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In-Situ Forming Biomimetic Hydrogels for Tissue Regeneration

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1. Introduction

Tissue loss or organ failure caused by injury or damage is one of the most serious and costly problems in human health care. Tissue engineering, proposed by Langer *et al.* in the early 1990's (Langer & Vacanti, 1993), is an emerging strategy of regenerative biomedicine that holds promise for the restoration of defect tissues and organs. The concept of tissue engineering is defined as "the application of the principles and methods of engineering and the 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" (Langer & Vacanti, 1993). In order to accomplish these goals by tissue engineering, three essential components are required, that is, cells for the generation of new tissues, scaffolds for supporting the cell growth and the regeneration of new tissues, and bioactive factors capable of stimulating biological signals *in vivo* for cell proliferation, dfferentiation and tissue growth. Among these, the scaffolds play an important role in the success of tissue regeneration since they serve as temporary temples to mimic the excellular matrix for cell growth and interim mechanical stability for tissue regneration and integration,.

Hydrogels are one of most used bio-scaffolds in the field of tissue enginereering. They are three-dimensional, water-swollen, crosslinked networks of hydrophilic polymers. Wichterle and Lim for the first time reported on hydrogels based on the hydroxyethyl methacrylate (HEMA) for biological use in 1960 (Wichterle & Lim, 1960). Due to their unique tissue-like properties, such as high water content and good permeability to oxygen and metabolites, hydrogels have been widely studied as biomimetic extracellular matrixes for tissue regeneration. Hydrogels may be used by implantation or injection, which corresponds to so-called preformed hydrogels or in-situ forming hydrogels. From the clinical point of view, in-situ forming hydrogels are highly desirable since they gain advantages over preformed hydrogels: (1) Enabling minimally invasive surgeries for implantation; (2) Formation in any desired shape in good alignment with surrounding tissue defects; (3) Easy encapsulation of bioactive molecules and progenic cells. Therefore, in-situ forming hydrogels have received much attention in recent years.

2. Strategies to design in-situ forming hydrogels

In-situ forming hydrogels are referred to as hydrophilic polymer networks that are in-situ formed in the body after the injection of liquid gel precursors. They are typically categorized into chemical hydrogels and physical hydrogels according to the mechanism underlying the network formation. Chemical hydrogels are those that are prepared by chemically covalent crosslinking of polymers. On the other hand, physical hydrogels are obtained by physical interactions of polymers, such as stereocomplex formation, hydrophobic interactions, and ionic interactions. So far, different crosslinking methods have been developed to prepare in-situ forming hydrogels, which are described in detail as follows.

2.1 Chemical crosslinking

Chemical crosslinking produces irreversible, also called permanent hydrogels. Generally, the hydrogels have robust mechanical properties and chemcial stability, which are favorable as supportive scaffolds for tissue engineering. Furthermore, covalent cross-linking is a good means to precisely control the cross-linking density of chemical hydrogels, thus controlling the hydrogels properties such as degradation time and mechanical strength.

2.1.1 Michael-type addition

Michael-type addition reaction is one of the commonly used approaches for the preparation of hydrogels, especially, in-situ forming hydrogels. By this approach, in-situ forming hydrogels can be obtained by mixing aqueous solutions of polymers bearing nucleophilic (amine or thiol) and electrophilic groups (vinyl, acrylate or maleimide) (Mather et al., 2006). For example, Feijen *et al.* prepared in-situ forming hydrogels based on vinyl sulfone-conjugated dextran and poly(ethylene glycol) (PEG) thiols through Michael-type addition (Hiemstra et al., 2007a). The gelation times can be tailored from 7 to 0.5 min when the drgree of vinyl sulfone subsitution increased from 4 to 13. Additionally, by varying the degrees of substitution, dextran molecular weights and polymer concentrations, the storage moduli of the hydrogels can be adjusted from 3 to 46 kPa, and the degradation time from 3 to 21 days. In another study, Hubbell et al. reported on smart hydrogels that were formed in-situ by the addition of thiol-containing oligopeptides to multi-arm vinyl sulfone-terminated PEG (Fig. 1) (Lutolf et al., 2003). Rheology test showed the pH condition plays an important role in the gel formation. With the increasing pH value from 7 to 8, the gelation time decreased from 24 to 4 min. Also, different thiol-bearing peptides (e.g., cysteine-bearing peptides) could be

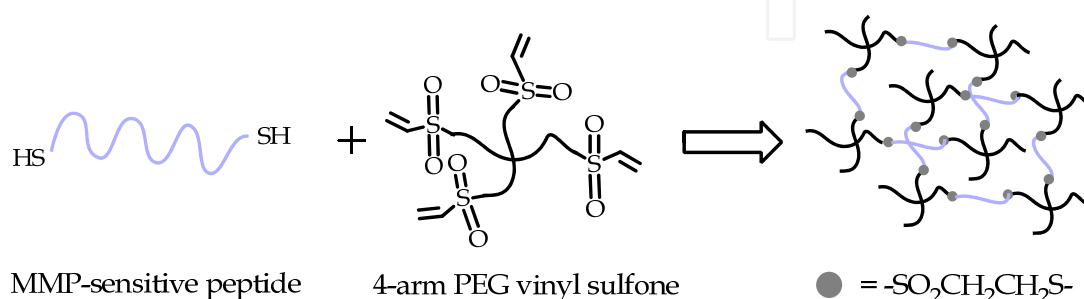MMP-sensitive peptide   4-arm PEG vinyl sulfone   ● = -SO$_2$CH$_2$CH$_2$S-

Fig. 1. In-situ forming cell-responsive hydrogels prepared from vinyl sulfone-functionalized 4-arm poly(ethylene glycol) and the MMP-sensitive bis-cysteine peptide.

incorporated to yield biofunctional or bioreponsive hydrogels, enhancing cell adhesion and matrix production (Seliktar et al., 2004). This indicates that Michael-type addition is an ideal method for the preparation of in-situ crosslinked hybrid hydrogels.

