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Injectable polymeric hydrogels for the delivery of therapeutic agents: A review

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ABSTRACT

Since drug delivery systems have become one of the most promising areas of human health related research, the applications of biomaterials such as hydrogels have been widely investigated. Possessing unique hydrophilic, biocompatible network structures and the ability to form solid-like gel states once administered, injectable hydrogels facilitate the encapsulation and release of therapeutic agents, including drugs, proteins, genes and cells, in a controllable manner. A wide and diverse range of techniques have been used to generate hydrogels, from chemical cross-linking, such as photo-polymerization, click chemistry, enzyme-catalyzed reactions, Schiff's base reactions, and thiol-based Michael reactions, to physical cross-linking induced by temperature, pH, ionic interaction, guest–host inclusion, stereo-complexation or complementary binding. This review covers the utilization of various injectable hydrogel systems for the delivery of therapeutic agents from the viewpoint of cross-linking methods.

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1. Introduction

As scientific research has grown, an enormous number of studies about biomaterials related to human health have been carried out. On the 50th anniversary of the European Polymer Journal, we are honored to give a brief review of hydrogels, one of the major subjects of biomaterials research. Since the first report of hydrogels by Wichterle and Lim as water-swollen three-dimensional networks, these materials have attracted remarkable interest from the scientific research community [1]. A hydrogel is a three dimensional structure that can absorb and contain a high amount of water or biological fluid [2]. Its polymer network structure can be formed by chemical cross-linking, physical cross-linking or both simultaneously. The chemical nature, network morphology and equilibrium swollen state of hydrogels are responsible for several important properties such as mechanical strength and internal and external transport [3]. Moreover, the large volume of water that they can absorb and their soft consistency are some of the main reasons for some of the advantageous properties of hydrogels, such as biocompatibility and an ability to mimic the extracellular matrix environment. Thanks to these favorable characteristic, hydrogels have become potential candidates for many biomedical and pharmaceutical applications [4–9]. Typical applications of hydrogels include tissue engineering [10–12], soft contact lenses [13], wound-healing [14], sensors [15], mucoadhesives [16] and bioactive factor delivery systems [17,18]. From their very early and relatively simple application as contact lenses, hydrogels have been widely developed and significantly used for more complicated applications, particularly in tissue engineering and the controlled delivery of therapeutic agents. Proteins or drugs can benefit greatly from hydrogel-based controlled release systems. Possessing a

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water-swollen porous structure, which can be controlled by the density of cross-links, hydrogels provide a well-designed and friendly environment for encapsulated bioactive factors. Hopefully, after loading into a gel matrix, proteins or drugs will be released with a concentration in the plasma that falls within the therapeutic range. Various delivery strategies were detailed in a recent review paper by Alsberg et al. [18].

Hydrogels can be classified in a number of ways, such as according to their original source (natural and synthetic polymer), structure (inter-penetrating network, copolymer network, homopolymer network, and double network), cross-linking method (chemically and physically), charge (anionic, cationic, amphoteric, and non-ionic) and biodegradability (non-degradable and degradable). Previously, hydrogel research mainly focused on chemically cross-linked networks that formed a permanent gel before administration [5]. In those implantable permanently cross-linked systems, the use of toxic catalysts and cross-linker monomers could damage and denature fragile molecules such as proteins, drugs or cells. Moreover, there was a demand for homogeneous encapsulation with biological molecules and a minimally invasive operation. Hence, to overcome these drawbacks, recent hydrogel developments have concentrated on *in situ*-forming hydrogels that can gelate spontaneously in physiological conditions after injection. As well as *in situ*-forming systems, a subcategory of preformed gels which is identified as shear-thinning hydrogel can also be employed by injection method, which is preferable for use in therapeutic agent delivery. Although this review focuses mostly on the applications of injectable *in situ*-forming systems, the utilizations of shear-thinning hydrogels are still addressed. *In situ*-forming hydrogels can be prepared by applying UV radiation, adding non-reversible covalent bonds or using self-assembling polymers. Among these, the most interesting are self-assembling hydrogels, which can be fabricated by various cross-linking methods, including physical gelling, chemical cross-linking and a dual mechanism. The cross-linking process significantly changes the properties of polymers, such as the molecular mass, mechanical strength, and resistance to heat and solvents [19]. Either physical or chemical cross-linking creates the three-dimensional structure of a hydrogel, which allows the encapsulation and release of drugs and biomolecules. Hydrogels from the viewpoint of cross-linking methods have been reviewed elsewhere [20]. Physically cross-linked hydrogels usually have poor mechanical properties that may not satisfy the requirements of complicated applications in which toughness and strength are demanded. In contrast, a drawback of *in situ*-forming chemical hydrogels is the possible dissolution of the hydrogel immediately after injection due to a slow gelation kinetic. To combine the mechanical strength of a chemical gel and the fast gel formability of a physical gel, synergistic dual gelling hydrogels have recently been developed. In this review, we present the recent progress in this state-of-the-art research field, with a particular focus on injectable chemical, physical and dual gelling hydrogels for therapeutic agent delivery. Moreover, our recent works on the development of pH/temperature-sensitive hydrogels are also described in this review.

2. Chemically cross-linked hydrogels

Chemically cross-linked hydrogels represent a hydrogel class that can change from a liquid state to a gel state by forming new covalent bonds in a polymer network through chemical reactions. These types of hydrogels have typically been used for implantable applications. Because injectable devices thereafter received considerable attention, chemical cross-linking techniques have also been applied in such systems, particularly, *in situ*-forming hydrogels. Reactions can take place by various mechanisms, such as redox reactions, photo-polymerization, click chemistry, Michael reactions, Schiff's base reactions, enzymatic reactions, or disulfide-forming reactions. New covalent bonds formed from the reaction construct a polymeric three-dimensional network structure in which water can be entrapped, and therapeutic agents or living cells can be encapsulated. Each type of reaction involves a preparation process, mechanical strength, a catalyst, hydrolytic stability and other specific properties of hydrogels. In this section, some of the main strategies for fabricating injectable chemically cross-linked hydrogels will be addressed.

2.1. Photocrosslinked hydrogels

Photopolymerization has been a cross-linking technique with several advantages, including low energy and free solvent requirements, a rapid reaction with mild conditions. Additionally, thanks to spatio-temporal control ability, photo-polymerizable hydrogels have been exploited for a decade in biomedical and pharmaceutical applications, mostly in tissue engineering [21,22]. Photo-crosslinked systems can be formulated from aqueous solutions of polymers containing photo-sensitive molecules and a catalyst for polymerization. Upon exposure to an external irradiation source such as UV or visible light, the photo-initiator can be decomposed, thus forming free radicals and catalyzing the polymerization. Polymers used for photocrosslinking reactions usually have methacrylate or acrylate groups, which undergo rapid polymerization in the presence of light irradiation (Fig. 1). This approach allows for the spatial control of the cross-linked network. Moreover, the gelation rate can also be controlled timely, resulting in the formation of patterned structured hydrogels for designed release profiles. Some studies on the controlled delivery of proteins, genes and drugs are reported in this section.

An early example of an injectable photocrosslinked hydrogel for bioactive agent delivery was introduced by Hubbell et al. in 1993 [23]. Polyethylene glycol was copolymerized with α-hydroxy acids such as polylactic acid (PLA) or polyglycolic acid (PGA) and modified with an acrylate group to yield a water-soluble copolymer that could be photocrosslinked under visible light in the presence of an initiator. Gelation occurred rapidly under mild conditions without any excess heating or local toxicity. The potential for protein delivery was studied using an albumin protein model and resulted in a continuous release of up to

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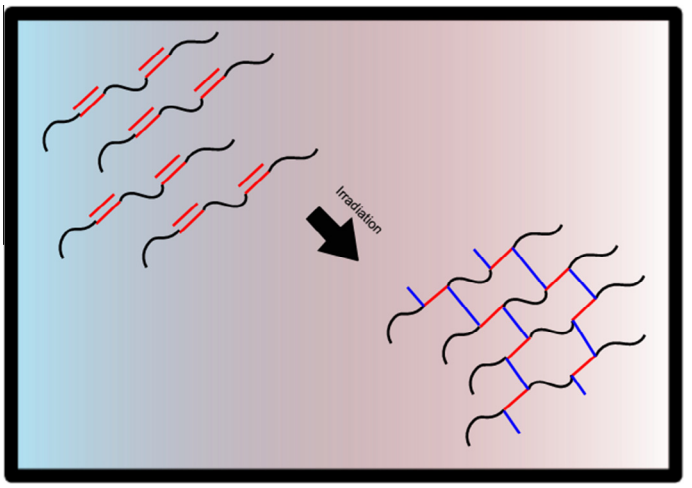

Fig. 1. Schematic mechanism of gelation by photocross-linking reaction of vinyl groups bearing polymers.

