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Chemically-modified biopolymers for the formation of biomedical hydrogels

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Abstract

Biopolymers are natural polymers sourced from plants and animals, which include a variety of polysaccharides and polypeptides. The inclusion of biopolymers into biomedical hydrogels is of great interest due to their inherent biochemical and biophysical properties, such as cellular adhesion, degradation, and viscoelasticity. The objective of this review is to provide a detailed overview of the design and development of biopolymer hydrogels for biomedical applications, with an emphasis on biopolymer chemical modifications and crosslinking methods. First, the fundamentals of biopolymers and chemical conjugation methods to introduce crosslinking groups are described. Crosslinking methods to form biopolymer networks are then discussed in detail, including i) covalent crosslinking (e.g., free radical chain polymerization, click crosslinking, crosslinking due to oxidation of phenolic groups), ii) dynamic covalent crosslinking (e.g., Schiff base formation, disulfide formation, reversible Diels-Alder reactions), and iii) physical crosslinking (e.g., guest-host interactions, hydrogen bonding, metal-ligand coordination, grafted biopolymers). Finally, recent advances in the use of chemically-modified biopolymer hydrogels for the biofabrication of tissue scaffolds, therapeutic delivery, tissue adhesives and sealants, as well as the formation of interpenetrating network biopolymer hydrogels, are highlighted.

Graphical Abstract

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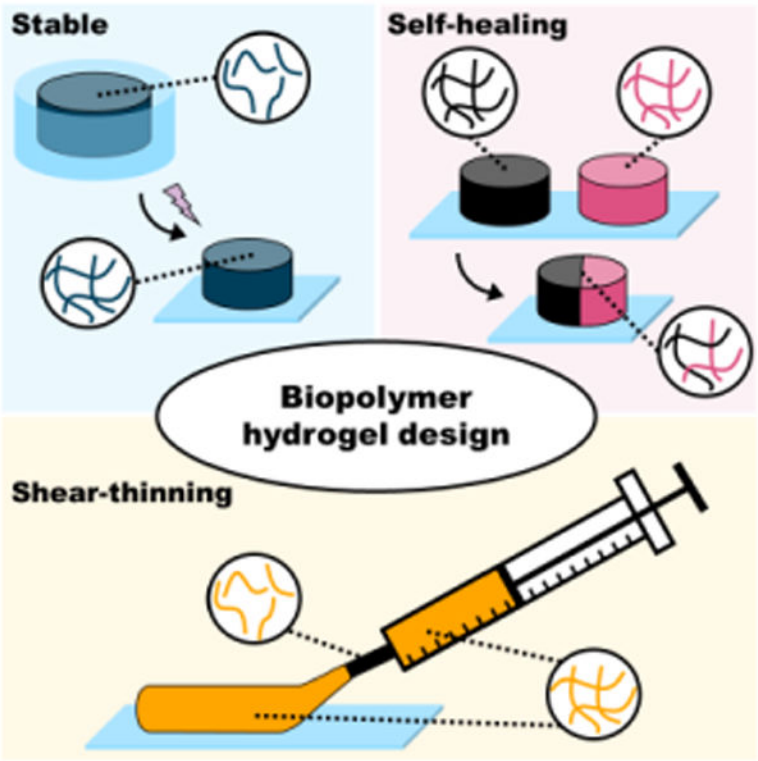

1. Introduction

Hydrogels are water-swollen polymer networks that have great utility for biomedical applications.[1] Hydrogels can mimic features of many tissues, and there have been great advances in the tailoring of hydrogel properties (e.g., mechanics, degradation) for widespread biomedical applications. Many important advances have been made with the use of synthetic polymers to construct hydrogels due to precise control over chemical structures, low batch variability, and ease of sourcing.[2] However, recent trends have included the fabrication of hydrogels from biological molecules, such as biopolymers, to introduce specific inherent biofunctionality to hydrogels.[3]

Biopolymers are natural polymers that are sourced from animals and plants, including a wide range of polysaccharides (e.g., sugars) and polypeptides (e.g., proteins). Representative examples of polysaccharides include hyaluronic acid, chondroitin sulfate, heparin, dextran, alginate, cellulose, chitin, and chitosan. Representative examples of polypeptides include gelatin, silk fibroin, albumin, elastin, keratin, and unique polypeptides engineered for specific functionality. The selection of specific polysaccharides or polypeptides introduces inherent properties to hydrogels, such as cell adhesion and degradability.

It is often necessary to chemically modify biopolymers to facilitate hydrogel formation. These modifications are performed on various chemical groups within the biopolymer repeat units (e.g., amines, hydroxyl groups, carboxylic acids) to allow for diverse methods of crosslinking (e.g., mixing, light, redox, thermal). The mechanical properties of formed

hydrogels are generally driven by the extent of biopolymer modification, the degree of crosslinking, the biopolymer concentration, and the type of crosslinking chemistry used. If a very stable hydrogel is desired, chemical groups that permit covalent crosslinking (e.g., free radical chain polymerization, click reactions) are often used (Figure 1). However, dynamic covalent crosslinking (e.g., Schiff base, disulfides) can also be implemented to combine hydrogel stability with features such as self-healing behavior (Figure 1). If a less-stable hydrogel is desired, physical crosslinking (e.g., hydrogen bonding, metal-ligand coordination) is typically used, which exhibits properties such as shear-thinning and disassembly over time (Figure 1). Various biopolymer networks can also be combined (e.g., interpenetrating networks) to further vary hydrogel properties to match the needs of specific applications.

The overall objective of this review is to provide the reader with an introduction to the use of biopolymers for the formation of biomedical hydrogels, with an emphasis on chemical modifications that facilitate hydrogel formation and control over hydrogel properties. There is great diversity in the modifications and resulting hydrogels properties, which is a strength to the use of biopolymers in hydrogel formation. Furthermore, specific examples of where biopolymer-based hydrogels are being used in biomedical applications of tissue engineering, biofabrication, and drug delivery are introduced, particularly where the use of a biopolymer and chemical modification was important to the hydrogel function.

2. Overview of biopolymers

Biopolymers are natural polymers that are derived from animals and plants. Biopolymers used for hydrogel formation generally fall into two classes of molecules: polysaccharides and polypeptides. Their repeat units consist of sugars or peptides, which guide the various biopolymer properties. Biopolymers inherently incorporate features that may be attractive in their use as biomaterials, including chemical compositions for cell interactions and degradation. Biopolymer hydrogels can be formed by polymer entanglement due to high molecular weight or high polymer concentration, by assembly (e.g., charge) due to the specific functionality of certain biopolymers, or by inter-polymer crosslinking due to chemical modifications of the biopolymer. In this section, we discuss the various biopolymers that are often chemically modified for hydrogel formation in the biomaterials field, including their general properties (e.g., molecular weight, adhesion to cells, degradability) and past use in commercial products.

2.1. Polysaccharides

Polysaccharides consist of monosaccharide or disaccharide repeat units and have important structural and biological functionality in living organisms (Table 1). For biomaterial applications, polysaccharides are often isolated from renewable sources such as plants and microorganisms. They may also be sourced from animal byproducts in the meat and fish industries. Many polysaccharides have been chemically modified to obtain natural hydrogels with a wide range of mechanical and biological properties.

2.1.1. Hyaluronic acid—Hyaluronic acid (HA) is a linear glycosaminoglycan (GAG) consisting of alternating D-glucuronic acid and *N*-acetyl-D-glucosamine repeat units that

are linked together by alternating β-1,4 and β-1,3 glycosidic bonds.[4,5] HA is a native component of the extracellular matrix (ECM) and is found throughout multiple tissues in the body, including cartilage, muscle, skin, and vocal folds.[6,7] In its naturally occurring state, the size of HA can range from ~100 kDa in serum to ~8000 kDa in vitreous fluid.[8] Through its structure and chemical properties, HA influences the mechanical and biological functionality of native tissues, as well as cellular responses in wound healing.[9] HA is very hydrophilic, and one of its major roles is the maintenance of viscoelasticity and low-friction tissue interfaces, such as in synovial and vitreous fluids.[5,10] Cells bind to HA through surface receptors such as the glycoprotein CD44.[11] HA-CD44 binding interactions are very important in many cellular processes, such as chondrocyte proliferation and matrix production in cartilage tissue.[12] HA can be degraded by oxidative species or enzymes such as hyaluronidase, glucuronidase, and hexosaminidase.[5]

Since the 1960s, HA has been utilized for many clinical applications including dermal fillers for soft-tissue augmentation,[13–15] wound dressings,[16] and intra-articular injections to manage symptoms of osteoarthritis.[17] For research and clinical use, HA is either derived from streptococcal fermentation cultures or from animal sources, such as rooster combs.[18,19] Unmodified HA can be crosslinked with 1,4-butanediol diglycidal ether (BDDE) or divinyl sulfone (DVS), either loosely to increase HA solution viscosity or more extensively to increase the mechanical integrity of HA hydrogels.[13] To increase the diversity in properties of HA hydrogels for biomedical applications, a range of chemical modifications have been introduced to HA.[4,20]

2.1.2. Chondroitin sulfate—Chondroitin sulfate (CS) is a linear sulfated GAG consisting of ~40–100 repeat units of alternating β-1,3-linked-*N*-acetyl-galactosamine and β-1,4-linked-glucuronic acid sugar residues.[21] CS is the main GAG found in aggrecan, a proteoglycan (PG) consisting of a protein core with GAG side chains.[22] Aggrecan, and thus CS, plays an important role in cartilage mechanics by influencing tissue hydration, swelling, and lubrication.[23] CS and PGs in general also play an important role in injury and disease recovery in the central nervous system.[24] PGs formed with CS interact with ECM and cell adhesion molecules.[25] CS can be degraded by enzymes such as chondroitinase ABC.[26] Clinically, CS has been delivered orally for management of pain in knee and hip osteoarthritis, as CS stimulates PG synthesis in the joint space, as well as exhibits anti-inflammatory properties.[27,28] For use in biomedical applications, CS is isolated from animal sources including bovine trachea, chicken keel, shark fins, and pig nasal septa.[29]

2.1.3. Heparin—Heparin is a linear GAG consisting of repeat units of uronic acid and D-glucosamine that are linked together by β-1,4 glycosidic bonds.[30] Heparin is found on the cell surface and in the ECM, and is known to play essential roles in tissue development, angiogenesis, and anticoagulation.[31] Heparin and heparan sulfate (HS), a sulfated derivative of heparin polysaccharides, interact with proteins to form PG coatings around cells,[31] which enable cells to interact with many signaling molecules.[31] Heparin binds to many biomolecules such as growth factors, cytokines, and adhesion proteins, including fibronectin.[32] The molecular weight of naturally occurring heparin can range from 5 to 1000 kDa.[30] Endothelial cells and macrophages preferentially bind to higher molecular

weight heparin.[33] Enzymes such as heparinase can degrade heparin and HS, which is important in ECM maintenance and remodeling.[32]

Clinically, heparin is used as a blood thinner to prevent the formation of blood clots. Heparin is one of only a few clinically approved polysaccharide drugs, and it is one of the oldest drugs still in clinical use.[34] The World Health Organization (WHO) identifies heparin as one of the world's Essential Medicines.[35] For biomedical use, heparin is isolated from animal sources, most often porcine intestine.[34] Heparin is often classified as either unfractionated heparin (UFH) or purified low molecular weight heparin (LMWH). As an anticoagulant, clinical use has shifted from UFH to LMWH for increased efficacy, as higher molecular weight heparin can adhere to endothelial cells and macrophages, and impede their anticoagulant ability.[33] As a tissue engineering scaffold, higher molecular weight heparin may be of interest due to its increased affinity for endothelial cell adhesion.

2.1.4. Dextran—Dextran is a highly branched polysaccharide consisting of α-1,6-linked-glucose monomers and α-1,3 branching.[36] Dextran is a major component of bacterial ECM, allowing for surface adhesion and biofilm formation.[37] Dextran has been extensively researched in the dental field, as streptococci bacteria secrete dextran to form gelatinous plaques on teeth.[38] Dextran can vary from molecular weights of ~10–150 kDa and ~5–30% degree of branching, depending on the bacteria and purification process used.[36,39] Most dextran in commercial use is produced from *Leuconostoc mesenteroides* bacteria with ~5% degree of branching.[39] Dextran can be degraded enzymatically by dextranase.[40]

Due to the ease of manufacturing and its biocompatibility, dextran has been widely used in many industries. Clinically, dextran is used as an antithrombotic agent to decrease vascular thrombosis by binding to erythrocytes, platelets, and vascular endothelium to reduce aggregation and make clots more easy to lyse.[36] Dextran is also used as a lubricant in eye drops and as an additive in intravenous fluids to solubilize other factors.[36] The clinical grades of dextran most often used include Dex-40 (40 kDa MW) and Dex-70 (70 kDa MW). The WHO includes Dex-70 on its List of Essential Medicines.[35] In addition to its uses in medicine, dextran is extensively used in food and cosmetic products, as well as in waste water treatment processes.[36] Due to the widespread availability and history of success in clinical use, dextran is a promising material for tissue engineering.

2.1.5. Alginate—Alginate is a linear polysaccharide consisting of repeat units of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues.[41] Alginate is found in the cell walls of brown algae (*Phaeophysceae*), providing a flexible mechanical structure to protect seaweed from damage due to strong water motion.[42] Alginate rapidly crosslinks in the presence of divalent cations (e.g., Ca^{2+}) due to ionic interactions with G residues. This ionic crosslinking mechanism has been used as a method to encapsulate biomolecules and cells for decades. For biomedical applications, alginate is frequently explored due to its biocompatible ionic gelation mechanisms, as well as its low cost and low toxicity.[43]

Purified alginate can be derived from brown algae cell walls as well as some bacterial strains, though commercially available alginate is derived exclusively from algal sources.[44] Alginate is available across a range of molecular weights from ~30–400 kDa.[43] Alginate has

been used in many products for clinical applications, including reduction of gastrointestinal reflux, accelerated wound healing, and defect filling in musculoskeletal tissues.[44] In addition to biomedical applications, alginate has been extensively used in the food industry as a thickening and gelation additive, colloid stabilizer, and sausage casing material.[45]

2.1.6. Cellulose—Cellulose is a linear polysaccharide consisting of repeating D-glucose units linked together by β-1,4 glycosidic bonds.[46] Cellulose is considered to be the most abundant organic polymer on Earth.[47] In plants, cellulose is a major component of the cell wall, where it forms strong microfibril crystal structures, leading to impressive mechanical properties.[48] Cellulose derived from plant sources is used extensively for the production of paper, lumber, and cotton textiles.[48] For biomedical applications, cellulose is typically derived from bacterial sources.[49] Some bacteria produce cellulose to form flocs and create a mechanically robust microenvironment.[48] Microbial-derived cellulose has been used in therapies for burns and ulcers, as well as in dental implants.[49] Cellulose is also commonly utilized as an emulsion stabilizer in cosmetic and food products.[49] Water-soluble derivatives of cellulose are manufactured by etherification reactions for use in food and medical industries.[50] The most common cellulose derivatives used in hydrogel formation are carboxymethyl cellulose (CMC) and hydroxypropyl cellulose (HPC).[50,51]

2.1.7. Chitin and Chitosan—Chitin is a linear polysaccharide with similar structure to cellulose, except the hydroxyl group is replaced with an acetamide group, resulting in *N*-acetyl-D-glucosamine repeat units.[52] Like cellulose, chitin monomers are linked by β-1,4 glycosidic bonds. The acetamide group allows for increased hydrogen bonding, resulting in increased strength in the chitin fibrillar matrix when compared to cellulose alone. Chitin is the primary component of exoskeletons of crustaceans and insects, and it is also found in fish scales, fungi cell walls, and cephalopod beaks.[53] Behind cellulose, chitin is the second most abundant natural biopolymer on Earth.[54] For biomedical applications, chitin is mostly sourced from shrimp and crab shells, which are waste products of the food industry.[55,56] In its native form, chitin is hydrophobic, which can be utilized to form hard materials for tissue engineering applications.[54] The electrical properties of chitin have also been explored for biomedical materials that benefit from electrical conductance.[54] For hydrogel formation, water-soluble derivatives of chitin are often used.

Chitosan is produced by either chemical or enzymatic deacetylation of chitin isolated from crustaceans.[57] It consists of glucosamine and *N*-acetyl-D-glucosamine repeat units.[58] The degradation rate and hydrophilicity of chitosan is influenced by the degree of deacetylation, which may range from 30–95%.[58] Lysozyme is the main enzyme that degrades chitosan in humans.[58] Clinically, chitosan has been used in chitosan-based hemostatic dressings,[59] as well as explored for use as a vaccine adjuvant.[60]

2.2. Polypeptides

Polypeptides are biopolymers consisting of amino acid repeat units, which are considered proteins when they consist of more than 50 amino acids (Table 2). Polypeptide- and protein-based hydrogels are of great interest in biomedical applications due to their potential to incorporate numerous cell interaction sites and to mimic native functions of the ECM.

Polypeptides may be isolated from human, animal, or plant sources, or synthetically engineered using recombinant protein production or peptide synthesizers. The precision and diversity in polypeptide materials are attractive for many biomedical applications.

2.2.1. Gelatin—Gelatin is a hydrolyzed and denatured form of collagen, which is the main structural protein in mammalian connective tissue ECM.[61] Collagen is the most abundant protein in mammals, making up 25–35% of the total protein content.[61] To produce gelatin, collagen proteins are extracted from the skin and bones of animal sources (most often porcine skin) by acid or alkaline treatments, followed by a thermal-driven process of protein separation.[62,63] Due to the heterogeneity in animal sources and gelatin isolation methods, the molecular weight of commercially available clinical-grade gelatin can range from ~10^3-10^6 Da.[64] Much of the triple-helix structure of native collagen is denatured in gelatin production; however, the chemical structure of gelatin remains similar to collagen.[63] Gelatin contains Gly-X-Y amino acid repeat sequences, where X is usually proline and Y is usually hydroxyproline.[65] Gelatin also contains the RGD sequence (Arg-Gly-Asp), which is a cell adhesion site and binds to integrins.[66] Adding gelatin (and thus RGD) to biomaterials has been shown to improve cell integration and tissue repair in many applications.[66] In the body, gelatin can be degraded by proteases such as collagenase and metallo-proteases.[67]

Gelatin has been widely used in many industries, including those related to food, photography, and pharmaceuticals. For example, in food science, gelatin is used as a stabilizer, thickener, texturizer, and emulsifier,[68] whereas in photography gelatin is used as a medium for making emulsions.[68] In clinical use, gelatin is a major ingredient in hard and soft capsules, as well as tablet preparation,[63,68] and gelatin sponges and particles have been widely used as hemostatic agents and to fill cartilage and bone defects.[69] Towards tissue engineering, a major advantage to using gelatin is that it has biological functionality (RGD sequence) and thus mimics native ECM functions.[70] Crosslinkers such as glutaraldehyde (GTA) and genipin can be used to directly form hydrogels with gelatin.[71] To improve mechanical performance and increase the range of possible mechanical properties, gelatin can also be chemically modified with functional groups to undergo hydrogel formation.

2.2.2. Silk fibroin—Silk is produced through a series of proteins found in the glands of some arthropods including silkworms, spiders, scorpions, and bees.[72] Silk is composed of two major proteins: silk fibroin, a semi-crystalline protein which provides structural stiffness and strength, and sericin, a glue-like protein that wraps around silk fibroin to hold fibers together.[72] In biomedical applications, silk fibroin is of interest due to its excellent mechanical strength, biodegradability, and widespread availability. Silk fibroin consists of semi-crystalline polypeptides that have a heavy chain (MW ~390 kDa) and a light chain (MW ~26 kDa) linked together via a single disulfide bond.[72] For clinical applications, silk fibroin is extracted from the *Bombyx mori* silkworm[73] and consists mainly of Gly (43%), Ala (30%), and Ser (12%) amino acids.[72] Silk fibroin contains hydrophobic domains that allow for the formation of stable anti-parallel β-sheet crystallites.[72]

Silk fibroin has been used in the textile industry for thousands of years.[74] Towards clinical applications, silk has been used as a suture material for centuries.[75,76] FDA-approved silk fibroin-based products include surgical meshes and ligament grafts.[76] It has been shown that

silk fibroin can be engineered for attachment and growth of human and animal cells.[77] *In vivo*, silk fibroin scaffolds can be degraded by enzymes and hydrolysis,[77] and the β-sheet crystalline content can be reduced in silk fibroin scaffolds to increase degradation rates.[78] Chemically-modified silk fibroin scaffolds are being widely explored for applications in tissue repair and regeneration.

