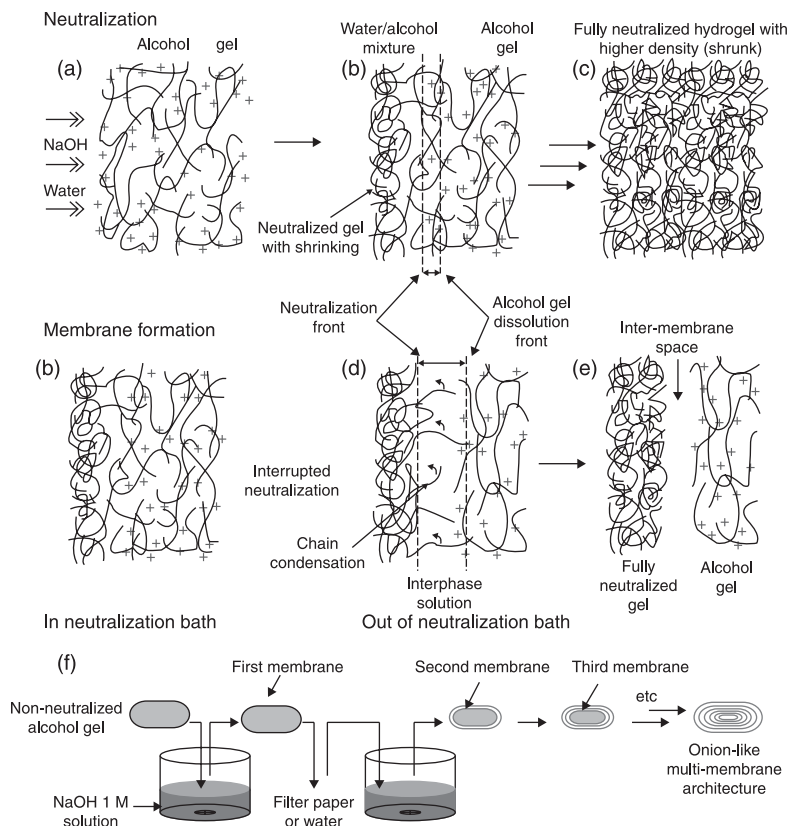
4.4.3 Multimembrane hydrogels

Methods have been developed to produce multimembrane hydrogels through a multi-step process involving interruptions to the process by which neutralization and dissolution fronts advance (see Fig. 4.1) (Ladet *et al.*, 2008). This technique holds the promise that hydrogel structures can be fabricated which more closely resemble the geometric organization of organs and tissue ultrastructures. The technique may permit the development of experimental organ or tissue models for cell culture and co-culture within compartmentalized scaffold structures. Multimembrane structures also open up the possibility of controlling release of growth factors, nutrients or other therapeutic agents from appropriate locations.

4.5 Photopolymerization and photopatterning

Preparation of hydrogels by photopolymerization involves the exposure of a polymer solution to a light source. It has advantages over conventional polymerization techniques, including spatial and temporal control, fast curing rates at room or physiological temperatures and minimal heat production (Nguyen and West, 2002). Photocrosslinkable hydrogels can be created *in situ* from aqueous monomers in a minimally invasive manner. This is attractive for biomedical applications as complex shapes can be formed that conform to tissue structures.

Photopolymerization *in vivo* offers the possibility of injectable gels which can be polymerized *in vivo* in conformation with local hard or soft tissues. For bulk photopolymerization of injected hydrogels, the photoinitiator is dissolved in the



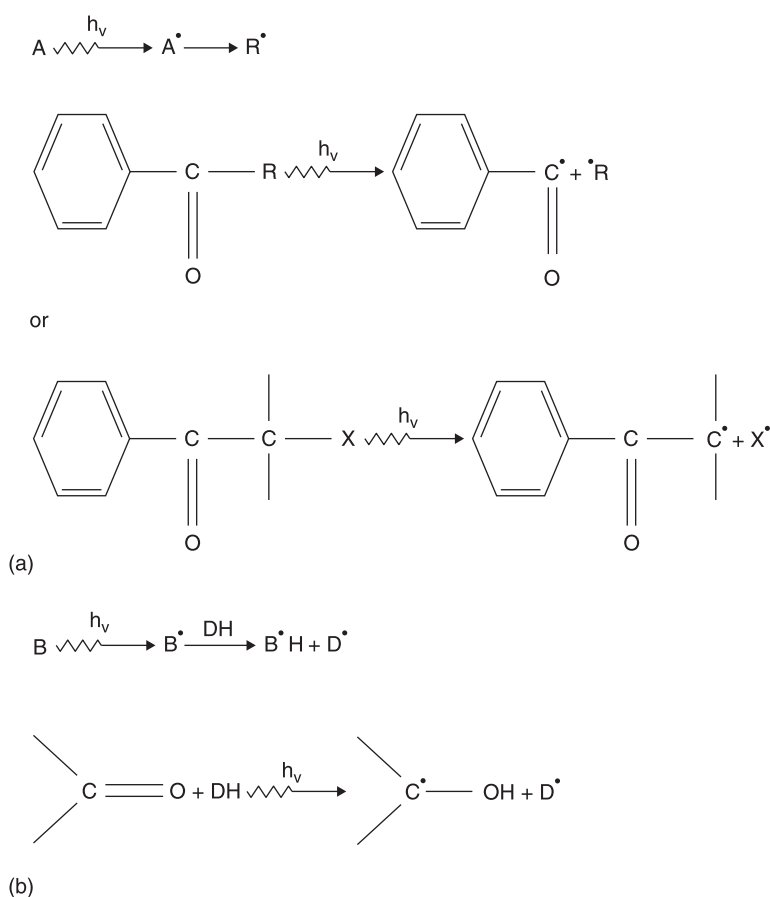
4.1 Neutralization of polyelectrolyte gel and generation of a multi membrane gel; (i) progression of gel neutralization front in bath in normal process (from right, steps a, b and c), (ii) formation of the interphase solution and collapse of polymer chains onto neutralized gel (following gel removal from bath), (step d), (iii) final formation of inter-membrane space after completion of condensation of residual polymer chains (step e), and (iv) repeated application of the process to produce onion-like membrane architectures (step f). (Reprinted by permission of Macmillan Publishers Ltd: *Nature*, Volume 452 (7183), Ladet, S., David, L., Dornard, A., Multimembrane Hydrogels, pp. 76–80, 2008.)

monomer solution which converts to a hydrogel polymer upon exposure to UV. For interfacial photopolymerization, hydrogel linings (<100 μm) are created by adsorption of a photoinitiator onto the surface of tissue or cells, which are then exposed to the hydrogel monomer solution and the appropriate light source.

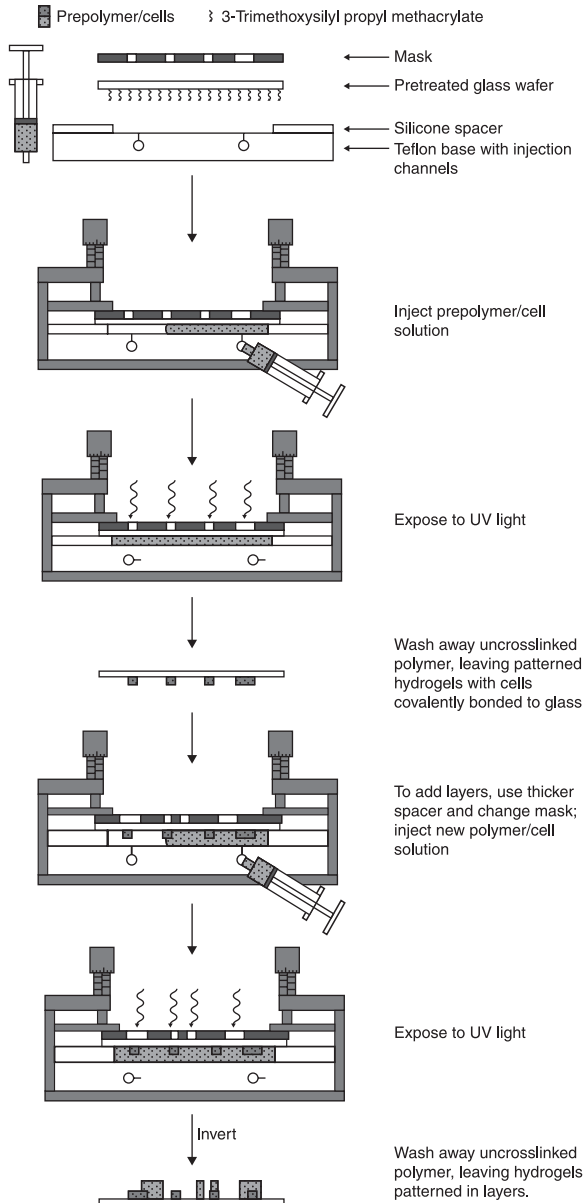
The formation of hydrogel networks by these methods generally requires the presence in a polymer solution of a photoinitiator with a high absorption at a specific wavelength to produce radical initiating species (Nguyen and West, 2002). In one mechanism, photoinitiators undergo cleavage at C-C, C-Cl, C-O or

C-S bonds to form radicals when exposed to UV (Fig. 4.2). In an alternative mechanism, aromatic ketones can undergo hydrogen abstraction from an H donor molecule to generate a ketyl radical and a donor radical (Fig. 4.3). A further mechanism, cationic photopolymerization, generates protonic acids and so is not considered appropriate for tissue engineering applications.

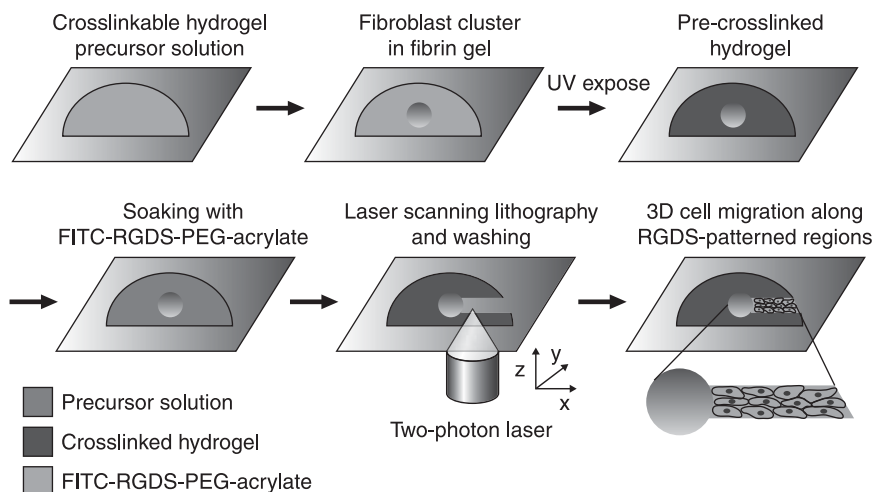
Photopatterned cell-laden hydrogels have been obtained, in which live cells suspended in a polymer solution are locally photoimmobilized in multiple cellular domains in a controlled hydrogel architecture (Liu Tsang and Bhatia, 2004). Uncrosslinked polymers and cells are washed away and the process repeated in the same layer or in additional layers containing different cell types, concentrations or different polymer mixtures. By increasing the height of the photocrosslinking



4.2 Photoinitiation mechanisms, (a) – Photocleavage, (b) – Hydrogen Abstraction (Reprinted from *Biomaterials*, Volume 23, Issue 22, Photopolymerizable hydrogels for tissue engineering applications, pp. 4307–14, 2002, with permission from Elsevier.)



4.3 Photopatterning of hydrogels containing living cells. A polymer/cell mixture is injected into a cavity separated from a UV light source by a glass wafer and an emulsion mask that allows UV to pass through only certain regions. (With kind permission from Springer Science+Business Media, *Biomedical Microdevices*, Three-dimensional photopatterning of hydrogels containing living cells, Volume 4, Issue 4, 2002, pp. 257–66, Liu, V.A., and Bhatia, S.N.)



4.4 Schematic of steps involved in guiding fibroblast cell migration in preformed hydrogels by two-photon laser scanning photolithography. A precursor solution is prepared, into which a fibroblast cluster is inserted. The hydrogel is crosslinked by UV exposure, then soaked with a cell adhesive ligand, which diffuses throughout. The two-photon laser is used to irradiate a 3D pattern of tethered biomolecules which guides 3D cell migration. (Reprinted from *Biomaterials*, Volume 29, Issue 20, Lee, S-H, Moon, J.J., West, J.L. Three-dimensional micropatterning of bioactive hydrogels via two-photon laser scanning photolithography for guided 3D cell migration, pp. 2962–8, 2008, with permission from Elsevier.)

chamber in between steps, additional layers can be built to create a 3D cellular hydrogel tissue scaffold. Figure 4.4 shows the photopatterning method used to build a 3D PEG-based hydrogel/cell network. It was reported that features as small as 50 μm containing cells are possible with structures containing three layers. The objective for development of these systems is to enable researchers to address the structural, multicellular and biochemical complexity found in many human organs.

4.6 Stereolithography

Hydrogels with spatially tailored properties and architectures have also been produced using photolithographic patterning methods. Stereolithography (SL) is a photopolymerization technique that directs light from a laser beam onto predefined regions of a layer of liquid polymer, causing selective solidification (Liu Tsang and Bhatia, 2004). The stage is then lowered, covered with a new layer of polymer solution and the process repeated. This technology has been used extensively in the prototyping of polymer-based tissue engineering scaffolds, and,

less commonly, for hydrogel scaffolds. Stereolithography has been shown to produce features as small as 70 μm in polymer applications.

Techniques have been developed in which cells suspended in a polymer solution are locally photoimmobilized in multiple cellular domains within a controlled hydrogel architecture (Liu Tsang and Bhatia, 2004). Uncrosslinked polymer and cells are washed away and the process repeated in the same layer or in additional layers containing different cell types, concentrations or different polymer mixtures. By increasing the height of the photocrosslinking chamber in between steps, additional layers can be built to create a 3D cellular hydrogel tissue scaffold. It was reported that features as small as 50 μm containing cells are possible with structures containing three layers. A particular limitation of this approach is the vertical resolution, and the limitations on the achievable three-dimensional geometries achievable imposed by the layerwise construction path. Further development of these systems to address these issues could enable researchers to address the structural, multicellular and biochemical complexity found in many human organs.

Recent progress related to sensors containing arrangements of living cells has been reported, utilizing photopatterning and electropatterning to produce arrangements of cells in a three-dimensional gel culture (Albrecht *et al.*, 2005). The need for high resolution patterning methods to define the micron scale features to which cells respond has been recognized. Photopatterning has been used to selectively crosslink hydrogel microstructures containing living cells, *albeit* at a resolution of the order of 100 μm , and further demonstrated the use of dielectrophoretic forces to localize single cells at positions within a fluid volume at micron-scale resolution (Albrecht *et al.*, 2006).

4.7 Two-photon laser scanning photolithography

Single photon laser absorption photolithography suffers from significant limitations on the complexity of structures which can be directly fabricated, most notably the need for geometric uniformity in vertical directions. Recent work with two-photon laser absorption photolithography has opened up the possibility of modifying the chemical and mechanical properties of preformed hydrogels along precisely defined internal pathways so that gel microarchitectures can be engineered in three dimensions (Hahn *et al.*, 2005, Hahn *et al.*, 2006a). The use of two-photon lasers in optical data storage and photolithography has been pursued for more than a decade but the application to solvated systems is much more recent.

A two-photon laser absorption based patterning approach in which a polyethylene glycol diacrylate (PEG-DA) hydrogel (pre-formed) is used as a template has recently been developed (Hahn *et al.*, 2005). The principle involves spatially controlled crosslinking of photosensitive monomer solutions which have been allowed to diffuse into an already formed hydrogel. The use of two-photon lasers allows photopolymerization of the monomer solution in geometrically

defined regions of variable depths and cross sections, facilitating the definition of complex three-dimensional patterns within the bulk hydrogel.

The pioneering work with this technology has demonstrated patterning inside a template hydrogel of PEG-DA which is biocompatible, and is photopolymerizable in the presence of photoinitiators (Hahn *et al.*, 2005). This preformed PEG-DA gel was soaked with either a low molecular weight PEG-DA or a fluorescently labelled monoacryloyl-PEG-peptide (ACRL-PEG-RGDS), and the laser focal point then passed through a pre-defined path to generate either regions of higher crosslink density (for the low MW PEG-DA), which affects local mechanical and transport properties, or preferred pathways for cell migration (in the case of the fluorescently labelled monoacryloyl-PEG-peptide, Fig. 4.5). Radiation exposure levels can also be controlled to create either spatial gradients of physical properties or concentration gradients of the patterned cell adhesion peptides. The capability of this technique to produce internal patterns of a variety of shapes, including those with non-uniform axial features within a standing hydrogel, has been shown (Hahn *et al.*, 2005, Lee *et al.*, 2008).

Further studies aimed at the three-dimensional migration of cells, using the two-photon laser absorption principle with an adapted confocal microscopy system, have created three-dimensional pathways of a cell adhesive ligand (RGDS) in an otherwise bioinert hydrogel (Lee *et al.*, 2008, Moon *et al.*, 2009).

Application to basic scientific investigations of such processes as wound healing, inflammation, embryogenesis and tumour cell metastasis are envisaged by the investigators. The implications for manufacture of scaffolds for guided tissue regeneration are also foreshadowed.

There has been great progress in creating three-dimensional hydrogel structures in which precise patterns of biomolecules and cell types can be positioned to more closely represent the structural complexity of tissues or organs and in which complex biological processes sensitive to physical and biochemical stimuli can be modelled and exploited. This enabling technology is sure to find wide application in research fields in the biological sciences.

4.8 Processing of multicomponent hydrogels

There have been many studies concerned with utilizing multicomponent hydrogels of various types to replicate the biological activities and multiscale organization of natural soft tissues such as cartilage or vascular tissue. These have been the subject of a recent comprehensive review (Jia and Kiick, 2009). Included in this are studies concerned with the synthesis of multiblock polymer/peptide hybrid polymers to mimic the molecular origins of elastin elasticity, and hydrogels responsive to environmental factors, such as the presence of specific antigens. The use of nanoparticles to control hydrogel structural and morphological parameters, and the applications of hydrogels with embedded nanoparticles in drug delivery are also reviewed (Jia and Kiick, 2009). In engineering implants for functional

biomechanical applications such as cartilage, hydrogel scaffolds able to withstand high mechanical loads are very desirable. In the past, this requirement has represented a significant challenge to many candidate materials, although polyvinyl alcohol hydrogels have attracted interest. In recent years, there have been interesting developments in the field of high strength hydrogels, most notably the development of surprisingly strong double network hydrogels (Gong *et al.*, 2003).

The initial work in this area reported compressive strengths in excess of 20 MPa for double network hydrogels of PAMPS and polyacrylamide (PAAm) hydrogels containing 60–90% water. They show high wear resistance due to their low coefficient of friction.

The two structural parameters that are crucial in obtaining these high strength gels are the molar ratio of the first to the second network and the crosslinking densities. A dramatic improvement in the mechanical strength of the gel is observed only when the molar ratio of the second network to the first network is in the range of several tens. Another substantial increase in strength is observed when the first network is highly crosslinked and the second is loosely crosslinked. This allows collagen or agarose to be used as the first network and synthetic polymers such as poly(2-hydroxyethyl methacrylate) (HEMA) and poly(N,N'-dimethyl acrylamide) (PDMAAm), which are used in contact lenses, as the second network. DN gels are produced simply by synthesizing the second network in the presence of the first network. The first network is immersed into the synthesizing medium containing the second network for 24 hours until equilibrium is reached.

Several follow-up studies have attempted to explain the underlying mechanism for the impressive strength of these hydrogels, but a clear understanding of the source of this strength has yet to emerge (Nakajima *et al.*, 2009). Nevertheless, the possibility of such high performance hydrogels, achievable through very specific combinations of solutions and polymerization parameters, is likely to lead to new opportunities for use of hydrogels in advanced applications (Calvert, 2009).

4.9 Future trends

As the requirements for engineering of biomedical hydrogel products grow more complex, the need for fabrication technologies capable of producing gels with microarchitectures and biochemical functionalities tailored in three-dimensional space will also increase. An area of ongoing focus is the need to improve the geometric precision of photolithography and stereolithography technologies which are commercially available for general rapid prototyping purposes. The development and automation of processes for developing hydrogels with the ability to represent the true structural complexity of organs or biological systems, including the encapsulation of cells in particular regions, would open up opportunities for more sophisticated experimental models of biological processes, and help pave the way for the engineering of complex organs.

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Regulation of novel biomedical hydrogel products

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Abstract: To ensure that patients and users benefit from novel biomedical hydrogels with medical applications, anyone involved in the design and development of these products should be aware of the increasingly strict regulatory requirements that these products must meet before they can be placed on the market. This chapter will discuss important issues that should be taken into consideration when hydrogels are regulated as medical devices, concentrating on the United States (US) and Europe. For example, the US and Europe regulate medical devices in different ways, each with its own regulatory framework, although efforts to harmonize these requirements are under way.

Key words: European regulation of medical devices, US regulation of medical devices, Global Harmonization Task Force, medical device authorization process, medical device post-market requirements.

5.1 Introduction

Novel biomedical hydrogels span an impressively wide range of medical applications, holding enormous potential for clinical benefit. To ensure that patients and users actually do benefit from the products, anyone involved in the design and development of the products should be aware of the increasingly strict regulatory requirements that they must meet before they can be placed on the market. This realization should begin at the earliest stage of research and development because the decisions made at this stage of the medical device lifecycle often lead to either success or failure of the products reaching the very patients and users that they are intended to benefit.

This chapter will discuss important issues that should be taken into consideration regarding hydrogels regulated as medical devices, concentrating on the United States (US) and Europe. For example, the US and Europe regulate medical devices in different ways, each with its own regulatory framework. Efforts to harmonize these requirements are under way. The regulation of medical devices is generally based upon the level of risk of product usage. Appropriate non-clinical testing needs to be performed. Sufficient clinical data should exist to justify product safety and effectiveness or performance. Marketing authorization, quality management systems and post-market requirements differ in important ways in the US and Europe. Medical device regulation is evolving in the US and Europe with important changes in process at the time of writing.

5.2 Regulatory jurisdictions

There are some similarities among various regions of the world regarding the regulation of medical devices; however, significant differences exist. For example, most jurisdictions regulate medical devices under systems that differ from the regulation of pharmaceutical products. In general, the stringency of regulatory requirements applied to particular types of devices is related to the risks posed by those devices. In spite of these similarities, the specific approach to regulation in various jurisdictions can be markedly different.

This chapter covers the US and Europe, but other regions of the world operate under systems that differ in important ways from both the US and European systems. In some cases, countries rely on the marketing authorization of other countries. It should never be taken for granted that the requirements in one jurisdiction will be recognized in another, or that requirements are so similar that meeting medical device requirements in one country or region will automatically mean that the requirements in another have been met.

The Global Harmonization Task Force (GHTF) was established in 1992 in an effort to encourage the international harmonization of medical device regulations. The GHTF is a voluntary group of representatives from national medical device regulatory authorities and the regulated industry. The purpose of the task force is to work toward greater uniformity among national medical device regulatory systems with two aims in mind, which are to enhance patient safety and increase access to safe and clinically beneficial medical technologies around the world. It is important to be aware of the GHTF because some of the guidance documents developed by the GHTF have been adopted by individual regulatory jurisdictions including the US and Europe. Information on this group and its harmonization activities can be found on its website at www.gh tf.org.

5.3 Regulatory frameworks

5.3.1 United States (US) framework

In the US, the regulation of medical devices is under the authority of the US Food and Drug Administration (FDA), which is a US public health, consumer protection and scientifically based law enforcement agency. The FDA is an agency within the US Department of Health and Human Services and consists of eight offices and centers. Detailed information on these offices and centers can be found on the FDA website at www.fda.gov/AboutFDA/CentersOffices/default.htm.

The Center for Devices and Radiological Health (CDRH) is responsible for ensuring the safety and effectiveness of medical devices. The FDA carries out its mission through the enforcement of the US Food, Drug and Cosmetic Act (FD&C Act) and several other public health laws. The FD&C Act is the basic food and drug law of the United States, which is intended to ensure that the products on the

US market that the FDA regulates, including medical devices, are safe and effective for their intended uses.

Manufacturers wishing to enter the US market must comply with all applicable FDA requirements before, during, and after the process of placing medical devices on the market. All establishments engaged in the manufacture, preparation, propagation, compounding or processing of a medical device for human use must register their establishments and list the device in conformance with the requirements in 21 CFR Part 807 before the product is placed on the market. This process requires the payment of a user fee. For example, the fee that must be paid during fiscal year (FY) 2010 (1 October 2009 to 30 September 2010) is \$2008. Firms located outside the US must submit the name, address and telephone number of a US agent as part of its initial and updated registration information.

In addition to registration and listing requirements, firms must comply with the Quality System Regulation (QSR) specified in Title 21 Code of Regulations (CFR) Part 820 (21 CFR 820) unless exempted; ensure that devices are labeled in compliance with US FDA labeling requirements; and obtain premarket clearance or approval unless exempted from these requirements. These requirements are discussed in more detail later in the chapter.

5.3.2 European framework

In Europe, the regulation of medical devices is harmonized, but not as centralized as it is in the US. That is, the European directives, which specify the regulatory requirements for medical devices, are issued by the European Commission; however, they are not directly in force. Instead, each European member state must transpose the requirements of the directives into national laws and regulations, which include the legally binding requirements that medical devices being placed on the market in a particular member state must meet. Fortunately, there are few important variations among member states regarding the requirements transposed from the directives; however, some variations exist. Some of the more important variations involve the languages that can be used on product labels and in instructions for use, the requirements for notifying the member state of clinical investigations, or whether or not it is required to register certain classes of devices with the member state when the devices are placed on the market.

Medical devices placed on the market in Europe must be in compliance with one of the European directives for medical devices: the Active Implantable Medical Device Directive (90/385/EEC) (AIMDD), the Medical Device Directive (93/42/EEC) (MDD) or the *In Vitro* Diagnostic Directive (98/79/EC) (IVDD). Other directives may also apply; however, these three directives are principal directives related to medical devices. Medical devices must also be affixed with the CE mark to indicate compliance with the applicable directive.

The CE mark, which is an abbreviation of 'Conformité Européenne' (European Conformity), may be found on many items other than medical devices, to indicate

compliance with European directives that apply to that specific type of product, including machinery, electrical/electronic equipment, pressure vessels and personal protective equipment. The CE mark allows products to be marketed throughout Europe. The AIMDD and MDD have recently been revised by Directive 2007/47/EC. Manufacturers and other involved parties needed to comply with the revised requirements as of 21 March 2010. Information on the revisions can be obtained from the European Commission website at http://ec.europa.eu/enterprise/medical_devices/index_en.htm.

The European directives are based upon a set of 'essential requirements', such as the protection of health and safety which goods, including medical devices, must meet when they are placed on the market. The requirements in the directives apply to the manufacturers; competent authorities, which are the government bodies in each member state responsible for implementing and enforcing the directives; notified bodies, which are designated by competent authorities to perform one or more of the conformity assessment procedures for medical devices in Class I that are sterile or have a measuring function; Class IIa, Class IIb and Class III; and authorized representatives, which are any natural or legal persons established in the Community who, explicitly designated by the manufacturer, act and may be addressed by authorities and bodies in the Community instead of the manufacturer with regard to the manufacturer's obligations under this directive.

The European standards bodies have been given the task of drawing up corresponding technical specifications for meeting the essential requirements of the directives. The technical specifications are contained in 'harmonized standards', which are European standards, adopted by European standards bodies, following a mandate issued by the European Commission after consultation of member states. Compliance with harmonized standards provides a presumption of conformity to the corresponding essential requirements of the European directives. It is important to recognize that compliance with harmonized standards is voluntary and manufacturers are free to choose any other technical solution that provides compliance with the essential requirements. In spite of this option, it is rare for manufacturers to choose it. Other standards, such as those developed outside Europe, may be useful in demonstrating compliance with the essential requirements of the directives, but conformity with these standards does not provide a presumption of conformity with the essential requirements.

Under the European regulatory framework, the manufacturer is responsible for affixing the CE mark to the product. Before doing so, an appropriate conformity assessment procedure must be completed and a Declaration of Conformity to the relevant directive drawn up. The manufacturer can select from various conformity assessment procedures ranging from product testing to the implementation of a full quality system. Device classification determines the conformity assessment procedures available to medical device manufacturers to affix the CE mark. For devices in higher risk categories, notified bodies must be involved in the conformity assessment procedure. These bodies, which can be private

organizations or government agencies, must carry out required evaluation and certification activities, which are specified in the various conformity assessment procedures. Most commonly, notified bodies evaluate medical device technical documentation and conduct quality management system certifications.

5.4 Risk-based device classification

5.4.1 US classification of medical devices

The classification of medical devices in the US depends on the intended use of the device and the perceived risk the device poses to the patient and/or user. The FDA classifies medical devices into one of three regulatory classes: Class I, Class II or Class III. Class I devices are in the lowest risk category, Class II devices are intermediate and Class III devices are in the highest risk category. Nearly 2,000 different generic types of device have been classified by the FDA.

Medical devices in all three classes are subject to baseline requirements called general controls, such as prohibition against adulteration and misbranding, manufacturing facility registration, device listing, submission of a marketing application, compliance with good manufacturing practices, and record keeping. Devices in Class II are subject to special controls in addition to general controls. Special controls may include conformity with mandatory or voluntary standards, conformity with FDA guidance documents, special labeling requirements, or the requirement to conduct post-market surveillance activities. Devices in Class III are subject to premarket approval. Class III devices are those for which insufficient information exists to assure safety and effectiveness solely through general or special controls.

The US classification regulations in 21 CFR Parts 862 through 892 contain the names and definitions of devices, their classification, and whether or not they are exempt from certain FDA requirements. It is important to note that most Class I devices and a limited number of Class II devices are exempt from the process of notifying the FDA of the intent to market a medical device in the US, that is, the 510(k) process. In addition, some devices are also exempt from the requirements of the US Quality System Regulation (QSR), which is discussed in section 5.8 of this chapter; however, 510(k) exemption does not automatically mean that devices are exempt from the QSR.

5.4.2 European classification of medical devices

In Europe, medical devices are not assigned to various classes by regulatory authorities. It is the manufacturer who is responsible for determining the correct classification of the device. Medical devices subject to the MDD are classified in accordance with the classification rules outlined in Annex IX of the Directive. Classification is based upon intended use, the risks associated with the use of the

medical device, whether the device is active or non-active, the duration of contact of the device with the patient, the degree of invasiveness of the device, and the part of the body affected by the use of the device. Under this system, there are four device classes: Class I, Class IIa Class IIb and Class III.

The IVDD places *in vitro* diagnostic (IVD) medical devices into several regulatory categories depending upon the risks associated with their use; however, the term 'classification' is not used. For example, IVD devices listed in Annex II, List A of the Directive include the highest risk IVD devices, such as those used for determining an ABO system, rhesus (C, c, D, E, e) anti-Kell blood groups. Annex II, List B includes high risk IVD devices, which are considered lower risk than those in List A. List B includes devices for the detection, confirmation and quantification in human specimens of markers of HIV infection (HIV 1 and 2), HTLV I and II, and hepatitis B, C and D. IVD devices used for self-testing are considered to be in another regulatory category. IVD devices that are not in any of the above categories are sometimes referred to as 'other IVD devices' or 'non-List A, B, self-testing IVD devices' and are subject to the least stringent controls applicable to IVD devices.

Under the AIMDD, there is only one device risk category.

5.5 Non-clinical testing

Manufacturers are responsible for demonstrating that their medical devices are safe and that the benefits of using these devices outweigh associated risks. They must also demonstrate that the devices perform as they intend the devices to perform as specified in device labeling, instructions for use or other printed or electronic materials or advertising media. In some jurisdictions, such as the US, the demonstration of effectiveness instead of performance is required. It is important to recognize that the requirements for demonstrating safety and performance or effectiveness may differ depending upon the regulatory jurisdiction and not solely on the type of medical device that is being placed on the market.

The methods used to demonstrate safety and performance or effectiveness of a medical device vary depending upon factors such as the intended use of the device, the risks associated with its use, the environment in which the device is used, whether or not the device will be used by a skilled healthcare professional or the patient, the availability of existing safety and performance or effectiveness data for the device or a device to which it is equivalent or similar, and perhaps additional factors.

When insufficient information exists to demonstrate safety and performance or effectiveness of a medical device, these data are generally developed by conducting bench or laboratory testing, animal testing and/or clinical studies, discussed later. Biological evaluation of medical devices is performed to determine the potential toxicity resulting from contact of device component materials with the body. The device materials should not, either directly or through the release of their material

constituents: (i) produce adverse local or systemic effects; (ii) be carcinogenic; or (iii) produce adverse reproductive and developmental effects. The international standards in the ISO 10,993 series, biological evaluation of medical devices, provide guidance for selecting the tests to evaluate the biological response to medical devices. These standards are accepted in the US and Europe; however, in some cases, the FDA requires additional testing than that specified in the standards.

In the US, the vast majority of regulatory submissions for medical device marketing authorization are premarket notifications, termed 510(k)s, which are required for most Class II devices, and are discussed later. Test data provided in a 510(k) are intended to demonstrate that a device to be marketed in the US is equivalent with respect to safety and effectiveness to a product that is already on the US market; the latter device is termed a 'predicate device'. The premarket approval (PMA) process, which is required for most Class III devices, requires sufficient valid scientific evidence to demonstrate that the device in its own right is safe and effective for its intended use(s); test data often represent an important part of this evidence.

When compared with other jurisdictions, including Europe, FDA has developed significantly more general and specific guidance documents, many of which describe the manner in which test data should be presented in US regulatory submissions. In addition, under the CDRH Standards Program, the FDA allows the use of recognized consensus standards in satisfying premarket review requirements. A distinct benefit of this program to companies marketing their products in both Europe and the US is that some of the recognized standards are the same as those adopted as European harmonized standards.

The European directives on medical devices, as transposed by member states, require that manufacturers are able to demonstrate compliance with the essential requirements related to safety and performance, which are listed in the first Annex of each of the medical device directives. In many cases, this includes test data. As discussed earlier, compliance with applicable European harmonized standards provides manufacturers with a presumption of conformity with the related essential requirements of the relevant directive. Some of these standards contain detailed technical specifications and test requirements for particular types of device and should be identified as early as possible in the design and development process.

5.6 Clinical data and studies

5.6.1 US clinical data requirements

The FDA states that clinical data are not needed for most devices cleared by the 510(k) process; however, the need for clinical data and clinical studies appears to be increasing. Section 807.92, Content and format of a 510(k) summary, requires

that if the determination of substantial equivalence is based not only on other data, but also on an assessment of clinical performance data, the 510(k) summary must include a brief discussion of clinical tests submitted, referenced or relied on in the premarket notification submission and how their results support a determination of substantial equivalence.

The clinical test discussion must include, where applicable, a description of the subjects upon whom the device was tested, a discussion of the safety or effectiveness data obtained from the testing, with specific reference to adverse effects and complications, and any other information from the clinical testing relevant to a determination of substantial equivalence. In addition, section 807.92(b)(3) requires that the summary include the conclusions drawn from the nonclinical and clinical tests (discussed above) that demonstrate that the device is as safe, as effective, and performs as well as or better than the predicate device.

In contrast with 510(k) submissions, clinical data are generally an important part of PMA applications for the highest risk category of devices. The PMA regulations (21 CFR Part 814), which apply to Class III devices, provide detailed information on clinical data, which must be included in the PMA application. Section 814.20(b)(8)(i) requires that the application include a bibliography of all published reports, whether adverse or supportive, known to the applicant concerning the safety or effectiveness of the device. These reports are in addition to, and not the same as, the data and information on laboratory studies and clinical investigations conducted by the applicant. Section 814.20(b)(8)(ii) requires an identification, discussion and analysis of any other data, information or report relevant to an evaluation of the safety and effectiveness of the device known to the applicant from any source, foreign or domestic. This includes information derived from investigations other than those proposed in the application and from commercial marketing experience.

In addition to understanding what is contained in the regulations, it is extremely important to check whether or not the FDA has developed any guidance documents that are applicable to a particular device for which a premarket submission is being prepared. For example, the Guidance on 510(k) Submissions for Keratoprostheses (3 March 1999) contains guidance on the elements that should be included in a clinical study protocol for a study of a permanent keratoprosthesis (artificial cornea). The recommended protocol elements are found in Appendix A. Unless a guidance document is very recent, the submission applicant should contact the FDA to determine if the guidance is still valid or if other concerns have developed, or if there is additional advice regarding the submission which has not yet been included in the published guidance document.

All clinical studies of investigational devices performed in the US, unless they are exempt, must have an approved investigational device exemption (IDE) before the study is initiated. The requirements for obtaining an approved IDE are in 21 CFR Part 812 – Investigational Device Exemptions. In addition, other requirements and expectations should be clearly understood before initiating

clinical study activities, including FDA expectations regarding the conduct of clinical studies that comply with good clinical practices (GCPs), which are defined in a series of regulations and guidance documents. Guidance documents and information sheets, which represent the FDA's current thinking on the conduct of clinical studies, can be obtained from the FDA website at www.fda.gov/ScienceResearch/SpecialTopics/RunningClinicalTrials/default.htm.

Some of the more important regulations related to medical device studies include:

- Electronic Records; Electronic Signatures (21 CFR Part 11)
- Protection of Human Subjects (Informed Consent) (21 CFR Part 50)
- Financial Disclosure by Clinical Investigators (21 CFR Part 54)
- Institutional Review Boards (21 CFR Part 56)
- Investigational Device Exemptions (21 CFR Part 812)
- Premarket Approval of Medical Devices (21 CFR Part 814).

Some manufacturers believe that all or most clinical investigations must be conducted in the US when the data are intended to support a US submission; however, FDA regulations allow otherwise. Section 814.15, Research conducted outside the United States, specifies the requirements for non-US clinical investigation data intended to support a US submission. This section should be reviewed by manufacturers interested in conducting non-US clinical investigations for such a purpose. For example, the regulation states that the FDA will accept studies conducted outside the US in support of a PMA if the data are valid and the investigator has conducted the studies in conformance with the 'Declaration of Helsinki' or the laws and regulations of the country in which the research is conducted, whichever accords greater protection to the human subjects.

Furthermore, a manufacturer may base a PMA solely on non-US clinical data, which otherwise meets the criteria for approval under Part 814, Premarket Approval of Medical Devices, if: (1) the foreign data are applicable to the US population and US medical practice; (2) the studies have been performed by clinical investigators of recognized competence; and (3) the data may be considered valid without the need for an on-site inspection by the FDA or, if the FDA considers such an inspection to be necessary, the FDA can validate the data through an on-site inspection or other appropriate means. The FDA advises, however, that applicants are encouraged to meet with FDA officials in a 'presubmission' meeting when approval based solely on foreign data will be sought. The requirements in section 814.15 also apply to non-US clinical studies conducted to support 510(k) applications.

5.6.2 European clinical data requirements

Under the European directives for medical devices, the confirmation of medical device safety and performance and the evaluation of undesirable side effects must

be based on adequate clinical data. There are important amendments made to the AIMDD and MDD by Directive 2007/47/EC on clinical data requirements and the related clinical evaluation process. For example, a new definition of clinical data has been introduced, which is defined as:

the safety and/or performance information that is generated from the use of a device. Clinical data are sourced from:

- clinical investigation(s) of the device concerned; or
- clinical investigation(s) or other studies reported in the scientific literature, of a similar device for which equivalence to the device in question can be demonstrated; or
- published and/or unpublished reports on other clinical experience of either the device in question or a similar device for which equivalence to the device in question can be demonstrated.

A new essential requirement in the MDD has been introduced, which requires that the demonstration of conformity with the essential requirements must include a clinical evaluation in accordance with Annex X. An analogous requirement has been introduced in the AIMDD. This means that a clinical evaluation is required for all classes of devices, not just those in the higher risk categories.

The conformity assessment procedures of the AIMDD and MDD also contain new requirements related to clinical data. For example, Annex II of the MDD now requires that manufacturers not only establish a process for reviewing post-market experience and reporting adverse events, but they also need to comply with the clinical data provisions in Annex X related to post-market clinical experience. This means that clinical evaluations must be actively updated with data obtained from post-market surveillance. In addition, the post-market surveillance plan will need to include a post-market clinical follow-up unless duly justified and documented.

Other amendments to the directives related to clinical data, clinical evaluation, the conduct and notification of clinical investigations to member state regulatory authorities have been introduced and should be reviewed to ensure that the new requirements will be met. As stated previously, these requirements became mandatory on 21 March 2010.

The European guideline, *Evaluation of Clinical Data: A Guide for Manufacturers and Notified Bodies* (MEDDEV 2.7.1 Rev.3, December 2009) provides guidance to manufacturers on the evaluation of clinical data, and to notified bodies on reviewing the results of this evaluation. This document was significantly revised from the previous version published in April 2003 and was based upon the GHTF guidance on clinical evaluation. Thus, the revised MEDDEV document not only updates the guidance on meeting the new European requirements for clinical evaluation, but also promotes harmonization with other regulatory jurisdictions.

When clinical investigations are conducted in Europe, it is important that the investigations are conducted in compliance with the European harmonized standards for medical device clinical investigations. This is because the intent of

these standards is to provide the procedures needed to comply with the requirements in the directives related to the conduct of clinical investigations. As such, competent authorities and notified bodies must presume compliance with the relevant requirements in the directives when manufacturers conform to these standards. The harmonized standard for medical device clinical investigations is ISO 14155:2003, Parts 1 and 2, which provides detailed guidance on the conduct of these types of clinical investigation. This standard has recently undergone an extensive revision and, at the time of writing, a final draft international standard (FDIS) has been published and is undergoing formal approval. It is hoped that publication of the final ISO standard will take place in the first quarter of 2011.

5.7 Marketing authorization processes

The marketing authorization process in various parts of the world has some common features, including the identification and description of the device to be placed on the market, the generation of sufficient safety and performance or effectiveness data, and development of proper documentation that demonstrates compliance with applicable regulatory requirements. In spite of these common characteristics, the specific processes that must be followed vary significantly depending upon the particular regulatory jurisdiction. The two primary routes for placing a product on the US market are the ‘premarket notification,’ or 510(k) process, and the ‘premarket approval’ (PMA) process. Both processes will be briefly described.

5.7.1 US marketing authorization process – 510(k)s

The term ‘510(k)’ is from the Section in the FD&C Act that specifies actions that must be taken when planning to introduce a medical device onto the US market. A premarket notification must be submitted at least 90 days before a medical device is intended to be placed on the US market:

- when a device is being introduced into commercial distribution for the first time
- when a device is being introduced into commercial distribution for the first time by a person who is required to register, or
- when a device is one that the person currently has in commercial distribution or is reintroducing into commercial distribution, but that is about to be significantly changed or modified in design, components, method of manufacture, or intended use.

The requirements for submitting a premarket notification are listed in 21 CFR Part 807, Subpart E – Premarket Notification Procedures. Detailed information on the 510(k) submission process can be obtained on the CDRH website at www.fda.gov/MedicalDevices/default.htm. The premarket notification or 510(k) application process is based upon the process of comparisons. The 510(k)

applicant must demonstrate that the device that is planned to be marketed is substantially equivalent to a legally marketed device, that is, one that was marketed before 28 May 1976, or one that was marketed after that date, which was found substantially equivalent through the premarket notification process.

A device is substantially equivalent if:

- in comparison with a legally marketed device, it has the same intended use and the same technological characteristics, or
- if it has different technological characteristics, but the submitted information does not raise new safety and effectiveness questions and the submitted information demonstrates that the device is as safe and as effective as the legally marketed device.

A number of relevant guidance documents on the premarket notification process are available from the CDRH website.

There is an important facet of ownership of 510(k) that should be known to manufacturers or others wishing to market medical devices in the US. When a distributor or an importer submits a 510(k), unless otherwise clearly stated in the 510(k) submission, the distributor or importer will be the 'owner' of the 510(k). This means that no other firm can market the medical device under the cleared 510(k) unless agreed with the owner of the 510(k) or unless the rights to the 510(k) are sold to the person wishing to use it to market a device. For these reasons, many firms use the services of third parties who have no interest in the commercial aspects of the process.

The FDA charges a fee for 510(k) review unless the submission is exempt. For example, 510(k)s submitted to third parties for review are exempt from any FDA fee. In this case, the third party will charge a fee. In addition, any application for a device intended solely for pediatric use is exempt from a user fee. Small businesses may qualify for a reduced fee, including those headquartered outside the US. Guidance on obtaining small business status can be obtained from the CDRH website. The standard 510(k) fee for fiscal year 2010 was \$4,007 and for small businesses it was \$2,004.

Under the FDA Accredited Persons Program, 'Accredited Persons' may conduct the primary review of 510(k)s for those devices that are eligible under the program. The 510(k) is submitted directly to the accredited person for the primary review. The accredited person then forwards its review, its recommendation, and the 510(k) to the FDA. A final determination must be issued by the FDA within 30 days after receiving the recommendation of an accredited person. Of course, the 510(k) can be submitted directly to the FDA if a submitter does not wish to use this program. The steps for using the program, list of accredited persons, list of eligible devices, and other useful information on the program can be found on the CDRH website.

Before a firm begins to market a device that has been cleared through the 510(k) process, it is critically important that the firm understands the US legal obligation

to be in full compliance with the QSR. If this requirement is not met, the products being marketed in the US may be considered to be adulterated and/or misbranded, which constitute violations of the FD&C Act. FDA enforcement actions will depend upon the nature and extent of the violation, but can include the issuance of a warning letter, injunction, detention, seizure, civil penalty and/or prosecution for US-based persons/companies. For a non-US company, serious violations could lead to a warning letter and/or a US import detention of the company's products.