2 months. Subsequently, a protein and oligonucleotide *in vitro* release study of this system was reported in 1995 [24]. This report also examined in detail the dependence of release ability on the diffusion and degradation process. Using a tissue plasminogen activator and anti-rev mRNA as the model protein and oligonucleotide, respectively, the release kinetics of these molecules could be tailored according to the PEG block length and composition of the hydrogel. Furthermore, the unique advantage of these materials was the ability to form gels *in situ* compared to previous hydrogel systems. An example of a PEG-based photocrosslinked hydrogel for long-term gene delivery was studied by Anseth et al. [25]. A polymer system was designed using PEG as the center of the backbone, coupled with PLA or PCL block to induce degradation, and functionalized with a methacrylate group to induce rapid photo-polymerization. The plasmid DNA release could be tuned by a wide range, from 6 to 100 days, with a nearly linear profile without an initial burst, by changing the polyester type and composition. Hence, thanks to its controlled release capability and gelation under mild conditions, this system showed versatility and adaptability for various gene delivery applications, causing minimal damage. In addition to biocompatible PEG-based hydrogels, other advantageous materials, which have typically been used for cell- and protein-related applications, are natural polysaccharides. Alsberg et al. introduced a biodegradable photocrosslinked hydrogel based on heparin/alginate for affinity-based growth factor delivery [26]. Different growth factors such as FGF-2, VEGF, TGF-b1 and BMP-2 were successfully released over 3 weeks in a sustained manner, which was attributed to the strong affinity interaction between heparin and the growth factor. Subsequently, released VEGF and BMP-2 were examined for their period of bioactivity, which was 3 and 2 weeks, respectively. For drug delivery applications, a study of the controlled release of paclitaxel using a chitosan-based photocrosslinked hydrogel was carried out by Ishihara et al. [27]. In that research, a well-mixed solution of azide-lactose-modified chitosan and taxol, containing paclitaxel, were photocrosslinked upon UV irradiation to form an insoluble hydrogel network. The *in vitro* release of paclitaxel in PBS was determined by HPLC, resulting in a half-time release of up to 45 h, with 35–40% of the paclitaxel being released from the hydrogel within 1 day. The tumor growth inhibition effect of this system was examined in tumor-bearing mice and lasted for 14 days after injection. This result was well matched with the anti-proliferation effect on tumor cells and showed the potential of a photocrosslinked chitosan hydrogel in a therapeutic drug delivery application. However, in addition to the advantages of photo-crosslinked systems, some issues need to be noted, including the use of this approach for sensitive living cells or pharmaceutical agents and the decrease of light irradiation intensity according to the depth of the targeted tissue layer.

2.2. Click reactions

Generally, the term "click" chemistry has been defined by Sharpless and coworkers as certain reaction types that have high efficiency, excellent specificity, bioorthogonality and mild reaction conditions [28]. Click chemistry has played a significant role in polymer synthesis and bioconjugation as a flexible and efficient method to connect functionalized molecules. Thanks to these advantages, click chemistry has been of great interest in the fabrication of hydrogels, nanogels and microgels as an emerging platform for tissue engineering and drug delivery [29]. Typically, a wide and diverse range of reactions, including Cu(I) catalyzed alkyne-azide cycloaddition (CuAAC) reactions (Fig. 2), catalyst-free alkyne-azide coupling reactions, Diels–Alder cycloaddition, radical-mediated thiol-Michael reactions and Schiff's base reactions, could be considered to be "click" chemistry. Among them, the reaction between alkyne and azide groups has become the most representative example of "click" chemistry, thanks to its two most important advantages, which are high efficiency at physiological conditions and extreme chemo-selectivity [30]. This section summarizes recent developments in which the alkyne-azide click reaction was applied to the design of hydrogels for therapeutic applications.

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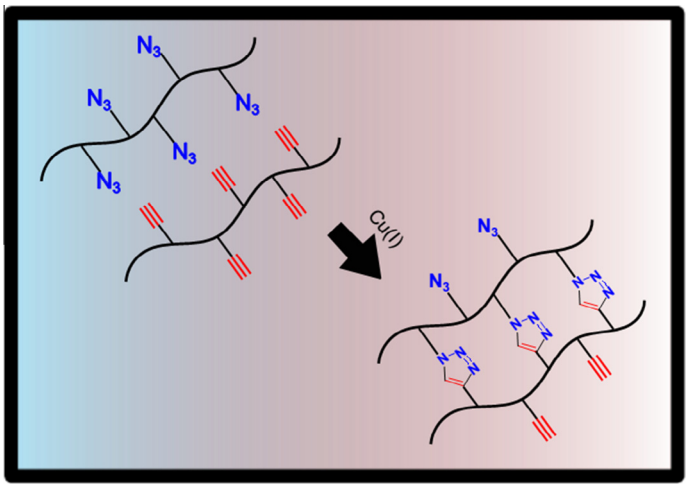

Fig. 2. Schematic mechanism of gelation by alkyne-azide click reaction with Cu(I) as catalyst.

The very first example of applying a CuAAC reaction to hydrogel formation was the work by Hilborn et al. [31]. Alkyne and azide groups were introduced into PVA and PEG chains via carbamate linkage to construct a hydrogel network. The preparation of the hydrogel took place by the following two approaches: mixing of alkyne-PVA with azide-PVA or mixing of alkyne-PVA with PEG diazide. Both methods led to the establishment of a hydrogel network within a minute after adding a Cu(I) catalyst. However, mixing two multiple-functionalized alkyne-PVA and azide-PVA has proven to be more effective in gel formation than using a bifunctional crosslinker. Increasing the concentration of the functional groups was found to first enhance gelation ability and then cross-link density. In addition to synthetic polymers, this procedure was also utilized to generate hydrogels from a natural source for drug delivery and tissue engineering in the work of Lamanna et al. [32]. Hyaluronic acid was modified with azide and alkyne groups by reacting these groups with 11-azido-3,6,9-trioxaundecan-1-amine and propargylamine, respectively. Thus, after adding an aqueous CuCl solution to the mixture of the two functionalized hyaluronic acid solutions, a stable gel state could be reached within a few minutes. Rheological measurement determined the gelation time of this system and proved fast gel formability within 130–160 s. Doxorubicin and benzydamine were utilized as model drugs to check the release potential of this system. The released amounts of doxorubicin and benzydamine were examined by UV–Vis spectrophotometry, which showed a rather rapid release of 70% benzydamine in 8 h and a slower release of 14–25% doxorubicin after up to 10 days, according to crosslink density. Liskamp et al. reported that an enzymatically degradable hydrogel could be yielded from PEG and a peptide via click chemistry [33]. With the compatibility of the CuAAC click reaction with most functional groups in proteins and peptides, this paradigm could be used to create peptide-based hydrogels. Four-arm PEG molecules modified with an alkyne group were incorporated with diazide peptide segments in an aqueous $CuSO_4$ solution as the catalyst to obtain a hydrogel network within a few minutes. Rheological and swelling properties could be engineered by changing the PEG molecular weight, polymer concentration and enzyme conditions. In addition, trypsin- and plasmin-catalyzed degradation were examined by immersing the gel in a PBS solution in the presence of these enzyme. The result indicated that only trypsin could degrade the hydrogel, whereas the bis-azido peptide could be hydrolyzed by both trypsin and plasmin. A copper-catalyzed click reaction may be a fast and efficient reaction, but toxicity caused by the metal catalyst might limit its application in areas requiring high biocompatibility. To overcome this drawback, Kiser et al. introduced the first use of a copper-free Huisgen cycloaddition reaction to fabricate a hydrogel from an azide-functionalized polymer and a dialkyne crosslinker [34]. The liquid to gel state transition occurred within 12 h after incubating a mixture of the two components at 37 °C. The slow gelation kinetic prevents the use of this material in most *in situ* applications. Consequently, another reaction between the azide and alkyne groups that does not need a catalyst was developed and named strain-promoted azide-alkyne coupling (SPAAC) by Bertozzi et al. [35]. This approach was applied by Song et al. to generate cells encapsulated by a crosslinked hydrogel based on PEG and polycarbonate [36]. A biocompatible gel with tunable properties was rapidly formed within 1 min and allowed the encapsulation of bone marrow stromal cells (BMSC). Another hydrogel system that used SPAAC was developed by Ito et al. [37]. A hyaluronan-based hydrogel was formed by a strain-promoted reaction between azide and cyclooctyne functional side-groups. Gelation time could be varied over a wide range of 5–100 min by changing the polymer concentration. Subcutaneous and intraperitoneal injection of this system in mice was carried out and resulted in inflammation but no peritoneal adhesion. Nevertheless, the use of a copper-free azide-alkyne click reaction has still been limited because of the difficulty of synthesizing cyclooctyne.