2.1.2 Radical polymerization

Radical polymerization is one of the most frequently used crosslinking methods to prepare robust and stable in-situ forming hydrogels. Radicals are created from initiator molecules through thermal, redox or photointiated mechanisms. Then, the radicals propagate through unreacted double bonds during polymerization to form long kinetic chains, and the chains react further with each other to form crosslinked polymeric networks (Ifkovits & Burdick, 2007). In general, macromers bearing vinyl groups are relatively biocompatible and more favourable as compared to monomers. Since the reaction takes place in aqueous solutions, the conversion of double bonds is high due to the high mobility of reacting species during gel formation. This also decreases the potential toxicity of the materials. PEG and PEG-based copolymers are commonly used synthetic biomaterials (Nguyen & West, 2002) (Fig. 2). They can be functionalized with acryl chloride and further crosslinked in the presence of free-radical initiators under a physiological environment to form hydrogels. Multi-arm polymers such as 4-arm and 8-arm PEG were also employed to increase the crosslinking density because of their increased functionality as compare to linear analogues. Other types of polymers are natural polymers such as dextran, hyaluronic acid and collagen (Dong et al., 2005; S.H. Kim et al., 1999; Y.D. Park et al., 2003). As compared to synthetic polymers, they have different functional groups (hydroxyl, amine or carboxylic groups) on their polymer backbones and are amenable to various chemical modifications. The number of double bonds introduced can be precisely controlled on demand. Thus, the properties of free-radical polymerized hydrogel such as gelation time, mechanical properties and degradation profiles can be adjusted for use in different tissue engineering.
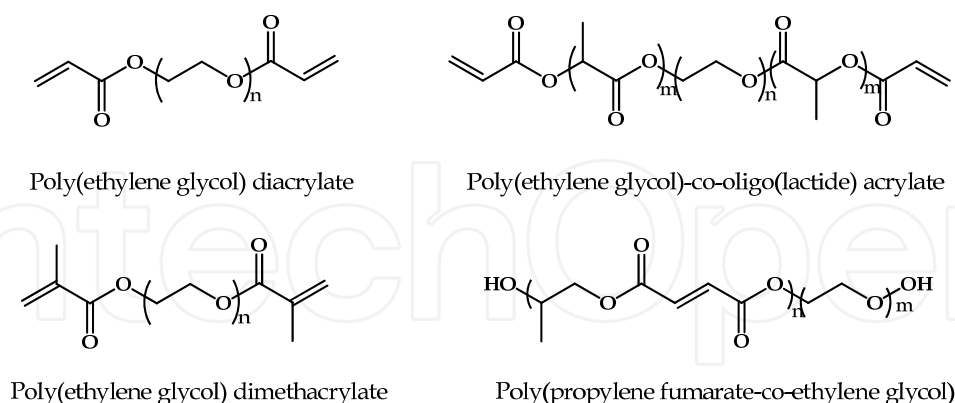

Poly(ethylene glycol) diacrylate

Poly(ethylene glycol)-co-oligo(lactide) acrylate

Poly(ethylene glycol) dimethacrylate

Poly(propylene fumarate-co-ethylene glycol)

Fig. 2. Commonly used poly(ethylene glycol)-based polymers for in-situ forming hydrogels

2.1.3 Enzymatic crosslinking

In-situ hydrogels formation using enzymes have emerged recently. Enzymes are known to exhibit a high degree of substrate specificity, which potentially avoids side reactions during crosslinking. Another advantage of the enzymatic crosslinking is of mild gelation conditions (e.g. physiological conditions), favourable for tissue regeneration.

Horseradish peroxidase (HRP) has been recently employed in the preparation of in situ forming hydrogels. HRP is a single-chain b-type hemoprotein that catalyzes the coupling of phenols or aniline derivatives in the presence of hydrogen peroxide (Kobayashi et al., 2001). Crosslinking reaction takes place via a carbon-carbon bond at the ortho positions and/or via a carbon-oxygen bond between the carbon atom at the ortho position and the phenoxy oxygen in the phenol moieties (Fig. 3a). For example, Feijen *et al.* reported on HRP-mediated in-situ forming dextran-tyramine hydrogels for cartilage tissue engineering (Jin et al., 2007). Tyramine was conjugated to dextran by first activation of the hydroxyl groups in dextran using p-nitrophenyl chloroformate and then treatment with tyramine by aminolysis. The gelation rates induced by enzyme-mediated crosslinking can be readily adjusted from minutes to seconds by varying the HRP concentrations. By the same approach, Jin and Lee *et al.* prepared the chitosan-phloretic acid and hyaluronic acid-tyramine hydrogels for cartilage tissue engineering (Jin et al., 2009) and protein delivery applications (F. Lee et al., 2009), respectively. The disadvantage of this approach is the use of hydrogen peroxide. It is reported that high concentration of hydrogen peroxide (>0.2 mM) may induce cell apoptosis (Asada et al., 1999). Therefore, it is important to control the amount of hydrogen peroxide used in a cell-favourable range.
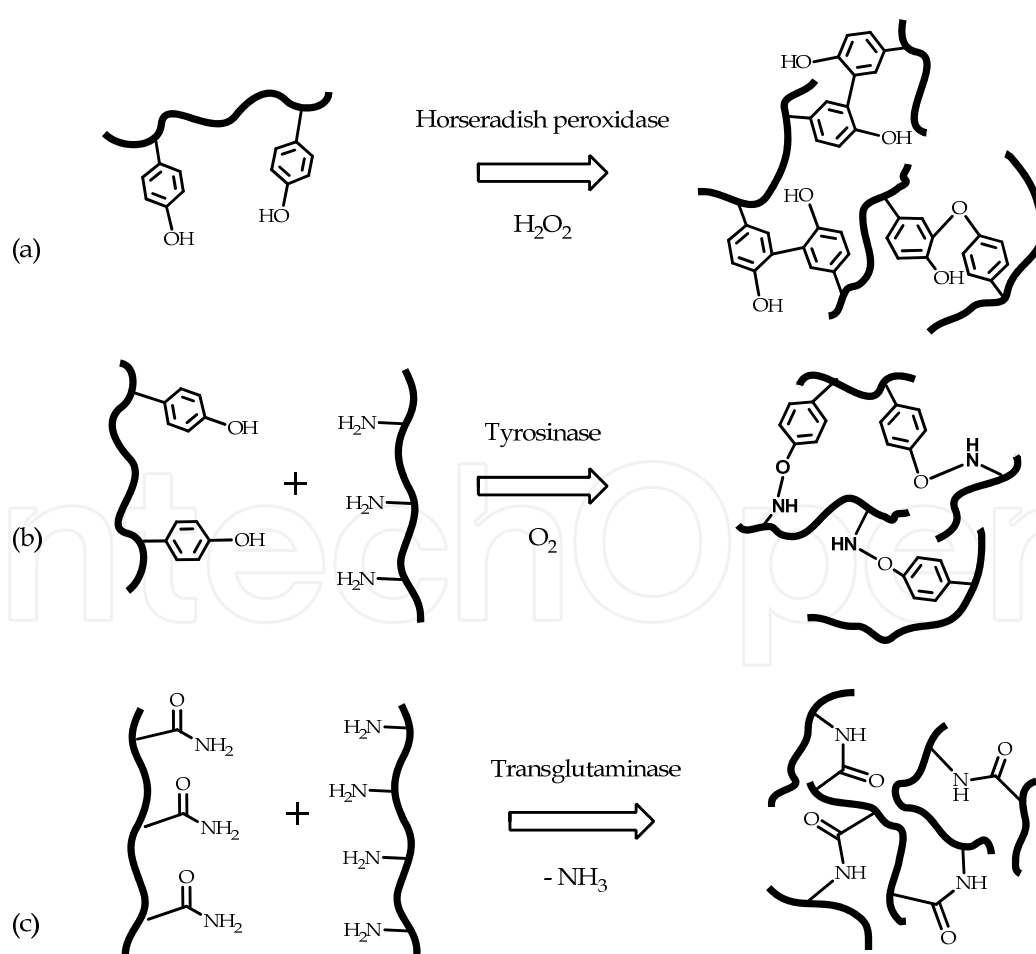

Fig. 3. Enzymatic crosslinking method to prepare in-situ forming hydrogels

Tyrosinase is another enzyme used to form in-situ forming hydrogels. Unlike HRP, the tyrosinase crosslinks phenol-containing polymers in the presence of oxygen instead of hydrogen peroxide (Fig. 3b). Besides, tyrosinase is an oxidative enzyme present in the animal or human body. These features imply milder gelation conditions and better cyto-biocompatibility of tyrosinase-crosslinked hydrogels as compared to HRP-crosslinked hydrogels. So far, only few studies have been conducted to construct hydrogels using tyrosinase. For example, Payne *et al.* reported on the hydrogels from the composites of chitosan and gelatin (Chen et al., 2003). The strength of tyrosinase-catalyzed gels could be adjusted by altering the gelatin and chitosan compositions. The author speculated that the gel system may be useful as emergency dressings for burns and wounds.

