Chapter 6

In Situ-Forming Cross-linking Hydrogel Systems: Chemistry and Biomedical Applications

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Additional information is available at the end of the chapter

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Abstract

With the development of chemical synthetic strategies and available building blocks, *in situ*-forming hydrogels have attracted significant attention in the biomedical fields over the past decade. Due to their distinct properties of easy management and minimal invasiveness via simple aqueous injections at target sites, *in situ*-forming hydrogels have found a broad spectrum of biomedical applications including tissue engineering, drug delivery, gene delivery, 3D bioprinting, wound healing, antimicrobial research, and cancer research. The objective of this chapter is to provide a comprehensive review of updated research methods in chemical synthesis of *in situ*-forming cross-linking hydrogel systems and their diverse applications in the biomedical fields. This chapter concludes with perspectives on the future development of *in situ*-forming hydrogels to facilitate this multidisciplinary field.

Keywords: chemical cross-linking, free radical polymerization, *in situ*-forming hydrogel, biomedical applications, hydrophilic polymers

1. Introduction

Hydrogels are a class of three-dimensional (3D) cross-linked polymeric structures capable of holding large amounts of water or biological fluids in their swollen state [1]. The first report of water-swollen cross-linking polymer network of 2-hydroxylethyl methacrylate and ethylene glycol dimethacrylate for applications of contact lenses was published by Wichterle and Lim in 1960 [2]. Due to their high water content and structure similarity to natural extracellular matrix (ECM) as well as their biodegradability and low immunogenicity, hydrogels have gained considerable interest in biomedical and pharmaceutical fields over the

past few decades, especially with the development of a wide variety of chemical building blocks and various synthetic strategies in polymer chemistry, organic chemistry, and bioconjugation chemistry. Up to date, hydrogels have been applied in a broad spectrum of biomedical applications including tissue engineering, 3D bioprinting, drug delivery, gene delivery, would healing, antimicrobial research, and cancer research [3]. Among these applications, *in situ*-forming hydrogels have been intensively investigated because of their easy formulation to encapsulate bioactive ingredients and/or cells into hydrogel by mixing followed by a cross-linking process. They can be delivered into the desired sites through minimal invasive injection, which improves patient compliance and curative efficacy [4, 5]. They can also be used in surgery as tissue adhesive to seal tissue defects [6].

With regard to biomedical applications, *in situ*-forming hydrogels must first meet the basic requirement of biocompatibility to provide the appropriate macro- and microenvironment for cell proliferation and tissue growth. Therefore, it is of great importance to develop *in situ*-forming hydrogels with minimized immunological rejection through thoughtful selection of the materials and incorporation of physicochemical and biological cues to replicate the natural ECM. Porosity of the hydrogel should be considered when selecting the hydrogel materials as it affects the viability and proliferation of cells in 3D culture as well as the drug release profile for delivery. In addition, rigidity of the *in situ*-forming hydrogel is crucial due to its influence on cell differentiation [7] and its mechanical strength to support 3D constructs in bioprinting and tissue engineering.

There are two strategies to synthesize *in situ*-forming hydrogels. One strategy is the polymerization of small molecules in the presence of initiators and cross-linkers. The other strategy is to directly cross-link either natural or synthetic hydrophilic polymers [3]. In general, synthetic polymers are hydrophobic and mechanically stronger compared to natural polymers, which results in slow degradation but high durability in hydrogels. Another property of synthetic polymers is their inert cellular environment that prohibits active cell binding, which results in low cell viability. To compensate, bioactive compounds such as peptides or growth factors need to be incorporated into the hydrogel network [8]. Natural polymers, on the other hand, have the advantages of low toxicity and biodegradability but their mechanical properties are weaker. The opposite properties between synthetic and natural polymers need to be balanced through optimal design for specific hydrogel applications [9].

The most used natural polymers for *in situ* hydrogels are hyaluronic acid (HA), collagen, gelatin, alginate, chitosan, fibrin, etc. Some commonly used synthetic polymers are poly(ethylene glycol) (PEG), poly(acrylic acid) (PAA), poly(acrylamide) (PAM), poly(vinyl pyrrolidone) (PVP), poly(vinyl alcohol) (PVA), poly(lactic acid) (PLA), and poly(lactic-co-glycolic acid) (PLGA). Detailed information about each polymer will not be covered in this chapter. Interested readers may refer to review articles for more information [10–12].

2. Chemistry of *in situ*-forming cross-linking hydrogels

Since hydrogels are simply hydrophilic polymer networks cross-linked in some fashion to produce an elastic structure, any strategy that can produce a cross-linked network could be

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used for *in situ* hydrogel synthesis [9]. Hydrogels can be classified into different categories based on various parameters such as preparation method, ionic charge, and mechanical and structural characteristics [13]. Based on cross-linking mechanisms, hydrogels can be divided into physically cross-linked hydrogels and chemically cross-linked hydrogels. Physically cross-linked networks are often called "reversible" or "physical" gels, as they can be dissolved by changing environmental conditions such as pH, ionic strength, or temperature [3]. They possess temporary connections either through polymeric chain entanglement or physically induced gelation through stimuli such as ion-ion interaction, hydrogen bonding, thermo-induced gelation, complementary binding, inclusion complex formation, and hydrophobic interactions [9]. Physically cross-linked hydrogels are of great interest for encapsulation of bioactive substance and cells [14], although they are not covered in this chapter. Chemically cross-linked hydrogels are also called "permanent" or "chemical" gels which are networks cross-linked by covalent bonds through chemical reactions to achieve cross-linking of macromolecular chains in solution. In order for in-depth discussion, this chapter will focus on chemically cross-linked *in situ* hydrogels by reviewing various chemical cross-linking strategies to synthesize *in situ*-forming hydrogels and their updated biomedical applications.

To be suitable for biomedical applications, it is preferred that the cross-linking occurs in aqueous and mild conditions. The reactions should not damage the cells or biofunctional molecules in the hydrogel matrix. Reactions employed should not generate toxic side products and should not require high temperatures or heavy metal catalysts that are toxic to the cells [15].

Chemical cross-linking is the most common and highly efficient method for the formation of *in situ*-forming hydrogels that have excellent mechanical strength. This section will review the updated chemistry and various cross-linking mechanisms in hydrogel synthesis including free radical polymerization, reactions of complementary groups, and enzyme-catalyzed reactions.