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2.3. Thiol-based Michael reactions

The term Michael reaction refers to the nucleophilic addition of a nucleophile to an α,β-unsaturated carbonyl compound. Particularly, nucleophile components are thiol- and amine-bearing molecules, whereas unsaturated carbonyl components are commonly associated with acrylate, methacrylate and vinyl sulfone groups. The Michael addition reaction has been extensively employed in different emerging applications including biomedicine, pharmaceutics, optoelectronics, composites, adhesives, and coatings [38]. Thanks to the mild conditions required, controllable reaction rate, high chemical yield and relative inertness to biomolecules, Michael reactions between thiol and vinyl groups have been extensively investigated to construct injectable *in situ* cross-linked hydrogels for therapeutic applications (Fig. 3). A very early study that utilized a Michael reaction to design a hydrogel for protein delivery was the work of Hubbell et al. [39]. After mixing a multiacrylated-PEG solution with a dithiol-PEG solution at an equivalent functionality ratio, gelation was obtained after 340–40 s, according to the concentration of PEG in the solution. The gel swelling ratio was analyzed initially or during the degradation process and showed dependence on the functionality and concentration of the polymer. Solid bovine serum albumin was used as a model protein to determine the release ability of this system. It was observed that the BSA was almost completely released in a zero-order kinetic after 8–12 days, depending on the functionality of the acrylated-PEG. Another application of thiolated-PEG in the preparation of hydrogels for protein delivery was conducted by Stein et al. [40]. Thiol-containing PEG was prepared and mixed with divinylsulfone-PEG to form a chemically cross-linked hydrogel structure. For an *in vitro* protein release study, FITC–BSA was encapsulated into a hydrogel network and resulted in a sustained released of up to 100% over 3 weeks. To demonstrate the potential of this system for clinical use, *in vivo* protein release experiments were carried out by using erythropoietin (EPO) in rabbit models and by using RANTES and its derivative conjugates in rat models. The blood plasma levels of the drug were kept nearly constant for at least 30 days in the case of subcutaneously injected RANTES contained hydrogel, whereas the half-life of these four proteins was only 15–360 min after being intravenously injected into rat models. Furthermore, EPO levels in the blood plasma of the rabbit models were maintained for at least 2 weeks after subcutaneous administration. Moreover, polyethylene glycol, hyaluronic acid and other natural materials were also reinforced by thiol Michael reactions to build hydrogels for the delivery of therapeutic agents. Prestwich et al. introduced a series of chemically crosslinked hydrogel networks based on thiol-modified heparin and thiolated hyaluronan or thiol-containing chondroitin sulfate, with polyethylene diacrylated acting as the crosslinker [41]. This hydrogel system resulted in the sustained release of a basic fibroblast growth factor for up to 35 days, which could be controlled by the concentration of heparin, a component that could bind to bFGF. Similarly, heparin- and hyaluronan-based hydrogels crosslinked by PEG diacrylate continue to be used for dual growth factor loading, such as vascular endothelial growth factor (VEGF) with angiopoietin-1 (Ang-1) or keratinocyte growth factor (KGF) or platelet-derived growth factor (PDGF) [42]. *In vivo* studies in mice models have shown that growth factor-containing hydrogel scan produce partially controlled vascularization, lessen necrosis rates, and reduce leukocytes and inflammation.

2.4. Schiff's base reactions

A Schiff's base is a compound that contains a double-bond of carbon–nitrogen, usually obtained when nucleophilic amines or hydrazides react with electrophilic carbon atoms of aldehydes or ketones (Fig. 4). Schiff's base reactions can occur without any chemicals or catalysts under physiological conditions and thus have attracted considerable interest for the fabrication of injectable *in situ*-forming hydrogels. Another advantageous characteristic of Schiff's base reactions is a controllable reaction

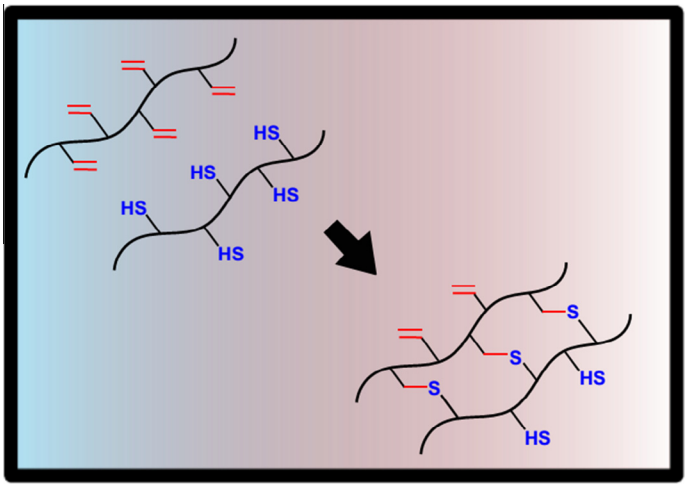

Fig. 3. Schematic mechanism of Michael addition reaction between vinyl and thiol groups.

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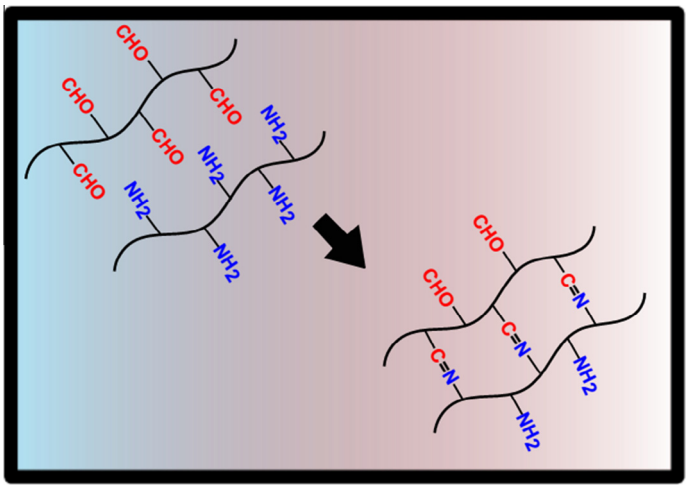

Fig. 4. Schematic mechanism of Schiff's base reaction between aldehyde and amine groups.

rate with respect to the pH of the medium environment [43]. However, aldehyde-containing molecules can affect bioactive factors or tissue extracellular matrix molecules by reacting with amine groups. An interesting system of a hyaluronic acid-based hydrogel crosslinked via a Schiff's base reaction was investigated by Kohane et al. for the delivery of bupivacaine as an anesthesia drug [44]. This approach could possibly replace the more common method that uses fibrin glue to deliver lidocaine. A hydrogel was formed 1–5 min after mixing the two solutions of aldehyde and hydrazide-modified hyaluronic acid, depending on the concentration of the polymer. The *in vitro* release of encapsulated bupivacaine was obtained at approximately 50% after 5 h, which was much slower than the release obtained with an aqueous solution. An experiment with a rat sciatic nerve blockade was carried out to prove the anesthetic effect of the released bupivacaine. The cross-linked hyaluronic acid-based hydrogel successfully increased the duration of the blockade for approximately twice as long compared with the results obtained with bupivacaine in an aqueous solution, unmodified hyaluronic acid and solid aldehyde or hydrazide-functionalized hyaluronic acid. In another study, a similar injectable hyaluronic hydrogel crosslinked by Schiff's base reaction was exploited to deliver therapeutic agents, specifically bone morphogenic protein-2 (BMP-2) for bone augmentation [45]. It was found that this hydrogel system could provide the sustained release of 86% of recombinant human BMP-2 for up to 28 days *in vitro*. An *in vivo* evaluation of the bone forming ability of the released bone growth factor injected via a BMP-2-loaded hydrogel was carried out via subperiosteal and subcutaneous injection in rat calvarial models. The results showed that new bone was promoted within 8 weeks, confirming the activity of the released bone growth factor. Another strategy for generating a natural source-based hydrogel crosslinked by Schiff's base reaction was demonstrated in the work of Jayakrishnan et al. [46]. In their report, an injectable hydrogel was prepared by a chemical crosslinking reaction between oxidized alginate and gelatin in the presence of a small amount of borax. The gelation time varied from a couple of seconds to less than one minute and could be adjusted according to the concentration of the mixture components. By changing the polymer concentration, this system exhibited an *in vitro* controlled release of primaquine. Furthermore, hepatocytes encapsulated in the hydrogel showed an increase in viability and albumin secretion after weeks, demonstrating the potential of this system to be used in tissue engineering applications. Recently, Kohane et al. reported a novel injectable Schiff's base cross-linked hydrogel with various formulations combining hyaluronic acid, dextran, carboxymethylcellulose and gelatin for protein delivery [47]. It was observed that the formulation using dextran considerably slowed down the release of FITC–BSA; additionally, the presence of gelatin prolonged the release time even more. The controlling effect of gelatin was more obvious in the case of another protein model, interleukin-2 (IL-2), which maintained >70% activity after three weeks. In addition to conventional approaches that use amine or hydrazide functional groups, another exciting trend for designing Schiff's base hydrogels is called "oxime chemistry". Oxime bonds exhibit better hydrolytic stability than hydrazone and imine bonds, which share the same pH-controllable reaction time. Thanks to its stability and biocompatibility, oxime cross-linking has been employed to prepare hydrogel systems that can be used in the transcatheter-delivery of treatment for post-myocardial infarction repair as well as in stem cell encapsulation [48,49].