Transglutaminase (TGase) is an enzyme frequently used in protein crosslinking. Recently, it has been employed in the preparation of in-situ forming hydrogels. TGase catalyzes an acyl-transfer reaction between the γ-carboxamide group of protein bound glutaminyl residues and the amino group of ε-lysine residues, resulting in the formation of ε-(γ-glutamyl)lysine isopeptide side chain bridges (Sperinde & Griffith, 1997) (Fig. 3c). McHale *et al.* designed and synthesized engineered elastin-like polypeptide (ELPs) hydrogels that are capable of undergoing enzyme-initiated gelation via tissue TGase (McHale et al., 2005). Two kinds of polymer solutions ELP[KV6-112] and ELP[QV6-112] were first mixed and the gels were formed within an hour after enzymes and $CaCl_2$ were subsequently added. In another study, Sanborn *et al.* investigated TGase-catalyzed gelation of peptide-modified PEG and the results showed that gelation times ranged from 9 to 30 min (Sanborn et al., 2002). To shorten the gelation time, Messersmith *et al.* attempted to rationally design the peptide substrates by increasing their specificity (Hu & Messersmith, 2003). It was found that the introduction of an N-terminal L-3,4-dihydroxylphenylalanine (DOPA) residue into the tripeptides resulted in ca. 2.4-fold increment in specificity. This facilitated the gel formation with shorter gelation time of 2 min.

2.1.4 Peptide ligation

Peptide ligation is often employed in the synthesis of proteins and enzymes, which is based on chemoselective reaction of two unprotected peptide segments. Recently, this reaction was explored by Grinstaff *et al* for the preparation of in-situ forming hydrogels due to the mild chemical reaction conditions. A typical peptide ligation reaction is based on the reaction of aldehyde groups in poly(ethylene glycol) derivatives and NH_2-terminal cysteine moieties in peptide dendrons, which can form thiazolidine rings (Wathier et al., 2004) (Fig.4a). The gelation process took place within a few minutes. However, these hydrogels were intact for short periods of time (about 1 week) due to the reversible thiazolidine ring formation. To overcome the problem, the same group developed stable hydrogels prepared from poly(ethylene glycol) with endcapped ester-aldehyde groups instead of aldehyde groups (Wathier et al., 2006) (Fig.4b). The ester-aldehyde groups firstly reacted with the NH_2-terminal cysteine moieties to form the thiazolidine ring, which then can undergo a rearrangement to give chemically stable pseudoproline ring. The mechanical properties of the hydrogels depend on the concentrations of the polymer solutions and different ratios of aldehyde to cysteine reactive functionality. Degradation studies demonstrated that the pseudoproline ring was more stable than the thiazolidine ring and the hydrogels retained their shape and size with less than 10% weight loss for more than 6 months.
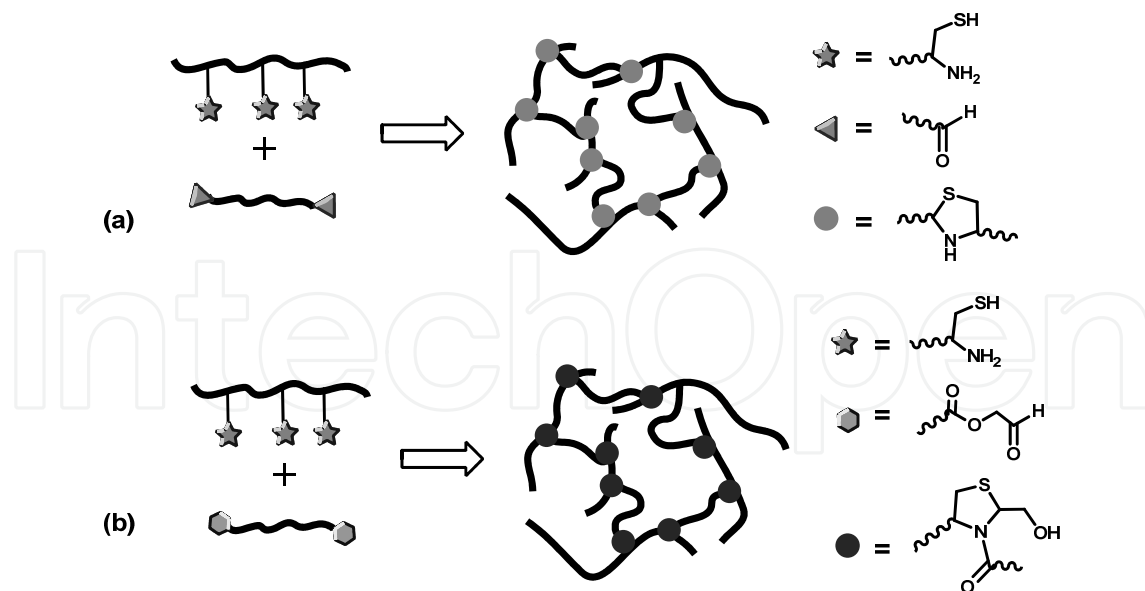
Fig. 4. Preparation of in-situ forming hydrogel via peptide ligation

2.2 Physical crosslinking

Much attention has been paid in the preparation of in-situ forming physical hydrogels. The advantages of physical crosslinking are relatively good biocompatibility and less toxicity since toxic crosslinking reagent or initiators are not used during crosslinking. However, physically-crosslinked hydrogels are gnerally unstable and mechanically weak. The changes in the environment such as pH, temperature and ionic strength may lead to the disruption of the gel network. Typical physical crosslinking methods include stereocomplexation, hydrophobic interactions and ionic interaction.

2.2.1 Stereocomplexation

A typical polymer used for stereocomplex formation is poly(lactide) (PLA). It is a kind of aliphatic polyesters, which are known to be biocompatible and render PLA-based hydrogels biodegradable. Lactide has three possible configurations, which refer to D-lactide, L-lactide and meso-lactide according to the arrangement of substituents around the chiral carbon. The corresponding polymers are defines as poly(L-lactide) (PLLA), poly(D-lactide) (PDLA) and poly(D,L-lactide) (PDLLA). The formation of stereocomplexes when mixing PLLA and PDLA was first reported by Ikada *et al.* (Ikada et al., 1987). The stereocomplexation not only occurs in the blends of PLLA and PDLA homopolymers, but also in water-soluble PLA and poly(ethylene glycol) (PEG) block copolymers, such as linear (Hiemstra et al., 2005) and multiarm PEG-PLLA and PEG-PDLA block copolymers (Hiemstra et al., 2006). This gives the possibility to design different PLA-conjugated materials in hydrogel preparation. For example, Hennink *et al.* reported on physical hydrogels based on the stereocomplexation of PLA-dextran conjugates (de Jong et al., 2000). L- and D-lactic acid oligomers were coupled to dextran to yield dex-(L)lactate and dex-(D)lactate. It was found that the degree of polymerization of lactic acid oligomers must be at least 11 to obtain the hydrogels. The stereocomplex crosslinking can be detected by X-ray diffraction (de Jong et al., 2002). Varying the degree of polymerization of oligomer, the degree of substitution of dex-lactate

and the water content of dex-lactate solutions, the properties of the hydrogels can be well modulated.