2.2.3. Albumin—Albumin is an endogenous protein produced mainly by the liver and secreted into the blood plasma.[79] Human serum albumin (HSA) is the most abundant protein in blood plasma, accounting for 50–60% of total protein content.[80] The primary role of HSA is the regulation of fluid distribution by providing ~80% of the total blood plasma oncotic pressure.[81] HSA is a globular protein consisting of 585 amino acids with a molecular weight of 66 kDa.[81] As determined by X-ray crystallography, the tertiary structure of HSA is a heart-shaped protein that is stabilized by 17 disulfide bridges formed between amino acids.[82] Clinically, HSA has been used as a plasma expander for decades to restore and maintain circulating blood volume in response to trauma, surgery, and blood loss.[81,83] HSA can be isolated from human blood plasma by many methods, including plasma fractionation followed by liquid chromatography.[82] For biomedical research, albumin may be sourced from human blood plasma; however, bovine serum albumin (BSA) is also being widely explored as a cheaper and more abundant alternative.[82]

2.2.4. Elastin—Elastic fibers are an important ECM structural component and are responsible for the resilience and elasticity in many vertebrate connective tissues, including skin and cartilage.[84,85] Elastic fibers are composed of ~90% elastin protein and a complex microfibrillar structure made of numerous other macromolecules.[84] Elastin is composed of tropoelastin precursors that accumulate on the microfibrillar skeleton.[86] The half-life of human elastin is around 70 years, making it an extremely durable biopolymer with low turn-over in healthy tissue.[87] Elastic fibers can be degraded with disease or age due to proteolytic elastase enzymes.[88] Many cell types interact with elastin, including through elastin receptors and integrins.[84] Elastin is insoluble in water due to the presence of multiple hydrophobic domains; however, for hydrogel formation, water-soluble elastin-based materials have been explored.[84]

Various elastin formulations have been developed that include α-Elastin, a water-soluble elastin derivative that has been solubilized with oxalic acid,[89] and tropoelastin, which is water soluble at low temperatures.[90] Elastin-containing materials may be isolated from animal sources or human cadavers and processed into water-soluble derivatives for hydrogel formation.[91] Using synthetic protein engineering, Elastin-like polypeptides (ELPs) have also been engineered for biomedical applications.[92] ELPs contain the hydrophobic motif Val-Pro-Gly-X-Gly (VPGXG), where X is any amino acid except for Pro.[93] VPGXG is one of the main hydrophobic motifs present in natural elastin that contributes to its unique mechanical properties. Elastin-based biopolymers can be crosslinked without chemical modification using crosslinkers such as GTA, disuccinimidyl suberate, and disuccinimidyl glutarate.[89] Towards forming hydrogel scaffolds for tissue engineering applications, elastin-based materials are of particular interest due to their diverse biological and mechanical properties, which arise from the unique resilient behavior of elastin polypeptides.[84]

2.2.5. Keratin—Keratin is a fibrous protein rich in cysteine residues and is naturally found in hard integuments of animals, including in skin, hair, nails, wool, feathers, scales, and horns.[94,95] Keratinous tissues serve structural and protective functions in a variety of animals.[95] In humans, keratin is found in many epithelial tissues, including the epidermis and corneal epithelium, contributing to their role as a protective barrier.[96] Keratin contains multiple cell adhesion sites, including RGD.[97] The rich cysteine content allows for the formation of disulfide bonds, giving keratinous tissue strong and resilient mechanical properties.[94] Keratins are often classified as either α-keratins (forming α-helices) or β-keratins (forming β-sheets).[94] In the textile industry, keratin has been used as a raw material for centuries.[94] For biomedical purposes, keratin may be extracted from numerous sources, including human hair,[98] wool,[99] and feathers.[100] Keratin is of growing interest for use as a sustainable and cheap raw material in the biomedical field, as it can be easily sourced from the millions of tons of wool and feathers that are produced annually as by-products in livestock industries.[94] In its native state, keratin is insoluble in most solvents, including water.[101] Post-processing must be used to form water-soluble keratin for hydrogel formation, often involving the breaking of disulfide bonds with the addition of a reducing agent.[98] Such a process results in free thiol groups on keratins that can be used for crosslinking or further functionalization.[98,99]

2.2.6. Engineered polypeptides—Advances in recombinant protein production and peptide synthesis have allowed for the design of engineered polypeptides that can be fabricated into hydrogels.[102] Engineered polypeptides can be designed to mimic biological functions of naturally occurring peptides. For example, resilin-like polypeptides (RLPs) have been recombinantly engineered to fabricate hydrogel scaffolds that mimic the highly resilient mechanical properties of resilin protein found in arthropods.[103] Using RLPs instead of native resilin allows for control over incorporation of other bioactive motifs into the polypeptide, such as MMP-sensitive and cell-binding sites.[103] As another example, the engineered peptide poly(γ-propargyl-L-glutamate) (PPLG) has been used in combination with poly(ethylene glycol) (PEG) to form hydrogels.[104] PPLG introduces cell-adhesion sites as well as nanoscale stiffness due to PPLG's rod-like tertiary folding structure.[104] Furthermore, self-assembling peptide hydrogels have been designed that result in nanofibrillar structures due to β-sheet formation.[105] Other examples of hydrogels formed from engineered polypeptides include the use of novel pH-responsive engineered peptide amphiphiles for the formation of injectable nanofibrous scaffolds,[106] and the use of engineered PEG-peptide copolymers for the formation of "Shear-thinning Hydrogels for Injectable Encapsulation and Long-term Delivery" (SHIELD).[107] While engineered polypeptides may be designed for self-assembly, chemical modification of the engineered polypeptides may also be utilized for hydrogel formation, such as for crosslinking by azide-alkyne cycloaddition.[108,109] Ultimately, the engineering of polypeptides expands potential hydrogel components well beyond those that are found in natural tissues and structures.

3. Conjugation reactions to modify biopolymers

As stated above, although many biopolymers have inherent inter-molecular interactions that can be used to form hydrogels, chemical modification is often needed for hydrogel

formation or to improve upon formed hydrogel properties. Fortunately, biopolymers possess various chemical groups (e.g., hydroxyl, carboxyl, amine, thiol) available for modification through standard conjugation procedures. For example, all polysaccharides have hydroxyl groups (-OH), as do the amino acids serine and tyrosine, which often contribute to the hydrophilicity and hydrogen-bonding capabilities of biopolymers. The carboxyl group (-COOH) is found on two amino acids, aspartic acid and glutamic acid,[110] and in numerous polysaccharides such as HA, alginate, and CS. Amines (-NH_2 or -NR_2) are common on biopolymers such as chitosan and within the amino acid lysine as a component of proteins and polypeptides.[110] Lastly, thiols (-SH) are found in the amino acid cysteine, and the oxidation of thiols can lead to formation of a disulfide bond, which is commonly used in biopolymer hydrogel formation and fabrication.[110]

The most common conjugation reactions to chemically modify biopolymers include the formation of esters, amides, ethers, and carbamates, which involve hydroxyl, carboxyl, amine, and thiol groups on biopolymers (Figure 2). Ester formation is accomplished via the condensation of hydroxyl and carboxyl groups, usually in the presence of dehydrating reagents and appropriate catalysts. A common bioconjugation method is to use a carbodiimide such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) to activate a carboxylic acid group for esterification, in combination with a catalyst, such as 4-dimethylaminopyridine (DMAP). Esterification can also be achieved by combining a carboxylic acid with an epoxy, resulting in a hydroxyester. In bioconjugation, it is also common to combine acid anhydrides with functional hydroxyl groups on biopolymers to form esters. For example, methacrylic anhydride can be used to chemically modify biopolymers containing an aliphatic hydroxyl group with a methacrylate.[111] Di-tert-butyl decarbonate (i.e., Boc anhydride, Boc_2O) combined with DMAP can also be used to accomplish esterification.[112]

Amide formation can be achieved by condensing a carboxylic acid with an amine group. To accomplish this, the carboxylic acid group is usually first converted into an activated ester compound. Carbodiimides such as EDC activate the carboxylate group, and molecules like *N*-hydroxysuccinimide (NHS),[113,114] hydroxybenzotriazole (HOBt),[115,116] or benzotriazol-1-yloxytris (dimethylamino)phosphonium hexafluorophosphate (BOP)[117] form activated ester compounds. The activated esters then readily form stable amide bonds with amine functional groups present in the reaction. Ethers can be formed by combining epoxide and hydroxyl functional groups under basic conditions. For example, this method is used to conjugate glycidyl methacrylate (which has an epoxide functional group) to biopolymers containing free hydroxyl groups,[118–120] often in the presence of DMAP as a base. Lastly, compounds containing isocyanate functional groups can form carbamate bonds (also referred to as urethane bonds) with hydroxyl groups or can combine with thiols to form thiocarbamate bonds.[121,122]

There are numerous other examples of modifications directly to biopolymers for hydrogel formation. For example, Michael addition reactions can be used to chemically modify thiols on biopolymers, as well as to crosslink modified biopolymers for hydrogel formation (as described later in Section 4).[123] Specifically, under basic conditions, thiolated molecules (Michael donors) can be combined with electron-deficient unsaturated compounds (Michael

acceptors, such as maleimides, vinyl sulfones, acrylates, and acrylamides) via thiol-Michael addition, leading to the formation of a thioether bond.[124] Polysaccharides can also be modified using ring-opening oxidation, resulting in free aldehyde groups on the biopolymer backbone, which can change degradation rates or be used for crosslinking (e.g., Schiff base formation).[125,126] A common method to introduce aldehydes is to use sodium periodate as the oxidizing agent.[127] Lastly, biopolymers such as keratin that have multiple disulfide bridges can be exposed to a reducing agent such as dithiothreitol (DTT) or mercaptoethanol to functionalize with free thiol groups,[128] allowing for further biopolymer modification or crosslinking.

4. Covalent crosslinking

Hydrogels can be formed by the covalent crosslinking of functional groups attached to biopolymers. Covalent crosslinking mechanisms often require catalysts or initiators to induce covalent bond formation. Due to the general stability of covalent bonds, covalently crosslinked hydrogels have the potential to remain stable over long timescales both *in vitro* and *in vivo*, although this may be dependent on the ability of the network to undergo degradation. Both the mechanical and biological properties of the hydrogel formed are influenced by various components of the biopolymer and hydrogel design, such as the concentration of biopolymer, the type of crosslinking group introduced, and the degree of modification of the biopolymer. While there are many methods to form covalently crosslinked hydrogels, this review will focus on the most common approaches utilized in biopolymer hydrogel formation, including free radical chain polymerization, click chemistry, and oxidation of phenolic groups.

4.1. Crosslinking via free radical chain polymerization

Free radical chain polymerization consists of three steps: 1) initiation, 2) propagation, and 3) termination. During the initiation step, free radicals are generated from initiators, typically with changes in temperature, light, or redox conditions. During propagation, free radicals interact with unsaturated double bonds, and the free radical active center is transferred to propagate the kinetic chain, leading to crosslinking of the modified biopolymers (Figure 3a). The crosslinking reaction is terminated by either combination, disproportionation, or chain transfer events that stop the radical from propagating further. There are a wide range of functional groups that are used for the crosslinking of biopolymers in free radical chain polymerization (Figure 3a).

Photoinitiation is a common method to generate free radicals in hydrogel formation.[129] In this approach, a photoinitiator molecule cleaves in response to certain wavelengths of light, resulting in the generation of free radicals. Examples of water-soluble, biocompatible photoinitiators used in crosslinking include ultraviolet (UV) light-responsive molecules such as 2-hydroxy-4-(2-hydroxyethoxy)-2-methylpropiophenone (e.g., Irgacure, I2959)[120,130] and visible light-responsive molecules such as lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP).[131] Parameters such as light intensity and exposure time, as well as initiator and biopolymer concentrations, will affect the rates of polymerization and the resulting hydrogel properties. Oxidative-reductive (redox)

mechanisms may also be used to generate free radicals. One of the most used redox initiators in biomedical applications is ammonium persulfate (APS) in combination with tetramethylethylenediamine (TEMED). Since radicals are generated upon mixing, injectable, *in situ*-forming hydrogels with tunable mechanical properties are possible with redox systems.[132] Lastly, there are a number of thermal initiators in which a change in temperature is used to generate free radicals.[133] Free radical chain polymerizations form stable hydrogels, as the kinetic chains formed are non-degradable, although degradable groups (e.g., hydrolytically degradable, enzymatically degradable) can be incorporated into the network to tailor erosion behaviors.

4.1.1. Meth(acrylates) and methacrylamides—To functionalize biopolymers for free radical chain polymerization, methacrylate groups are often conjugated to biopolymers. This can be accomplished through various reactions, including esterification with methacrylic anhydride[134] and etherification with glycidyl methacrylate (GMA).[135] Dextran was first modified with methacrylate groups by etherification between GMA and hydroxyl groups on the dextran backbone, forming GMA-Dex.[118] GMA-Dex hydrogels were crosslinked *in situ* in the presence of APS/TEMED redox radical initiators.[118] Kim et al. synthesized methacrylated dextran by esterification with methacrylic anhydride under basic conditions.[136] It was demonstrated that model drugs such as fluorescently-labeled dextran and doxorubicin could be released in a sustained fashion from methacrylated dextran hydrogels crosslinked with UV light *in vitro*.[137] To introduce micro- and macro-porous structures, PEG has been mixed into methacrylated dextran hydrogels, and liquid-liquid phase separation created different morphologies and porosities.[119] In a similar approach, Ferreira et al. modified dextran with acrylate groups to form hydrogels containing tethered RGD and vascular endothelial growth factor (VEGF) encapsulated in poly(lactic-co-glycolic acid) (PLGA) microspheres for use as a scaffold for human embryonic stem cell vascular differentiation.[138]

HA modified with methacrylate moieties has been widely used in tissue engineering and drug delivery. Smeds et al. demonstrated that HA could be methacrylated (MeHA) using esterification with methacrylic anhydride and subsequently photocrosslinked to form HA hydrogels for sustained release of model drugs.[134] Stiffness, swelling ratio, and degradation rates can be varied in HA hydrogels formed from MeHA as a function of the degree of substitution, polymer concentration, and photoinitiation conditions.[111] In another chemical modification approach, Leach et al. showed that HA could be modified through etherification between hydroxyl groups on the polymer backbone and GMA (GMA-HA), and subsequently photocrosslinked using UV light.[120] Furthermore, BSA was released as a model drug in a sustained fashion from GMA-HA for several weeks.[139] Hydrogels from methacrylated HA have been used for a wide range of applications, including vocal fold tissue engineering,[140] controlled human embryonic stem cell differentiation,[141] and bioprinting.[142]

Numerous other polysaccharides have been modified with methacrylates for free radical chain polymerization, including cellulose,[143] alginate,[144] and CS.[145] By altering reaction conditions, such as reagent concentrations and temperature, the degree of substitution of methacrylate groups on cellulose could be controlled, which later influenced hydrogel

mechanical properties.[143] HPC has been modified with methacrylates through reaction with methacrylic anhydride[146] and processed with photolithography to create patterned hydrogel structures for diagnostics and tissue engineering applications.[147] Towards cartilage tissue engineering, methacrylated alginate, methacrylated HA, and methacrylated CS have been used for chondrocyte encapsulation and proliferation.[148–154]

Chitin has been modified with methacrylate groups via esterification between methacrylic anhydride and free hydroxyl groups on water-soluble carboxymethyl chitin, resulting in a photocrosslinkable hydrogel.[155] In another approach, chitin has been functionalized with methacrylate groups by carbamate bond formation between hydroxyl moieties on chitin and 2-isocyanatoethyl methacrylate.[156] The modification resulted in a photocrosslinkable chitin hydrogel that could be micropatterned for controlled guidance of cells.

Beyond methacrylates, reactive methacrylamide groups have been used for free radical chain polymerization of modified biopolymers. For example, HA was modified with methacrylamides using amidation reactions in the presence of EDC.[157] Park et al. showed that diacrylated PEG (PEGDA) could be incorporated into these methacrylamide-HA hydrogels to increase mechanical properties, and RGD could be tethered to the hydrogels to allow for cell adhesion and proliferation.[157] Gelatin has also been used extensively after modification for free radical chain polymerization, mostly commonly through esterification with methacrylic anhydride,[158] or by amidation with methacrylamide to form GelMA.[159] GelMA has been shown to be a useful material for photopatterned, cell-laden microtissues and microfluidic devices.[160] While GelMA has been a widely explored polypeptide for hydrogel formation, other polypeptides have also been explored. Kim et al. demonstrated that hydrolyzed silk fibroin could be methacrylated to form photocrosslinked hydrogels in the presence of UV light and LAP photoinitiator, where crosslinking is aided by β-sheet formation.[161]

4.1.2. Styrene—Although not used extensively, styrene moieties contain alkene groups that can be used for free radical chain polymerization. Styrene has been introduced to gelatin, HA, heparin, and albumin by either esterification with 4-vinylbenzoic acid or amidation with 4-vinylaniline.[162] Styrenated gelatin has been explored for cartilage tissue engineering as a hydrogel for chondrocyte delivery.[163] Furthermore, styrenated gelatin microspheres have been fabricated in a batch emulsion and subsequently explored for adipose tissue engineering.[164]

4.1.3. Degradable hydrogels from free radical chain polymerization—For some applications, hydrogels with high mechanical properties and low degradability may be preferred; however, more rapid hydrogel degradation may be desired for other applications. To introduce control over hydrogel degradation, hydrolytically degradable groups (e.g., esters) can be incorporated in between the biopolymer backbone and conjugated reactive groups. For example, hydroxyethyl methacrylate (HEMA) has been conjugated to many biopolymers, including dextran[130] and HA,[132] to modulate hydrogel degradation behavior. To increase hydrogel degradation rates, multiple lactic acid groups can be introduced.[135] Sahoo et al. demonstrated that this could be achieved with HA, and that ECM distribution increases when mesenchymal stromal cells (MSCs) were cultured in these hydrolytically

degradable hydrogels.[165] The incorporation of lactic acid can result in hydrogels that degrade too quickly, resulting in cell clustering and altered cell morphology.[166] To overcome this, caprolactone can be used instead as a hydrolytically degradable group with slower degradation rates. It has been shown that incorporating caprolactone groups between the HA backbone and methacrylate moieties allows for the tuning of hydrogel degradation rates to match the ECM deposition rates of MSC-laden hydrogels towards superior neocartilage formation *in vitro*.[166] Beyond hydrolysis as a method of degradation, Wade et al. introduced protease-degradable peptides between the HA backbone and methacrylates, so that formed hydrogels respond to proteases (Figure 3b).[167] These modified HA biopolymers were processed into degradable electrospun fibrous scaffolds, where degradation was dependent on the protease-sensitivity of the peptide sequence and the protease concentration.

4.2. Crosslinking via click chemistry

Click chemistry refers to a set of often biocompatible chemical reactions that result in the rapid formation of covalent bonds. Click chemistry reactions occur in a one-pot system, have a high thermodynamic driving force (greater than 20 kcal/mol), are not disturbed by water, have high specificity, and generate minimal byproducts.[168] Due to the biocompatibility, reliability, and specificity of click chemistry reactions, they are often used in biomedical applications such as drug discovery and biomaterials engineering.[169,170] Herein, we review some of the most common click chemistry reactions used for crosslinking of biopolymer hydrogels, including thiol-ene radical additions, thiol-ene Michael additions, azide-alkyne reactions, and tetrazine-norbornene cycloaddition.

4.2.1. Thiol-ene radical addition—Thiol-ene radical additions form a covalent thioether bond between an alkene and a thiol in the presence of a radical initiator. This click reaction is a powerful biomaterials tool due to its high yield, mild reaction conditions, regiospecificity and stereospecificity, and biorthogonality.[171] Radical initiators convert thiols into thiyl radicals, which subsequently form thioether bonds with electron-deficient or strained enes (e.g., norbornene) and can be used to form hydrogels (Figure 4a). Although methacrylates, acrylates, styrenes, and maleimides can undergo both thiol-ene step growth and radical chain growth homopolymerization, norbornenes and vinyl ethers only undergo thiol-ene step growth, which permits better control over hydrogel formation.[171] Thus, towards biopolymer hydrogel formation, the thiol-ene radical addition of thiols and norbornenes is most commonly used.

Norbornene is a bridged cyclic hydrocarbon with a strained carbon-carbon double bond. Many biopolymers have been functionalized with norbornene groups to undergo thiol-norbornene radical addition crosslinking, including HA,[112,117,131,172] alginate,[173,174] cellulose,[114,175,176] gelatin,[177–180] and silk fibroin.[181] Biopolymers can be functionalized with norbornene derivatives using esterification[112,177] or amidation[117,174] reactions. DTT,[112,181] multi-functional PEG dithiols,[178] and enzymatically degradable dithiols[114,173] have been explored as crosslinkers. While photoinitiator systems are commonly employed, redox-mediated radical initiators such as APS/TEMED can also be utilized for radical generation and thiol-norbornene crosslinking.[176] Biopolymer, crosslinker, and initiator concentrations can be used to tune hydrogel mechanical properties across orders of

magnitudes (~1–100kPa);[112,114] however, the range of hydrogel stiffnesses that can be achieved may be hindered by limited chain mobility as the reaction progresses, resulting in "maximum possible" stiffnesses despite increased polymer or crosslinker concentrations.[175]

Gramlich, et al. demonstrated that HA could be functionalized with norbornene (NorHA) using esterification between 5-norbornene-2-carboxylic acid and the secondary alcohol group on HA.[112] NorHA hydrogels could be spatiotemporally patterned by conjugating thiolated peptides to remaining free norbornenes using photomasks, demonstrating the ability to independently tune biochemical and mechanical properties.[112] Vega, et al. further demonstrated the photopatterning capabilities of NorHA (synthesized through BOP coupling of 5-norbornene-2-methalamine to carboxylic acid via amidation) by encapsulating MSCs in a NorHA hydrogel with photopatterned gradients of thiolated- RGD and other peptides created using a sliding opaque photomask.[117] In a single hydrogel, over 100 distinct biochemical formulations could be formed and screened for cartilage formation, demonstrating the promising application of thiol-norbornene radical addition in screening potential hydrogel formulations for tissue engineering.[117] NorHA can also be utilized with other scaffold biofabrication techniques, including bioprinting[131] and microgel formation.[172]

Other biopolymers have been modified with norbornenes for hydrogel formation. For example, norbornene-functionalized alginate hydrogels have been explored for many applications, including tissue engineered implantable constructs. Leuckgen et al. showed that norbornene-modified alginate hydrogels crosslinked with dithiolated enzymatically degradable crosslinkers allowed for cell and tissue infiltration *in vivo* after 8 weeks in a subcutaneous mouse study.[173] Furthermore, Ooi, et al. demonstrated that norbornene-functionalized alginate could be used as a cell-laden bioink for bioprinting of tissue engineering scaffolds.[174] CMC functionalized with norbornenes for thiol-ene radical addition crosslinking has also been explored for tissue engineering applications.[114,175,176,182] Ji et al. showed that CMC can be modified with norbornene groups using either amidation or esterification reactions.[182] Norbornene-modified CMC was combined with DTT to undergo thiol-ene photocrosslinking, and subsequently used as a cell-laden bioprinting ink (Figure 4b). Furthermore, Dadoo et al. showed that norbornene-modified CMC could be crosslinked with a thermally responsive dithiol-terminated poly(N-isopropyl acrylamide) crosslinker for spatiotemporal control over hydrogel swelling upon targeted temperature regulation.[114] In another strategy, cellulose nanofibrils could be functionalized with norbornene to allow for conjugation with different thiolated molecules to create nanofibril hydrogel suspensions with a wide range mechanical properties.[176] Lastly, Ryu, et al. demonstrated that silk fibroin modified with norbornenes could be combined with 4-arm PEG norbornene and DTT to create PEG hydrogels with embedded silk fibroin microgels, including with adenocarcinomic human alveolar basal epithelial cells.[181]

Gelatin can also be functionalized with norbornene groups using amidation between amines on the collagen backbone and carbic anhydride.[177–179] Munoz, et al. demonstrated that norbornene-modified gelatin hydrogels supported more rapid and extensive cell spreading of encapsulated human MSCs (hMSCs) when compared to GelMA.[177] This may be due to radical-mediated damage to proteins and cells due to kinetic chain growth in GelMA hydrogels, as well as the limited control over mesh size and molecular transport within

GelMA hydrogels.[177] Greene et al. demonstrated temporal control over crosslinking and thus mechanical properties of norbornene-modified gelatin hydrogels using intermittent light exposure, which was used to study hepatocellular carcinoma cell fate as a function of hydrogel matrix properties *in vitro*.[178] While functionalizing biopolymers with norbornene groups is a common approach, biopolymers can also be functionalized with thiols and subsequently crosslinked with multi-arm norbornene crosslinkers. Holmes, et al. functionalized amine groups on collagen with thiols using 2-iminothiolane in the presence of DTT as a reducing agent.[180] Thiolated collagen was then crosslinked with a multi-arm norbornene crosslinker for hydrogel formation. In another example, Yue et al. used thiol-modified keratin, along with a multi-arm PEG norbornene and Eosin Y photoinitiator, to create hydrogel constructs upon exposure to visible light.[98] The keratin hydrogels could encapsulate cells with high viability and exhibited tunable compressive moduli up to 45 kPa.