5.7.2 US marketing authorization process – premarket approval (PMA)

The PMA process is the most stringent US marketing authorization process for medical devices. The process is based on a determination by the FDA that the PMA application contains sufficient valid scientific evidence that provides reasonable assurance that the device is safe and effective for its intended use or uses. An approved PMA is a private license granting the applicant (or owner) permission to market the device. A PMA applicant must receive FDA approval of its PMA application before marketing the device in the US. Details regarding the PMA submission and review process are provided on the CDRH website.

The regulations specifying the requirements for the PMA process are listed in 21 CFR Part 814 – Premarket Approval of Medical Devices. In addition, numerous guidance documents on the PMA process can be found on the CDRH website. PMAs are required for the majority of Class III medical devices, which are those that support or sustain human life, are of substantial importance in preventing impairment of human health, or which present a potential, unreasonable risk of illness or injury. Because of the level of risk associated with Class III devices, the FDA has determined that general and special controls alone are insufficient to assure their safety and effectiveness.

The PMA applicant or an authorized representative must sign the PMA application. If the applicant does not reside or have a place of business within the US, the PMA must be countersigned by an authorized representative residing or maintaining a place of business in the US and must identify the representative's name and address.

The technical sections of the PMA need to contain data and information that will allow the FDA to determine whether to approve or disapprove the application. The non-clinical laboratory studies section of a PMA, depending upon the specific product, will include information on microbiology, toxicology, immunology, biocompatibility, stress, wear, shelf life, and other laboratory or animal tests. Non-clinical studies for safety evaluation should be conducted in compliance with 21 CFR Part 58, Good Laboratory Practice for Non-clinical Laboratory Studies. The clinical investigations section will include study protocols, safety and effectiveness data, adverse reactions and complications, device failures and

replacements, patient information, patient complaints, tabulations of data from all individual subjects, results of statistical analyses, and any other information from the clinical investigations. Readers should refer to the PMA regulations and applicable guidance documents concerning the contents of a PMA application.

The PMA regulations state that the FDA has up to 180 days to review the PMA and make a determination; however, the review time is usually longer. The FDA may refer the PMA to an FDA advisory panel, which is an outside panel of experts. In general, this is done for applications of a first-of-a-kind device; however, if the FDA believes that the pertinent issues in determining the safety and effectiveness for the type of medical device are understood and that the FDA has developed the ability to address those issues, an advisory panel opinion is not sought for future PMAs for devices of that type unless a particular application presents an issue that can best be addressed through panel review. Additional information on advisory panels can be found on the CDRH website.

After notifying the applicant that the PMA has been approved or denied, the public is notified of the FDA decision. If the PMA is approved, the summary of the safety and effectiveness data upon which the approval is based is made available on the CDRH website.

The FDA charges a fee for the review of PMAs; however, the user fee is waived for the first PMA from a small business with gross receipts or sales less than \$30 million. The fee is also waived for any application for a device intended solely for pediatric use. As in the case for 510(k)s, small businesses may qualify for a waived or reduced fee, including those headquartered outside the US. The standard PMA review user fee in US dollars for FY2010 is \$217787 and for a small business it is \$54447. Companies submitting applications in subsequent years should always check the CDRH website to determine the correct fee.

It should be noted that the FDA will often conduct a manufacturing facility inspection to determine compliance with the QSR before authorizing a PMA.

5.7.3 European marketing authorization process

The routes available for manufacturers to be able to affix the CE mark to their medical devices depend on a number of factors, including risk classification, type of product, internal quality management system and human/technical resources. For medical devices in higher risk classes, the CE mark can be affixed only after involvement of a notified body, to either verify compliance of the product with the Directive's essential requirements and/or the manufacturer's quality system with the provisions of the conformity assessment procedures, which are included in various annexes of the directives.

The major steps in the CE marking process, which may proceed in parallel or in a different order than presented below, include:

- determination that the product meets the definition of a medical device or *in vitro* diagnostic medical device
- determination of the applicable medical devices directive
- determination of the device classification or category
- identification of the conformity assessment route
- contracting with a notified body, if applicable
- identification of applicable essential requirements (in Annex I of each directive)
- identification of applicable European harmonized standards
- conduct of device risk analysis
- conduct product verification and validation testing
- conduct of clinical evaluation
- generation of any clinical data, if required
- complete essential requirements checklist and risk management documentation
- complete selected conformity assessment procedure (including quality system certification, if applicable)
- complete technical documentation
- establish system and procedures for reporting adverse events for medical devices and conducting post-market surveillance
- draw up Declaration of Conformity
- affix CE mark to the device.

Guidance documents on compliance with the European directives for medical devices, which can also assist in the CE marking process, can be found on the European Commission website at http://ec.europa.eu/enterprise/sectors/medical-devices/index_en.htm.

5.8 Quality system requirements

The FDA requires that manufacturers establish and follow quality systems to help ensure that their products consistently meet applicable requirements and specifications. The quality systems for FDA-regulated products (food, drugs, biologics and devices) are known as current good manufacturing practices (CGMPs). The CGMP requirements for devices are to be found in the QSR in 21 CFR Part 820.

The QSR, which replaced the first good manufacturing practices regulation for medical devices, is based upon ISO 9001:1994, Quality Systems – Model for Quality Assurance in Design, Development, Production, Installation, and Servicing, and the Committee Draft version of ISO 13485:1996, Quality Systems, Medical Devices, Particular Requirements for the Application of ISO 9001. These standards were based upon 20 quality elements, but the QSR contains additional requirements.

Since the time of publication of the QSR, ISO 13485 was revised to be a stand-alone standard incorporating medical device requirements and the requirements of ISO 9001:2000 into one standard. However, the revised standard is no longer based on the 20 quality system elements, but on the process approach on which

ISO 9001:2000 is based. The 'process approach' is considered to be the application of a system of processes within an organization, together with the identification and interactions of those processes and their management. As a result, the structure of the QSR and the structure of ISO 13485 are different. In spite of the structural differences, the QSR and ISO 13485:2003 have many common requirements. This is because the developers of the revised ISO 13485 standard made every effort to maintain the same level of quality system requirements for medical devices that had already been established. Nonetheless, important differences exist between the QSR and ISO 13485:2003; therefore, it is critical that manufacturers understand that conformity with ISO 13485 alone will not fully satisfy US QSR requirements.

The QSR applies to finished device manufacturers who intend to commercially distribute medical devices in the US unless the device is exempt from the requirements of the QSR. A finished device is defined in 21 CFR 820.3(l) as any device or accessory to any device that is suitable for use or capable of functioning, whether or not it is packaged, labeled or sterilized. Certain components such as blood tubing and diagnostic X-ray components are considered by the FDA to be finished devices because they are accessories to finished devices. A manufacturer of accessories is subject to the QSR.

In some cases, medical devices are exempt from the requirements of the QSR. This status is specified in the classification regulations published in the US Federal Register and codified in 21 CFR 862 to 892. It should be noted, however, that exemption from the QSR does not exempt manufacturers of finished devices from keeping complaint files (21 CFR 820.198) or from general requirements concerning records (21 CFR 820.180). It is also important to recognize that medical devices manufactured under an investigational device exemption (IDE) are not exempt from design control requirements under 21 CFR 820.30 of the QSR.

Information on quality system requirements, including the critically important element of design controls, as well as human factors and incorporating human factors engineering into risk management, can be found on the CDRH website.

In Europe, compliance with the European harmonized standard EN ISO 13485:2003, Medical devices – Quality management systems – Requirements for regulatory purposes, is used to demonstrate compliance with the quality assurance requirements of the medical devices directives. Although manufacturers are not obligated to obtain quality system certification to this voluntary standard, the vast majority of manufacturers choose to do so. This is because, as mentioned previously, competent authorities and notified bodies must presume compliance with the related requirements in the directives, in this case, quality assurance requirements, when manufacturers conform to harmonized standards.

Under the European directives, certification of quality systems indicating compliance with the directives must be provided by notified bodies. For this reason, manufacturers are wise to select the same notified bodies to issue certificates to the voluntary standard, EN ISO 13485. Certification is then confirmed on either an

annual or semi-annual basis by means of “surveillance audits” from the same notified body.

5.9 Post-market requirements

5.9.1 Introduction

Medical device requirements are not limited to requirements applicable to the premarket phase. Very important post-market requirements apply and if not addressed can jeopardize the ability of a product to remain on a particular market. Some post-market requirements apply to all medical devices, while other requirements apply only to some types of medical devices. Each regulatory jurisdiction has its own requirements, which may be similar or differ in important ways from other jurisdictions, and include such activities as:

- fulfilling medical device registration or notification requirements
- reporting serious adverse events
- managing and, where required, reporting medical device recalls
- conducting post-market clinical studies
- tracking devices from their manufacture through the distribution chain.

5.9.2 Post-market requirements – US

Under the US Medical Device Reporting (MDR) regulation (21 CFR Part 803), manufacturers, importers and users are required to report adverse events in which a device may have caused or contributed to a death or serious injury. Certain types of malfunction must also be reported. This applies to all devices, regardless of device class.

Another important US post-market regulation that applies to all device classes is the regulation on Corrections and Removals (21 CFR 806), which requires that manufacturers and importers submit a report to the FDA of any correction or removal of a medical device if the correction or removal was initiated to reduce a risk to health posed by the device or to remedy a violation of the FD&C Act caused by the device that may present a risk to health. Where a manufacturer or importer fails to voluntarily recall a device that is a risk to health, the FDA may oblige the manufacturer to do so under the authorities specified in 21 CFR Part 810, Medical Device Recall Authority. Manufacturers should also be aware of important FDA policies regarding recalls that are not related to risk to health. These policies are listed in 21 CFR Part 7.

In addition, post-market requirements may also apply to only some types of device. For example, section 522 of the FD&C Act grants the FDA the authority to require a manufacturer to conduct post-market surveillance of a Class II or Class III device that meets any of the following criteria:

- its failure would be reasonably likely to have serious adverse health consequences
- it is expected to have significant use in pediatric populations
- it is intended to be implanted in the body for more than one year
- it is intended to be a life-sustaining or life-supporting device used outside a device user facility.

The 522 Postmarket Surveillance Studies Program covers the design, tracking, oversight and review responsibilities for studies mandated under section 522 of the US FD&C Act. Information on this program can be found on the CDRH website.

The FDA has also been granted authority to require sponsors to perform a post-approval study or studies at the time of approval of a PMA, humanitarian device exemption (HDE), or product development protocol application to help assure continued safety and effectiveness, or in the case of an HDE, continued probable benefit, of the approved device. The CDRH Post-Approval Studies Program covers the design, tracking, oversight and review responsibilities for these types of studies. Information on this program can be found on the CDRH website.

5.9.3 Post-market requirements – Europe

The conformity assessment annexes of the European directives for medical devices require manufacturers to establish a system for reviewing experience gained from devices during the post-market phase. Manufacturers are also required to take appropriate corrective actions when problems are identified. The requirement also includes the obligation to notify the competent authorities of serious incidents. In April 2007, the European Commission published a medical device vigilance guidelines document, *Guidelines on a Medical Devices Vigilance System* (MEDDEV 2.12–1 rev 5, April 2007), which replaced the 2001 version. The 2007 document includes reporting terminology and concepts such as ‘periodic summary reporting’ and ‘trend reporting’. Terms used in the 2001 version, including ‘advisory notice’, ‘near incident’ and ‘recall’, have been eliminated or replaced by the other terms. As with the 2001 version, the current version refers to the incorporation of the views of the GHTF into the European context. This indicates a continuing support of the regulatory harmonization initiatives of the GHTF. The document can be obtained from the European Commission website.

The European Commission issued a guidance document, *Guidelines on Postmarket Clinical Follow-Up*, in May 2004. This document can be obtained from the European Commission website. The document provides guidance on the role of post-market clinical follow-up (PMCF) in meeting European post-market surveillance requirements. It makes the point that clinical evidence is an essential element of the premarket conformity assessment process; however, there are limitations inherent to premarket clinical investigations. That is, the type of data, which can be obtained in the premarket phase, cannot detect infrequent

complications or problems, which become apparent only after widespread use, nor do they enable the detection of long-term performance problems. For this reason, a suitable post-market surveillance program as part of the manufacturer's quality system is needed to identify and investigate the risks associated with the use of medical devices placed on the market.

It is noteworthy that the PMCF guidance document states that PMCF studies and registries have an important role in the post-market strategies that should be developed. This is because an important amendment to the AIMDD and MDD has been introduced regarding the conduct of PMCF studies. The conformity assessment procedures now include a new requirement, namely that post-market surveillance programs include certain new provisions in the annex on clinical evaluation. In the AIMDD, this is Annex 7, and in the MDD it is Annex X. These provisions require that the clinical evaluation and its documentation must be actively updated with data obtained from the post-market surveillance. Furthermore, the new requirement states that where a PMCF study as part of the post-market surveillance plan for the device is not deemed necessary, this must be duly justified and documented. This is an extremely important amendment which will clearly lead to the conduct of more PMCF studies in order to meet European post-market requirements.

5.10 Future trends

The regulation of medical devices is continuing to evolve in the US, Europe and worldwide. While true harmonization of medical device regulations has not yet been reached, significant efforts are continuing and progress is being made.

For example, since the formation of the GHTF, European and US officials have taken an active role in the development of documents that are intended to be used by regulatory authorities as a basis for their own regulations. For example, in 1997, the FDA published *Design Control Guidance for Medical Device Manufacturers*, acknowledging significant input from the GHTF. In a recent presentation on quality system requirements, the FDA mentioned three GHTF quality management system guidance documents: *Implementation of Risk Management Principles and Activities Within a Quality Management System* (2005), *Quality Management Systems – Process Validation Guidance* (2004) and *Quality Management System – Medical Devices – Guidance on the Control of Products and Services Obtained from Suppliers* (2008). In addition, CDRH is encouraging submitters of premarket approval (PMA) applications and 510(k)s to participate in the ongoing voluntary STED Pilot Program. The Summary Technical Document (STED) format for regulatory submissions is a harmonized submission format developed by the GHTF. Information on this program can be obtained from the CDRH website.

Since the formation of the GHTF, Europe has been very active in the GHTF. For example, the 2009 Work Programme for Medical Devices included continuing active participation in the GHTF steering committee and working groups. The

European Commission website directs visitors to the GHTF website to obtain relevant guidance on regulatory auditing of quality systems of medical device manufacturers. The European guidance document on clinical evaluation was revised using the GHTF guidance document on clinical evaluation as a basis.

5.11 Sources of further information and advice

The Internet is a fundamental resource for information on regulations in many regulatory jurisdictions. The following website links should be useful to anyone seeking information on the regulation of medical devices:

Government

European Commission – Medical devices: http://ec.europa.eu/enterprise/sectors/medical-devices/index_en.htm

United Kingdom: www.mhra.gov.uk

United States Center for Devices and Radiological Health: www.fda.gov/MedicalDevices/default.htm

Japan Pharmaceuticals and Medical Devices Agency: www.pmda.go.jp/english/index.html

International harmonization organizations

Global Harmonization Task Force: www.ghtf.org

Standards organizations

American National Standards Institute (ANSI): www.ansi.org

American Society for Testing & Materials (ASTM): www.astm.org

European Committee for Standardization (CEN): www.cenorm.be

European Committee for Electrotechnical Standardization (CENELEC): www.cenelec.org

International Electrotechnical Commission (IEC): www.iec.ch

International Standardization Organization (ISO): www.iso.org

Associations

Regulatory Affairs Professional Society (RAPS): www.raps.org

AdvaMed: www.advamed.org

Association for the Advancement of Medical Instrumentation: www.aami.org

EUcomed: www.eucomed.org

European Diagnostic Manufacturers Association (EDMA): www.edma-ivd.be

Medical Device Manufacturers Association (MDMA): www.medicaldevices.org

Spinal disc implants using hydrogels

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Abstract: Low back pain, a common cause of disability in individuals – especially between 20 and 50 years old – with enormous socioeconomic consequences, may be strongly associated with the degeneration of the intervertebral disc (IVD). The traditional IVD treatments, such as spinal fusion, even though they provide amelioration of the pain, present different drawbacks; consequently there is a lot of research interest in replacing the damaged disc with an artificial one. In this chapter, after an introductory part on IVD and the pathologies and treatment related to it, an overview of the IVD traditional prostheses is given, followed by the presentation of new hydrogels-based prostheses designed according to a biomimetic approach. Finally, the hydrogels-based systems aimed to replace the nucleus pulposus (NP) and to act as scaffolds to carry cells to engineer the IVD tissues are described.

Key words: hydrogels, intervertebral disc prostheses, nucleus pulposus, tissue engineering.

6.1 Introduction

Low back pain represents the most common cause of disability in individuals, especially between 20 and 50 years old, and has enormous socioeconomic consequences. Even though there are several causes of back pain, it may be strongly associated with the degeneration of the intervertebral disc (IVD). Consequently, disorders of the IVD have been some of the most widely investigated mechanisms for low back pain (Nachemson, 1985; Alini *et al.*, 2003).

Mechanisms that may contribute to the IVD degeneration encompass reduction in nutrient supply, loss of notochordal cells, diminished cell viability, cell senescence and cell apoptosis, leading to biochemical alterations in the composition and structure of the IVD extracellular matrix and loss of its functionality, which can also result in damage of other spinal structures (Holm *et al.*, 1981; Bernick and Cailliet, 1982; Taylor and Twomey, 1988; Roberts *et al.*, 1993; Roberts *et al.*, 1996; Gruber and Hanley, 1998; Heathfield *et al.*, 1998; Aguiar *et al.*, 1999; Ayotte *et al.*, 2000; Lotz and Chin, 2000; Alini *et al.*, 2003; Cole *et al.*, 1985).

Furthermore, disc tissue alterations are also influenced by genetic factors and associated with or aggravated by mechanical factors (Kazarian, 1975; Keller *et al.*, 1987; Kiviranta *et al.*, 1987; Ohshima *et al.*, 1995; Paassilta *et al.*, 2001; Alini *et al.*, 2003).

Some chronic back-pain patients may benefit from spinal fusion, a surgical procedure in which adjacent vertebrae are fused together, thus immobilizing the spinal segment. This is usually done through application of a device or bone graft that promotes new bone to grow between the vertebrae. However, even though the amelioration of the pain is provided, the loss of flexibility can be considered the main drawback of this approach (Hukins, 2005; Sebastine and Williams, 2007; Gloria, 2008).

There is a growing body of opinion that restoration of flexibility, by replacing the damaged disc with an artificial one, may be preferable to fusion (Hukins, 2005).

Currently there are many devices for replacing the whole disc; some have been implanted in patients, whilst others are still at the design stage. Thus, an artificial intervertebral disc seems to be the gold standard, since it should restore the structure and function of the healthy disc (Sebastine and Williams, 2007). If annulus and endplates are relatively healthy and still functional, the nucleus replacement technique, performed with a synthetic material, could be another interesting approach to restore disc height and spine mobility. In this context great efforts have been devoted to designing a biocompatible and safe material as nucleus substitute and in particular injectable systems, to reduce surgical invasiveness. Moreover, recently tissue engineering strategies have also been used to engineer the different tissues of the disc.

6.2 Intervertebral disc

6.2.1 Composition and structure

The spine can be considered as a cooperative system of elements, and its function is to provide trunk flexibility, while protecting the spinal cord and nerve roots that pass through the spinal canal and foramen (Rothman and Simeone, 1992). As for the structure, the human spinal column is a complex structure that consists of 24 individual vertebrae plus the sacrum.

The IVDs represent the joints of the spine, and they lie between two vertebral bodies, being separated from them by a cartilage endplate. The IVD is a complex and load-bearing structure that consists of an inner structure, the nucleus pulposus, which contains chondrocyte-like cells and the annulus fibrosus (AF), an outer rim, which contains fibroblast-like cells (Gan *et al.*, 2003; Di Martino *et al.*, 2005). As to the nucleus, it is mainly composed of water, whose content varies from 90% at birth to less than 70% at over 60 years of age, and proteoglycans, forming a gel-like matrix. The annulus presents a layered structure where each layer is reinforced by a regular pattern of collagen fibers embedded in a proteoglycan-water gel, running in opposite directions in adjacent layers (Markolf and Morris, 1984; Cassidy *et al.*, 1989). The IVD is characterized by a structurally graded architecture, as in the concentric lamellae of the annulus the orientation of the collagen fibers vary from 62° at the periphery to 45° in the vicinity of the nucleus,

with respect to the spinal axis (Cassidy *et al.*, 1989). It is well known that the type and orientation of collagen fibers in the IVD play an important role on how load is distributed. As previously described, the IVD is covered on the upper and lower surfaces by cartilaginous microporous endplates through which the exchange of water, nutrients and products of metabolism occur (Bao *et al.*, 1996). The intervertebral disc (IVD) plays a crucial role in the biomechanics of the spine since its mechanical function results in absorbing shock and distributing axial loads while providing flexibility.

6.2.2 Pathologies and surgical treatments

It is well known that the IVD aging is a normal process since at approximately 30 years there is a gradual change in the types of proteoglycans and a loss of the overall water content. Consequently, IVD degeneration usually involves the dehydration of the nucleus. This is often accompanied by small tears in the annulus, since the load on the nucleus decreases while the load on the annulus increases (Bao *et al.*, 1996; Hukins, 2005; Tsantrizos *et al.*, 2005).

Like an automobile tire, if inflated the multi-layered structure of the annulus performs well, and it is susceptible to damage and delamination when it is flat. Thus, radial tears and cracks first occur within the annulus. Some of these may heal through the appropriate environment; however, if healing does not occur, the nucleus could migrate from the center of the disc to the periphery through the tears (Bao *et al.*, 1996). The nucleus migration between and within the fibers of the annulus causes stretching and delamination of the annular structure, thus leading to back pain through the stimulation of the sinu-vertebral nerve. When the nucleus transgresses all retaining layers of the annulus, the 'disc herniation' occurs. The herniated disc material may deform a nerve root, providing a 'radicular pain'.

Disc herniation may vary in severity from disc protrusion to disc extrusion, and finally to disc sequestration. Different definitions of disc herniations can be found in the literature (Mink, 1989; Onik and Helms, 1991; Czervionke, 1993; Silverman *et al.*, 1995). Onik and Helms (1991) have defined as a protrusion a disc herniation that is contained by the annulus fibrosus or the posterior longitudinal ligament, whilst a disc herniation that is not contained by the posterior longitudinal ligament as an extrusion. On the other hand, Czervionke (1993) has suggested the term protrusion to define a disc herniation when it is intra-annular, while defining as an extrusion the disc material that has passed through the annulus fibrosus. Moreover, a dehydrated IVD loses its central pressure and collapses, reducing its height. The reduction of the disc height results in the loss of its shock absorber ability and less space in the neuroforamen for the exiting nerve root, compromising spine stability and increasing motion between vertebrae. Accordingly, as the spine is a cooperative system of elements, any alteration in its structure and mechanics at one location significantly increases the stresses experienced at the adjacent locations, thus causing a 'degenerative cascade'.

Discectomy and fusion represent the two most common spinal surgery approaches; however, they are far from the ideal treatment for disc diseases (Bao *et al.*, 1996; Bao and Yuan, 2000). The primary purpose of discectomy is to excise any disc material which compresses the spinal nerve. Discectomy provides good short-term results in relieving low back pain, but it also causes reduction of the disc height in almost all patients and increases the instability of the treated disc. Hence, the consequences of these changes in anatomy and structural stability may be considered twofold (Goel *et al.*, 1986; Hanley and Shapiro, 1989).

Spinal fusion means immobilization of the joint between two vertebrae using particular devices such as baskets, cages and threaded inserts, which are mainly made of metals or bioceramics. These devices are designed and manufactured so that tissues can grow into them, thus ensuring rigid anchoring of prostheses to the bone (Gloria *et al.*, 2008). The use of bone graft is often considered to promote bone ingrowth and eventual fusion of the vertebral bodies.

However, by eliminating motion, fusion also significantly alters the normal biomechanics of the spine, as a single-level IVD fusion increases stress and strain on the discs at adjacent levels (Bao and Yuan, 2000). There are a great number of other drawbacks related to fusion, including loss of spinal mobility, graft collapse resulting in suboptimal sagittal balance, autograft harvest site pain, and alteration of muscular synergy. To overcome these drawbacks, most research attention has been focused on the design of suitable prostheses to replace the damaged IVD.

6.3 Disc implant

6.3.1 Traditional prostheses

The artificial IVD should be designed to replace the entire disc, restoring the dynamic and physiological motion of the healthy structure. However, the complex structure and function of the IVD have provided many difficulties, preventing the design of a total prosthesis that emulates all the mechanical properties of a natural disc. Ideally an artificial disc should show functions and properties that are similar to those of the natural structure (Ramakrishna *et al.*, 2001; Gloria *et al.*, 2010). For this reason, in designing IVD prostheses the attention has been focused on the biocompatibility and long-term mechanical performance. Consequently, over the past years, metals, polymers, ceramics and combinations of metallic and non-metallic materials have been taken into consideration to design IVD prostheses. To date many examples of artificial IVDs have been designed and developed; they range from ball bearing prostheses to those made of porous coated metal endplates sandwiching elastomeric cushions (Martz *et al.*, 1997).

The most widely investigated all-metal IVD prostheses are made up of two Ti-6Al-4V springs pocketed between two forged or hot isostatically pressed cobalt-chromium-molybdenum alloy plates with a posterior hinge to allow flexion and extension (Hedman *et al.*, 1991; Hellier *et al.*, 1992; Gloria *et al.*, 2010).

Long-term mechanical performance was assessed by performing fatigue tests on the individual components undergoing 100 million cycles without failure (Hedman *et al.*, 1991; Hellier *et al.*, 1992; Gloria *et al.*, 2010). Even though results obtained from the sheep model highlighted that in the short term fibrous tissue did not grow between the hinges or around the coils, such ingrowth could interfere with the implant, thus leading to potential risks. For this reason, to date it has never been implanted in humans.

The high fatigue strength represents the main advantage of using a metal IVD prosthesis, but the great benefit of a non-metal design may be its mechanical properties that are closer to the natural disc.

With regard to the non-metal category, the most widely studied device is that developed by Lee and colleagues (1990). Basically, it is made up of a soft elastomeric core to reproduce the function of the nucleus, reinforcing fiber sheets with specific alternating fiber orientation in six to fifteen laminae embedded in a second elastomer, which emulates the function of the annulus, and two stiff plates. This device is able to reproduce both the compressive modulus and the compressive-torsional stiffness of the natural disc, but the lack of adequate implant and vertebra fixation is thought to be the great obstacle to its clinical use (Bao and Yuan, 2000).

In order to take advantage of both metal and non-metal materials, overcoming the drawbacks related to the use of either of them individually, many researchers have combined both types of material in their designs, thus realizing a metal-polymer-metal sandwich design.

The LINK SB Charité consists of two metal endplates with spikes or teeth that allow them to be anchored to the vertebral bodies, and a polymer core (Buttner-Janz, 1992; Traynelis, 2002). In particular, in its third generation, it is the most widely implanted IVD prosthesis (Traynelis, 2002). The Charité III consists of an ultrahigh-molecular weight polyethylene (UHMWPE) spacer surrounded by a radiopaque ring for radiographic localization, and two endplates made up of cobalt-chromium-molybdenum alloy. In order to promote bone bonding, these endplates are also coated with titanium and hydroxyapatite.

Other prostheses with a metal-polymer-metal sandwich design are available. Examples are ProDisc, which consists of two cobalt-chromium-molybdenum alloy endplates with vertical wings coated by a pure titanium surface to improve osteointegration, and a monoconvex polyethylene core that is inserted in the caudal endplate, or Acroflex prosthesis, made up of a hexene-based polyolefin rubber core vulcanized to two titanium endplates (Traynelis, 2002; Gloria *et al.*, 2010).

As highlighted, current IVD prosthetic implants available on the market usually consist of a polymer core interposed between two metallic plates, and are characterized by standard sizes. These devices frequently undergo failure, mainly due to wear or mismatch between mechanical proprieties of the device implanted and the natural tissue; moreover, they do not possess the hydrophilic feature and they do not mimic functions and behavior of the natural structures (Shikinami *et al.*, 2004; Gloria *et al.*, 2007; Gloria *et al.*, 2010).

6.3.2 Hydrogel-based prostheses

To overcome the limitations of traditional prostheses, an approach involving hydrogel-based composite materials has been considered in order to design nucleus/annulus synthetic substitutes with suitable mechanical, biological and transport properties (Ambrosio *et al.*, 1996; Ambrosio *et al.*, 1998; De Santis *et al.*, 2004; Gloria *et al.*, 2007; Gloria *et al.*, 2010).

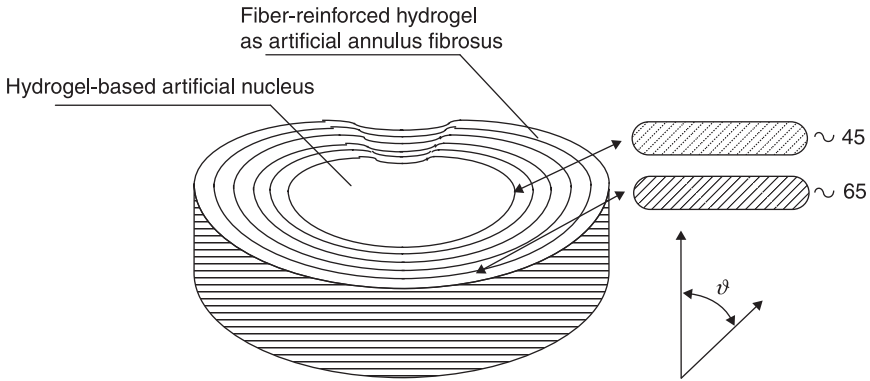
Hydrogels possess the advantages to be biocompatible, highly permeable and hydrophilic (Netti *et al.*, 1993; Peppas *et al.*, 2000; Hoffman, 2002). The main drawback of hydrogels is that, for biomedical applications where high mechanical performances are required, their mechanical properties in the hydrated state are not appropriate (Netti *et al.*, 1993; Ambrosio *et al.*, 1996; Ambrosio *et al.*, 1998).

The mechanical behavior of the hydrogels can be enhanced by incorporating hydrophobic components, such as poly(caprolactone) (PCL), and polymeric fibers (Davis *et al.*, 1991; Ambrosio *et al.*, 1996; Ambrosio *et al.*, 1998; De Santis *et al.*, 2004).

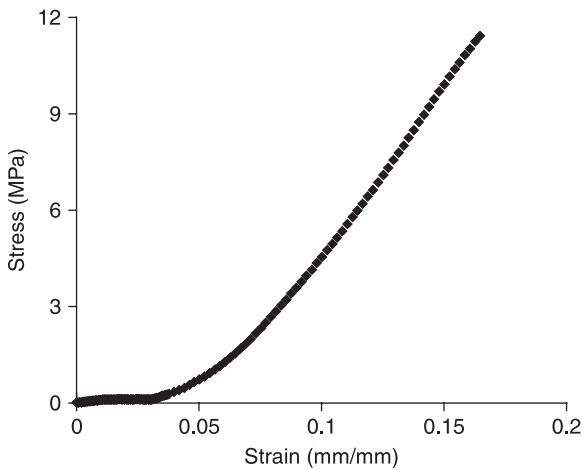
Based on this, to mimic the natural structure of IVD the prosthesis design was based on a fiber-reinforced composite hydrogel that is able to match the mechanical properties of natural IVDs and the surrounding tissues (Ambrosio *et al.*, 1996; Ambrosio *et al.*, 1998; De Santis *et al.*, 2004).

In particular, poly(2-hydroxyethylmethacrylate) (PHEMA)-based networks of composite hydrogels reinforced with poly(ethylene-terephthalate) (PET) fibers have been considered (Ambrosio *et al.*, 1996; Ambrosio *et al.*, 1998). Furthermore, the PHEMA-based network was reinforced with PCL or with a biostable polymer, poly(methylmethacrylate) (PMMA), thus obtaining semi interpenetrating network (s-INP). These fiber-reinforced composite hydrogels were prepared using filament winding and molding technologies. Using a biomimetic approach and composite materials science, it was possible to design a hydrogel-based nucleus/annulus substitute for an IVD prosthesis consisting of a more hydrophilic inner core and a stiffer and less hydrophilic outer fibrous part (Fig. 6.1) (Ambrosio *et al.*, 1996; Ambrosio *et al.*, 1998; De Santis *et al.*, 2004; Gloria *et al.*, 2007; Ambrosio *et al.*, 2007; Gloria *et al.*, 2010).

Static and dynamic compressive tests were performed on the designed fiber-reinforced hydrogels. The swollen PHEMA-based fiber-reinforced hydrogels highlighted an initial J-shaped stress-strain curve in compression (Fig. 6.2) that is typical of natural IVDs. The initial upward concavity, which is known as ‘toe region’, evidences a relatively high flexibility at low strain levels (i.e. low modulus and hence high compliance), while displaying a high compressive strength (Gloria *et al.*, 2007; Gloria *et al.*, 2010). The toe region is related to the properties of the hydrogel-based matrix and the realignment of fibers which reorient themselves in the transverse direction. Figure 6.2 shows that during the loading process the influence of the fibers sharply increases, hence the linear region may be ascribed to the fibers straightening.



6.1 Schematic representation of a nucleus/annulus substitute for an alternative intervertebral disc prosthesis: a hydrogel-based nucleus and a peripheral structure made of fiber-reinforced hydrogel (Gloria et al., 2007; Ambrosio et al., 2007).



6.2 Stress-strain curve in compression for a swollen PHEMA/PMMA 80/20 (w/w) s-IPN composite hydrogel reinforced with PET fibers having a winding angle from 45° to 65°, tested up to a stress level of 11.4 MPa without breaking (Gloria et al., 2007; Gloria et al., 2010).

The main feature of this approach is the possibility to tailor the hydrophilicity and the mechanical properties of the composite structure by varying the composition of the hydrogel-based matrix, the winding angle and the amount of the PET fibers.

Results from compression tests carried out on swollen PHEMA/PCL s-IPNs composite hydrogels reinforced with PET fibers have evidenced that the mechanical properties are a function of concentration PCL content in the s-IPN and the

winding angle (Ambrosio *et al.*, 1996; Ambrosio *et al.*, 1998). In particular, a decrease of maximum strain (from 0.52 to 0.40 mm/mm) associated with increases of the compressive modulus (from 30 to 73 MPa) and maximum stress (from 12 to 17 MPa) was found with increasing PCL content for PHEMA/PCL 90/10, 80/20 and 70/30 (w/w) s-IPNs composite hydrogels reinforced with 40% by volume of PET fibers having a winding angle from 45° to 65°. Furthermore, PHEMA/PCL 70/30 s-IPN reinforced with 50% by volume of PET fibers with a winding angle varying from 45° to 90° showed compressive modulus and maximum stress of 129 and 20 MPa, respectively (Ambrosio *et al.*, 1996). Moreover, fatigue tests carried out on PHEMA/PMMA s-IPNs reinforced with PET fibers imposing a sinusoidal compressive load ranging from 200 to 2200 N at 2 Hz, have evidenced a high long-term performance as it underwent 10 million cycles without failure.

It is worth noticing that previous studies on IVDs have highlighted a dependence of the mechanical properties upon the spine location (Cassidy *et al.*, 1990). The modulus in the linear region generally increases over the length of the spinal column, varying from 32.0 MPa at C2-C3 level to 115.0 MPa at L6-L7 one, while the maximum stress spans from 8 to 19 MPa (Cassidy *et al.*, 1990).

Thus, the approach used in designing the fiber-reinforced hydrogels has allowed to modulate the mechanical properties of the hydrophilic composite structures for optimizing its characteristics at several locations along the spinal column.

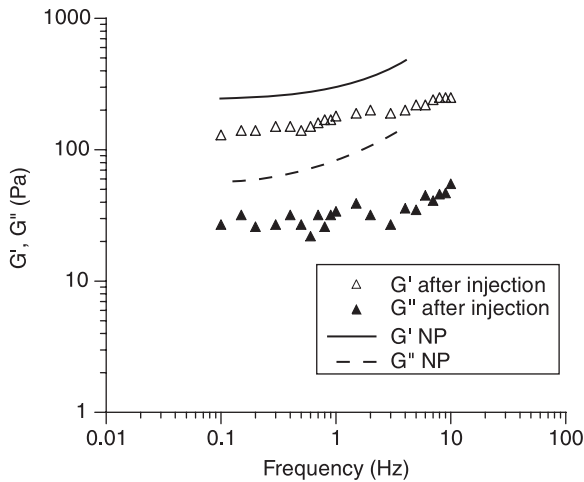
6.3.3 Injectable hydrogel

If annulus and endplates are relatively healthy and still functional, the nucleus replacement technique, performed with a synthetic material, could be another interesting approach to restore disc height and spine mobility (Buckwalter, 1995; Traynelis, 2002). One of the most commercially available nucleus substitutes is the prosthetic disc nucleus (PDN), developed by RayMedica, which consists of a pair of hydrophilic polymer implants. A flexible, inelastic jacket of woven polyethylene encases each dehydrated, compressed hydrogel implant, while allowing fluid exchange. One of the shortcomings of the PDN is that it cannot completely fill the cavity left by the removed nucleus, whereas complete filling is necessary to avoid an incorrect stress distribution (Shim *et al.*, 2003). In this context great efforts have been devoted to designing a biocompatible and safe material as nucleus substitute and in particular injectable systems, to reduce surgical invasiveness. Injectable hydrogels have been proposed for nucleus replacement since they are able to swell, maintain the hydrostatic pressure and mimic the mechanical properties of native NP, and are highly biocompatible. Several chemically or physically crosslinked hydrogel-based materials have been studied (Oka *et al.*, 1990; Gopferich, 1996).

Bao and Hinghaam (1999) have developed a nucleus pulposus replacement based on poly(vinyl alcohol) (PVA), with the aim to restore the function of the intervertebral disc by mimicking both the mechanical and physiological properties of the disc. They report that the hydrogels contained 70% of water and acted

similarly to the nucleus in that it absorbed and released water depending on the applied load. The hydrogels, furthermore, had a compressive strength sufficient for the application. Since PVA may not be stable within the physiological environment, because PVA is a semicrystalline, hydrophilic polymer that can undergo dissolution, PVA-based hydrogels have been stabilized by physical and chemical crosslinking. As to the former approach, hydrogel based on a blend of PVA and poly(vinyl-pyrrolidone) (PVP) have been studied (Thomas *et al.*, 2003; Joshi *et al.*, 2006). The network is stabilized by physical crosslinking between these two polymers, i.e. hydrogen bonding interactions between the carbonyl group of the PVP and the hydroxyl group on PVA. The hydrogels were optimized in terms of PVA/PVP composition as well as PVA and PVP molecular weight. The blend that had the best combination of network stability under physiological conditions and a stable, crosslinked network was prepared with 99% PVA (143 K) and 1% PVP (40K). On the other hand, chemical crosslinked PVA-based systems were obtained by photopolymerization of glycidyl methacrylate modified PVA and they displayed viscoelastic behavior similar to NP (Bader and Rochefort, 2007). Moreover, hydrogels based on copolymers of NVP/HEMA (N-vinyl-2-pyrrolidone and 2-hydroxyethylmethacrylate) crosslinked with allyl-methacrylate showed water content, fatigue resistance and recovery after loading suitable for NP substitute (Boelen *et al.*, 2005). In the search for naturally based systems, polysaccharide-based hydrogels have also been studied as a nucleus substitute. Hydrogels based on amidic derivative of alginate crosslinked with 1.3 diaminopropane have been prepared and characterized as potential NP substitutes (Leone *et al.*, 2007). Because hyaluronic acid (HA) has been identified among the glycosaminoglycans present in the NP, HA-based hydrogels have been widely investigated. HA is found ubiquitously in the extracellular matrix (ECM) of virtually all mammalian connective tissues and it plays a major role in tissue growth and remodeling, interacting specifically with endogenous receptors such as CD 44 and ICAM-1 (Ambrosio *et al.*, 1999). HA regulates trafficking of macromolecules within interstitial space and it plays a pivotal role in the lubrication of joints and contributes to mechanical strength in compression of soft tissue. Thanks to its physical chemical and biological properties it is already used in several biomedical applications (ophthalmological surgery; orthopedic field). However, the short residence time due to a fast degradation process of HA and the poor mechanical characteristics strongly limit the possibility of broadening its range of biomedical applications including NP substitution. Consequently, several chemical modifications of native hyaluronan have been devised to provide mechanically and chemically robust materials and expand its range of application (Borzacchiello *et al.*, 2007; Maltese *et al.*, 2006; Borzacchiello *et al.*, 2010).

HYAFF120[®] is a photo-linkable ester obtained by linking the HA molecule with a compound that initiates polymerization upon exposure to UV light; consequently its solutions form chemical crosslinked hydrogels after UV irradiation. From a rheological point of view HYAFF120[®] showed a 'gel'



6.3 Mechanical spectra of HY120 at 50 mg/ml after injection through 16 G catheter compared to those of natural porcine NP, adapted from reference (Causa *et al.*, 2002).

behavior, as can be observed in Fig. 6.3, in which the dependence of the dynamic elastic and viscous modulus as function of frequency, the so-called mechanical spectra, is reported. For these materials the elastic modulus (G') is higher than the viscous one (G'') in the frequency range analyzed, and both moduli curves are parallel to each other. A similar behavior is also displayed by the NP (Fig. 6.3); for this tissue, indeed, the interactions – chemical, physical and topological – among its macromolecules, such as proteoglycans, HA and collagen, lead to the formation of a network that exhibits predominantly an elastic character ($G' > G''$) (Causa, 2002; Ross-Murphy, 1991). HYAFF120[®] based systems can, then, mimic the viscoelastic behavior of nucleus tissue. Using HYAFF120[®] as injectable material, results evidenced a successful repair of nucleotomized IVDs (Ambrosio *et al.*, 2007).

Up to now, the hydrogel systems proposed possess some of the requirements as NP substitute but none of them seems to mimic completely the required behavior. In the search for new strategies, tissues engineering (TE) approaches appear particularly promising. To form the different tissues of the disc both the scaffold-free and the scaffold-containing approaches have been developed, but it has been found that the presence of the scaffold can be crucial for the success of IVD engineering (Kandel *et al.*, 2008), in particular for stem cell-based therapy (Richardson and Hoyland, 2008). To date a variety of hydrogel-based systems have been used to support the formation of the different IVD components, such as chitosan, collagen, alginate, HA and its derivatives, just to list a few.

Hyaluronic acid derivatives have been widely exploited for TE strategies, such as nucleus pulposus TE and they showed properties that could be successful for these

applications (Borzacchiello *et al.*, 2007). Among these, HYADD3[®], a dodecylamide derived from HA that, due to the presence of the aliphatic chains, can form physical crosslinked gels in water suitable as a vehicle or scaffold to carry cells. HYADD3[®], indeed, shows a rheological behavior typical of gel material and analogous to that of NP that can be related both to topological interactions, such as entanglements, among the polymer chains and to physical crosslinks, such as hydrophobic associations, determined by the presence of alkyl chains which results in the formation of an elastic three-dimensional network (Borzacchiello *et al.*, 2010). Indeed, different from native HA macromolecules: in HYADD3[®] the presence of hydrophobic groups can result in further hydrophobic interactions. The latter drastically reduce the mobility of the macromolecules: they cannot flow individually and their principal response mode to the applied stress is by network deformation. HYADD3[®] systems showed very promising results in tissue engineering of the disc; indeed, evidence of nucleus regeneration were verified after injection of these HA derivatives loaded with homologous bone marrow stem cells in pigs after six weeks. Due to its presence among the NP macromolecules, collagen has also been considered and in particular as injectable cell-seeded scaffolds, atelocollagen-based hydrogels have been studied. Atelocollagen is a collagen gel that has an advantage in safety, as the antigenic telopeptide region is removed by pepsin digestion. Atelocollagen is in liquid form at 4°C, but forms physically crosslinked gels after incubation at 37°C. Using atelocollagen type I and type II as scaffold for NP tissue engineering, NP-like tissues were generated *in vitro* by four weeks' culture of NP human cells (Sakai *et al.*, 2006). To mimic the composition of the NP extracellular matrix, collagen-based systems containing HA and/or other glycosaminoglycans have also been studied (Xin *et al.*, 2004; Halloran *et al.*, 2008; Alini *et al.*, 2003). Hydrogels based on atelocollagen type II, non-crosslinked and enzymatically crosslinked, and containing varying concentrations of aggrecan and hyaluronic acid have been used as scaffold to seed NP cells. Collagen was crosslinked using microbial transglutaminase, and the crosslinking did not cause any negative effect on cell viability and proliferation. The crosslinked scaffolds retained the highest proteoglycans synthesis rate and the lowest elution of the glycosaminoglycans; moreover, in terms of volume reduction they provided a more stable structure for the cells compared with the non-crosslinked scaffolds. These results showed that Ha/collagen-based systems possess suitable properties for developing injectable cell-seeded scaffolds for NP treatment (Halloran *et al.*, 2008).

6.4 Conclusion

Hydrogel-based systems have played a major role in the design of intervertebral disc prostheses following a biomimetic approach and in the future their importance is going to increase as they can act as scaffolds to carry cells for engineering the intervertebral disc tissues.

6.5 References

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Hydrogels for intraocular lenses and other ophthalmic prostheses

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Abstract: The eye is unique among all organs in that it has large volumes which are completely avascular. These avascular tissues are inherently isolated from the normal immune response of the body. This ‘immunological privilege’ makes the eye an excellent candidate for permanent prostheses using hydrogels which mimic the eye’s natural soft, transparent hydrogel materials. Both the lens and vitreous humors are natural hydrogels. They transmit light from the environment to the retina, necessitating transparency. However, their mechanical function requires some elasticity. Understanding the optical and mechanical interplay in these tissues will allow successful permanent prostheses using biomimetic hydrogels. The eye is also a focal point for the investigation of hydrogel-based tissue adhesives. Since many eye surgeries require the surgeon to cut holes in the cornea, a transparent method for sealing the hole will allow continued visual function during rehabilitation. Selecting a suitable adhesive may also allow a more rapid regeneration of surrounding cells and less chance of long-term effects from the surgical procedure.