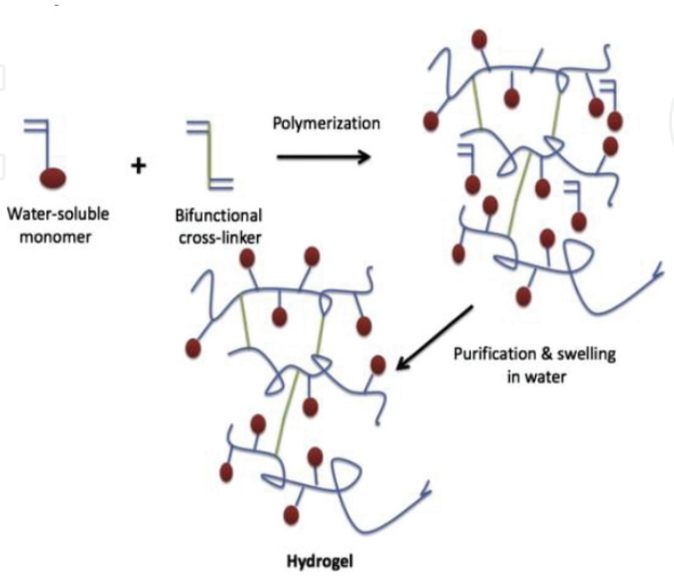

Figure 1. Hydrogels by 3D polymerization.

There are two commonly used strategies to prepare chemically cross-linked hydrogels which are illustrated in **Figures 1** and **2** [3]. The first strategy is called "3D polymerization," which is achieved by polymerization of hydrophilic molecules, such as acrylates and vinylic monomers, in the presence of multifunctional cross-linkers. The drawback of 3D polymerization is the significant amount of unreacted monomers and other small molecules, which could be toxic and have to be removed by extensive purification processes. The second strategy is to directly crosslink hydrophilic polymers so that extensive purification can be avoided as there are not as many toxic small molecules remained in the system. Water-soluble polymers such as PAA, PVA, PEG, PAM, and polysaccharides are commonly used systems for biomedical and pharmaceutical applications due to their nontoxicity and biocompatibility [3].

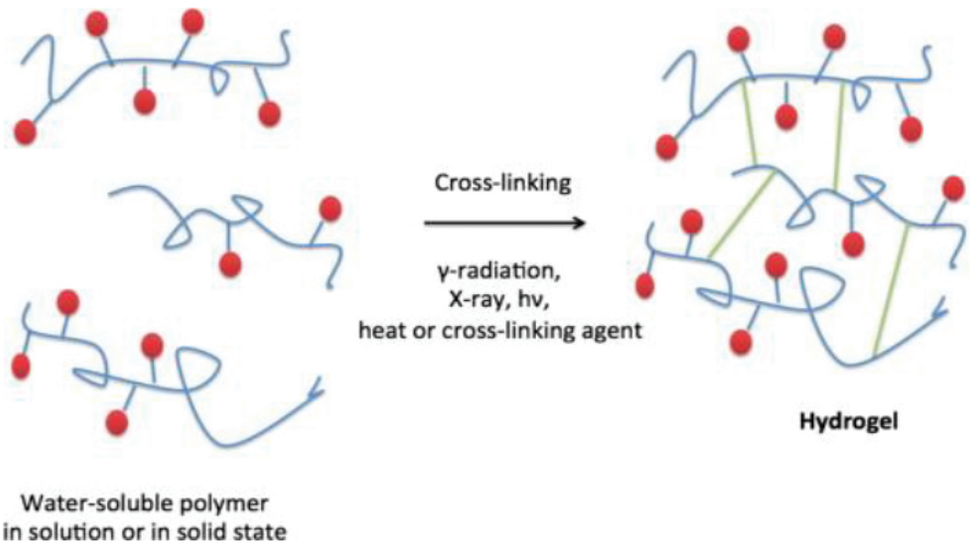

Figure 2. Hydrogels by cross-linking of biopolymers.

2.1. Cross-linking by free radical polymerization

Free radical polymerization is the most commonly used cross-linking strategy for hydrogel synthesis due to its advantages over other polymerization methods. First, it is highly reactive, which results in polymers of high molecular weights and cross-linking density. Second, free radical polymerization tolerates a variety of functional groups and it occurs in mild conditions, even in aqueous conditions. This makes it a facile approach for cross-linking hydrogel synthesis [16]. Free radical polymerization can be further classified as homopolymerization, copolymerization, and multipolymer interpenetrating polymeric hydrogels by hydrogel composition [9].

2.1.1. Hydrogels by homopolymerization

Homopolymeric hydrogels are hydrogel systems originated from polymerization of a single monomer species in the presence of an initiator and a cross-linker. For example, homopoly-

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merization of *N*-acryloylglycinamide through radical polymerization with 2,2′-azo-bisisobutyronitrile (AIBN) as an initiator and *N*,*N*'-methylene-bis-acrylamide (MBAm) as a chemical cross-linker yields a hydrogel [17]. Similarly, *N*-vinyl-2-pyrrolidone is homopolymerized in the presence of a free radical initiator AIBN and a cross-linker MBAm to produce a pH-responsive hydrogel for *in vitro* delivery of propranolol hydrochloride [18].

2.1.2. Hydrogels by copolymerization

Copolymerization hydrogels are synthesized by polymerization of two or more different monomers with at least one hydrophilic component. Depending on the structure of the polymer chain, copolymers are further classified into random, block, or alternating copolymers. A recent report described the synthesis of pH temperature dual stimuli-responsive smart hydrogel for drug release. In this research, PEG was reacted with methyl ether methacrylate to afford methacrylate terminated PEG, which copolymerized with *N*,*N*'-dimethylaminoethyl methacrylate. The copolymer solution is mixed with α-cyclodextrin (α-CD) to form a pH-thermo dual sensitive hydrogel allowing the release of a model drug 5-fluorouracil to be effectively controlled by temperature and pH [19]. Copolymerization allows for the combination of properties from both ingredients with varied ratios.

2.1.3. Hydrogels of interpenetrating polymer networks

Interpenetrating polymer networks (IPNs) and semi-interpenetrating polymer networks (semi-IPNs) have emerged as innovative biomaterials for biomedical applications. IPNs are a family of hydrogels that contain two independent hydrogel components with each component being a cross-linked hydrogel from synthetic and/or natural polymers. Semi-IPNs contain two independent hydrogels with one being cross-linked hydrogel and the other noncross-linked hydrogel [9]. The purpose of two hydrogel components is to provide tuned physical properties or stimuli responsiveness. For example, Reddy et al. fabricated cyclotriphosphazene-based IPN hydrogels through free radical polymerization of mono (methacryloyl-2-ethoxy)-pentakis(N^1,N^1-dimethylpropane-1,3-diamino)-cyclotriphosphazene and acrylamide, and cross-linked by MBAm in the presence of pectin. The synthesized semi-IPN hydrogels exhibited dual responsiveness for pH and temperature which triggered the delivery of 5-fluorouracil [20].

2.2. Cross-linking by chemical reaction of complementary groups

In situ-forming hydrogels can be prepared by direct cross-linking of natural or synthetic polymers through chemical reactions of complementary functional groups. In consideration of biomedical applications, chemical reactions employed in cross-linking hydrogels should be achievable in aqueous solutions without generating toxic by-products. The reaction should be very efficient with few reactants and active functional groups remaining. For that purpose, click chemistry, Michael additions, thiol-ene/yne coupling, Diels-Alder reaction, disulfide formation, Schiff-base formation, and epoxide reactions are examples of suitable reactions which will be discussed in detail. A summary of cross-linking reactions via complementary groups is depicted in **Table 1**.