2.2.2 Hydrophobic interaction

The hydrophobic interaction provides another driving force of physical gelation. Some amphiphilic copolymers can undergo a sol-gel transition via this mechanism. Typical amphiphilic polymers are block copolymers based on poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO), also called as Pluronics® (Fusco et al., 2006). It was found that, when increasing temperature, the PEO-PPO polymers can undergo a dehydration process. This leads to the formation of hydrophobic domains and, in turn, transition of an aqueous liquid to a hydrogel. The drawbacks of PEO-PPO hydrogels include rapid erosion, potential cytotoxicity (Khattak et al., 2005), and non-biodegradability. Alternatively, polyester-based copolymers that are biodegradable received much attention. For example, Jeong and co-workers described thermosensitive, biodegradable hydrogels based on poly(ethylene oxide) and poly(lactic acid) (Jeong et al., 1997). Solutions of the diblock copolymers were shown to be in solution state at 45°C, but gel state at body temperature. However, the encapsulation of drugs at an elevated temperature might lead to denaturation of bioactive agents such as therapeutic proteins or growth factors. The same group subsequently reported on a series of triblock copolymers of poly(ethylene oxide) and poly(lactic acid)/poly(glycolic acid) (Jeong et al., 1999). This thermosensitive hydrogel system is inverse to the hydrogel based on poly(ethylene oxide)-co-poly(lactic acid) diblock polymers, that is, poly(ethylene oxide)-b-(D,L-lactic acid-co-glycolic acid)-b-poly(ethylene oxide) triblock copolymers were found to be in a solution at room temperature, but form a hydrogel when the temperature is increased to 37°C. This makes the gel system easy to handle and favourable for tissue engineering applications (Jeong et al., 2000). Moreover, the sol–gel transition temperature and degradation properties can be adjusted by the polymer concentration, molecular weight of poly(ethylene oxide) and the lactic acid/glycolic acid ratio in the poly(lactic acid-co-glycolic acid) blocks. In another study, Ding *et al.* reported that the end groups have a surprising effect on the hydrogel formation (L. Yu et al., 2006). The results showed that the transition temperature increased with a decreasing hydrophobicity of the end groups. Importantly, it is noted that sol-gel transition takes place only when the hydrophobic interactions are strong enough to induce the large-scale self-assembly of micelles. However, over hydrophobicity and higher temperature lead to precipitation of polymers as a result of the break of micelle structure.

Polypeptide or peptide-conjugated polymers is another type of polymers that can be used to form hydrogels via hydrophobic interactions. The formation of hydrogels from polypeptides are based on coil-coil interaction, triggered by the self-assembly of peptide sequences. The coiled-coil interaction is one of the basic folding patterns of native proteins and consists of two or more helices winding together to form a superhelix (Y.B. Yu, 2002). A series of hydrogels based on peptides or peptide/synthtic polymer hydrids were made. Typical examples are synthetic N-(2-hydroxypropyl)methacrylamide (HPMAm) copolymer grated with coiled-coil protein motifs (C. Wang et al., 1999; J. Yang et al., 2006b). The gelation time can be adjusted by the length and the number of coiled-coil grafts per chain an ranged from a few minutes to several days (J. Yang et al., 2006a). Besides, it was found that at least 4 heptads were needed to achieve hydrogels formation. In another study, Xu *et al.*

reported on the hydrogels based on genetically engineered protein block copolymers with 2 coiled-coil domains in a random coil polyelectrolyte (Xu & Kopeček, 2008). The self-assmebly process between coil-coils was influenced by the protein concetration, pH and temperature. Changes in the peptide sequence of the coil-coil domains endow hydrogels with different stability.

2.2.3 Ionic interaction

Ionic interaction is another route to construct in-situ forming hydrogels. For example, the hydrogels can be formed by ionic interactions between water-soluble charged polymers and their di- or multi-valent counter-ions. As a typical example, alginate, a naturally occurring polysacchride, can form a hydrogel network in the presence of calcium ions under physiological conditions. The mechanism underlying the ionic crosslinking is ion exchange of sodium ions by calcium ions in the carboxylic groups and subsequent formation of an egg-box structure (Gombotz & Wee, 1998). The hydrogel is degradable slowly with the diffusion of calcium ions out of the hydrogels and finally excreted from the kidney. Further studies showed that alginate-based hydrogels with $CaSO_4$ usually reveal a heterogeneous structure due to the difficulty in the control of gelation kinetics (Kuo & Ma, 2001). This phenomenon also occured for the hydrogels using $CaCl_2$ (Skjak-Brvk et al., 1989). In contrast, $CaCO_3$ can give homogeneous alginate hydrogel, while its low solubility is unfavourable for further biomedical application. Ma et al. reported on crosslinked alginate hydrogels using $CaCO_3$-GDL (D-glucono-d-lactone) and $CaSO_4$-$CaCO_3$-GDL systems (Kuo & Ma, 2001). Gelation rates and mechanical properties of the alginate hydrogels could be controlled by varying the composition of calcium compound systems and alginate concentration, thereby giving rise to structurally uniform hydrogels.

In-situ forming hydrogels can also be prepared by ionic interactions between polycations and polyanions. For example, Hennink et al. reported on self-gelling hydrogels based on oppositely charged dextran microspheres (Tomme et al., 2005). These charged dextran-microspheres were prepared by radical polymerization of hydroxyethyl methacrylate-derivatized dextran (dex-HEMA) with methacrylic acid (MAA) or dimethylaminoethyl methacrylate (DMAEMA). Hydrogels could be formed as a result of ionic interactions between oppositively-charged microspheres. The networks of hydrogels were disrupted either by applied stress, low pH or high ionic strength. Reversible yield point from rheological analysis indicated that this hydrogel system can be applied for controlled delivery of pharmaceutically active proteins and tissue engineering. However, a main disadvantage of ionically-crosslinked hydrogels is that their mechanical strength is far from satisfactory when they are served as scaffolds for tissue regeneration.

3. Biomimetic hydrogels

The success of tissue engineering depends on biomimetic hydrgoel scaffolds that possess controlled structures and on-demand properties to modulate specific cellular behaviors. The development of suitable synthetic methods encompassing chemistry and molecular biology open a new way for the design of biomimetic hydrogels mimicking basic processes of living systems. In general, biomimetic hydrogels can be categorized into bioactive, bioresponsive, and biofunctional hydrogels.