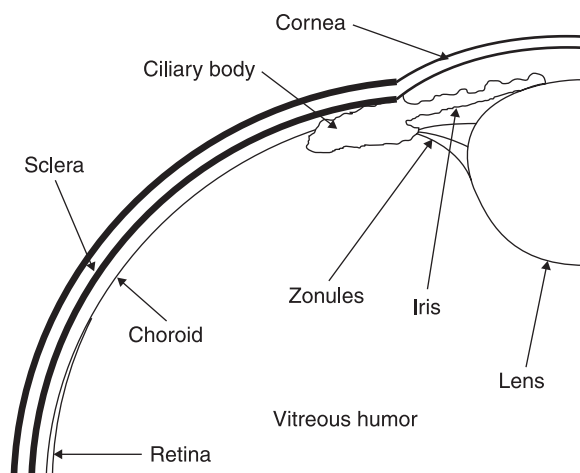
Key words: intraocular lens, lens refilling, vitreous substitute, tissue adhesive, hydrogel, ocular prostheses.

7.1 Introduction

The purpose of the human eye is to form a clear image of the environment on the retina. Since light must pass through several tissues to reach the retina, these tissues must be transparent. Transparency implies avascularity, which in turn implies that the transparent tissues are immunoisolated. This makes the transparent tissues of the eye ideal candidates for replacement with hydrogels.

The anatomy of the anterior portion of the eye is summarized in Fig. 7.1. Light enters the eye through the cornea – the transparent portion of the eye’s outer shell. The cornea has a large and fixed refractive power. After light passes through the cornea, it travels through the aqueous humor, a saline-like fluid filling the anterior chamber of the eye. The lens, which provides the fine focusing ability of the human eye, separates the anterior and posterior chambers of the eye. The retina surrounds the vitreous humor in the posterior chamber.

The cornea is unique among the three transparent tissues in that it directly interfaces with vascularized tissue (the sclera) at its periphery. Therefore, it is not entirely immunoisolated. However, many maladies of the cornea have been



7.1 Ocular anatomy.

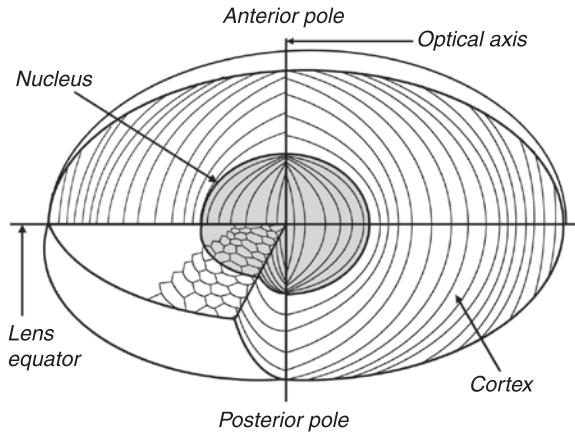
successfully treated with corneal transplants such that synthetic prostheses are unnecessary. Therefore, primary focus will be placed on potential for hydrogels as lens and vitreous prostheses.

7.1.1 Lens

The lens (Fig. 7.2) is entirely ectodermal in origin, and the cells at its center are formed during the early development of the embryo. The lens cells differentiate and grow throughout life via proliferation of the epithelial cells near the lens equator. Epithelial cells form a monolayer across the anterior lens surface. These cells produce an amorphous collagen capsule which surrounds the entire lens. The capsule is attached to an annular muscle, the ciliary muscle, via fibrillin fibers called zonules. Thus, the lens and its capsule are not in contact with any vascular tissues. Contraction and relaxation of the ciliary muscle are responsible for altering the refractive power of the eye – a process known as accommodation.

The lens's continued growth throughout life, which occurs in spite of a cessation of growth of the whole eye at approximately 13 years of age, leads to a degradation of lens performance with age. This loss of refractive power is termed presbyopia, which literally means 'old eye'. Presbyopia gradually affects all humans by causing the near focal point to recede. This is generally noticed clinically when the near point approaches one meter – longer than the normal human arm. This occurs between the ages of 35 and 50 years and generally requires the use of reading glasses or bifocals for correction.

The pathogenesis of presbyopia is likely to be complex and multifactorial. Generally, researchers have attributed its onset to an increase in lens volume and/or an increase in lens stiffness over time. More recent work has indicated the



7.2 Lens anatomy.

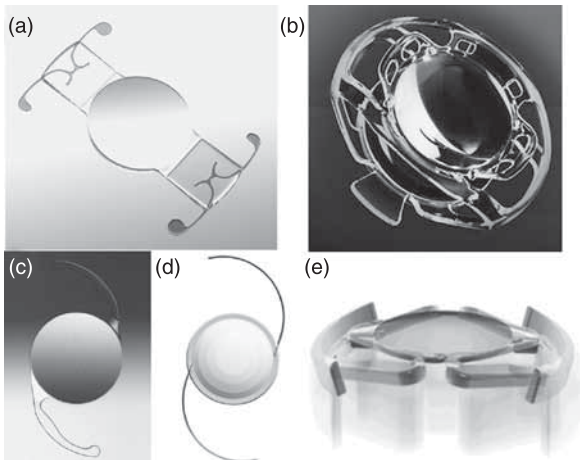
presence of an age-dependent mechanical property gradient within the lens which may play a critical role in accommodation.

An additional problem with the lens is the formation of opacities called cataracts. Depending on the size, type and location of the cataract, it may impair vision or even cause blindness. While the causes of cataracts are complex and not well understood, an extremely successful treatment has been developed. In this procedure, the anterior portion of the lens capsule is removed. The lens cells are then destroyed and removed using phacoemulsification. A hard polymer disc, called an intraocular lens (IOL), is then injected into the remainder of the capsule. Generally, IOLs use haptics to press against the equatorial capsule and hold the IOL in position. Various newer IOLs attempt to return some accommodation using a wide variety of designs (Fig. 7.3).

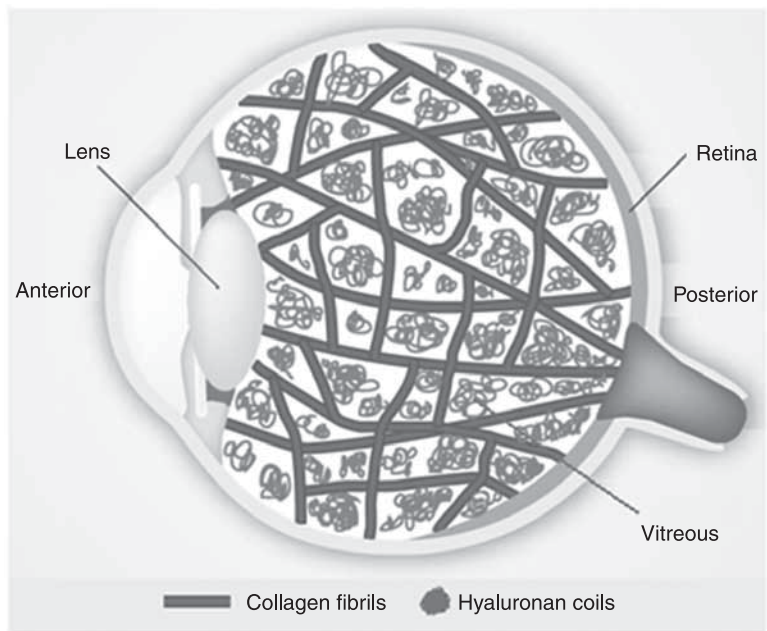
7.1.2 Vitreous

The posterior chamber is filled with the vitreous humor (Fig. 7.4), which is a soft, natural hydrogel comprising collagen, hyaluronic acid and approximately 99% water. The vitreous gradually liquefies with age. This liquefaction is correlated with increased occurrence of retinal detachments and ocular injury. This correlation may be linked to a decrease in mechanical pressure exerted on the retina by the vitreous and a degradation of vitreous viscoelastic properties.

Vitreous substitutes are required to maintain ocular volume during posterior ocular surgeries after removal of the natural vitreous. Current substitutes include air, saline solution and silicone. In addition to requiring the patient to remain face down for weeks after surgery, these substitutes have short retention time in the eye and are highly correlated with developments of post-surgical cataracts. Silicones are also implicated in retinal toxicity. The vitreous chamber eventually



7.3 New intraocular lens designs which attempt to restore accommodation: (a) Bausch & Lomb crystalens® translational accommodating intraocular lens; (b) Visiogen Synchrony dual-optic intraocular lens; (c) Alcon AcrySof® ReSTOR® multifocal intraocular lens; (d) Abbott Medical Optics ReZoom® dual haptic multifocal intraocular lens (image provided courtesy of Abbott Medical Optics); (e) Acuity C-Well translational accommodating intraocular lens.



7.4 Ocular anatomy with schematic of vitreous structure. (Adapted from *Expert Rev. Ophthalmol.* 2(2), 255–265 (2007) with permission from Expert Reviews Ltd [Swindle and Ravi, 2007].)

fills with physiological saline solution after the loss of these temporary vitreous substitutes.

Replacement of the vitreous with an appropriate biomimetic hydrogel would allow the patient to be fully mobile shortly after surgery. This could be achieved by designing the prosthesis such that it exerts a mechanical pressure on the retina to tamponade and prevent detachment during recovery. Further, a hydrogel which is covalently crosslinked after insertion in the eye should have a very long retention time and may serve as a permanent substitute, protecting against subsequent ocular trauma via viscoelastic damping.

7.1.3 Tissue adhesives

Many ocular surgeries require entrance through the cornea. The cornea is also commonly affected by trauma. Transparency, shape and structural integrity of the cornea are essential for both optical and mechanical functions of the eye. Therefore, sutures are inappropriate for mending the cornea after trauma or surgery. Tissue adhesives, such as cyanoacrylate and fibrin glues, have been used with success to repair the cornea while maintaining transparency and shape. These adhesives often offer superior biocompatibility, reduced inflammation and decreased surgical trauma associated with the insertion of sutures.

7.1.4 Hydrogels in ophthalmology

Ophthalmic applications are uniquely suited to permanent hydrogel prostheses for a variety of reasons. The lens and vitreous are immunologically privileged: due to the avascularity of their domains, they are not subjected to the body's immune system. Thus, rather than simply serving as a temporary scaffold or filler material, the properly formulated hydrogel may be a permanent implant. The transparency offered by some hydrogels allows maintenance of function that could not be achieved using other materials.

Here, we will discuss the use of hydrogels as permanent implants in the eye. Currently, three major areas – IOLs, vitreous substitutes and retinal adhesives – are the focus of research in this area.

7.2 Intraocular lenses

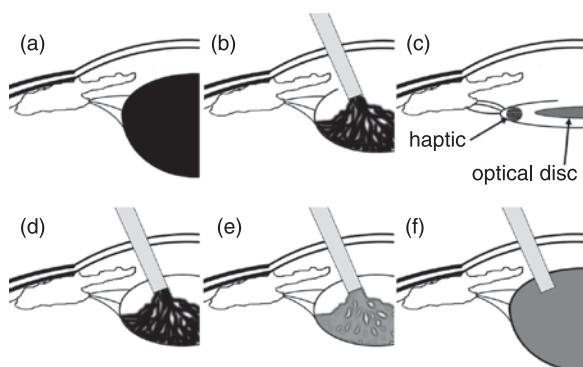
7.2.1 Current treatments

The intraocular lens was serendipitously discovered by Sir Harold Ridley while working as an ophthalmologist in the Royal Air Force during World War II. He observed that flecks of aircraft windshields, made of poly(methyl methacrylate) (PMMA), did not evoke an immune response when embedded in the eyes of patients. This observation of the 'immunological privilege' of

avascular eye tissues led him to create the first intraocular lens (IOL), which he implanted in 1949. He implanted a permanent IOL the following year. The procedure was not approved by the United States' Food and Drug Administration until 1981, fully 29 years after the first procedure was performed on American soil. IOLs are implanted (Fig. 7.5 A–C) by creating a large capsulorrhexis in the central anterior lens capsule. Phacoemulsification is used to destroy the lens architecture. The lens cells are then aspirated and the IOL is injected through the capsulorrhexis such that its haptics press against the equatorial portion of the lens capsule.

Traditional IOLs made using PMMA are very stiff – essentially rigid in the eye. Thus, they may be used to give clear vision at only a single distance (usually at optical infinity) such that the recipient must use reading glasses for near vision. At intermediate distances, objects are never truly in focus.

To improve on this fixed-focus design, several alternative treatments have been developed. Fixing one eye at near and one eye at far by implanting IOLs of different optical powers is one approach. IOLs have also been developed with different optical zones such that each zone focuses at a different distance. Most recently, 'accommodating' IOLs have been introduced, functioning on various optical principles such as axial translation. The shortcomings of other IOL designs have recently been reviewed (Menapace *et al.*, 2007). While these devices may offer some benefit to the patient, they are obviously inferior to the young, natural eye which grants clear vision at all distances via accommodation.



7.5 Schematic of cataract surgery with phacoemulsification and intraocular lens injection: (a) the intact cataractous lens; (b) phacoemulsification and removal of lens cells through a large, central, anterior capsulorrhexis; (c) placement of a typical intraocular lens within the remaining capsular bag. Schematic of cataract surgery with phacoemulsification and lens refilling: (d) phacoemulsification through a small peripheral capsulorrhexis; (e) injection of the refill material; (f) the fully refilled capsular bag.

7.2.2 Experimental treatments

Julius Kessler began experimenting with ‘lens refilling’ in the 1960s (Fig. 7.5 D–G). By creating a small hole in the capsule and removing the lens fiber cells via suction, he left the lens capsule largely intact. The empty capsular bag could then be filled with a suitable material (Kessler, 1964).

Mechanical considerations

This possibility sparked renewed interest in understanding the mechanism of accommodation, particularly regarding mechanics. Various experiments have since shown that the lens fiber cells are viscoelastic in nature (Itoi *et al.*, 1965; Ejiri *et al.*, 1969; Soergel *et al.*, 1999; Weeber and van der Heijde, 2007a, 2007b; Reilly *et al.*, 2008a, 2008b), that the overall accommodative mechanism may be modeled as linear viscoelastic subunits (Beers and van der Heijde, 1994; Reilly *et al.*, 2008a), and that the lens capsule is essentially hyperelastic (Krag and Andreassen, 1996, 1997). More recent experiments have also shown that the mechanical properties of the lens matrix and the lens capsule depend on position as well as age (Krag and Andreassen, 2003; David *et al.*, 2007; Heistand *et al.*, 2007; Heyes *et al.*, 2004; Weeber and van der Heijde, 2007a; Reilly and Ravi, 2009). Evidence from both experiments (Weeber and van der Heijde, 2007a; Reilly and Ravi, 2009) and simulations (Weeber and van der Heijde, 2007b) indicate that these mechanical property gradients may be essential to the mechanism of accommodation such that the age-related changes are a key component of presbyopia. Neural simulations of accommodation feedback loop dynamics indicate that the dynamic behavior of the lens is critical to the stability of the visual system (Schor *et al.*, 2007). Changes in lens viscoelasticity with age may, therefore, be another important component in the pathogenesis of presbyopia (Weeber *et al.*, 2005).

This extensive body of work seems to suggest that a successful lens refill material should mimic the mechanical properties of the healthy young lens not only in its elastic properties but also in its dynamic (viscoelastic) behavior. Since the mechanical properties of the lens also vary with position, the problem of formulating a suitable synthetic material with these properties is formidable. Recent testing of the lens using microindentation has indicated that the lens may achieve its mechanical property gradient via a gradient in the density of cytoskeletal crosslinks (Reilly and Ravi, 2009). Thus, one possible method for successfully replacing the lens is to develop a system which mimics the natural crosslink density gradient.

Tight control over the volume of refill material is a key consideration in optomechanical performance of the lens prosthesis (Nishi *et al.*, 1997; Koopmans *et al.*, 2003a; Reilly *et al.*, 2009b). Therefore, another important consideration when selecting a potential prosthesis material is its dimensional stability. In

particular, hydrogels are prone to swelling when water infiltrates the polymer network. The degree of swelling depends on many parameters, such as the affinity of the polymer and solvent, molecular weight between crosslinks, and the conformational state of the polymer at gelation. While swelling behavior is predictable (Swindle *et al.*, 2008), surgical considerations may require that the successful lens prosthesis remains at its initial injected volume to prevent poor optomechanical performance.

Optical considerations

Adding to this difficulty are the exceptional optical property requirements of the lens. The natural lens also exhibits a gradient in refractive index. This RI gradient has a maximum at the center of the lens and a minimum at the equator (Pierscionek and Chan, 1989; Keenan *et al.*, 2008), and is important in reducing higher-order optical aberrations which would occur in a lens with a uniform RI (Siedlecki *et al.*, 2004), as well as improving lens transparency by minimizing the loss of light due to spectral scattering which occurs at sharp discontinuities in RI (Pierscionek, 1995). The RI of the lens also changes with age and may be a contributor to presbyopia (Smith *et al.*, 1992). The young human lens has a refractive index of approximately 1.435 (Hermans *et al.*, 2008).

Obtaining a high refractive index material is generally accomplished by increasing the concentration of the polymer or protein to a sufficient level. The natural lens achieves a high refractive index while maintaining transparency through a complex mixture of crystallin proteins and their interaction with cytoskeletal proteins found in the lens fiber cells (Bloemendal *et al.*, 2004). These crystallin proteins have several unique properties which maintain lens transparency while granting a sufficiently high refractive index (Benedek, 1983). Increasing the concentration of crystallins in solution decreases transparency to a critical point, above which transparency dramatically increases (Delaye and Tardieu, 1983; Xia *et al.*, 1996). This and other findings indicate that the charges on the individual protein molecules give the crystallins a short range order which grants not only the necessary optical properties, but also some desirable mechanical properties (Reilly *et al.*, 2008b).

Compartmental model of lens optomechanics

Considering the mechanical and optical phenomena of the lens from this molecular perspective gives unique insight into the method nature has used to construct a lens which is both soft and optically dense, despite having a fairly limited selection of materials. This body of work seems to indicate that the elastic portion of the lens's mechanical properties primarily arise from the insoluble cytoskeleton, whereas its viscous and optical properties arise primarily from the soluble (crystallin-rich) phase. This allows the lens to overcome the physical limitations

of single-component systems, again giving insight into how one might approach the design of potential prosthesis materials: the polymer matrix may be dilute, such that the elastic modulus of the resulting lens is low, and it can then be loaded with a nanoparticle at a much higher concentration to improve the refractive power of the overall prosthetic.

However, most experimental lens refill materials thus far attempt to replace the lens with a single homogeneous material. This is due to a variety of factors, certainly including simplicity and the recent availability of the aforementioned studies. Therefore, this section's focus is on homogeneous lens material studies, though information on more complex materials is included when available.

7.2.3 Lens refilling studies

Silicone

Initial studies of lens refilling utilized silicone due to its ready availability, an extensive body of work regarding its biocompatibility for breast augmentation, and its inherent viscoelasticity and high refractive index (Parel *et al.*, 1986; Haeffliger and Parel, 1994). Additional work using silicones attempted to improve the technique by utilizing a variety of devices. Balloons of various shapes were inserted into the capsule and filled with the silicone polymer (Nishi *et al.*, 1997). Silicone plugs were used to prevent leakage after refilling (Nishi and Nishi, 1998; Nishi *et al.*, 1998). However, these devices added additional difficulty to the surgical technique while actually decreasing the accommodative amplitude in a primate model. Even this diminished accommodation was lost over time and was accompanied by complications such as post-surgical cataract (Nishi and Nishi, 1998).

More recently, Koopmans *et al.* investigated lens refilling using a proprietary formulation of silicone. This material is said to have a refractive index of 1.428 and an elastic modulus of 800 Pa (Koopmans *et al.*, 2003a, 2006), though the methods through which this value was obtained are unclear. *In vitro* testing indicated that the change in optical power due to stretching was highly dependent on the volume of the refill material used (Koopmans *et al.*, 2003b). *In vivo* testing in five-year-old (i.e. pre-presbyopic) rhesus monkeys indicated that the amplitude of accommodation was significantly lower in the post-surgical eye. Significant difficulties with post-surgical cataracts were also encountered in one group, though treatment of the capsule with a solution designed to prevent early post-surgical capsular opacification significantly improved short-term performance. Significant opacification still occurred during the six-week examination period despite the use of this solution (Koopmans *et al.*, 2006).

These and other silicone refilling experiments were recently reviewed by Nishi *et al.* (2009).

Thus, while silicone was very promising from an optical and elastic property viewpoint, its poor biocompatibility within the ocular system has resulted in

abandoning it as a feasible material for lens refilling. Hydrogel systems have therefore become the focus for future study.

Hydrogels

Preformed – Owing to the obvious shortcomings of silicone, several groups began investigating hydrogels for lens refilling. Initial attempts on this front simply used poly(acrylamide) due to the ease with which such gels may be made. However, injecting a preformed gel through a small-gauge needle causes shear degradation of the gel, resulting in a decreased elastic modulus, fragmentation and loss of gel form. Thus, the mechanical performance of the hydrogel would not be the same after the injection, nor would the post-injection polymer still necessarily retain its pre-injection gel structure and properties (Chirila and Hong, 1998).

In situ forming – To overcome this difficulty, inventive methods for inducing post-injection gelation were considered. Each of these methods involved injecting a linear polymer into the evacuated capsular bag. One method for *in situ* gelation is photopolymerization. This method is potentially problematic in the eye due to the extreme sensitivity of the retina to relevant wavelengths (Sloney, 1983), as well as the potential for leakage of potentially toxic uncrosslinked material prior to the onset of gelation (Kwon *et al.*, 2005). De Groot *et al.* (2001) examined this method using two model systems: poly(ethylene glycol diacrylate) (PEGDA) and modified copoly(N-vinylpyrrolidone/vinyl acetate) (NVP/VA). They noted that the PEGDA solution's viscosity was too low to remain within the lens capsule prior to crosslinking and was opaque at concentrations high enough to give a suitable refractive index. The NVP/VA copolymer was successfully crosslinked within the capsule while maintaining radiation levels which were safe for the retina. Less than 1% change in volume due to swelling was observed. However, to achieve a suitable refractive index, a 50% solution of NVP/VA in water was used, likely giving an elastic modulus significantly higher than would be appropriate for restoring accommodation via lens refilling. Kwon *et al.* (2005) utilized a photoinitiator to crosslink a poloxamer hydrogel in rabbits. They noted significant complications linked to all of the photoinitiators investigated. The elastic modulus of the resulting gels was very high (20 kPa) and the refractive index was low (1.36), indicating that this hydrogel is inappropriate for restoring accommodation. Murthy and Ravi (2001) explored the possibility of endocapsular gelation by mixing a chemical initiator with the polymer upon injection. A poly(ethylene glycol)-methacrylate model system was used to demonstrate the viability of this process. Various formulations were synthesized, yielding a range of viscoelastic properties similar to those of the natural lens. However, the presence of unreacted, potentially toxic, monomers within the gel would have resulted in poor biocompatibility. To overcome this pitfall, a disulfide linkage was introduced into a poly(acrylamide) backbone. The disulfides were reduced to thiol groups to recover a linear polymer.

The linear polymer was thoroughly washed to remove any low molecular weight materials using dialysis. The purified polymer was then injected into the capsular bag. Diffusion of oxygen into the capsule after injection resulted in spontaneous regelation as the thiol groups oxidized to reform the disulfide crosslinks. This method allowed the benefits of photopolymerization without the introduction of potentially toxic initiators (Aliyar *et al.*, 2004). The same method was used to refill six-month-old porcine lenses which were tested *in vitro*. The gels were analyzed using dynamic mechanical analysis (DMA), indicating an elastic modulus of 700 Pa, similar to the cortical elastic modulus of the porcine lens (Reilly and Ravi, 2009). Both natural and refilled lenses were tested using a robotic stretching device. Lenses were refilled with a range of volumes encompassing that of the natural porcine lens. Optical performance of the refilled lens was generally superior to the natural porcine lens, though the performance was highly volume-dependent. A multivariate regression model predicted that a lens which was refilled with a volume similar to that of the natural lens would perform similarly, while those with lower volumes could have improved performance to a point. Dynamic force measurements also indicated that the refilled lens responded more slowly to applied displacements *in vitro* (Reilly *et al.*, 2009a). This was likely due to the use of an entropic poly(acrylamide) system, whereas the lens itself is a highly organized structure which may behave primarily as an entropic gel.

Elastic gradient materials – Poly(acrylamide) hydrogels with elastic property gradients have been available for over a decade (Pelham and Wang, 1997). These gels are photopolymerized in the presence of a linear gradient photomask, resulting in a gradient in crosslink density. Obviously, this method would fail in the lens due to its constant exposure to light.

Gellan is a natural polysaccharide which undergoes a reversible conformational change from a random coil to a triple helix when subjected to increases in temperature or the presence of cations. In the helical conformation, gellan will spontaneously form crosslinks with other gellan molecules at very low concentrations (<0.1 wt%). Thus, by controlling the temperature or cation concentration profile within the sample, the elastic modulus profile may also be controlled (Zhang *et al.*, 2009; Reilly *et al.*, submitted). Its helical conformation also reduces the entropic degrees of freedom in the gel, yielding rapid relaxation on the same timescale as the natural porcine lens (Hamilton *et al.*, 2007). However, since the crosslinking mechanism is reversible, this method may not produce permanently graded gels.

Nanocomposites – The necessity of tailoring both optical and mechanical properties in parallel, subject to polymer scaling laws, may require a multicomponent system for successful lens prostheses (Ravi *et al.*, 2005). The natural lens appears to utilize this multicomponent design principle to achieve high optical power in a soft material. Highly concentrated (~40 wt%) crystallin proteins are present in integer proportions and with various electrostatic charges

(Bloemendal *et al.*, 2004). This gives rise to a solution with short-range order, preserving transparency in a very optically dense system (Delaye and Tardieu, 1983). These proteins are found in the cytoplasm of the lens fiber cells, often interacting with structural cytoskeletal proteins (Bloemendal *et al.*, 2004). These cytoskeletal proteins form a sparsely crosslinked, gel-like structure with a low elastic modulus (Reilly and Ravi, 2009).

Only preliminary work has been done on producing a transparent, optically dense, soft nanocomposite gel. However, this model system gave promising results, indicating that a two-component nanocomposite may be able to successfully mimic the behavior of the natural lens. The concentrations of the two components were successfully varied to yield a transparent system with both elastic modulus and RI similar to those of the young porcine lens (Ravi *et al.*, 2007).

Tissue engineering – One method for overcoming all of the pitfalls of purely synthetic systems discussed above is to utilize the lens's cells to regenerate themselves after cataract removal. The extensive problems encountered with secondary cataracts are the result of residual lens epithelial and/or fiber cells which were not successfully removed during the procedure. These cells may regenerate a healthy lens if provided with the proper environment and stimuli.

Extensive *in vitro* work has shown that the lens may spontaneously regenerate and remain transparent under certain conditions (Greenburg and Hay, 1982; O'Connor and McAvoy, 2007; O'Connor *et al.*, 2008). These studies have been carried out to examine the role of certain growth factors in the development of cataracts rather than the possibility of regenerating the lens. However, they give useful insights into the conditions necessary for the healthy, transparent regeneration of a natural lens.

Since the removal of lens opacities necessarily leaves a void within the capsular bag, attempts to regrow the natural lens structure must fill this void. Arlene Gwon has published extensively on methods to regenerate a healthy, transparent lens *in vivo* with progressively improved results. Gwon *et al.* (1990) showed that it is residual epithelial cells, not fiber cells, which regenerate the lens. The timescale of this regeneration was on the order of months in rabbits and was shorter in younger animals. Regeneration requires the presence of both the anterior and posterior capsules (Mayer, 1832), likely due to their importance in generating forces required for mechanotransduction, which guides the differentiation process (Bito *et al.*, 1965).

7.3 Vitreous substitutes

7.3.1 Introduction

For years, vitreous substitute research dealt primarily with looking for a biocompatible fluid capable of approximating the retina to the posterior of the

eye. This has led to the development of several short-term vitreous substitutes. However, they are not appropriate for long-term or permanent vitreous substitution due to migration from the eye, toxic reactions, and other unsuitable properties (Giordano and Refojo, 1998).

It would be more appropriate to design a vitreous substitute that mimics the physical and mechanical properties of the natural vitreous humor. Porcine, bovine and human vitreous have been tested by rheological methods to determine their viscoelastic properties, and the results have recently been summarized (Swindle and Ravi, 2007). It has been determined that the vitreous behaves as a viscoelastic solid with higher elasticity than viscosity.

Accordingly, the focus of recent vitreous substitute research has been on polymeric hydrogels. Hydrogels are hydrophilic polymers that form a gel network when crosslinked and are capable of absorbing several times their weight in water. The result is typically a clear viscoelastic gel that strongly resembles the natural vitreous humor. Hydrogels are more favorable vitreous substitutes because they are clear, tend to be biocompatible, and can act as a viscoelastic damper much like the natural vitreous (Chirila *et al.*, 1994). Additionally, hydrogels exhibit controllable swelling in aqueous solution, which enables the substitute to push the retina into place by exerting osmotic pressure while swelling (Peppas and Brannon-Peppas, 1990).

The main problem with preformed polymeric hydrogels as vitreous substitutes is that they irreversibly shear upon injection into the eye during vitrectomy. This irreversible destruction of the network causes the hydrogels to lose some of their elasticity and become more fluid-like and viscous. Additionally, shearing of the hydrogels through injection breaks the crosslinks in the gels, potentially decreasing biocompatibility due to the uncrosslinked polymer chains infiltrating the posterior segment and causing irritation (Chirila and Hong, 1998).

This problem has been addressed by our process of *in situ* regelation, in which a solution of linear polymers is injected into the eye, then gels under physiological conditions. Our group has achieved regelation *in situ* with disulfide chemical crosslinks, which are found in natural biopolymers such as proteins. These disulfide crosslinks form when the polymer comes into contact with oxygen. There are other methods of *in situ* gel formation such as thermally reversible gels and ionic gels (Suri and Bannerjee, 2006). However, the formation of chemical crosslinks is preferable because it improves biocompatibility, increases retention in the eye and mimics the natural vitreous.

The vitreous humor is an avascular network occupying the majority of the volume of the eye. The vitreous fills the space between the retina and the lens, allows for clear passage of light, holds the retina in place and dampens eye movements. A schematic of the eye is shown in Fig. 7.4.

The vitreous humor undergoes liquefaction or transformation from a formed gel to a phase-separated fluid with advancing age, and in some cases it causes retinal detachments that can lead to blindness. The vitreous is removed during

some surgical procedures and replaced with a vitreous substitute. No permanent vitreous substitutes are currently available and vitrectomy accelerates the formation of cataracts in the elderly (Federman and Schubert, 1988).

It is known that the vitreous is a natural hydrogel composed of 99% water and a framework of collagen and hyaluronic acid. Even in a normal eye the vitreous undergoes syneresis or degradation. The collection of collagen fibers is frequently referred to as 'floaters', which may interfere with vision. Liquefaction of the gel structure can cause degeneration or detachment of the vitreous. Retinal detachment occurs when the neurosensory retinal segments separate from the retinal pigment epithelium. A number of vision-threatening phenomena, such as macular holes, retinal detachments and vitreous hemorrhage are associated with this transition (Los *et al.*, 2003).

In recent years, research of vitreous substitutes has focused on polymeric hydrogels and these have been reviewed extensively (Chan *et al.*, 1984; Chirila *et al.*, 1994; Soman and Banerjee, 2003; Swindle and Ravi, 2007). However, these preformed equilibrium-swollen hydrogels disintegrate when injected and sheared through a small gauge needle (Chirila and Hong, 1998). We have previously designed, synthesized and characterized water-soluble copolymers that gel in the presence of oxygen (Foster *et al.*, 2006). Thus, prior to injection, they exist in polymeric form. The use of a disulfide crosslinker in the initial formation of the hydrogel enables exhaustive purification and subsequent chemical reduction to a substantially pure thiol containing copolymer that can now regel under physiological conditions in the eye.

The primary hindrance to advancement in this field is the lack of a vitreous substitute with viscoelastic and physicochemical properties similar to that of the vitreous humor. Overcoming this difficulty will enable ophthalmologists to better treat retinal detachments with new vitreous substitutes that act like the youthful vitreous humor.

The biomechanical properties of the porcine vitreous humor have been characterized because the porcine vitreous has been shown to be most similar to the human vitreous (Lee *et al.*, 1994). The data was used to design novel *in situ* gelling polymeric vitreous substitutes that match the viscoelastic properties and refractive index (RI) of the natural vitreous humor. The development of improved vitreous substitutes will significantly improve the quality of life of patients requiring vitrectomy. We have previously shown that polymers containing a disulfide crosslinker can gel *in situ* in porcine eyes by air oxidation (Swindle *et al.*, 2006).

The vitreous humor exerts an osmotic pressure, which may keep the retina attached to the posterior of the eye. The vitreous humor is a natural hydrogel, composed of 99% water and biopolymers. It has been shown that polymeric hydrogel vitreous substitutes shear thin when injected into the eye through a small-gauge needle (Chirila and Hong, 1998). Therefore, we hypothesize that a polymeric solution that gels *in vivo* would be an ideal vitreous substitute due to its

similar composition to the natural vitreous, biocompatibility, and ability to swell and exert an osmotic pressure, thus providing a mechanism to reattach the retina to the posterior of the eye during retinal detachment surgery.

7.3.2 Biomechanics of vitreous humor

The vitreous humor is a viscoelastic gel, which means that it exhibits both solid- and liquid-like behavior. The vitreous has a higher storage modulus (G') than loss modulus (G''), which indicates its viscoelastic solid behavior. G' represents the elastic or recoverable component, whereas G'' represents the viscous component or dissipated energy. Its viscosity is highest in the posterior and decreases toward the anterior segment (Lee *et al.*, 1992).

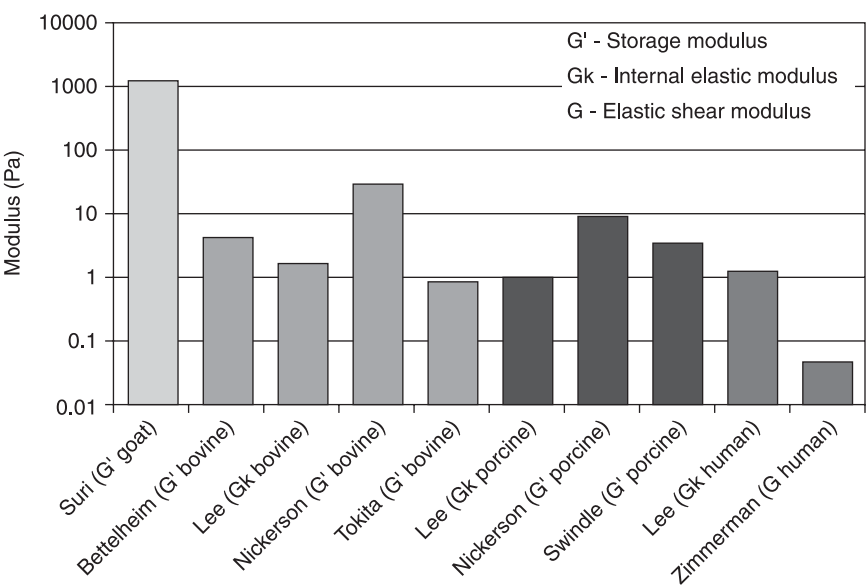
The mechanical properties of the vitreous humor have been studied by several groups (Fig. 7.6). Beginning in 1976, Bettelheim and Wang tested the viscoelastic properties of bovine eyes by inserting compression chucks in the vitreous cavity. A dynamic viscoelastometer applied sinusoidal compression via electromagnetic transducers. In bovine vitreous, the storage and loss moduli were found to be 4.2–4.7 Pa and 1.9–3.7 Pa, respectively. They hypothesized that hyaluronic acid contributed to the viscosity and collagen contributed to the elasticity. Their results showed that the elastic and viscous components were of the same magnitude, but the elasticity was slightly higher (Bettelheim and Wang, 1976). The two biopolymers in the vitreous interact to form a stable hydrogel without syneresis or mechanical collapse when subjected to conditions that would normally destroy collagen networks (Chirila *et al.*, 1994).

In 1980, Zimmerman measured the viscoelasticity of the human vitreous *in vivo* by light scattering. He reported an elastic shear modulus of 0.05 Pa (Zimmerman, 1980). Tokita *et al.* used a torsional pendulum to measure the complex shear modulus of bovine vitreous at low frequencies, giving a shear modulus value of 0.5 Pa (Tokita *et al.*, 1984).

In the early 1990s, a magnetic microrheometer was developed by Lee *et al.* (Lee *et al.*, 1992) because typical rotational rheometers may destroy the vitreous structure. The fluid was stressed by moving a microscopic iron sphere in a horizontal direction under the influence of magnetic force magnets. They then used an empirical four-parameter viscoelastic model to calculate the creep compliance of human, bovine and porcine vitreous. Several conclusions could be drawn from their work. The human vitreous has lower retardation times than the bovine or porcine vitreous, indicating faster recovery in the human eye. The vitreous humor is most viscous at the posterior in order to protect the retina and is less viscous at the anterior in order to allow rapid accommodation. The mechanical properties of the human vitreous are more similar to the porcine than to the bovine vitreous and the human vitreous most closely resembles that of the central region of the porcine vitreous. Their results indicate that the porcine vitreous would serve as a suitable animal model for the human vitreous humor (Lee *et al.*, 1994).

Nickerson *et al.* developed a cleated geometry to overcome wall slip in shear rheometry. Initial G' and G'' values were 30 Pa and 16 Pa for bovine vitreous, and 9.5 Pa and 3.6 Pa for porcine vitreous. The final steady-state values were 6.5 Pa and 2.0 Pa for bovine, and 2.6 Pa and 0.65 Pa for porcine. They reported storage moduli higher than all other sources, and postulated that the moduli are even higher *in vivo* due to the noticeable decrease in modulus with time outside the eye. The hyaluronan trapped in the vitreous *in vivo* increases the modulus by placing the collagen network under internal tension as it swells to its equilibrium state. The release of tension would provide a driving force for modulus reduction and fluid expulsion when the vitreous was removed from the eye and hyaluronan was no longer trapped (Nickerson *et al.*, 2005).

As shown in Fig. 7.6, the human vitreous humor acts as a viscoelastic polymeric hydrogel. The high molecular weight elements, such as collagen and hyaluronic acid, provide a system that absorbs stress and protects eye tissues during eye movement and activity (Balazs, 1989). The combination of collagen and hyaluronic acid creates a mesh with primary stress supported by collagen fibrils and hyaluronic acid coils protecting the network from collapse (Balazs, 1989). It is evident that a vitreous substitute should mimic the natural vitreous by being viscoelastic and transparent.



7.6 Literature values for modulus of vitreous humor. (Reproduced from *Expert Rev. Ophthalmol.* 2(2), 255–265 (2007) with permission from Expert Reviews Ltd).

7.3.3 Vitreous substitutes

A vitrectomy is a surgical procedure where the vitreous is cut and aspirated, and this is normally followed by replacement with an artificial substitute. A vitrectomy is usually performed for relief of traction and removal of blood from the ocular cavity. Currently, gases (air, sulfur hexafluoride or perfluoropropane), perfluorocarbon liquids, fluorosilicone oil or silicone oil (polydimethylsiloxane) are used as temporary vitreous substitutes to tamponade the detached retina against the posterior of the eye. These substitutes are not satisfactory for several reasons. For example, depending on the location of the retinal tear, these substitutes may require the patients to position themselves face down for days (Colthurst *et al.*, 2000). Silicone oil can be difficult to remove, has shown toxicity to intraocular structures, is capable of emulsification, and has been associated with glaucoma and corneal decompensation, both of which can lead to blindness (Leaver and Billington, 1989; Giordano and Refojo, 1998; Jonas *et al.*, 2001). Silicone oil is only 70% effective in retinal reattachment (Jonas *et al.*, 2001), and often the patient has to undergo subsequent cataract surgery after use of silicone oil as a tamponade (Leaver and Billington, 1989). Most importantly, none of these clinically available substitutes can be left in the eye safely for more than a few months (Giordano and Refojo, 1998). While silicone oil has been successful in retinal reattachment in some severe cases, it is evident that a better long-term vitreous substitute is needed. Silicone oils, perfluorocarbon liquids and gases as vitreous substitutes have been extensively reviewed (Leaver and Billington, 1989; Giordano and Refojo, 1998; Jonas *et al.*, 2001; Colthurst *et al.*, 2000; Wolf *et al.*, 2003; Sparrow *et al.*, 1990; Versura *et al.*, 2001), and will not be discussed here.

Initial research on vitreous substitutes focused on replacing the vitreous humor with vitreous from animals. However, due to the degradation of the vitreous outside the eye, these failed as vitreous substitutes (Deutschmann, 1906; Cutler, 1947). Next, researchers focused on developing vitreous substitutes from the natural components. However, the failure of collagen (Pruett *et al.*, 1972, 1974; Nakagawa *et al.*, 1997; Nayak, 1999) and hyaluronic acid (Nakagawa *et al.*, 1997; Pruett *et al.*, 1979) to mimic the natural vitreous led to research in synthetic polymers. Work with synthetic polymers began in the 1950s after poly(methyl methacrylate) was used in lens and cornea prostheses (Refojo, 1971). A number of uncrosslinked polymers were evaluated as vitreous substitutes, but these were ultimately unsuccessful due to inflammatory response, diffusion through the eye, and toxicity of the low molecular weight components. These have also been reviewed extensively (Chirila *et al.*, 1994; Swindle and Ravi, 2007). Crosslinking hydrophilic polymers to form hydrogels is the most promising method of vitreous substitution, and these hydrogels are discussed below.

In 1968, Refojo demonstrated biocompatibility of poly(glyceryl methacrylate) (PGMA) hydrogels in the eye using *in vitro* studies with bovine vitreous and *in vivo* studies with rabbits. PGMA gel was not noticed to have changes in swelling

characteristics when mixed with bovine vitreous. It was tested as a substitute in rabbit eyes with gel swelling in surrounding fluid in cavity. There was no inflammatory reaction after seven months. Implanting dehydrated gels caused surgical trauma and swelling took too long (Daniele *et al.*, 1968). Hogen-Esch *et al.* later developed injectable PGMA in 1976 and had satisfactory results in the rabbit vitreous cavity. They used a low degree of crosslinking leading to a gel-sol transition point. There was a negligible loss in transmittance of this viscoelastic gel upon injection. The gels swelled to 4–5% polymer concentration in isotonic saline, were soft, transparent, and had RI 1.3364, which closely matches that of the vitreous. Tissue culture and preliminary studies in rabbits were satisfactory (Hogen-Esch *et al.*, 1976). However, PGMA was injected after crosslinking to form a gel, so shear thinning would occur and destroy the gel structure.

Poly(2-hydroxyethyl methacrylate) (PHEMA) was found to be tolerated by rabbit eye tissues by Refojo in 1971. These hydrogels were inserted into the eye as solid implants. They were found to maintain their positions better than liquid vitreous substitutes. The hydrogels were insoluble in most solvents and swell in the eye. PHEMA could also be heat sterilized to improve biocompatibility (Refojo, 1971). However, PHEMA was not evaluated further as a vitreous substitute, and the surgical procedure of implanting a solid gel would be traumatic.

In 1984, poly(2-hydroxyethyl acrylate) (PHEA) hydrogels were evaluated as vitreous substitutes. The gels were dialyzed for one month and autoclaved before injection into rabbit eyes. The hydrogel was transparent with injectability and cohesiveness. However, the material promoted fibrin formation and cellular activity with membrane formation for two to three weeks. Lens opacification occurred in one eye. Histopathology showed a disorganized retina with glial cell proliferation and chorioretinal scarring. One eye had lens rupture due to accumulation of epithelia and plasma cells while another eye showed chronic nongranulomatous inflammation with plasma cells and lymphocytes. This material was not suitable as a vitreous substitute (Chan *et al.*, 1984).

In 1990, pluronic polyol F-127 (PF-127) was evaluated as a vitreous substitute. PF-127 is a clear physical gel that is liquid at cool temperatures and solid at body temperatures. Its specific gravity is 1.032, and it has been used for slow drug delivery. It was evaluated as a vitreous substitute in nine rabbits. The polymer was adsorbed slowly from the rabbit vitreous in spite of its high molecular weight. It was injected into the vitreous of rabbits and the electroretinogram (ERG) amplitude decreased in the treated eyes 24 hours after surgery. There was an atrophy of outer segments with vacuolization of the retinal pigment epithelium after 24 hours. There was an obvious decrease in retinal function and toxicity of PF-127. This would not be acceptable for clinical use (Davidorf *et al.*, 1990). Rheologically, this material was shown to significantly shear thin, with its viscosity significantly decreasing, showing that the gel was degraded during injection into the eye (Dalton *et al.*, 1995).

Chirila *et al.* used poly(methyl 2-acrylamido-2-methoxyacetate) (PMAGME) in 1991 to produce gels susceptible to external stimuli, such as pH and temperature. The homopolymer was tested, along with copolymers with 2-hydroxyethyl methacrylate, diacetone acrylamide and 2-acetoacetoxyethyl methacrylate. Their best vitreous replacement material was formed by homopolymerization of the monomer at 20% in water. These vitreous substitutes were developed as preformed hydrogels that would be injected into the eye. Fragmentation upon injection was negligible, but mild to severe postoperative inflammatory reactions were apparent in rabbit eyes. There was total atrophy of the optic nerve after one year. *In vivo* studies indicated that PMAGME was toxic to neural tissue (Chirila *et al.*, 1994).

Yamauchi published his results on poly(vinyl alcohol) (PVA) hydrogels that were tested in rabbits in 1991. The PVA gel had a similar refractive index and water content to the natural vitreous. Light transmittance was similar to that of the vitreous. Little or no inflammation of the anterior globe was observed and the fundus was clear after two weeks. Histological examination showed no retinal abnormalities after two months. However, the hydrogel was prone to aggregation, which could cause light scattering and opacity formation (Yamauchi, 1991).