Entry	Reaction	Complementary groups	Conditions	Ref.
1	Click reaction	Alkyne + azide	Cu(I), aqueous	[23–25]
		Alkyne + azide	aqueous, 37 °C	[26]
		Oxanorbornadiene + azide	aqueous, 37 °C	[22, 27]
		Cyclooctyne + azide	aqueous, 37 °C	[28–31]
2	Michael addition	Maleimide + thiol	aqueous, 37 °C	[15, 36]
		Vinyl sulfone + thiol	aqueous, 37 °C	[37]
		Acrylate + thiol	aqueous, 37 °C	
		Methacrylate + thiol	aqueous, 37 °C	
3	Thiol-ene/yne coupling	Alkene + thiol	aqueous, 37 °C	[38–43]
		Norbornene + thiol	Photo, radical catalyzed	
		Alkyne + thiol		
4	Diels-Alder reaction	Furan + maleimide	aqueous, 37 °C	[44, 45]
		Tetrazine + norbornene	aqueous, 37 °C	[46]
		Tetrazine + *trans*-cyclooctene	aqueous, 37 °C	[47, 48]
5	Disulfide formation/ exchange	Thiol-thiol	aqueous, H_2O_2	[49, 50]
		Pyridyl disulfide + thiol	aqueous, 37 °C	[52, 53]
6	Epoxide coupling	Epoxide + amine	aqueous, 37 °C	[54, 55]
		Epoxide + hydroxyl	acidic or basic	[56]
		Diepoxide + amine	aqueous, carbon black	[57]
7	Schiff-base formation	Aldehyde + amine	aqueous, 37 °C	[59]
		Aldehyde + hydrazide	aqueous, 37 °C	[61]
		Aldehyde + hydroxylamine	aqueous, 37 °C	[60, 61]
8	Condensation	Amine or hydroxyl + acid derv.	aqueous, 37 °C	[33]
		Amine or hydroxyl + isocyanate	DMSO, 35 °C	[62]
		Boronic acid + amine/hydroxyl	aqueous, pH 4.8	[65]
	Genipin coupling	Amines + genipin	0.1 M acetic acid, 4 °C	[63]
	Photo-induced crosslink	Alkenes	UV, photoinitiator	[64]
9	Staudinger-ligation	Azide + ester derivative of triphenylphosphine	aqueous, $BaCl_2$, 37 °C	[70]
	Tetrazole-photoclick	Alkene + tetrazole	aqueous, UV light	[71]
	Quadricyclane-ligation	Quadricyclane + Ni bis(dithiolene)	aqueous, pH 4.5	[72]

Table 1. Summary of cross-linking reactions of complementary groups.

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2.2.1. Click reactions

Click reactions refer to the cycloaddition of azide and alkyne to form a linkage through a triazole ring. It was proposed by Sharpless and co-workers in 2001 as copper-catalyzed azide-alkyne cycloaddition (CuAAC), aiming for efficient chemical synthesis with minimized byproducts and purification effort [21]. Over the past decade, click chemistry has gained significant application in a broad range of chemical synthesis of small molecules, polymers, dendrimers, biomacromolecules, and bioconjugation. Click chemistry has been widely used in cross-linking hydrogels due to its high selectivity and efficiency without generating by-products in aqueous conditions, plus the bioorthogonality of the components without interaction with the environment of biological or biomedical systems [22]. To date, click reactions of different versions have been developed not only to build materials that are biologically compatible, highly functional and organized in structure, but also to produce highly complex patterns of biofunctionalities within a single cellular scaffold [23]. For example, HA-based hydrogels were cross-linked by CuAAC reaction to produce a thermo-responsive hydrogel with tailorable mechanical properties [23, 24]. Kaga et al. fabricated "clickable" hydrogels using dendron polymer-based triblock polymers [25].

To avoid the toxicity of copper, metal-free click chemistry has been developed to eliminate heavy metal residue in hydrogel matrix. Truong et al. synthesized chitosan-PEG hydrogels by copper-free azide-alkyne click reaction [26]. Chitosan and HA-based hydrogel were cross-linked after functionalization by oxanorbornadiene and azide [22, 27] (**Table 1**, entry 1). Another good example of metal-free click reaction is strain-promoted azide-alkyne cycloaddition (SPAAC) that involves a difluorinated cyclooctyne moiety. Due to the ring strain and the electron-withdrawing difluoride, the alkyne functionality is greatly activated for a cycloaddition without a catalyst [28]. This reaction has been shown to be very efficient with high chemoselectivity even for *in vivo* applications, making it suitable for *in situ* cross-linking hydrogel [29, 30]. DeFrost et al. synthesized PEG-based hydrogel by cross-linking PEG-tetraazide and a difluorinated cyclooctyne functionalized cleavable peptide. The reaction occurred in aqueous conditions at 37 °C in the presence of cells, which enabled independent and *in situ* tuning of biochemical properties of biomaterials [31]. It is worth noticing that the cross-linking of azide and alkyne functionalized polymers with metal-free click reactions is normally too slow to be used in most *in situ* applications [29]. In order for this cross-linking strategy to be useful, the gelation kinetics needs to be improved by increasing the electron deficiency of alkynes and electron density of azide structures. Interested readers may refer to review articles for more information [32, 33].

2.2.2. Michael addition

Michael addition is a 1,4-addition of nucleophiles to α,β-unsaturated ketones or esters. It occurs in high efficiency under aqueous conditions without any side products, making it a suitable approach for cross-linking in hydrogel synthesis. The common nucleophiles are macromolecules that are functionalized with multiple terminal amine or thiol groups, which cross-link with electrophilic macromolecules functionalized with alkene groups with adjacent electron withdrawing groups, such as vinyl sulfone, acrylate, or methacrylate [15]. Surfactants can be

used to promote the kinetics of the Michael addition when the nucleophilic and the electrophilic macromolecules exhibit significant differences in hydrophilicity [34].

Like the cross-linking of alkyne and azide in SPAAC, the cross-linking kinetics of Michael addition depends greatly on the electron deficiency of the alkenes. Our research group has systematically studied the gelation kinetics and mechanical property of poly(amidoamine) (PAMAM) dendrimer-HA cross-linking hydrogels. PAMAM dendrimers are a family of synthetic polymers with well-defined structures and ample surface groups for conjugation of bioactive functionalities. They are widely used as a platform to deliver bioactive molecules into biological systems due to their water solubility, nontoxicity and nonimmunogenicity [35]. The combination of PAMAM and HA allows for easy chemical modification of dendrimer structures to modulate the physical and mechanical properties of the cross-linking hydrogel. In this research, PAMAM dendrimers are functionalized with maleimide, vinyl sulfone, acrylic, methacrylic, and a normal alkene group. When alkene functionalized PAMAM dendrimers are cross-linked with thiolated HA at experimental concentration, the gelation time displayed a large range from 8 seconds to 18 hours, and modulus from 36 to 183 Pa depending on the alkene group attached to the dendrimer. ^{1}H NMR study revealed that the gelation time is governed by the electron deficiency of alkenes [36]. Introduction of a RGD peptide in hydrogel greatly enhanced the cell attachment, viability, and proliferation of both bone marrow stem cells and human umbilical vein endothelial cells [37].