In addition to norbornene modification, biopolymers including gelatin,[183] chitosan,[184] and starch[185] have been modified with allyl groups through reaction with allyl glycidyl ether (AGE) or allyl chloride[186] for thiol-ene radical addition crosslinking. AGE-modified gelatin (Gel-AGE) hydrogels crosslinked with DTT have been explored for bioprinting tissue engineered scaffolds for the encapsulation of chondrocytes.[187] Kiliona et al. demonstrated that chitin nanocrystals and nanofibrils (nanochitin) could be functionalized with allyl groups by reaction with 10-undecenoyl chloride.[188] When mixed with thiolated PEG, allyl-modified nanochitin was used to form organogels in the presence of UV light and photoinitiators.[188] Hilderbrand et al. demonstrated that allyl-functionalized collagen mimetic peptides (CMPs) can be combined with thiolated PEG to fabricate a photocrosslinkable hydrogel for 3D cell culture.[189] In another approach, biopolymers can be modified with pentenoate to functionalize with ene groups in order to undergo thiol-ene radical addition. For example, Mergy et al. modified both dextran and HA with pentenoate groups via esterification with pentenoic anhydride to undergo thiol-ene photocrosslinking in the presence of thiol crosslinkers.[190] Further, pentenoate-modified gelatin and thiolated gelatin have been combined to form a photocrosslinkable hydrogel for cell encaulstion.[191]

4.2.2. Thiol-ene Michael addition—In Michael addition crosslinking, thiol-ene reactions can occur readily between thiols (Michael donors) and electron-deficient enes (Michael acceptors) without the need for radical initiators (Figure 5a).[171] Thiol-ene Michael addition reactions can be either base-catalyzed or nucleophile-catalyzed.[124] Common ene groups used for hydrogel crosslinking include maleimides, vinyl sulfones, acrylates, and methacrylates, in order of decreasing reactivity towards thiol-ene Michael addition (Figure 5b).[124] Variations in the ene group, the pH, and the biopolymer and crosslinker concentrations allow for tuning of gelation times from a few seconds to several hours. Biopolymers may be functionalized with ene or thiol groups for crosslinking using esterification or amidation reactions. Biopolymer hydrogels crosslinked using Michael addition reactions are commonly utilized as injectable, *in situ* forming hydrogels due to the ability to tune gelation kinetics to clinically relevant timescales.

Thiol-ene Michael addition reactions have been used for *in situ* crosslinking of injectable HA hydrogels for drug delivery and tissue engineering applications.[192–194] Hahn et al. showed that MeHA crosslinked through Michael addition with DTT could be used as an

injectable hydrogel for sustained release of erythropoietin.[192] Gelation time varied from 30 minutes to 3 hours, and erythropoietin could be released over a 7 day period in a rat *in vivo* model.[192] Vanderhooft et al. also demonstrated a wide range of gelation times (30 seconds – 2 hours) for hydrogels consisting of thiolated HA, thiolated gelatin, and various ene-functionalized PEG crosslinkers, including PEGDA and PEG-dimaleimide.[193] Furthermore, the storage modulus of thiolated HA hydrogels crosslinked with PEGDA by Michael addition ranged from tens to thousands of Pa.[195] Forgoing the need for small molecules or synthetic polymer crosslinkers, HA has been modified with methacrylates, acrylates, vinyl sulfones, and maleimides, and subsequently mixed with thiolated HA to crosslink via a Michael addition reaction, with gelation times ranging from instantaneous (maleimide) to ~45 minutes (methacrylate) as a function of the ene group present, pH, and polymer concentration.[196] Towards cardiac tissue engineering, MeHA and thiolated HA were selected as an *in situ* crosslinking, injectable hydrogel formulation for mechanical stabilization of myocardial tissue after infarction.[196] Michael addition crosslinking in HA hydrogels has been used for numerous applications, including cartilage tissue engineering[197,198] and neural tissue engineering.[199,200]

A range of chemical modifications have been applied to dextran hydrogels for thiol-ene Michael addition hydrogel formation. Injectable, *in situ* crosslinking hydrogels consisting of thiolated dextran and either acrylated PEG or vinyl-sulfonated dextran have been developed with a wide range of mechanical and degradation properties.[201] Degradation timescales can vary significantly (days to weeks) as a function of polymer concentration, Michael donors and acceptors selected, and spacing between conjugated functional groups and biopolymer backbone.[201,202] For spatiotemporal control over degradation, Peng et al. chemically modified dextran with an acrylate functional group that contained a photolyzable *o*-nitro-benzyl moiety between the acrylate and dextran backbone.[203] The hydrogel was crosslinked with a dithiolated PEG crosslinker using Michael addition, and subsequent exposure to UV light resulted in controlled degradation and release of model drugs.[203]

Many other biopolymers have been modified with thiols and/or ene functional groups for thiol-ene Michael addition crosslinking. For example, an injectable, thiolated chitosan hydrogel was developed by crosslinking with an acrylated PEG, resulting in tunable gelation times between ~10 seconds to 20 minutes.[204] Cell attachment and spreading was demonstrated *in vitro* upon addition of an RGD peptide.[204] Kim et al. showed that thiolated heparin could be mixed with PEGDA for Michael addition crosslinking to form a hydrogel useful for the encapsulation and *in vitro* culture of primary hepatocytes (Figure 5c).[205] Furthermore, thiolated gelatin hydrogels mixed with PEGDA are promising for the rapid encapsulation of MSCs for wound repair applications.[206] Xu et al. showed that, when applied to a full thickness wound rat model, these hydrogels supported accelerated wound closure, re-epithelialization, and vascularization.[206] In another example, Zhang et al showed that thiolated keratin can be mixed with 4-arm PEG-vinyl sulfone (PEG-VS) to undergo Michael Addition crosslinking.[99] The keratin hydrogel showed promise as a flexible strain sensor for future applications in wearable electronics.

4.2.3. Azide-alkyne [3+2] cycloaddition—Azide-alkyne [3+2] cycloaddition, also called Huisgen 1,3-dipolar cycloaddition, is a powerful click chemistry tool that is widely

used in bioconjugation to form strong covalent bonds in a one-pot reaction.[207] To perform the reaction under physiologically relevant conditions, the reaction is often catalyzed by Cu(I) or Cu(II).[208] Towards hydrogel formation, biopolymers can be modified with azide or alkyne moieties to undergo [3+2] cycloaddition crosslinking, potentially in the presence of copper catalysts (Figure 6a). Li et al. fabricated a thermo-responsive albumin hydrogel by conjugating propargyl maleimide to thiol groups on BSA cysteine residues.[209] The combination of alkyne-functionalized BSA with poly(N-isopropylacrylamide) (PNIPAAm) end-terminated with azide groups in the presence of Cu(II) catalysts yielded an azide-alkyne hydrogel.[209] Gelatin hydrogels formed via azide-alkyne reactions have been engineered by conjugating propolic acid to lysine residues to add azide functionality and crosslinking with either 4,4′-diazido-2,2′stilbenedisulfonic acid or 1,8-diazidooctane, both of which are di-functionalized with alkyne groups.[210] Upon exposure to Cu(II) catalysts, the gelatin hydrogels reached compressive moduli between 50 and 390 kPa.[210] Other biopolymers that have been crosslinked by copper-catalyzed azide-alkyne cycloaddition reactions include HA,[170,211,212] cellulose,[213] and alginate.[214]

Copper-catalyzed azide-alkyne cycloadditions allows for rapid gelation; however, copper catalysts are often cytotoxic, limiting the ability for copper-catalyzed reactions to be used in cellular systems.[215] To overcome this, strain-promoted [3+2] azide-alkyne cycloaddition (SPAAC) can be utilized by combining azides with strained cyclooctynes (Figure 6b).[215] Wang et al. engineered metal-free, azide-alkyne crosslinked, injectable dextran hydrogels by modifying dextran with either azadibenzocyclooctyne (ADIBO-Dex) or azides (Dex-N_3).[216] Upon mixing the two components, gelation occurred within 1 to 10 minutes, resulting in hydrogels with storage moduli between 2 and 6 kPa.[216] The hydrogels supported chondrocyte growth and cartilaginous tissue formation *in vitro*.[216] In another approach to fabricate metal-free azide-alkyne hydrogels, chitosan was functionalized with azides by modification with azidopentanoic acid and subsequently mixed with 3-arm PEG-propiolate, a multifunctional alkyne crosslinker.[217] The resulting hydrogel formed crosslinks within 5 to 60 minutes and reached compressive moduli between ~40 and 80 kPa.[217] Lastly, ELPs have been functionalized with either azide or bicyclononyne moieties to undergo SPAAC crosslinking (Figure 6c).[109] SPAAC-ELP hydrogels crosslinked within minutes.[108,109] These hydrogels were used to rapidly encapsulate hMSCs and murine neural progenitor cells *in vitro* with high viability and phenotypic maintenance.

4.2.4. Tetrazine-norbornene—Tetrazine-norbornene reactions are useful to rapidly form irreversible covalent bonds. Hydrogels can be crosslinked by tetrazine-norbornene mechanisms for the encapsulation of cells and therapeutics (Figure 7a).[218] Tetrazine-norbornene click chemistry offers similar advantages to metal-free azide-alkyne cycloaddition without the burden of the high cost of strained cyclooctyne groups.[218] Biopolymers can be functionalized with tetrazine and norbornene groups to engineer injectable hydrogels that undergo *in situ* crosslinking.[219,220] Desai et al. demonstrated that alginate could be functionalized with either benzylamino tetrazine or norbornene methylamine groups by EDC/NHS amidation.[221] Cytocompatible alginate hydrogels could then be formed via tetrazine-norbornene click chemistry, with gelation times of approximately a few minutes and storage moduli between ~0.5 and 3 kPa.[221,222]

Furthermore, Lueckgen et al. showed that tetrazine-norbornene biopolymer networks containing oxidized alginate allowed for cell infiltration in an *in vivo* subcutaneous injection mouse model.[222] HA has also been modified with tetrazine and norbornene moieties using EDC/NHS amidation mechanisms.[219] The resulting HA hydrogels were engineered as an injectable delivery vehicle for the sustained release of protein therapeutics.[219] Gelatin modified with tetrazine and norbornene groups has been explored for tissue engineered scaffold formation and as a delivery vehicle for contrast agents.[220,223] For example, Koshy et al. developed a tetrazine-norbornene crosslinked gelatin hydrogel that supported cell growth and matrix remodeling *in vitro,* as well as cell infiltration in an *in vivo* subcutaneous mouse model (Figure 7b).[223]

4.3. Crosslinking via oxidation of phenolic groups

Phenols are aromatic hydrocarbons that contain one or more hydroxyl groups. Oxidative environments lead to the generation of phenolate radicals that form covalently bonded phenol dimers.[224] This mechanism can be used to form crosslinks between biopolymers modified with phenolic moieties. Two of the most common approaches for hydrogel formation include the enzymatically driven crosslinking of tyramine and oxidation of catechol groups.

4.3.1. Tyramine—Tyramine is a naturally occurring amine derived from the tyrosine amino acid. In the presence of horse radish peroxidase (HRP) and hydrogen peroxide (H_2O_2), tyramine groups are converted into phenolate radicals that form either carbon-carbon bonds or di-tyramine adducts which can be used for hydrogel crosslinking. For example, radical photoinitiators can be combined with keratin, which contains tyrosine amino acids, to form a photocrosslinkable hydrogel upon exposure to UV light.[225]

Biopolymers can be modified with tyramine functional groups, usually by amidation between tyramine and carboxyl groups on biopolymer backbones, and subsequently covalently crosslinked by the addition of HRP and H_2O_2. (Figure 8a). Tyramine-based enzymatic crosslinking occurs rapidly within a few seconds to minutes, but can be tuned by varying concentrations of tyramine, HRP, and H_2O_2 in solution.[116,226] While enzymatic crosslinking of tyramine moieties is most common, it has also been shown that tyramine can be crosslinked using visible light and a photoinitiator.[227,228]

Using carbodiimide chemistry, tyramine groups can be conjugated to HA (HA-Tyr) by amidation with free carboxyl groups.[116,229] Simultaneous injection of two solutions, one containing HA-Tyr and H_2O_2 and one containing HRP, can be used for rapid gelation *in vivo* upon mixing.[116] It has been demonstrated that HA-Tyr hydrogels can be used for sustained delivery of proteins and anticancer therapeutics.[230,231] For example, Ueda, et al. showed that HA-Tyr hydrogels could be used to rapidly encapsulate interferon-alpha (IFN-α) for sustained release as an immunotherapy treatment (Figure 8b).[231] The HA-Tyr hydrogel was able to prolong the biological half-life of IFN-α and improve anticancer effects *in vivo* when evaluated in a human renal cell carcinoma xenograft mouse study.

Towards tissue engineering applications, Loebel et al. demonstrated the versatility of HA-Tyr hydrogels, comparing the influence of enzymatic crosslinking and visible light

photocrosslinking on encapsulated MSCs.[227] It was found that when HA-Tyr hydrogel stiffness was constant, enzymatically crosslinked biopolymer networks resulted in increased cell spreading and greater focal adhesion strength when compared to photocrosslinked hydrogels; however, photocrosslinked hydrogels resulted in increased cellular tractions.[227] This highlights the importance of considering how crosslinking methods influence cell behavior. Furthermore, it has been shown that additional biopolymers, such as silk fibroin[232] and tyramine-modified CS,[233] can be added to enzymatically crosslinked HA-Tyr hydrogels to create multifunctional hydrogels for tissue engineering.

Using amidation, alginate can also be modified with tyramine groups (Alg-Tyr) for enzymatic crosslinking.[234] It has been shown that enzymatically crosslinked Alg-Tyr hydrogels can retain their ability to undergo additional ionic crosslinking upon exposure to calcium (Ca^{2+}).[234,235] Furthermore, enzymatic crosslinking of Alg-Tyr hydrogels results in more stable hydrogels, overcoming the potential dissolution that occurs with ionic crosslinking during long-term cultures. In addition to Alg-Tyr, alginate modified with catechol moieties allow for HRP/H_2O_2 enzymatic crosslinking.[236] Hou et al. showed that enzymatically crosslinked alginate hydrogels with catechol moieties results in improved tissue adhesiveness when compared to Alg-Tyr hydrogels.[236]

One method to functionalize dextran with tyramine groups is to first modify dextran with *p*-nitrophenyl chloroformate and to subsequently conjugate tyramines by urethane bond formation.[121,226] In an alternative strategy, dextran can be modified with di-glycolic anhydride and subsequently functionalized with tyramine groups by amidation.[121] Both synthesis methods result in tyramine-modified dextran hydrogels that can undergo enzymatic crosslinking; however, the latter method results in a hydrogel with increased hydrolytic degradability.[121] Tyramine-modified dextran hydrogels have been promising towards cartilage repair, especially when combined with tyramine-modified heparin.[226,237,238]

Many other biopolymers have been modified with tyramine groups for enzymatic crosslinking, including cellulose derivatives,[228,239] CS,[240] pullulan,[240] and chitin.[241] Many polypeptides, such as silk fibroin, contain tyrosine residues for HRP/H_2O_2 enzymatic crosslinking.[232,242] However, polypeptides can also be further modified with additional tyramine groups for increased enzymatic crosslinking in hydrogel formation.[243,244]

4.3.2. Catechol—Catechol, the ortho isomer of benzene diol, is a versatile functional group that can undergo crosslinking by the formation of covalent bonds, metal-ligand coordination, and hydrogen bonding.[245] Catechol moieties occur widely in nature, with the famous example being mussel adhesion to dynamic wet surfaces due to the secretion of fluids rich in catechol groups.[245,246] Inspired by mussels, tissue adhesive hydrogels containing catechol groups have been explored.[246,247] Under oxidative conditions, catechol groups can form covalently crosslinked catechol dimers, which have been widely used in hydrogel formation (Figure 9a).[245] Dopamine contains a catecholic moiety and has been conjugated to many biopolymers by EDC/NHS amidation reactions.[248,249]

Alginate,[247] chitosan,[250] and HA[249] have all been modified with dopamine functional groups for hydrogel formation and tissue-adhesive applications. Lee et al. developed a catechol-modified alginate hydrogel that covalently crosslinks upon exposure to sodium periodate ($NaIO_4$).[248] The storage modulus could be tuned from 300 to 6000 Pa, depending on polymer concentration and degree of substitution.[248] Furthermore, catechol-alginate hydrogels exhibited increased cytocompatibility when compared to ionically crosslinked alginate.[248] Hong et al. demonstrated that catechol-modified HA (HA-CA) hydrogels exhibited strong adhesion to wet surfaces in acidic environments and increased mechanical stability due to the formation of covalent crosslinks in basic environments.[249] Covalently crosslinked HA-CA hydrogels have been shown to adhere to both wet and beating tissues *in vivo*.[251] Shin et al. engineered an HA-CA hydrogel for tissue adhesion applications (Figure 9b).[251] The hydrogel adhered to liver and heart tissue for at least 1 month in an *in vivo* rat model. While exposing catechol-modified biopolymers to basic conditions yields rapid covalent crosslinking, Sato et al. showed that HA-CA hydrogels can undergo auto-oxidation and covalent crosslinking under physiological conditions (pH ~7.4) over a period of a few hours.[252]

5. Dynamic covalent crosslinking

Dynamic covalent crosslinking consists of a subset of reactions that allow for the formation of reversible covalent crosslinks between biopolymers. The dynamic nature of the covalent bonds may introduce shear-thinning and self-healing properties into the hydrogel while maintaining high mechanical moduli for structural integrity. Furthermore, dynamic covalent bonds can introduce stimuli-responsiveness in hydrogel assembly and disassembly. Gelation via dynamic covalent crosslinking may be achieved by one-pot mixing of reactive components. When compared to covalently crosslinked biopolymer networks, biopolymer networks crosslinked via dynamic covalent chemistries may experience faster degradation times and increased cell infiltration both *in vitro* and *in vivo*. Mechanical and biological properties of dynamic covalent biopolymer networks will be influenced by biopolymer and crosslinking group concentrations, as well as bond strength and bond formation kinetics. Dynamic covalent crosslinking in hydrogels has been explored for many applications, including tissue engineered scaffolds, drug delivery vehicles, and bioprinting inks. This review will focus on dynamic covalent crosslinking mechanisms commonly used towards biomedical applications, including Schiff base reactions, disulfide formation, and reversible Diels-Alder reactions.

5.1. Schiff base crosslinking

The Schiff base reaction was discovered by Hugo Schiff in 1864 and has been widely used as a click chemistry tool.[253–255] Schiff bases are a type of imine, which has the structure $R_2C{=}NR'$ and are either secondary aldimines or ketimines, where $R' \neq H$. The condensation of carbonyl and primary amine groups results in Schiff base formation, with water as the only byproduct. The reversible reaction can proceed under mild conditions and is pH-responsive.[253] For hydrogel formation, Schiff base crosslinks are formed by mixing aldehyde-functionalized and amine-functionalized biopolymers.[254,255] Crosslink stability can be influenced by the neighboring atoms attached to the primary amine groups used in

Schiff base formation. Imine crosslinks are formed when a carbonyl group condenses with a primary amine group attached to a hydrocarbyl group (Figure 10a). Hydrazone crosslinks, which are more stable than imine crosslinks, are formed when a carbonyl group condenses with a primary amine group attached to a nitrogen atom (Figure 10b).[127,253] Oxime crosslinks are formed when a carbonyl condenses with a primary amine group attached to an oxygen atom and are more stable than both hydrazone and imine crosslinks.[256,257] Hydrogel degradation can be tuned by selecting the type of Schiff base used in crosslinking (e.g., imine, hydrazone, oxime).[127,256] For biopolymer hydrogel formation, imine and hydrazone functionalities are most commonly utilized to achieve dynamic crosslinking behavior.