In 1992, Peyman *et al.* evaluated the potential of silicone gel. The gel was injected as a liquid and polymerized in the eyes of monkeys. Gel formation required 15–30 minutes. Preparation A, a proprietary formulation of the silicone gel, had minimal inflammation for one week and remained clear. No gels were emulsified. However, Preparation B, another proprietary formulation, stayed hazy. Neither gel became encapsulated, nor did any cell adherence or leakage occur. ERGs were normal for both gels, indicating no retinal toxicity. The gel was safely left in the eye up to 13 months without contacting the cornea. However, silicone is hydrophobic and lighter than water, so it complicates the surgery (Peyman *et al.*, 1992).

Crosslinked poly(vinyl pyrrolidone) hydrogels were then tested *in vitro* in enzyme solution and *in vivo* in rabbit eyes. The hydrogel was clear, viscoelastic, cohesive, and showed minor fragmentation upon injection. The storage modulus was greater than the loss modulus, similar to the natural vitreous. The gel did not change properties when incubated with enzymes *in vitro*. In four weeks, 50% of the gel disappeared from the vitreous cavity by diffusion and macrophages were present with large vacuoles with polymers incorporated in the phagolysosomes. Phagocytosis of the hydrogels was common and gel syneresis occurred *in vivo* (Hong *et al.*, 1998). PVP was mixed with β -chitosan and tested as a hydrogel for general biomedical applications. *In vitro* haemorheological tests using cultured mouse (Balb/c-3T3) fibroblasts showed good biocompatibility with no noticeable changes in contact. These semi-interpenetrating network membranes could potentially be tested as vitreous substitutes in the future (Hong *et al.*, 1996). PVP was polymerized with divinyl glycol (DVG) crosslinker and the gels were clear, transparent and cohesive. The material was found to be cytostatic. Half the rabbit eyes showed opacities which inhibited vision. Other rabbits showed traction of

the blood vessels with large white patches. Biodegradation of the polymer occurred *in vivo*, possibly due to fragmentation upon injection. The retina was intact, but choroidal veins and capillaries were dilated. However, the polymer had a relative viscosity of 1.7–1.8 and refractive index of 1.3390, which are close to the values of the human vitreous (Vijayasekaran *et al.*, 1996).

In other work by the same group, the 1-vinyl-2-pyrrolidinone monomer was polymerized with varying amounts of 2-hydroxyethyl methacrylate (HEMA) and crosslinked with various crosslinkers to make PVP gels. Hydrogels were selected if the elastic modulus was higher than the storage modulus and if the resiliency was high. The selected hydrogels used divinyl glycol (DVG), a hydrophilic crosslinker. Gels of 5% HEMA with 0.5–0.75% diallyl ether (DAE) or 1–2% HEMA with 1% DVG showed mechanical properties close to that of the natural vitreous. However, injection of hydrogels resulted in breakage of crosslinks and entanglements, causing a decrease in the elasticity of the substitutes (Chirila and Hong, 1998). While these substitutes appeared promising, they could not feasibly be injected through a small-gauge needle during surgery and retain their mechanical properties.

De Jong *et al.* evaluated Adcon-L *in vivo* as a vitreous substitute in 2000. All rabbits tested had ocular inflammation after one day. Adcon-L is a translucent carbohydrate-based hydrogel currently used in neurosurgery. After one week, only the posterior synechiae, or adhesion of the iris to the lens, remained. After two weeks, there was a corneal opacity in one rabbit and lens capsules were thickened in all rabbits. After three weeks, additional corneal opacity and thicker lens capsules were observed. The hydrogel was no longer visible due to cataract formation. ERGs indicated a decrease in retinal activity or toxicity to the retina. Clearly, this is not a suitable vitreous substitute (De Jong *et al.*, 2000).

In 2004, Cavalieri *et al.* evaluated polymeric hydrogels for ocular tissue replacement. Methacrylated poly(vinyl alcohol) (PVA-MA) formed clear hydrogels. The hydrophobicity of the network was increased by the methacrylate addition. Partial degradation occurred at low degrees of cross-linking *in vitro* in bovine serum albumin. This gel network had a molecular weight of 13 kDa. These could be photocrosslinked upon injection into the vitreous cavity. The mechanical properties were evaluated, but the storage modulus was two orders of magnitude higher than that of the natural vitreous. The solutions would not gel at polymer concentrations lower than 4%, which caused the gels to have a higher modulus than that of the vitreous humor (Cavalieri *et al.*, 2004).

Recently, *in vivo* results were published on poly(vinyl alcohol) hydrogel vitreous substitutes. They investigated sterilized PVA hydrogels consisting of 99% water in rabbit eyes. The polymer had good optical properties, but inflammation and vitreous opacity occurred more frequently with PVA than with saline solution. UV spectrometric techniques were used to evaluate penetration rates. A mixture of PVA with chondroitin sulfate had a higher transparency than PVA alone, absorbed more water, and could not be broken down as easily, but was

found to be less biocompatible. The *in vitro* long-term retention was slightly worse than saline, but was judged acceptable by the authors as a temporary vitreous substitute (Maruoka *et al.*, 2006).

Also in 2007, Suri and Banerjee used gellan gum mixed with hyaluronic acid (HA) as a short-term vitreous substitute. Gellan gum is an exocellular microbial heteropolysaccharide secreted by *Sphingomonas paucimobilis*, and is commonly used in the food industry. Gellan gum is capable of forming a physical gel *in situ* when cooled to room temperature and maintains its gel structure when reheated to body temperature. Gellan, in its deacylated form, and HA were mixed in ratios of 9:1 and 4:1 w/w and were tested *in vitro*. The addition of calcium chloride to the gellan/HA mixture causes chemical crosslinking to occur, forming a stronger hydrogel. The wettability and light transmittance of these semisynthetic substitutes were comparable to those of the natural vitreous. However, their rheological properties showed storage and loss moduli two orders of magnitude greater than those of the natural vitreous humor. *In vitro* cytotoxicity studies with L929 mouse fibroblast cells showed viability of 92% after 48-hour incubation for the most promising formulation. Further *in vivo* tests and modification of the rheological properties to match those of the natural vitreous would be necessary prior to clinical trials. Additionally, this proposed substitute would not be capable of long-term use because it degrades after a week in biological fluids *in vitro* (Suri and Banerjee, 2006).

Ravi *et al.* have developed reversible hydrogels from acrylamide and bis(acryloyl)cystamine (BAC), a disulfide crosslinker. The disulfide bonds were reduced to thiol groups, enabling purification and removal of all unreacted toxic monomer and low molecular weight polymers. Gel elasticity was maintained after injection in human cadaver eyes (Foster *et al.*, 2006) and porcine eyes (Swindle *et al.*, 2006) *ex vivo*. The patient would not have to remain face down for extended periods of time as is required for vitrectomy with traditional materials (Aliyar *et al.*, 2004). This work confirmed that these gels may be formed in the eye and that it is possible for a hydrogel to produce osmotic pressure in the vitreous cavity (Foster *et al.*, 2006). The addition of a hydrophobic monomer, N-phenylacrylamide (NPA), to the acrylamide and BAC greatly improved biocompatibility. Toxicity tests on Chinese hamster ovary (CHO) cells showed a viability of approximately 100% after 5 days at a concentration of 15 mg/mL (Swindle *et al.*, 2006). Additionally, rheological testing showed that the storage and loss moduli of this hydrogel formulation matched those of the natural porcine vitreous (Swindle *et al.*, 2008). Preliminary *in vivo* studies in rabbits showed that the purification steps enabled by use of the reversible disulfide crosslinker produced a biocompatible formulation that gelled upon injection (Swindle-Reilly *et al.*, in press).

In Ravi's most recent work, statistical experimental design was employed to rapidly screen for *in situ* forming hydrogel vitreous substitute candidates that would match the optomechanical properties of the natural vitreous humor (Swindle-Reilly and Ravi, 2009). Since the natural vitreous humor attaches to the retina at several points and it would be desirable to replace the vitreous with a

hydrogel that could adhere to the retina and could mimic those attachment points, poly(acrylic acid) was selected as a potential vitreous substitute due to its adhesive property. By acquiring the properties of the vitreous humor, better vitreous substitutes were developed that are capable of forming *in situ* due to the incorporation of the disulfide crosslinker. Several hydrogel formulations were developed that matched the refractive index and mechanical properties of the vitreous humor (Swindle-Reilly and Ravi, 2009).

7.3.4 Conclusion

The future of vitreous substitutes is to find a formulation that can be left in the eye long term. Additionally, it would be preferable to mimic the mechanical properties, water content, and light transmittance of the natural vitreous humor. Silicone oil, currently the most commonly employed vitreous substitute, accomplishes none of these things. Polymeric hydrogel vitreous substitutes developed and tested experimentally have proven capable of matching these properties. Rheological testing can help match the mechanical properties of the polymeric substitutes to those of the natural vitreous. Furthermore, the process of *in situ* gelation is key to viable vitreous substitutes because polymers that are injected as gels rather than liquids fragment due to shear, lose their elasticity and can cause inflammatory reactions in ocular tissues. In the future, there will be further research conducted on *in situ* gelling polymeric vitreous substitutes. Polymers can be tailored to alter their mechanical properties to match those of the natural vitreous humor. The goal is to match those properties, have a transparent hydrogel that is 99% water and find a substitute that will not cause cytotoxic reactions and will be retained in the eye. Vitreous substitute candidates can be screened rapidly for these characteristics by utilizing statistical experimental designs.

Here are some of the key issues in the area of vitreous substitute research that should be considered. Silicone oil, although it is used most commonly today, is not a viable, long-term vitreous substitute because of complications that include cataract formation and because its properties differ greatly from those of the natural vitreous. Ideal vitreous substitutes should mimic the physical and mechanical properties of the natural vitreous, including light transmittance, water content, viscoelasticity and biocompatibility. Properly formulated polymeric hydrogels show water content, physical and mechanical properties close to those of the natural vitreous humor. *In situ* gelation of polymeric substitutes prevents shear thinning and degradation of the gel structure when injected into the eye.

7.4 Tissue adhesives

Tissue adhesives are used extensively in ophthalmology, particularly after surgical interventions which involve cutting the cornea or sclera. Cataract and refractive surgeries require holes or flaps in the cornea to give access to the lens or the

posterior surface of the cornea, respectively. Procedures involving the vitreous and retina are performed via holes in the sclera. Sutures are not an ideal method for repairing these tissues due to the proximity to low-viscosity ocular fluids, inflammation and interference with ocular movements. Tissue adhesives have also been investigated as ocular drug delivery vehicles: by creating a glue-based solution containing the drug and injecting it into direct contact with a lesion, localized treatment is possible without accompanying concerns of side-effects common with systemic chemical treatments.

7.4.1 Adhesives to replace sutures

Corneal wounds are generally repaired using nylon sutures. However, sutures are undesirable because of the additional trauma to the cornea; potential for infection; inflammation; scarring; neovascularization; post-operative instability or breakage; the necessity of post-surgical removal of the sutures, which offers a new opportunity for infection and other complications, and the likelihood of uneven healing leading to imperfect remodeling of the corneal surface, resulting in distorted vision (Grinstaff, 2007).

Initial efforts in repairing the cornea with adhesives utilized cyanoacrylate glues due to their ready availability in emergency rooms (Refojo *et al.*, 1968; Hirst *et al.*, 1979, 1982; Weiss *et al.*, 1983; Trott, 1997). Cyanoacrylates have several desirable characteristics that make them suitable for corneal adhesion. They are largely transparent, prevent re-epithelialization and collagenase production and have bacteriostatic activity (Eiferman and Snyder, 1983). However, they are toxic to corneal epithelial cells, though this toxicity may be controlled somewhat by increasing the length of alkyl chains (Weiss *et al.*, 1983). Therefore, cyanoacrylate glues are only suitable for temporary treatment when the integrity of the globe is compromised (Vote and Elder, 2000).

It was recognized early on that fibrin glues are a superior alternative to cyanoacrylate adhesives, though they were not as readily available in trauma situations (Vote and Elder, 2000). They have been used to seal corneal perforations arising from a variety of circumstances with a high degree of success (Gauthier and Lagoutte, 1989; Bonatti *et al.*, 2007). These glues reduced inflammation and scarring while maintaining transparency relative to sutures, in addition to reducing operating time (Berdahl *et al.*, 2009). Very recently, fibrin adhesives were applied to pterygium surgery (Srinivasan *et al.*, 2009). Fibrin glues, rather than sutures, were used to manage the conjunctiva, resulting in reduced inflammation without compromising stability.

7.4.2 Additional ocular uses

Retinoblastoma has been treated successfully using external beam radiation (EBR) in adults (Murphree *et al.*, 1996). However, EBR has been shown to

induce secondary cancers in pediatric patients (Eng *et al.*, 1993; Kaye and Harbour, 2004). Therefore, local delivery of prolonged doses of chemotherapeutics in pediatric patients is a promising alternative. Local chemotherapeutic agent fibrin delivery vehicles for the treatment of retinoblastoma have recently been reviewed (Martin *et al.*, 2008).

Additional basic science work in this area is attempting to improve treatment options further. Doraiswamy *et al.* (2009) have investigated the possibility of printing three-dimensional hydrogel-based tissue adhesives using a microelectromechanical valve-based inkjet printer. Their device allowed detailed specification of the desired hydrogel design using computer aided design software prior to printing mussel adhesive proteins. Elvin *et al.* (2009) have investigated using photocrosslinked fibrinogen as a tissue adhesive. Unmodified fibrinogen may be photochemically crosslinked to form a hydrogel with at least a five-fold improvement in elastic strength relative to fibrin glues. In addition, the fibrinogen gel cured in less than 20 seconds and was non-toxic *in vitro*.

7.5 Conclusions

The eye offers unique opportunities and challenges for the use of hydrogels. Hydrogels may be applied as permanent prostheses to treat presbyopia, cataract and vitreous liquefaction. Further, tissue adhesives have already been used to improve patient outcomes in ocular surgeries, as well as offering new methods for combating retinoblastoma. In all cases, the use of hydrogels in place of more traditional silicone or organic polymer systems may improve functionality, biocompatibility, ease of use and improved surgical recovery times.

7.6 Acknowledgements

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7.7 References

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Cartilage replacement implants using hydrogels

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Abstract: Articular cartilage (AC), a highly specialised tissue that provides low friction and allows for efficient load bearing and distribution, can undergo disruption for traumatic or degenerative processes. Conservative treatment with various medications gives only temporary relief of symptoms rather than cure. Since 1960 several surgical procedures have been tested without significant improvement of AC regeneration. Actually, third generation tissue engineering applied to the cartilage tissue regeneration is giving promising results. The right combination of cell types, scaffolds and culture media can represent the only way to cure degenerated cartilage tissue. However, several critical issues need to be overcome.

Key words: articular cartilage, tissue engineering, arthroscopic techniques, scaffolds, gene therapy.

8.1 Introduction

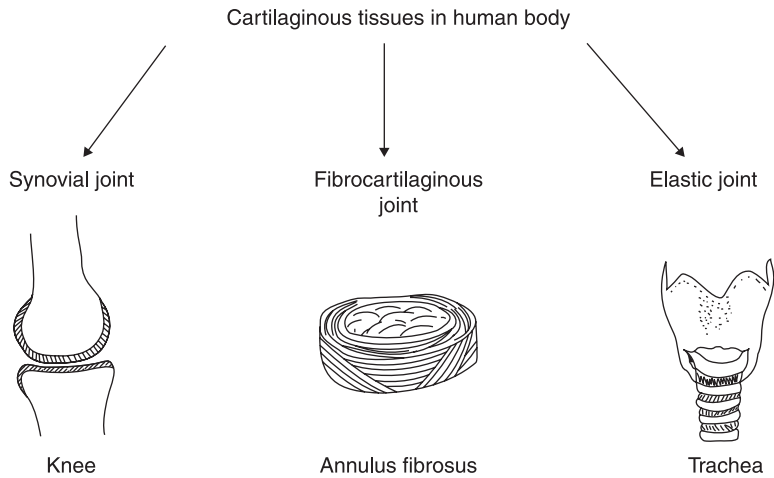
The materials classed as cartilage exist in various forms and perform a range of functions in the body. Depending on its composition, cartilage is classified as elastic cartilage, fibrocartilage or articular cartilage (also known as hyaline) (Fig. 8.1).

Elastic cartilage helps to maintain the shape of structures such as the ear and the trachea. In joints, cartilage functions as either a binder or a bearing surface between bones. The annulus fibrosus of the intervertebral disc is an example of a fibrocartilaginous joint with limited movement (an amphiarthrosis).

In the freely moveable synovial joints (diarthrosis) articular cartilage is the bearing surface that permits smooth motion between adjoining bony segments. Hip, knee and elbow are examples of synovial joints and these freely moveable joints are the most easily subjected to alteration (Mansour, 2003).

8.1.1 Articular cartilage composition and structure

Articular cartilage (AC) is a 0.5 to 5 mm thick layer of hydrated soft tissue that covers the articulating extremities in diarthrodial joints (Netti and Ambrosio, 2002). Articular cartilage serves to support and distribute applied loads (Mow *et al.*, 1992) and functions biomechanically as a multiphasic fibre-reinforced material with anisotropic, inhomogeneous, non-linear and viscoelastic properties (Mow *et al.*, 1980; Soltz and Athesian, 2000; Woo *et al.*, 1979). These complex mechanical properties provide the essential mechanism for pressurisation of the



8.1 Examples of different kinds of cartilage tissue in the human body.

interstitial fluid of the tissue under stress, allowing for the crucial load-support and low-friction properties of the tissue (Moutos *et al.*, 2007).

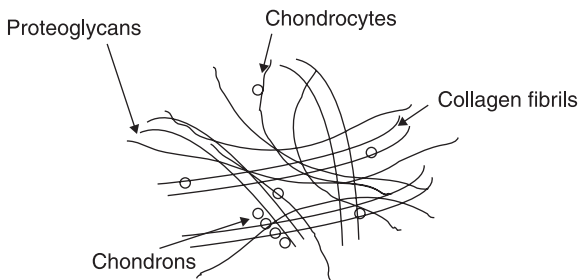
The major constituents, reported in Table 8.1, comprise specialised cells, chondrocytes, embedded in highly hydrated (water content: 60–80%) and organised extracellular matrix (ECM) consisting of collagens, fibres and proteoglycans (Fig. 8.2).

The predominant cell type is the chondrocyte (5% wet weight), which is responsible for extracellular matrix (ECM) production. They are spherical in shape, surrounded by lacunae, but they become more flattened as they get closer to the superficial zone, where they assume a fibroblastic shape. They often clump together in columns forming chondrons (2–4 cells), which are orientated along collagen fibres (Fig. 8.2). The ECM is made up of collagen fibres (25% wet weight) of which Type II predominates (95%), but also types VI, IX and XI with type X are present in the calcified layer. These fibres are anchored to the calcified layer running perpendicularly to it, crossing each other in arcs at the superficial zone. The horizontal cellular orientation, combined with the collagen network in the superficial zone, provide resistance to shear forces. Chondrocytes secrete lubricin (also known as superficial zone protein), a molecule which is responsible for reducing the coefficient of friction and thus providing cartilage with such favourable tribological properties.

In the spaces formed by the crosslinking of collagen fibres, large negatively charged hydrophilic proteoglycan molecules are present, essentially aggrecan and hyaluronan. The dense collagen network restricts the hydration of these molecules to about 40–60%. As a result, the swelling pressure which is generated provides the compressive stiffness of cartilage. Smaller glycoproteins also exist, including

Table 8.1 Composition of cartilage tissue

H ₂ O (60–80%)	
Proteoglycans	• Aggrecan • Hyaluronan
Small glycoproteins	• Fibronectin • Cartilage Oligomeric Protein (COMP)
Cells: Chondrocytes (5%)	• Spherical shape: inner zone • Flat ‘fibroblast-like’ shape: superficial zone • Chondrons: 2–4 cells oriented along collagen fibres
Collagen (25%)	• Type II (95%) • Type VI, IX and X (especially in calcified cartilage)
Regulating factors	• Bone morphogenic proteins (BMPs) • Matrix metalloproteinases (MMP-3; MMP-8; MMP-9; MMP-13; aggrecanases 4; aggrecanases 5) • Tissue inhibitors of metalloproteinases (TIMPS)



8.2 Ultrastructure of cartilage tissue.

fibronectin and cartilage oligomeric protein (COMP), which have a role in cell adhesion. In addition there are growth factors present, such as bone morphogenetic proteins (BMPs), whose role is under investigation. Tissue turnover is mostly governed by a balance between the matrix metalloproteinases (MMP-3, MMP-8, MMP-9, MMP-13 and aggrecanases 4 and 5) and the tissue inhibitors of metalloproteinases (TIMPS) (Murphy and Lee, 2005).

AC can be thought of as a peculiar organ, since it is avascular and aneural, and it receives its nutrition from the synovial fluid, as a result of mechanical movement of the tissue producing a diffusion gradient. Furthermore, AC is immunoprivileged. In fact, it does not contain immune cells; therefore chondrocytes secrete lysozyme to counteract microorganisms (Getgood *et al.*, 2009). During the early degenerative process, when the collagen fibres are disrupted, the proteoglycans can become more hydrated, causing the cartilage to soften.

8.1.2 Pathogenesis

As said before, articular cartilage has no blood vessels, it is not innervated and normal mechanism of tissue repair perform poorly to form only fibrocartilaginous tissue. Articular cartilage defects may result from injury or osteochondral pathology, such as osteonecrosis and osteochondritis dissecans. In adults, these defects heal poorly and progress to catastrophic degenerative arthritis (Chiang and Jiang, 2009). Degenerative disease can be further sub-classed as either primary or secondary. The aetiology of secondary arthrosis is multifactorial and many different risk factors have been implicated (Jordan *et al.*, 2003). Mechanical factors such as direct trauma, instability, malalignment and loss of meniscal chondroprotection have a role, as well as metabolic factors such as diabetes, alcohol abuse and obesity. The aetiology and the depth of injury deeply affect the AC defects' behaviour, and traumatic partial thickness cartilage defects do not spontaneously heal. The paucity of chondrocytes within the ECM, their inability to migrate to the zone of injury, and their relative inability to regenerate large amounts of ECM, promote these defects. On the contrary, full thickness defects, which penetrate the subchondral bone, have the potential for intrinsic repair due to the communication gained with the marrow cavity and the mesenchymal stem cell (MSC) population. Nevertheless, the regenerated tissue tends to be fibroblastic in origin.

In early osteoarthritis (OA) an increase in matrix molecule synthesis is often recognised. However, once loss of matrix eventually exceeds that which is deposited, a loss of ECM results. The chondrocytes proliferate and form clusters, and cell hypertrophy is often observed. Loss of chondrocytes in the superficial zone occurs followed by fibrillation, fissuring, erosion, subsequent denudation of bone and finally deformity (Getgood *et al.*, 2009). In this pathogenic process, the subchondral bone plays a key role (Radin and Rose, 1986) even though the extent of its involvement in modulating disease progression is not completely clarified yet.

Inflammatory mediators such as cytokines play a role in this process with Interleukin 1 (IL-1) known as one of the main protagonists. IL-1 and tumour necrosis factor alpha (TNF- α) have been shown to stimulate chondrocytes to produce nitric oxide, MMPs and aggrecanases (ADAMTS), and suppress the synthesis of aggrecan and collagen.

8.2 Historical background in cartilage repair and injury: existing therapies

The existing therapies for cartilage repair are limited and physicians are often obliged to wait until the cartilage degeneration reaches the point where a partial or total joint replacement can be applied as a treatment. In fact, the clinical finding that articular cartilage 'once destroyed is not repaired' has not been changed since it was firstly observed by Hunter in 1743 (Hunter, 1743).

Conservative treatment with various medications gives only temporary relief of symptoms rather than cure, and clinicians have instead tried surgical strategies (Kheir and Shaw, 2009).

The first attempt to treat surgically cartilage injuries was made by Magnasson, who described the arthroscopic lavage and debridement more than six decades ago (Magnasson, 1946). This involves arthroscopic washout of debris and debridement of friable inflammatory tissues as well as removal of unstable chondral flaps which were proposed as the main cause of symptoms (Harwin, 1999). Good or excellent short-term results were achieved in 52% of the patients following arthroscopic joint lavage alone (Baumgaertner *et al.*, 1990). When combined, arthroscopic lavage and debridement appear to improve results and provide a better sustainable outcome (Hubbard, 1996). This treatment option is the least expensive and not technically demanding. This technique does not provide any reparative cartilage formation and cannot be used in large osteochondral defects. The outcomes after arthroscopic lavage or arthroscopic debridement in arthritic knees have been reported to be no better than those after a placebo procedure (Moseley *et al.*, 2002).

An initial surgical attempt to restore the normal articulating surface of joint cartilage was made with the introduction of Pridie's resurfacing technique. This chondral repair technique utilised the disruption of subchondral bone to induce bleeding from the bone marrow, thus promoting the regular wound-healing mechanism in the cartilage defect site (Suh and Matthew, 2000). In particular, Pridie drilled a series of holes into the subchondral bone using a quarter-inch drill. These drill holes allowed the vascular bone beneath to provide regenerative cells to the surface and the osteocartilage defect becomes filled with fibrocartilage (Pridie, 1959; Insall, 1974). Since this first tentative step, several subchondral disruption techniques have been tried in an attempt to improve the healing mechanism of damaged tissue. In particular, Johnson introduced arthroscopic abrasion arthroplasty in 1986 (Johnson, 1986). This contemporary technique has been ameliorated by Steadman (Steadman *et al.*, 1999), who uses specially designed punches to produce microfractures in the area of cartilage loss. Steadman's microfracture of subchondral bone may allow more bone marrow to enter the site of cartilage defect where more variety of marrow content can accumulate. Actually, this technique is the most utilised one (Chiang and Jiang, 2009).

The microfracture procedure is done arthroscopically. The surgeon visually assesses the defect and performs the procedure using special instruments that are inserted through three small incisions on the knee. After assessing the cartilage damage, any unstable cartilage is removed from the exposed bone. The surrounding rim of remaining articular cartilage is also checked for loose or marginally attached cartilage. This loose cartilage is also removed so that there is a stable edge of cartilage surrounding the defect. The process of thoroughly cleaning and preparing the defect is essential for optimum results. Multiple holes, or microfractures, are then made in the exposed bone about 3–4 mm apart. Bone marrow cells and blood from the holes

combine to form a 'super clot' that completely covers the damaged area. This marrow-rich clot is the basis for the new tissue formation. The microfracture technique produces a rough bone surface which the clot adheres to more easily. This clot eventually matures into firm repair tissue that becomes smooth and durable. Since this maturing process is gradual, it usually takes two to six months after the procedure for the patient to experience improvement in the pain and function of the knee. Improvement is likely to continue for about two to three years.

This last technique is relatively safe and does not pose a risk of thermal necrosis. It is now the most popular treatment option for osteochondral defects (OCD). Nevertheless, there are two main disadvantages to this technique. First, it is designed to stimulate primitive mesenchymal cells to differentiate into fibrocartilage, and the biochemical properties of the repair fibrocartilage are inferior to those of hyaline cartilage. In fact, several experimental animal studies on full-thickness cartilage repair have revealed that subchondral breaching techniques created a fibrin clot (Shapiro *et al.*, 1993). The second disadvantage is that following microfracture the extent of fill has been reported to be rarely more than 75% of the total volume of the chondral defect (Freedman *et al.*, 2003; Buckwalter *et al.*, 1994).

More recent surgical strategies aim to replace the defect with patches or grafts. Different kinds of grafts have been tested. Firstly, allografts harvested from cadaveric donors have been utilised. This technique consists of implanting the osteochondral patches which can be harvested from cadaveric donors (Ghazavi *et al.*, 1997). In fact, several scientists suggested that osteochondral allografts could represent a biological alternative to knee replacement (Fitzpatrick and Morgan, 1998). The advantage of osteochondral allografts is the ability to provide fully formed articular cartilage without specific limitations with respect to defect size. In addition, there is no concern for donor site morbidity. Subjective improvement in 75% to 85% of patients after osteochondral allograft implantation treatment of properly selected chondral lesions has been reported (Bugbee and Convery, 1999). Disadvantages of fresh osteochondral allograft include the relative paucity of donor tissue, complexities in procurement and handling and the possibility of disease transmission through the transplantation of fresh tissue (Gross *et al.*, 2002). Furthermore, if implanted freshly, cartilage allograft is challenged by immune reaction from the immersing synovial fluid, which was once considered insignificant for the avascular nature of articular cartilage (Phipatanakul *et al.*, 2004). Several studies have also confirmed a significant immunologic response in humans to histocompatibility (class I and II) presented by frozen osteochondral allografts (Strong *et al.*, 1996; Friedlaender *et al.*, 1999). This favours the use of decellularised cartilage to minimise the risk of immunologic rejection.

Autografting appears as a more promising procedure (Redman *et al.*, 2005). This technique involves the transfer of intact hyaline cartilage and the underlying subchondral bone to replace an OCD (Kish *et al.*, 1999). The success of this technique is dependent on chondrocyte viability because only living chondrocytes

can produce and maintain the extracellular matrix of proper load-bearing capacity (Buckwalter, 1997). Several studies have been conducted regarding the ideal donor site and plug size (Outerbridge *et al.*, 1995; Bobic, 1996; Hangody *et al.*, 2001; Duchow *et al.*, 2000; Feczko *et al.*, 2003).

Grafts taken from the most marginal (medial or lateral) zone of the patellar groove have been shown to provide a significantly better topographic match than grafts taken from the central intercondylar notch or from the side of the notch. It was also shown that the most inferior donor sites on the medial and lateral edges of the patellar groove provided a better match to the weight-bearing portions of the condyles than the more superior sites of the patellar groove (Bartz *et al.*, 2001). Bottomed plugs were found generally to provide much more stability than unbottomed ones. Short-bottomed plugs were more stable than long-bottomed plugs. Thus, in clinical practice it is advisable to use short-bottomed plugs (Kock *et al.*, 2006).

This technique, also called mosaicplasty, is easily applied and the grafts self-secure to the subchondral bone without additional fixing procedures or devices (Brittberg *et al.*, 1994). This is a major advantage because cartilage-only grafts are difficult to fix to the recipient site. Usually, the graft is secured to the surrounding native cartilage by sutures. This is a technically demanding and time-consuming procedure and may further damage the native tissue. In addition, the thin patch of graft without a secure osteochondral adhesion may easily detach from the underlying bone as a result of the shearing force during joint motion. The mosaicplasty grafts are firmly implanted and can be applied using minimally invasive arthroscopic procedure (Chow *et al.*, 2004). Finally, such osteochondral transplantation concurrently replaces the pathological subchondral bone that frequently exists with cartilage defects, such as those of the osteochondritis dissecans and osteonecrosis. The main disadvantage of this technique is significant donor site morbidity after osteochondral harvest. Furthermore, some studies pointed out that a significant decline in knee function appeared following osteochondral graft harvesting. Nevertheless, this technique has yielded better clinical outcomes than other surgical modalities, including abrasion arthroplasty, Pridie drilling and microfracture (Feczko *et al.*, 2003; Giannini *et al.*, 2002; Hangody and Fules, 2003; Hangody *et al.*, 2008). Unfortunately, the utility of mosaicplasty has been largely limited by the extreme shortage of autogenous donor sources in the human body (Chiang and Jiang, 2009). Consequently, mosaicplasty is valid only for smaller-sized cartilage defects (Jakob *et al.*, 2002).

Artificially constituted extra cartilage is needed to repair large defects. For this reason, an increasing interest in the use of periosteum or perichondrium is observed. Periosteum or perichondrium has been largely considered as a potential tissue to generate articular cartilage and to patch cartilage defects (O'Driscoll *et al.*, 1986; O'Driscoll *et al.*, 1988). In fact, periosteum can differentiate to produce new bone (Ritsila *et al.*, 1994). In the case of using a periosteal graft for full thickness chondral defects reconstruction, the cells may originate either from periosteal chondrocyte precursor cells or from bone marrow mesenchymal cells.

The progenitor cells residing on the cambium layer of the periosteum are induced by environmental factors at the recipient site to present as chondrocytes, whereas the periosteum itself serves as a scaffold to accommodate these cells (O'Driscoll, 1999; Ito *et al.*, 2001). Periosteal patching grafts cartilage defects with progenitor cells rather than mature chondrocytes, and the mechanism and efficiency of transformation of progenitor cells to chondrogenic cells remain unclear. Success is enhanced by knee motion after transplantation of the periosteum, but the abrasion force during motion may cause early suture failure and a subsequent unsuccessful outcome (Mukherjee *et al.*, 2001). This technique is performed in a limited number of centres, and there are few reported outcomes that have validated its use (Seradge *et al.*, 1984; Jaroma and Ritsila, 1987; Hoikka *et al.*, 1990; Homminga *et al.*, 1990). Some success has been reported but the results are less favourable than for mosaicplasty (Trzeciak *et al.*, 2006; Kon, 1981).

All these clinical experiences have led to the development of a new technique that transplants laboratory-expanded chondrocytes. This technique, called Autologous Chondrocytes Implantation (ACI), involves two stages: first a hyaline cartilage biopsy is harvested arthroscopically and the cells are cultured *in vitro*; the second stage involves re-implantation of the cultured chondrocytes into the chondral defect underneath a periosteal patch. The first reported ACI in humans was described by Brittberg in 1994 (Brittberg *et al.*, 1994). They reported that the number of cells in the initial biopsy was the key factor affecting success. To date the precise number of cells required for a successful clinical implantation of chondrocytes has not been studied sufficiently (Chen *et al.*, 1997).

In a 2004 randomised trial, comparing autologous chondrocyte implantation with microfractures showed that the improvement with microfractures was significantly better in comparison with autologous chondrocytes implantation, even though a mid- or long-term follow-up was needed to determine if one method was better than the other for generating long-lasting hyaline cartilage and alleviating symptoms (Knutsen *et al.*, 2004).

The previously reported surgical treatments are summarised in Table 8.2.

Table 8.2 Main surgical treatments for cartilage diseases from 1940 to present

	Surgical treatments
Marrow stimulation	1946: Magnasson: Arthroscopic lavage and debridement 1959: Pridie: Subchondral drilling 1986: Johnson: Arthroscopic abrasion arthroplasty 1999–present: Steadman: Microfracture
Patch grafting	Osteochondral allografts Autografts patch or Mosaicplasty Periosteal patch grafting Autologous Chondrocytes Implantation (ACI)

A natural evolution of ACI, which can be considered as the tissue engineering first generation, was achieved combining the use of ACI with a support to potentiate the development of implanted chondrocytes. This combination represents the underlying concept of tissue engineering.

8.3 First and second generation tissue engineering

A formal definition of tissue engineering has been formulated in the following terms: ‘the application of the principles and methods of engineering and life sciences towards the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes that restore, maintain or improve tissue function’ (Abatangelo *et al.*, 2002; Shalack and Fox, 1988).

It becomes clear that the main goal of tissue engineering is the reconstruction of living tissues to be used for the replacement of damaged or lost tissues/organs of living organisms. To achieve such an aim it is necessary to combine the use of cells together with natural or synthetic scaffolds in or onto which cells must develop, organise and behave as if they were in their native tissue (Abatangelo *et al.*, 2002).

In particular, the aim of cartilage engineering is the development of new cartilage as an autograft to overcome the shortage of donor material. Tissue engineering and cartilage engineering, in particular, have evolved over generations to improve the efficiency of tissue regeneration (Table 8.3).

Table 8.3 Tissue engineering evolution, from its origin to present

Tissue engineering evolution			
	Principle	Advantage	Disadvantages
First generation ACI	CELLS	Introduction of cell-based therapy	Complex suture technique Possibility of chondrocytes dedifferentiation Stiffness of joint
Second generation	CELLS+ SCAFFOLD	Support cell	Material safety requirement
Third generation	CELLS+ SCAFFOLD+ GROWTH FACTORS	Preservation of chondrogenic ability	Limited clinical information

8.3.1 First generation

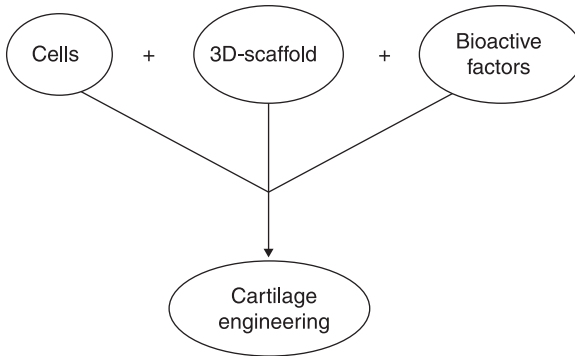
As stated previously, ACI procedure can be considered as first generation tissue engineering. In fact, the first generation cartilage engineering consisted of implanting chondrocytes or chondrogenic cells, with or without preceding culture to multiply their number into the cartilage defect, where these cells deposit ECM to constitute cartilage repair. In particular, the technique consisted in the detachment of a piece of periosteal patch which was, subsequently, sutured to the cartilage defect. Then, cultured expanded chondrocytes, suspended in aqueous culture media, were injected to the space beneath the patch. The importance of this technique – ACI technique – relied on the fact that it firstly introduced the concept of cell-based therapy to replace the conventional simple tissue grafting. Its importance is counteracted by several disadvantages. First of all, it needed to harvest periosteal patch and required complex suture techniques. Secondly, the water-seal around the patch might not maintain the chondrocytes in the defect site. Thirdly, chondrocytes could dedifferentiate during culture process and, finally, the technique needed arthrotomy and time-consuming surgery, causing stiffness of the joint (Chiang and Jiang, 2009). This first generation cartilage engineering was substituted rapidly by the second generation cartilage engineering.

8.3.2 Second generation

The passage from the first to the second generation represented a great change in the concept of cartilage regeneration. In fact, a support to permit chondrocytes to proliferate was introduced. In particular, chondrocytes were seeded onto a porous absorbable scaffold that supported the chondrocytes during the culturing process and early post implantation stage. The main aspect of this generation relies on the introduction of biodegradable scaffold to ensure cellular inhabitation at the defect site and to support cellular presentation. Furthermore, some scaffolds could be applied through minimally invasive surgery or arthroscopy to decrease post-operative joint stiffness. The major weakness of this generation is the lack of long-term follow-up clinical reports, which is partially due to the fact that few biomaterials have been approved by the FDA and consequently tested in surgery (Chiang and Jiang, 2009).

8.4 Third generation tissue engineering

A further improvement in cartilage engineering was represented by the third generation approach, in which the three elements of tissue engineering are applied: the cells; the scaffold that supports cells; and an appropriate cultivation environment (Fig. 8.3). All three elements play a key role in the development of an optimal cartilage substitute.



8.3 The three fundamental elements of third generation tissue engineering.

8.4.1 Cells

The optimal cell source for cartilage tissue engineering is still being identified. Chondrocytes, fibroblasts, stem cells, and genetically modified cells have all been explored for their potential as a viable cell source for cartilage repair.

Chondrocytes are the most obvious choice since they are found in native cartilage and have been extensively studied to assess their role in producing, maintaining and remodelling the cartilage ECM (Chung and Burdick, 2008). Fibroblasts are easily obtained in high numbers and can be directed toward a chondrogenic phenotype (Nicoll *et al.*, 2001). Actually, research is focused on stem cells, which have multi-lineage potential and can be isolated from several tissues. These progenitor cells can be expanded through several passages without loss of differentiation potential. Additionally, they can be modified genetically to induce or enhance chondrogenesis. The aim is to find an ideal cell source that can be easily isolated, is capable of expansion and can be cultured to express and synthesise cartilage-specific molecules (e.g., type II collagen and aggrecan) (Chung and Burdick, 2008).

Chondrocytes

Differentiated chondrocytes are characterised by a rounded morphology and by the production of ECM molecules such as type II collagen and sulfated glycosaminoglycans (GAGs). Chondrocytes maintain and remodel cartilage matrix tissue by a careful balance of catabolic and anabolic processes involving matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). Preserving these characteristics is crucial for chondrocytes to be used as a cell source for cartilage repair (Murphy and Lee, 2005; Chung and Burdick, 2008).

Many studies have focused on the use of articular chondrocytes as a viable cell source for cartilage repair. However, the harvesting of joint cartilage is a highly

invasive procedure accompanied by the potential for donor site morbidity and loss of function. In addition, low cell yields, low mitotic rates and low bioactivity can further limit the use of articular chondrocytes in a clinical setting. Other potential autologous chondrocyte sources in the body, including auricular, nasoseptal and costal cartilage, are being investigated, even though differences among these chondrocyte sources in terms of function, structure, and composition are known and make each unique in elaborating an ECM with discrete biochemical, physical and biomechanical properties (Chung and Burdick, 2008).

Human auricular chondrocytes were tested for cartilage repair (van Osch *et al.*, 2004; Yamaoka *et al.*, 2006). Compared with articular cartilage, auricular chondrocyte isolation resulted in cell yields twofold higher and cell proliferation rates four times faster, while retaining chondrogenic potential when cultured in alginate beads (van Osch *et al.*, 2004). With *in vivo* culture, constructs exhibited proteoglycan-rich matrices with positive type II collagen staining and faint elastin staining. In addition, auricular chondrocyte samples produced neocartilage with greater biochemical and histological similarity to that of native cartilage than articular counterparts when implanted *in vivo* (Panossian *et al.*, 2001).

Adult nasal chondrocytes have also been investigated for applications in craniofacial and plastic surgeries. They are capable of generating a matrix with high collagen II/I ratio and GAG accumulation (Kafienah *et al.*, 2002). In addition, nasal chondrocytes proliferate four times faster than articular chondrocytes in monolayer and can be seeded at very low seeding densities with an 838-fold expansion in one passage without dedifferentiation (Hicks *et al.*, 2005). Nasal chondrocytes have been successfully cultured once seeded on several scaffolds, such as collagen microcarriers (Shikani *et al.*, 2004), and in a number of systems such as PEGT/PBT block copolymer (Miot *et al.*, 2005), methylcellulose (Vinatier *et al.*, 2007), and HYAFF®11, a hyaluronic acid (HA) ester (Aigner *et al.*, 1998). These cells show good viability and produce an ECM-rich tissue with high expression of type II collagen under appropriate culture conditions. Additional studies show that nasal chondrocytes respond to growth factors such as TGF- β 1, FGF-2, bone morphogenic protein-2 (BMP-2), and IGF-1 (Richmon *et al.*, 2005; van Osch *et al.*, 2001), in serum-free culture with enhanced proliferation and/or matrix deposition.

In chondrocyte source comparison studies, bovine nasal, articular, costal and auricular chondrocytes were grown on a scaffold obtained by blending poly (L-lactic acid) and poly- ϵ -caprolactone for four weeks (Isogai *et al.*, 2006). Growth rates and gene expression varied with cell type and the highest expression of type II collagen and aggrecan was found for costal chondrocytes, followed in order of decreasing expression by nasoseptal, articular and auricular chondrocytes. The construct size also varied, with auricular constructs having the largest diameter and costal constructs the greatest thickness. Another study looked at the effect of growth factors (GFs) on auricular, nasal and costal chondrocytes and showed that all cell types exhibited increased proliferation, GAG/DNA content

and up-regulation of type II collagen expression after GF supplementation. However, re-differentiation was only achieved in auricular and nasal chondrocyte cell pellets (Tay *et al.*, 2004) even if articular, auricular and costal chondrocytes were able to form new cartilaginous matrix when cultured in fibrin glue-cartilage composites *in vivo* (Johnson *et al.*, 2004).

As mentioned previously, efforts in cartilage regeneration have focused also on chondrocytes isolated from immature animals. These neonatal and young chondrocytes have faster growth rates, the capacity for rapid *in vitro* expansion and greater chondrogenic potential (increased Sox 9 and type II collagen expression) over chondrocytes from older donors (Hidaka *et al.*, 2006). Although these traits are advantageous for expanding chondrocytes and producing ECM-rich neocartilage, the use of immature cartilage in a clinical setting for older patients may not be possible. Thus, the proliferative and chondrogenic potential of adult, osteoarthritic and even cryogenically preserved chondrocytes are also explored as alternative cell sources. However, research is still needed to optimise culture techniques for these aged cells and to define their limitations and potential use in a clinical setting (Barbero *et al.*, 2004; Giannoni *et al.*, 2005; Terada *et al.*, 2005; Tran-Khanh *et al.*, 2005; Mesa *et al.*, 2006; Marlovits *et al.*, 2003).

Osteoarthritic (OA) chondrocytes have also been investigated for their potential in cartilage repair. Both *in vitro* and *in vivo* culture of OA cells on HYAFF®11 yielded positive staining for type II collagen and sulphated proteoglycans and negative staining for type I collagen (Wenger *et al.*, 2006). Furthermore, OA articular chondrocytes can be transduced with Sox 9 via adenoviral and retroviral vectors to stimulate type II collagen expression and deposition in both monolayer and alginate bead cultures (Li *et al.*, 2004).

Finally, cryogenically preserved cells may provide an alternative source for cartilage regeneration. Septal chondrocytes frozen for three years showed evidence of hyaline growth on knitted polygalactin 910 woven mesh scaffolds after six weeks of culture in a slowly turning lateral vessel (Gorti *et al.*, 2003).

Fibroblasts

Skin presents a minimally invasive and relatively abundant source of fibroblasts for tissue engineering. Fibroblasts can be redirected towards a chondrocytic phenotype when cultured under the appropriate conditions. Several evidences of this behaviour have been proved (French *et al.*, 2004; Yates *et al.*, 2004; Lee *et al.*, 2001). When injected into cartilage, defects showed evidence of newly formed hyaline cartilage after six weeks (Lee *et al.*, 2001).