2.5. Enzymatic reactions

Different from other chemical cross-linking methods that are physically or chemically catalyzed, an enzymatic reaction occurs biologically in the presence of an enzyme. Due to this main characteristic, enzymatic reactions have received remarkable interest for cell-friendly applications. Enzymatically catalyzed cross-linking requires mild reaction conditions, including a neutral pH, aqueous environment and mild temperature. Another important

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advantage of an enzymatic reaction is the substrate specificity of the enzyme, which can prevent toxicity caused by side reactions. Horseradish peroxidase, transglutaminase, tyrosinase, phosphopantetheinyl transferase, and lysyl oxidase have been used as enzyme catalysts to prepare hydrogel systems, especially for tissue engineering [50]. In this section, some examples of the enzymatic approach being used for hydrogel formation for therapeutic agent delivery will be illustrated.

Peroxidase, especially horseradish peroxidase (HPR), has been the most attractive candidate for enzymatically catalyzing cross-linked hydrogels, thanks to its high stability and easy purification, and the fact that its use results in high mechanical strength hydrogels with fast gelation. Horseradish peroxidase catalyzes the oxidative coupling of phenol components in the presence of an oxidant such as hydrogen peroxide (H_2O_2) (Fig. 5). HPR and H_2O_2 catalyst systems have been widely applied in many hydrogel systems using natural materials such as chitosan [51], hyaluronic acid [52], dextran [53], gelatin [54] and the combination of these [55], predominantly for tissue engineering. In another work, Kurisawa and coworkers reported an injectable enzymatically catalyzed hydrogel based on hyaluronic acid for therapeutic protein delivery [56]. In that study, hyaluronic acid was functionalized with tyramine moieties, followed by fluorescent labeling with aminofluorescein. First, fluorescently labeled BSA and lysozyme were loaded into a hyaluronic-tyramine hydrogel to analyze gel formation. The result indicated that enhancement of the HRP concentration could increase the storage modulus and decrease the subcutaneous gelation time. Additionally, positively charged lysozyme and negatively charged α-amylase were employed as model proteins for examining the release ability. Sustained release of the protein could be engineered by changing the cross-linking density and/or ionic strength of the environment and by the use of hyaluronidase as an enzymatic degradation agent. Another example of applying a hyaluronic-tyramine hydrogel system in therapeutic agent delivery was the work of Hahn et al. [57]. In this research, dexamethasone (DMT), an anti-inflammatory agent, was encapsulated and delivered by a hydrogel system to treat rheumatoid arthritis. The preparation of the hyaluronic-tyramine hydrogel system was fairly similar to the abovementioned work of the Kurisawa group. Dexamethasone showed sustained controlled released *in vitro* and *in vivo* within 4 weeks, showing potential for use in rheumatoid arthritis (RH) treatment. The therapeutic effect of dexamethasone released from hyaluronic-tyramine with/without an HPR system on an RA animal model was affirmed by comparing the concentrations of PGE_2 and IL-6 in the blood plasma of the control group of normal SD rats with those of the PBS-treated group. The statistically lower concentrations of PGE_2 and IL-6 in the PBS-treated group compared with the negative control group within 4 weeks demonstrated the controlled release capability of the hydrogel system. Moreover, the recuperation of cartilage 4 weeks after treatment with the DMT-loaded hyal-tyr hydrogel was equal to that of the control groups, which undoubtedly proved the potential of this system in RH treatment.

Transglutaminase is another widely used enzyme catalyst that provides mild reaction conditions, fast gelation and high cytocompatibility and can act as "biological glue". Several groups have exploited transglutaminase in the preparation of hydrogels based on PEG [58], polypeptide [59], and gelatin–chitosan conjugate [60]. Among them, Li et al. introduced a microbial transglutaminase-catalyzed cross-linked hydrogel based on casein for the controlled delivery of Vitamin B12 [61]. Casein is a phosphoprotein that has advantageous properties such as high hydrophilicity, biocompatibility, as well as reactive side groups for modification. By either changing the temperature or concentration of transglutaminase, the gelation time of the casein hydrogels could be adjusted within a range of 20–115 min. The *in vitro* release experiment of encapsulated Vitamin B12 was executed under physiological conditions and resulted in a decreased release rate when transglutaminase was added and increased. This

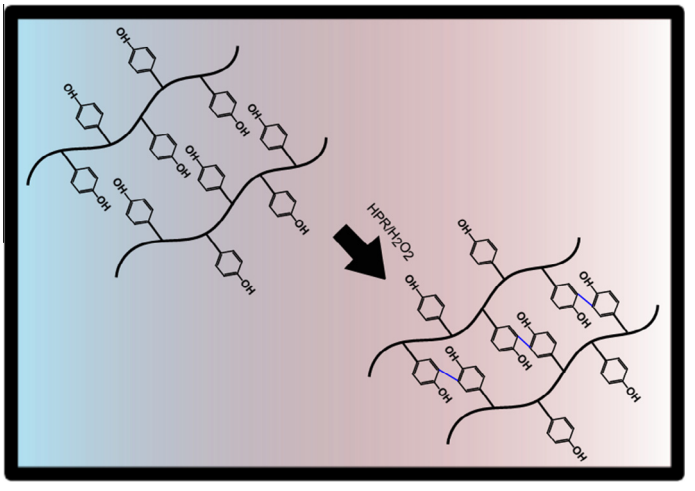

Fig. 5. Schematic mechanism of enzymatic reaction with horseradish peroxidase and hydrogen peroxide as catalyst system.

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observation affirmed the potential use of enzymatically catalyzed casein hydrogels in therapeutic controlled delivery.

2.6. Other chemically cross-linked hydrogels

In addition to the more popular trends summarized in previous sections, other chemical cross-linking methods have also been utilized to produce hydrogels for biomedical applications, including disulfide formation, epoxy reactions, genipin cross-linking and native chemical ligation. Though these strategies satisfy the basic requirements for hydrogel formation such as high chemical yield and mild reaction conditions in an aqueous environment, there has still been a limited focus on therapeutic agent delivery applications. Several typical examples of these chemically cross-linked hydrogels are briefly addressed in this section. Bernkop-Schnurch et al. prepared an *in situ*-forming hydrogel obtained by disulfide formation after mixing oxidizing agents with a chitosan-thioglycolic acid conjugate [62]. Subsequently, Sinko et al. developed a multi-arm PEG-based hydrogel system that crosslinked by oxidizing multiple thiol groups to deliver doxycyline for dermal wound healing of mustard injuries [63]. A hydrogel matrix that allowed the stable growth and proliferation of articular canine chondrocytes based on epoxide-crosslinked chitosan was introduced by Lin et al. [64]. Recently, another epoxy-cross-linked hydrogel reported by Lendlein et al. provided the controlled release of triamcinolone and was applied as a retinal patch for preventing cell migration and proliferation after a retinal break [65]. In the work of Yan et al., a simple yet interesting hydrogel based on a cross-linking reaction between genipin and casein was successfully prepared and enabled the controlled release of bovine serum albumin over 4 days [66]. Genipin was also used as a natural cross-linker for catechol-chitosan mucoadhesive hydrogels in a study by Cerruti et al. for buccal lidocaine delivery [67]. Possessing fantastic chemo-selectivity and endogenous thiol functionality, native chemical ligation (NCL) between cysteine-terminated and thioester-functionalized molecules is a modern and desirable approach for peptide-related or cell-friendly hydrogels. Messersmith et al. described the development of PEG-based hydrogels cross-linked by NCL reactions that could be functionalized with RGD peptide for adhesion of human mesenchymal stem cells [68]. Thereafter, this group applied their NCL cross-linked and peptide-modified hydrogel systems to encapsulate pancreatic islet cells for the treatment of type I diabetes mellitus [69]. Another promising class of NCL is oxo-ester mediated reaction, which has been used to develop biologically compatible hydrogel [70]. This system shared the same biocompatibility property and showed faster gelation rate in physiological pH compared to general NCL chemistry-based hydrogels.