5.1.1. Imine crosslinks—Imine crosslinking is widely used in biopolymer hydrogel formation (Figure 10a).[255] Sugar rings in polysaccharide backbones can be oxidized with sodium periodate to form dialdehyde-functionalized biopolymers. Furthermore, many biopolymers, such as chitosan and gelatin, have primary amine groups that can be used for imine crosslinking.[258–260] Qu et al. developed an injectable *N*-carboxyethyl chitosan hydrogel crosslinked by imine formation between amino groups on chitosan and dialdehyde PEG crosslinkers (Figure 10c).[258] The hydrogels were self-healing and showed promise as drug delivery vehicles for hepatocellular carcinoma therapy. In other examples, chitosan has been combined with many oxidized polysaccharides including cellulose,[261,262] CS,[263] and HA[126,264] for hydrogel formation. Gelatin has been combined with many oxidized polysaccharides including alginate[265,266] and pectin[260] for hydrogel formation. The number of amine groups available for imine crosslinking can be increased by coupling gelatin with ethylenediamine using carbodiimide chemistry.[265] Hydrolysis of the imine crosslinks results in hydrogel degradation.[126] The dynamic nature of the imine crosslink formed can result in injectable hydrogels with shear-thinning and self-healing properties.[126,259,263] To improve mechanical properties, imine-crosslinked hydrogels have also been reinforced with methacrylate-based covalent photo-crosslinking[265] or with the incorporation of microgels into the hydrogel structure.[263]

5.1.2. Hydrazone crosslinks—Hydrazone crosslinks are more stable than imine crosslinks, and thus can be used to increase hydrogel stability.[253] Hydrazone crosslinks can be formed by mixing oxidized polysaccharides with hydrazide-modified biopolymers (Figure 10b). Hydrazide modification is often accomplished using carbodiimide chemistry to conjugate adipic dihydrazide (ADH) to free carboxylic acid groups on biopolymer backbones.[267,268] Due to the ease of chemical modification, HA has been extensively explored for hydrazone-based crosslinking.[269–271] Wang et al. demonstrated that oxidized HA (HA-ALD) could be combined with adipic dihydrazide-modified HA (HA-ADH) to form a shear-thinning, self-healing hydrogel for bioprinting applications.[271] Furthermore, combining HA-ALD/HA-ADH networks with a thiol-ene crosslinkable NorHA increased hydrogel storage moduli 3-fold, and allowed for thiol-norbornene photo-patterning of thiolated peptides within the bioprinted scaffolds.[271] Domingues et al. demonstrated that injectable hydrazone-crosslinked HA networks could be strengthened by incorporating aldehyde-modified cellulose nanocrystals into the network.[272] Hydrazone crosslinking has been explored for many other biopolymers in addition to HA, including alginate,[270] cellulose,[273] dextran,[268] and xanthan gum.[274]

The oxidization of polysaccharide sugar rings using sodium periodate is a simple way to functionalize biopolymers with aldehydes; however, it can result in decreased biopolymer molecular weight. To improve hydrogel mechanical properties and stability, it may be desired to functionalize biopolymers with pendant aldehydes rather than oxidizing sugar rings in the polymer backbone. Biopolymers can be modified with pendent aldehydes using carbodiimide coupling of 3-amino-1,2-propanediol to carboxylic acid groups on the biopolymer backbone, followed by brief (~5 min) exposure to sodium periodate in order to oxidize the pendant diol for aldehyde formation.[115] To further improve stability, biopolymers can be modified with carbohydrazide (CDH) instead of ADH for hydrazone-crosslinked hydrogel formation.[127] Hozumi et al. showed that hydrogels formed by combining pendant aldehyde-modified HA and CDH-gelatin were stable for ~30 days, whereas hydrogels formed from combining ADH-gelatin and oxidized HA degraded within ~5 days.[127]

5.2. Disulfide crosslinking

Disulfide bonds (i.e., SS-bonds, disulfide bridges) are dynamic covalent interactions that can be cleaved and reformed in response to chemical or physical stimuli.[275] Protein folding and structure rearrangement rely on the formation and shuffling of disulfide bonds, mostly involving cysteine residues containing thiol groups, which can undergo disulfide bond formation under oxidative conditions.[276] Hydrogels crosslinked by disulfide bonds can exhibit shear-thinning, self-healing properties, while maintaining increased crosslink stability when compared to physical supramolecular interactions.[275,277]

Biopolymers have been functionalized with thiol groups for the formation of dynamic covalent disulfide crosslinks (Figure 11a). For example, HA has been modified with dithioiso(propanoic dihydrazide) (DTP) using carbodiimide amidation and subsequent reduction with DTT to result in HA-DTPH biopolymers for hydrogel formation.[278] Shu et al. demonstrated that HA-DTPH hydrogels could be oxidized by exposure to ambient air for the formation of dynamic covalent disulfide crosslinks.[278] In a later study, HA-DTPH and Gel-DTPH were combined to form a synthetic ECM hydrogel scaffold crosslinked by disulfide bonds for *in vitro* cell culture.[279] Bermejo-Velasco et al. demonstrated that disulfide bond formation kinetics and stability could be increased by modifying HA with thiol moieties containing electron-withdrawing groups at the β-position (Figure 11b).[280] HA modified with either cysteine or *N*-acetyl-cysteine groups formed disulfide biopolymer networks at neutral pH within minutes to hours, whereas thiolated HA was unable to form stable disulfide crosslinks at neutral pH within 24h.[280] Alginate hydrogels crosslinked with disulfide bonds have been fabricated by modifying carboxyl groups on alginate with either cysteine or cysteamine via amidation.[281] Zhao et al. demonstrated that these alginate hydrogels were pH responsive and underwent disassembly of disulfide crosslinks in the presence of DTT.[281] In another strategy, self-healing cellulose hydrogels have been fabricated by chemically modifying cellulose nanocrystals to enable dynamic disulfide bond formation.[282]

Polypeptide hydrogels can be crosslinked by forming disulfide bridges between cysteine amino acids. Sun et al. showed that injectable albumin hydrogels could be easily fabricated

by combining BSA with H_2O_2.[283] Wang et al. used a similar approach by combining keratin from chicken feathers with H_2O_2 to induce disulfide bond formation between free thiols on keratin.[100] The resulting keratin hydrogel was explored for wound healing applications. To increase crosslinking, polypeptides can also be modified with excess thiol groups. Thi et al. fabricated a gelatin-based hydrogel with dual crosslinking functionality by mixing hydroxyphenyl propionic acid-modified gelatin (GH) with thiolated gelatin (GS).[284] GH was crosslinked with HRP/H_2O_2, and the adhesive strength of the hydrogel increased 6-fold upon addition of GS due to the formation of disulfide crosslinks within the hydrogel and at the hydrogel-tissue interface.[284] Engineered polypeptides have also been designed to form hydrogels via disulfide bonds. Shen et al. designed am artificial protein crosslinked by leucine zipper domains.[285] It was shown that incorporating thiol groups within the leucine zipper structures allowed for the stabilization of crosslinks due to the formation of disulfide bonds.[285]

5.3. Reversible Diels-Alder crosslinking

Diels-Alder reactions, which are [4+2] cycloadditions between dienes and a dienophiles, are a widely used class of click chemistry tools (Figure 12a).[286] For biomedical applications, the dynamic Diels-Alder reaction between furan and maleimide is of particular interest due to its reversibility at 100°C (i.e., retro-Diels-Alder).[287] Hydrogels crosslinked with furan-maleimide Diels-Alder reactions degrade via hydrolysis of the maleimide groups as well as occurrence of the retro-Diels-Alder reaction.[288] Synthetic polymers and crosslinkers containing furan and maleimide functionalities have been explored to engineer dynamic hydrogels with thermo-responsive behavior.[289,290] To engineer hydrogels, many biopolymers have been modified with furan moieties and subsequently crosslinked with multifunctional maleimide crosslinkers.

Amidation reactions have been used to conjugate furfurylamine groups onto many biopolymers including alginate,[291] cellulose,[292] chitosan,[293] HA,[294] and gelatin.[295] Etherification and imine formation have also been explored to conjugate furan moieties to chitin[296] and chitosan,[297] respectively. Furan-modified biopolymers can then be crosslinked by synthetic bismaleimide crosslinkers to form hydrogels.[291,296,298] These hydrogels can be thermo- and pH- responsive, and have been explored for tissue engineering scaffolds, drug delivery, and anti-microbial coatings.[296,297,299–301] By tuning concentrations, furan-maleimide crosslinked biopolymer hydrogels can be injectable and undergo *in situ* crosslinking within minutes-to-hours.[295,296] While synthetic bismaleimide crosslinkers are commonly used, maleimide groups can also be conjugated to the biopolymer backbone. For example, hydrogels consisting of HA modified with both furan and maleimide groups have been explored for sustained drug release.[302]

Bi et al. showed that chitin could be modified with furfuryl glycidyl ether to engineer a unique dual-gelation system (Figure 12b).[296] At 37°C, physical interactions between chitin molecules result in immediate gelation. When combined with a PEG-based maleimide crosslinker, further gelation occurs over the period of hours-to-days due to long-term Diels-Alder formation. In another strategy, Yu et al. showed that HA could be modified with both furan and tyramine functional groups.[303] Subsequent addition of HRP/H_2O_2 and a PEG-

based maleimide crosslinker resulted in immediate gelatin due to enzymatic crosslinking, followed by further gelatin due to the Diels Alder reaction over 24 hours. Bai et al. created a dual-crosslinking system consisting of furan-modified CS, maleimide-modified Pluronic F127, and PEG-based maleimide.[300] The formulation underwent rapid gelatin at 37°C due to Pluronic, followed by long-term gelation due to furan-maleimide Diels Alder reactions. The hydrogel showed promise in the field of bone tissue engineering.

6. Physical crosslinking

Physical crosslinking can be achieved by physical interactions between biopolymer chains modified with crosslinking groups. The reversible nature of physical interactions often leads to hydrogels with shear-thinning and self-healing properties, which may allow for injectable hydrogels. While mechanical moduli may be lower relative to hydrogels crosslinked with covalent bonds, utilizing physical crosslinks may allow for increased cell migration and diffusivity within a hydrogel system. Hydrogel assembly through physical interactions often occurs through one-pot mixing of two or more components, allowing for rapid gelation and facile encapsulation of cells or drugs. Such systems also typically do not require catalysts or initiators, which may improve cytocompatibility. Furthermore, some physical interactions can be designed to be stimuli-responsive in order to allow for controlled hydrogel assembly and disassembly or to bind to drugs to control their release from hydrogels. The mechanical and biological properties of physically crosslinked hydrogels depend on the concentrations of biopolymers and crosslinking groups, as well as the binding affinity between chemical groups. In this section, various types of physical crosslinking mechanisms that have been utilized with chemically-modified biopolymers are discussed, including the introduction of guest-host interactions, hydrogen bonding, metal-ligand coordination, and interactions between synthetic polymers grafted to biopolymers.

6.1. Guest-host interactions

Guest-host interactions involve the formation of inclusion complexes between a “host” macrocycle with a hydrophobic interior and one or more hydrophobic “guest” moieties (Figure 13).[304] For biopolymer hydrogel formation, naturally-derived cyclodextrins and synthetic cucurbit[*n*]urils are the most commonly utilized host molecules due to their water-solubility, chemical diversity, low toxicity, cytocompatibility, and history of use.[255,305,306] Numerous guest moieties have been explored for hydrogel assembly, some of which include stimuli-responsive properties to allow for controlled assembly and disassembly of the guest-host inclusion complex.[305]

6.1.1. Cyclodextrins—Cyclodextrin (CD) is a cyclic macromolecule formed from either 6 (α-CD), 7 (β-CD), or 8 (γ-CD) glucopyranoside units, which has a long history of use for solubilization of drugs.[307] The hydrophobic inner cavity of CD forms guest-host inclusion complexes with a variety of guest molecules including adamantane (Ad), cholesterol, azobenzene (Az), and ferrocene (Fc).[255] CDs have been incorporated into hydrogels to act as host molecules for the sustained delivery of hydrophobic drugs.[308–311] To form shear-thinning, self-healing hydrogels, many biopolymers have been modified with either CDs or guest moieties and subsequently mixed to allow for rapid guest-host mediated

assembly (Figure 13a). Examples of biopolymers that have been chemically modified with CD groups include alginate,[312,313] cellulose,[314,315] chitosan,[316] dextran,[123] HA,[317,318] and keratin.[319] Furthermore, gelatin has been mixed with synthetic polymers containing β-CD to form hydrogels due to the guest-host interactions between β-CD and aromatic amino acid residues.[320]

Ad-CD guest-host interactions have been widely investigated and are known to have an association constant of about 10^5 M^{-1}, which is relatively high for guest-host complexation.[304] HA has been modified with either Ad (AdHA) or CD (CDHA) to form shear-thinning, self-healing guest-host hydrogels upon mixing for injectable tissue scaffolds, drug delivery, and bioprinting inks.[321] Many methods have been utilized to conjugate Ad or CD to HA. For example, Rodell et al. demonstrated that carboxylic acid-derivatives of Ad can be conjugated to HA via esterification, and amine-derivatives of CD can be conjugated to HA via amidation.[318] Once combined, AdHA and CDHA form injectable, self-healing guest-host hydrogels. Properties of AdHA-CDHA hydrogels depended on the concentration of modified HA, degree of modification, and the molar ratio of guest and host functional groups.[318] Furthermore, Ad-CD guest-host networks can be combined with secondary covalent crosslinking to increase network mechanics and stability.[196] There are many other guest-host pairings for CD that have been explored for hydrogel formation. In one example, alginate modified with β-CD has been combined with Pluronic F108, forming inclusion complexes between β-CD and the hydrophobic poly(propylene glycol) (PPG) block on the Pluronic.[322] The resulting hydrogel had shear-thinning, self-healing properties in addition to increased thermo-responsive gelation due to the presence of the Pluronic.[322]

To increase the functionality of CD-containing guest-host hydrogels, stimuli-responsive guest molecules have also been explored. Az acts as a guest molecule for CD and undergoes *trans*-to-*cis* isomerization in response to UV light. While *trans*-Az has a high affinity for CD, *cis*-Az has a low affinity.[323] Thus, hydrogels crosslinked with Az-CD inclusion complexes can undergo UV light-responsive disassembly, and subsequent re-assembly upon removal of the UV light.[324] For biomedical applications, this system can be used to control scaffold degradability and drug release. UV-responsive Az-CD hydrogels have been formed with chemically-modified alginate,[325] dextran,[123] and HA.[326] For example, Peng et al. showed that dextran can be modified with either β-CD or *trans* Az for guest-host hydrogel formation (Figure 13c).[123] Upon exposure to UV light, Az undergoes a *trans* to *cis* isomerization, resulting in hydrogel disassembly and photoresponsive protein release. Fc can also be used as a stimuli-responsive guest molecule for CD.[327] Tan et al. demonstrated that β-CD modified alginate could be mixed with Fc modified Pluronic F127 for hydrogel formation.[328] NaOCl was used to oxidize Fc to form Fc^+, which resulted in disassembly of guest-host complexes.[328]

6.1.2. Cucurbit[n]urils—Cucurbit[*n*]urils (CB[*n*]) are a class of cyclic macromolecules consisting of *n* glycoluril units that form a hydrophobic cavity with two openings.[329] The strength of guest-host interactions between CB[*n*] and guest molecules depends on the binding affinity and CB[*n*] cavity size.[329] For biomedical applications, CB[6], CB[7], and CB[8] are most often used,[330] which have cavity sizes of 164, 279, and 479 Å,

respectively.[331] CB[8] host molecules provide a large enough cavity to bind two aromatic guest molecules simultaneously.[331]

Biopolymers have been modified with CB[*n*] derivatives and guest moieties for hydrogel formation. Park et al. developed an HA hydrogel crosslinked by guest-host complexation between CB[6] and polyamine chemical modifications for application as a tissue scaffold.[332] Both diaminohexane (DAH) and spermine modifications were explored as guest molecules due to their high binding affinity with CB[6] (10^{10}-10^{12} M^{-1}).[332] In a follow up study, CB[6]/DAH-HA hydrogels were used to encapsulate MSCs for potential use as an artificial ECM.[154] CB[6]/DAH-HA guest-host hydrogels exhibited increased cell viability and retention when compared to Matrigel after 60 days *in vivo* in an subcutaneous mouse model.[154] Furthermore, Sohail et al. engineered a tunable photoluminescent guest-host hydrogel consisting of CB[7] derivatives and alginate modified with dequalinium chloride hydrate guest moieties.[333]

CB[8] host molecules can accommodate two guest molecules due to the relatively large cavity size. Towards hydrogel formation, biopolymers can be chemically modified with guest moieties and subsequently mixed with free CB[8] compounds to form shear-thinning, self-healing hydrogels (Figure 13b).[122,334] For example, cellulose derivatives have been modified with naphthalene and phenylalanine guest molecules for hydrogel formation with CB[8] hosts.[122,335] HA modified with phenylalanine has also been shown to form injectable guest-host hydrogels with CB[8].[334] Rowland et al. conjugated phenylalanine to HA using Michael addition between methacrylate-functionalized HA and free thiols on a cysteine-phenylalanine compound to form biopolymer hydrogels that showed promise for injectable drug delivery to the brain.[336]

6.2. Hydrogen bonding

Hydrogen bonding is a physical attractive interaction between hydrogen atoms and electronegative atoms such as oxygen and nitrogen. Many biological processes such as protein folding and DNA base-pairing are driven by hydrogen bonding interactions.[337] For hydrogel formation driven by hydrogen bonding, biopolymers can be modified with functional groups such as ureidopyrimidone or gallol (Figure 14).

6.2.1. Ureidopyrimidone—Ureidopyrimidone (UPy) is a quadruple hydrogen bonding motif that has been used as a dynamic crosslinker to prepare pH- and temperature-responsive injectable hydrogels (Figure 14a).[338,339] The quadruple binding motif allows for transient network formation independent of stoichiometry.[339] Injectable dextran hydrogels crosslinked by UPy hydrogen bonding have been developed for application in musculoskeletal tissue engineering, where isocyanate-functionalized UPy was conjugated to the hydroxyl groups on dextran (Figure 14c).[340] The UPy-dextran hydrogel was able to sustain doxycycline drug release for a week and BSA release for more than a month.[340] Gelatin has also been functionalized with UPy through urethane bond formation between isocyanate-modified UPy and amino groups on gelatin to create injectable hydrogels.[341,342] Alavijeh et al. developed a UPy-modified gelatin hydrogel that demonstrated shape memory behavior for applications as a controlled drug delivery matrix.[341] Furthermore, hydrogels

have been formed using UPy-modified cellulose nanocrystals as well as UPy-modified heparin binding peptides.[343,344]

6.2.2. Gallol—Gallol is an aromatic ring structure with three hydroxyl groups that is associated with fruit browning.[345] Gallol moieties can be conjugated to biopolymers for the formation of dynamic hydrogen bonds resulting in shear-thinning, self-healing injectable hydrogels (Figure 14b).[346,347] Upon exposure to oxidative conditions (i.e., $NaIO_4$), gallol moieties can convert to quinones for covalent stabilization of networks. Shin et al. developed an ECM-mimetic bioink by modifying both gelatin and HA with gallol moieties via EDC/NHS coupling between biopolymer carboxyl groups and hydroxydopamine.[347] Hydrogen bonding between gallol functional groups resulted in a printable hydrogel ink, whereas slow spontaneous oxidation post-printing resulted in covalent stabilization of gallol crosslinks.[347]

6.3. Metal-ligand coordination

Metal-ligand coordination complexes consist of a central metallic atom surrounded by bounded molecules referred to as ligands. Many biological processes such as self-assembly and adhesion rely on the formation of metal-ligand complexes.[348] For example, mussels make use of reversible metal-ligand complexes between catechol moieties and metal ions to form a self-healing protective fluid coating capable of protection against turbulent tidal motion.[349] Hydrogel biomaterials inspired by reversible mussel adhesive chemistry have been explored for many applications, including engineered hydrogel actuators and bioprinting.[350,351] The reversibility of metal-ligand complexation allows for the formation of shear-thinning, self-healing supramolecular hydrogels.[352] Furthermore, hydrogels crosslinked via metal-ligand coordination can have pH-responsive behaviors and reach mechanical moduli near that of covalently bonded hydrogels.[353]

One of the most common approaches to introduce metal-ligand complexation into biopolymer hydrogels is to first conjugate catechol to the biopolymer backbone, which can then form metal-ligand complexes with many metallic atoms including Fe(III), Cu(II), and Al(III) (Figure 15a).[352,354] Chitosan has been modified with catechol moieties and subsequently crosslinked via metal-ligand coordination to form shear-thinning, self-healing hydrogels.[352] Injectable, self-healing catechol-chitosan hydrogels crosslinked by Fe(III) metal complexation have been developed as a promising drug delivery vehicle for breast cancer, showing capabilities of sustained release of anti-cancer drugs *in vivo* in a mouse model for more than 40 days (Figure 15b).[355] Furthermore, catechol-modified chitosan hydrogels crosslinked with Fe(III) exhibit pH-responsiveness, adding another degree of tunability to hydrogel functionality.[356,357]

HA hydrogels crosslinked with metal-ligand coordination chemistry have also been developed.[351,358] Lee et al. modified HA with catechol moieties using amidation between amino groups on dopamine and carboxyl groups on HA (HA-CA).[358] HA-CA was crosslinked by the addition of Fe(III), as well as by oxidative conversion of catechol groups to covalently crosslinked quinones.[358] Furthermore, HA-CA/Fe(III) crosslinks exhibited pH-responsiveness.[358] Shin et al. developed granular hydrogels by jamming covalently

crosslinked HA microgels containing gallol moieties.[351] The microgels were mixed with silver nanoparticles (AgNPs) and the gallol groups formed metal-ligand coordination complexes between microgels with the AgNPs[351]. The resulting injectable hydrogel was conductive due to the presence of AgNPs, with potential applications in bioprinting of electroactive tissue constructs.[351]

6.4. Grafted biopolymers

To increase the range of functionalities in biopolymer hydrogels, synthetic polymers may be grafted to biopolymer backbones so they may interact with each other for assembly into hydrogels (Figure 16). For biomedical applications, two of the most common synthetic polymers grafted to biopolymers are poly(N-isopropylacrylamide) (PNIPAAm) and Pluronic.