Stem cells

Recently, stem cells have been indicated as an alternative to autologous chondrocytes. Stem cells are pluripotent cells that can be differentiated down

multiple cell lineages given the appropriate cues. In 1998, bone-marrow derived stem cells were found to undergo chondrogenesis when cultured in cell aggregates in the presence of TGF- β 1 (Johnstone *et al.*, 1998). More recently, several sources of stem cells investigated for repair cartilage have been found. Among them, adipose tissue (Zuk *et al.*, 2001), muscle (Nawata *et al.*, 2005; Adachi *et al.*, 2002), synovium (Park *et al.*, 2005; Sakaguchi *et al.*, 2005; Yokoyama *et al.*, 2005) and periosteum (Fukumoto *et al.*, 2003) have been identified as sources of stem cells.

Finally, embryonic stem cells (ESCs), which are obtained from the inner cell mass of blastocyst stage embryos, have been tested for cartilage repair. These cells are capable of many doublings and have the ability to differentiate into all somatic cell types. Although ESCs are appealing as a cell source for their vast proliferation capabilities, difficulties in ESC selection and purity, as well as antigenicity and ethical issues, may hinder their clinical use (Toh *et al.*, 2007; Vats *et al.*, 2006).

8.4.2 Materials

Several materials have been used for cartilage regeneration. Scaffolds are able to furnish a three-dimensional environment, which is fundamental for the production of cartilaginous tissue. An ideal scaffold should have specific requirements:

- controlled degradation (slow degradation may impede new cartilaginous ECM production, while fast degradation may compromise structural support and shape retention)
- promote cell viability, differentiation and ECM production
- allow for the diffusion of nutrients and waste products
- adhere and integrate with the surrounding native cartilage
- span and assume the size of the defect
- provide mechanical integrity depending on the defect location (Chung and Burdick, 2008).

In the last few decades, an enormous range of natural and synthetic materials have been investigated as scaffold for cartilage repair. Among the natural polymers explored as bioactive scaffolds for cartilage engineering, the most analysed are alginate, agarose, fibrin, hyaluronic acid (HA), collagen, gelatin, chitosan, chondroitin sulphate and cellulose. The main advantage offered by natural polymers is that they can interact with cells via cell-surface receptors and regulate or direct cell function. However, due to this interaction, these polymers may also stimulate an immune system response; thus, antigenicity and disease transfer are the main disadvantages when using these biomaterials. In addition, natural polymers may be mechanically inferior and subject to variable enzymatic host degradation.

Synthetic polymers show a more controllable and predictable behaviour, since their chemical composition and properties can be easily modified, altering, as a consequence, their mechanical and degradation characteristics. Synthetic

polymers currently explored for cartilage repair include: poly (α -hydroxy esters), PEG, poly(NiPAAm), poly(propylene fumarates) and polyurethanes. However, synthetic polymers do not benefit from direct cell-scaffold interactions, which play a role in adhesion, cell signalling, directed degradation and matrix remodelling. In addition, degradation by-products may be toxic or induce an inflammatory response (Chung and Burdick, 2008). Finally, scaffold architecture also plays a major role in dictating cellular behaviour. Scaffolds can be categorised into hydrogels, sponges and fibrous meshes.

Hydrogels

Hydrogels are water-swollen networks which can be used as injectable scaffolds since they easily fill defects of any size and shape and may be implanted in a minimally invasive manner (Leone *et al.*, 2008). Hydrogels easily permit the diffusion of nutrients and waste. Furthermore, they represent an ideal three-dimensional environment in which cells can be suspended, retaining a rounded morphology that may induce a chondrocytic phenotype. Hydrogels are also capable of transducing mechanical loads to exert controlled forces on encapsulated cells, similar to physiological conditions. PEG, which is a relatively inert polymer, is able to induce chondrogenesis when crosslinked into hydrogels (Bryant and Anseth, 2002). Further modifications to PEG have improved cartilage tissue growth (Bryant and Anseth, 2002; Bryant and Anseth, 2003; Bryant *et al.*, 2003; Rice and Anseth, 2004; Lee *et al.*, 2006).

HA, a linear polysaccharide found in native cartilage, can also be crosslinked to obtain a scaffold for cartilage tissue regeneration. It functions as a core molecule for the binding of keratin sulphate and chondroitin sulphate in forming aggrecan in cartilage and degrades primarily by hyaluronidases found throughout the body. HA plays a role in cellular processes such as cell proliferation, morphogenesis, inflammation and wound repair (Chen and Abatangelo, 1999), and may function as a bioactive scaffold. Cell surface receptors for HA (CD44, CD54 and CD168) allow for cell/scaffold interactions. Furthermore, HA can be easily modified with different functional groups and, by varying them, a wide range of properties can be obtained (Nettles *et al.*, 2004; Smeds *et al.*, 2001; Burdick *et al.*, 2005).

Chondroitin sulphate, another major constituent of cartilage, can also be crosslinked to produce hydrogels able to support viable chondrocytes (Li *et al.*, 2004).

Fibrin glue, a natural polymer formed from the polymerisation of fibrinogen with thrombin, elicits good biocompatibility as a wound adhesive and can facilitate cell–matrix interaction via integrin binding (Mosesson, 2005). It is attractive as a natural scaffold because it can be made from autologous blood. Fibrin glue has also been combined with other polymers, such as polyurethane, and improved cell seeding viability and distribution, increasing the expression of aggrecan and type II collagen (Lee *et al.*, 2005).

Type I and type II collagen scaffolds have also been realised. A type I collagen gel seeded with autologous chondrocytes has been used to treat full thickness defects in rabbits, with newly regenerated cartilaginous tissue formation seen after six months and tissue organisation after 12 months (De Franceschi *et al.*, 2005).

Gelatin, which is derived from collagen, is also biocompatible and can be crosslinked with visible light but it showed some diffusion limitations (Hoshikawa *et al.*, 2006).

Alginate is a polyanionic polymer found in brown algae which can be crosslinked with bivalent cations to form stable ionically crosslinked gels. Different formulations have been tested showing good ability in supporting chondrocytes and inducing chondrogenesis (Awad *et al.*, 2004; Erickson *et al.*, 2002; Lin *et al.*, 2005; Connelly *et al.*, 2007; Iwasaki *et al.*, 2004; Wayne *et al.*, 2005). Despite its advantages for studying *in vitro* chondrogenesis, limitations to alginate gels include low mechanical properties and slow degradation rates.

Agarose is a linear polysaccharide derived from seaweeds that forms gels in water when cooled. It has been widely used to study chondrocyte response to deformational loading since it is able to transmit applied mechanical forces to cells during compression (Genes *et al.*, 2004). Chitosan, a biosynthetic polysaccharide derivative of chitin, and chitosan hybrid hydrogels support normal chondrocyte phenotypes. In fact, as described by Chen and Cheng, a thermo-responsive chitosan-graft-PNIPAM (CPN) copolymer hydrogel with comb-like structure can be obtained by conjugating carboxylic acid-ended poly(N-isopropylacrylamide) (PNIPAM-COOH) to the amine group of chitosan, in the presence of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS). This chitosan-based hydrogel shows beneficial effects on cell phenotypic morphology, maintenance of cell viability and stimulation of initial cell-cell interaction (Chen and Cheng, 2006).

Some synthetic copolymers have also been investigated as thermoreversible hydrogels, showing gelation above their lower critical solution temperature (LCST). In particular, N-isopropylacrylamide and acrylic acid copolymer p(NiPAAm-co-AAC) and poly(propylene fumarate-co-ethylene glycol) (p(PF-co-EG)) are capable of retaining chondrocyte phenotype and viability (Au *et al.*, 2003; Fisher *et al.*, 2004).

Finally, self-assembling peptides constitute another class of biomaterials that can be made into hydrogels, and form by amino acid sequences of alternating ionic hydrophobic and uncharged hydrophilic side groups. These self-assembling peptide hydrogels form stable β -sheets of apparent interwoven nanofibres when exposed to an electrolyte solution and are capable of rapidly encapsulating chondrocytes at physiological electrolyte concentrations and pH levels. In particular, RAD-, ELK- and EAK-based peptides form strong β -sheet secondary structures in aqueous solutions (Holmes, 2002; Zhang *et al.*, 1993; Kisiday *et al.*, 2002).

To promote and ensure cellular adhesion and proliferation a sufficient porosity in the scaffold must be present. Porous hydrogels can be considered and are called sponges.

Sponges

To date, several methods have been employed to manufacture sponges, including porogen leaching, freeze-drying and gas foaming. Numerous materials have been used to fabricate sponge scaffolds, including poly (α hydroxy esters) (Sato *et al.*, 2004; Chen *et al.*, 2004), alginate (Miralles *et al.*, 2001), polyglactin/polydioxanone (Barnewitz *et al.*, 2006), HA (Barbucci and Leone, 2004) and gelatine (Xia *et al.*, 2004). A new biodegradable elastomer scaffold from poly(1,8 octanediol citrate) (POC) has been obtained by a salt leaching procedure and supported the growth of chondrocytes *in vitro* (Kang *et al.*, 2006). This POC scaffold is capable of complete recovery from compressive deformation, and may provide good structural support in the mechanically loaded knee environment.

Chitosan can also be formed into sponges via freeze-drying and lyophilisation (Nettles *et al.*, 2002). Chitosan and chitin hybridised scaffolds in various compositions were investigated as potential scaffolds (Kuo and Lin, 2006). They showed type II collagen, elastic fibres and GAG production, with total GAG content around 90% of that found in native cartilage (Xia *et al.*, 2004).

Silk fibroin is composed of a filament core protein called fibroin with a glue-like coat of sericin proteins. Sponges can be formed from silk fibroin by a solvent casting/salt leaching method that supports chondrocytes (Hofmann *et al.*, 2006; Wang *et al.*, 2006).

Collagen and collagen hybrid sponges have also been formed. They are able to support chondrocyte growth and phenotype retention (Vickers *et al.*, 2006). In particular, chondrocytes seeded on type II collagen-GAG scaffolds with low crosslinking densities experienced cell-mediated contraction, an increase in cell number, enhanced chondrogenesis and increased degradation rates (Vickers *et al.*, 2006).

Meshes

Finally, natural and synthetic polymers can be fabricated in the form of meshes, which are networks of woven and non-woven fibres. Non-woven meshes have high void volumes and surface areas that are well suited for tissue regeneration, whereas woven meshes exhibit greater strengths and can be made in a wide range of porosities. In general, these prefabricated forms can be used to culture cells *in vitro* to create mechanically stable scaffolds and then be implanted *in vivo* for complete repair. A drawback to prefabricated scaffolds is a difficulty in filling irregularly shaped defects, where incomplete contact with surrounding cartilage may hinder complete integration. Three-dimensional fibre deposition is one

technique used to form scaffolds with regulated patterns (Chung and Burdick, 2008). A scaffold with biomechanical properties comparable to bovine articular cartilage using three-dimensional fibre deposition of poly(ethylene oxide) terephthalate/poly(butylene) terephthalate (PEOT/PBT) was obtained by Moroni (Moroni *et al.*, 2006).

Recently, electrospinning has generated much interest to produce biomaterials with nano-scale polymer fibres that mimic collagen fibrils in cartilage ECM (Li *et al.*, 2003). Fibres are generated as the surface charge of the polymer droplet overcomes its surface tension in an applied electric field, causing an instability that creates jets of polymer that can then be collected as solvent evaporates. Advantages in using electrospun scaffolds include high surface area to volume ratios and fully interconnected pores and the ability to create aligned fibres. By collecting the nanofibres on a rotating mandrel, aligned fibrous scaffolds can be fabricated and can mimic the anisotropic morphology of some tissues. These nanofibrous scaffolds support chondrocytes and stem cells (Li *et al.*, 2003; Li *et al.*, 2005).

The most commonly used meshes are made of poly(α -hydroxy esters). These meshes have been used since the early 1990s for cartilage regeneration and include poly(lactic acid) (PLA), poly(glycolic acid) PGA and their copolymers (PLGA). PGA is the most hydrophilic of this group and degrades into a natural metabolite that is completely resorbed through metabolic pathways. On the other hand, PLA, with an additional methyl group, is more hydrophobic, resulting in slower degradation. Copolymers of PLA and PGA can be optimised for mechanical and degradation properties (Chung and Burdick, 2008).

Poly(caprolactone) (PCL) is another member of the poly(α hydroxy ester) family with slower degradation kinetics. Recently, PCL has been electrospun to form nanofibrous scaffolds capable of supporting proliferating chondrocytes that produce proteoglycan-rich matrices (Li *et al.*, 2003).

Several natural materials have also been processed as fibrous scaffolds, including cellulose (Muller *et al.*, 2006) and HA derivatives (Giroto *et al.*, 2003; Grigolo *et al.*, 2002; Radice *et al.*, 2000). Non-woven HA esters, HYAFF® derivatives, are semisynthetic, resorbable meshes that support cell adhesion, proliferation, and production of cartilage-specific ECM *in vitro* (Grigolo *et al.*, 2002; Radice *et al.*, 2000) and *in vivo* (Radice *et al.*, 2000). In a clinical setting, Hyalograft® C (a graft composed of autologous chondrocytes grown on a HYAFF®11 scaffold) has been used to treat a number of human articular cartilage defects (Gobbi *et al.*, 2006; Hollander *et al.*, 2006). Hyalograft® C repaired cartilage showed significant improvements over pre-operation assessments with cartilage regeneration even in joints with progressed osteoarthritis (Li *et al.*, 2003; Hollander *et al.*, 2006).

8.4.3 Culture conditions

Several factors can be selected and adjusted to promote cell adhesion and proliferation. In particular, the friction caused by surface motion, compressive

stress, oxygen tension, hydrostatic force and dynamic mechanical stimulation should be considered. Among them a crucial role is played by mechanical stimuli. In fact, under physiological conditions, articular cartilage is subjected to various mechanical stimuli, such as hydrostatic pressure, as well as compressive and shear strain. The composition and structural organisation of the ECM of articular cartilage are responsible for its biomechanical properties. The type II collagen network confers to cartilage its strength against tensile forces and the highly hydrated proteoglycans provide its compressive resistance (McMahon *et al.*, 2008). Interestingly, physiological loading is a pivotal factor influencing the chondrogenic differentiation of MSCs during articular cartilage development. In addition, mechanical stimuli on chondrocytes have been reported to be essential for the maintenance of cartilage integrity (Grodzinsky *et al.*, 2000). Varying loads that have been applied on cartilage tissue engineering constructs resulted in a different structural organisation of cartilage ECM proteins, such as collagens and glycosaminoglycans (Arokoski *et al.*, 2000). Given that mechanical stimuli widely influence cartilage formation, they are an important factor to take into account in the development of cartilage engineering products. Consequently, to address this issue, all the above-cited parameters have been integrated into bioreactors, in which specific physicochemical parameters, mechanical stimuli and fluid flow can be controlled and applied to cell-seeded scaffolding biomaterials. The different bioreactor systems used to mechanically stimulate tissue engineering constructs have been reviewed by Schulz (Schulz and Bader, 2007). A bioreactor that is able to accommodate the environmental factors mentioned above would be a crucial tool for cartilage engineers and would help to move tissue engineering from the laboratory to the bedside.

Apart from an appropriate scaffold, in the case of MSC use, growth and differentiation factors are required to induce differentiation and the maintenance of chondrocyte phenotype.

Growth factors

Several growth and differentiation factors that are involved in regulating cartilage development and homeostasis of mature articular cartilage have been identified. However, long-term successful cartilage repair still requires identification and application of those specific growth factors that are able to induce and maintain the chondrocytic phenotype. Five families of factors are particularly relevant for cartilage formation (Vinatier *et al.*, 2009).

The transforming growth factor- β superfamily

The transforming growth factor (TGF)- β family of polypeptides includes TGF- β , bone morphogenetic proteins (BMPs), activins and inhibins. These molecules initiate signalling from the cell surface by interacting with type I and type II

receptors, depending on the ligands they bind. The TGF- β family includes five members (TGF- β 1–5), which are predominantly produced in bone and cartilage. Active TGF- β 1, 2 and 3 are generally considered to be potent stimulators of proteoglycans and of type II collagen synthesis in chondrocytes and are able to induce the chondrogenic differentiation of MSCs *in vitro*. *In vivo*, TGF- β 1 can induce the chondral differentiation of MSCs to form ectopic cartilage and was able to repair a full-thickness cartilage defect by improving chondrocyte integration into the endogenous tissue (Canalis *et al.*, 2003; Grimaud *et al.*, 2002; Fan *et al.*, 2006). BMPs constitute a large sub-class of 20 polypeptides that have essential roles in chondrogenesis and osteogenesis during skeletal development. Several BMPs, including BMP-2, -4, -6, -7, -13 and -14, can stimulate the chondrogenic differentiation of MSCs (Sekiya *et al.*, 2005), and enhance the synthesis of collagen II and aggrecan by chondrocytes *in vitro* (Grunder *et al.*, 2004). The use of BMP-2, -4 and -7 has been approved for some clinical applications (Garrison *et al.*, 2007), but their potential to enhance cartilage repair still needs to be validated in humans.

Fibroblast growth factor family

In vertebrates, the fibroblast growth factor (FGF) family comprises 22 structurally related proteins that bind one of four FGF receptors (FGFRs). The importance of FGF signalling in skeletal development is highlighted by the number of dysplasias that have been attributed to specific mutations in the genes encoding the FGFR1, -2 and -3. Genetic studies have also identified defects in chondrogenesis in mice lacking FGF18 (Ornitz, 2005). One study also reported that FGF18 might stimulate repair of damaged cartilage (Moore *et al.*, 2005). The contradictory results for the potential role of various FGFs in chondrogenesis highlight the need for a better characterisation of the signalling pathways that are activated by FGFs to be able to fully understand how they affect FGF activity.

Insulin-like growth factor family

The insulin-like growth factor (IGF) family comprises the ligands IGF-1 and IGF-2, the receptors IGF1R and IGF2R, at least six different IGF-binding proteins (IGFBPs) and multiple IGFBP proteases, which regulate IGF activity. IGF-1 is considered an essential mediator of cartilage homeostasis through its capacity to stimulate proteoglycan synthesis and to promote chondrocyte survival and proliferation (Davies *et al.*, 2008). IGF-1 also induces the differentiation of MSCs towards the chondrocytic phenotype (Uebbersax *et al.*, 2008).

Wingless family

In vertebrates, the wingless (Wnt) family contains more than 20 members that exhibit distinct functions in development. Wnts bind the receptors Frizzled (Fzd)

and cooperate with the transmembrane molecules, low density lipoprotein (LDL)-receptor-related protein 5 (LRP5) and LRP6 (Clevers, 2006). Various Wnt members are involved in both early and late skeletal development and have a role in the control of chondrogenesis (Church *et al.*, 2002). Overall, it seems that the Wnt network has dual roles in cartilage. In fact, it is an important regulator of chondrocyte development but at the same time deregulation of Wnt signalling might lead to disease, in particular to osteoarthritis.

Hedgehog family

In mammals, the hedgehog (Hh) family comprises three members, Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh). In concert with other signalling molecules, Ihh has been found to function as a central regulator of endochondral ossification, coordinating chondrocyte proliferation, differentiation and ossification of the perichondrium (St-Jacques *et al.*, 1999).

Some other nonproteinaceous chemical factors have also been shown to promote chondrogenesis, including prostaglandin E2, thyroxine, 1,25 dihydroxy vitamin D, ascorbic acid, dexamethasone, ethanol, staurosporine, dibutyryl cAMP, concanavalin A and vanadate (Biddulph *et al.*, 2000; Miyamoto *et al.*, 2003; Siebler *et al.*, 2002; Tsonis, 1991; Harmand *et al.*, 1984; Farquharson *et al.*, 1998; Kulyk, 1991; Revillion-Carette *et al.*, 1986; Mikhailov and Gorgolyuk, 1988; Kato *et al.*, 1987). These chemicals are less labile, with a longer half-life than the protein-based factors, and are thus advantageous for prolonged *in vitro* culture over several weeks.

Regardless of the nature of the cells, standard culture conditions require the presence of serum, basically of bovine origin. The risk of undesired pathogen transmission has been debated when the cells are implanted to humans. Autologous serum-supplemented culture medium has become the state of the art for ACI, but serum-free culture is more attractive (Dumont *et al.*, 1999). The avascular condition of natural cartilage does not suggest that serum is needed to support the chondrocytes. One study has even indicated that serum hinders the chondrogenic ability of chondrocytes (Malpeli *et al.*, 2004). Serum-free culture is worthy of further development to develop regenerated cartilage for clinical application.

The third generation cartilage engineering approach is summarised in Table 8.4. In Table 8.5 the products actually used and firms involved in the production of the materials for cartilage regeneration are reported.

8.5 Future trends

8.5.1 Fourth generation: gene therapy

To date there is a very high number of patents (more than 8000) concerning gene therapy for cartilage regeneration. In the opinion of many scientists, healing and organ replacements may not work without the introduction of additional properties

Table 8.4 Third generation tissue engineering

	Cell type	Source	
CELLS	Chondrocytes	Human articular chondrocytes	
		Human nasal chondrocytes	
		Human costal chondrocytes	
		Human auricular chondrocytes	
		Bovine nasal chondrocytes	
		Neonatal/young chondrocytes	
		Aged chondrocytes	
		Osteoarthritic chondrocytes	
		Cryogenically preserved chondrocytes	
	Fibroblasts	Adipose	
SCAFFOLDS	Stem cells	Muscle	
		Synovium	
	Embryonic cells	Periosteum	
		Inner cell mass of blastocyst stage embryos	
	Origin	Material	Form
	Natural	Alginate	Hydrogel; Sponge
		Agarose	Hydrogel
		Fibrin	Hydrogel
		HA	Hydrogel; Sponge
		Collagen	Hydrogel
		Gelatin	Sponge
		Chondroitinsulphate	Hydrogel
		Cellulose	Hydrogel; Meshes
		Silk Fibroin	Sponge
		PLA	Meshes
	Synthetic	PGA	Meshes
		PLGA	Meshes
		PCL	Meshes
		PEOT/PBT	Meshes
		p(NiPAAm-co-AAC)	Hydrogel; Sponge
		(p(PF-co-EG))	Hydrogel; Sponge
		self-assembling peptides: RAD-, ELK-, and EAK-based peptides	Hydrogel; Sponge
		poly (α hydroxy esters)	Meshes; Sponge
		polyglactin/ polydioxanone	Sponge
		poly(1,8 octanediol citrate) (POC)	Sponge
Nature	Physico-chemical factors	Chitosan	Hydrogel; Sponge
		HYAFF®	Meshes
		PEG	Hydrogel
		Stymul	
		Friction	
		Compressive stress	
		Oxygen tension	
		Hydrostatic force	

(continued)

Table 8.4 Continued

	Cell type	Source
CULTURE ENVIRONMENT	Protein-based factors	Dynamic mechanical stimulation transforming growth factor (TGF)- β family fibroblast growth factor (FGF) family insulin-like growth factor (IGF) family Wingless (Wnt) family Hedgehog (Hh) family
	Non-protein-based factors	prostaglandin E2 thyroxin 1.25 dihydroxy vitamin D dexamethasone staurosporine ethanol dibutyl cAMP vanadate concanavalin A ascorbic acid

Table 8.5 Main companies involved in the production of cartilage substitutes

Company	Product
Biomet (Cartilix)	Chondun®
Histogenics	NeoCart®
	VeryCart®
Genzyme Corporation	Synvisc®
	MACI®
Osiris	Chondrogen®
Mesoblast	Chondroelect®
Anika Therapeutics, Inc., distributed by DePuy Mitek, Inc	Orthovisc®
Smith & Nephew	Supartz®
Ferring Pharmaceuticals Inc	Euflexxa™
Sanofi-Aventis	Hyalgan®

supplanted onto the genome of the implanted cells or tissues (Gaissmaier *et al.*, 2008). There is a wide range of approaches, from activation of hypermetabolism for a certain period of time to accelerating regenerative processes to introduce genes that compensate for genetic defects responsible for patients' diseases. Problems in cartilage regeneration centre around the paucity of cells typical for joint and intervertebral disc cartilage and their usually bradytrophic metabolism during adulthood.

There are two possible solutions to the problem: finding cells that possess high metabolic properties or hormones that speed up regenerative metabolism within the repaired defect. The first solution could be answered by either applying mesenchymal precursor cells that multiply and differentiate under the guidance of introduced conditions (hormones, mechanical stimuli, etc), or using foetal or pre-puberty donor chondrocytes that possess an inherently high proliferative and extracellular matrix-building capacity (Mirmalek-Sani *et al.*, 2006; Murphy *et al.*, 2002; Parsch *et al.*, 2001). Utilisation of immature chondrocytes from very young human donors is an option that is currently being tested in an FDA-approved clinical trial started in November 2006 (Gaissmaier *et al.*, 2008). A foreseeable even higher shortage than for adult donor tissue may not make this procedure a standard option for future methodology, aside from the significant ethical implications. The second solution, hormonal stimulation, will either need topical application of hormones or topical expression of hormones in competent cells, the cells ideally being implanted or residual cells within the defect area. Most technical problems centring on gene delivery (route of transfection) may be resolved in the near future. Key issues that remain are controlling the number of copies delivered to a single cell, site of integration into the host-cell genome, promoter-steered control of expression levels and time of residence or activity for the supplanted DNA within the host genome.

However, gene therapy measures will always remain orphan drug approaches since the genetic background of each patient differs. Taken together, while the scientific rationale is most convincing, the approach is technically very demanding and the necessary background knowledge exceeds present experience. The high risk currently associated with gene therapy causes an imbalance between the impact of the disease on the patient and the health risks associated with the therapy.

Currently, the answer would not be given by scientists and physicians but by legislation: producing synthetic implants to be introduced into a human body is much less regulated and scrutinised than even autologous cell/tissue-based constructs. Consequently, development and production of entire joint replacement sets from metallic, ceramic and plastic materials are much more profitable than the approach to biological repair of joint surface, cruciate ligament or intervertebral disc damage. In fact, when the constructed cartilage tissue is considered for clinical use, the safety of the whole process has to be considered. Furthermore, it has to be conducted with expensive laboratory facilities that meet the high standard of good tissue practice. In addition, all reagents involved in the process should be proven as safe for human use. When developing a system to regenerate cartilage for clinical application, we should always consider its safety and simplicity, even if it is necessary to compromise the quality of the regenerated tissue.

Nevertheless, cartilage tissue engineering is a potentially very important technique for the regeneration of cartilage tissue damaged due to the disease. The final aim is that joint replacement procedures are based on individually reconstructed biological joint parts.

8.6 References

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Hydrogels for wound healing applications

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Abstract: A great deal of effort has been directed during the last few years towards the development of new artificial wound coverings which will meet the requirements faced in the treatment of major skin wounds. Research has mainly been focused on achieving the specifications of an ideal wound dressing. This chapter discusses the use of hydrogels, both natural and synthetic, that can be used for wound healing applications. This chapter also reviews the pH and thermosensitive smart polymers that may be used as wound dressings. The various novel techniques in this fascinating area are also being described.

Key words: hydrogels, grafting, blending, wound dressing, wound healing, gene therapy, stem cell therapy, skin engineering, pH and thermosensitive polymers.

9.1 Introduction

Healthcare is an essential aspect of human survival. Several biopolymers have generated interest in a number of biomedical applications. Wound management is one such area where management of cuts, ulcers and sores needs dressings which help in promoting rapid wound healing in order to obtain both functional and cosmetic results.¹ There are many kinds of products used for wound management, such as staples or sutures, dressings or bandages, surgical sealants and adhesives, skin substitutes and other biomaterials.²

Human skin provides an effective barrier to microbial penetration and subsequent infection. However, once the wound has been developed in this barrier, the infection chance increases. In the case of chronic wounds, the colonization and infection potential increases as the result of the presence of avascular eschar, which provides an environment for the uninhibited growth of microorganisms.³ The rate of infection is related to the type of wound, general wound care and general health of the patient.^{4,5} For avoiding infection, good clinical practices are needed. The management of chronic wounds is a very costly practice and it also places an enormous drain on healthcare resources; studies have calculated the cost of wounds to the NHS to be about £1 billion a year.⁶ So for lowering this cost, wound management products are needed that are more economical and effective. In this chapter, we will discuss the wound dressings that will provide an optimal healing environment to the wound. A wound dressing is an adjunct used for application to a wound in order to promote healing and prevent infection and further damage. It is designed to be in direct contact with the wound, so it is different from a bandage

in the manner that bandages are normally used to hold a dressing in place. A wound is a break in the epithelial integrity of skin and may be accompanied by disruption of the structure and function of underlying normal tissue. Wounds can be divided into four categories based on their appearance and stage of healing: necrotic, sloughing, granulating and epithelializing wounds.⁷ Wounds cause discomfort and are more prone to infection and other troublesome complications.⁸ Some diseases such as diabetes and ischaemia and conditions such as malnourishment, ageing, local infection and local tissue damage due to burn lead to delay in wound healing. Infection is a major complication of burn injury and is responsible for 50–75% of hospital deaths.⁹

Human skin has one of the greatest abilities to regenerate itself compared to all other tissue in our body. It continuously replaces old cells with new ones, enabling it to repair itself. Wound healing is a complex, physiologic process, which consists of three overlapping phases: inflammatory, proliferative and remodeling phases. The normal healing process starts as soon as the tissue is injured. As the blood components come at the site of injury, the blood platelets come into contact with collagen and extracellular matrix and release clotting and growth factors. During the inflammation process, neutrophils are the first leukocytes which come at the site of injury to rid it of bacterial contamination. The monocytes and their conversion to macrophages initiate tissue repair by releasing a number of biologically active substances and growth factors that are necessary for the initiation of the tissue formation process. In the third process, fibroblasts proliferate and migrate into the wound space and start the deposition of the loose extracellular matrix. Endothelial cells grow into a wound simultaneously with fibroblasts and undergo angiogenesis. Finally, tissue remodeling takes place to reconstruct the basement membrane by the differentiation of keratinocytes as well as the formation of follicle cells.^{10,11,12}

During the process of wound healing, scars develop. This mainly occurs after a surgical incision or the healing of a wound. Scarring is less when the damaged outer layer of skin is healed by rebuilt tissue. When the thick layer of tissue beneath the skin gets damaged, deep rebuilding is more complicated. In order to cover it, our body lays down collagen fibers and this results in a highly obvious scar, i.e., a permanent reminder of the injury. Thus, a dressing that can induce scarless healing is needed.

Historically, a dressing was a piece of material, sometimes cloth, but the use of cobwebs, leaves and honey has also been described, while modern wound dressings include gauzes, gels, foams, hydrocolloids, alginates and hydrogels. Wound dressings are passive, active or interactive. Passive dressings simply provide cover, while active or interactive dressings are capable of modifying the physiology of the wound environment. Interactive dressings include hydrocolloids, hydrogels, alginates, foam dressings and antimicrobial dressings.^{13,14,15}

Traditionally dry wound dressings are considered to be good for healing wounds, i.e., the wound should be covered with gauze or left open. But it has been observed

by Winter¹⁶ that when a wound is left open to air without any dressing, a scab, i.e., a dry layer, covers the wound and decreases the rate of epithelialization. On the other hand, if a moist dressing is used in place of dry dressings, the scab will not be formed and the rate of healing would increase as moist dressings provide low oxygen tension, which helps in wound healing. These dressings not only keep cells viable to enable them to release growth factors while maintaining contact between them and the healing tissues, but may also modulate or stimulate their proliferation. These dressings decrease the pain at rest, during ambulation and during dressing changes. At the same time, the moist environment allows rapid and efficient delivery of any added antimicrobial agent, thus preventing the wound from becoming infected. Dressings that create and maintain a moist environment are now considered to provide the optimal conditions for wound healing.

9.2 Requirements of an ideal wound care system

The following characteristics should be present in the ideal wound care system. First, it should be capable of maintaining high humidity at the wound site, as surface drying not only impedes delivery of nutrients and immune defenses to the wound surface but also markedly impedes the ability of cells to migrate across the wound surface. Epithelial cells need a moisture layer to migrate and spread because cell growth needs moisture. Cells can grow, divide and migrate at an increased rate to optimize the formation of new tissue. Moist wound treatment is known to prevent formation of a scab, allowing epithelial cells to spread horizontally outwards through the thin layer of wound exudate to rapidly close the wound. Also, up to 50% faster wound healing (epithelialization and dermal repair) occurs in a moist environment. The environment should be non-toxic and non-allergenic so that it would not cause any infection at the wound site. It can be removed without causing trauma to the wound, i.e., painless removal. It should be impermeable to bacteria, i.e., antimicrobial, thermally insulating and soft to the touch. It should allow proper gaseous and water vapor exchange. It should also promote tissue reconstruction processes and, last but not least, it should be cost effective.^{14,17,18} Out of all the dressings – hydrocolloids, alginates and hydrogels – each one has its own advantages and limitations, but hydrogels are best and have all the characteristics that are needed in an ideal wound dressing. All of the above mentioned characteristics can be achieved in hydrogel wound dressings.

9.3 Hydrogels for wound healing applications

Hydrogels are either natural or synthetic cross-linked polymers used in a variety of biomedical fields. It consists of a matrix of insoluble polymers with about 96% water content. These hydrogels can donate water to the wound site and thus help in maintaining a moist environment, which helps in faster wound healing. These are used in the formation of drug-delivery vehicles, wound dressings, contact

lenses and as electrodes or sensors.¹³ Examples of hydrogels include Aquaform, Intrasilite, GranuGel, Nu-Gel, Purilon and Sterigel.

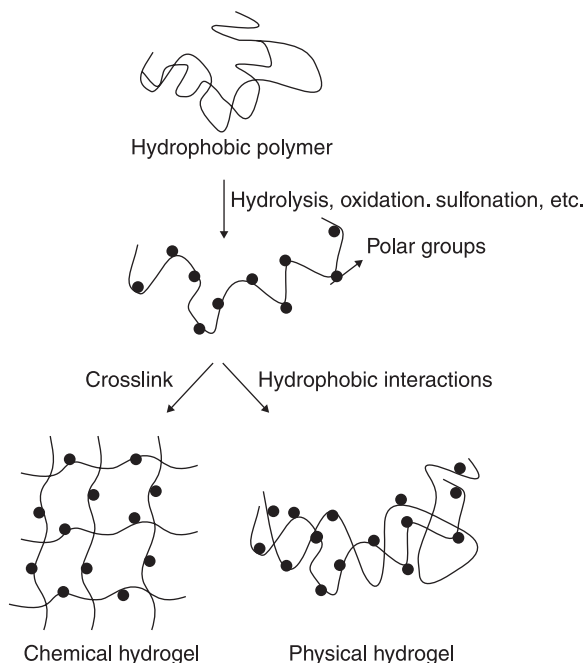
These hydrogels also have the ability to absorb a degree of wound exudate. They transmit moisture vapor and oxygen, but their bacterial and fluid permeability is dependent on the type of secondary dressing used.¹⁴ Hydrogels swell or shrink in aqueous solutions due to the association, dissociation and binding of various ions to polymer chains. These systems may swell in water until an equilibrium state is reached and retain their original shape. The interactions responsible for water sorption by hydrogels include the process of hydration, which is connected to the presence of such chemical groups as -OH, -COOH, -CONH₂, -CONH-, and -SO₃H and the existence of capillary areas and differences in osmotic pressure. The forces that make hydrogel dissolution impossible are presence of covalent bonds between individual polymer chains, hydrophobic and electrostatic interactions.¹⁹

These are hydrophilic polymer networks which may absorb 10–20% (an arbitrary lower limit) and up to thousands of times their dry weight in water. These may be chemically stable or they may degrade and dissolve. They are called ‘reversible’ or ‘physical’ gels when the polymer networks are held together by molecular entanglements, and/or secondary forces such as ionic and H-bonding.^{20,21,22} Hydrogels are called ‘permanent’ or ‘chemical’ gels when they are covalently crosslinked networks as shown in Fig. 9.1.

Hydrogels can be made by high energy radiation, freeze-thawing or chemical methods. Out of all the methods, radiation such as gamma rays, electron beams, X-rays or ultraviolet light are considered as a suitable tool for the formation of hydrogels because there is easy control of processing, and no need of adding initiators or cross-linkers which may be harmful. Moreover, irradiation brings the possibility of formation and sterilization in one step. However, hydrogels formed by this method have poor mechanical strength. Nowadays, a freeze thawing technique is used to prepare hydrogels to have good strength and stability with no added crosslinkers and initiators. The main disadvantage of freeze thawing is that the hydrogels have an opaque appearance and limited swelling and thermal stability.²³

In comparison with the traditional gauze therapy, the application of a hydrogel seems to significantly stimulate wound healing.²⁴ Various natural and synthetic polymers with good biocompatibility are used to develop hydrogel wound dressings. These polymers include natural polymers such as alginate, chitosan, gelatin and collagen and synthetic polymers, such as polyurethane, poly(ethylene glycol), polycaprolactone, poly(vinyl pyrrolidone), poly(lactide-*co*-glycolide), polyacrylonitrile, poly(amino acid), etc. Table 9.1 shows different hydrophilic polymers that are used to synthesize hydrogel matrices.

Hydrogels may be classified as homopolymer hydrogels, copolymer hydrogels, multipolymer hydrogels, and interpenetrating polymeric hydrogels. Homopolymer hydrogels are crosslinked networks of one type of hydrophilic monomer unit,



9.1 Schematic method for formation of physical and chemical hydrogels.²⁰ (Reprinted from *Advanced Drug Delivery Reviews*, 54, Allan S. Hoffman, 'Hydrogels for biomedical applications', 10, 2002, with permission from Elsevier.)

Table 9.1 Hydrophilic polymers used to synthesize hydrogel matrices²⁰

Natural polymers and their derivatives ($\pm$ crosslinkers)

Anionic polymers: HA, Alginic acid, Pectin, Carrageenan, Chondroitin sulfate, Dextran sulfate

Cationic polymers: Chitosan, Polylysine

Ampipathic polymers: Collagen (and gelatin), Carboxymethyl chitin and Fibrin

Neutral polymers: Dextran, Agarose, Pullulan

Synthetic polymers ($\pm$ crosslinkers)

Polyesters: PEG-PLA-PEG, PEG-PLGA-PEG, PEG-PCL-PEG, PLA-PEG-PLA, PHB, P(PF-co-EG) $\pm$ acrylate end groups, P(PEG/PBO terephthalate)

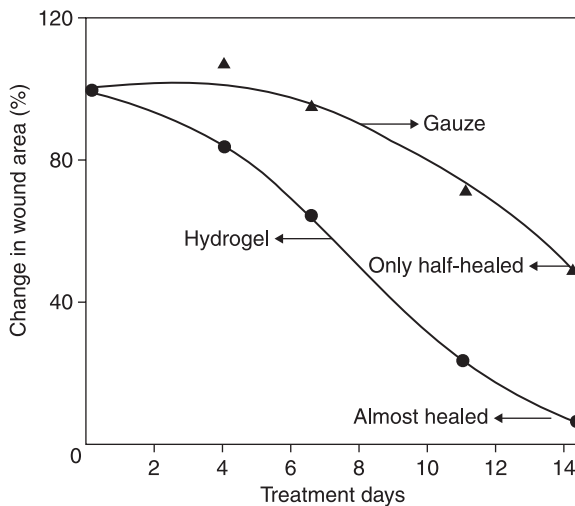
Other polymers: PEG-bis-(PLA-acrylate), PEG $\pm$ CDs, PEG-g-P(AAm-co-Vamine), PAAm, P(NIPAAm-co-AAc), P(NIPAAm-co-EMA), PVAc, PVA, PNVP, P(MMA-co-HEMA), P(An-co-allyl sulfonate), P(biscarboxy-phenoxy phosphazene), P(GEMA-sulfate)

Combinations of natural and synthetic polymers

P(PEG-o-peptides), alginate-g-(PEO-PPO-PEO), P(PLGA-co-serine), collagen-acrylate, alginate acrylate, P(HPMA-g-peptide), P(HEMA/Matrigel®), HA-g-NIPAAm

Reprinted from *Advanced Drug Delivery Reviews*, 54, Allan S. Hoffman, 'Hydrogels for biomedical applications', 10, 2002, with permission from Elsevier

whereas copolymer hydrogels are produced by the crosslinking of two comonomer units, one of which must be hydrophilic. Multipolymer hydrogels are produced by the crosslinking of more than three monomers. Finally, interpenetrating polymeric hydrogels are produced by the swelling of a first network in a monomer and the reaction of the latter to form a second intermeshing network structure.^{19,25} It has been shown that the blending of a natural polymer with a synthetic polymer seems to be an effective method for obtaining materials, having required mechanical and thermal properties in comparison with pure components. It is also a simple method by which suitable shapes such as films, sponges and hydrogels can be obtained easily to realize a variety of biomedical devices. Figure 9.2 shows healing is faster with a hydrogel dressing than with a gauze dressing. A wound area covered by hydrogel decreases faster, with an increasing healing period. On the contrary, the wound covered by gauze dressing reduces by only half a percent even after 14 days.⁶³



9.2 Healing of wound by gauze (dry) and hydrogel (moist) dressings.⁶³ (Reprinted from *Radiation Physics and Chemistry*, 55, F. Yoshii, Y. Zhanshan, K. Isobe, K. Shinozaki, K. Makuuchi, 'Electron beam crosslinked PEO and PEO/PVA hydrogels for wound dressing', 6, 1999, with permission from Elsevier.)

9.4 Natural hydrogels for wound healing applications

Natural polymers, such as chitin, chitosan, alginate, collagen, elastin, genipin, gelatin, cellulose, etc., have been used for dressing wounds because they play an important role in the healing process.²⁵ Chitosan is a partially deacetylated form of chitin. Chitin as Beschitin®, Unitika, is also commercially available as dressing in Japan.²⁷ It is biocompatible, biodegradable, haemostatic, fungistatic²⁸ and

non-toxic and can be successfully used as gels, films and fibers. This polymer also shows antibacterial properties and possesses good wound healing properties.^{29,30,31} It has many applications as a wound dressing, drug delivery device and as scaffold for tissue engineering.^{32,33} Some examples of wound dressings which use chitosan as one of the main components are given below.

Asymmetric chitosan membranes have been developed by using immersion-precipitation phase-inversion method.^{34,35,36} These asymmetric chitosan membranes are homogeneous and have a porous structure. This membrane was prepared by preheating casted chitosan solutions in an oven for different time periods for dry phase separation and then the materials are immersed in a coagulant tank for wet phase separation and subsequently freeze-drying. The skin layer acts as the rate controlling barrier for the release of the drug and the porous layer provides mechanical support to the skin layer. The water vapor transmission rate, gas permeability, PBS solution absorption, *in vitro* degradation, cell culture, bacterial penetration and wound healing tests of this dressing were carried out. These membranes are effective in controlling evaporative water loss, showed excellent oxygen permeability and also are antibacterial in nature. They are also found to be urgent hemostats, i.e., the chitosan hydrogels could effectively stop bleeding from a cut by forming bonds with platelets and red blood cells to form a gel-like clot which seals a bleeding vessel. In one of the papers, wound healing experiments using a mouse model have been carried out and it was found that the photocrosslinkable chitosan hydrogel application on an open wound induced wound contraction and accelerated the wound closure and healing.⁶⁰

In another study, silver sulphadiazine was incorporated as an antimicrobial agent to this asymmetric dressing. The release behavior of both silver and sulphadiazine ions was studied and found to be significantly different from one another. Silver ions displayed a slow release behavior while sulphadiazine ions exhibited a burst effect on the first day of the drug release and then slowly tapered off. This is because of the interaction of silver with the amino group of chitosans leading to their slow release throughout, whereas the sulphadiazine ions were free to diffuse through the membrane to reach the wound site; thus they showed a burst release. The membranes were further found effective against *P. aeruginosa* and *S. aureus*.