2.2.3. Thiol-ene/yne coupling

Thiol-ene reaction is a radical-mediated mechanism at room temperature and in aqueous conditions even in the presence of biological cargos such as proteins or cells, thus making it a good technique for hydrogel cross-linking. Unlike the traditional free radical polymerization, the radical thiol-ene reactions are relatively not oxygen sensitive [38]. The radicals that initiate the thiol-ene reaction can be generated using thermal, oxidation-reduction, or photochemical process based on initiator selection [39]. Fairbanks et al. devised photoinitiated thiol-ene reaction between four-armed PEG tetra-norbornene and dicysteine-terminated peptide to form *in situ*-forming hydrogels [40]. In another study, tetra-acetylene functionalized PEG and pentaerythritol are cross-linked in the presence of trimethylamine under moderate temperatures to form a robust hydrogel network [41]. Interested readers are referred to other recent articles for more information [42, 43].

2.2.4. Diels-Alder reaction

The Diels-Alder reaction is a robust cross-linking strategy for biopolymer-based hydrogels as it is rapid, efficient, versatile, and selective. It proceeds with high efficiency in aqueous conditions for hydrogel cross-linking or covalent immobilization of functional biomolecules. The most commonly employed functional groups for Diels-Alder cross-linking are furan and maleimide groups. As an example, furan and maleimide functionalized HA were cross-linked in 2-(*N*-morpholino)ethanesulfonic acid buffer at various volume ratios for delivery of dexamethasone which is an adipogenic factor [44]. Using the Diels-Alder reaction, Fisher et al. cross-linked furan-modified HA with bismaleimide enzyme-cleavable peptide cross-linkers

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to study MDA-MB-231 breast cancer invasion [45]. Recently, inverse electron demand Diels-Alder reaction has been employed in hydrogel cross-linking with tetrazine as the diene for cycloaddition with an alkene or alkyne (**Table 1**, entry 4). Desai et al. synthesized tetrazine and norbornene functionalized alginate to form cross-linking hydrogel using inverse electron demand Diels-Alder reaction without the external input of energy, cross-linkers, or catalyst [46]. Tetrazine and *trans*-cyclooctene are also a good pair of functional groups for inverse demand Diels-Alder reactions. Jung and others have employed tetrazine and *trans*-cyclooctene for protein conjugation using a tobacco mosaic virus template assembled with hydrogel microparticles for protein sensing applications [47]. Zhang et al. reported the interfacial bioorthogonal cross-linking of tetrazine modified HA with bis-*trans*-cyclooctene cross-linker to produce pattern biomaterials through diffusion-controlled gelation at the liquid-gel interface [48]. Similar to SPAAC, the cycloaddition between tetrazine and cyclooctene is also promoted by the strain in the *trans*-cyclic structure and the rate constant was determined to be $k_2 > 10^5\ M^{-1}\ S^{-1}$ [48].

2.2.5. Disulfide formation/exchange

Disulfide bonds are usually formed from oxidation of thiol groups. For example, thiolated HA can be chemically synthesized with varied degrees of thiolation and cross-linked through oxidation in the air or using hydrogen peroxide [49]. In another report, HA and gelatin were chemically modified using 3,3'-dithiobis(propionic hydrazide) followed by treatment of dithiothreitol. The thiol derivatives of HA and gelatin bearing thiol groups were mixed to form a disulfide cross-linking hydrogel in the presence of hydrogen peroxide. The hydrogel can be degraded by hyaluronidase [50]. Zhang et al. synthesized elastin-like polypeptide hydrogels for wound repair by disulfide bond cross-linking in the presence of ultraviolet (UV) light [51].

Macromolecules with pyridyl disulfide can react with thiol functionalized polymer through disulfide exchange, eliminating pyridine-2-thione as a by-product. Kannan et al. developed PAMAM dendrimer-PEG hydrogels for the sustained release of amoxicillin through disulfide exchange of a pyridyl disulfide functionalized PAMAM dendrimer generation 4 and an eight-armed thiolated PEG [52]. Similarly, an HA-based cleavable hydrogel was synthesized by cross-linking pyridyl disulfide functionalized HA with PEG dithiol [53]. The disulfide exchange reaction has been found to exhibit fast cross-linking kinetics and cytocompatibility as hydrogels can be synthesized in minutes under physiological pH in the presence of many cell types, with tunable rheological and physical properties. The limitation of the disulfide cross-linking approach is that the hydrogels may exhibit low stability by degradation especially in the presence of hyaluronidase or reducing agents such as glutathione [53].

2.2.6. Epoxide coupling

Water-soluble epoxides are highly reactive electrophiles that readily react with nucleophiles such as amines, alcohols, and even carboxylic acids, and the reactions are not oxygen sensitive. Due to the difference in nucleophilicity, the reaction rate is fast with amines and slow with alcohols. PEG diepoxide, 1,2,3,4-diexpoxbutane, and 1,4-butandiol diepoxide are commonly used epoxide sources [15]. HA was reported to react with diepoxide to form cross-

linking hydrogels under either basic or acidic conditions [54, 55]. However, epoxides may suffer from some degree of hydrolysis under basic conditions. Binetti and others cross-linked PVA with PEG diglycidylether (PEGDGE) through epoxide coupling to form PVA/PEG hydrogels for injectable nucleus replacement [56]. Calvert et al. made epoxy hydrogels as hydrogel sensors for glucose by epoxide coupling of PEGDGE and Jeffamine in aqueous conditions [57].

2.2.7. Schiff-base reaction

A Schiff base is usually achieved by reaction of amines, hydrazides, or hydroxylamines with aldehydes or ketones to form an imine, hydrazone, or oxime linkage. Schiff-base formation can occur in aqueous conditions without using extra chemicals or catalysts. It also displays controllable reaction rates depending on the pH. Therefore, it becomes a facile approach to produce *in situ*-forming hydrogels. The disadvantage of Schiff-base reaction is that aldehyde-containing compounds could affect bioactive factors or extracellular matrix molecules through reaction with amine groups [58]. In one study, aldehyde functionalized alginate was prepared by reaction of oxidized alginate and borax, followed by cross-linking with amine groups in gelatin. The hydrogel formation time ranged from a couple of seconds to less than one minute by varying the concentration of the components [59]. Similarly, aldehyde and oxyamine functionalized PEG was cross-linked through Schiff-base chemistry [60]. The hydrazone linkage is known to be labile and reversible due to hydrolysis, which may cause instability to hydrogels. A recent report demonstrated a hydrazone bond that was 15-fold more stabilized than regular hydrazone by fine tuning of the charge distribution over the hydrazone moiety, thus producing a stable hydrogel for tissue engineering [61]. The oxime linkage exhibits better hydrolytic stability than the hydrazone or imine linkage [58].