3. Physically cross-linked hydrogels

Different from chemical cross-linking mechanisms, physically cross-linked hydrogels achieve a gel state by changing intermolecular forces such as hydrogen boding, hydrophobic interaction, electrostatic ionic force or intermolecular assemblies such as guest–host inclusion, stereo-complexation and complementary binding. These changes can occur by the internal arrangement of the polymers themselves or can be induced by external stimuli such as heat, pH, ionic strength electric fields, light, pressure, sound or the presence of specific molecules [71]. Because the addition of possibly toxic crosslinkers or catalysts can be avoided, physical cross-linking methods provide simple and safe approaches to preparing injectable *in situ*-forming hydrogels. Due to the various assembly mechanisms, each class of physically cross-linked hydrogels possess their own characteristics in mechanical strength, gelation time and degradation properties. This section focuses mainly on temperature-responsive and pH-sensitive hydrogels. The works of our group on pH/temperature-sensitive hydrogels for some specific applications are also presented herein.

3.1. Temperature sensitive hydrogels

Temperature-sensitive hydrogels were extensively exploited in early generations of physically cross-linked hydrogels, thanks to their simple administration and fast gelation. Temperature can induce a change in the solubility of a whole polymer network, thereby causing the sol–gel phase transition. Generally, the structure of a temperature-sensitive hydrogel often contains hydrophobic and hydrophilic components. Shifting the hydrophilicity–hydrophobicity balance determines the macroscopic soluble–insoluble state transition in an aqueous solution (Fig. 6). Gelation of these hydrogels can be achieved via various microscopic mechanisms depending on particular temperature-sensitive groups. There are several main classes of these hydrogels, classified by their temperature-sensitive moieties. Many excellent review articles of commonly used types as well as new directions in thermo-responsive hydrogels have been published elsewhere [72–77]. Several typical trends involving these types of hydrogels will be discussed herein.

Poly(N-isopropylacrylamide) (PNIPAAm) was probably the most extensively studied temperature-sensitive hydrogel when such hydrogels were first discovered. PNIPAAm is a thermal-sensitive polymer that undergoes a hydrophilic–hydrophobic transition when the temperature is raised above the lower critical solution temperature (LCST). This transition behavior is caused by the change of the macromolecules from an expanded coil state to a collapsed globule state. PNIPAAm can connect with a hydrophilic acrylic acid (AAc) group or hydrophobic butyl methacrylate (BMA) group to generate negative or positive temperature-responding hydrogels, respectively [71]. When the temperature is raised above the LCST, the shrinking behavior of the hydrogel indicates negative feedback, whereas swelling indicates a positive response. It has been found that when PNIPPAm, as the LCST monomer, was cross-linked with bis-vinyl-terminated polydimethylsiloxane (VTPDMS), as the more hydrophobic macromer, a heterogeneous thermal-sensitive hydrogel was formed [78]. The hydrogel showed fast temperature-responding shrinking-swelling

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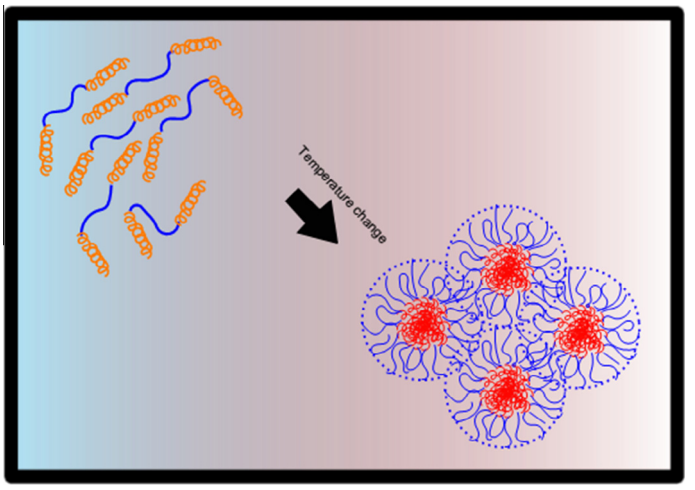

Fig. 6. Schematic mechanism of gelation driven by shifting of hydrophobic interaction under the change of temperature.

behavior at the transition temperature, similar to the LCST point of PNIPAAm, even with a feed composition of up to 50% (w/w) VTPDMS. The *in vitro* release of encapsulated hydrophobic drugs such as progesterone was observed for up to 12 days and showed a zero-order kinetic as a function of the composition ratio between PNIPAAm and VTPDMS. In another work, creation of a macroporous hydrogel from PNIPAAm for protein loading and delivery was presented [79]. Using aqueous sodium chloride solutions as the media for the polymerization reactions, a series of fast temperature-response macroporous hydrogels were produced. The presence of sodium chloride in the reaction medium greatly affected the swelling ratio as well as the water retention ratio in the deswelling process of the hydrogel network. Thus, a comparison of the BSA release ability at both 25 °C and 37 °C was carried out and showed an enhancement of the release rate with the macroporous network using sodium chloride in the reaction medium. Additionally, the release rate of BSA is a function of temperature due to the thermal sensitivity of PNIPAAm.

Similar to PNIPAAm, another widely used non-biodegradable temperature-sensitive hydrogel from early during the research on these types of hydrogels is the Pluronic hydrogel. Pluronic F127 (also known as Poloxamer 407) is the commercial name of the ABA-type triblock hydrogel consisting of polypropyleneoxide (PPO) as the hydrophobic A block and polyethyleneoxide (PEO) as the hydrophilic B block. The aqueous solution of this triblock copolymer exhibited both sol-to-gel and gel-to-sol phase transitions when heated from a low to a high temperature. The sol state, gel state and precipitated state matched with areas from low temperature to lower boundary, between two boundaries and upper boundary to beyond temperature in phase transition diagrams, respectively. The gel-state region in the sol–gel transition diagram can be adjusted according to the concentration or composition of the polymer. When the concentration of the polymer in an aqueous solution is higher than a particular critical value, aggregation and bridges of micelles can induce the gelation of the whole solution. In the work by Blanchard et al., the incorporation of additive reagents such as polyethylene glycol (PEG), polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA), methylcellulose (MC) and hydroxypropyl methylcellulose (HPMC) into Pluronic F127 formulations effectively controlled the dissolution rate as well as the *in vitro* release of pilocarpine hydrochloride (PHCL) [80]. It has been found that MC- or HPMC-incorporated formulations significantly prolong the amount of PHCL released by up to 3–4 times more than in control groups. To achieve biodegradability, grafting with hyaluronic acid has also been utilized in the work of Cho et al. and Park et al. [81,82]. The resulting hydrogels were employed as carriers for the delivery of ciprofloxacin and human growth hormone. The controlled release of bioactive factors from Pluronic F127 is only maintained over a few days, which makes Pluronic hydrogels more suitable for short-term delivery applications. A detailed table of the applications of Pluronic F127 for drug delivery over the past few years can be found in a review paper by Bae et al. [83].

Because there was a clear need for the biodegradability of hydrogels after administration in the body, a large family of synthetic biodegradable and biocompatible hydrogels was developed by coupling poly(α-hydroxy ester) blocks from glycolic acid, lactic acid, ε-caprolactone with polyethylene glycol. Well-controlled thermo-responding gelation properties or biodegradability can be obtained by changing the polyester type and composition. An early example of these injectable and biodegradable hydrogels was introduced by Jeong et al. [84]. Monomethoxy polyethylene glycol was copolymerized with L-lactic acid to obtain diblock PEG–PLLA or further coupled with hexamethylene diisocyanate to yield a triblock PEG–PLLA–PEG copolymer. With a rapid thermal-inducing sol–gel transition *in vitro* and *in vivo*, these systems were used in an FITC-dextran release test, which showed their potential for use in the local delivery of bioactive agents. Subsequently, the addition of a glycolic acid component into the PEG–PLLA–PEG chain enabled researchers to avoid

the use of heat before administration. The newly formed PEG–PLGA–PEG was found to form a gel state at 37 °C from a free-flowing sol state at room or low temperature. This triblock copolymer was exploited for the delivery of the hydrophilic drug ketoprofen [85], hydrophobic drug spironolactone [85] as well as plasmid DNA [86]. Later, by inverting the hydrophilic PEG in the middle of the triblock of the two arms of PLGA, a PLGA–PEG–PLGA compound was designed that achieved a much lower critical gellable polymer concentration. These compounds were employed as carriers for the controlled delivery of insulin [87], testosterone [88], lysozyme [89], 5-fluorouracil [90] and indomethacin [90]. Instead of glycolic acid, ε-caprolactone was also copolymerized with L,D-lactide and polyethylene glycol to obtain a PCLA–PEG–PCLA triblock polymer that subsequently functionalized with an RGD sequence for sustained doxorubicin delivery [91].