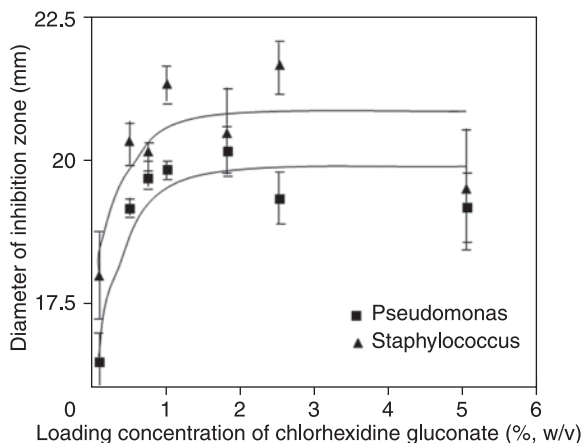
Wound dressings may also be formed using chitosan films and Minocycline Hydrochloride (MH) and commercial polyurethane film (Tegaderm) as a backing. This is also a useful formulation for the treatment of severe burn wounds. Water vapor and oxygen can permeate the Tegaderm film but water cannot. The Tegaderm film supports the polymer membrane.³⁷ A silver nanocrystalline chitosan (SNC) wound dressing composed of nano-silver and chitosan has been constructed by self-assembly and nanotechnology and used for treating deep partial-thickness wounds. In this, sterility and pyrogen testing were performed to ensure biosafety. These dressings promote wound healing and combat infection, and also decrease the risk of silver absorption in comparison with silver sulphadiazine (SSD) dressings.³⁸

In another method of forming, the wound dressing was composed of chitosan, i.e., the formation of polyelectrolyte complex of gum kondagogu (GKG) and chitosan. This complex is formed by the electrostatic interaction between a carboxyl group of gum and an amine group of chitosan. This method is more advantageous as it avoids the use of organic solvents and chemical crosslinking agents and thus reduces the toxicity and undesirable side effects. In this, diclofenac sodium is used as model drug. The diclofenac loaded complex of gum kondagogu and chitosan shows drug release which changes with change in pH. The drug release was higher at pH 6.8 as compared to pH 1.2, due to higher swelling of the complex at higher pH. This holds a great potential as a natural polymer-based delivery device for controlled delivery of drugs like diclofenac sodium for two reasons: (i) to reduce dosing frequency and (ii) to lower the gastric toxicity.³⁹

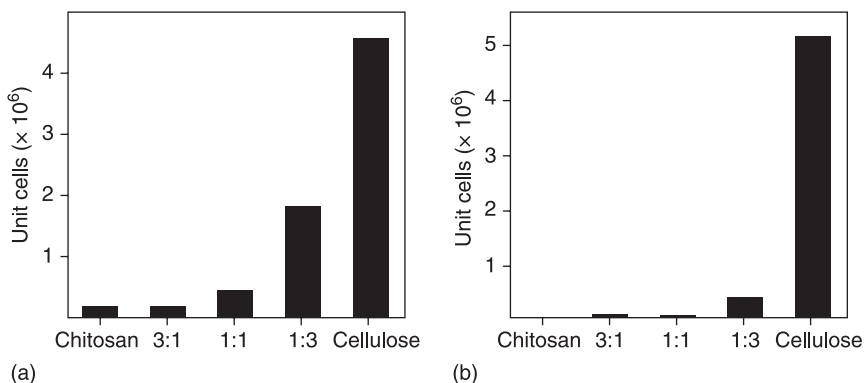
Semi-interpenetrating polymer networks (SIPNs) composed of chitosan (CS) and poloxamer were prepared in order to improve the mechanical strength of CS. The WVTR was found to be $2508.2 \pm 65.7 \text{ gm}^{-2} \text{ day}^{-1}$, i.e., these can maintain a moist environment at the wound site which enhances epithelial cell migration. Also, the *in vitro* assessment of SIPNs showed proper biodegradation and low cytotoxicity. *In vivo* assessments were carried out on experimental full thickness wounds in a mouse model and found that the wounds covered with these were completely filled with new epithelium without any visible adverse reactions after three weeks.¹⁷ The chitosan-gelatin sponge (CGSWD) has also been evaluated as having an antibacterial property. The *in vitro* test showed that the antibacterial effect of CGSWD on *E. coli* K88 is better than that of penicillin, and the effect on *S. aureus* is also better than that of cefradine.⁴⁰

In another effort, the wound dressing has been prepared consisting of two separate layers, in which the upper layer is a swellable hydrogel material that can absorb exudates and also serve as a mechanical and microbial barrier, while the lower layer is chitosan acetate foam incorporated with the antimicrobial agent chlorhexidine gluconate.⁴¹ The antimicrobial activity is checked by the Bauer–Kirby Disk Diffusion Test: inhibition zones can be clearly seen around the discs of chitosan acetate foams incorporated with chlorhexidine gluconate, in culture plates inoculated with either Gram-negative or positive bacteria, showing that the dressing is antimicrobial in nature. It is clear from Fig. 9.3 that antimicrobial capability of the dressing was observed to be more effective against the Gram-positive bacteria, i.e., *S. aureus*, as indicated by the bigger inhibition zone.

Blending is a convenient and effective method to improve physical and mechanical properties of hydrogels. So modification of chitosan is done by blending with other polymers such as cellulose.⁴² In this, *E. coli* and *S. aureus* were used as the test bacteria to examine the antibacterial properties of chitosan, cellulose and chitosan/cellulose blends. The numbers of colonies of these bacteria formed on the test membranes are shown in Fig. 9.4(a) and 9.4(b). It was noted that the numbers of colony of all test bacteria formed on the chitosan/cellulose



9.3 Relationship between inhibition zone diameter and chlorhexidine gluconate loading concentration used in the formulation of wound dressing.⁴¹ (Reprinted from *Journal of Biomedical Materials Research*, 53, Loke Weng-Keong, Lau Sok-Kiang, Yong Lim Lee, 'Wound dressing with sustained anti-microbial capability', 10, 2000, with permission from John Wiley and Sons.)



9.4 Bacteriostatic effects of cellulose, chitosan and the chitosan/cellulose blends on the growth of (a) *Escherichia coli* and (b) *Staphylococcus aureus*.⁴² (Reprinted from *Carbohydrate Polymers*, 57, Yu-Bey Wu, Shu-Huei Yu, Fwu-Long Mi, Chung-Wei Wu, Shin-Shing Shyu, Chih-Kang Peng, An-Chong Chao, 'Preparation and characterization on mechanical and antibacterial properties of chitsoan/cellulose blends', 6, 2004, with permission from Elsevier.)

blend membranes were decreased with the increase of chitosan concentration. These blends are more effective against *E. coli* than that of *S. aureus*, as indicated by the lower colony unit. Thus these dressings are suitable to use as an antimicrobial wound dressing.

Chitosan, due to its structural properties, has the ability to heal wounds without scar formation.⁴³ Since chitosan is composed of *D*-glucosamine, which is also the component present in the disaccharide subunits of hyaluronic acid, chitosan tries to structurally mimic hyaluronic acid and exert similar effects.⁴⁴ It has been known that the fetal wound healing takes place without fibrosis or scar formation due to the presence of hyaluronic acid.⁴⁵ Chitosan, as a semi-permeable biological dressing, maintains a moist environment and prevents the wound site from dehydration and contamination. In addition, digital color separation analysis of donor site scars demonstrated an earlier return to normal skin color at chitosan-treated areas, as shown in Fig. 9.5.⁴⁶

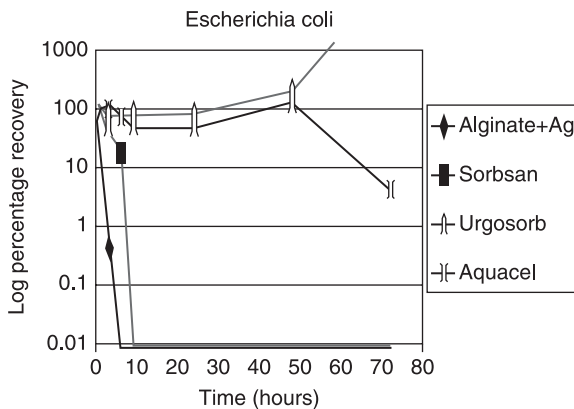
Collagen is also a biopolymer that is used as a polymer for making wound dressing and drug delivery devices as it is non-inflammatory, non-toxic and biocompatible. It is a biological skin substitute, i.e., natural, easily available, ready to use, non-immunogenic and non-pyrogenic. A new collagen dressing with gentamycin or amikacin was prepared in one of the research works and these followed the basic requirement of antibacterial bandages. The dressing is composed of two collagen biomaterials – membrane and sponge – and both are non-immunogenic when implanted subcutaneously in mice and non-toxic. These dressings released antibiotics slowly and showed the antibacterial treatment in experimentally infected superficial wounds in mice. Thus, it can be used for the treatment of infected wounds in humans.⁴⁷ As discussed previously, both chitosan



9.5 Appearances of a Mepitel control donor site scar (left) and the adjacent chitosan-treated scar (right) at two months (the sites of punch biopsy can be seen centrally within each area).⁴⁶ (Reprinted from *British Journal of Plastic Surgery*, 53, C. A. Stone, H. Wright, T. Clarke, R. Powell, V. S. Devaraj, 'Healing at skin graft donor sites dressed with chitosan', 6, 2000, with permission from Elsevier.)

and collagen are excellent materials that can be used as wound dressing materials and both can be used simultaneously. It is found that the wound dressings composed of chitosan crosslinked collagen sponge (CCCS) enhance the diabetic wound healing. Collagen crosslinked with chitosan showed several advantages required for wound dressing, including the uniform and porous ultrastructure, less water imbibition, small interval porosity and high resistance to collagenase digestion and slow release of fibroblast growth factor (FGF) from CCCS/FGF.⁴⁸

Following the moist healing concept, alginates have become one of the most important materials for wound management because these materials are able to absorb exudates from wounds, a prime requirement in an ideal dressing.^{28,49,50–52} In this particular field, the properties of alginate fibers are unparalleled in many respects. Alginate-based products form a gel and provide painless removal from the wound as compared to cotton and viscose fibers.⁵¹ Also, the alginate fibers are non-toxic, non-allergic, haemostatic, biocompatible and easily processable. Sorbsan™ was first commercialized in 1981 and after that many dressings were launched. The alginate fibers can be converted into wound dressings by using a number of textile processes. Non-woven is the main form of alginate wound dressings because of its high absorbency.⁵³ When the non-woven structure of a piece of calcium alginate wound dressing is in contact with wound exudate, absorption takes place in two ways. First, some of the wound fluid is held by capillary forces between the fibers in the non-woven textile structure. This action is rapid and the liquid is held by physical forces. Second, as the fibers are wet, water is drawn into the fiber structure by chemical forces.⁵⁴ So in the case of alginate wound dressings a large amount of water is absorbed into the fiber structure. The antimicrobial action of alginate dressing can be seen as in Fig. 9.6,



9.6 Antimicrobial action of silver containing alginate fibers against *E. coli*.⁵⁴ (Reprinted from *Polymers for Advanced Technologies*, 19, Qin Yimin, 'The gel swelling properties of alginate fibers and their applications in wound management', 9, 2008, with permission from John Wiley and Sons.)

which shows the antimicrobial action of silver-containing alginate fibers against *E. coli*. There was 100% reduction in bacteria count within 5 hours after the fibers were placed in contact with solutions containing the bacteria. Sorbsan alginate fibers showed some antimicrobial activity while Aquacel™ (made of carboxymethyl cellulose) does not show any antimicrobial effect.⁵⁴

Gelatin is widely found in nature and is the major constituent of skin, bones and connective tissue. Gelatin can easily be obtained by a controlled hydrolysis of the fibrous insoluble protein, collagen.⁵⁵ This is also used in a number of biomedical applications such as wound dressings. Hydrogel wound dressings from gelatin, oxidized alginate and borax were prepared and it was found that the composite matrix promotes wound healing because of alginate, has haemostatic effect of gelatin and is antiseptic because of borax. The water vapor transmission rate (WVTR) of the hydrogel was found to be 2686 ± 124 g/m²/day, indicating that this hydrogel can maintain a proper fluid balance at the wound site, which helps in cell migration.⁵⁶

Genipin has been used to crosslink chitosan membranes to control swelling ratio and mechanical properties. It increased its ultimate tensile strength but significantly reduced its strain-at-fracture and swelling ratio. It had significantly less cytotoxicity for human fibroblasts and slower degradation rate compared to the glutaraldehyde-crosslinked membrane. This genipin-crosslinked chitosan membrane can be successfully used as a wound dressing.⁵⁷

Cellulose is the basic material of all plant substances and is the most abundant polysaccharide found in nature. Cellulose derived from plants is unpurified while bacterial cellulose (BC) is nearly purified cellulose. It can be extracellularly synthesized into nano-sized fibrils by the bacteria *Acetobactor xylinum*, using glucose as a common substrate. BC is another natural polymer consisting of microfibrils containing glucan chains bound together by hydrogen bonds. Since the chemical structure of chitosan backbone is very similar to that of cellulose, it was expected that chitosan could be miscible with cellulose. The blending improved the chemical, physical, mechanical and biological properties of the developed film, thus these two biopolymers together can form an ideal material for dressing wounds.^{58,59} These are antibacterial and scar preventive in nature too.

Natural polymers have been considered limited in their applications as wound-dressing materials because of their low mechanical properties and difficult processing and so we move towards the synthetic polymers that can be used for wound healing applications.

9.5 Synthetic and other hydrogels for wound healing applications

9.5.1 Synthetic hydrogels

Synthetic polymers are also being used successfully in biomedical applications as one of the materials because of their wide range of mechanical properties,

suitability for easily forming into a variety of different shapes and low fabrication costs. In an ideal dressing both the characteristics, i.e., antimicrobial ability and moist healing environment, should be present. Therefore, in order to prevent the wound from dehydration and bacterial penetration a new dressing composed of polyurethane was designed in such a way that the upper layer of the dressing was microporous (pore size $< 0.7 \text{ }\mu\text{m}$) supported by a highly porous lower layer containing micropores (pore size $< 10 \text{ }\mu\text{m}$) as well as macropores (pore size: $50\text{--}100 \text{ }\mu\text{m}$). The pores of both layers were interconnected and formed a continuous structure in the membrane. Membranes according to this design were prepared either by means of a two-step or by means of a one-step casting process. Both fabrication methods are based on phase inversion techniques.⁶¹ These membranes are transparent, thus the wound healing can be monitored easily. These dressings were evaluated on the backs of guinea pigs and found to be occlusive to such an extent that they prevent the wound from dehydration and microbial penetration. The high drainage capacity of both types of polyurethane wound dressings can be explained by the fact that the micropores in the top layers were interconnected. Therefore multiple channels were formed which allow the flow of fluids from the macropores of the sublayer through the micropores in the top layer. Furthermore the wound dressing remained firmly adhered to the wound surface and could be left on the wound until full regeneration of the skin was achieved.

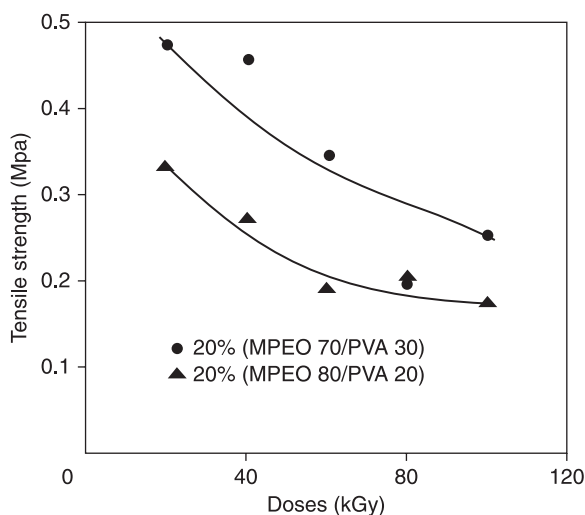
Polyvinyl pyrrolidone (PVP) is one of the most widely used synthetic polymers in medicine because of its solubility in water and its extremely low cytotoxicity. Hydrogels prepared by radiation crosslinking of an aqueous solution of polyvinyl pyrrolidone (PVP) have been used as wound dressing.⁶² Polyvinyl alcohol (PVA) is a well-known polymer because it is biocompatible in contact with the body fluids and has the required mechanical properties. Polyethylene oxide (PEO) is a hydrophilic semicrystalline polyether which is biocompatible, non-toxic, non-polar, non-antigenic and non-immunogenic and is highly desirable in most biomedical applications requiring contact with physiological fluids. A hydrogel composed of PEO for wound dressing was prepared and PVA was added to give toughness to the hydrogel by electron beam irradiation, and it was found that these hydrogels showed satisfactory properties for wound dressing, having been evaluated by creating wounds on the back of marmots.⁶³ The hydrogel gives a wet environment to wounds which causes faster healing compared with the gauze dressing with a dry environment. The weight of the hydrogel increases quickly at the earlier stages, up to four days, due to absorption of effusion produced from the wound as shown in Table 9.2. After that, the production of effusion from the wound ceases and the weight of the hydrogel decreases due to evaporation of the water in the hydrogel. This means that the healing of the wound proceeds smoothly with time. The hydrogel can be peeled off easily from the wound at the time of removal.

The toughness of PEOs hydrogel is improved by the addition of PVA and it was found that tensile strength decreases with increasing dose because of the enhancement in crosslinking.²⁶ This can be seen in Fig. 9.7.

Table 9.2 Absorption of effusion from wound of dressing during healing⁶³

	Healing time	Hydrogel (g)	Gauze (g)
1st Time	Initial day	3.59	0.69
	4 days	5.43	1.98
2nd Time	4 days	3.60	0.68
	7 days	3.83	2.23
3rd Time	7 days	3.62	0.67
	11 days	2.11	1.81
4th Time	11 days	3.55	0.67
	14 days	1.31	1.07

Reprinted from *Radiation Physics and Chemistry*, 55, F. Yoshii, Y. Zhanshan, K. Isobe, K. Shinozaki, K. Makuuchi, 'Electron beam crosslinked PEO and PEO/PVA hydrogels for wound dressing', 6, 1999, with permission from Elsevier.



9.7 Tensile strength of PEO/PVA blended hydrogel.⁶³ (Reprinted from *Radiation Physics and Chemistry*, 55, F. Yoshii, Y. Zhanshan, K. Isobe, K. Shinozaki, K. Makuuchi, 'Electron beam crosslinked PEO and PEO/PVA hydrogels for wound dressing', 6, 1999, with permission from Elsevier.)

Various synthetic polymers as discussed above are used for wound dressing applications. There are very few dressings that are composed of biological materials as compared to synthetic polymers. But it has been known that these biomaterials are typically extracellular matrix components and are produced in nature by plants and living organisms, participate in natural biocycle and are eventually degraded and reabsorbed in nature. Owing to increased environmental awareness, growing public health and environmental regulations, biopolymers

such as polysaccharides, cellulose, starch, chitosan and chitin along with synthetic polymers are better options to make a hydrogel with the desired physical, mechanical and biological properties. Some of the polymer blends exhibit superior and rare properties, unexpected from homopolymers.

9.5.2 Blended hydrogels

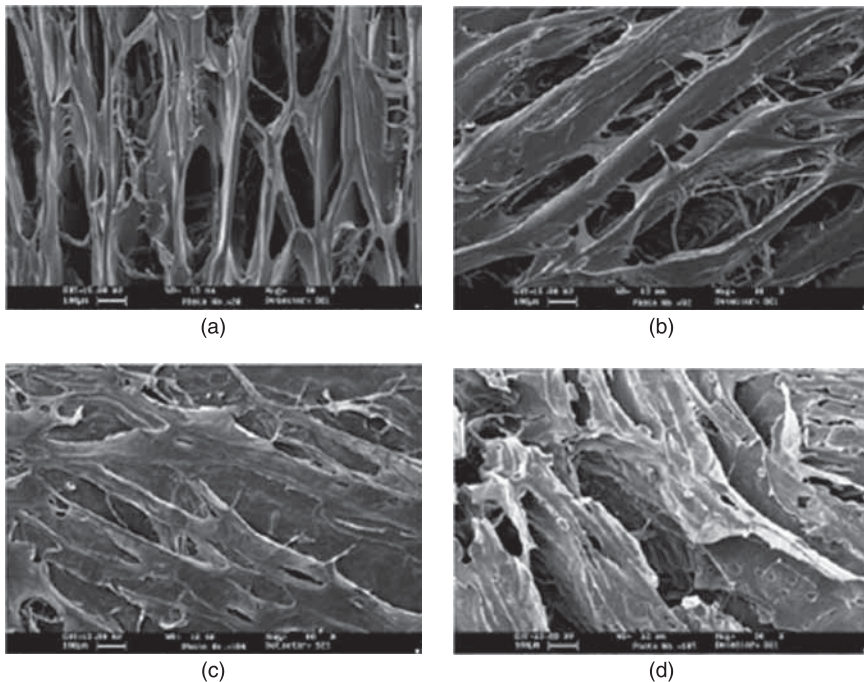
Since both the natural and synthetic polymers have their own advantages and disadvantages, so a combination of natural and synthetic polymers can endow the optimal properties necessary for wound repair.²⁵ The combination of natural and synthetic polymers is used in the biomedical, bioengineering and biotechnology field nowadays because of their great potential. Blending of polymers is one of the simplest ways to get a variety of physical, mechanical and biological properties of different polymers. The polymers that are blended can be natural, synthetic or both natural and synthetic. The physical, chemical and radiation methods can be applied to prepare polymer blends. The property of the blend depends on the degree of compatibility and miscibility of the polymers at a molecular level. A blended hydrogel composed of polyvinyl alcohol/polyvinyl pyrrolidone and charcoal was prepared by single 'freezing and thawing' or two-step 'freezing and thawing' and γ -ray irradiation and applied as wound dressing. It was found that the absorption of *S. aureus* and *P. aeruginosa* by charcoal/PVA/PVP hydrogels was larger than those by PVA/PVP hydrogels; this is due to the absorption and attachment capability of bacteria by charcoal.⁶⁴

The most classical way of fabricating a chitosan-based wound dressing has been to design an asymmetric composite structure. In this method, the cotton fabric was coated with chitosan (CS) and polyethylene glycol (PEG) followed by freeze-drying. The outer dense layer helps in preventing microbial passage across the dressing and provides a rate controlling barrier for water vapor permeation, while the inner porous layer provides a high surface area for the exudates absorption. For the absorption of wound exudates porosity is the prime requirement in a dressing. It has been found that these dressings have porosity of 54–70% and the pore size was in the range of 75–120 μm .¹ The increase in the PEG content in the blend composition led to an enhanced destabilization of pores, causing an increase in the pore size with elongated morphology. There seems to be phase separation between the two components, which is an important factor for the observed behavior of the porous structure. Cotton fabric has been used as the support layer for the CS–PEG layer and results in very thin and lightweight structures. The structure of the dressing has been designed in such a way that it leads to high porosity of the bulk structure. The thickness of CS coating plays an important role in the development of the porosity on the surface.

PEG addition to CS makes significant alteration in the surface morphology of this CS–PEG/cotton membrane (freeze-dried), henceforth known as CPC membrane. There is a distinct trend in the loss of inherent elongated porous

structure in membranes and formation of the partially collapsed porosity takes place due to the PEG addition. This suggests that a very limited interaction between CS and PEG exists, which is reflected in the observed surface morphology. It has been observed that the higher the amount of PEG, the higher the pore destabilization will be leading to larger pores. This is evident from the morphology of the CPC membrane at 50% PEG-20 content, as shown in Fig. 9.8.¹

On the above matrix, the addition of PVP and drugs followed by coating on the cotton fabric and freeze drying of the coated matrix is also carried out. It has been found that the drugs undergo fast release with time in phosphate buffer saline (PBS) solution reaching saturation within two days. This is the indication that these dressings will be excellent materials for wound care management where the dressings need to be replaced every day or every alternative day. Samples with CS do not show zones of inhibition. However, in other samples containing drugs, the zone of inhibition has been observed indicating that these dressings will not allow bacterial growth in their surroundings.⁶⁵ A semi-interpenetrating polymer network

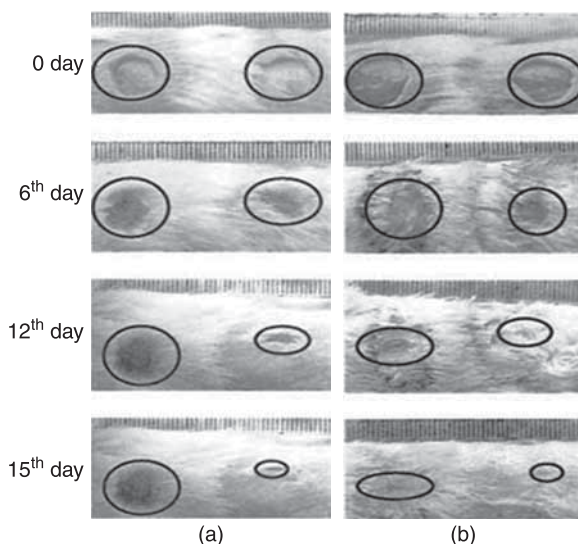


9.8 SEM micrographs of freeze-dried (a) CS coated cotton membrane and CPC membranes with (b) 10% PEG-20; (c) 30% PEG-20; and (d) 50% PEG-20.¹ (Reprinted from *Polymers for Advanced Technologies*, 20, B Gupta, A Arora, S Saxena, M S Alam, 'Preparation of chitosan-polyethylene glycol coated cotton membranes for wound dressings: preparation and characterization', 8, 2009, with permission from John Wiley and Sons.)

(IPN) system may also be prepared in which CS crosslinking network acts as matrix, linear polymer PEG acts as domain. The formation of the porous structure takes place by extraction with hot water, dispersion phase. PEG had been effectively extracted and the porous network retained.⁶⁶

In one of the studies, aloe vera was added to a mixture of PVA/PVP and these hydrogels are prepared by freezing and thawing, γ -ray irradiation, or a two-step method of freezing and thawing and γ -ray irradiation. The swelling degree of hydrogels obtained from the irradiation process was much higher than those obtained from freezing and thawing or the two-step method of freezing and thawing and irradiation. The swelling degree increased as the concentration of aloe vera in PVA/PVP/aloe vera increased and as the radiation dose and repeated cycles of freezing and thawing decreased. The degree of water evaporation increased rapidly up to 5 h, continued to rise steadily up to 15 h, and then leveled off. The PVA/PVP/aloe vera hydrogel had a better curing effect than no dressing and the commercial urethane membrane that can be seen in Fig. 9.9.¹⁹

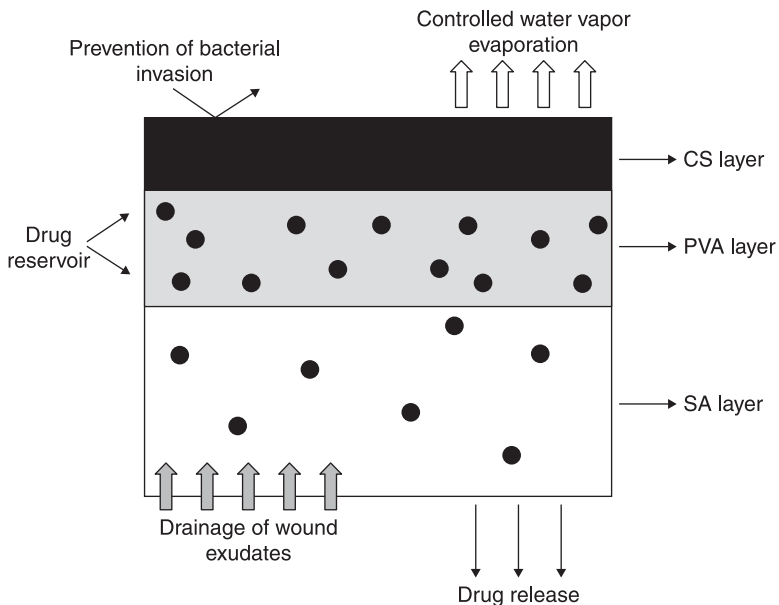
Both natural and synthetic polymers are used for wound healing applications. In one of the studies, a new chitosan-polyvinyl alcohol-alginate (C-P-A) film had been developed by the casting/solvent evaporation method. This new type of C-P-A film consists of a chitosan top layer and sodium alginate sublayer separated



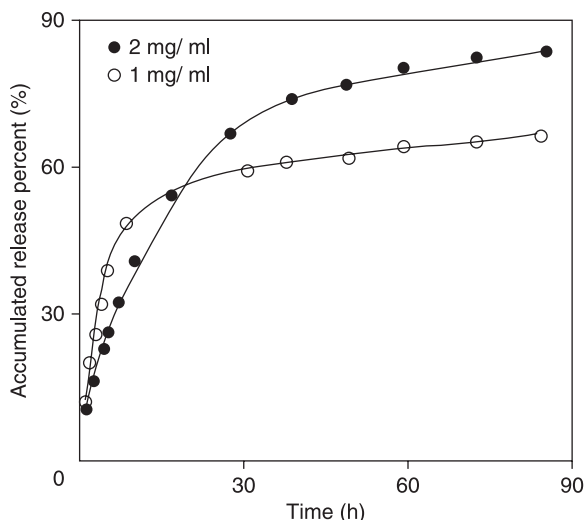
9.9 Healing process of wound with (a) no dressing (left) and PVA/PVP/aloe hydrogel (right) and (b) Tegaderm (left) and the PVA/PVP/aloe hydrogel (right).¹⁹ (Reprinted from *Journal of Applied Polymer Science*, 90, Park Kyoung Ran, Nho Young Chang, 'Preparation and characterization by radiation of poly(vinyl alcohol) and poly(N-vinylpyrrolidone) hydrogels containing aloe vera', 9, 2003 with permission from John Wiley and Sons.)

by an ornidazole-incorporated poly(vinyl alcohol) layer. The schematic representation of the dressing can be seen in Fig. 9.10. The films had excellent light transmittance, water vapor transmission and fluid drainage ability. The *in vitro* release studies showed that about 90% of OD was released from the composite films within 60 minutes, and no significant difference was observed in cumulative release percentage with increases in the drug content. The film at low concentration of OD (1.0 mg/cm^2) showed effective antimicrobial activity against *S. aureus* and *E. coli* in culture plates.⁶⁷

Agar can also be incorporated into a PVP/PEG mixture and the hydrogel is prepared by electron beam irradiation technique. The maximum swelling decreases with increasing irradiation dose, but increases with increasing the PEG concentration. Initially the swelling rate is very high, and water can penetrate easily into the polymer network.⁶⁸ A new type of medicated dressing composed of poly(vinyl alcohol)/ poly(*N*-vinyl pyrrolidone)/ chitosan hydrogels was prepared by a low temperature treatment and subsequent ^{60}Co γ -ray irradiation and then was medicated with ciprofloxacin lactate (an antibiotic) and chitosan oligomer (molecular weight – 3000 g/mol).⁶⁹ The drug and chitosan oligomer release behavior was studied at 37°C in a modified Franz diffusion cell, which could



9.10 Schematic explanation of the structure of the C-P-A composite film.⁶⁷ (Reprinted from *Journal of Biomedical Materials Research*, 85A, Pei Hong Na, Chen Xi Guang, Li Yan, 'Characterization and ornidazole release *in vitro* of a novel composite film prepared with chitosan/ poly(vinyl alcohol)/alginate', 7, 2008 with permission from John Wiley and Sons.)

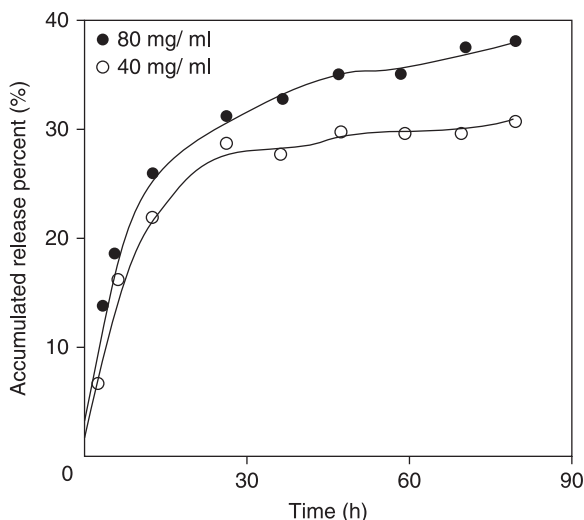


9.11 Release behavior of ciprofloxacin lactate loaded in the PVA/PVP/Chi-1 hydrogel with an initial drug loading concentration of (■) 2.0 or (○) 1.0 mg/mL.⁶⁹ (Reprinted from *Journal of Applied Polymer Science*, 101, Yu Haijun, Xu Xiaoyi, Chen Xuesi, 'Medicated wound dressings based on poly(vinyl alcohol)/poly(N-vinyl pyrrolidone)/chitosan hydrogels', 11, 2006 with permission from John Wiley and Sons.)

simulate actual wound conditions. The *in vitro* drug and chitosan-3000 release behaviors are shown in Fig. 9.11(a) and Fig. 9.12. The drug release was very quick at the beginning and then became slower and slower. In the first 20 h, about 60% of the drug was released. At the end of the 90 h release experiment, the total amounts of the drug released were 85% and 65% for initial drug contents of 2.0 and 1.0 mg/mL, respectively. As shown in Fig. 9.12, chitosan-3000 was released similarly, and the total amount released in 90 h was also dependent on the initial content of the chitosan oligomer. On the other hand, the total percentage of the oligomer released from the hydrogel was lower than that of the drug. This may be because of its stronger interaction with the hydrogel and its higher molecular weight.⁶⁹

9.5.3 Modified hydrogels by grafting

Graft copolymerization is an attractive technique of modifying the chemical and physical properties of polymers for widening their practical use. The properties of the resulting graft copolymers are controlled by the characteristics of the side chains, including molecular structure, length and number. Graft copolymers are the materials which consist of homopolymer backbone and have branches of other types of polymer. It provides an excellent way to introduce desired functionalities onto the

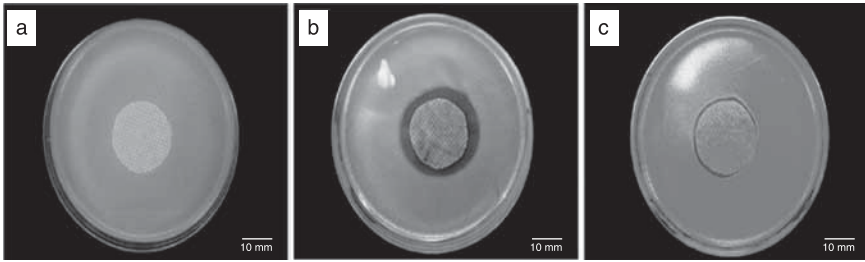


9.12 Release behavior of chitosan-3000 loaded in the PVA/PVP/ Chi-1 hydrogel with an initial chitosan oligomer loading concentration of (■) 80.0 or (○) 40.0 mg/mL.⁶⁹ (Reprinted from *Journal of Applied Polymer Science*, 101, Yu Haijun, Xu Xiaoyi, Chen Xuesi, 'Medicated wound dressings based on poly(vinyl alcohol)/poly(N-vinyl pyrrolidone)/chitosan hydrogels', 11, 2006 with permission from John Wiley and Sons).

chitosan backbone by covalent bonding with a molecule. This can be achieved by methods such as by chemical, photochemical and γ -initiation. It has been observed that the UV-initiation leads to much lower levels of grafting as compared to the other methods, while the γ -radiation producing very high level of grafting.⁷⁰ This grafting technique is used to prepare wound dressings using different natural and synthetic polymers. As in the case of acrylic, acid-grafted and chitosan/collagen-immobilized polypropylene non-woven fabric (PP-AAg-CCi) are produced.⁷¹

Figure 9.13(a), (b) and (c) shows photographs of the bacteria inhibition zone for PP, PP-AAg-PGi and (c) PP-AAg-PGCi, respectively. The zone around the sample is caused by the bacteria inhibition of anti-bacterial agent chitosan.

Semi-interpenetrating polymer network (semi-IPN) hydrogels were prepared by UV irradiation of water soluble *N*-carboxylethyl chitosan (CECS) and 2-hydroxyethyl methacrylate (HEMA) aqueous solutions in the presence of D-2959 as photoinitiator. SEM showed that semi-IPN hydrogels displayed a porous surface and therefore had a high surface area. Cytotoxicity results suggested that semi-IPN hydrogels had good biocompatibility. In this work, a water-soluble *N*-carboxylethyl chitosan was synthesized by Michael addition reaction, and then CECS/poly (HEMA) hydrogels were prepared by the photopolymerization technique. The CECS/poly (HEMA) hydrogels could be potentially used as transdermal drug delivery matrix or wound dressing materials.^{72,73,74,75}



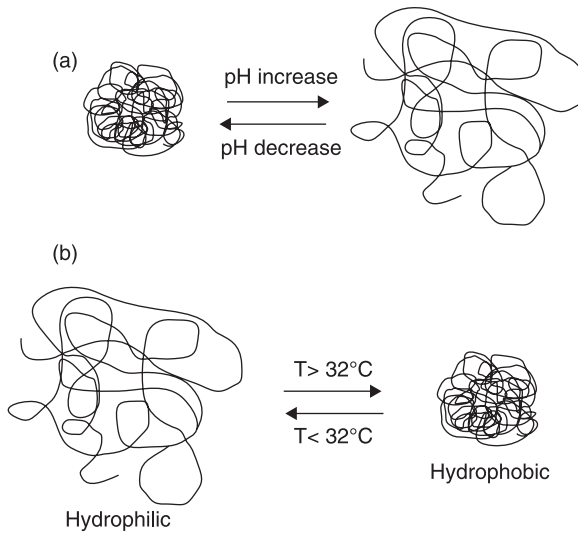
9.13 Photographs of the bacteria inhibition zone for (a) PP, (b) PP-AAg-PGi and (c) PP-AAg-PGi, respectively.¹¹ (Reprinted from *Materials Science and Engineering: C*, 29, Chyung-Chyung Wang, Ching-Hua Su, Jong-Pyng Chen, Cheng-Chi Chen, 'An enhancement on healing effect of wound dressing: Acrylic acid grafted and gamma-polyglutamic acid/chitosan immobilized polypropylene non-woven', 10, 2009, with permission from Elsevier.)

In one of the studies, a series of environmentally friendly hydrogel films was prepared from dihydroxypropyl chitosan (DHP-chitosan) using irradiation technique without any bifunctional crosslinking compounds. Ionizing radiation usually allows the combination of the synthesis and sterilization of polymeric materials in a single technological step, which reduce cost and production time. The desired elasticity and flexibility of DHP-chitosan hydrogels meet various demands from the applied science of biomedical application, such as wound dressing and tissue engineering.⁷⁶

9.5.4 Smart hydrogels, i.e., pH and temperature sensitive hydrogels

Smart polymeric materials respond with a considerable change in their properties to small changes in their environment. Environmental stimuli include temperature, pH, chemicals and light. These can be natural, alginate, chitosan and κ -carrageenan or synthetic, poly(*N*-isopropylacrylamide) and methylmethacrylates polymers,^{77,78} or a combination of both, such as collagen-acrylate and poly(polyethylene glycol *co*-peptides).²⁰ Thermosensitive hydrogels can be classified as positive or negative temperature-sensitive systems. A positive temperature-sensitive hydrogel has an upper critical solution temperature (UCST), i.e., these contract upon cooling below the UCST. Negative temperature-sensitive hydrogels have a lower critical solution temperature (LCST), i.e., these contract upon heating above the LCST.^{79,80} This phenomenon of transition from a solution to a gel is commonly referred to as sol-gel transition. The schematic diagram of smart polymers can be seen in Fig. 9.14.

Yin *et al.*⁸¹ synthesized copolymers by a reversible addition fragmentation transfer (RAFT) method, using different NiPAAm and polyacrylic acid (PAA)



9.14 Schematic diagram showing the pH and temperature sensitive hydrogels.

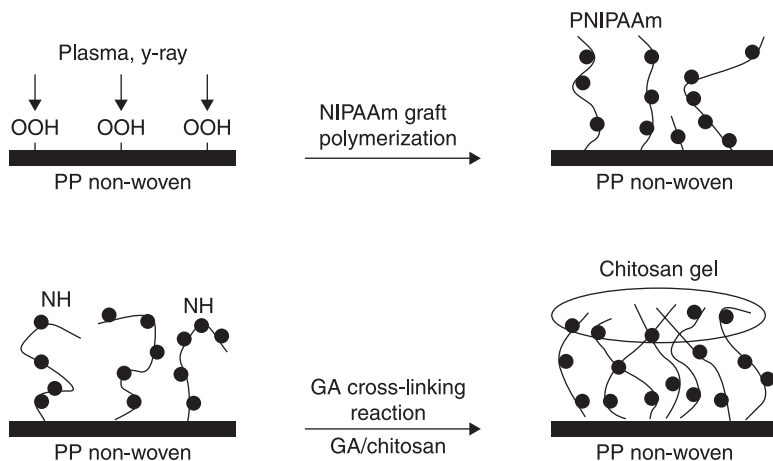
ratios. They showed that even small changes in pH can have a big effect on the LCST of the hydrogel. This feature can be useful for applications such as drug delivery.⁷⁹ Hydrogels based on poly(*N*-isopropylacrylamide), PNIPAAm, show a volume phase transition around 32°C. At higher temperatures, these hydrogels shrink and expel water from gel networks. At lower temperatures, the interaction between water and polymer chains becomes favorable, and the hydrogels swell after absorbing water into the gel network.^{82,83,84} Many researchers reported innovative pH and temperature responsive interpenetrating polymer network (IPN) hydrogels composed of PVA and poly(acrylic acid) (PAAc).^{85,86,87,88,89,90}

In one of the studies, polypropylene (PP) non-woven fabric (NWF) was modified by direct current pulsed plasma followed by grafting with acrylic acid (AAc) to improve its surface hydrophilicity and to introduce carboxylic acid groups. To incorporate the thermosensitive nature, PP-g-collagen NWF was further modified with poly(*N*-isopropylacrylamide) (PNIPAAm).⁹¹ During the change of a wound dressing where separation of the dressing material from the tissue is required, a low temperature treatment below the LCST of PNIPAAm to the dressing material will make the polymer swell and become hydrophilic by absorbing water. In such cases, the NWF dressing could be readily removed from the wound without causing any harm.⁹¹ One more wound dressing was prepared by immobilizing chitosan on a PNIPAAm/polypropylene non-woven composites surface for wound dressing applications.⁹² Using the thermosensitive nature of PNIPAAm, a multilayer membrane wound dressing system was designed. The first layer was a porous polypropylene (PP) non-woven fabric to provide mechanical support. The second layer was *N*-isopropyl acrylamide or acrylic acid

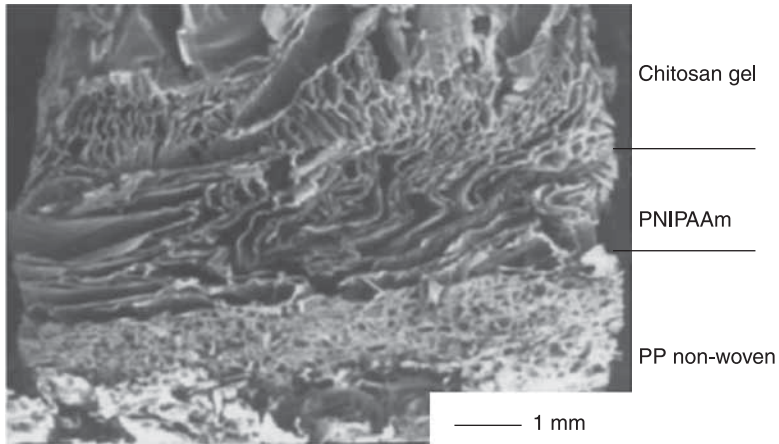
grafted material to furnish the hydrophilic surface for the adhesion of chitosan and collagen, which was the third layer of the dressing.⁹³

Triblock copolymers poly(ethylene oxide)-b-poly(propylene oxide)-b-poly(ethylene oxide) (PEO-PPO-PEO), known also as Pluronic[®] or Poloxamers, are another group of synthetic polymers with thermoreversible behavior in aqueous solutions. These copolymers have been extensively used in applications such as drug and gene delivery, inhibition of tissue adhesion and burn wound covering.^{79,94,95} Chitosan may also be immobilized on PNIPAAm gel/ PP NWF by using cross-linking agent, glutaraldehyde (GA) (Fig. 9.15). The plasma-activation treatment and subsequently UV-light graft polymerization is followed to prepare the dressing. The result showed that CS hydrogels displayed antibacterial ability to *E. coli* and *S. aureus*.⁹²

In the above study, the complex structure was characterized by SEM and it was found that the PNIPAAm grafted layer attached well to the plasma pretreated non-woven as compared to untreated non-woven, due to the increase in wettability between hydrogel and substrate.⁹² However, due to this complicated entangled structure between NWF and CS, the non-woven was difficult to strip. Consequently, an easy-stripped interface layer was really required for preparing an ideal wound dressing. Therefore, a PNIPAAm hydrogel interface was chosen to solve the entanglement problem due to its temperature sensitivity and high hydrophilic property. This tri-layer wound dressing can be a promising approach for tissue engineering applications, whose SEM can be seen in Fig. 9.16.



9.15 Schematic diagram for the chemical reaction of photo-induced grafting polymerization.⁹² (Reprinted from *Materials Science and Engineering: C*, 25, Ko-Shao Chen, Yuan-An Ku, Chi-Han Lee, Hong-Ru Lin, Feng-Huei Lin, Tim-Mo Chen, 'Immobilization of chitosan gel with cross-linking reagent on PNIPAAm gel/PP nonwoven composites surface', 7, 2005, with permission from Elsevier.)



9.16 SEM of tri-layer wound dressing structure by freeze dried at -80°C .⁹² (Reprinted from *Materials Science and Engineering: C*, 25, Ko-Shao Chen, Yuan-An Ku, Chi-Han Lee, Hong-Ru Lin, Feng-Huei Lin, Tim-Mo Chen, 'Immobilization of chitosan gel with cross-linking reagent on PNIPAAm gel/PP nonwoven composites surface', 7, 2005, with permission from Elsevier.)

9.5.5 Antimicrobial dressings

Silver, in ionic or nanocrystalline form, has for many years been used as an antimicrobial agent particularly in the treatment of burns (silver sulfadiazine cream). Iodine also has the ability to lower the microbial activity in chronic wounds in two forms: (a) as povidone-iodine (polyvinylpyrrolidone-iodine complex), an iodophor; and (b) as cadexomer iodine (a three-dimensional starch lattice containing 0.9% iodine). Some of the examples of antimicrobial dressings are Acticoat, Actisorb Silver 200, Aquacel Ag, Arglaes, Avance, Inadine, Iodoflex, Iodosorb and Metrotop Gel.¹⁴ Nowadays many medicinal plants and chemicals such as aloe vera and curcumin are incorporated into the dressings to provide an antimicrobial nature to them.