2.2.8. Cross-linking by other reactions

Other chemical reactions suitable for hydrogel cross-linking include condensation reactions that involve hydroxyl or amino groups reacting with carboxylic acid derivatives such as activated esters or isocyanates [33, 62], and genipin coupling through which nucleophiles such as amine or alcohol-containing polymers react with a natural cross-linker genipin [63]. Reactants that contain a photo-activated functional group can be cross-linked by photoirradiation [64]. The reaction between boronic acid and amine or hydroxyl group has been employed as a cross-linking approach to make pH responsive hydrogels [65].

2.2.9. Cleavable cross-linking

It is worth noticing that the stability of cross-linking is not always preferable in some applications when the encapsulated payload in hydrogel needs to be released at the target site. In that case, reversible cross-linking with labile linkages can be introduced so that the hydrogel is broken down in response to stimuli and the cargo can be released for function. The most common cleavable cross-links are cleavable to pH, photo, redox, enzyme, etc. Interested readers should refer to relevant articles for details [33, 66].

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2.2.10. Bioorthogonal chemistry

Despite the various cross-linking strategies for synthesis of different hydrogel networks and immobilization of functional cues within a cellular scaffold, techniques are needed to introduce different functionalities in specific locations and at different times to produce spatiotemporally complex and yet well-defined biochemical cues in hydrogels for specific applications or studies [67]. For that purpose, a strategy has been established to use two or more orthogonal reactions in a sequential fashion with the first reaction to form the cross-linking hydrogel and the second or later reactions to introduce biochemical functionalities. **Figure 3** illustrates how bioorthogonal strategy increases spatiotemporal diversity in hydrogel networks.

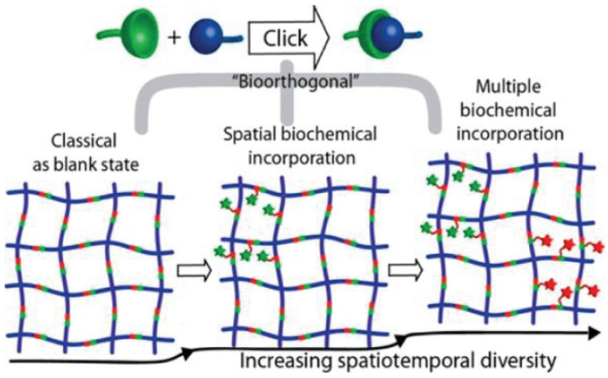

Figure 3. Bioorthogonal chemistry to increase spatiotemporal diversity in hydrogels [67].

The reactions employed in bioorthogonal chemistry must be nontoxic to cells. In addition, the reaction should be selective and the fidelity of the reaction should not be affected by other functionalities present. Among the cross-linking reactions for *in situ*-forming hydrogels, the azide-alkyne click chemistry, the SPAAC, thiol-ene reaction and thiol-based Michael addition are widely used to achieve orthogonality due to their virtues of simplicity, mild reaction conditions, availability of functional precursors, and versatility for spatiotemporal manipulation. For example, DeForest et al. demonstrated the bioorthogonal chemistry of a four-armed PEG-tetraazide and a degradable peptide which was double functionalized with dicyclooctyne and a pendant allyl moiety. The cross-linking occurred between the dicyclooctyne in peptide and the azide in PEG through SPAAC. The allyl moiety in the hydrogel matrix allowed for subsequent postgelation, photopatterning, or attachment of bioactive molecules such as RGD sequence at a different time and location through orthogonal photoinitiated thiol-ene reaction [68]. This hydrogel system created niches for 3T3 fibroblast cell culture and displayed a remarkable impact on dynamic cellular behaviors such as cell attachment, proliferation, and morphology in defined regions controlled by photoexposure. This system demonstrated the possibility to manipulate cell functions by spatially tuning the material properties through chemistry. In another study, a tough hydrogel was synthesized by two orthogonal cross-linking reactions that reinforce each other. First, a loose network was formed through an electron-demand Diels-Alder reaction of norbornene-functionalized chitosan and ditetrazine-functionalized PEG. Subsequent postfunctionalization through thiol-ene cross-

linking of a four-armed PEG-tetraalkyne and a linear PEG-dithiol resulted in a dense and biocompatible hydrogel network that exhibited high viability in 3D culture of human mesenchymal stem cells [69].

In spite of all the recent advancements, bioorthogonal strategy is still open for expansion in chemical synthesis of biomaterials for specific applications. Staudinger ligation, tetrazole photoclick reaction, and quadricyclane ligation are new orthogonal reactions that are potentially useful for synthesizing *in situ*-forming hydrogels with bioorthogonality [70–72] (**Table 1**, entry 9). In addition, reactions facilitated by thermal- or photochemical stimuli also hold potential as bioorthogonal reactions for hydrogels. Thiol-yne photoclick reactions are yet to be investigated for bioorthogonal chemistry in hydrogel [73]. Reactions of ultrasound-mediated reversible cycloadditions are also candidates for bioorthogonal chemistry [74].

2.3. Cross-linking by enzyme catalysis

Enzymes can be used to cleave or establish a chemical bond with greater efficiency than other methods. Due to their short reaction times and specificity, enzymes have been used for catalytic cross-linking to form hydrogels without interference with other chemical functional groups in macromolecules. Enzymatic cross-linking occurs under mild reaction conditions such as an aqueous environment, a neutral pH, or mild temperatures [58]. The most studied enzymes to prepare hydrogels are horseradish peroxidase, transglutaminase, tyrosinase, phosphopantetheinyl transferase, and lysyl oxidase [75]. Themolysin, galactosidase, and esterase have also been used to prepare *in situ* hydrogels through the enzymatic cross-linking reactions or decreased aqueous solubility of the polymer or the compound [15].

Overall, enzyme catalyzed reactions are a new approach in hydrogel formation. The reactions provide exceptional control over hydrogel formation, promoting higher complexity, noncytotoxicity and noninvasiveness. Despite the major advantages of enzymatic reactions, the challenges of this approach include instability of some of the enzymes and the insufficient mechanical properties of the gels formed. Interested readers may refer to more recent reports for a comprehensive review [76, 77].