6.4.1. PNIPAAm—PNIPAAm is a synthetic polymer that is characterized by having both hydrophilic amide moieties and hydrophobic propyl moieties.[359] PNIPAAm undergoes a reversible low critical solution temperature (LCST) around 34°C. At low temperatures, the amide groups are solvated by water, allowing for the existence of a PNIPAAm aqueous solution.[359] Upon exposure to elevated temperature (e.g., >34°C), interactions between propyl hydrophobic groups strengthen, leading to the formation of a physically crosslinked network.[359]

PNIPAAm has been grafted to many biopolymers including alginate,[360] HA,[361] CS,[362] chitosan,[363] silk fibroin,[364] and gelatin[365] for the formation of thermo-responsive hydrogels (Figure 16a). For example, Zhu et al. grafted PNIPAAm onto keratin by first using thiol-ene radical addition to conjugate NIPAAm monomers onto free thiol groups on keratin, and subsequently polymerizing PNIPAAm off the keratin backbone using free radical kinetic chain polymerization.[366] The resulting thermo-sensitive keratin-g-PNIPAAm hydrogel exhibited gelation around 28–32°C, depending on the grafting ratio (Figure 16c).[366] The keratin-g-PNIPAAm hydrogel was explored for brain injury repair applications *in vivo*. In another approach, PNIPAAm has been grafted to HA by amidation between PNIPAAm end-terminated with carboxyl groups (PNIPAAm-COOH) and aminated HA.[367] Tan et al. demonstrated that the injectable AHA-g-PNIPAAm hydrogels exhibited rapid gelation above 30°C.[367] The hydrogel showed promise for adipose tissue engineering applications both *in vitro* and *in vivo*.[367] Chitosan has also been functionalized with PNIPAAm using amidation between PNIPAAm-COOH and amine groups on chitosan using EDC/NHS carbodiimide chemistry.[368] Yuan et al. combined chitosan-g-PNIPAAm with PEG to fabricate electrospun nanofibers for drug delivery and tissue engineering applications.[363] In another example, gelatin-g-PNIPAAm hydrogels have been explored for use as an injectable delivery vehicle for intracameral delivery of the antiglaucoma drug, pilocarpine, in a rabbit glaucoma *in vivo* model.[369]

6.4.2. Pluronics—Pluronics are A-B-A triblock copolymers consisting of PEG-PPG-PEG that are manufactured by BASF.[370] Pluronics have a critical micelle temperature (CMT) between 25–40°C, forming micelles with a hydrophobic core.[370] Biopolymers grafted with Pluronics can form injectable hydrogels that undergo thermo-responsive

gelation upon exposure to physiological temperatures (Figure 16b).[371] The most common Pluronic used in hydrogel formation is Pluronic F127 (PEG_{100}-PPG_{65}-PEG_{100}) due to its hydrophilicity at low temperatures.[370,371] Many biopolymers have been modified with Pluronics for scaffold formation and drug delivery, including HA,[372] chitosan,[373] heparin,[374] and gelatin.[375] Lee et al. demonstrated that Pluronic F127 capped with amine groups could be grafted to carboxyl groups on HA using EDC/HOBt amidation.[372] Bovine chondrocytes were encapsulated in the HA-g-F127 hydrogel for application as an injectable cell carrier for cartilage regenartion.[372] Chitosan-g-F127 hydrogels have also been explored as an injectable hydrogel for cartilage repair.[376] Park et al. showed that chitosan-g-F127 hydrogels reached gelation at a temperature of 25°C and could reach a storage modulus of 10^4 Pa.[376]

7. Recent applications of biopolymer hydrogels to biomedicine

Biopolymer hydrogels from chemically-modified biopolymers have been widely used across biomedical applications for several decades. In previous sections, we briefly mentioned some of these applications in the context of specific biopolymer modifications and crosslinking methods. In this section, we review representative recent advances (last 5 years) that have been made using chemically-modified biopolymer hydrogels, including for the biofabrication of tissue scaffolds, therapeutic molecule delivery, tissue adhesives and sealants, and the formation of interpenetrating network hydrogels. Although it is not possible to be comprehensive with all of the biomedical examples of biopolymer hydrogels, the studies discussed throughout this section were selected to highlight diverse biopolymers and chemical modifications and to emphasize examples where these biopolymer modifications were integral to the application.

7.1. Biofabrication of hydrogel scaffolds

7.1.1. Bioprinted scaffolds—Bioprinting has rapidly evolved as a widely adopted biofabrication technique to engineer tissue constructs with complex microarchitectures. Chemically-modified biopolymer hydrogels have been developed as bioinks to embed cells and bioprint tissue constructs, particularly with photocrosslinkable biopolymers to obtain stable tissue constructs.[377]

Traditional extrusion-based bioprinting utilizes viscous, shear-thinning photocrosslinkable bioinks to print constructs that are crosslinked by light exposure post-deposition.[377] GelMA has been widely explored as a bioink for extrusion-based bioprinting. For example, Bejleri et al. bioprinted a cardiac patch composed of GelMA, decellularized cardiac ECM, and human cardiac progenitor cells.[378] The addition of cardiac ECM to the GelMA bioink improved printability and the pro-angiogenic potential of embedded cells. The bioprinted patches were evaluated in an *in vivo* rat model and supported vascularization after 14 days. Towards musculoskeletal tissue engineering applications, Cidonio et al. engineered a bioink composed of GelMA, Laponite® nanoclay, and hMSCs for bone defect repair.[379] The incorporation of nanoclay improved print fidelity when compared to GelMA alone, and the addition of VEGF in the scaffold resulted in increased vascularization in an *ex vivo* chick chorioallantoic model.

HA bioinks have also been widely adopted in bioprinting. For example, Petta et al. developed bioinks from HA-Tyr for extrusion-based bioprinting.[380] Various cell types, including hMSCs and bovine chondrocytes, were encapsulated in HA-Tyr bioinks that were lightly enzymatically crosslinked with HRP/H_2O_2 before printing. After deposition, constructs were stabilized by secondary photocrosslinking and supported high cell viability with culture. In another approach, Kuss et al. combined MeHA, GelMA, polycaprolactone (PCL), and hydroxyapatite to engineer an extrusion bioprinted scaffold for craniofacial defect repair.[381] Hybrid scaffolds were fabricated with layers of PCL/hydroxyapatite and then MeHA/GelMA containing embedded stromal vascular fraction of adipose tissue. After short-term culture in hypoxic conditions *in vitro*, the scaffolds were subcutaneously implanted into an *in vivo* mouse model and demonstrated microvasculature formation 4 weeks post-implantation. In another approach, Kesti et al. combined HA-g-PNIPAAm with MeHA to create a dually-crosslinked hydrogel.[382] The rapid gelation of printed constructs occurred upon contact with a substrate at 37°C, providing immediate structural support for the constructs. Subsequent free radical chain polymerization of methacrylate groups provided long-term mechanical stability.[382]

Numerous other biopolymers have been chemically modified for use as extrusion-based 3D printing bioinks. For example, methacrylated alginate, methacrylated chondroitin sulfate, and GelMA have been combined with a graphene oxide nanofiller for use as a multicomponent photocrosslinkable bioink, which was explored for cartilage tissue engineering.[383] Recently, norbornene-modified cellulose bioinks were engineered by functionalizing cellulose with a norbornene group using either amide or ester bond formation (Figure 4).[182] The bioink was photocrosslinkable by thiol-ene radical addition and showed high cell viability for cell-laden bioprinted scaffolds.

One of the challenges with traditional extrusion-based bioprinting is the need for high bioink viscosity or rheological additives to ensure high print fidelity, which limits the use of many materials.[377] To combat this, extrusion bioprinting with *in situ* crosslinking has emerged as a promising technique to permit extrusion-based printing of non-viscous bioinks.[131,384] In this approach, bioinks are cured by photocrosslinking during the deposition process through the use of photopermeable capillary tubes. Ouyang et al. demonstrated that a wide variety of non-viscous bioinks, including GelMA, MeHA, and NorHA, could be printed using *in situ* crosslinking.[384] Fibroblasts embedded in the non-viscous biopolymer inks exhibited high viability (>90%) post-printing. Galarraga et al. later showed that MSCs could be encapsulated in non-viscous NorHA bioinks to fabricate bioprinted constructs with *in situ* crosslinking for cartilage tissue engineering.[131] Bioprinted constructs were cultured in chondrogenic media for 56 days, allowing for the formation of cartilaginous tissue. In a recent study, gelatin additives were used to process a wide variety of modified biopolymer bioinks, where the gelatin could be subsequently removed through heating after the printing was complete.[385] This approach was used to bioprint soft hydrogels that were favorable for cell culture, but that would have been challenging to print otherwise. In another approach, Heo et al. developed a carbohydrazide-modified gelatin (Gel-CDH) bioink that was extruded into an oxidized alginate (OAlg) bath.[386] Upon deposition of the bioink, hydrazide bonds formed *in situ* to stabilize the construct, allowing for good mechanical integrity of the scaffold as well as high cell viability when a cell-laden Gel-CDH bioink was used.

Beyond extrusion-based printing, lithography-based bioprinting has emerged as a promising biofabrication technique for printing constructs with non-viscous bioinks.[387] Lithography-based bioprinting allows for the fabrication of constructs with high accuracy and precise spatial patterning.[387] Dynamic light processing (DLP) is a technique that uses a digital light projector to selectively photocrosslink a bioresin on a computerized stage.[377] The stage moves stepwise in the z direction to allow for layer-by-layer fabrication of constructs with significantly higher spatial resolution that extrusion-based techniques.

Biopolymers with photocrosslinkable groups have been explored as non-viscous bioinks for DLP-based biofabrication of tissue constructs. Zhu et al. engineered a bioink consisting of GelMA and GMA-HA with embedded human umbilical vein endothelial cells (HUVECs) and MSCs.[388] DLP biofabrication was used to create prevascularized constructs by patterning complex microarchitectures into the HA/gelatin scaffold. Scaffolds exhibited high cell viability over 1 week *in vitro*. Constructs were subcutaneously implanted *in vivo* in a mouse model, and significantly improved vascular density was observed 2 weeks post-implantation when compared to a non-prevascularized control. In another example, Bertlein et al. developed a thiol-ene clickable Gel-AGE scaffold.[187] The Gel-AGE bioink was used to bioprint constructs with high print fidelity using both extrusion-based and DLP bioprinting.

Kim et al. engineered a methacrylated silk fibroin bioink for scaffold formation using DLP biofabrication (Figure 17a).[389] By tuning bioink composition, bioprinted scaffolds with a compressive modulus greater that 120 kPa were achieved. The use of DLP biofabrication allowed for the printing of precise, complex structures, including brain, ear, trachea, lung, and heart-shaped scaffolds. It was shown that fibroblasts could be encapsulated in the silk-based bioink and maintained with high viability throughout cultures up to 14 days. In another approach, Placone et al. engineered a photocrosslinkable keratin hydrogel scaffold using DLP biofabrication, which showed good print resolution and high biocompatibility.[390]

Stereolithography (SLA) is another lithographic printing approach that uses a focused laser light to crosslink specific areas in a resin vat in order to build a 3D structure.[377] Smith et al. used methacrylated BSA (MA-BSA) to form an albumin-based scaffold via SLA biofabrication that demonstrated high fibroblast cell viability.[391] Further, the albumin constructs were subject to thermal incubation in order to disrupt native albumin structure and introduce physical crosslinks between denatured albumin chains in addition to chemical crosslinks between methacrylate groups.

7.1.2. Granular hydrogel scaffolds—Granular hydrogels consist of hydrogel microparticles that are agglomerated into a jammed state.[392] Hydrogel microparticles may be fabricated by various methods, including microfluidic devices, batch emulsions, lithography, and mechanical fragmentation.[392] Granular hydrogels are injectable, microporous, and modular, making them a promising tool for engineering tissue scaffolds.[392] Furthermore, interparticle crosslinks can be introduced between hydrogel microparticles to further stabilize granular hydrogel scaffolds.[392] Granular hydrogel scaffolds fabricated from PEG have been explored for multiple applications, including wound closure.[393,394] Recently, hydrogel microparticles fabricated from chemically-

modified biopolymers have shown promise for the fabrication of granular hydrogel scaffolds.

HA microparticles have been widely explored for the fabrication of granular hydrogels. For example, acrylated HA microparticles have been formed by Michael addition in the presence of MMP-degradable dithiol crosslinkers using a microfluidic device.[200,395] The HA microparticles could be annealed by enzymatic crosslinking, free radical chain polymerization, or amidation reactions between microparticles.[395] The resulting granular hydrogel is termed a microporous annealed particle (MAP) scaffold: a granular hydrogel consisting of hydrogel microparticles annealed by interparticle crosslinking.[393] HA MAP scaffolds annealed by enzymatic interparticle crosslinking have been shown to decrease scar formation and inflammation when injected into an *in vivo* mouse stroke model.[200] MAP scaffolds have also been fabricated by NorHA microparticles with tetra-PEG-tetrazine, resulting in tetrazine-norbornene crosslinks between microparticles.[396,397] The microparticles were fabricated in a water-in-oil batch emulsion and subsequently filtered to obtain a narrow size distribution. These tetrazine-norbornene annealed granular hydrogel scaffolds have been used to study polyplex-mediated gene delivery *in vitro* and have shown promise in stroke recovery applications.[396,397]

To fabricate injectable HA granular hydrogels, NorHA modified with Ad (AdNorHA) has been used to fabricate microparticles in a microfluidic device.[398] AdNorHA microparticles were subsequently jammed by vacuum filtration in the presence of CDHA to create a shear-thinning, self-healing granular hydrogel with guest-host interparticle crosslinks.[398] The hydrogel was used to study the disease-dependent behavior of hydrogel degradation in the myocardial infarction microenvironment using an *in vivo* rat model.[398] Furthermore, it has been shown that granular hydrogels formed from jamming NorHA microparticles can be used as bioinks for bioprinting of microporous tissue structures.[172]

Many other biopolymers have been explored for granular hydrogel formation. Injectable granular hydrogels fabricated from cell-laden, norbornene-modified gelatin microparticles have been explored for hyaline cartilage tissue engineering.[399] It was shown that the incorporation of 4-arm PEG-NHS between microparticles led to interparticle crosslinking for scaffold stabilization due to the formation of amide bonds.[399] GelMA microparticles have been fabricated in a microfluidic device and subsequently annealed following additional UV light-mediated free radical crosslinking between microparticles.[400] Within the granular hydrogels, stiffness and porosity could be tuned independently.[400] It was shown that HUVEC cell migration into the GelMA granular hydrogel *in vitro* was significantly higher than for bulk GelMA controls.[400] Furthermore, granular hydrogels have been fabricated by mixed microparticles formed from GelMA and methacrylated chitosan (ChitoMA), where ionic interactions between negatively-charged GelMA microparticles and positively-charged chitosan microparticles increased scaffold stability and mechanical properties (Figure 17b).[401] The GelMA-ChitoMA granular hydrogels were used to support Schwann cell migration and axon growth in an *in vivo* sciatic nerve defect rat model.[401]

In another strategy, bulk hydrogels made from crosslinked cellulose nanofibrils (CNFs) were extruded through a microscale mesh to create granular gels with microscale porosity.[402]

The granular CNF gel exhibited increased fibroblast migration throughout the scaffold when compared to the control bulk hydrogel.[402] In a recent study, granular hydrogels were formed by extruding bulk hydrogels through a mesh to create hydrogel microstrands, which were combined with cells for use as a cell-laden bioink.[403] The microstrand fabrication process was explored using multiple biopolymers, including HA, gelatin, and carrageenan, a linear sulfated polysaccharide extracted from red seaweed.

7.1.3. Electrospun hydrogel scaffolds—Electrospinning is a method to fabricate fibrous materials by ejecting charged polymer solutions through a spinneret under a high-voltage electric field. The polymer solution solidifies through solvent evaporation, resulting in a stable nanofiber filament. Towards biomedical applications, electrospun biopolymers are of interest due to their ability to mimic the native fibrillar properties of the ECM.[404] Hydrogels can be formed by crosslinking electrospun biopolymer fibers to form cellular scaffolds and drug delivery vehicles.[404,405] There are many examples of hydrogels formed by crosslinking electrospun fibers made from non-modified biopolymers. For example, alginate fibers can be ionically crosslinked by immersion in Ca^{2+} solutions,[406] fibrin fibers can be enzymatically crosslinked in the presence of thrombin,[406] and silk fibroin fibers can be crosslinked by β-sheet formation.[407]

Towards the fabrication of chemically-modified biopolymer scaffolds made from electrospun fibers, GelMA and MeHA have been widely used to form fibrous scaffolds via free radical chain polymerization for crosslinking within and between fibers.[152,408–412] For example, Sun et al. fabricated electrospun GelMA fibers that were subsequently photocrosslinked to form a fibrous hydrogel scaffold.[410] The scaffold was evaluated *in vivo*, demonstrating increased microvasculature formation when compared to controls. In another example, Chen et al. fabricated an electrospun GelMA fibrous hydrogel scaffold for spinal cord repair and regeneration.[411] Song et al. fabricated electrospun MeHA hydrogel scaffolds to investigate the influence of fibrous matrix stiffness on meniscal cell migration.[412] Fibers were electrospun from MeHA with different degrees of modification to make soft (30% modification) and stiff (97% modification) MeHA fibrous hydrogel networks.[412] The stiffer MeHA scaffolds supported enhanced cell invasion and collagen deposition when compared to the softer MeHA scaffolds. In another approach, Zhou et al. fabricated an electrospun scaffold from methacrylated chitosan that could be post-crosslinked using UV light to form a stable construct for potential use as a skin repair matrix.[413]

Other crosslinking chemistries have been explored to create biopolymer hydrogels from electrospun fibers. Zhang et al. fabricated electrospun fibers from solutions of alginate and thiolated-HA.[406] The fibers were subsequently dually crosslinked by immersing in a solution of $CaCl_2$ (ionic crosslinking) and PEGDA (for Michael addition crosslinking). Furthermore, NorHA has been electrospun and crosslinked with thiol-ene radical addition to form hydrogels.[414] Davidson et al. developed a multifiber hydrogel network consisting of electrospun NorHA that was further modified with either hydrazide groups (NorHA-Hyd) or aldehyde groups (NorHA-Ald) (Figure 17c).[415] NorHA-Hyd and NorHA-Ald were co-electrospun to create a multifiber network. Post-electrospinning, norbornene groups were crosslinked within and between fibers through thiol-ene radical addition in order to stabilize the fibrous scaffolds. Dynamic covalent interactions due to hydrazone crosslink formation

between fibers resulted in increased scaffold stiffness and plastic deformation, permitting scaffold self-adhesion and the formation of layered and tubular constructs.

7.2. Therapeutic delivery

Injectable hydrogels have been widely used for localized and sustained delivery of therapeutics, including small molecules, nucleic acids, and proteins.[416,417] To achieve sustained delivery by limiting passive diffusion, therapeutic molecules are often either physically bound to a hydrogel or encapsulated in nano- or microparticles within a hydrogel. However, larger therapeutic molecules, such as proteins, may be directly encapsulated within hydrogels for localized and sustained delivery.

CD guest-host complexation has been explored for sustained release of hydrophobic molecules.[418] In addition, therapeutics such as nucleic acids can be modified with hydrophobic groups such as cholesterol to undergo guest-host complexation with CD.[419] The reversible physical association between CD and hydrophobic therapeutic molecules allows for sustained release over days to weeks. Injectable HA hydrogels with β-CD moieties have been engineered for sustained release of cholesterol-modified RNAs.[420,421] For example, shear-thinning, self-healing guest-host HA hydrogels were formed by mixing CDHA and AdHA, and cholesterol-modified miRNA formed guest-host inclusion complexes with unoccupied CD hosts.[420] The injectable hydrogel sustained the delivery of miRNA to promote cardiomyocyte proliferation in an *in vivo* mouse myocardial infarction (MI) model. In another approach, dynamic covalent HA hydrogels were formed by mixing hydrazide-modified HA (HA-ADH) with aldehyde-modified HA (HA-ALD).[421] The HA-ALD polymers also contained a CD (CD-ALD-HA) moiety for guest-host complexation with cholesterol-modified siRNA for sustained release. The hydrogel was used to deliver siRNA against MMP2 in order to prevent pathological remodeling in a rat MI model. Towards sustained release of small molecule drugs, Thi et al. engineered a tyramine-modified gelatin hydrogel that underwent enzymatic crosslinking in the presence of HRP/H_2O_2.[422] Oxidized β-CD molecules were encapsulated within the hydrogel, as well as model hydrophobic drugs including dexamethasone and curcumin. The hydrophobic model drugs formed inclusion complexes with the oxidized β-CD, and the aldehyde groups on the oxidized β-CD formed dynamic imine crosslinks with free amino groups on gelatin. Furthermore, incorporation of aldehyde groups from oxidized β-CD resulted in significantly increased tissue adhesion.

In another approach to achieve sustained delivery, therapeutics may be entrapped within a nanoparticle, which is then encapsulated in a biopolymer hydrogel. Segovia et al. developed a dextran-based hydrogel for nanoparticle-mediated delivery of siRNA as an anticancer therapeutic.[423] The siRNA formed nanoparticles by aggregation with poly(amidoamine) (PAMAM) dendrimers. The nanoparticles were mixed with oxidized dextran to form a dynamic covalent hydrogel with imine linkages between aldehyde groups on dextran and amine groups on the PAMAM nanoparticles. The hydrogel was used to deliver anti-luciferase siRNA in an *in vivo* xenograft mouse model of human breast cancer that contained luciferase-expressing tumor cells. Sustained release of anti-luciferase siRNA resulted in 70% luciferase silencing after 6 days, reaching improved transfection efficiencies

when compared to commercially available siRNA-delivery agents. In a later study, the injectable aldehyde-dextran hydrogel was used to complex miRNA-PAMAM nanoparticles for sustained release of miRNA, leading to nearly 90% tumor shrinkage 2 weeks post-implant in a triple negative murine breast cancer model.[424]

To facilitate sustained delivery, therapeutic molecules may be encapsulated in a hydrogel microparticle. Chen et al. demonstrated that interleukin 10 (IL-10) could be encapsulated in NorHA microgels formed in a microfluidic device by photo-mediated thiol-ene radical addition.[425] The NorHA microgels were incorporated into a CDHA-AdHA guest-host hydrogel to form a composite injectable delivery vehicle for sustained release of IL-10 in an *in vivo* rat MI model. Reduced macrophage density, as well as improved scar thickness and ejection fraction, were observed 4 weeks post-injection.

As an example of encapsulating and releasing molecules directly from hydrogels, Schirmer et al. engineered a heparin-based hydrogel for sustained delivery of interleukin 4 (IL-4) to facilitate improved wound healing.[426] The hydrogel was formed via Michael addition by mixing maleimide-modified heparin with thiol-modified 4-arm star PEG. Heparin was selected due to the physical complexation that occurs between heparin and IL-4. Heparin acts as a stabilizer to protect IL-4 against thermal and proteolytic degradation, resulting in more sustained delivery of the therapeutic. In an *in vitro* study, primary murine macrophages adopted the pro-wound healing M2 phenotype upon exposure to the heparin hydrogels containing IL-4. In another example of hydrogel-based protein delivery, Turabee et al. developed a novel polypeptide triblock copolymer that undergoes thermal- and pH-responsive gelation.[427] The polypeptide triblock copolymer contained a PEG block, a temperature-sensitive poly(γ-benzyl-L-glutamate) polypeptide block, and a pH-responsive oligo(sulfamethazine) block. The triblock copolymer formed a viscoelastic hydrogel upon subcutaneous injection into a rat model. Sustained release of lysozyme, a model protein, was demonstrated over a 1-week period *in vivo*.

7.3. Tissue adhesives and sealants

Hydrogel-based tissue adhesives and sealants have been researched for decades as alternatives to staples and sutures in order to promote improved wound closure and incision sealing.[428] These hydrogels must be able to adhere to wet surfaces, withstand dynamic forces, and exhibit biocompatibility.[428,429] Ideally, adhesives and sealants should be engineered to meet the needs of specific tissue and wound types.[429] Many tissue adhesives fabricated from biopolymers have had clinical success, such as Tisseel (fibrin), Evicel (fibrin), PreveLeak (BSA), BioGlue (BSA), and LifeSeal (gelatin).[429] Recent advances in hydrogels fabricated from chemically-modified biopolymers have furthered research into new tissue adhesives and sealants.