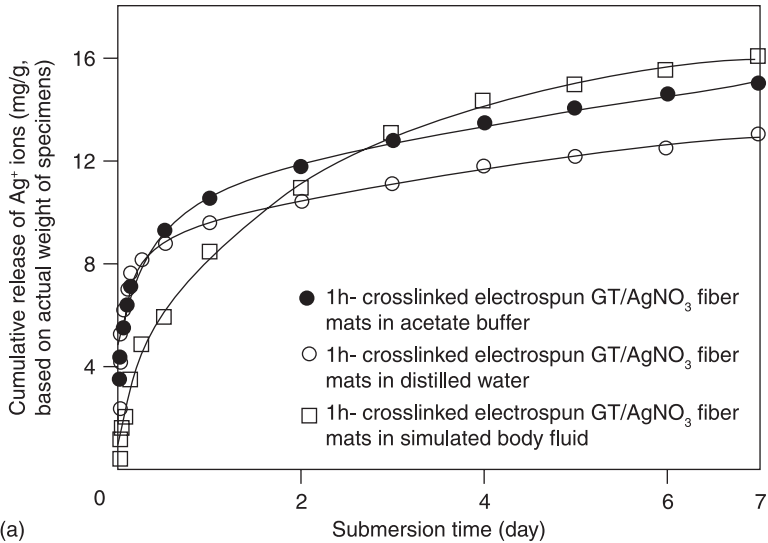
Among all the antimicrobial agents, silver (Ag) is known to have strong antimicrobial activities.⁹⁷ Silver impregnated textiles are used as wound dressings for infected wounds.⁹⁸ Antimicrobial yarns⁹⁹ can be produced from cotton, silk, polyester, nylon and their blends. The nanosilver particles showed effective antimicrobial activity against various bacteria, fungi, etc. The mechanism of antimicrobial action of silver is widely known. It enters the wound and becomes absorbed by bacteria and fungi. There it interacts with enzymes and proteins important for respiration and transport of important substances across the cell membrane. It also interacts with the DNA of bacteria, thereby inhibiting cell division. They are bound to the cell wall and outer cell, thereby changing or inhibiting the functionality of the cell membrane. In this way, it kills microbes, resulting in treatment

of infected wounds.^{100,101} Historically, a number of polymer-based materials have been fabricated into electrospun fibers containing nAg like polyacrylonitrile (PAN),^{102,103} cellulose acetate (CA),^{104,105} poly(*N*-vinylpyrrolidone) (PVP)¹⁰⁶ and poly(vinyl alcohol) (PVA).^{100,106,107}

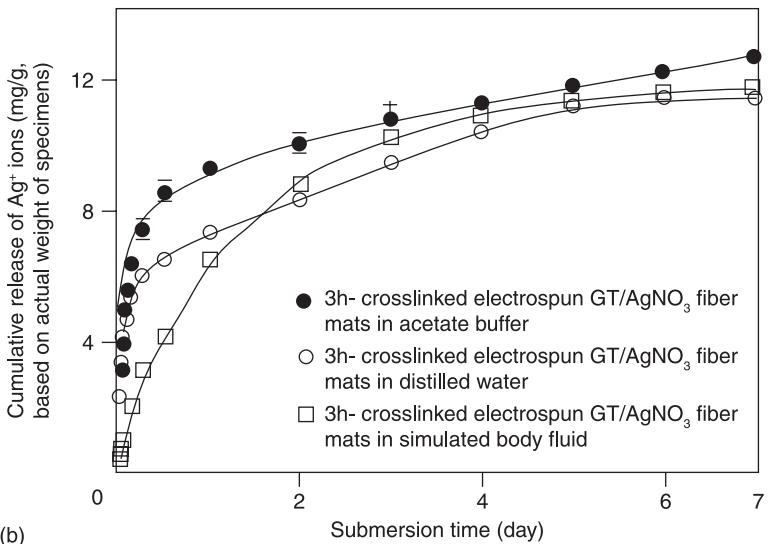
A novel wound dressing material was prepared by electrospinning polyvinyl alcohol and silver nitrate aqueous solution into non-woven webs and then treating these non-woven webs by heat or UV radiation. It was found that both treatments reduced the Ag⁺ ions in the electrospun web into Ag nanoparticles. Also, heat treatment improved the mechanical properties and thus these electrospun fiber web was an effective wound dressing.¹⁰⁰ Some of these nAg-loaded e-spun fiber mats were tested for their antibacterial activity against *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. aureus*.^{100,105,106,107} In one of the studies, mats of gelatin fibers containing nAg were prepared by e-spinning and these are used as wound dressing pads.¹⁰⁹ Ag⁺ ions were reduced directly into nAg through a series of steps, including nuclei formation, crystal growth via diffusion mechanism to give primary particles, and spontaneous self-organization of primary particles to form clusters.¹¹⁰ The cumulative release of Ag⁺ ions from the samples in the acetate buffer and distilled water occurred rather rapidly during the first 60 min after submersion in the releasing medium, and increased gradually afterwards; while those in SBF occurred more gradually over the testing period, as shown in Fig. 9.17.

Aloe vera, a succulent plant with many biologically active ingredients that help in the healing and sealing of wounds, is an important product for assistance in the healing of cuts, scrapes and even skin ulcers. Thus it is supposed that aloe vera leaf gel extract has the ability to be used as a component of wound dressing materials.¹¹¹ This wound healing ability of aloe vera is because it stimulates fibroblasts directly. Fibroblasts, the skin cells responsible for manufacture of collagen, play an important role in fiber formation of wounds in repair by protein synthesis and associative enzyme activity.^{111,112} Aloe vera also contains mannose-6-phosphate, which increases the macrophage activity and promotes wound healing and may also promote wound healing in this way.^{113,114,115,116} Also, the increased presence of oxygen caused by the aloe vera improves microcirculation, which should greatly enhance the wound healing process.^{111,117} Aloe vera has been added to a mixture of PVA/PVP and had a better curing effect than no dressing and the commercial urethane membrane, as discussed in Fig. 9.9.¹⁹

A commercial bandage of Johnson & Johnson containing aloe vera and Vitamin E is on the market and has the following advantages: (a) aloe vera helps soothe, protect and promote ‘natural’ healing, (b) is easy to apply, (c) antiseptic pad helps to kill germs and the side seals help prevent further infection and (d) hypo-allergenic. Another commercial bandage – Gentell Hydrogel Aloe Vera Wound Dressing from ActiveForever.com – is an aloe vera-based, hydrating wound gel which protects the wound bed and enhances the moist environment. Curcumin (diferuloylmethane), a polyphenol, is an active component of the perennial herb *Curcuma longa* (commonly known as turmeric). It has an anti-oxidant, anti-cancer



(a)



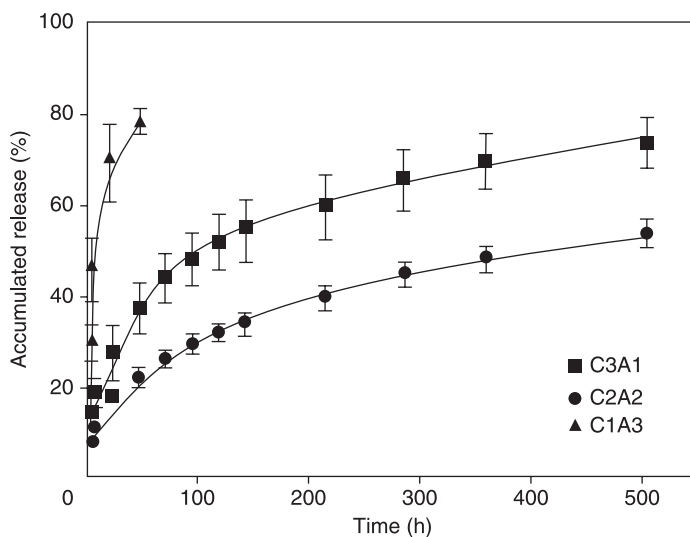
(b)

9.17 Cumulative release profiles of Ag^+ ions from 1- and 3 h-cross-linked nAg containing e-spun gelatin (GT) fiber mat specimens reported as the weight of Ag^+ ions released (in mg) divided by the actual weight of specimens (in g) in three types of releasing medium, i.e., (a) acetate buffer (pH 5.5), (b) distilled water (pH 6.9), at the skin temperature of 32 °C, and (c) simulated body fluid (pH 7.4), at the physiological temperature of 37 °C.¹⁰⁹ (Reprinted from *Polymer*, 49, Pim-on Rujitanaroj, Nuttaporn Pimpha, Pitt Supaphol, 'Wound-dressing materials with antibacterial activity from electrospun gelatin fiber mats containing silver nanoparticles', 10, 2008, with permission from Elsevier.)

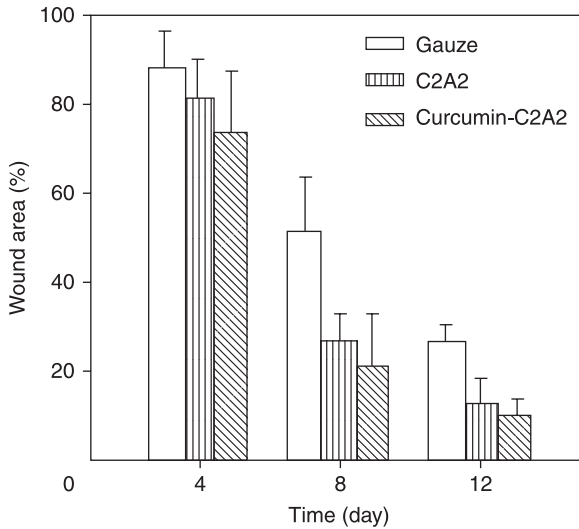
and anti-inflammatory action.⁹⁶ The antiseptic activity of an aqueous extract of turmeric was used by Johnson & Johnson in Band-Aid®, a turmeric-based bandage (patent), available in the market over recent years.¹¹⁸ The application of poly (caprolactone) (PCL) nanofibers as a delivery vehicle of curcumin for wound healing applications has been observed. The fibers showed sustained release of curcumin for 72 h and it could be made to deliver a dose much lower than cytotoxic concentration while remaining bioactive. The *in vivo* wound healing capability of the curcumin loaded PCL nanofibers was investigated in a streptozotocin-induced diabetic mouse model and an increased rate of wound closure proved.¹¹⁹

In one of the studies, curcumin was incorporated into the chitosan and alginate sponge to deter wound infection.¹²⁰ In this, the crosslinked sponges based on chitosan and alginate were successfully prepared at the various conditions of mixing ratios 3:1, 2:2, 1:3 and the samples formed are C3A1, C2A2 and C1A3 respectively. The drug release behavior can be seen in Fig. 9.18. Based on the results of drug release of curcumin, it was found that the C2A2 sponges exhibit a sustained release behavior for up to 20 days. This shows that the C2A2 sponge could be a good drug support to be employed for sustained release.

Figure 9.19 shows the total wound area of skin over time. It can be seen that on the twelfth postoperative day, the C2A2 and C2A2-Curcumin sponges showed a better wound healing effect.



9.18 The *in vitro* release behaviors of curcumin from chitosan alginate sponges.¹²⁰ (Reprinted from *Journal of Biomedicine and Biotechnology*, Mei Dai, XiuLing Zheng, Xu Xu, XiangYe Kong, XingYi Li, Gang Guo, Feng Luo, Xia Zhao, Yu Quan Wei, and Zhiyong Qian, 'Chitosan-Alginate Sponge: Preparation and Application in Curcumin Delivery for Dermal Wound Healing in Rat', 8, 2009, with permission from Elsevier.)



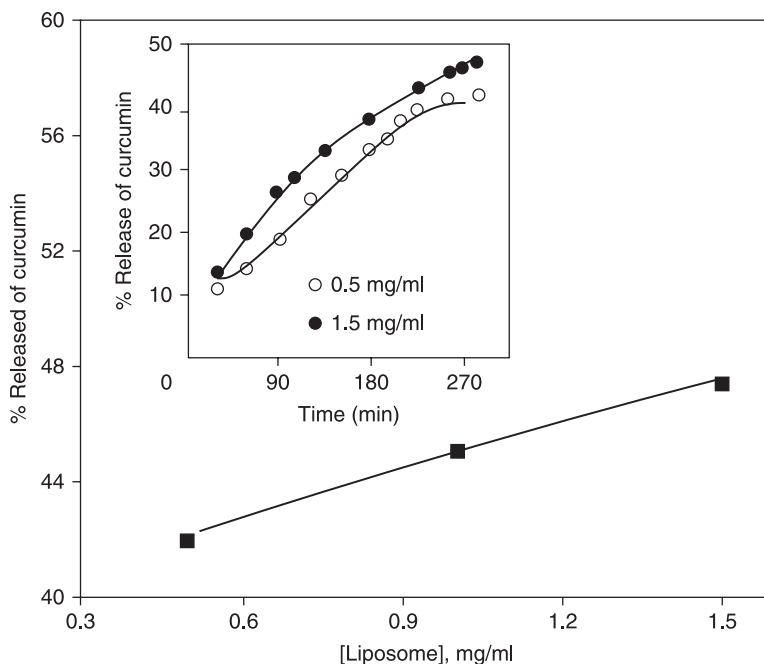
9.19 Total wound area of skin over time as a percentage of original wound size.¹²⁰ (Reprinted from *Journal of Biomedicine and Biotechnology*, Mei Dai, XiuLing Zheng, Xu Xu, XiangYe Kong, XingYi Li, Gang Guo, Feng Luo, Xia Zhao, Yu Quan Wei, and Zhiyong Qian, 'Chitosan-Alginate Sponge: Preparation and Application in Curcumin Delivery for Dermal Wound Healing in Rat', 8, 2009, with permission from Elsevier.)

In one of the studies, aqueous PVA solution could easily be crosslinked to form a hydrogel loaded with curcumin. The results show that with increase in the concentration of curcumin, more curcumin gets loaded in the hydrogel. This study is a model for future biological applications of curcumin hydrogel. Figure 9.20 shows the percentage of curcumin released on incubation of liposome solutions with curcumin-loaded hydrogel.¹²¹

9.5.6 Textile-based wound dressings

Biomedical textiles are textile products used for medical and biological applications. They are used for first aid and for clinical or hygienic purposes. Some of the examples of their application are in the form of wound dressings, bandages, pressure garments, etc. The importance of textiles for wound care applications is shown by their strength, flexibility, air and moisture permeability and three-dimensional structures.

Nowadays, electrospinning arouses much interest as an attractive technique for producing polymer fibers with diameter in the range from several micrometers down to tens of nanometers. Because of the presence of high specific area they are used in a wide variety of applications. The nanofibers are usually obtained in non-woven form, which is very suitable for applications such as wound dressings.¹²²



9.20 Plot of percentage of curcumin released (from hydrogel loaded with 250 μM curcumin solution) as a function of liposome concentration at equilibrium. (Inset) Percentage of curcumin released in 0.5 and 1.5 mg/ml liposome solution from the curcumin-loaded hydrogel obtained by equilibrating with 250 μM curcumin solution as function of time.¹²¹

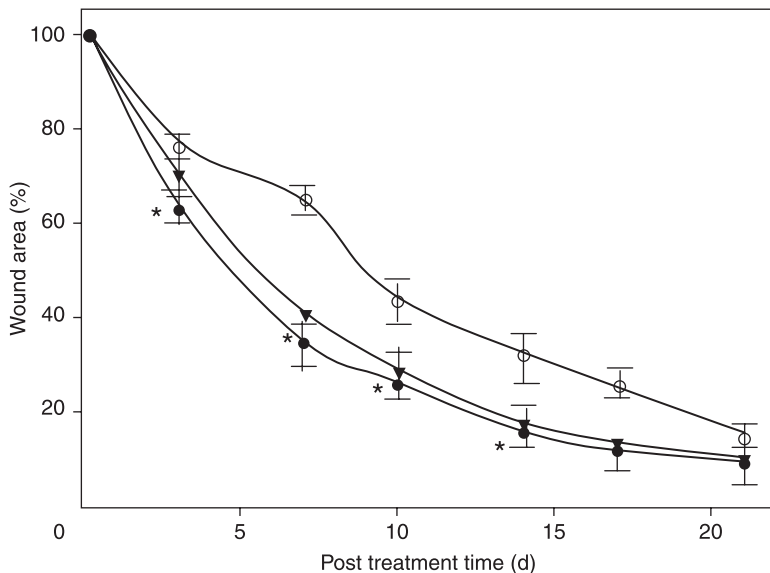
Biopolymers, such as chitin, chitosan, alginates, along with textile materials have been presented as versatile candidates in the area of wound dressings. These provide all the specifications required for an ideal wound dressing. They are the smart dressings that provide microbial protection, water and air permeability and capability of covering the wound, and also adhere well to the wound and provide painless removal without causing any harm to the newly formed tissues, etc. Textile-based hydrogel dressings are non-toxic and biocompatible too. Non-woven fabric (NWF) serves as an excellent dressing material with its high porosity and larger surface area, which provides an open structure for drainage of exudates, and reduces the risk of second infection. The fabric has been used as the support layer for the hydrogel layer and leads to very thin and lightweight structures.

Nowadays nanofibers are also found to be suitable in the area of wound dressings. Nanofibers containing poly(vinyl pyrrolidone)–iodine complex (PVP–iodine) were formed by electrospinning. The average diameters of the fibers were in the range of 150–470 nm. When it comes in contact with skin and mucous membrane, the complex behaves as an iodophor, i.e., it gradually releases active iodine. The broad-spectrum microbicidal activity of PVP–iodine is related to the released

non-complexed, freely mobile iodine. The active iodine reacts with enzymes of the respiratory chains and with amino acids from the cell membrane proteins, resulting in destruction of the well-balanced protein tertiary structure and in irreversible damage to the microorganisms. Thus it can be used as a component of non-woven textile for external antibacterial applications as required in wound dressings.¹²²

Electrospinning is also used to fabricate the composite nanofibrous membranes (NFM) of type I collagen, chitosan and polyethylene oxide. This NFM is then further crosslinked by glutaraldehyde vapor. NFMs showed no cytotoxicity toward the growth of 3T3 fibroblasts and had good *in vitro* biocompatibility. From animal studies, it can be concluded that these NFMs are better than gauze and commercial collagen sponge wound dressing in the rate of wound healing. This novel electrospun matrix will have potential as a wound dressing for skin regeneration. The electrospun membrane is also important for cell attachment and proliferation in wound healing.¹²³

Figure 9.21 shows the changes in wound areas at different healing times using NFM, gauze and a commercial collagen sponge wound dressing. The wound areas decreased gradually and reached about 5% after 21 d when wound dressings



9.21 Wound healing tests of (o) gauze, (▼) commercial collagen sponge and (•) NFM (* $p < 0.05$ compared with gauze; the data are presented as mean $\pm$ S.D) ($N=4$).¹²³ (Reprinted from *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 313–314, Jyh-Ping Chen, Gwo-Yun Chang, Jan-Kan Chen, 'Electrospun collagen/chitosan nanofibrous membrane as wound dressing', 6, 2008, with permission from Elsevier.)

were used. NFM was found to be better than gauze ($p < 0.05$) and collagen sponge in promoting wound healing.

New textile dressings containing dibutylchitin (DBCH) or regenerated chitin (RC) have been prepared by coating a polypropylene non-woven material with films of DBCH or RC. The sterilized dressings were subjected to biological evaluation. DBCH and RC caused no cytotoxic effects or primary irritation either *in vitro* or *in vivo* and both had a positive influence on the wound healing process conducted on 16 albinos. Microscopic assessment showed that the wounds covered with the dressing containing DBCH healed fastest.¹²⁴

9.6 Commercial dressings

Many commercial dressings such as Melolin®, Telfa®, Perfron®, Lotus®, Micropad®, Mesoft®, Regal® and Vernaïd® have one unsatisfactory feature, i.e., they cause fresh damage to the wound when removed because of their adherence to the wound surface.¹²⁵ So, these are hard to peel off from the wound. In order to prevent harm, new hydrogel dressings are required that will provide a moist environment at the wound site.

'Hydron' is a commercial dressing based on poly(2-hydroxyethyl methacrylate) and polyethylene glycol that is formed *in situ* on the wound by spraying.^{56,126,127} Omiderm (Omikron Scientific Ltd., Rehovot, Israel), a new synthetic transparent wound covering based on hydrophilized polyurethane, is synthesized by grafting acrylamide on a polyurethane film. The membrane thus obtained is a hydrophilic, flexible, transparent material, capable of absorbing water up to about 100% of its weight, i.e., highly permeable to water. Its WVTR value is 5000 g/m²/day in comparison to 1400 and 500 g/m²/day for Biobrane (Hall, Woodroof Inc., Santa Ana, CA) and Op site (Smith and Nephew Ltd.), respectively. It is highly permeable to water and prevents fluid accumulation. The topical antimicrobials can be incorporated into these dressings to make it more effective against bacterial growth.¹²⁸

One more hydrogel dressing is LUOFUCON™ Medical Hydrogel dressing. It is a swelling polymer made from polyethylene oxide and polyvinyl alcohol by moderate crosslinking. It has a three-dimensional structure with high hydrophilic groups on the networks and thus provides a moist environment to the wound. It can absorb water and release moisture. The product is semi-transparent, so it is convenient to observe the healing conditions of the wound. It will not stick to the wound and can be removed easily.

Lupin Ltd. supplies Calgigraf Ag Foam wound dressing, which comprises silver alginate, calcium alginate and Maltodextrin matrix. It provides slow extended release of ionic silver for broad antimicrobial effectiveness and helps to prevent contamination from external bacteria. It maintains a moist environment and absorbs a lot of exudates coming out of the wound. Calcium alginate accelerates hemostasis promotes cell proliferation and also enhances granulation

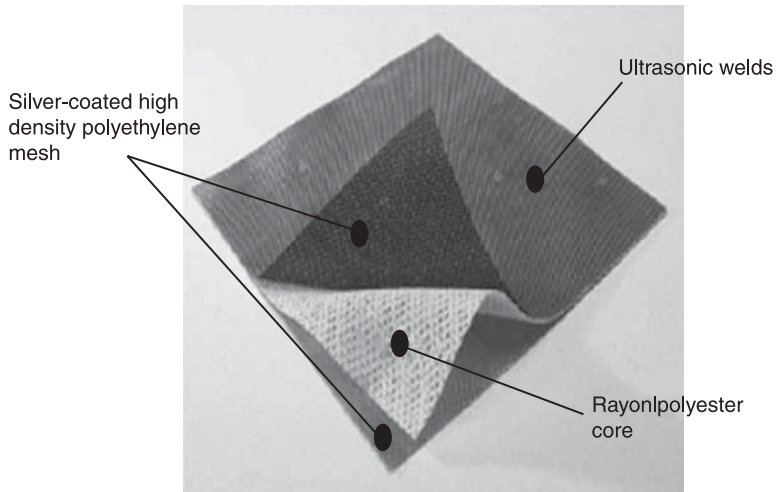
tissue formation. Another essential feature is that Maltodextrin is a chemotactic agent that creates an environment which helps the body's own cells to carry out the formation of granulation tissue while simultaneously aiding the elimination of odor from the wound. Also, this foam promotes wound healing through inhibition of matrix metalloproteinases. There is also a reduction of pain and inflammation. It does not promote microbial resistance. Its antimicrobial spectrum involves *Pseudomonas* and Methicillin-resistant *Staphylococcus aureus* (MRSA) as well. Moreover, it is non-cytotoxic, non-irritating and non-staining.

Smith and Nephew are selling various wound dressings under the names of Acticoat™, Primapore, Intrasite gel and Bactigras®. Johnson & Johnson and 3M Companies also have many patent applications detailing novel applications of hydrogels. 3M's intellectual property is more focused on delivery technologies such as fine mists and micro beads, while Johnson & Johnson has contributed towards the application of newer synthetic polymeric blends and enzyme additives. Others like Coloplast, Beiersdorf AG, Hollister and Convatec have also significantly contributed in the area of wound care.

3M's dressing Tegaderm™ consists of a thin polyurethane membrane coated with a layer of an acrylic adhesive. This dressing is permeable to both water vapor and oxygen and is impermeable to microorganisms; once in position, it provides an effective barrier to external contamination, whilst producing a moist environment at the surface of the wound by reducing water vapor loss from the exposed tissue. The main reason for using a Tegaderm dressing is for extended wound management. Being able to see the wound and the healing process is a major incentive for using this dressing. Tegaderm keeps the wound sterile and moist. This speeds up the healing process and reduces the likelihood of scarring.

Cuticell™ Plus, marketed by BSN medical Pvt. Ltd., is an antibiotic tulle dressing which protects, soothes and helps to heal. They facilitate dressing changes while supporting a moist wound environment to aid healing.

Primapore* is an adhesive non-woven wound dressing which combines an absorbent pad with a soft and conformable fixative layer for the simple and effective management of sutured wounds. It has different features such as a soft breathable cover, highly absorbent pad, low allergy adhesive and comfortable pad and cover. Bactigras® is medicated chlorhexidine paraffin gauze dressing and has features such as a soft paraffin base and sterile leno weave presentation, and contains Chlorhexidine acetate 0.5%, an antiseptic with a broad spectrum. Also it is demonstrated to be effective *in vitro* and *in vivo* against MRSA. Intrasite gel provides high water content, thus creating a moist wound surface and debrides wounds by hydration. The dressing promotes autolysis and can absorb large amounts of exudates but the limitation of this gel is that it is not appropriate for heavily exuding wounds. Acticoat™ is a commercial textile dressing containing silver nanocrystals, shown in Fig. 9.22, and is used as a wound dressing good for burn patients. It contains silver nanocrystals that kill



9.22 Commercial textiles Dressing Acticoat™ with silver nanocrystal.

bacteria *in vitro* in as little as 30 minutes, i.e., two to five times faster than other forms of silver.

Acticoat™ possesses effective antimicrobial activity *in vitro* and *in vivo* capable of reducing colonization and preventing contamination by microorganisms. Its release mechanism ensures a continuous distribution of 70 to 100 mg/L of ionized silver over more than 48 hours and a rapid start of action (within 30 minutes of application) in optimal moisture conditions. It reduces pain and this benefit can be intensified if dressings are changed only every three days, as recommended by the manufacturer.^{129,130}

Acticoat can be applied to any anatomical location including the face and has proved a successful alternative to traditional silver sulfadiazine preparations in dedicated units with demonstrated less cost than silver sulfadiazine over the same treatment period, reducing the requirements for grafting.^{131,132}

Myskin™ (Fig. 9.23) is the first product to be launched in 2005 by CellTran for the active treatment of chronic wounds.¹³³ It comprises flexible medical-grade silicone coated with a chemically controlled plasma polymer film which supports the growth of skin cells. After culturing, Myskin™ is applied to the damaged area so that cells are in contact with the wound bed. The polymer film is engineered to promote cell growth and subsequent release when triggered by exposure to the wound. For obtaining autologous cells a small skin biopsy is taken from the patient (usually from the thigh). The cells delivered by this dressing have a high proliferative capacity and are able to survive an aggressive wound site, and ultimately provide epidermal cover. This promotes the healing of wounds, assisted by the increased expression of natural growth factors that the cultured autologous cells provide.



9.23 Commercial textile dressing Myskin™.¹³³

9.7 Future trends

There are a few new approaches being developed that will help in the healing of wounds in such a way that it will counteract the major problem of scarring of burn wounds. There are some novel areas of research and new trials are under way.

9.7.1 Gene therapy

Gene therapy aims to help the cell to help itself by providing it with specific genes. Genes, once incorporated in the cell, affect the cell and its milieu through their products of expression. Those in the context of wound healing are growth factors, their receptors, adhesion molecules and inhibitors of proteases.⁵² Introduction of the gene rather than growth factor is cheaper and more efficient for treating chronic wounds. Initially gene therapy was developed for the treatment of congenital defects but now it seems to be a new option for enhancing wound repair.¹³⁴

9.7.2 Stem cell therapy

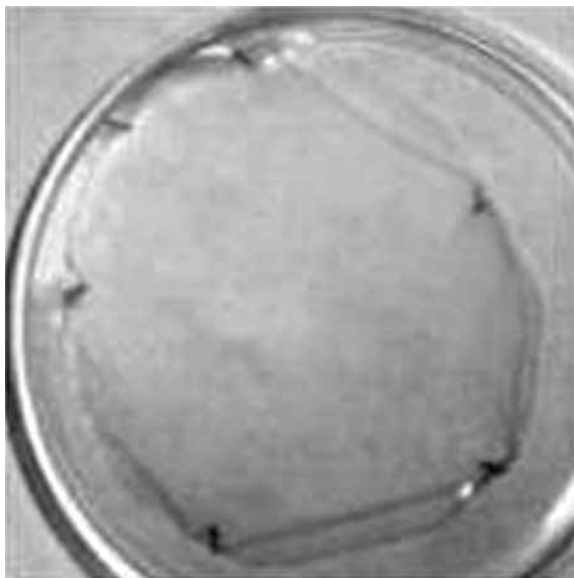
Another novel method is stem cell therapy. Bone marrow derived stem cells are pluripotent, being capable of differentiating into a variety of cells. This property

can be used in the wound healing environment.⁵² Embryonic and adult stem cells have a prolonged self-renewal capacity with the ability to differentiate into various tissue types. A variety of sources, such as bone marrow, peripheral blood, umbilical cord blood, adipose tissue, skin and hair follicles, have been utilized to isolate stem cells to accelerate the healing response of acute and chronic wounds.¹³⁴ Nowadays, gene and stem cell therapy in combination have emerged as a promising approach for the treatment of chronic wounds.

9.7.3 Skin engineering

Skin is an important tissue engineering target for reconstructive surgery of burns victims, but it can also help in healing of diabetes-related ulcers. The three main developments in tissue engineering of the skin are EpiCel[®],¹³⁵ Apligraf[®] and Dermagraft[®],¹³⁶ and Integra[®].¹³⁷ The first skin substitutes developed at Sheffield were cultured epithelial autografts; these can be seen in Fig. 9.24. They are thin sheets of keratinocytes taken by biopsy from a patient and multiplied in the laboratory and have been used since 1981.

The sheets of cells prepared in the laboratory were fragile and take 13 days to prepare. The collaboration between clinical scientists and materials scientists at Sheffield made the first big improvement on this technique – the development of flexible synthetic surfaces on which keratinocytes could easily be cultured *in vitro*. The synthetic support medium allows rapid culture, reducing waste, and



9.24 First skin substitutes developed at Sheffield.¹⁰⁸

makes the tissue very much easier to handle. The studies have shown that cells migrate from the dressing to the wound and greatly accelerate healing rates. This technology has also been successful in treating severe burn patients.¹⁰⁸ But the process automation cost is too high so future developments will depend very much on public and professional support for further research.

9.8 Conclusion

The data present an overview which shows the important developments in the field of wound dressings, from fibers to finished products to hydrogels and new techniques. Many natural and synthetic polymers are used as hydrogels for wound healing applications but it is found that all have their own advantages and disadvantages, so a combination of both natural and synthetic polymers is generally used for preparing wound dressings that will be ideal and has all the characteristics needed in an ideal wound dressing. Many methods, such as blending and grafting, are used to synthesize the hydrogels with the required characteristics. This chapter also describes the use of silver nanoparticles in the biomedical field. These nanoparticles when incorporated into the hydrogel dressings provide an optimal healing environment and prevent the wound from infection.

The chapter also discusses the textile-based dressings in which the textile fabric provides support to the hydrogel. The electrospinning technique is also described here, which helps in the formation of nanofibers which are used as they provide a large surface area. Some commercial and antimicrobial dressings are also mentioned. pH and temperature sensitivity characteristics of polymers are used in the preparation of drug delivery devices and wound dressings.

As technologies advance, our ability to perfect the healing process and create aesthetically pleasing results increases. However, in many respects the new technologies are improving outcomes in several different ways. As a result, it is expected that there will be room for various technology leaders to develop and bring to market advanced moist dressing solutions in the coming years. Fundamental research in the area of wound healing management and scar-free tissue rebuilding increases the hope that eventually we will be able to restore the appearance and function of skin completely after the wound healing process.

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9.10 Appendix: list of abbreviations

EG, ethylene glycol
HA, hyaluronic acid
HEMA, hydroxyethyl methacrylate
PAAc, poly(acrylic acid)
PAAm, poly acrylamide
PBO, poly(butylene oxide)
PCL, polycaprolactone
PEG, poly(ethylene glycol)
PEMA, poly(ethyl methacrylate)
PF, propylene fumarate
PHB, poly(hydroxy butyrate)
PHPMA, poly(hydroxypropyl methacrylamide)
PLA, poly(lactic acid)
PLGA, poly(lactic-co-glycolic acid)
PNIPAAm, poly(*N*-isopropyl acrylamide)
PNVP, poly(*N*-vinyl pyrrolidone)
PPO, poly(propylene oxide)
PVA, poly(vinyl alcohol)
PVAc, poly(vinyl acetate)
PVamine, poly(vinyl amine).

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Abstract: This chapter discusses the application of various imaging technologies for *in situ* characterization of hydrogel implants. It begins with the motivation for imaging implants *in situ* and then introduces the most relevant imaging modalities. The second half of the chapter addresses the challenges of imaging hydrogels *in situ* and discusses the use of contrast agents to improve the detection of hydrogels. The state of the art for the use of imaging techniques to characterize hydrogel implants and the healing responses they induce is then presented. Finally, the chapter concludes with a commentary on future trends and provides resources for additional information.

Key words: optical coherence tomography, magnetic resonance imaging, bioluminescence imaging, multiphoton microscopy, animal models, wound healing, contrast agents.

10.1 Introduction

Hydrogels represent an interesting class of implantable materials due to their composition and structure as well as their ability to serve as delivery systems for biologically active molecules. Their high water content and viscoelastic properties often mimic tissue properties of the native extracellular matrix (ECM). However, once implanted, hydrogels often undergo changes, either by designed degradation and remodeling or by the effects of mechanical loading or cellular responses, and thus characterizing or evaluating hydrogel implant behavior *in situ* is of critical importance. Yet, it is a challenging endeavor. The need for noninvasive and nondestructive techniques for implant characterization has led to the application of various imaging technologies to this problem. While both imaging hydrogel materials and imaging *in vivo* push at the limits of existing technologies, the potential for further developments in these areas is high. This chapter begins with the motivation for imaging implants *in situ* and continues with a description of the most relevant imaging modalities as well as their advantages and disadvantages. The second half of the chapter addresses the challenges of imaging hydrogels *in situ* and discusses means of improving the detection of hydrogels within images through the use of contrast agents. The state of the art for the use of imaging techniques to characterize hydrogel implants themselves both *in vitro* and *in vivo* as well as to characterize the healing responses induced by hydrogel implants *in vivo* is then presented. Finally, the chapter concludes with a commentary on future trends in imaging hydrogel implants and provides resources for additional information on the topic.

10.2 Rationale for imaging implants *in situ*

10.2.1 Imaging as a characterization method for hydrogel materials

Most standard methods for characterizing hydrogel materials are destructive, whether one uses chemical or mechanical testing to measure material properties or one uses standard microscopic analysis of fixed and stained histological sections to examine implant and tissue morphology. However, noninvasive imaging offers a promising alternative. Generally, imaging techniques have been used to determine structural information about a sample, either by allowing one to view micro- or nano-scale features or to view internal structures within nontransparent materials. Additional nonstructural information can sometimes be gained from imaging data, through the correlation of measured imaging parameters with material properties¹ or through the incorporation of sensitive markers into a sample providing functional readouts,² as two examples. Thus, imaging techniques have the potential to provide multiple metrics for characterizing a sample from data collected during one measurement session. Further, the nondestructive nature of most imaging methods allows one to follow the temporal evolution of morphological features. At present, sophisticated systems have been developed to provide real-time imaging of materials *in vitro*. For example, microfabrication techniques have been used to create a hydrogel-based three-dimensional (3D) cell culture platform utilizing optical microscopy with time-lapse image acquisition to examine cellular proliferation and migration within the hydrogels.³ Looking towards *in vivo* applications, the presence of many of the above features in biomedical imaging systems makes them powerful tools for the characterization of implanted materials.

10.2.2 The necessity of *in situ* characterization

In situ evaluation has relevance in both the clinical use and the preclinical testing of hydrogel implants. In patients who have received an implant, future assessment should be conducted with a minimum of discomfort and certainly without a second invasive surgical procedure. Further, even a small biopsy for histological evaluation can potentially disrupt the implant. In preclinical animal models, noninvasive imaging techniques also minimize pain and distress to the animals. Additionally, the nondestructive nature of biomedical imaging systems allows one to repeatedly image the same specimen, thus reducing the number of animals used in longitudinal studies and increasing the statistical power. Imaging can also be used in the *in vitro* evaluation of materials, where additional (often destructive) tests can be performed in parallel to fully characterize the materials. These *in vitro* tests can be used to establish correlations with aid in the interpretation of *in vivo* imaging data, providing a means of characterizing an implant without disturbing

it. In this chapter, sections 10.6 and 10.7 present several examples of imaging methods applied for the *in situ* characterization of hydrogel implants *in vitro* and *in vivo*. But first, the imaging technologies themselves are considered.

10.3 Imaging modalities and their advantages and disadvantages for the *in situ* imaging of hydrogel implants

Many biomedical imaging technologies exist for *in vivo* application; however, for various reasons, not all are suitable for imaging hydrogels. The most relevant imaging systems, including optical coherence tomography, multiphoton microscopy, bioluminescence imaging, magnetic resonance imaging, and several others, are discussed here. The principles of each technology are described, focusing on the relevant parameters for evaluating imaging systems with respect to hydrogel implants. Some of these key parameters include the imaging penetration depth in nontransparent tissue, which is essential for noninvasive imaging *in vivo*; the resolution, which should allow one to distinguish microstructural features; and the source of contrast for the imaging technology, which may limit what types of materials are able to be “seen” in an image.

10.3.1 Optical coherence tomography

Optical coherence tomography (OCT) has been applied to the imaging of biological samples since the 1990s and utilizes the echo time delay of low coherence light to generate an image based on the optical scattering of microscale structures within a material or tissue.⁴ Since the speed of light is too fast to measure this delay directly, principles of low coherence interferometry are used. An incident beam, typically with a wavelength of 800–1300 nm for better tissue penetration, is split so that half is sent to the sample and half to a reference mirror of known position. Light reflections in the sample occur due to its scattering properties and at interfaces of structures with different optical characteristics. At the detector, interference between the two reflected beams indicates that light reflected by a structure within the sample travels over the same distance as light reflected by the reference mirror, thus giving its position. The reference mirror can be scanned to obtain a depth profile of the sample (A-scan). Two-dimensional (2-D) and three-dimensional (3-D) images can be obtained by rastering the imaging beam across the sample. OCT offers an excellent spatial resolution of <10 μm and a penetration depth of up to 2–3 mm in biological tissue.⁵ While hydrogel materials are optically transparent and generally would not generate a signal in OCT images, encapsulated materials or interfaces with tissue can easily be detected because of the refractive index mismatch.⁶ Thus, OCT has been used to characterize hydrogel implants in different settings.^{7,8,9,10,11}

Further, ultrahigh resolution and polarization sensitive OCT techniques have been developed. Ultrahigh resolution systems utilize lasers with large spectral bandwidths, such as Ti:sapphire femtosecond lasers, resulting in a coherence length or axial resolution $<4\ \mu\text{m}$ in air.^{12,13} Ultrahigh resolution OCT has been used to image skin wound healing in mice¹² as well as to image skin equivalents consisting of keratinocytes and fibroblasts grown in collagen gels.¹³ Morphological features, such as the dermal-epidermal junction, could be observed in both cases. Polarization sensitive OCT (PSOCT), which measures the polarization of the backscattered light, can be used to image birefringent materials, such as collagen.¹⁴ This technique has been validated to determine collagen orientation in *ex vivo* tissues.¹⁵ The incident beam is first sent through a polarizer and a polarizer modulator before being split; after recombination, the beam is again sent through a polarization beam splitter, and the interference fringes are detected with horizontal and vertical polarization detectors. The birefringence can be calculated from the depth, source wavelength and phase retardation of several A-scans, using methods such as those developed by de Boer and Milner,¹⁶ and provides a measure of tissue organization.

10.3.2 Multiphoton microscopy

Multiphoton microscopy (MPM) utilizes differences in the local nonlinear optical properties of tissue to generate an image. In MPM, contrast can result from two-photon excitation fluorescence (TPEF), second harmonic generation (SHG), and/or third harmonic generation (THG), depending on the imaging setup used.¹⁷ TPEF is an excellent technique for obtaining high-resolution 3-D images of samples that are autofluorescent or that can be labeled with a fluorescent tag and, like OCT, has been applied since the 1990s to image samples of biological relevance.¹⁸ TPEF exploits the near-simultaneous absorption of two lower energy photons to excite a fluorophore in a single quantum event and thus can use a laser in the infrared (IR) range, allowing for good tissue penetration. Because the probability of dual absorption is highest at the focal point, excitation is restricted to a small volume resulting in high resolution. Likewise, as fluorescent excitation is localized to the focal volume, little out-of-focus fluorescence is generated, allowing the use of high-sensitivity detectors to catch all of the emitted fluorescence and resulting in minimal photobleaching in other areas of the sample. 2-D and 3-D images can be obtained by scanning the excitation beam across a sample (within the focal plane) and by performing a depth scan of the sample (through the focal plane). TPEF has been used to detect fluorescently labeled hydrogels, such as polyacrylic acid microparticles, which were shown to adhere to human corneal surfaces *ex vivo*.¹⁹

SHG²⁰ and THG²¹ are based on the second- and third-order nonlinear light-matter interactions induced at the focus of a high numerical aperture microscope objective, also resulting in high resolution optical sectioning. The excitation

wavelength of the incident light is typically in the IR to avoid absorption by biological tissues and to provide deeper penetration, which can reach a few hundred microns within highly scattering tissue or even a millimeter within hydrogels.²² Again, the beam can be rastered across the sample to generate 2-D and 3-D images. SHG results in the emission of light at one-half of the incident wavelength and is produced by noncentrally symmetric molecules. Examples of molecules that can generate a good SHG signal include starch granules, other polysaccharides, striated muscle, chloroplasts, and importantly, collagen (due to its triple helical secondary structure).¹⁷ On the other hand, THG is sensitive to inhomogeneities in a sample and is produced at the interface between materials with different refractive indices or third-order susceptibilities.¹⁷ Through the simultaneous detection and correlation of TPEF, SHG, and/or THG images, it is possible to obtain both structural and molecular information about a sample.^{1,22,23}

10.3.3 Bioluminescence imaging

Bioluminescence imaging (BLI) cannot be used for viewing hydrogel materials themselves; however, it is a powerful technique for noninvasive imaging over time in small animals. BLI, taking advantage of the fact that most cells and tissues are not luminescent, utilizes light emitted from the interaction of the enzyme luciferase with its substrates for the noninvasive *in vivo* imaging of cells or molecules.^{24,25,26} BLI can be performed on both cells in culture and on whole (small) animals.^{27,28} Generally, BLI is used for tracking cells, studying the effects of genetic manipulations to cells, or examining protein expression and function. As examples, BLI has been used to noninvasively image luciferase expressing cells in hydrogels implanted subcutaneously,^{29,30} and it has been used to measure gene expression for hydrogel delivery vehicles.^{2,31}

The luciferase used for BLI can come from several origins. In bacteria, the operon *lux* encodes luciferase and all proteins required for substrate biosynthesis. For nonbacterial luciferases, such as firefly luciferase, *Renilla reniformis* luciferase, and *Gaussia princeps* luciferase, the substrate (D-luciferin or coelenterazine) must be exogenously added. For typical *in vivo* imaging, cells are first transfected with a plasmid for luciferase, and then they are implanted. The substrate is injected intravenously immediately before the imaging session, and the light produced is detected by an ultrasensitive CCD camera. The choice of which luciferase to use can be limited by its emission spectra. Firefly luciferase is often chosen due to its broad emission spectrum, including a large component at wavelengths greater than 600 nm, which has better transmission through skin.³² A 2-D map of light intensity can be obtained and overlaid with a photograph of the animal for localization. BLI has several advantages, including low background signal and no need for an external light source. Luminescence can be detected for as few as 10^3 cells *in vivo* and scales linearly with cell number,³⁰ making BLI a sensitive measurement technique. However, light generated by luciferase can

only be detected to depths of a few centimeters in tissue, the signal may be further attenuated by implant materials,²⁹ and the spatial information is generally limited to 2-D. Moving towards 3-D imaging, recent work has utilized tomographic techniques to determine the depth of light-emitting sources.^{33,34}

10.3.4 Magnetic resonance imaging

Magnetic resonance imaging (MRI)³⁵ utilizes an electromagnetic field to align the magnetic moments of protons (hydrogen atoms). When the field is turned off, the protons decay, and a photon is released because of the difference in energy. Protons in different tissues decay at different rates, which can be detected and used to form an image. Further, the alignment of the magnetic field can be adjusted during scanning to obtain positional information. Both 2-D slices and 3-D reconstructions can be obtained from MRI data. MRI works well in tissues with high water content, as water molecules contain two protons. The two basic parameters of image acquisition, the echo time (T_E) and the repetition time (T_R), lead to different MRI scan sequences. Spin-lattice relaxation time (T_1)-weighted scans use a short T_E and short T_R and provide good contrast between white matter and grey matter in brain tissue whereas spin-spin relaxation time (T_2)-weighted scans use a long T_E and long T_R and result in a bright signal from fluid-containing tissues.

Unlike other imaging technologies, hydrogels can easily be resolved in MR images due to their water content.^{36,37,38} Further, as T_2 derives from molecular motion/water mobility, this parameter can be used in the characterization of implanted hydrogels. For example, in chondral defects treated with a photopolymerizable hydrogel, a decrease in T_2 was shown to correlate with an increase in repair tissue (presumably replacing the hydrogel).³⁹ Contrast agents (described in detail in section 10.5), such as superparamagnetic iron oxide (SPIO) nanoparticles and ferritin nanoparticles,⁴⁰ have been developed that affect T_2 . The magnetic properties of the ferritin nanoparticles can be varied with their distribution in a nanofibrous hydrogel network, leading to changes in the T_2 relaxation times.⁴¹ On the other hand, the contrast agent gadolinium (Gd) has been used to allow T_1 -weighted scans of hydrogels, providing information on protein distribution profiles⁴² and glycosaminoglycan (GAG) content of engineered cartilage,⁴³ as two examples.