3. Biomedical applications of *in situ*-forming cross-linking hydrogels

Hydrogels are suitable for biomedical applications due to their favorable properties such as biocompatibility, structural similarity to ECM, and biodegradability. Among the hydrogel family, *in situ*-forming hydrogels are specifically attractive as they can be injected by minimally invasive techniques and exhibit sol-to-gel phase transition under external physical or chemical cross-linking [78]. Among any possible applications, we reviewed the most promising applications of hydrogels, such as tissue engineering, 3D bioprinting, drug delivery, gene delivery, antimicrobial hydrogels, wound healing, and cancer research.

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3.1. Tissue engineering

The objective of tissue engineering is the fabrication of living parts for the body due to the tremendous need for organs and tissues [79]. Hydrogels have been widely used in tissue engineering because their properties are similar to those of natural ECM, which is essential for these purposes [48]. Many strategies employ material scaffolds to engineer tissues. These scaffolds serve as a synthetic ECM to provide a 3D architecture for cells and direct the growth and formation of desired tissues. Those scaffolds can be used for space filling agents, bioactive molecule delivery, and cell/tissue delivery.

Hydrogels made from natural polysaccharides are ideal scaffolds, as they resemble the native ECM of tissues which are comprised of various glycosaminoglycans. An injectable electroactive and antioxidant hydrogel based on a tetra-aniline functional copolymer and α-CD has exhibited excellent biodegradation, cell proliferation, and regeneration properties in tissue engineering [80]. *In situ*-forming metal-free chitosan/hyaluronan hydrogels also showed biocompatible and biodegradable properties and are good for soft tissue engineering. The hydrogel could support cell survival and proliferation of human adipose-derived stem cells (hADSCs) [27].

One major drawback of hydrogels is the lack of mechanical strength; hence, maintaining and improving the mechanical integrity of the processed scaffolds has become a key issue regarding 3D hydrogel structures [81]. Many researchers focus on the development of hydrogels using synthetic biomaterials that may have enhanced mechanical properties. Schuurman et al. prepared gelatin-methacrylamide (GelMA) hydrogels with tunable mechanical properties by manipulating the cross-linking parameters [82]. Hu et al. designed and synthesized a library of supramolecular hydrogels inspired by collagen. Those hydrogels exhibited promising potential for tissue engineering as they mimic properties of collagen [83]. Readers may refer to reviews of hydrogel scaffolds for tissue engineering [13, 76].

Lack of vascular networks in engineered tissues thicker than 200 μm puts a limitation on the nutrition of encapsulated cells. Therefore, advances in tissue engineering *in vitro* have generated a necessity for a parallel development and design of 3D vascular networks [84]. Current approaches in vascular network bioengineering mainly use natural hydrogels as embedding scaffolds that have drawbacks of poor mechanical stability and suboptimal durability. The search for improved hydrogels has become a priority in tissue engineering. Chen et al. developed a vascular network generated in photo-cross-linkable GelMA hydrogels, in which human blood-derived endothelial colony-forming cells and bone marrow mesenchymal stem cells generate extensive capillary-like networks *in vitro* [85].

3.2. 3D Bioprinting

3D bioprinting technologies enable the automated biofabrication of cell-laden constructs through the layer-by-layer deposition of biochemicals (termed as "bioinks") both *in vitro* and *in vivo*. Among the wide range of biofabrication techniques available to generate cellular constructs for tissue engineering, 3D bioprinting is currently one of the most fascinating because of its ability to print multiple biomaterials, cells, and bioinks in precise spatial locations

with high resolution and accuracy, which is important to the precise shape of the 3D structures as well as the self-development of cells in the structures [86, 87].

The full implementation of bioprinting strongly depends on the development of novel biomaterials exhibiting fast-cross-linking kinetics, appropriate printability, cell-compatibility, and biomechanical properties. Different biomaterials have been used in 3D bioprinting, such as alginate [88], HA, PEG [89], gelatin, collagen [90], and thermo-responsive biodegradable polyurethane [91]. Various cells have been printed on those materials, such as tumor cells, neural cells, and stem cells [88–91]. Hsieh et al. investigated two thermo-responsive water-based biodegradable polyurethane dispersions (PU1 and PU2) [91]. The stiffness of the hydrogel could be easily fine-tuned by the solid content of the dispersion. Neural stem cells (NSCs) were embedded into the polyurethane dispersions before gelation and showed excellent proliferation and differentiation in 25–30% PU2 hydrogels. Therefore, the newly developed 3D bioprinting technique involving NSCs embedded in the thermo-responsive biodegradable polyurethane ink offers new possibilities for future applications of 3D bioprinting in neural tissue engineering.

Photo-cross-linkable hydrogels are attractive materials for bioprinting as they provide fast polymerization under cell-compatible conditions and exceptional spatiotemporal control over the gelation process. Photo-cross-linkable GelMA and PEG dimethacrylate (PEGDA) were used as example hydrogels to demonstrate the feasibility and effectiveness of the approach. An array of GelMA/PEG hydrogels encapsulating human periodontal ligament stem cells (PDLSCs) with a gradient of material composition was printed, and the responses of human PDLSCs in the hydrogel array were investigated. The approach may be helpful for human PDLSCs-ECM screening and other cell-ECM systems. Please refer to the review for most recent developments on 3D bioprinting of photo-cross-linkable biodegradable hydrogels for tissue engineering [86].

3.3. Drug delivery

The use of hydrogels in drug delivery applications has been a significant subject in recent research due to the unique physical properties of hydrogels, the biodegradability, and the changes in gel structure according to environmental stimuli such as temperature, pH, and/or ionic strength. Their highly porous structure can easily be tuned by controlling the density of cross-links in the gel matrix and the affinity of the hydrogels for the aqueous environment in which they are formed. Their porosity also permits the loading of drugs into the gel matrix and subsequent drug release through the gel network [92]. The hydrogel systems have been developed for delivery of biomolecules ranging from small molecular drugs to large biomacromolecules such as nucleic acids, peptides, and proteins [93, 94]. Readers are directed to some recent reviews on drug delivery [95, 96].

There are several important areas in the field of hydrogels for drug delivery. First, development of *in situ*-forming hydrogel systems for drug delivery is an area of great interest. Fan et al. developed *in situ* injectable biocompatible poly(glutamic acid)-based hydrogels that are potential candidates in cell encapsulation and drug delivery [97].

http://dx.doi.org/10.5772/63954

Second, the development of strategies to increase the loading rate and capacity and to control the release of drugs from the hydrogel is an important area of interest. There are generally two ways to load drugs into hydrogels, e.g., soaking formed hydrogels in drug solution and forming hydrogels in the presence of drugs. Both ways have their limitations and often result in a modest amount of drugs loaded into hydrogels. Ray et al. prepared an IPN hydrogel based on PVA networking with PAA (PVA-co-PAA)/NaCl microspheres. The hydrogels were loaded with diltiazem hydrochloride (DL) and showed comparatively higher DL entrapment (79%), better control over DL release up to 24 h, and were more effective in reducing blood pressure to 40.1% [98]. Josef et al. investigated a composite gel system formulated from microemulsions (ME) embedded in alginate hydrogels. These hydrogels appeared to be a promising drug delivery system since they were capable of loading several hydrophobic compounds with a wide range of aqueous solubility and exhibited a release of 6–8 hours in water [99].