Adhesives and sealants fabricated from gelatin hydrogels show promise in research and clinical settings. It has been shown that photocrosslinked GelMA hydrogels can be used as tissue adhesives and sealants that exhibit a low inflammatory response, degrade quickly, and allow for rapid wound healing *in vivo*.[430] In an *in vivo* rat lung incision model, GelMA sealant was able to recover healthy lung burst pressures 7 days after application.[430] Tayafoghi et al. showed that combining GelMA with methacrylated alginate resulted

in an injectable, photo-crosslinkable tissue adhesive hydrogel.[431] The incorporation of an ionically crosslinked alginate network resulted in a 600% improvement in hydrogel toughness when compared to GelMA alone.[431] Wei et al. engineered a mussel-inspired gelatin adhesive hydrogel, where gelatin was modified with isothiocyanate-functionalized dopamine by thiocarbamate bond formation.[432] The resulting gelatin hydrogel contained both catechol and thiourea functional groups, where the thiourea groups crosslinked catechol moieties and also reduced oxidized quinone groups on catechol.[432] The mussel-inspired gelatin hydrogel achieved the same adhesion strength as commercially available Tisseel adhesives in a T-peel porcine pericardium tissue assay.[432]

Chemically-modified HA has also been researched for applications in tissue adhesives and sealants. Kim et al. engineered an injectable and sprayable HA-Tyr hydrogel tissue adhesive.[433] The hydrogel was biocompatible in an *in vivo* mouse subcutaneous model, and strong adhesion strengths were observed in an *ex vivo* mouse skin adhesion assay.[433] In another approach, Bermejo-Velasco et al. modified HA with either enolizable or non-enolizable aldehyde groups to form a tissue adhesive hydrogel.[434] Combining the two biopolymers resulted in rapid gelation due to the formation of aldol crosslinks.[434] Residual aldehydes remaining after crosslinking resulted in tissue adhesive properties, as demonstrated by successful bonding of bone tissues *ex vivo*.[434]

Other biopolymers are being explored for fabrication of tissue adhesives and sealants. Hong et al. recently demonstrated that alginate could be modified with boronic acid derivatives to undergo pH-responsive curing and adhesion.[435] The hydrogel showed strong adhesive strength to mouse intestinal tissue in an *ex vivo* study.[435] Azuma et al. demonstrated that methacrylated chitin combined with chitin nanofibers could achieve faster tissue adhesion than some commercially available tissue adhesives *in vivo*.[436] Annabi et al. engineered a methacrylated tropoelastin tissue adhesive from human recombinant tropoelastin.[437] The hydrogel successfully sealed rat arteries and lungs in an *in vivo* model.[437] Rat lungs sealed by the hydrogel exhibited higher burst pressure over lungs sealed with Evicel and Cosseal.[437] In another approach, Wang et al. fabricated an adhesive, photocrosslinkable hydrogel by modifying chitosan with both methacryloyl and catechol groups.[438] The hydrogel was explored for use as an antibacterial wound dressing.

7.4. Interpenetrating network biopolymer hydrogels

Most of the hydrogels discussed above consisted of single networks of biopolymers that are crosslinked together. Although different biopolymers may have been combined (e.g., CDHA and AdHA to form a guest-host network), the resulting hydrogel was formed from a single network. To build additional complexity into biopolymer hydrogel design, the development of interpenetrating polymer networks (IPNs) allows for further modulation of hydrogel behavior by combining properties of more than one network (Figure 18a). Utilizing IPNs increases the range of possible chemical, mechanical, and biological behaviors within a single hydrogel material. IPN hydrogels involve the combination of multiple independent polymer networks to form hydrogels, where the individual networks are inter-mixed, but not linked together. IPN hydrogels from biopolymers are reviewed in detail elsewhere.[439]

Biopolymers and synthetic polymers may be combined in an IPN hydrogel to take advantage of favorable properties from both networks. For example, Gan et al. engineered an IPN for nucleus pulposus replacement with both natural and synthetic polymer components.[440] The primary network consisted of oxidized dextran mixed with amine-modified gelatin crosslinked by imine bond formation, which allowed for hydrogel bioactivity. The secondary network consisted of photocrosslinkable acrylated PEG, which allowed for increased mechanical stability. In another approach, Zhao et al. combined a synthetic catechol-modified polymer network that crosslinked by metal-ligand coordination with UPy-modified gelatin, which crosslinked by quadruple hydrogen bonding, to create an adhesive wound dressing with fast shape adaptability and self-healing properties.[441] The catechol-modified synthetic polymer network allowed for strong adhesion, and the UPy-modified gelatin allowed for integration of biological signals and rapid self-healing behavior. As a last example, Abandansari et al. fabricated an IPN consisting of bis-maleimide-PEG, furan-modified gelatin (Gel-Furan), and Chitosan-g-Pluronic (CP) (Figure 18b).[442] The physically-crosslinked CP network allowed for immediate stabilization of the injected hydrogel as well as thermo-responsive behavior, while the Mal-PEG-Mal and Gel-Furan network allowed for long-term covalent stabilization due to Diels-Alder crosslinking after multiple hours. The hydrogel showed promise for use as a thermosensitive, injectable cell delivery vehicle.

IPNs can also be designed such that both networks are formed from biopolymers. For example, Suo et al. combined photocrosslinkable GelMA and physically crosslinked chitosan into a single network.[443] In another strategy, Chen et al. combined a modified collagen network crosslinked by azide-alkyne cycloaddition with a modified HA network crosslinked by Michael addition for use as an injectable, *in situ*-forming corneal defect filler.[444]

IPNs have been useful for the formation of complex stimuli-responsive hydrogels. In this approach, one network provides the responsiveness to stimuli, whereas the other network maintains hydrogel stability or controls hydrogel properties. For example, heparin was incorporated into IPN hydrogels with PNIPAAm to allow the formation of thermo-responsive hydrogels for the controlled release of growth factors.[445] As another example, protease-sensitive crosslinks can be introduced into one network of an IPN hydrogel to allow for enzyme-responsive behavior.[446,447]

One specific class of IPN hydrogels are double network (DN) hydrogels, which consist of two networks that have asymmetric and contrasting properties and where the molar concentration of the secondary network is often >20 times that of the primary network. The first network is sacrificial and brittle, while the second network is ductile, allowing for the formation of hydrogels with high strength and toughness. This is due to the protection of the secondary network from fracture by the first network via energy dissipation. Although not with biopolymers, the first reports of DN hydrogels were from Gong and colleagues.[448]

A wide range of biopolymers have been processed into DN hydrogels, using the chemical modifications described above. For example, HA has been modified with either catechol or methacrylate groups and processed into DN hydrogels, through mussel-inspired and

reversible Fe^{3+}-catechol interactions and free-radical chain polymerization, respectively.[449] In another strategy, Xiao et al. combined silk fibroin and methacrylated HA to fabricate a DN hydrogel for cell encapsulation.[450] In another example, chitosan networks were combined with GelMA networks to form DN hydrogels that were explored for cartilage repair.[443] There is significant potential to increase the diversity of properties in biopolymer hydrogels through the formation of IPNs.

8. Concluding remarks and future outlook

Our intent with this review was to motivate the use of biopolymers, their chemical modifications, and their crosslinking to form hydrogels for various biomedical applications. Numerous conjugation techniques have been utilized to introduce a range of chemical modifications onto biopolymers, where the type of modification, extent of modification, and concentration of components drive the resulting hydrogel properties. For example, we classify the types of crosslinking as covalent, dynamic covalent, and physical, with generally reduced mechanics and stability across these classifications.

Biopolymer hydrogels have been implemented in diverse biomedical applications, and we provide examples throughout on their use as cell culture substrates, scaffolds for tissue engineering, drug delivery vehicles, and tissue adhesives, where the selection of biopolymer and chemical modification drives the hydrogel utility. Evolution in biopolymer hydrogel complexity and control will advance their usefulness in these applications, as well as expand their utility into other applications. As one example, IPN biopolymer hydrogels are allowing for the combination of features of independent hydrogel networks. Further, the engineering of new polypeptides may introduce new properties that are not attainable with sourced biopolymers.

Despite these advances, we must pay attention to the balance between complexity and simplicity in biopolymer hydrogel design, particularly with regards to translational use. Bearing this in mind, there are a number of factors that should be considered when designing a chemically-modified biopolymer hydrogel for biomedical applications, particularly with translation in mind. For example, when selecting a biopolymer for biomedical hydrogel formation, one may consider whether cell adhesion sites are required for successful hydrogel function. If so, biopolymers such as HA, gelatin, and keratin that contain native cell adhesion sequences may be favorable. While cell adhesion peptides such as RGD may be easily conjugated to other biopolymers in a laboratory setting, such a step would require more elaborate processing on a larger scale, potentially hindering translation.

Batch variability is another important factor to consider when selecting a biopolymer for hydrogel formation. When extracting biopolymers from any natural source, either plant or animal, there is concern for batch-to-batch variability between sources. Biopolymers that can be mass-produced from microbial manufacturing, such as dextran, HA, and cellulose, may mitigate concerns over batch variability. In addition, one may consider the environmental sustainability of the biopolymer source. Biopolymers such as cellulose, chitin, keratin, and alginate are naturally abundant, providing plentiful green resources for hydrogel raw materials. In contrast, biopolymers isolated from animal sources are viewed

as less sustainable. For example, commercial gelatin is mostly isolated from porcine and bovine sources and relies heavily on production in the meat industry. There is growing concern over the unsustainable nature of meat production.[451] Thus, there is a need to develop alternate sustainable methods for manufacturing biopolymers that are traditionally obtained from animal sources.[452,453]

Another aspect to consider is the water solubility of the biopolymer. For example, some biopolymers such as cellulose, chitin, silk fibroin, and keratin are hydrophobic in their native states. Thus, extensive processing is required to create water-soluble biopolymer derivatives that can be processed into a hydrogel. While some of these processes have been successfully implemented on a large scale, there is growing concern over the environmental impact of these processes, motivating the need for the eco-friendly synthesis of water-soluble derivatives of such biopolymers.[454,455]

When selecting a chemical modification, it is important to consider the simplicity of the chemical modification process. For example, many reactions to functionalize hydrophilic biopolymers with methacrylates can be completed in a one-step aqueous reaction by addition of water-soluble molecules such as methacrylic anhydride or glycidyl methacrylate.[145,456] In contrast, the functionalization of hydrophilic biopolymers with hydrophobic groups, such as norbornenes or β-CD, currently require additional processing steps, which may hinder production scale-up.[322,457]

As another consideration, the crosslinking mechanism used must be evaluated for feasibility in the application of interest. For instance, covalent crosslinking techniques that require a radical photoinitiator may work well for the fabrication of implantable, pre-formed scaffolds. However, injectable hydrogels requiring the application of light within the body for hydrogel formation may raise concerns over cytotoxicity, considering the generation of free radicals and potential damage to native cells. As such, hydrogels formed by mixing two components (e.g., guest-host interactions, hydrazone formation) may be more favorable for applications requiring injection. In another approach, advanced delivery systems can be developed for *in situ* application of light in order to photocrosslink hydrogels.[458] However, translation of such an approach requires production scale-up of both the hydrogel and delivery device.

Storage and sterilization are important to consider in the design of biopolymer hydrogels for clinical translation. For example, temperature-sensitive biopolymers and crosslinking chemistries may require additional storage procedures. Further, the application of FDA-approved sterilization treatments, such as autoclaving and γ-irradiation, can have adverse effects on the biological and mechanical behavior of chemically modified hydrogel properties,[459] and thus, must be considered and characterized.

Most biopolymer-based hydrogels that have been implemented in clinical applications involve either unmodified biopolymers or very simple modifications and at low levels.[460] Chemical modifications introduce added scrutiny during translation. For successful clinical translation, it is important to consider that every component of a hydrogel system must be able to meet current good manufacturing practices (cGMP), including biopolymers,

crosslinkers, initiators, and encapsulated therapeutics.[460] Thus, when engineering a hydrogel for clinical translation, it is vital for a researcher to consider how each added complexity in a chemically-modified biopolymer hydrogel system is essential for successful hydrogel function.

With these considerations in mind, there is still great potential for the field of biomedical hydrogels to grow and succeed. In the past few decades, there have been nearly 30 injectable hydrogels that were FDA- and/or EMA-approved for clinical use.[460] Of those injectable hydrogel products, 11 contain HA, 7 contain collagen, 4 contain CMC, and 1 contains alginate.[460] Further, there are hundreds of hydrogels in clinical trial for biomedical applications ranging from injectable therapeutic delivery, wound dressings, regenerative medicine, and tissue sealants, many of which are formed from biopolymers.[460] Ultimately, the next decades are likely to see increased translation of new biopolymer hydrogels to clinical use.

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Jason A. Burdick, PhD is the Robert D. Bent Professor of Bioengineering at the University of Pennsylvania. Dr. Burdick's research involves the development of hydrogels through techniques such as photocrosslinking and self-assembly and their processing using approaches such as electrospinning and 3D printing. The applications of his research range from controlling stem cell differentiation through material cues to fabricating scaffolding for regenerative medicine and tissue repair. Jason currently has over 260 peer-reviewed publications and he is on the editorial boards of *Journal of Biomedical Materials Research A*, *Biofabrication*, and *Advanced Healthcare Materials*, and is an Associate Editor for *ACS Biomaterials Science & Engineering*. He has been recognized through numerous awards such as a Packard Fellowship in Science and Engineering, an American Heart Association

Established Investigator Award, the Clemson Award for Basic Science through the Society for Biomaterials, and the *Acta Biomaterialia* Silver Medal Award. Lastly, Jason has been elected as a Fellow to the American Institute for Medical and Biological Engineering and the National Academy of Inventors and has founded several companies to translate technology developed in his laboratory.

Glossary

Biopolymers
natural polymers that are sourced from animals and plants, including a wide range of polysaccharides (e.g., sugars) and polypeptides (e.g., proteins).

Click chemistry
a set of biocompatible chemical reactions that result in the rapid formation of covalent bonds.

Covalent crosslinking
the process of chemically linking polymer chains together via covalent bonds, primarily to form a polymer network.

Dynamic covalent crosslinking
a subset of crosslinking reactions that allow for the formation of reversible covalent crosslinks between biopolymers.

Free radical chain polymerization
a reaction in which either a polymer or crosslinks between polymers are formed via the propagation of radical species in the form of a growing kinetic chain.

Guest-host assembly
the formation of physical inclusion complexes between a “host” macrocycle with a hydrophobic interior and one or more hydrophobic “guest” moieties

Interpenetrating network (IPN)
the combination of multiple independent polymer networks, where the individual networks are inter-mixed, but not linked together.

Metal-ligand coordination
the formation of complexes consisting of a central metallic atom surrounded by bounded molecules (e.g., ligands).

Michael addition
an addition reaction that can occur readily between thiols (e.g., Michael donors) and electron-deficient enes (e.g., Michael acceptors) without the need for radical initiators.

Photocrosslinking
the use of light to facilitate a crosslinking reaction between polymers, primarily to form a polymer network.

Photoinitiator
a molecule that cleave in response to certain wavelengths of light, resulting in the generation of free radicals.

Physical crosslinking
the process of forming a polymer network by physical (e.g., non-chemical) interactions between polymer chains.

Abbreviations

Ad	adamantane
APS	ammonium persulfate
BSA	bovine serum albumin
CB[*n*]	cucurbit[*n*]uril
CD	cyclodextrin
CMC	Carboxymethyl cellulose
CS	chondroitin sulfate
DMAP	4-dimethylaminopyridine
DN	double network
DTT	dithiothreitol
ECM	extracellular matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
ELP	elastin-like peptide
GAG	glycosaminoglycan
GelMA	methacrylated gelatin
GMA	glycidyl methacrylate
HA	hyaluronic acid
HA-CA	catechol-modified hyaluronic acid
HA-Tyr	tyramine-modified hyaluronic acid
HOBt	hydroxybenzotriazole
HRP	horse radish peroxidase
I2959	Irgacure, 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone
IPN	interpenetrating network

LAP	lithium phenyl-2,4,6-trimethylbenzoylphosphinate
MeHA	methacrylated hyaluronic acid
MSCs	mesenchymal stromal cells
NHS	N-hydroxysuccinimide
NorHA	norbornene-modified hyaluronic acid
PEG	poly(ethylene glycol)
PEGDA	diacrylated poly(ethylene glycol)
PG	proteoglycan
PNIPAAm	poly(N-isopropylacrylamide)
TEMED	tetramethylethylenediamine
UPy	ureidopyrimidone
UV	ultraviolet
β-CD	β-cyclodextrin

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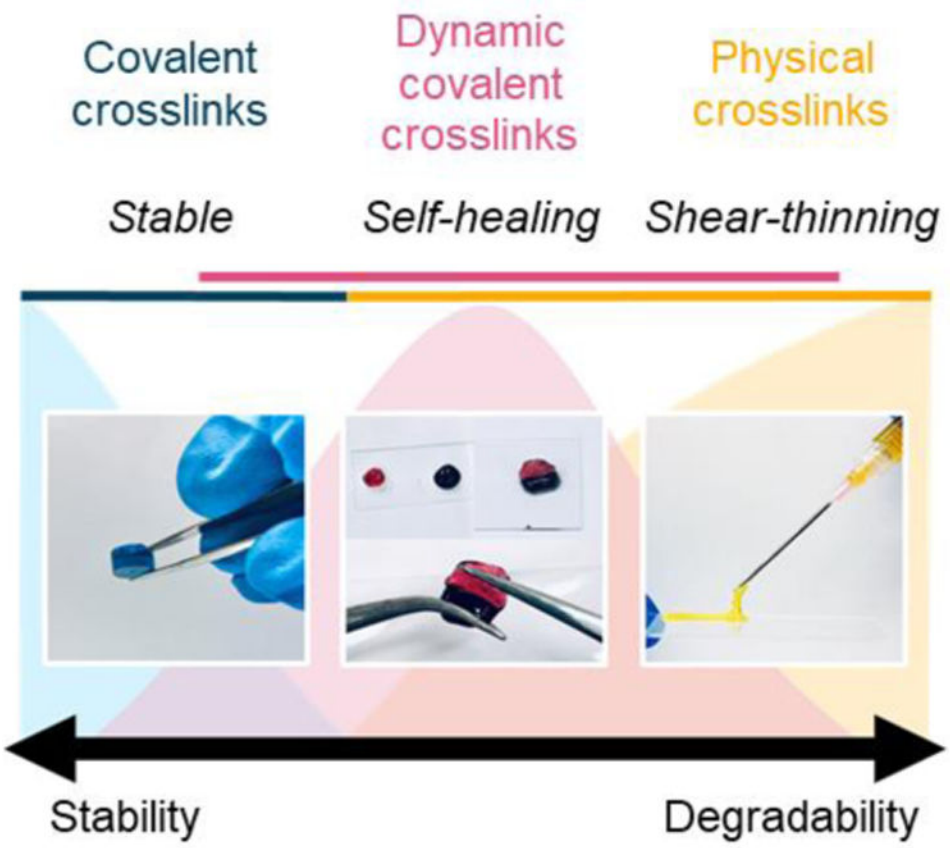


Figure 1. General hydrogel properties as a function of crosslink type.

Schematic illustrating representative images of hydrogels formed from different crosslinking mechanisms (i.e., covalent [blue], dynamic covalent [pink], physical [yellow]).

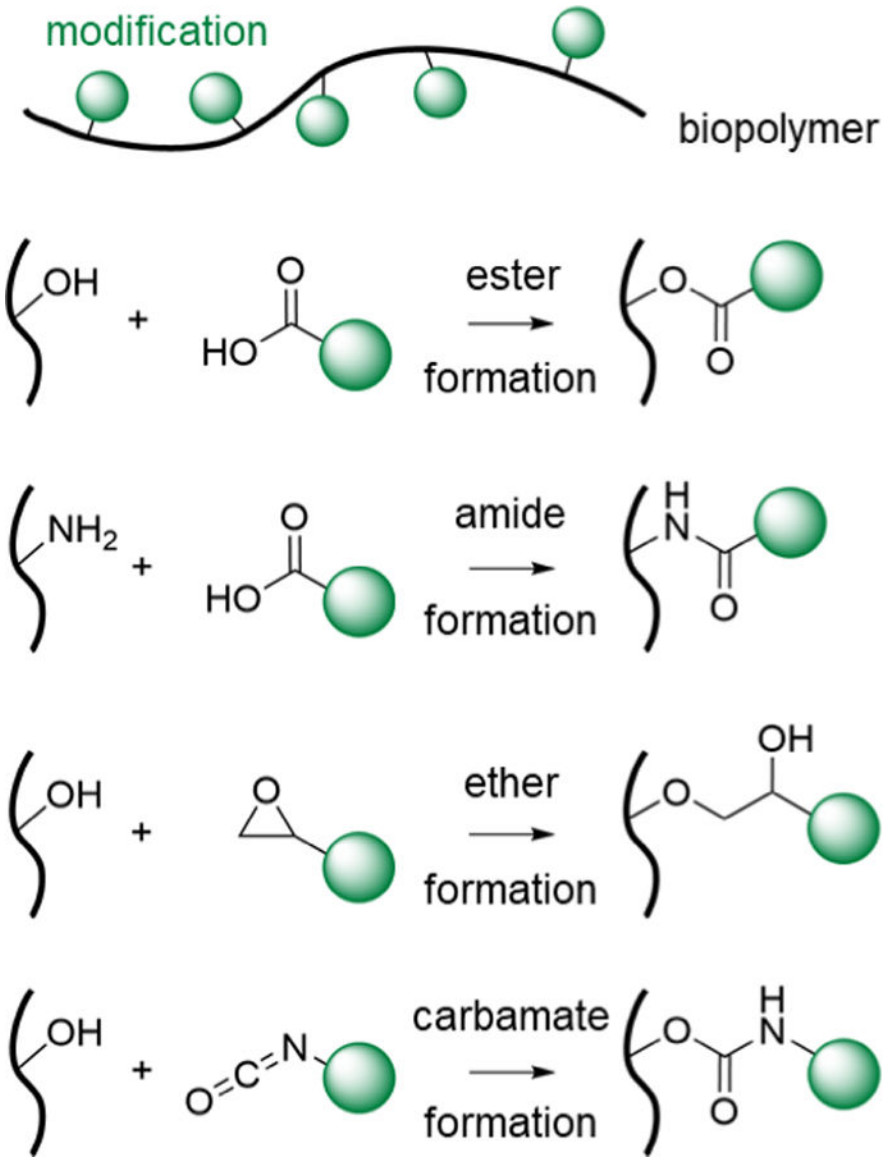


Figure 2. Common chemical reactions for modification of biopolymers.