10.3.5 Other imaging technologies

Several other imaging technologies exist for biomedical applications. Not all are particularly suited to imaging hydrogels, yet some may provide useful insights into the tissue response to implants. These technologies include laser Doppler imaging (LDI), laser speckle contrast imaging, sonography, and micro-computed tomography (microCT). LDI⁴⁴ utilizes a monochromatic laser, such as a Helium

Neon laser, and measures the Doppler shift of backscattered light from moving particles. This shift in frequency is proportional to the velocity of the particles. If these moving particles are cells such as erythrocytes, LDI can be used to measure blood flow in vessels, even as small as capillaries.⁴⁵ With an imaging penetration depth of about 1 mm in skin, LDI has become a common method to measure cutaneous perfusion.⁴⁶ LDI can also measure perfusion in small animal models and has been used to quantify angiogenesis induced by hydrogel scaffolds in a mouse model of hindlimb ischemia.⁴⁷ Laser speckle contrast imaging⁴⁸ can also be used to measure blood flow; however, it is presently limited to superficial tissues, such as the skin and the brain (accessible in small animal models by thinning of the skull over the region of interest). In this technique, speckle refers to the random interference pattern generated when coherent light is scattered in a random medium. Temporal changes in the speckle pattern result from movements of scattering particles within the medium. The time-integrated speckle pattern can then be analyzed to obtain measures of blood flow, as was first demonstrated by the imaging of the retina.⁴⁹

Sonography (or ultrasonography) utilizes the echo time delay of ultrasound waves to image an object. Ultrasound frequencies are any above the audio range, although most medical scanners use frequencies in the several megahertz range. Image contrast comes from the interface of materials with different acoustical impedances, with the intensity of the echo indicative of the acoustic impedance mismatch. Doppler measurements can also be used to enhance the information obtained from sonography. However, hydrogel materials do not image well with this technique, and the overall resolution of ultrasound imaging systems is rather low. Ultrasound is often used to measure heart function in echocardiography, and this technique has been used to demonstrate improvements in heart function for rats that had been treated with an alginate hydrogel after myocardial infarction.⁵⁰ In another interesting application, ultrasonography was used to measure ocular lengths of chicks that received different ocular hydrogel implants, which affected scleral growth.⁵¹

MicroCT utilizes X-rays for high resolution tomographic imaging. Image contrast comes mainly from materials that are radioopaque; therefore, for medical applications, microCT has primarily been applied to image bone. Vascular casting approaches have been used with radioopaque polymers to allow microCT imaging of the vasculature.⁵² While hydrogel materials would not appear in a microCT image, microCT has the potential to provide high resolution 3-D imaging of blood vessel⁴⁷ or bone^{53,54} growth into an implant. Further, microCT imaging systems have been designed to allow live imaging of small animals, allowing for longitudinal studies.

10.4 Challenges of imaging *in situ*

As discussed in the previous section, many biomedical imaging technologies exist; however, not all are suitable for imaging hydrogel implants *in situ*. One reason is

that many of the most promising imaging methods are inherently limited for *in vivo* imaging in humans. MRI is capable of imaging the whole body, but most other techniques (OCT, MPM, BLI) are limited in imaging penetration depth. However, these latter techniques often provide higher resolution images, which can be essential for observing effects at the cellular and molecular level. Recent work to miniaturize imaging systems to allow endoscopic imaging is discussed in section 10.8. A second challenge for imaging implants *in situ* is the inability of many imaging systems to detect hydrogels. In most cases, without the use of exogenous tags, hydrogels would not be visible in the images. Notable exceptions are the use of SHG to image collagen hydrogels and the potential visibility of hydrogels in T_2 -weighted MR images due to their water content. The use of contrast agents to enhance the detection of hydrogels in images is presented in section 10.5. A final consideration is the imaging speed of the system. Biological processes can occur on very fast time scales. If one wanted to characterize these processes, rapid image acquisition is essential. Further, with imaging *in vivo*, motion artifacts can affect image quality. Fast imaging speeds and/or image processing to remove motion artifacts may be necessary to result in high quality images.

10.5 Contrast enhancement

To address one limitation of imaging technologies for evaluating hydrogel implants, contrast enhancement mechanisms can be used to aid in the visualization of structures or molecules within a sample. Some imaging modalities take advantage of naturally occurring sources of contrast, such as that which results from the structure of collagen, while others require the use of exogenous contrast agents. These exogenous contrast agents range from fluorescent molecules to protein-based reporter systems to magnetic particles.

10.5.1 Natural sources of contrast

A number of naturally occurring molecules can provide contrast for imaging technologies. The amino acids tryptophan, tyrosine and phenylalanine; vitamin derivatives such as retinol and riboflavin; nicotinamide adenine dinucleotide; the pyridolamine crosslinks in elastin and collagens; and indoleamines are all fluorophores that can be found natively in tissue.⁵⁵ Collagen, microtubules and myosin are natural noncentrosymmetric molecules that can be detected using SHG.⁵⁶

Of interest because it can form hydrogel networks, collagen serves as a source of contrast for both TPEF and SHG. By utilizing long excitation wavelengths, it is possible to generate a backscattered SHG signal that does not overlap with TPEF emission.⁵⁷ Collagen SHG is dependent on fiber orientation and dimensions, and it has been hypothesized to emanate from the fibril shell.⁵⁶ Fibers smaller than the 385 nm SHG wavelength emit in the forward direction while fibers greater

than 385 nm tend to backscatter SHG.²² The ratio of forward to backscattered emission is also influenced by the ionic strength of the collagen solution.⁵⁶ High resolution SHG imaging systems are able to use the SHG signal to distinguish between type I and type III collagens.⁵⁸ The birefringent properties of collagen also allow it to be imaged using polarization sensitive techniques, such as PS-OCT (described in section 10.3.1). The birefringence of collagen is affected by fiber size and orientation, and thus has been used to examine collagen orientation in both engineered tissue constructs¹⁴ and native skin tissue.⁵⁹

10.5.2 Protein reporter systems

Protein reporter systems exist for almost all of the above mentioned imaging modalities. These systems can directly be used as the protein form, or the gene can be delivered as DNA to cells to either label the cells or provide a marker of gene expression levels. Common reporter systems include green fluorescent protein (GFP) and its analogues for fluorescence-based imaging, luciferase for BLI (see section 10.3.3), ferritin for MRI, and Herpes Simplex Virus-1 thymidine kinase for positron emission tomography (although this imaging technique is not discussed here).

Several protein expression systems for MRI detection exist, including tyrosinase,⁶⁰ beta-galactosidase,⁶¹ transferrin receptor,⁶² lysine-rich protein⁶³ and ferritin.⁴⁰ To discuss one system in more detail, the iron-binding protein ferritin provides a protein-based source for enhanced MRI contrast, acting to reduce the transverse relaxation of water (affecting T_2).⁴⁰ Iron has been extensively used as a contrast agent for MRI.⁶⁴ Naturally, ferritin helps to maintain iron homeostasis by chelating free iron; its expression is regulated by the iron regulatory RNA-binding proteins (IRP1 and IRP2) and the iron-responsive element present in the untranslated region of ferritin mRNA. Ferritin forms a shell consisting of 24 heavy and light chains that are folded in a four-helix bundle with threefold and fourfold channels and can bind up to 4500 iron atoms in the form of ferrihydrite ($5\text{Fe}_2\text{O}_3 \cdot 9\text{H}_2\text{O}$).⁶⁵ The iron core gives ferritin its antiferromagnetic and superparamagnetic properties, and the iron loading of ferritin and its aggregation state can influence its effects on relaxivity in MRI. As a contrast agent for hydrogels, ferritin has been incorporated into nanofibrous poly(vinyl alcohol) (PVA)-based formulations.⁴¹ Clustering of the ferritin particles was achieved by varying the temperature during electrospinning of the PVA/ferritin, which resulted in differences in the superparamagnetic properties of the ferritin and thus in different T_2 relaxation times. PVA/ferritin hydrogels electrospun at 80 °C provided enough enhanced contrast to be able to be detected *in vivo* by MRI.⁴¹

10.5.3 Inorganic contrast agents for MRI

The element gadolinium (Gd; atomic number 64) can be used as an MRI contrast agent due to its paramagnetic properties. Complexed with the chelating agent,

diethylenetriamine penta-acetic acid (DTPA), Gd-DTPA²⁻ affects the T_1 MRI signal. Because of its charge, Gd-DTPA²⁻ has been used to measure the fixed charge density (FCD) for cartilage, which correlated with FCD measurements of explanted cartilage made using other techniques⁶⁶ as well as with the dynamic modulus and the sulfated glycosaminoglycan (GAG) content of bioreactor-cultured cartilage.⁶⁷ Further, Gd-DTPA²⁻ has been used to measure the FCD of engineered cartilage constructs consisting of chondrocytes cultured in a poly(ethylene oxide) diacrylate hydrogel.⁴³ In another application, Gd-DTPA²⁻ has been used to label albumin to allow quantification of protein concentration profiles using MRI.⁴² In contrast to Gd-DTPA²⁻, iron oxide serves as a superparamagnetic MRI contrast agent, which shortens T_2 relaxation times. Iron oxide nanoparticles have been used to label stem cells for *in vivo* detection by MRI.⁶⁸

10.5.4 Other contrast mechanisms

Depending on the imaging method used, additional contrast agents can be employed to enhance the signal from implanted hydrogels or regenerating tissues. While the contrast agents described thus far in this section are by no means exhaustive, the following final examples were chosen to highlight contrast agents for two additional imaging technologies, OCT and microCT.

The scattering of light that generates a signal in OCT images derives from the optical scattering properties of the material being imaged and can also occur at the interfaces of two materials with different optical properties. Changes in the scattering or turbidity of a hydrogel suspension have been used to affect the OCT signal.⁸ In this case, the turbidity of the hydrogel suspension could be altered by the binding of concanavalin A (ConA) to glucose moieties on hydrogel particles. In a solution without free glucose, ConA remained bound to the particles resulting in a high scattering coefficient. However, upon the addition of glucose in solution, ConA dissociated from the particles, and the scattering coefficient (and OCT signal) was reduced.⁸

Contrast for microCT mainly comes from materials that are radioopaque. While this results in good images of mineralized tissues, such as bone, it is much more difficult to detect hydrogels or soft tissues. One contrast enhancing method is to utilize radioopaque contrast agents, and this has been done with great success using Microfil MV-122, a radioopaque silicon-based contrast agent containing lead chromate, for vascular casting.^{47,52,69} Microfil casting, tissue fixation, and subsequent decalcification resulted in better image contrast than a barium-sulfate based contrast agent for microCT imaging of collateral vessels in a mouse model of ischemia.⁵² Microfil perfusion was again used to examine vascularization of enzymatically degradable PEG scaffolds releasing VEGF as an angiogenic factor that was implanted subcutaneously.⁴⁷ MicroCT was performed, and image analysis could be used to quantify the volume of blood vessels that had grown into the implant.⁴⁷

10.6 Characterization of implants (*in vitro* and *in vivo*)

A main application of the *in situ* imaging of implants is to characterize the hydrogel materials themselves, whether *in vitro* or *in vivo*. As first mentioned in section 10.2, imaging methods are typically applied to examine the structure of a material; however, analysis of certain imaging parameters can give additional information on material properties, such as mechanical properties. This section presents several examples of structural and mechanical characterization of hydrogel implants using different imaging methods. Examples of imaging techniques used to characterize hydrogel drug delivery systems are also given.

10.6.1 Structure

Most imaging methods were principally developed to provide structural information about a sample; therefore, many have been applied to examine hydrogel structure or composition. The particular hydrogel material, preferably with minimal modification or labeling to avoid disturbing the gel network, influences the choice of imaging technique to best obtain the desired structural information. In the following two examples, different imaging methods were utilized to obtain structural information about hydrogel scaffolds *in vitro*. The noninvasive techniques used for imaging also have the potential to be applied once these hydrogels are implanted *in vivo*.

In a first example, PSOCT was used to examine the alignment of collagen fibers in tissue-engineered tendons that were exposed to varying growth conditions.¹⁴ Rat tenocytes were embedded in collagen hydrogels and exposed to daily cyclical loading in a Flexcell system. Phase retardation analyses were performed on PSOCT images, which were taken at several time points during the course of the study, to quantify the birefringence of the collagen (which is related to its orientation). There was a significant increase in phase retardation with culture time, suggesting a cell-mediated increase in collagen density.¹⁴

In a second example, Gd exclusion MRI was utilized to measure the GAG content of engineered cartilage.⁴³ Bovine chondrocytes were seeded in a photopolymerizable poly(ethylene oxide) diacrylate hydrogel and cultured *in vitro* up to 50 days. The matrix FCD, derived from the negative charges of the GAGs and indicative of compressive resistance, was estimated from the T_1 -weighted post-Gd MR images and correlated with the GAG content, measured by biochemical and histological assays of the constructs. This correlation was found to be statistically significant, suggesting the potential application of this method for the *in vivo* monitoring of the cartilage constructs.⁴³

10.6.2 Mechanical properties

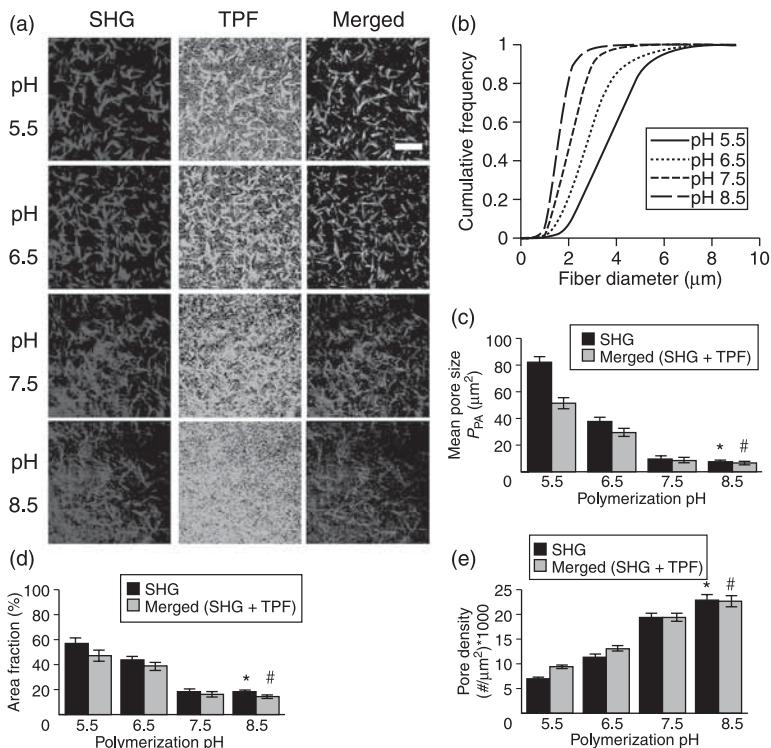
Several methods have been developed that utilize imaging techniques for the characterization of hydrogel mechanical properties, whether by imaging structural

changes during mechanical testing or by utilizing changes in imaging parameters that can be correlated with mechanical properties. In a novel method for the *in situ* and nondestructive mechanical characterization of hydrogels, a micro-indentation technique was combined with OCT imaging.⁷ The deformation of hydrogels was accurately measured in high resolution OCT images where deformation was induced at constant force using small (1–3 mm diameter) spherical indenters, and the Young's modulus was determined from the indentation depth by the Hertz contact theory. The OCT-based method measured values for the Young's modulus that were similar to values measured using traditional depth-sensing micro-indentation and further could be used for creep measurements to determine viscoelastic properties.⁷

MPM, specifically TPEF and SHG, can be used to image a collagen gel microstructure, which correlates with its mechanical properties. Raub *et al.* have shown that the bulk optical properties for collagen hydrogels change as the mechanical properties change.²² By changing collagen polymerization conditions, the hydrogel fiber diameter and porosity could be changed, resulting in changes in the mechanical properties. Interestingly, when decreasing the polymerization temperature, the mean segmented SHG signal increased with decreasing storage modulus whereas, when increasing glutaraldehyde crosslinking (which results in the formation of fluorescent intramolecular pyridinium-type crosslinks), the TPEF signal increased with a higher storage modulus.²² In a second study, image correlation spectroscopy (ICS) on MPM, scanning microscopy and darkfield microscopy images also correlated with the collagen mechanical properties, and in particular, the pore area fraction measured from SHG images decreased with increasing modulus.¹ Further, the ICS characteristic fiber size correlated with hand-measured fiber diameters for each polymerization condition.¹ Examples of SHG and TPEF images of the collagen hydrogels are seen in Fig. 10.1.

10.6.3 Hydrogel delivery systems

Hydrogels are frequently used as drug delivery systems themselves or as tissue mimetics for measuring drug delivery *in vitro*, and imaging methods provide a nondestructive means for hydrogel characterization. MRI has been utilized to examine structural changes (i.e., hydrogel formation and swelling) of polymeric dosage forms during release studies *in vitro*.^{36,37} Hydrodynamically balanced systems consisting of hydroxypropylmethylcellulose (HPMC) in a hard gelatin capsule were imaged by MRI in a custom-designed flow-through cell for dissolution studies. Solvent penetration into the hydrodynamically balanced system and hydrogel formation could be observed in the images after capsule dissolution.³⁶ Further, dimensional changes in the hydrogel layer could be quantified from segmented images, and these were found to vary in different dissolution media.³⁷ In a second example, *in situ* OCT imaging was used



10.1 (a) SHG, TPEF, and merged images of collagen hydrogels polymerized at different pH. Scale bar is 50 μm . (b) Fiber diameter frequency distributions, (c) mean pore size, (d) pore area fraction, and (e) pore density for collagen hydrogels polymerized at different pH. Pore size, area fraction, and density were determined from noise thresholded and inverted SHG images and from merged (SHG and TPEF) images. *,# indicate statistical significance. (Reprinted with adaptation with permission from Raub *et al.*¹ (© 2008 by the Biophysical Society).)

to quantify morphological changes in poly(lactide-co-glycolide) (PLGA) microspheres that were suspended within hyaluronic acid hydrogels as a controlled delivery system.⁶ In an *in vitro* release study using bovine serum albumin (BSA) as a model protein, an increase in particles observed to have a hollow morphology, indicative of polymer dissolution and protein release, was seen for samples made with a faster degrading PLGA. The rate of protein release from these hydrogels was also faster.⁶ The potential to make the same measurements *in vivo* was demonstrated by implanting a microsphere-loaded hydrogel in a rat calvarial defect model and performing OCT imaging through a ‘window chamber’ attached to the skull over the defect.⁶

As tissue mimetics, agarose hydrogels have been used as phantoms to measure the convection-enhanced delivery of proteins *in vitro* using MRI.⁴² Gd-DTPA-

labeled albumin (Gd-albumin) was utilized to provide enhanced MRI contrast, and the Gd-albumin distribution within the hydrogel could be determined from the MR images.⁴² The concentration profiles were determined from the signal enhancement in T_1 -weighted scans upon Gd-albumin infusion, using a calibration curve derived from measuring known concentrations of Gd-albumin. The MRI-derived concentration profiles correlated well with concentration profiles measured by optical methods as well as with simulated data.⁴² This method allows both the spatial and temporal progression of diffusion to be measured in a nondestructive manner and has potential for the *in vivo* monitoring of protein distribution after infusion into a tissue.

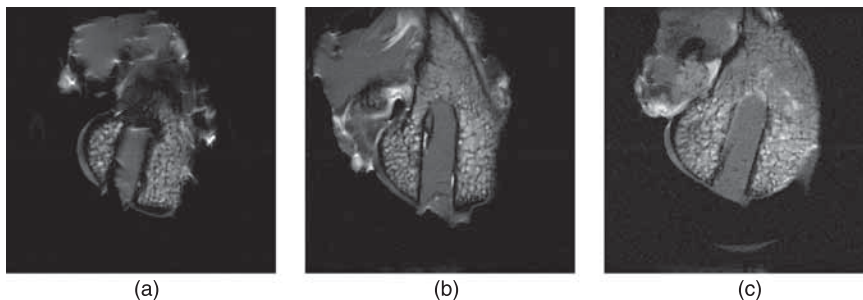
10.7 Characterization of *in vivo* healing

In addition to measuring material properties, a second application of *in situ* imaging for hydrogel implants is to characterize the healing response they can induce *in vivo*. This section highlights several examples of the state of the art for imaging this response. The healing process can cause changes to the hydrogel implant itself as well as to cells or drugs that are encapsulated within the hydrogel. Further, hydrogels can be used to deliver therapeutic agents or to induce tissue regeneration, and the effects on the surrounding tissue can be imaged.

10.7.1 Changes to hydrogel implants

Imaging methods can be applied to characterize implant placement as well as changes to the implant *in vivo*. Different imaging modalities can be used, depending on the type of implant, contrast mechanisms available and anatomical location of the implant. As two examples, TPEF has been used to image attachment of fluorescently labeled polyacrylic acid hydrogel nanoparticles to the human cornea *ex vivo*,¹⁹ and MRI has been used to image poly(ethylene glycol) (PEG) based hydrogels implanted in an *ex vivo* rabbit osteochondral defect model.³⁸ In this second study, the PEG gels were prepared by photocrosslinking injectable copolymer macromers that contained different metabolites, resulting in different structural and mechanical properties.³⁸ MRI was used to demonstrate that the crosslinked hydrogel continued to fill the entire defect volume after dynamic mechanical loading, as can be seen in Fig. 10.2.³⁸

Novel methods utilizing imaging techniques are also being developed to measure changes in hydrogel properties after implantation. In one example, changes in the scattering properties of a glucose sensitive hydrogel were measured using OCT, leading to potential application as an implantable glucose sensor.⁸ The sensor relied on the affinity binding of concanavalin A (ConA) to glucose residues on macroporous hydrogel particles. In the absence of soluble glucose,



10.2 MR images (sagittal plane) of hydrogels formed from poly(glycerol beta-alanine)-PEG macromers in a rabbit osteochondral defect after loading with a 3 kg weight. (a) 5% w/v hydrogel. (b) 10% w/v hydrogel. (c) 20% w/v hydrogel. Reprinted with adaptation with permission from Degoricija *et al.*³⁸ (© 2008 by the American Chemical Society).

this interaction increased the scattering coefficient of the particles (with ConA bound to the particles), whereas the presence of soluble glucose decreased the scattering coefficient of the particles (with ConA bound to free glucose). Changes in the turbidity of the hydrogel microsphere suspension *in vitro* in response to changes in soluble glucose levels were imaged using OCT. The scattering coefficient was affected by hydrogel particle size, particle size distribution and ConA concentration, and a dynamic response of the sensor was demonstrated. Further, the feasibility for using this technique *in vivo* was examined by studying the effects on the OCT signal when a tissue phantom was placed between the sensor and the imaging system.⁸

10.7.2 Changes to cells, drugs, etc. within hydrogels

While hydrogels hold some interest as implantable materials on their own, they have further application for regenerative medicine and tissue engineering such as drug delivery systems and cell encapsulation vehicles. In these cases, characterizing changes in the hydrogel materials is secondary to characterizing the delivery or survival of its cargo. BLI and MRI allow tracking of implanted, labeled cells that are encapsulated within hydrogels, and several examples are provided here.

As described in section 10.3.3, the luciferase-luciferin system can be used to label cells for tracking via BLI. Cells can be transfected with a luciferase plasmid *in vitro*, and luminescence after luciferin exposure can be used to quantify and localize labeled cells both *in vitro* and *in vivo*. Logeart-Avramoglou *et al.* demonstrated a correlation between cell number and bioluminescence for cells in suspension and then for cells on scaffolds.²⁹ Cell survival and proliferation were measured for labeled cells seeded on a porous translucent hydrogel, formed from a polymer composed of acrylonitrile and sodium methallyl sulfonate, and

even on a porous opaque ceramic material derived from coral. Further, BLI intensity measured for the scaffolds *in vitro* correlated well with BLI intensity measurements of the constructs implanted subcutaneously in nude mice despite some signal attenuation; *in vivo* cell proliferation was also observed using BLI.²⁹

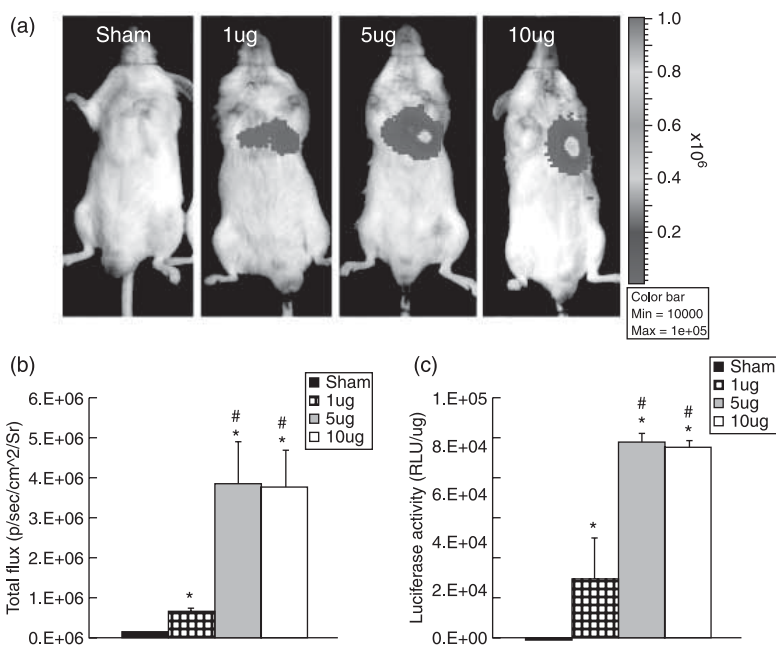
In addition to subcutaneous sites, BLI has been used to image hydrogels containing cells that are implanted intramuscularly.³⁰ Mouse embryonic fibroblasts were transfected with a luciferase plasmid and encapsulated in nanofibrillar hydrogels formed from the self-assembling peptide RAD16-I[Ac-(RADA)₄-NH₂; single letter amino acid abbreviation]. Composites consisting of hydrogel-encapsulated cells inside a microporous biorubber scaffold were also examined. For cells alone, the BLI signal correlated well with the cell number. The RAD16-I hydrogel supported cell survival and proliferation whereas the biorubber composite showed poor cell survival *in vitro*. *In vivo*, hydrogel encapsulation enhanced the survival of cells implanted intramuscularly.³⁰

MRI can also be used to track implanted cells *in vivo* when they are labeled with appropriate contrast agents. Syková *et al.* utilized iron-oxide nanoparticles or magnetic microbeads to label cells for detection by MRI.⁶⁸ Embryonic or mesenchymal stem cells were implanted in cortical or spinal cord lesions or delivered intravenously in rats. The cell therapy resulted in increases in function and hind leg sensitivity, and the transplanted cells could be visualized within the lesion using MRI.⁶⁸

10.7.3 Delivery of therapeutic agents

Gene therapy represents a powerful approach to the sustained delivery of therapeutic agents, and hydrogel materials are being tested to further enhance or prolong gene transfer and, ultimately, expression. BLI has been utilized for the noninvasive monitoring of gene expression *in vivo* in longitudinal animal studies. Two examples are given here in which BLI was applied to measure polymeric hydrogel-based gene transfer, in the heart² and in the skin.³¹

Kwon *et al.* utilized a thermo-responsive amphiphilic polymer based on poloxamer and di(ethylene glycol) divinyl ether, called multi-block copolymer 2 (MBCP-2), to encapsulate plasmid DNA.² This formulation carrying a plasmid encoding for luciferase was injected into the myocardium of a mouse, and gene expression was quantified using BLI (see Fig. 10.3). First, luciferase intensity in the BLI images was correlated with protein expression measured by standard assays. The dose dependence and temporal effects of gene expression were then explored, and the hydrogel formulation was found to both enhance initial gene expression and extend its duration compared to naked plasmid.² The hydrogel/DNA complex was then utilized to deliver a therapeutic gene, vascular endothelial growth factor (VEGF), which resulted in increased capillary density and larger vessel formation in a myocardial infarct model.²



10.3 (a) BL images (two days after gene delivery) for animals treated with 0, 1, 5, or 10 μ g luciferase plasmid, delivered to the heart. (b) Optical imaging flux. (c) Luciferase activity in homogenized heart tissue. *indicates $p < 0.05$ versus control. #indicates $p < 0.05$ versus 1 μ g treatment group. (Reprinted with adaptation with permission from Kwon *et al.*² (© 2009 by Elsevier B.V.).)

A second polymer hydrogel system, consisting of 2% agarose, was utilized to deliver DNA compacted with polylysine intradermally in rats.³¹ Quantification of gene expression was performed with BLI, and the superficial location of the injections resulted in a strong BLI signal with minimal light absorption. BLI intensity could be converted to luciferase concentration in the tissues by using a standard curve. Luciferase expression was both increased and prolonged with the gel formulation (>35 days) compared to the unencapsulated DNA complexes (<7 days).³¹

10.7.4 Evaluation of tissue morphology

As implants are frequently intended to induce tissue regeneration or remodeling, it is relevant to consider imaging techniques that can evaluate tissue morphology growing into or adjacent to hydrogel implants. Four examples, which span different imaging modalities and tissue types, include the *in vivo* LDI and *ex vivo* microCT imaging of angiogenic blood vessels,⁴⁷ the *ex vivo* MRI of chondral

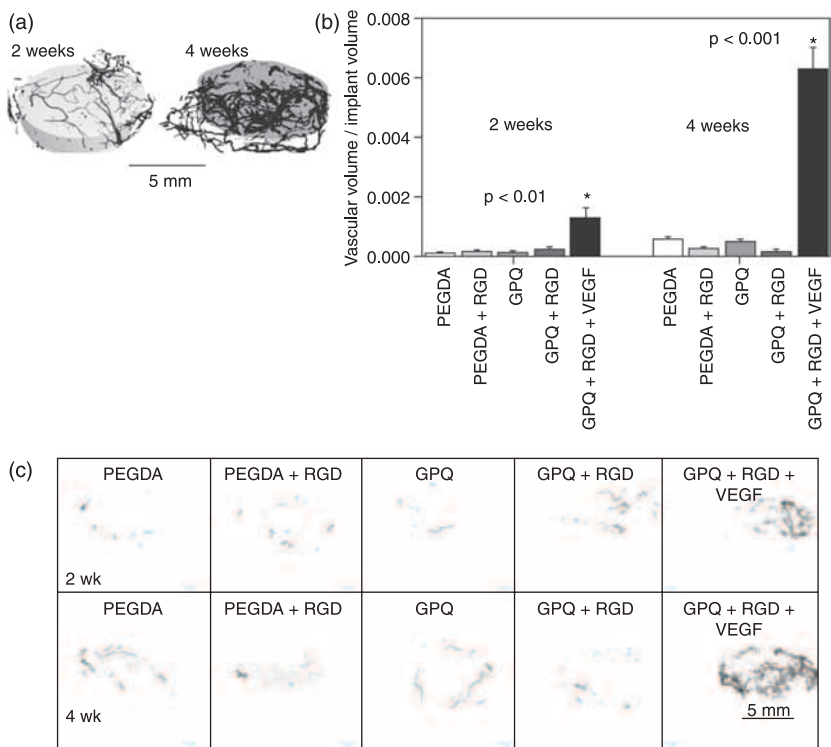
tissue repair,³⁹ the *in vivo* OCT imaging of cartilage repair,⁹ and the *in vivo* OCT imaging of skin wound healing.^{10,11}

Vessel ingrowth into poly(ethylene glycol) (PEG) hydrogels delivering VEGF was characterized by several imaging techniques *in vivo*.⁴⁷ LDI and microCT images are shown in Fig. 10.4. A PEG-diacrylate (DA) macromer containing a matrix metalloproteinase (MMP) sensitive peptide sequence was photocrosslinked with mono-acrylated PEG-VEGF and mono-acrylated PEG-RGD to form a cell-responsive hydrogel. (RGD is the cell-adhesive peptide ligand arginine-glycine-aspartic acid.) *In vivo* release of VEGF was determined by measuring the fluorescent signal in a Xenogen imaging system for animals that were treated with VEGF tagged with indocyanine green (ICG), a fluorescent dye. In a mouse hindlimb ischemia model, LDI showed increased reperfusion to both the feet and the legs for animals treated with PEG/RGD/VEGF compared to PEG or VEGF alone.⁴⁷ The same hydrogel system was evaluated in a rat subcutaneous implant model, and blood vessel ingrowth was viewed by *ex vivo* microCT imaging of the tissues after perfusion with Microfil, a radioopaque contrast agent. Similar to the results with the ischemia model, the degradable PEG/RGD/VEGF treatment resulted in an increase in the ratio of vascular volume to implant volume compared to nondegradable constructs and constructs without VEGF.⁴⁷

In a rabbit chondral defect model, a photocrosslinkable PEGDA/hyaluronic acid (HA) hydrogel was used as a treatment. After five weeks of healing, the animals were sacrificed, and *ex vivo* MRI of the distal femurs was performed. A high field (9.4 T) MRI was used, and the transverse relaxation time (T_2) was measured for each defect site. The amount of repair tissue filling the defect was measured by histomorphometry, and a correlation between T_2 and percent tissue fill was determined.³⁹ In this case, the hydrogel itself could not be distinguished in the MR images, as there was insufficient contrast between the hydrogel and the solution the tissue sample was placed in to remain hydrated during imaging. The authors of the study proposed that the correlation they observed using MRI T_2 data could be extended to measure chondral tissue repair *in vivo* as well as to examine the fate of *in vitro* tissue engineered cartilage after implantation. A second study utilizing the rabbit chondral defect model examined the effects of the implantation of chondrocytes embedded in collagen gels using OCT.⁹ Morphological features, such as the bone and cartilage interface, chondrocyte hypertrophy, surface fibrillations, and gaps between the repair tissue and host cartilage, were observed in the *ex vivo* OCT images and correlated with histological analysis.⁹ Again, this technique could be applied to *in vivo* imaging by integrating OCT with arthroscopic examination.

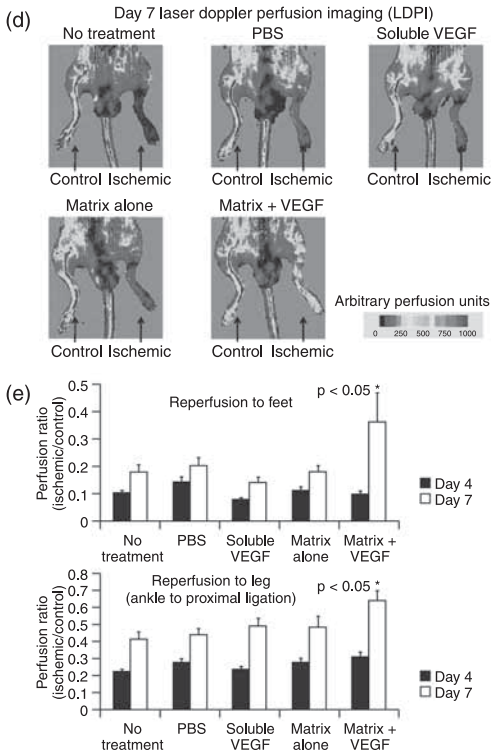
In perhaps the most exciting demonstration of *in vivo* imaging of hydrogel implants to date, Yuan *et al.* utilized high resolution OCT to monitor skin wounds treated with biodegradable hydrogels.¹⁰ An *in situ* forming hydrogel consisting of partially oxidized dextran, chitosan and HA was made in the wound beds after dorsal full-thickness excisional dermal wounds, in both normal and diabetic mice.

Important tissue features, such as ECM, connective tissue, re-epithelialization, granulation tissue, and dermis, could be correlated between the OCT images and standard histological sections after various healing times. An example image is shown in Fig.10.5. Quantitative measures of tissue morphology, such as the granulation tissue thickness or ECM thickness, could be determined from the OCT images, which correlated well with values measured using histological sections. Further, differences in tissue morphology between the normal and diabetic mice seen in the histological sections could also be observed in the OCT



10.4 (a–c) MicroCT imaging after perfusion with Microfil of PEG scaffolds implanted subcutaneously in rats. (a) Degradable PEG hydrogels functionalized with RGD cell adhesive ligand and VEGF showing vasculature surrounding and growing into implant (gray volume is the hydrogel). (b) Vascular volume/implant volume for different treatment groups and time points. (c) Representative microCT images at 2 and 4 weeks for (from left to right): nondegradable hydrogels, nondegradable hydrogels with cell adhesive ligand, degradable hydrogels, degradable hydrogels with cell adhesive ligand, and degradable hydrogels with cell adhesive ligand and VEGF.

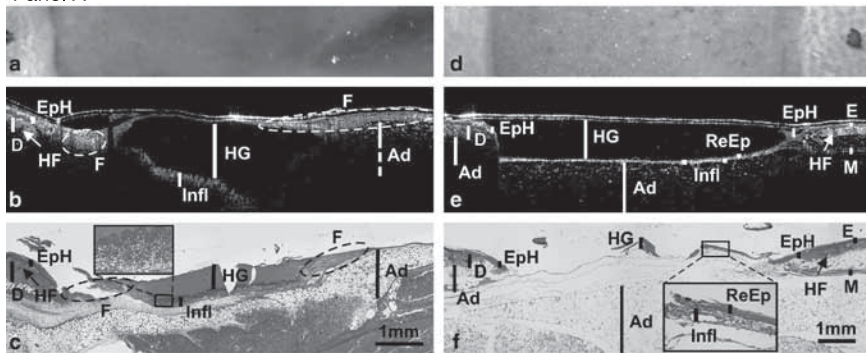
(continued)



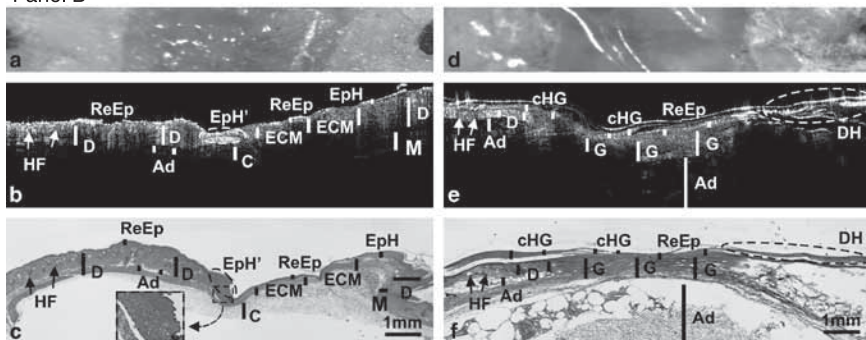
10.4 (d–e) LDI of hindlimb perfusion in mice after ligation of femoral artery and vein. (d) LD images after 7 days for groups: no treatment, PBS injection, soluble VEGF injection, degradable hydrogel with cell adhesive ligand, and degradable hydrogel with cell adhesive ligand and VEGF. (e) Perfusion ratios of normal leg versus ischemic leg after 4 days and 7 days. (Reprinted with adaptation with permission.⁴⁷ (© 2010 by PNAS).)

images. Importantly, the hydrogel implant could clearly be localized in early OCT images as a clear region with relatively sharp borders, and morphological changes during the healing process, such as cell-mediated hydrogel degradation and accelerated re-epithelialization, could be followed in time using OCT.¹⁰ This study extended previous results from the same group utilizing OCT to image dermal wounds treated with collagen implants.¹¹ There, OCT was used to detect inflammation, re-epithelialization and resorption of the collagen implant with high penetration depth (> 1.5 mm) into the wound bed.¹¹ Additional information can be obtained by the utilization of MPM, as has been shown *in vitro* in a study using OCT, TPEF and SHG to image a skin equivalent consisting of fibroblasts embedded in a collagen hydrogel.⁷⁰ There, regions of burn injury could be distinguished in the OCT and SHG images while fibroblast migration could be observed by TPEF.⁷⁰

Panel A



Panel B



10.5 Images of dermal wound healing after treatment with an *N*-carboxyethyl chitosan, oxidized dextran, and hyaluronic acid hydrogel after 3 days (Panel A) and 10 days (Panel B). Each panel consists of surface (a,d), cross-sectional OCT (b,e), and histological (c,f) images for normal (a–c) and diabetic (d–f) mice. Abbreviations: adipose tissue (Ad), cell-infiltrated hydrogel implant (cHG), dehydrated hydrogel (DH), epidermal hyperplasia (EpH), epidermal hyperplasia at a later-stage (EpH'), extracellular matrix (ECM), fibrotic exudates (F), granulation tissue (G), hair follicles (HF), hydrogel implant (HG), inflammatory reaction (Infl), and muscle layers (M). The fibrotic exudates, epidermal hyperplasia, cell-infiltration into the hydrogel, granulation tissue, and dehydrated hydrogel all resulted in high or increased scattering in the OCT images. (Reprinted with adaptation with permission from Yuan *et al.*¹⁰ (© 2010 by Mary Ann Liebert, Inc.).)

10.8 Conclusions

This chapter has provided an introduction to the state of the field for the *in situ* imaging of hydrogel implants. Biomedical imaging technologies provide a means for the noninvasive and nondestructive characterization of materials *in vivo*, and imaging systems are beginning to be applied to visualize hydrogels. Several common imaging methods, including OCT, MPM and MRI, have been directly

applied for the imaging of hydrogel materials. Additional techniques, such as BLI, LDI, laser speckle contrast imaging, sonography and microCT, may not be ideal for imaging hydrogels *per se* but can provide information about the tissue responses induced by hydrogels. The basic principles for each of these imaging technologies were discussed with the purpose of highlighting the advantages and limitations of each system in the context of imaging hydrogel implants.

At present, limited examples exist for the application of imaging technologies to the *in situ* imaging of hydrogels. In part, this is due to the youth of the biomedical imaging field, with many of the above techniques dating from the 1980s or 1990s. However, there are also technical challenges that limit *in situ* imaging. These challenges include the limited penetration depth of several high resolution imaging techniques (OCT, MPM, BLI), the poor contrast and detection of hydrogel materials without the use of exogenous labels, and the speed of imaging systems required to detect biological processes or limit motion artifacts *in vivo*. To provide better detection of hydrogels or regenerating tissues within images, several mechanisms for contrast enhancement exist, including fluorescent tags, protein reporter systems, magnetic particles and radioopaque materials.

While limited, the examples given for the *in vitro* and *in vivo* characterization of hydrogel implants and for the characterization of *in vivo* healing hint at the potential for this field. Techniques such as OCT, MRI and MPM have been applied for *in vitro* characterization of hydrogel structure, composition and/or mechanical properties. These methods can be used to make the same measurements *in vivo* and are primarily limited by the penetration depth of the imaging technique. Once implanted, changes can occur to the hydrogel implant or to its cargo. Again, OCT, MRI and MPM have been used to evaluate hydrogels *in vivo* whereas BLI and MRI have allowed tracking of labeled cells that were encapsulated in hydrogels. Finally, in many cases, the hydrogel implant is used as a delivery system or scaffold, and the desired outcome measure is the tissue response induced by the implant. This also represents the most advanced application of imaging methods for *in situ* imaging, with BLI used to detect *in vivo* gene expression, OCT used to detect skin and cartilage repair, and LDI and microCT used to quantify angiogenesis, all mediated by hydrogel systems.

The opportunities for progress in the *in situ* imaging of implants are vast. As many imaging systems are now commercially available (listed in the following section), it is only a matter of time before they are applied to characterize more materials and different tissues. Research is also being conducted to develop better contrast agents and to increase the versatility of imaging systems (by increases in resolution, imaging penetration depth, imaging speed, and so on). Two areas of exciting new research are the miniaturization of imaging systems for incorporation into endoscopes and the development of real-time imaging systems for imaging in awake and freely moving animals. As one example of an endomicroscope, a miniature compound lens and multimode-fiber collector

was integrated into a fiber-optic based system to allow TPEF and SHG imaging, which was demonstrated with fluorescent beads and rat tail tendon, respectively.⁷¹

In the field of neuroscience, real-time imaging of transient responses to behavioral changes has led to the development of several imaging systems that can be attached to moving animals. Fiber-optics can be used to allow fluorescence imaging in freely moving animals.^{72,73,74} One fiber-optic approach utilized a microendoscopic technique, which was coupled to a fluorescence microscope for detection.⁷⁴ The calcium (Ca^{2+}) sensing protein, yellow cameleon 3.60 (YC3.60), was used to detect Ca^{2+} activity in the brain, and discontinuities in the fluorescence trace (indicative of changes in bulk Ca^{2+} signals) could be seen when the animals changed behavior, such as starting movement.⁷³ Utilization of a different imaging modality could enhance the resolution of this system. For example, a miniature head-mounted two-photon microscope has been used to provide high-resolution imaging of the brain in freely moving animals.⁷⁵ Also applying fiber-optics, the images were obtained by scanning the fiber tip using a piezoelectric element, and capillaries filled with fluorescently labeled blood could be observed in the brain.⁷⁵ Recent advances have developed an ultra-compact two-photon microscope with a headpiece that weighs less than a gram.⁷⁶ To eliminate the need for the fiber-optic cable, another group has worked to develop a head-mountable miniature optical imaging system.⁷⁷ In the future, they hope to incorporate ultra-wide band telemetry to allow real-time analysis.

10.9 Sources of further information and advice

The purpose of this chapter has been to present different biomedical imaging techniques in the context of imaging hydrogel implants. Therefore, the description and theory behind each of the imaging methods have been limited to the most relevant parameters for this application. For additional information on these techniques, the reader is referred to the references given in the text. Current reviews as well as fundamental papers establishing the technique were selected for each imaging system.

Techniques for the imaging of biological samples as well as their functional implementation are increasingly being discussed in conferences and journals of leading optics and imaging societies. These include:

- The Optical Society (OSA): <http://www.osa.org>
- SPIE: <http://spie.org>
- The Institute of Electrical and Electronics Engineers: <http://www.ieee.org>
- The Society for Molecular Imaging: <http://www.molecularimaging.org>
- The International Society for Magnetic Resonance in Medicine:
<http://www.ismrm.org/>

Finally, many of the imaging systems discussed in this chapter are commercially available. Examples of commercially available systems include:

- OCT – Michelson Diagnostics (<http://www.md-ltd.co.uk>)
- MPM – Leica Microsystems (<http://www.leica-microsystems.com>)
- MRI – GE Healthcare (<http://www.gehealthcare.com>); Philips Healthcare (<http://www.healthcare.philips.com>); Siemens Healthcare (<http://www.medical.siemens.com>)
- BLI – Caliper LifeSciences (formerly Xenogen) (<http://www.caliperls.com/products/preclinical-imaging/>)
- LDI and laser speckle contrast imaging – Moor Instruments (<http://www.moor.co.uk>)
- Sonography – VisualSonics (<http://www.visualsonics.com>)
- MicroCT – SCANCO Medical (<http://www.scanco.ch>)

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