Third, there is a great interest in the development of stimuli-responsive hydrogels that are sensitive to temperature, pH, sugar, ionic strength, etc. These hydrogels are good candidates for controlled delivery systems that would release drugs to match a patient's physiological needs at the proper time and/or site [100]. Zhou et al. synthesized a series of pH-temperature dual stimuli-responsive copolymers [19]. Altering the temperature and the pH values of the environment could effectively control the release of the model drug. Li et al. synthesized pH and glucose dually responsive injectable hydrogels through the dynamic covalent imine bond and phenylboronate ester based on phenylboronic modified chitosan and oxidized dextran [101]. The rapid gelation and biocompatible cross-linking chemistry were appropriate for the incorporation of drug molecules and cells by *in situ* gel formation. Either decreasing the pH from physiological to mildly acidic or increasing the concentration of glucose around the gels could accelerate the rate of drug release from the gels. This hydrogel system potentially represented a versatile material platform for anticancer drug delivery.

3.4. Gene delivery

Gene delivery via hydrogels provides a fundamental tool for a variety of clinical applications including regenerative medicine, gene therapy for inherited disorders, and drug delivery [102]. Hydrogels serve the purpose of gene delivery by preserving activity of viral or nonviral vectors and shielding vectors from any host immune response. Hydrogels can also be injectable and environmentally responsive. Therefore, hydrogels hold great promise for gene delivery. There are two major areas that attracted great attention, vectors and biomaterial delivery system optimization.

Hydrogels used in gene delivery often need higher strength for extended use in order for internalization and transgene expression to occur. A hydrogen bonding strengthened hydrogel was prepared by radical copolymerization of PEG methacrylated β-CD and 2-vinyl-4,6-diamino-l,3,5-triazine monomer [103]. Kidd et al. investigated the delivery of lentiviral gene therapy vectors from fibrin hydrogels containing hydroxyapatite nanoparticles that can interact with both fibrin and the lentivirus [104]. The interaction of the hydroxyapatite with the fibrin may stabilize the hydrogels that will influence the rate of cell infiltration and vector release. The interaction of hydroxyapatite with lentiviral particles can enhance and localize

gene transfer within the hydrogel. These studies demonstrate the potential of fibrin hydrogels to serve as a material support for regenerative medicine and as a vehicle for the localized delivery of lentiviral vectors *in vivo*.

By choosing the proper vector and biomaterial system, gene delivery can be controlled for improved transgene expression. The interactions between scaffolds and vectors should be optimized so that vectors are adequately retained, but undergo dissociation, which enable vectors to interact with nearby cells and internalize [102]. The diffusion rate must be balanced to maintain spatial localization of gene transfer. The addition of interconnected macropores into the hydrogels can further increase the probability that infiltrating cells will internalize vectors and thereby improve transgene expression.

3.5. Wound healing

Wound healing is a complex process that implies equilibrium between inflammatory and vascular activity in the connective tissue and epithelial cells. The regenerative process needs the assistance of important elements to activate the natural processes of angiogenesis, activation of growth factors, and regeneration in a well-structured and biomimetic sequential process [105]. Hydrogel wound dressing has been widely researched because hydrogels promote wound healing by moisture retention to maintain a homeostatic environment. Ajji et al. prepared hydrogel wound dressings composed of PVP, PEG, and agar [106]. The gel fraction increased with increasing PVP, and decreased with increasing PEG. The hydrogel dressings could also be considered a good barrier against microbes. Reyes-Ortega et al. reported a new system based on the sequential release of two complementary bioactive components for application in the healing of compromised wounds [105]. The internal layer was a highly hydrophilic and biodegradable film loaded with the proangiogenic, anti-inflammatory, and antibacterial peptide, proadrenomedullin N-terminal 20 peptide for release. The more stable and less hydrophilic external layer was loaded with resorbable nanoparticles of bemiparin to promote the activation of growth factors and to provide a good biomechanical stability and controlled permeability of the bilayer dressing. This system demonstrated high efficacy of the early steps of the regenerative process in the wound site.

Traditional hydrogel dressings are inconvenient in applications as they need some degree of expertise and cause pain during changes. The thermo-sensitive hydrogels avoid the necessity of repeated and complicated application. Lee et al. investigated the ability of a thermo-sensitive hydrogel made of a triblock copolymer, PEG-PLGA-PEG, with TGF-β1 to treat the wound surface [107]. Results showed that the thermo-sensitive hydrogel provided excellent wound dressing activity and delivered plasmid TGF-β1 to promote wound healing in a diabetic mouse model. Hassan et al. developed a stem cell hydrogel system, in which the hADSCs were encapsulated *in situ* in the water-soluble, thermo-responsive hyperbranched PEG-based copolymer with multiple acrylate functional groups in combination with thiolated HA [108]. The hADSCs were successfully encapsulated *in situ* with high cell viability for up to 7 days in hydrogels and secrete proangiogenic growth factors with low cytotoxicity. This stem cell hydrogel system could be an ideal living dressing system for wound healing applications.

http://dx.doi.org/10.5772/63954

Supramolecular hydrogels are formed by noncovalent cross-linking of polymeric chains in water and can be developed specifically for biomedical applications [95]. Supramolecular hydrogels prepared by incorporating uranium chelating agents to eliminate uranium ions from the radionuclides contaminated wound sites of mice [109]. D-Glucosamine-based supramolecular hydrogels assist wound healing and prevent the formation of scars [110].

3.6. Antimicrobial hydrogels

The infectious diseases caused by pathogenic microorganisms such as bacteria, viruses, and parasites are still a public health problem despite the major development in health care and medical technology. Treatment with conventional antibiotics of infectious diseases often leads to the development of antibiotic resistance [111]. Recently, a new strategy to treat infectious diseases has been developed using antimicrobial hydrogels. The hydrogels act on the entire cellular membrane, which leads to cell membrane rupture, followed by a leakage of cytoplasmic contents and cell death. Different types of antimicrobial hydrogels have been developed in recent research.

Some hydrogels possess antimicrobial properties that include natural and synthetic polymeric hydrogels, and peptide-based hydrogels. Mohamed et al. prepared hydrogels by chitosan cross-linked with different amounts of pyromellitimide benzoyl thiourea moieties [112]. The hydrogels were extremely porous and exhibited a higher antibacterial activity and antifungal activity. The swelling ability of hydrogels and their antimicrobial activity increased with cross-linking density. Peng et al. developed novel cellulose-based hydrogels that showed superabsorbent property, high mechanical strength, good biocompatibility, and excellent antimicrobial efficacy against *Saccharomyces cerevisiae* [113]. The results showed possible use of these hydrogels for hygienic application. Synthetic polymeric hydrogels often have good mechanical properties and have been widely studied. Liu et al. developed a series of *in situ*-forming antimicrobial and antifouling hydrogels generated from cationic polycarbonate and four-armed PEG [114]. Peptide-based antimicrobial hydrogels with excellent inherent antibacterial activity have also been reported in recent years. Salick et al. designed a β-hairpin hydrogel scaffold based on the self-assembling of 20-residue peptide MAX1 that possessed intrinsic broad-spectrum antibacterial activity [115].