Schematic representation of chemically modifying biopolymers using common mechanisms. From top to bottom: esterification, amidation, etherification, and carbamate formation. The green circle denotes various chemical groups introduced onto biopolymers for potential use in hydrogel formation.

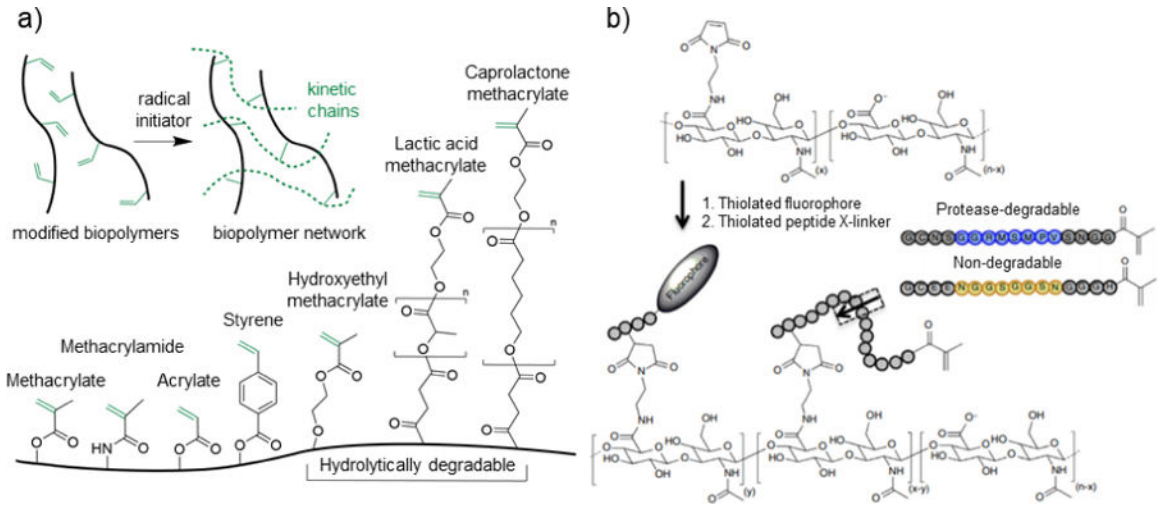


Figure 3. Crosslinking via free radical chain polymerization.

a) Schematic representation of the general crosslinking of modified biopolymers in the presence of an initiator to induce the formation of kinetic chains through the propagation of radical species (top), as well as common reactive groups used for biopolymer modification and hydrogel formation (bottom). b) Hyaluronic acid (HA) modified with maleimide groups to react with thiolated fluorophores and thiolated protease-degradable peptides capped with methacrylate groups for free radical chain polymerization. Peptide sequences are designed to be either protease degradable (blue) or non-degradable (yellow). Adapted with permission from Wade, et al.[167] Copyright, 2015 Springer Nature.

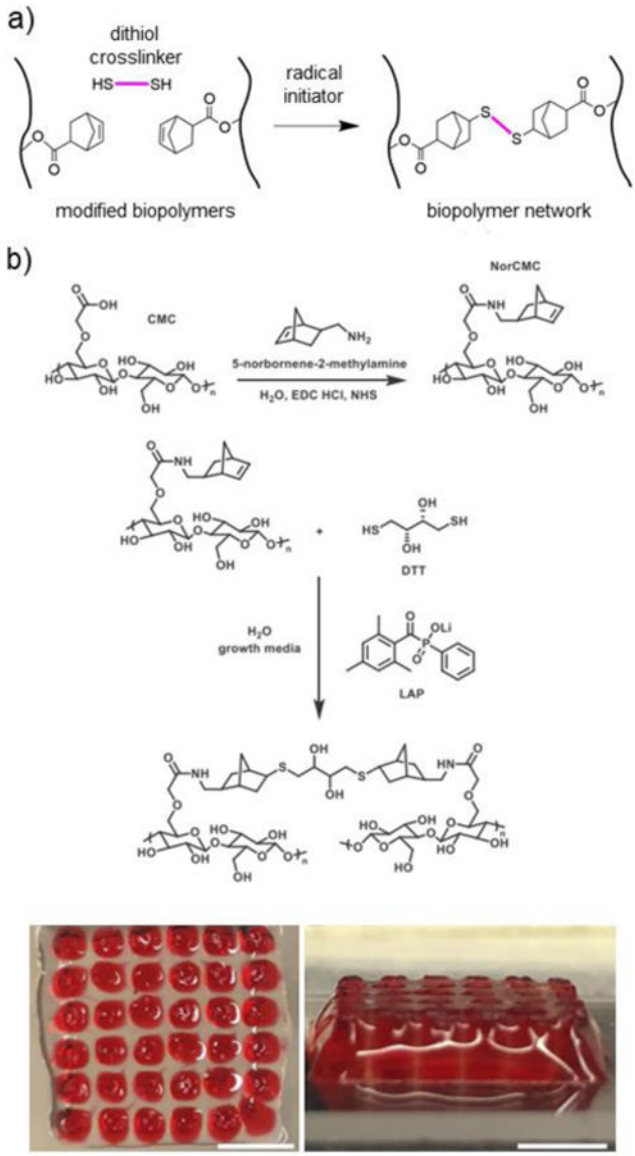


Figure 4. Crosslinking via a thiol-ene radical addition.

a) Schematic representation of norbornene-modified biopolymers (black) crosslinked with a dithiol crosslinker (pink) in the presence of a radical initiator. **b)** Thiol-norbornene crosslinked CMC hydrogels for bioprinting, showing (top) schematic representation of amidation reaction to synthesize norbornene-modified CMC (NorCMC), (middle) schematic representation of photocrosslinking reaction, and (bottom) bioprinted NorCMC scaffolds (clear) filled with Pluronic (red). Scale bars represent 5mm. Adapted with permission from Ji, et al.[182] Copyright, 2020 Elsevier.

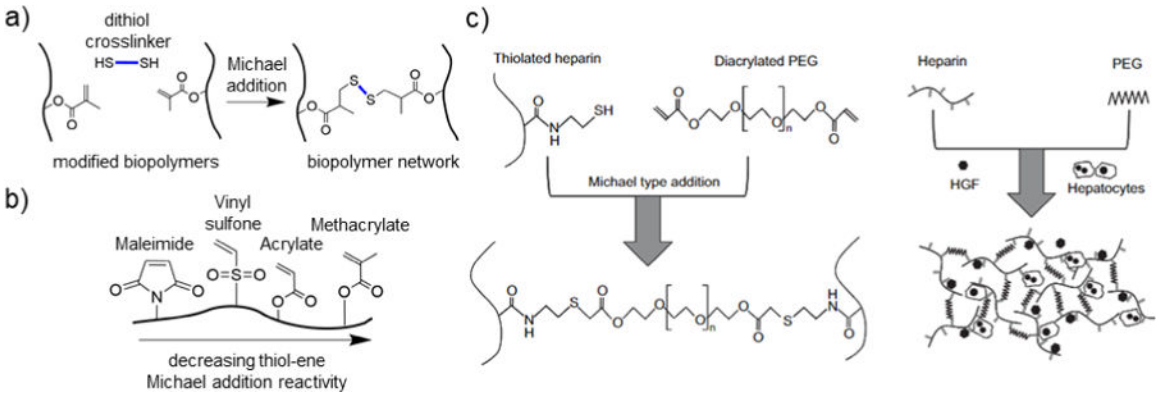


Figure 5. Crosslinking via thiol-ene Michael addition.

a) Schematic representation of methacrylate-modified biopolymers (black) crosslinked with a dithiol crosslinker (blue) under Michael addition conditions. **b)** Schematic representation of a biopolymer modified with multiple ene groups that can undergo thiol-ene Michael addition. From left to right, in decreasing order of Michael addition reactivity: maleimide, vinyl sulfone, acrylate, and methacrylate. **c)** Thiolated heparin is crosslinked with diacrylated PEG (PEGDA) via a thiol-ene Michael addition reaction, which was used for the culture of primary rat hepatocytes and hepatocyte growth factor (HGF). Adapted with permission from Kim, et al.[205] Copyright, 2010 Elsevier.

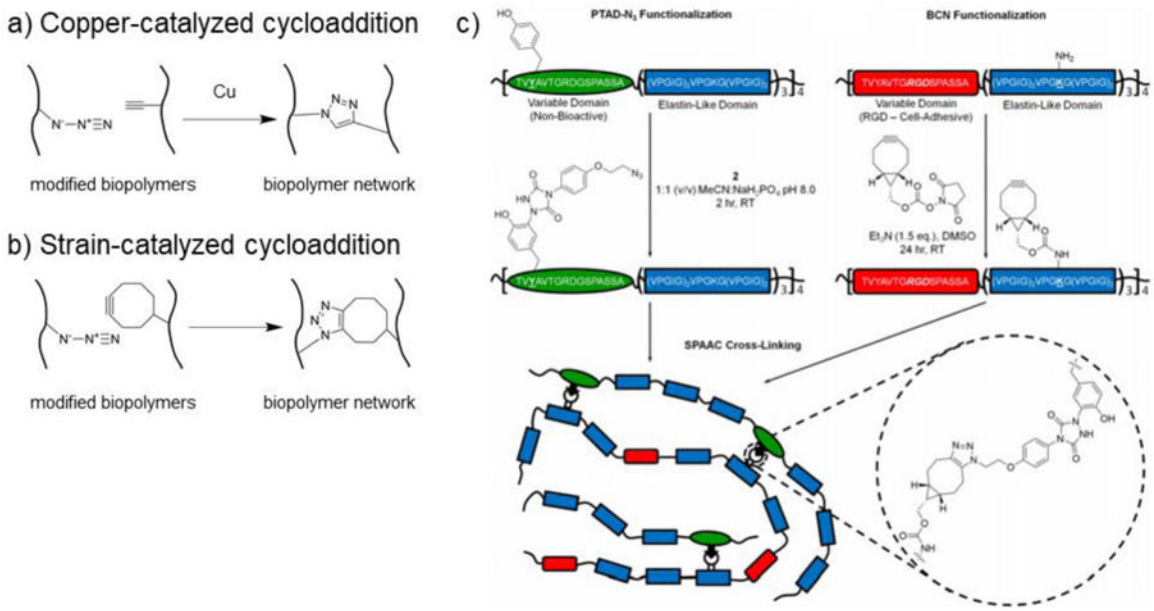


Figure 6. Crosslinking via azide-alkyne cycloaddition.

a) Schematic representation of copper-catalyzed azide-alkyne cycloaddition crosslinking of biopolymers. Biopolymers are modified with either azide or alkyne functional groups and upon combination in the presence of a copper catalyst, crosslinks form by azide-alkyne cycloaddition. **b)** Schematic representation of strain-catalyzed azide-alkyne cycloaddition crosslinking of biopolymers. Biopolymers are modified with either azide or strained alkyne (i.e., cyclooctyne) groups and upon combination, crosslinks form by azide-alkyne cycloaddition. **c)** Elastin-like polypeptides (ELPs) functionalized with either azide or bicyclononyne (BCN) groups for bio-orthogonal crosslinking due to the strain-promoted [3+2] azide-alkyne cycloaddition (SPAAC) reaction. Adapted with permission from Madl, et al.[109] Copyright, 2017 American Chemical Society.

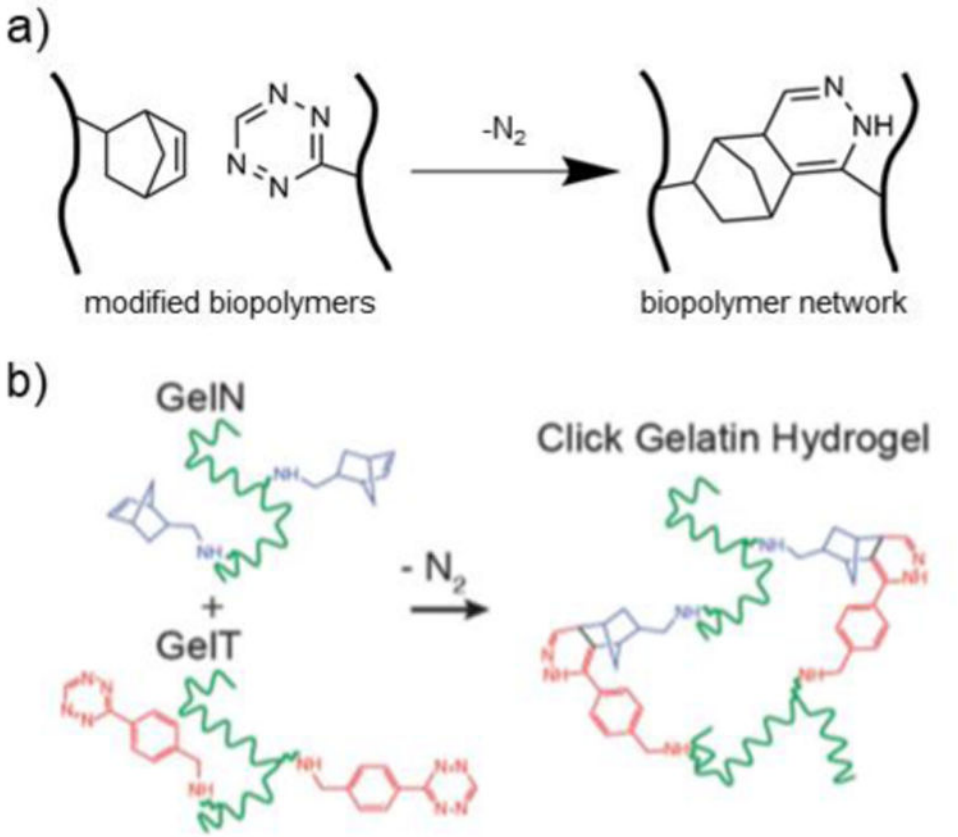


Figure 7. Crosslinking via tetrazine-norbornene reactions.

a) Schematic representation of biopolymers modified with either norbornene or tetrazine groups. Upon combination, crosslinks form by a tetrazine-norbornene reaction. **b)** Gelatin modified with either norbornene (GelN) or tetrazine (GelT) are mixed to form a tetrazine-norbornene click biopolymer network, which was cell-adhesive and degradable for use in cell encapsulation. Adapted with permission from Koshy, et al.[223] Copyright 2016, Wiley.

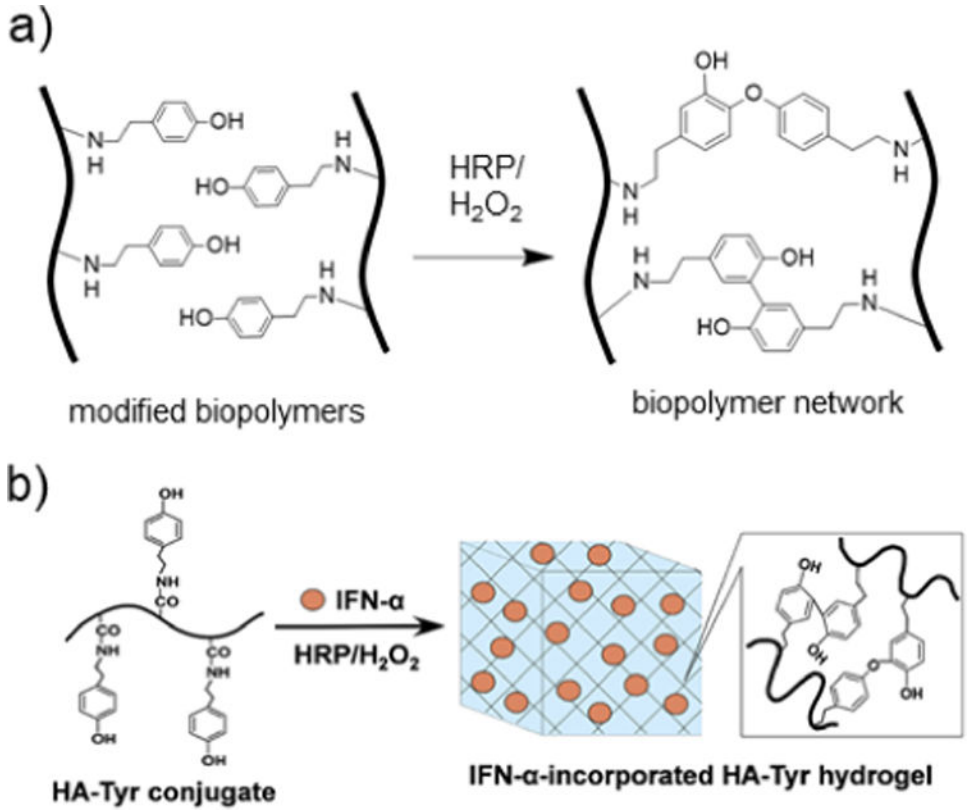


Figure 8. Crosslinking via tyramine enzymatic reactions.

a) Schematic representation of biopolymers modified with tyramine that crosslink in the presence of HRP and H_2O_2 to form dityramine adducts. **b)** HA is modified with tyramine and subsequently exposed to horse radish peroxidase (HRP) and H_2O_2 to undergo enzymatic crosslinking by oxidation of tyramine groups, forming covalent dityramine adducts. Interferon-α (IFN-α) is encapsulated in the hydrogel for use as a prolonged-release delivery vehicle for renal carcinoma treatment. Adapted with permission from Ueda, et al.[231] Copyright, 2016 Elsevier.

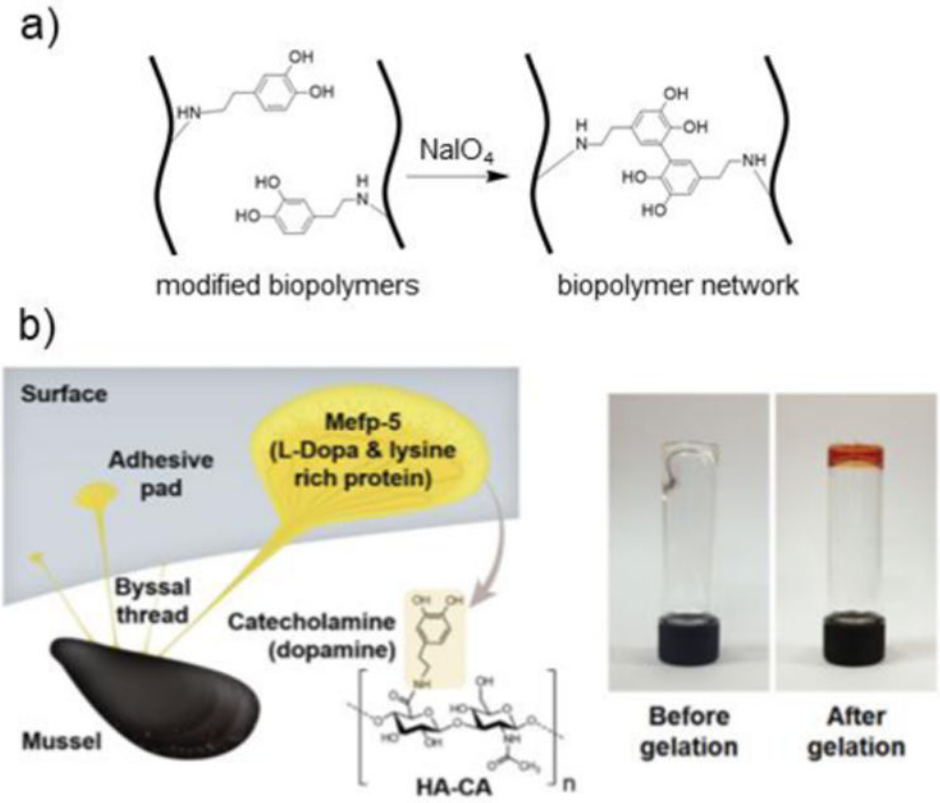


Figure 9. Crosslinking via catechol reactions.

a) Schematic representation of biopolymers modified with catechol and crosslinking in the presence of $NaIO_4$ to form dicatechol adducts. **b)** A mussel-inspired, HA hydrogel is formed by modifying HA with catechol moieties (HA-CA). HA-CA is covalently crosslinked in the presence of sodium periodate ($NaIO_4$). Image shows HA-CA hydrogel before (clear) and after (red) gelation. Adapted with permission from Shin, et al.[251] Copyright, 2015 Wiley.

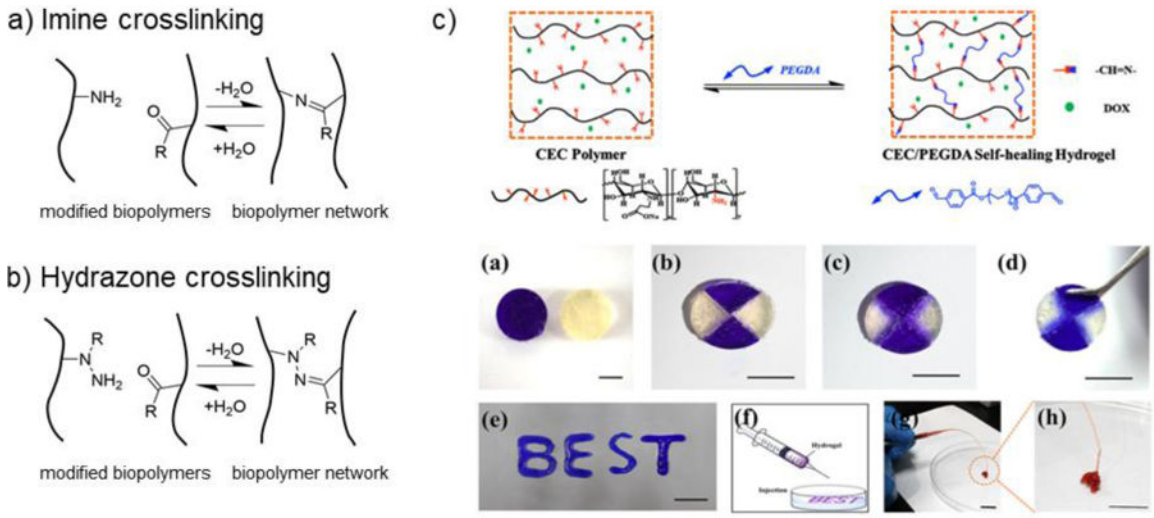


Figure 10. Crosslinking via Schiff base formation.

a) Schematic representation of imine dynamic covalent crosslinking by combining biopolymers modified with either primary amine or aldehyde groups. **b)** Schematic representation of hydrazone dynamic covalent crosslinking by combining biopolymers modified with either hydrazide or aldehyde groups. **c)** *N*-carboxyethyl chitosan (CEC) is combined with dialdehyde PEG (PEGDA, blue), where imine dynamic covalent crosslinks are formed between the amine groups on CEC and the aldehyde groups on PEGDA. Images show the self-healing (a-d) and shear-thinning (e-h) properties of the hydrogel. Adapted from Qu, et al.[258] Copyright, 2017 Elsevier.