3.1 Bioactive hydrogels

Much effort has been made in the design of bioactive hydrogels which can instruct cell behaviors and promote tissue regeneration. A well-know bioactive ligand is cell-adhesive peptides, e.g., Arg-Gly-Asp (RGD). It was revealed that RGD-modified PEG diacrylate hydrogels could induce enhanced cell attachment and mineralized matrix deposition of osteoblasts as compared to RGD-free hydrogels (Burdick & Anseth, 2002). Natural proteins such as collagen and its analogs may also serve as bioactive ligands due to inherent nature of biological recognition. Seliktar *et al.* reported on the preparation of proteins (collagen, albumin and fibrinogen) conjugated with acrylated PEG and subsequent hydrogel formation by photopolymerization (Gonen-Wadmany et al., 2007) (Fig. 5). The modified protein maintained its cell-adhesive properties and supported proteolytic degradability based on the specific characteristics of the protein backbone. In another study, Lee and coworkers reported on the collagen mimetic peptide-conjugated poly(ethylene glycol) hydrogels (H.J. Lee et al., 2006). The collagen mimetic peptide (CMP) with a specific amino acid sequence, -(Pro-Hyp-Gly)x-, forms a triple helix conformation that resembles the native protein structure of natural collagens. CMP was first conjugated with acrylated PEG, which copolymerized with poly(ethylene oxide) diacrylate to create a novel PEG hydrogel. The modified protein can maintain their cell-adhesive properties and support proteolytic degradability based on the specific characteristics of the protein backbone. The biochemical analysis showed that chondrocytes-encapsulated hydrogels revealed an 87% increase in glycosaminoglycan content and a 103% increase in collagen content compared to that of control PEG hydrogels after 2 weeks. These results indicate that the CMP enhances the tissue production of cells encapsulated in the PEG hydrogel by providing cell-manipulated crosslinks and collagen binding sites that simulate natural extracellular matrix.
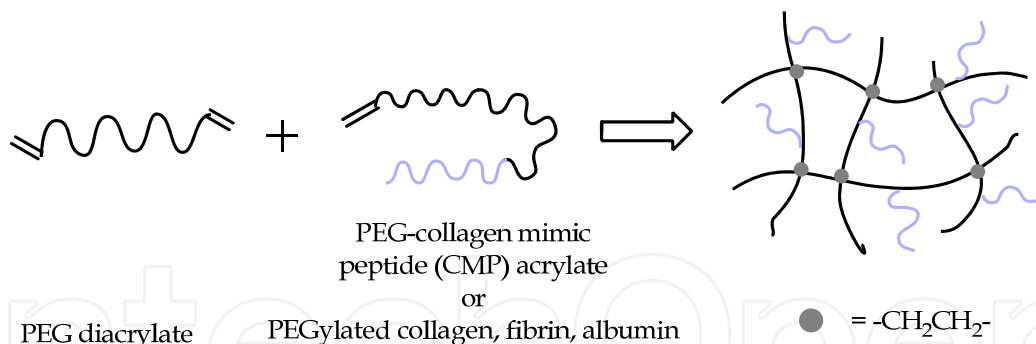

Fig. 5. Bioactive hydrogels prepared from poly(ethylene glycol) and proteins/peptide.

3.2 Bioresponsive hydrogel

Biomimetic hydrogels can response to biological components, such as enzymes, receptors and antibodies. After the hydrogels undergo a macroscopic transition (gelation, enzymatic degradation and swelling/shrinkage), this in turn directly leads to microscopic response of living cells (cell migration, differentiation, cell division and matrix production). For example, Lutolf *et al.* developed cell-responsive hydrogels that can degrade in response to local protease activity such as matrix metalloproteinase (MMP) at the cell surface. MMP is a protease family extensively involved in tissue development and remodeling. The hydrogel systems were made from vinyl sulfone-functionalized multiarmed PEG and the bis-cysteine

peptide crosslinker which contained the sequence sensitive to matrix metalloproteinases (Lutolf et al., 2003). The hydrogels were proteolytically degraded via the invasion of primary human fibroblasts. The invasion process depended on MMP substrate activity, adhesion ligand concentration, and network crosslinking density. By mimicking the MMP-mediated invasion of the natural provisional matrix, the hydrogels were shown to assist tissue regeneration. These results indicate potential applications of the cell-responsive hydrogels in tissue engineering and regenerative medicine.

3.3 Biofunctional hydrogels

Mechanical modulus is in the range of 10 kPa–350 MPa for soft tissues and 10 MPa–30 GPa for hard tissues (S. Yang et al., 2001). Depending on intended application, hydrogel should provide sufficient mechanical strength so as to protect seeded cells and developing neo-tissue as well as to withstand the physiologic load. However, most of hydrogels reported so far are not qualified especially for bone or cartilage tissue regeneration due to the lack of a high mechanical strength. Thus, robust hydrogels have been developed with on-demand mechanical properties. Mechanical moduli of hydrogels are generally increasing with increasing crosslinking density. Two types of robust hydrogel systems can be classified.
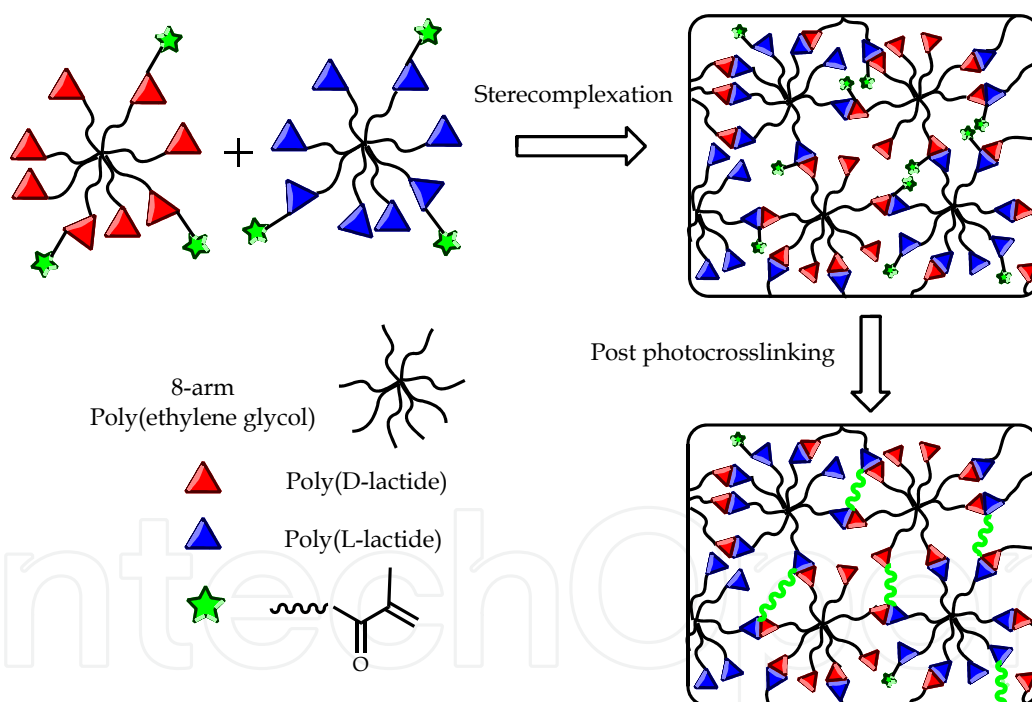

Fig. 6. Hydrogel prepared via the combination of stereocomplexation and photocrosslinking

First, hydrogels can be fabricated by double crosslinking methods. By this approach, the hydrogels have increased crosslinking density, thus improving the mechanical properties without comprising other properties such as permeability and biocompatibility. For example, Feijen and coworkers reported on the in situ hydrogels crosslinked by combining stereocomplexation and photopolymerization (Hiemstra et al., 2007b). Stereocomplexed hydrogels were first formed upon mixing solutions of an 8-arm PEG–PLLA and an 8-arm PEG–PDLLA whichh are partly functionalized with methacrylate groups (40%). These

hydrogels can be postcrosslinked by UV-irradiation (Fig. 6). These double-crosslinked hydrogels showed increased mechanical moduli and prolonged degradation times compared to the hydrogels that were formed only by stereocomplexation. The photopolymerization takes place at much lower initiator concentrations (0.003 wt%) than conventional photocrosslinking systems (0.05 wt%), which greatly reduces the possibility of heating effects that can damage cells.