Despite the tremendous ability of antimicrobial hydrogels in breaking down multidrug resistant microbes, the interactions between antimicrobial polymers and microbial cell membranes are nonspecific which, in most cases, cause mammalian cell death above certain concentrations [111]. One solution is to combine antibiotics and antimicrobial hydrogels so that less antimicrobial hydrogel is used and the associated toxicity is minimized.

Another type of interesting hydrogels contains antimicrobial metal nanoparticles. The use of silver ions and silver nanoparticles in hydrogels has obtained substantial advances in wound treatment [111]. The silver nanoparticles supported within PVA/cellulose acetate/gelatin was successfully synthesized. The hydrogels have antimicrobial activity against various fungi and bacteria [116]. The toxicity of silver and other metal salts is a disadvantage for this type of hydrogels. Efforts have been made to reduce the toxicity.

3.7. Cancer research

Hydrogels have been used in cancer research among many other applications. However, many drugs are hydrophobic and cannot be efficiently loaded and released from hydrogels. There are two ways to improve the loading and releasing, incorporating hydrophobic domains into hydrogels and introducing nanoparticles which encapsulate hydrophobic compounds [99].

Recent trends have indicated significant and growing interest in developing nanocomposite hydrogels (NCH) for various biomedical applications. NCH are hydrated polymeric networks, cross-linked with each other and/or with nanostructures [117]. Some of the nonocarriers have been successfully incorporated in gel networks, such as carbon nanotubes, ME, dendrimers, metal, ceramic, and polymeric nanoparticles [99, 117]. The NCH has nanocarrier stabilization, shape regulation, improved composite viscoelasticity, and mechanical properties with optimized drug release kinetics on top of the conventional hydrogel characteristics [118]. Abdel-Bar et al. developed a cisplatin ME hydrogel for controlled cisplatin release and improved cytotoxicity with decreased side effects [119]. The NCH containing nanosized carriers allowed a zero order drug release for 14 days and enhanced cytotoxicity. The higher animal survival rate and lower tissue toxicities proved the decreased toxicity of cisplatin nanocomposite compared to its solution. This system could help in achieving better outcomes and quality of life during use of chemotherapy for cancer treatment by intraperitoneal administration.

Another great interest of hydrogels in cancer research focuses on the cancer cell invasion in hydrogels. Fisher et al. studied the HA-based cross-linked hydrogels in breast cancer cell invasion [45]. The results showed that increased crosslink density correlates with decreased breast cancer cell invasion whereas incorporation of enzyme-cleavable sequences within the peptide cross-linker enhances invasion. This study provides a platform that recapitulates variable tissue properties and elucidates the role of the microenvironment in cancer cell invasion by independently tuning the mechanical and chemical environment of ECM mimetic hydrogels. Zhang et al. created covalently cross-linked hydrogel materials through a rapid reaction at the gel-liquid interface [48]. The interfacial cross-linking was then used to encapsulate prostate cancer cells. The cells obtained 99% viability, proliferated readily, and formed aggregated clusters. Such *in vitro* models are critically needed for drug testing and discovery.

4. Conclusions and perspectives

In this chapter, we have discussed recent progress in polymerization, various strategies for cross-linking of natural and synthetic biopolymers for preparation of *in situ*-forming hydrogels, and updated applications in biomedical fields. There are a number of points that should be emphasized for future direction of design and synthesis of *in situ*-forming hydrogels for biomedical applications.

From a chemistry point of view, the reactions selected should occur in an aqueous environment under mild reaction conditions without damaging the encapsulated biofunctional

http://dx.doi.org/10.5772/63954

molecules and cells. The chemicals used for cross-linking such as monomers, initiators, cross-linkers, and catalysts should be carefully selected to minimize toxicity to cells. Attention should also be paid to biocompatibility between polymers and incorporated bioactive species such as cells and proteins. Introduction of reactive functional groups to hydrogel materials will surely promote the cross-linking of hydrogels, but the active groups may also show off-target reactivity to incorporated bioactive species or cells. For example, amino groups and thiol groups on proteins can react with vinyl sulfone and acrylate groups used in Michael addition, aldehyde groups used in Schiff-base formation, or diepoxide groups in coupling reaction for hydrogel cross-linking. These undesired reactions may damage proteins, reduce drug efficacy, or induce immunogenicity. Therefore, reactions are preferred to be highly efficient and selective for cross-linking so that all groups intended for cross-linking are reacted, but no undesired reactions occur between the hydrogel and incorporated biofunctionalities or cells.

Because of the widespread biomedical applications, research on *in situ*-forming hydrogels is increasingly intensive. However, the potential of hydrogels has not been fully explored yet. There are future challenges in each area of the applications. For example, one of the most important challenges in tissue engineering is how hydrogels can be used to stimulate the blood vessel network formation through angiogenic factors and endothelial cells in the desired tissue [120]. In addition, many tissues such as bone and muscle require high mechanical properties that most current hydrogels lack. The mechanical properties of hydrogels originate from the intrinsic rigidity of the polymers and the cross-linking density. Therefore, higher mechanical properties may be achieved by increasing the component of synthetic polymers in hydrogel. Orthogonal cross-linking reactions could be employed to enhance mechanical properties by increasing cross-linking density. Other strategies to develop strong hydrogels include double networks for which two polymeric networks with contrasting properties are combined to achieve much higher mechanical properties than the two independent networks, topological gels in which the long polymer chains are topologically interlocked by cross-linkers to achieve high tensility, and nanocomposite hydrogels in which the main hydrogel networks are incorporated with high-strength inorganic nanostructures [121]. In drug delivery, hydrogels will become a large portion of drug delivery systems in the future. Hydrogel systems that administer drugs with a controlled release rate at the desired sites are to be investigated. Specifically, more research on hydrogels for delivery of therapeutic proteins and peptides is expected [3].

In general, a deeper understanding of material properties for the development of *in situ*-forming hydrogels that replicate the complex nature of tissue will facilitate these efforts. To meet these goals, both physicochemical and biological cues should be applied with spatiotemporal control in hydrogel. Novel hydrogel materials such as stimuli-responsive smart hydrogels, hydrogels prepared with bioorthogonality, enzyme-mediated cross-linking, or a combination of diverse chemistries should result in hydrogels that are precisely controllable, diverse, and biomimetic in order to perform specific requirements of biomedical applications in the future.

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