Despite the above advantageous characteristics, the degradation products of polyester-based hydrogels are usually acidic monomers that might cause harmful side-effects to surrounding environments. To overcome this drawback, a novel class of thermal-sensitive hydrogels based on polyphosphazene has drawn considerable interest recently, thanks to its non-toxic biodegradable products [92]. Moreover, various types of functional substituents can be introduced into the backbone of polyphosphazene to adjust the mechanical strength, degradation rate and drug interaction. In 2006, a conventional delivery approach that simply mixes a drug into a polyphosphazene hydrogel matrix was used in the work by Song et al. [93]. Over more than 20 days, the release rate of doxorubicin was successfully controlled by altering the mechanical strength of polyphosphazene. Later, Song and colleagues exploited a conjugation strategy involving doxorubicin and paclitaxel as therapeutic agents coupled to a polyphophazene backbone for local tumor treatment [94,95]. Another method of conjugation in polyphosphazene hydrogels involves a modification with protamine or poly-L-arginine for the delivery of human growth hormone (hGH) [96], which results in poly-L-arginine- or protamine-modified poplyphosphazene releasing hGH for up to 5 days *in vivo* or 7 days *in vitro*, respectively.

In addition to the abovementioned synthetic systems, there have been a number of natural thermal-sensitive hydrogels used for therapeutic agent delivery. These temperature-responsive materials have been built with gelatin, polysaccharide derivatives, amylose, amylopectin, carrageenans, cellulose derivatives, xyloglucan, chitosan and glycerophosphate [74,83].

3.2. pH-sensitive hydrogels

It should be noted that even though temperature-sensitive hydrogels have been widely used, thanks to their promising characteristics, there have been drawbacks that have limited the utilization of thermal-sensitive hydrogels in more specific applications. These limitations include possible blockages during injection through a syringe, a lack of ionic groups to complex with charged biomolecules, and difficulty in dissolving and storing materials [97–99]. Efforts to prepare a new class that can avoid possible gelation inside a needle, are easier to dissolve in water and have better ionic interaction with the protein/drug/gene led to the development of pH- or pH/temperature-sensitive hydrogels. pH is a notable environmental parameter that can be used for a stimuli-sensitive system because each site in the human body possesses a different pH value. A well-designed pH-responding system can be applied to the delivery of bioactive agents to any site in the body, such as the stomach, intestine, liver tumor, blood vessels and vagina. pH-triggered phase transition between soluble–insoluble mainly occurs via the protonation–deprotonation of ionizable groups around the pK_a value (Fig. 7). pH-sensitive polymers are usually weak polyelectrolytes based on either acidic moieties such as carboxylic acid, sulfonamide or basic tertiary-amine groups that ionize at high or low pH, respectively. In this section, our recent studies on sulfonamide- and tertiary-amine-based hydrogels for bioactive factor delivery will be presented.

In 2005, our group presented an early example of a biodegradable, biocompatible pH/temperature-sensitive hydrogel based on sulfonamide groups [100]. Oligomers of acidic sulfonamide pendant groups were coupled with a biodegradable triblock copolymer PCLA–PEG–PCLA to obtain the pH/temperature sensitive pentablock copolymer. An aqueous solution of this hydrogel underwent the phase transition process from a freely flowing sol state at a slightly high pH and room temperature to a gel state at a neutral or acidic pH and physiological temperature. The sol–gel transition behavior could be precisely tailored by altering the hydrophobic–hydrophilic block ratio, block length and total molecular weight of the polymer [101]. In another report, the biocompatibility and biodegradability of an OSM–PCLA–PEG–PCLA–OSM pentablock copolymer was studied [102]. The application of these hydrogels in therapeutic agent delivery was examined by incorporation with paclitaxel, a model anticancer drug [103]. PTX was successfully encapsulated and had a sustained release over 1 month *in vitro*. The *in vivo* antitumor activity of PTX-loaded OSM hydrogels after subcutaneous administration in tumor-bearing mice was shown to be maintained over 2 weeks. On the other hand, these hydrogels were also applied as scaffold depots to encapsulate human mesenchymal stem cells (hMSC) and recombinant human bone morphogenetic protein-2 (rhBMP-2) for bone tissue engineering [104].

Later, we prepared another class of biodegradable stimuli-sensitive hydrogels based on the corporation of a basic pH-sensitive poly(β-amino ester) with a temperature-sensitive triblock PCL–PEG–PCL for the controlled release of insulin [105]. In contrast to the OSM–PCLA–PEG–PCLA–OSM pentablock hydrogel, this system changed from a sol-state at a low pH into a gel-state at a neutral or high pH. Additionally, the positive charge of the protonated amino groups on the polymer backbone could electrostatically interact with the negative charges in anionic drugs, proteins or genes. Thus, the release profile of bioactive molecules can be controlled not only by diffusion or degradation but also by ionic interaction, which might reduce the chance of an initial burst phenomenon. The prolonged release of insulin from this system was significantly better than that obtained with the temperature-sensitive triblock

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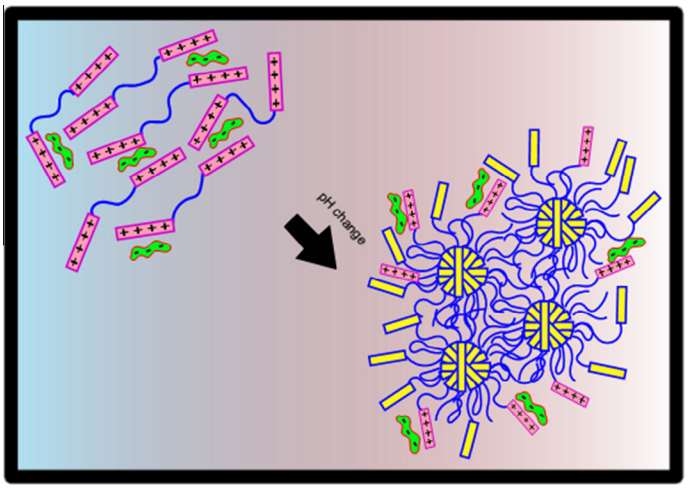

Fig. 7. Schematic mechanism of gelation driven by pH-inducing protonation–deprotonation transition of cationic hydrogel.

copolymer PCL–PEG–PCL both *in vivo* and *in vivo*. Due to this dual chemically and diffusion-controlled release mechanism, insulin had a sustained release *in vitro* of almost 36 days, and it maintained a constant concentration in the blood plasma for over 15 days. Furthermore, the amount of insulin released in the serum from this system could be effectively controlled by the concentration of the polymer in the aqueous solution as well as by the concentration of loaded insulin [106]. Similar to the OSM–PCLA–PEG–PCLA–OSM pentablock hydrogel, a sol–gel phase transition diagram of PAE–PCL–PEG–PCL–PAE could be easily adjusted by changing the molecular weights or compositions of the components of the polymer chain [107].

In subsequent studies, we developed various types of pH- and pH/temperature-sensitive hydrogels based on tertiary-amine groups. The architecture of these hydrogels could be a star-shaped multiblock, linear multiblock, or triblock copolymer or even a low molecular weight oligomer. A number of pH-sensitive moieties were used, including poly(β-amino ester), poly(amino urethane), poly(amidoamine), poly(amino ester urethane), and poly(amino urea urethane). The whole series exhibited a similar trend in the pH-triggered sol–gel transition, which was governed by the protonation–deprotonation of the amine groups. Any differences in solubility, biodegradability, temperature-sensitivity, mechanical strength, gelation critical concentration, adhesive properties and cytotoxicity were revealed to be attributable to the functional bonding, composition or structure of the polymer. The appearance of the PCL–PEG–PCL triblock compartment in the multiblock copolymer based on PAU may lead to difficulty in dissolving the polymers in an aqueous solution even at a soluble pH value, in comparison to the multiblock copolymer $(PEG–PAU)_x$ [108,109]. The addition of urea bonding enhanced the mechanical strength as well as prolonged the degradation time, which was promising for long-term applications [110]. In contrast, ester bonding in the multiblock and triblock poly(β-amino ester urethane) served as the hydrolytically degradable factor, which enhanced the degradation rate of the hydrogels [111,112]. Using 4-arm polyethylene glycol to build the star-shaped copolymer led to gelation even with low molecular weight polymers [113]. The poly(amidoamine) block induced adhesion to rat tissue, rapid *in vivo* gelation and a considerable change in viscosity from the sol to gel state, thanks to a high density of positively charged amines; however, this block also produced a mild inflammatory response in surrounding tissues [114]. Similarly, bioadhesion between the PAE–PEG–PAE triblock copolymer and mucin mussel has been attributed to the ionic interaction between the positive charges of amine-bearing polymers and the negative charge of mucin carboxylic acid [115]. In contrast to previously mentioned copolymers that have had rather complex structures, the recently developed oligomeric gelators have the advantage of a simpler synthesis process. Oligoamidoamine (OAA) and oligo(amidoamine/β-amino ester) (OAAAE) have been synthesized via a one-step Michael addition reaction, whereas oligo(β-amino ester urethane) has required one more reaction for the formation of urethane bonds [116–118]. Aqueous solutions of these short chain gelators have exhibited a pH- and temperature-responsive sol–gel transition, with the gel windows occurring under physiological conditions. For therapeutic applications, these polymers and oligomers may be potential carriers for the delivery of various types of drugs or proteins such as doxorubicin [111,118], human growth hormone [112], lidocaine [115], chlorambucil [109,113], FITC–BSA [110], paclitaxel [108], and insulin [117].