Second, robust hydrogels are produced that consist of two interpenetrated polymeric networks. The hydrogels with double networks contain a subset of interpenetrating networks (IPNs) formed by two hydrophilic networks, one highly crosslinked, the other loosely crosslinked. The double network structure can be obtained by pre- and post-crosslinking through exploiting the disparity of their reaction times. For example, a double netwok composed of two mechanically weak hydrophilic networks based on N, N-dimethylacrylamide and glycidyl methacrylated hyaluronan, provides a hydrogel with outstanding mechanical properties (Weng et al., 2008). Hydrogels containing more that 90% water possessed a compressive modulus and a fracture stress over 0.5 MPa and 5.2 MPa, respectively, demonstrating both hardness and toughness. Besides, it is found that both the concentrations of monomers and crosslinkers are important parameters related to the mechanical strength of double network gels. Therefore, it is easy to control the mechincal properties such as hardness and toughness independently by adjusting the compositions of of the gels for practical applications.

4. Tissue engineering applications

4.1 Cartilage tissue regeneration

Cartilage is a flexible, connective tissue in which chondrocytes are sparsely distributed in the extracellular matrixes rich in proteoglycans (PGs) and collagen fibers. Cartilage has a limited capacity for self-repair due to its avascular nature and low mitotic activity of chondrocytes. In articular cartilage, chondrocytes are the only cell type and responsible for the synthesis and maintenance of resilient extracellular matrix. Chondrocytes may undergo a dedifferentiation process during monolayer culturing and lose their phenotype. However, once cultured in hydrogels, dedifferentiated chondrocytes are able to redifferentiate (Benya & Shaffer, 1982), as indicated by their rounded morphology and the production of ECM molecules such as type II collagen and sulfated glycosaminoglycans.

4.1.1 Factors influencing cartilage regeneration

In-situ forming hydrogels enable a perfect match with irregular cartilage defects and good alignment with the surrounding tissues. Therefore, they are promising materials that can function as scaffolds for chondrocyte culturing and cartilage regeneration. Several factors may influence the cell viability, recovery or the maintenance of the chondrocytic phenotype, and correspondingly play an important role in cartilage tissue engineering.

Chemical compositions of hydrogels have been studied to explore their influence on cartilage regeneration. For example, Elisseeff et al. studied the cellular toxicity of transdermal photopolymerization on chondrocytes (Elisseeff et al., 1999). There was a significant decrease in the cell viability when the initiator concentration was increased from

0.012% to 0.036% or higher. In another study, Chung *et al.* noticed that a higher macromer concentration potentially compromised cell viability and growth (Chung et al., 2006). Besides, a higher polymer concentration also resulted in a decreased accumulation of matrix components such as proteoglycans and collagen type II (Sontjens et al., 2006).

Recent studies showed that the degradation properties of the gels may have a significant influence on the matrix production and distribution as well. Degradable hydrogels induced a more homogenous distribution of GAG than non-degradable hydrogels (Bryant & Anseth, 2002, 2003; Bryant et al., 2003; Martens et al., 2003). However, in fast degrading hydrogels void spaces are generally present before new matrix formation has taken place (Bryant & Anseth, 2003; Martens et al., 2003). Therefore, the degradation rate of hydrogels needs to be tailored by the combination of degradable main chain linkages and crosslinks.
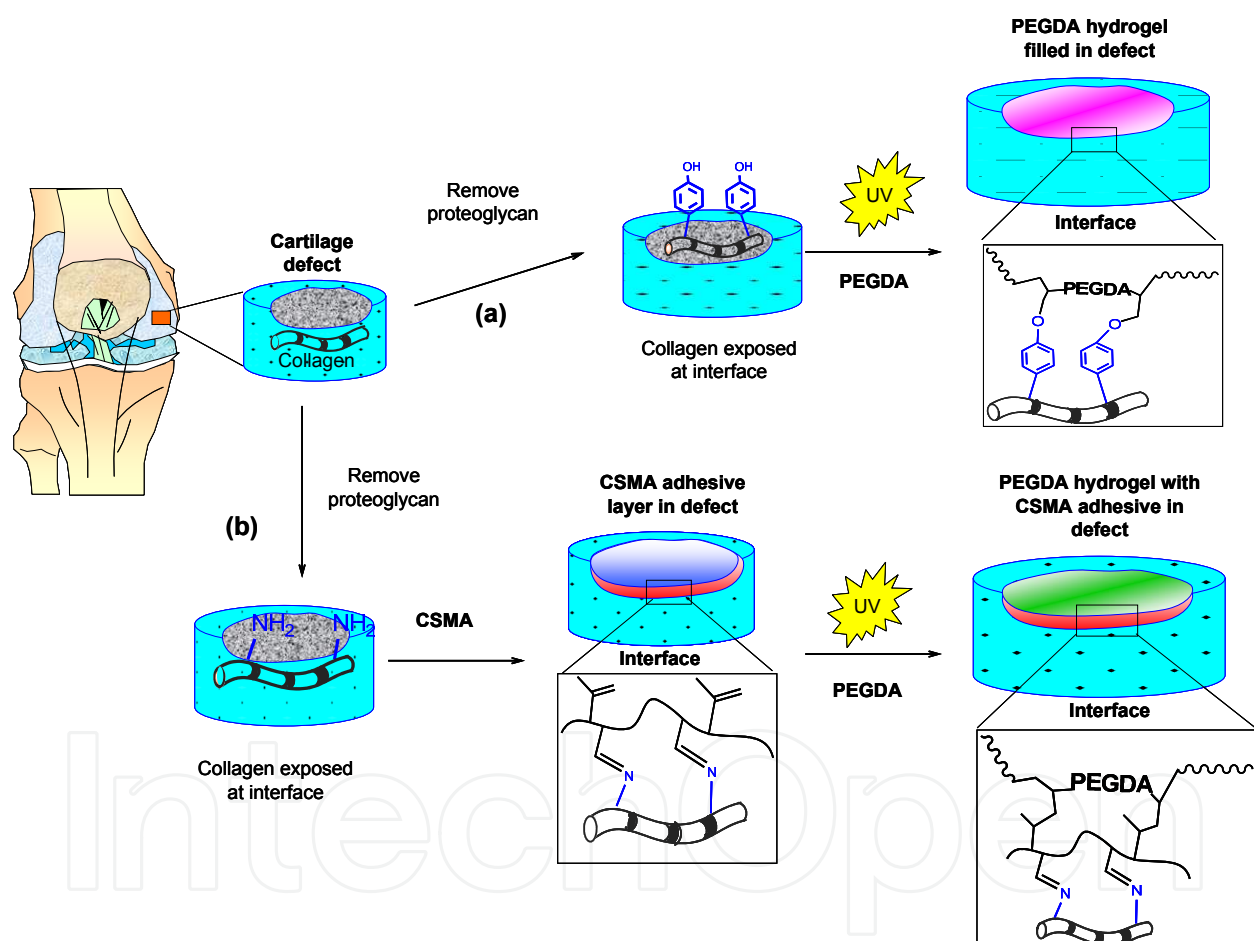

Fig. 7. Hydrogel-cartilage integration by (a) tissue-initiated photopolymerization or (b) Schiff-base formation.