Our recent effort to combine positively charged tertiary-amine with negatively charged sulfamethazine into one copolymer chain has led to the development of an innovative amphoteric copolymer [119]. Having both types of charges, this polymer was capable of forming a dually cationic and anionic hydrogel system with a special closed-loop reversible sol–gel–sol transition. Gelation was thus obtained *in vivo* after subcutaneous injection into SD rats under both mildly acidic and basic pH conditions. The controlled delivery of anionic human growth hormone

was also examined and resulted in a high concentration of released hGH in the blood plasma over 3 days without an initial burst.

3.3. Other physically cross-linked hydrogels

In spite of similar ion-related gelation mechanisms, pH-sensitive hydrogels can be formed by ionization–deionization, whereas ion-inducing complexation is caused by the opposite ionic electrostatic interaction (Fig. 8). An early example of this motif was the work on calcium ion-cross-linked alginate-based hydrogels by Cohen et al. [120]. α-L-guluronic acid moieties in alginate could electrostatically interact with calcium ions, resulting in the formation of an inhomogeneous gel. This *in situ* gelling alginate system was explored as an ophthalmic drug carrier for the delivery of pilocarpine. Another utilization of calcium-activated gelling alginate for ophthalmic drug delivery was introduced by Pan et al., with the addition of HPMC, a viscosity-enhancing agent [121]. The alginate/HPMC mixture underwent the sol–gel transition on the ocular surface and prolonged the release of gatifloxacin for over 8 h *in vitro*. Alginate could also be grafted to hyaluronate backbones to generate an ionically crosslinkable compound that could form hydrogels in the presence of calcium ions [122]. It was observed that a new extracellular matrix was formed, and transplanted cells maintained their chondrogenic phenotypes *in vivo,* which proved the potential of these systems for *in vivo* cartilage engineering. Interestingly, ionic gelation can also be achieved by mixing solutions of oppositely charged hydroxyethyl- methacrylated-derivatized dextran microspheres to yield a reversible shear-thinning hydrogel system [123]. The *in vitro* release of three model proteins, including lysozyme, BSA and IgG, demonstrated the diffusion-controlled release capability of this system. Recently, another ion-induced self-assembly system was prepared based on multi-domain peptide nanofibers [124]. These $E_2(SL)_6E_2$GRGDS peptide nanofibers induced the formation of hydrogels in the presence of Mg^{2+} ions. The peptide hydrogel underwent shear-thinning with appropriate viscosity and recovered rapidly, which was well suited for delivery via injection. Embryonic stem cell (ESC) secretomes as well as drug delivery potential were also demonstrated.

A supramolecular inclusion complex can be formed when particular groups or chains from guest-molecules are fitted into the cavity of cyclodextrin (CD)-bearing host molecules (Fig. 9). It is noteworthy that the unique geometry of cyclodextrin has led to a broad application range of their derivatives in nasal drugs, opthalmic drugs, and peptide and protein delivery [125]. Recently, inspired by CD-based supramolecular complexation, a large category of physically crosslinked hydrogels has been considerably expanded. In a report by Li et al., an inclusion complex-based hydrogel was yielded when the PEO segment of a PEO–PHB–PEO triblock polymer came in contact with α-cyclodextrin rings [126]. It was known that α-CD forms a necklace-like supramolecular structure with molecules of PEO in an aqueous solution. Additionally, the micellization of hydrophobic PHB blocks can also help gelation. As a result, an aqueous solution containing 13 wt% of PEO–PHB–PEO and 9.7 wt% of α-CD can form a gel at room temperature. Delivery of FITC-dextran as a model drug resulted in a significant enhancement in the *in vitro* release time of the PEO–PHB–PEO/α-CD complex compared with the PEO/α-CD system. Guest–host interaction between PEG and α-CD was also used in another system based on a natural material heparin [127]. MPEG was grafted onto heparin backbones via EDC/NHS chemistry and then formed a supramolecular hydrogel in the presence of α-CD. This hydrogel system was used *in vitro* for BSA and conjugated heparin release, blood clotting and hemolysis experiments. Conjugated heparin was released in a much slower manner than BSA because of the inclusion complexation between heparin-MPEG and the α-CD molecules. Heparin released from the hydrogel still maintained anticoagulant properties when in contact with fresh human blood. Different from α-CD, the pocket of the β-CD structure was employed as a reversible binding site for inclusion complexation with adamantane- or cholesterol-bearing molecules [128,129].

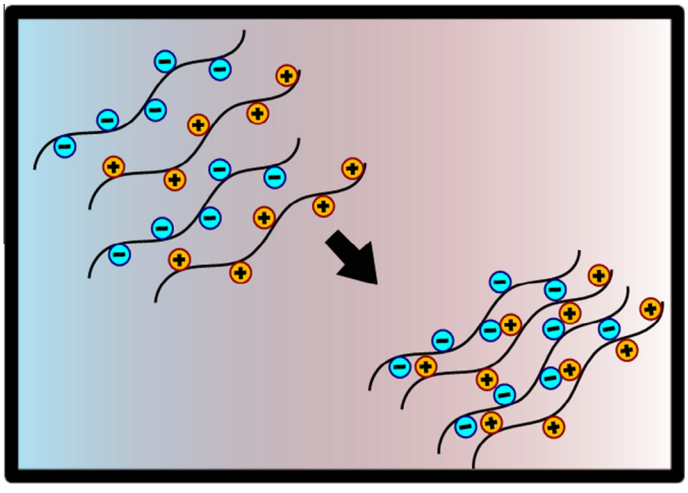

Fig. 8. Schematic mechanism of gelation by ionic interaction.

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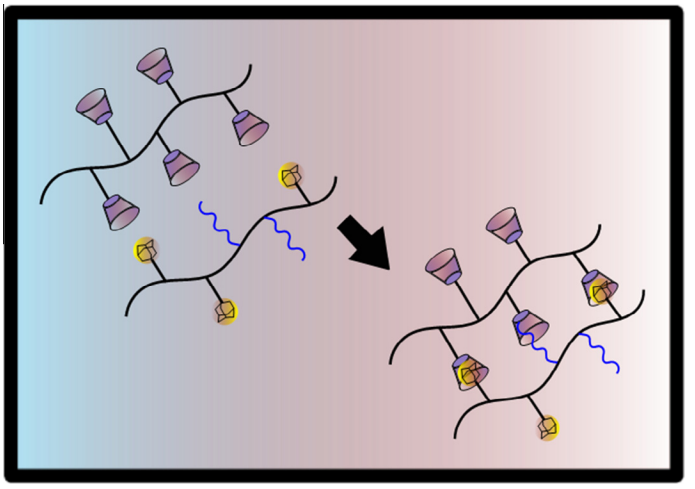

Fig. 9. Schematic mechanism of gelation by guest–host inclusion complexation between cyclodextrin groups and adamantane groups or PEG chains.

A double network of hydrogels was designed by combining adamantane-modified molecules with β-cyclodextrin-functionalized hyaluronic acid [128]. An almost immediate formation of a pseudoplastic hydrogel occurred upon mixing an aqueous solution of Ad-HA with CD-HA. The formed hydrogel exhibited an almost immediate recovery following shear-thinning delivery and a controlled release of BSA over 60 days. Lastly, Hennink et al. introduced a hydrogel material composed of β-CD- and cholesterol-derived 8-arm star-shaped PEG as a matrix for protein delivery applications [129]. The gel showed a sustained release of three model proteins, including BSA, lysozyme, and IgG, over a period of 9 days. The release profile of the small peptide bradykinin from this hydrogel might be effectively controlled via a hydrogel surface erosion mechanism.

Another recent strategy for forming physically crosslinked hydrogels was explored by using stereo-complexation between polymers of opposite chirality. For example, when enantiomers of L-lactic and D-lactic acid are mixed together, a stereo-selective interlocking system can be formed (Fig. 10). Therefore, a significant enhancement in mechanical strength, melting point and hydrolytic stability can be obtained in a PLA stereo-complex, in comparison to its enantiomers. Stereo-complexation has been found in various types of polymer pairs, but the most prevalent utilization in the biopolymer field has been for non-biodegradable poly(methyl methacrylate) (PMMA) and biodegradable PLA [130]. Applying this scheme, the Hennink group developed a physically crosslinked hydrogel system based on a stereo-complex assembly of L- and D-lactate oligomer-grafted dextran [131]. The releases of the encapsulated model proteins (lysozyme and IgG) were both sustained for over 6 days, and the different protein sizes determined the gel characteristics. In another approach by Hennink et al., dextran-HEMA was radically polymerized to yield microspheres, which were then grafted to activated oligolactate [132]. The formation of a macroscopic hydrogel was achieved by the self-assembly of dex-HEMA-L-lactate and dex-HEMA-D-lactate

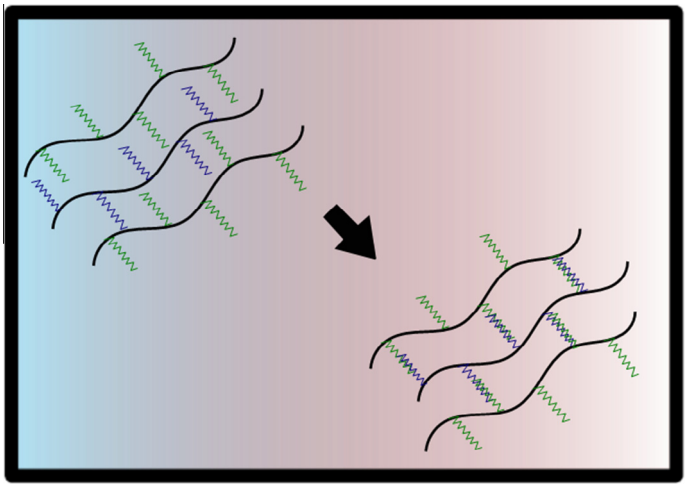

Fig. 10. Schematic mechanism of gelation by stereo-complexation between L-lactide and D-lactide bearing polymers.

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microspheres. After simply mixing with the microspheres in solution, the lysozyme was continuously released at up to 80% over 30 days, which was significantly slower than in previously mentioned reports. Stereo-complexation between enantiomeric polylactic acids was also used to create PEG-based hydrogels, which used star-shaped 8-armPEG [133] The therapeutic recombinant human IL-2 (rhIL-2) protein was loaded into a mixture of PEG–$(PLLA)_8$/PEG–$(PDLA)_8$ before the formation of a stereo-complexed hydrogel network. *In vitro* results showed an almost zero-order release of incorporated rhIL-2 from the hydrogel for up to 7 days, independent of the polymer concentration, which was attributed to the smaller size of the protein compared to the hydrogel mesh size. The *in vivo* therapeutic effect was examined by observing tumor size reduction in treated SL2-lymphoma-bearing DBA/2 mice. The cure rate of rhIL-2 loaded complexed hydrogels was 30%, showing a therapeutic effect compared to the negative control groups, but was still lower than the rate of the positive control group treated with free-rhIL-2 (70%), which provided a slower and more constant release of rhIL-2 from the hydrogel systems.

In addition to the previously mentioned common types of binding, other strategies could also be used to design three dimensional hydrogels for therapeutic agent delivery. Peptide-based hydrogels could be achieved via the association of complementary β-hairpin, β-sheet motifs [134,135] or the coiled-coil assembly of α-helix motifs [136,137]. Using a rather similar scheme, self-assembled DNA hydrogels were prepared via the base-pairing interaction of complementary DNA strands [138–140]. In other promising approaches, biomolecules-responsive bioconjugated hydrogels were obtained through ligand-receptor interaction between heparin and growth factors [141], glucose and concanavalin A [142], or antigen–antibody binding between rabbit IgG and goat anti-rabbit IgG [143,144]. Lastly, metal–ligand coordination between iron(II) and bipyridine [145,146], nickel(II) and terpyridine [147], or iron(III) and catechol [148] were exploited to produce metallo-hydrogels.

4. Outlook of dual gelling hydrogels

Because both physically and chemically cross-linked hydrogels possess their own limitations, there has been an obvious demand for a combination solution. Thus, dual gelling hydrogels have become the promising answer. Once injected into the body, these hydrogels undergo rapid gelation via physical interactions and subsequently stabilize due to the formation of covalent bonds. Fast gelation at the first step can prevent hydrogels from dissolving early, and then, post-gelation crosslinking can strengthen the three-dimensional network. A variety of choices are available for the design of dual gelling systems. In a common scheme, temperature-sensitive polymers based on PNIPAAm, PEO–PPO–PEO and poly(organophosphazene) were modified with various chemically functional groups for self-occurring post-gelation reactions including Michael additions, Schiff's base reactions, enzyme-mediated cross-linking, click reactions, epoxy reactions with amines or phosphates and native chemical ligation [149–158]. Photocrosslinking reactions of vinyl groups have been another solution to secondarily crosslink the thermal-sensitive hydrogels of PEGMEMA–PPGMA–EGDMA copolymers or the newly developed shear-thinning dock-and-lock hydrogels of peptide-modified hyaluronic acid [159,160]. Recently, series of dual crosslinking, hybrid systems from PEG-based hyperbranched thermoresponsive copolymers and cell-friendly hyaluronic acid have been developed for wound healing, cell delivery and tissue engineering [161–165]. In this section, several utilizations of dual gelling systems for therapeutic factor delivery will be addressed.

Benzaldehyde en-capped Pluronic F-127 underwent a secondary chemical crosslinking step with amine groups of glycol chitosan to yield a dual gelling system [156]. Schiff's base reaction was accelerated in high pH, and the gel thus had pH-sensitive characteristics, which were demonstrated in a sol–gel diagram. pH driven chemical crosslinking allowed for easier administration of the material. Moreover, the release of both hydrophobic and hydrophilic drugs (such as doxorubicin and prednisolone) could be pH/temperature controlled in this system. Schiff's base reaction was also applied in another simultaneous physical and chemical hydrogel composite system by Hoare et al. [150]. Hydrazide groups on thermo-sensitive PNIPAAm surface-modified superparamagnetic nanoparticles (SPIONs) were reacted with aldehyde groups on the dextran chain via condensation reactions. The resulting hydrogel was *in situ*-formed after being injected into a silicone mold by a double-barrel syringe. Bupivacaine hydrochloride as a therapeutic drug was encapsulated in this hydrogel system and examined by a pulsed-induction release experiment. It was observed that a notable enhancement in the release rate of bupivacaine occurred immediately after each pulse of the external magnetic field, which was attributed to the presence of SPIONs. In another study, an attempt was made to reinforce the poor mechanical properties of a Pluronic hydrogel by the utilization of a post-gelation enzyme-catalyzed reaction that was known to be biocompatible [155]. With the chemical reaction, the critical gelation concentration of the dual gel was effectively reduced, in comparison to a Pluronic–tyramine hydrogel without enzymatic crosslinking. Additionally, after the chemical reaction took place, the temperature still affected the sol-to-gel transition ability of this system, which might be attributed to the partial formation of dimers. This enzymatic-Pluronic-tyramine system provided a highly sustained release of FITC-dextran and much more controlled erosion rate over 13 days compared with those systems without enzymatic treatment.

5. Conclusion and perspectives

Overall, this review has highlighted examples of injectable hydrogels for therapeutic agent delivery based on various cross-linking mechanisms. It should be noted that each class of physical, chemical, and dual gelling systems possesses its own merits and drawbacks, depending on the particular type of application. Over the past several decades, numerous attempts have been made to design

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and facilitate hydrogels with the properties desired for specific aims. Nevertheless, in our opinion, there still have been some common yet notable concerns warranting further investigation as well as persistent challenges to be overcome. First, the compatibility between hydrogel systems with fragile molecules or cells has to be carefully examined. Once encapsulated into the network structure of a hydrogel, cells should be kept healthy with maintained proliferation, whereas proteins, DNA, peptides or oligonucleotides need to be protected from denaturation to remain active. Second, cytotoxicity attributed to degraded products from the hydrogel as well as residues of unreacted components, catalysts or crosslinkers should be closely monitored. Moreover, inflammatory responses after administration need to be avoided by understanding the interaction between the hydrogel materials and the surrounding tissues. Third, the complexity or simplicity of the materials' chemical structures or formulations are also important issues. As hydrogel development continues to progress, more complicated systems are being developed for more specific applications. However, complexity might be related to poorly defined structure and the low reproducibility of properties and activities. Better materials will need to have simple structures yet high efficacy. Fourth, the requirements of different applications for the employed system should obviously be considered. These include the appropriate gelation rate, viscosity during the injection process, mechanical strength after gelation, degradation period and release profile of bioactive factors. Fifth, the distance and difference between concepts and reality should be shortened. That is, the *in vivo* therapeutic effect of drug/protein/gene delivery should be studied as much as possible to confirm the practical efficacy of each developed system for further clinical research. After all, bioactive factors can greatly benefit from controlled release systems, which can lead to the development of a wide array of injectable hydrogels, from the conventional classes to the more recent novel structures. These innovative systems include all types of hybrid hydrogels, composite systems of drug/protein-encapsulated nano/microparticles inside hydrogel matrices, gene/protein/organ tissue-based hydrogels, multi-stimuli-responsive short molecular hydrogels, biomolecules-recognition hydrogels, and programmed releasing systems of drug-hydrogel conjugates. We expect these novel designs to play important roles in the bright future of hydrogels in controlled delivery applications.

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