A major problem for cartilage regeneration is poor integration of neocartilage with native cartilage tissue. To solve this problem, the gel precursor molecules were modified with functional groups that can react with collagen type II, a molecule present in native cartilage. For example, improved tissue adhesion and integration was achieved by tissue-initiated polymerization between acrylate groups in polymerizable PEGDA macromers and tyrosine groups in collagen when exposed to light and an oxidative reagent like H_2O_2 (D.A. Wang et

al., 2007) (Figure 7a). In another approach, methacrylated chondroitin sulfate (CSMA) was functionalized to endow aldehyde moieties which was covalently attached to collagen via Schiff-base formation (D.A. Wang et al., 2004) (Figure 7b). The CSMA layer was further polymerized by photo-crosslinking of PEGDA to give a gel/cartilage integrated scaffold.

4.1.2 Hybrid hydrogels for cartilage regeneration

Understanding of the tissue structure and composition can lead to a rational material design, targeted towards mimicking the underlying biological cues and specific chemistry of cartilage. Generally, in-situ forming hydrogels have been prepared from synthetic polymers, natural polymers or their hybrids. The latter has gained increasing attention in recent years because they combine the advantage of both synthetic and natural polymers, that is, tightly defined physical, chemical and biological properties. Table 1 lists typical examples of in-situ forming hybrid hydrogels for cartilage tissue regeneration.

Synthetic polymer	Natural moiety	Comments	Ref.
PEG	MMP-sensitive peptide	Proteolytic degradability, enhanced gene expression of type II collagen and aggrecan	(Y. D. Park et al., 2004)
PVA	Chondroitin sulfate	Balance between modulus and swelling of hydrogels, enhanced matrix production	(Bryant et al., 2005)
PEG	Collagen-mimic peptide	Retention of ECM production inside hydrogel via collagen binding, enhanced chondrogenesis	(H.J. Lee et al., 2008)
Pluronic F127	Hyaluronic acid, RGD	Improved cellular adhesion and proliferation, increased matrix production	(H. Lee & Park, 2009)
PEG	Heparin	Promoted chondrocyte proliferation, while maintaining chondrogenic nature	(M. Kim et al., 2010)

Table 1. Typical examples of in-situ forming hybrid hydrogels for cartilage regeneration

4.1.3 Growth factor

During the cartilage regeneration process growth factors play a crucial role in regulating cellular proliferation, differentiation, migration, and gene expression. Besides, they have large influences on the communication between cells and their microenvironment. A number of growth factors have been studied and include bone morphogenetic protein (BMP), transforming growth factor (TGF), insulin-like growth factor (IGF) and basic fibroblast growth factor (bFGF). Their main functions in cartilage regeneration are summarized in Table 2. For example, the BMP family can stimulate mitosis and matrix production by chondrocytes and induce chondrogenesis of mesenchymal cells, triggering them to differentiate and maintain a chondrogenic phenotype (Yuji et al., 2004). TGF-β not only enhances chondrocyte proliferation, but also increases the synthesis of proteoglycans (H. Park et al., 2005).

Growth factor	Function	Ref.
BMP	Inducing chondrogenesis; Stimulating cartilage formation	(Y. Park et al., 2005)
TGF-β	Regulation of cell proliferation and differentiation; Stimulating production of proteoglycans and other matrix components	(H. Park et al., 2005)
IGF	Promotion of cartilage tissue formation	(Elisseeff et al., 2001)
bFGF	Potent modulator of cell proliferation, motility, differentiation, and survival; initiation of chondrogenesis	(K.H. Park & Na, 2008)

Table 2. Delivery of growth factors using injectable hydrogels for cartilage regeneration

4.1.4 *In vivo* studies

Many in-situ forming hydrogels so far prepared for cartilage tissue engineering have been studied in vitro, however, only a few reports have appeared on their performance in vivo. Before clinical application, in-situ forming hydrogels need to be systematically evaluated in animal models. Early *in vivo* studies generally focused on the ability of cell-seeded injectable hydrogels to generate cartilaginous tissue after subcutaneous implantation/injection in mouse models. For example, Elisseeff *et al.* implanted chondrocyte-incorporated PEO-based hydrogels, and showed that the chondrocytes survived during the photopolymerization process and proliferated without any sign of necrosis (Elisseeff et al., 1999). However, partial chondrocyte dedifferentiation and undesired fibro-cartilaginous tissue formation were observed in these gels after 6 weeks' in vivo. To retain the chondrocyte phenotype and improve cartilage regeneration, ECM components and bioactive molecules were incorporated into hydrogels. Na *et al.* showed that cartilage-specific ECM production was significantly higher in poly(N-isopropylacrylamide-co-hydroxyethyl methacrylate) hydrogels containing HA and TGF-β3 compared to those without the growth factor or HA (K.H. Park & Na, 2008). Recent studies have been directed to in-situ forming hydrogels for cartilage regeneration in animal models like rabbit and goat. Hoemann *et al.* tested the residence of in-situ forming hydrogels in rabbit joints and showed that in-situ forming chitosan/glycerol phosphate gels could reside at least 1 day in a full-thickness chondral defect, and at least 1 week in a mobile osteochondral defect (Hoemann et al., 2005). Liu et al. described osteochondral defect repair in a rabbit model using a synthetic ECM composed of hyaluronic acid and gelatin (Liu et al., 2006). At 12 weeks, the defects were completely filled with elastic, firm, translucent cartilage and showed good integration of the repair tissue with the surrounding cartilage. A summary of in vivo studies on in-situ forming hydrogels for cartilage regeneration is presented in Table 3.

Hydrogel (+/-cell)	Animal model	Outcome	Ref.
Chitosan-GP-glucosamine (+ primary calf chondrocytes)	Subcutaneous injection in nude mice	Chitosan gels supported cartilage matrix accumulation by cells 48 days after injection.	(Hoemann et al., 2005)
Hyaluronic acid (+ swine auricular chondrocytes)	Subcutaneous implantation in nude mice	Neocartilage was produced and evenly distributed in the gels after 6 weeks. The P0 and P1 chondrocytes produced neocartilage tissue that resembled native auricular cartilage after 12 weeks.	(Chung et al., 2006; Ifkovits & Burdick, 2007)
PEGDA with methacrylated chondroitin sulfate as adhesive	Goat chondral defects	Defects treated with chondroitin sulfate adhesive and hydrogel showed improved cartilage repair compared to an empty, untreated defect after 6 months.	(D.A. Wang et al., 2007)
Chitosan-GP-glucosamine	Rabbit (osteo)chondral defects	Chitosan gel can reside at least 1 day in a full-thickness chondral defect and for at least 1 week in a mobile osteochondral defect.	(Hoemann et al., 2005)
Hyaluronic acid-gelatin-PEGDA (+ autologous MSC)	Rabbit osteochondral defect	Defects were completely filled with elastic, firm, translucent cartilage at 12 weeks and showed superior integration of the repair tissue with the cartilage.	(Liu et al., 2006)
Elastin-like polypeptide	Goat chondral defects	ELP formed stable, well-integrated gels and supported cell infiltration and matrix synthesis 3 months after injection. These hydrogels degraded rapidly.	(Nettles et al., 2008)

Table 3. In-situ forming hydrogels for *in vivo* cartilage regeneration

4.2 Would healing

Wound healing is a complicated process which requires coordination of complex cell and biomaterial interactions. Desirable properties of biomaterials involve formability in situ from aqueous solutions, good adhesion to tissues at one surface (tissue surface) and resistance to adhesion to the other (free surface), and degradability without induction of inflammation (Hubbell, 1996). In-situ forming hydrogels are attractive biomaterials in the application for would healing due to their ability of adjusting the moisture of the wound tissue (wetting the dehydrate tissue and absorb exudation) and conformability of the dressing on wounds (Jones & Vaughan, 2005).

4.2.1 Chitosan-based hydrogels

Polysaccharides, e.g. chitosan, represent a class of hydrogels used as would healing materials. Native chitosan has low solubility above pH 6. Modifications on chitosan can improve its solubility and make it suitable as in-situ forming materials. Ono et al. reported potocrosslinked chitosan as a dressing for wound occlusion (Ono et al., 2000). The modified chitosan (Az-CH-LA) containing both lactose moieties and azide groups exhibited a good

solubility at neutral pH. Application of ultraviolet light (UV) irradiation to Az-CH-LA produced an insoluble hydrogel within 60 s. The results showed that the chitosan hydrogels could completely stop bleeding from a cut mouse tail within 30 s and firmly adhere two pieces of sliced skins of mouse to each other. The wound healing efficacy of hydrogel was evaluated in experimental full-thickness-round wounds of skin using a mouse model and it is showed that chitosan hydrogels could significantly induce wound contraction and accelerate wound closure and healing in both db and db+ mice (Ishihara et al., 2002). Incorporation of fibroblast growth factor-2 further accelerated wound closure in db mice, however, not in db+ mice (Obara et al., 2003).

4.2.2 Alginate-gelatin hydrogels

Gelatin is a degraded form of collagen and alginate is derived from brown seaweed, both of which are biocompatible. Balakrishnan *et al.* reported on the evaluation of alginate-gelatin hydrogels for wound dressing (Balakrishnan et al., 2005). Hydrogels were prepared from oxidized alginate and gelation in the presence of borax. It was found that the hydrogel have the ability to prevent accumulation of exudates on the wound bed due to fluid uptake. In vivo experiment showed the wound covered with hydrogel was completely filled with new epithelium after two weeks using a rat model. In addition, incorporation of dibutyryl cyclic adenosine monophosphate (DBcAMP) into the in-situ forming hydrogels and sustained release of DBcAMP led to the enhancement in the rate of healing as well as re-epithelialization of the wounds (Balakrishnan et al., 2006). Complete healing was achieved within 10 days associated with mild contracture of some of the wounds.

4.3 Delivery system

From the clinical point of view, the success of a scaffold for tissue engineering is judged by its ability to regenerate tissue in both the onset and completion of tissue defect repair. During this process, the presence of growth factors in the hydrogel-based scaffolds usually helps to govern neo-tissue formation and organization. However, growth factors generally have short half-life time and are easy to lose their bioactivity(Edelman et al., 1991). Moreover, some unexpected adverse effects may occur which could be caused by initial burst release of growth factors. Therefore, the appropriate mode to deliver growth factors, make them available at the site of action and effectively controlled release of them to exert their maximum efficacy is of great important.

Direct administration of growth factors is commonly associated with problems such as a short biological half-life and easy diffusion. In-situ forming hydrogels offer significant opportunities for controlled local delivery of such biomolecules. These bioactive agents can be easily incorporated into hydrogels prior to gelation and their release kinetics can be adjusted on demand by the crosslinking density and stability of the networks. The disadvantages of in situ incorporation (Fig. 8a), however, is the potential damage of proteins during the gelation process (Sperinde & Griffith, 1997) or the occurrence of an initial burst release. To circumvent these disadvantages, growth factors can be incorporated into microparticles which can be added to the hydrogel precursor solutions (Fig. 8b). Microparticles can be prepared either from synthetic polymers (e.g. PGA, PLA, and PLGA) or from natural polymers (e.g. gelatin) (S.H. Lee & Shin, 2007). For example, Holland et al.

reported on TGF-β1-loaded-gelatin particles which were incorporated in oligo(poly(ethylene glycol) fumarate) hydrogels (Holland et al., 2003). In vitro release experiments showed a suppressed burst release and prolonged delivery of TGF-β1. Besides, when chondrocytes were embedded, an increased cellular proliferation and enhanced chondrocyte-specific gene expression was observed for the hydrogels containing TGF-loaded-gelatin particles (H. Park et al., 2005).
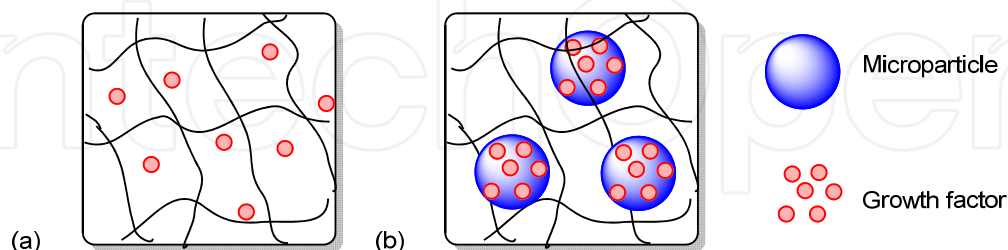

Fig. 8. Schematic representation of methods for encapsulating growth factors either by (a) direct incorporation or (b) preloading into microparticles

Another example to control the release of growth factors is to use heparin-containing hydrogels. It is known that heparin is able to bind basic fibroblast growth factor (bFGF) by the formation of a stable complex (Yayon et al., 1991). bFGF can be prevented from denaturation and proteolysis meanwhile maintaining its biological activity after its release. Besides, the use of hparin can efficiently control the release rate of bFGF. However, large quantity use of heparin induces side effects such as thrombocytopenia, thrombosis, and hemorrhage (Silver et al., 1983). To solve this problem, Prestwich et al. proposed an in-situ forming glycosaminoglycan (GAG) hydrogel based on hyaluronan (HA) and chondroitin sulfate (CS) (Cai et al., 2005). Crosslinking occur between thiol-modified HA or CS (HA-DTPH or CS-DTPH), thiol-modified heparin (HP) and poly(ethylene glycol) diacrylate (PEG-DA) to generate copolymers, containing only a small percentage of co-crosslinked thiol-modified HP, which is capable of controlled release of basic fibroblast growth factor. Notably, the diffusion of bFGF in the hydrogels can be substantially slowed down only with 1% (w/w) covalently bound heparin (relative to total glycosaminoglycan content). In vivo studies, carried out on the Balb/c mice using the hydrogels (HA-DTPH+HP-DTPH and CS-DTPH+HP-DTPH) with/without bFGF, showed that the implanted hydrogels containing bFGF enhanced the production of new blood vessels to a high extent than equal amount of injected free bFGF, indicating that covalently crosslinked HP was necessary to enhance bFGF activity and promote neovascularization.

5. Conclusion

Novel crosslinking methods provide significant opportunities for the design of in-situ forming hydrogels with multifunctional properties on demand for tissue regeneration. The fast progress in molecular biology inspires researchers to design biomimetic in situ forming hydrogels. Polymer composition and structures, hydrogel forming methods, degradation properties, mechanical strength and biocompatibility are of significant importance. Artificial extracellular matrices combining in situ forming hydrogel scaffolds, cells and growth factors hold great promise for tissue engineering, and pave the way for regenerated tissue.

6. Acknowledgment

7. References

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