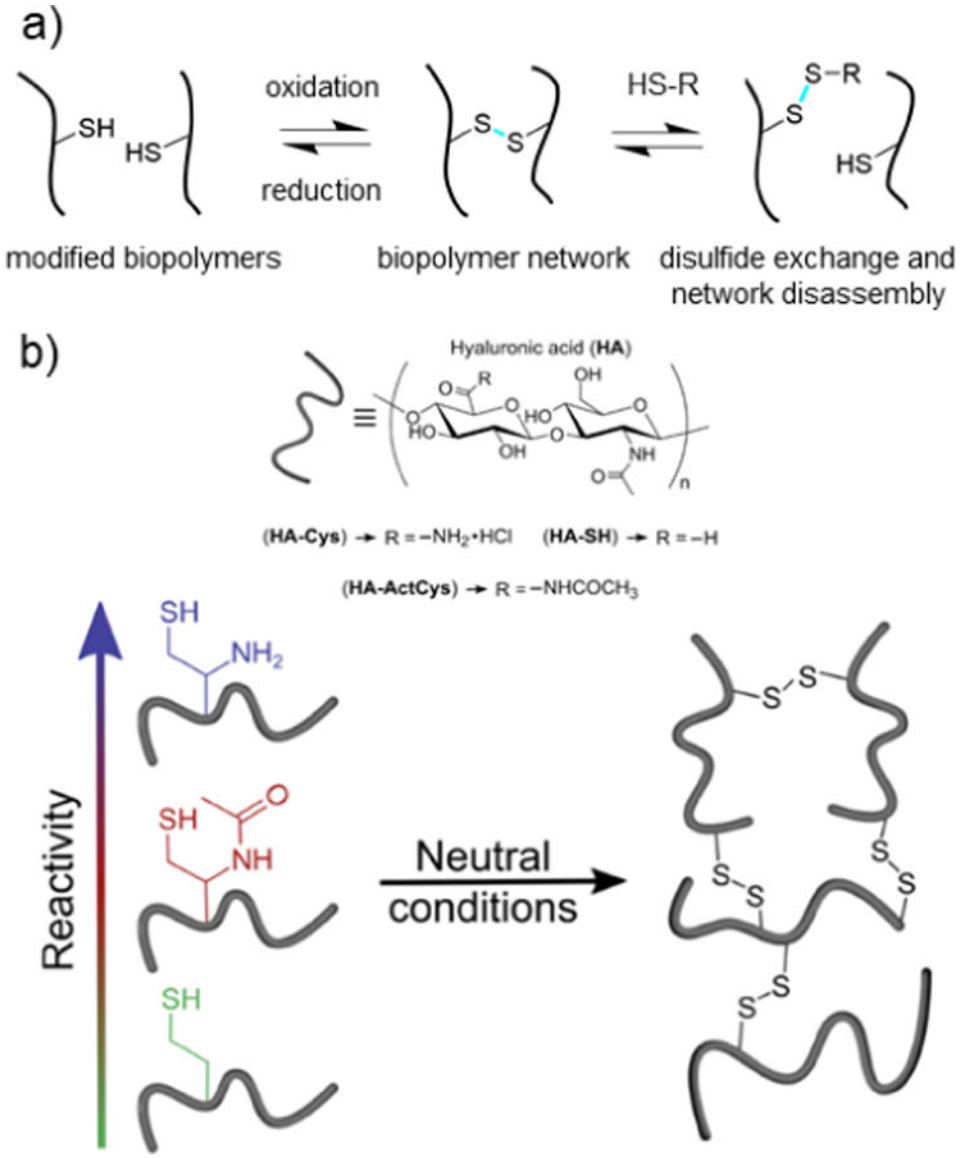


Figure 11. Crosslinking via disulfide bond formation.

a) Schematic representation of thiolated biopolymers forming dynamic covalent crosslinks by disulfide bond formation (blue) under oxidative conditions. Upon the addition of a mono-thiolated component, disulfide exchange can result in disassembly of the hydrogel. **b)** Various thiolated HA biopolymers are synthesized for disulfide dynamic covalent crosslinking, including: HA-thiol (HA-SH, green), HA-acetyl cysteine (HA-ActCys, red), and HA-cysteine (HA-Cys, blue). Among the thiol groups, HA-Cys has the strongest electron-withdrawing group in the β-position, resulting in the most disulfide bond formation under neutral conditions. Adapted with permission from Bermejo-Velasco, et al.[280] Copyright, 2019 American Chemical Society.

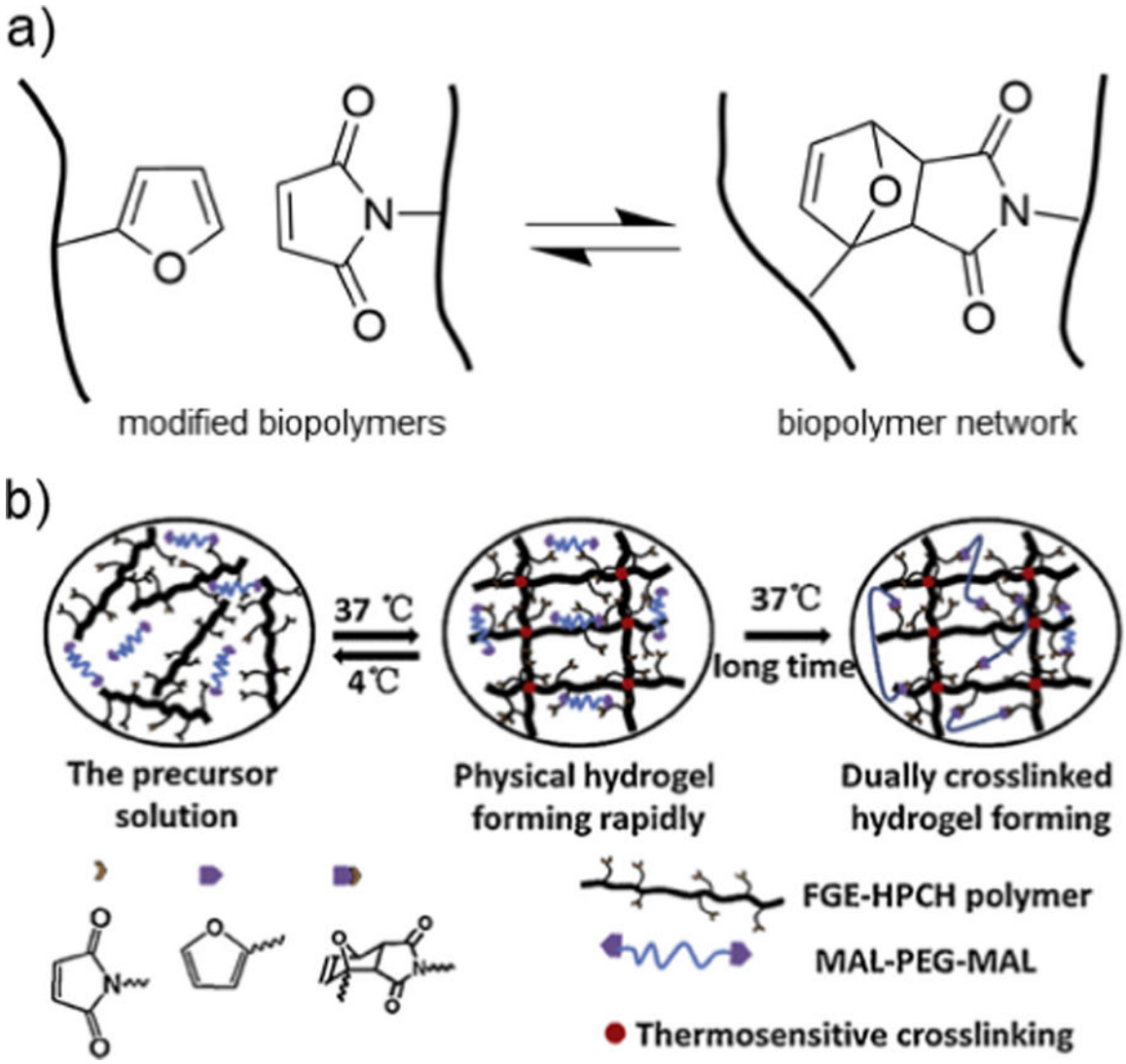


Figure 12. Crosslinking via reversible Diels-Alder reactions.

a) Schematic representation of dynamic covalent crosslinks formed by combining biopolymers modified with either furan or maleimide groups. **b)** Hydroxypropyl chitin (HPC, black) modified with furan groups and combined with PEG-bismaleimide crosslinks for hydrogel formation. Immediately, a thermo-responsive physical hydrogel forms due to interactions between HPC molecules, and over time, reversible Diels-Alder crosslinks form to stabilize the hydrogel structure. Adapted from Bi, et al.[296] Copyright, 2019 Elsevier.

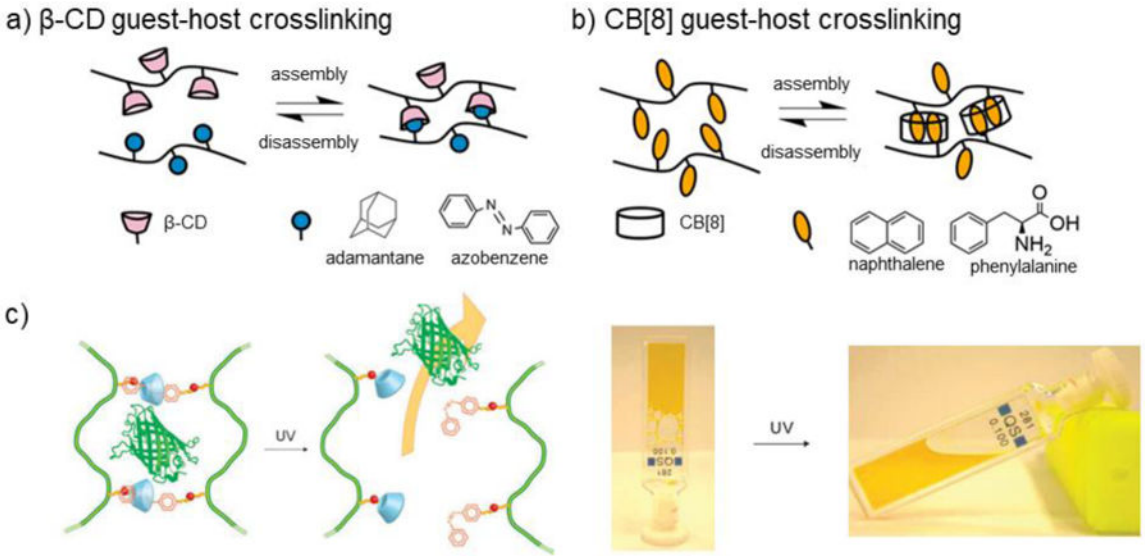


Figure 13. Crosslinking via guest-host complexation.

a) Schematic representation of biopolymers (black) modified with either host β-cyclodextrin (β-CD, pink) or guest (blue) functional groups to undergo reversible crosslinking due to guest-host complexation. Common guest groups for β-CD include adamantane (Ad) and azobenzene (Az). **b)** Schematic representation of biopolymers (black) modified with guest groups (yellow) and combined with host cucurbit[8]uril (CB[8]) to undergo reversible crosslinking due to guest-host complexation. CB[8] has a large host cavity to accommodate two guest groups, which commonly include naphthalene and phenylalanine. **c)** Dextran (green) is modified with either β-CD (blue) or *trans* Az (red). Upon mixing, hydrogel formation occurs due to guest-host complexation between β-CD and *trans* Az. Upon exposure to UV light, Az groups convert from *trans* to *cis* state, resulting in hydrogel disassembly and photoresponsive release of encapsulated proteins. Adapted from Peng, et al.[123] Copyright, 2010, Royal Society of Chemistry.

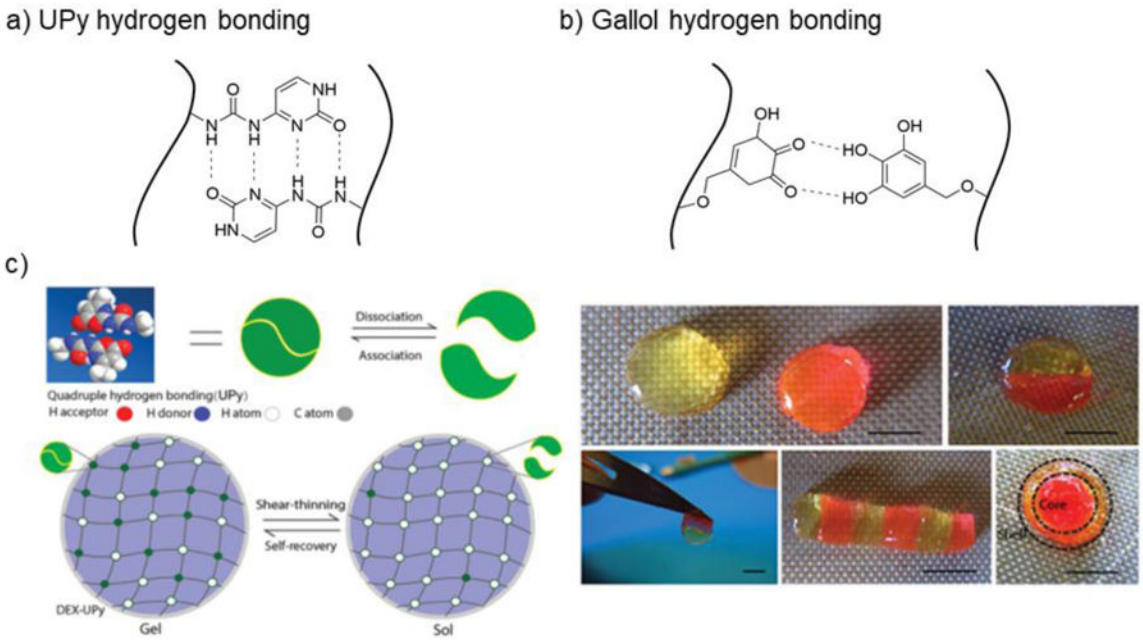


Figure 14. Crosslinking via hydrogen bonding.

a) Schematic representation of biopolymers modified with ureidopyrimidone (UPy) and crosslinking due to hydrogen bonding. **b)** Schematic representation of biopolymers modified with gallol groups and crosslinking due to hydrogen bonding. **c)** Dextran is modified with UPy (Dex-UPy) to undergo hydrogel formation due to hydrogen bonding. The resulting hydrogel is shear-thinning and self-healing. Images show self-healing behavior of Dex-UPy hydrogels. Adapted with permission form Hou, et al.[340] Copyright, 2015 Wiley.

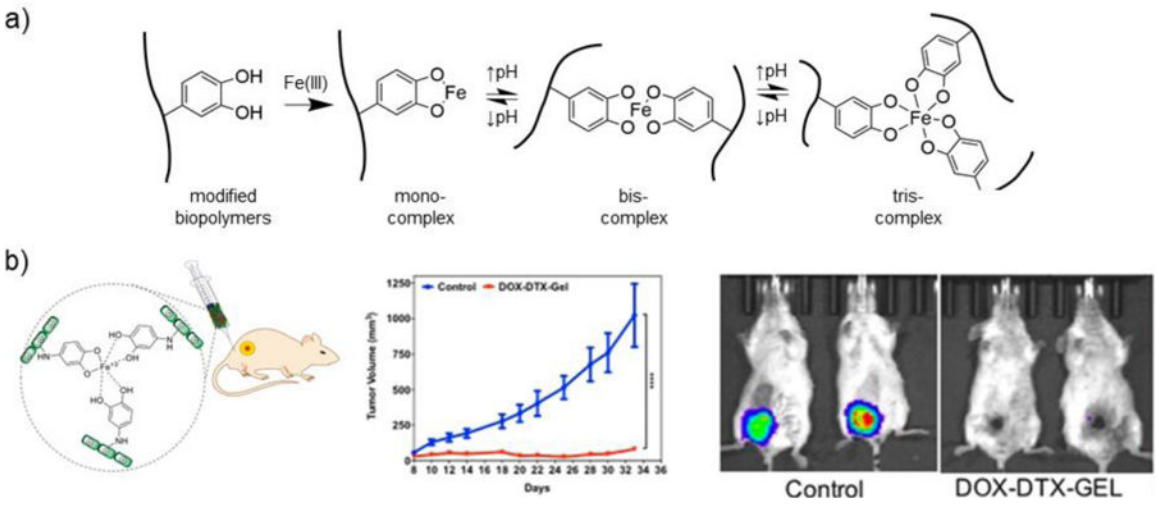


Figure 15. Crosslinking via metal-ligand complexation.

a) Schematic representation of biopolymers modified with catechol groups forming metal-ligand complexes with Fe(III). As pH increases, bis- and tris-complexation occurs, resulting in crosslink formation. **b)** Chitosan (green) is modified with catechol groups and forms a hydrogel metal-ligand complexation with Fe(III). The injectable hydrogel was used as a localized delivery vehicle for multiple chemotherapeutics. An increase in median survival rate was observed in murine lung and breast cancer models upon localized delivery of anticancer drugs from the hydrogel. Adapted with permission from Yavvari, et al.[355] Copyright, 2017 American Chemical Society.

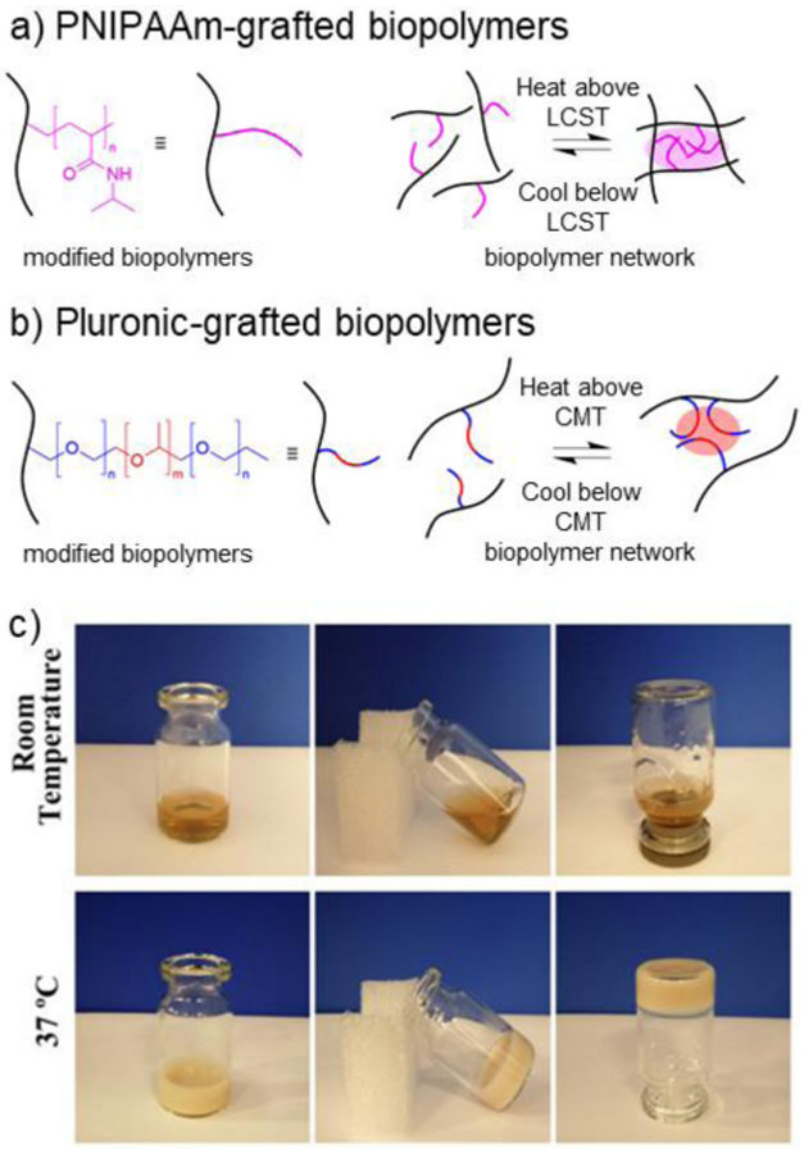


Figure 16. Crosslinking via interactions between synthetic polymers grafted to biopolymers.
a) Schematic representation of poly(N-isopropylacrylamide) (PNIPAAm, pink) grafted to a biopolymer (black) (left). The grafted biopolymer can undergo reversible physical crosslinking above the lower critical solution temperature (LCST) (~30°C) of PNIPAAm due to hydrophobic interactions between PNIPAAm groups (right). **b)** Schematic representation of Pluronic grafted to a biopolymer (black). Pluronic is an A-B-A triblock copolymer consisting of poly(ethylene glycol) (PEG) blocks (blue) and poly(propylene glycol) (PPG) blocks (red) (left). The grafted biopolymer can undergo reversible physical crosslinking above the critical micelle temperature (CMT) (~25–40°C) of Pluronic due to hydrophobic interactions between PPG blocks. (right) **c)** A biopolymer of PNIPAAm grafted to keratin (keratin-g-PNIPAAm) exhibited an LCST around 28–32°C, resulting in thermo-responsive hydrogel formation due to hydrophobic interactions between PNIPAAm

groups. Keratin-g-PNIPAAm was explored for applications in brain injury repair. Adapted with permission from Zhu, et al.[366] Copyright 2019, Elsevier.

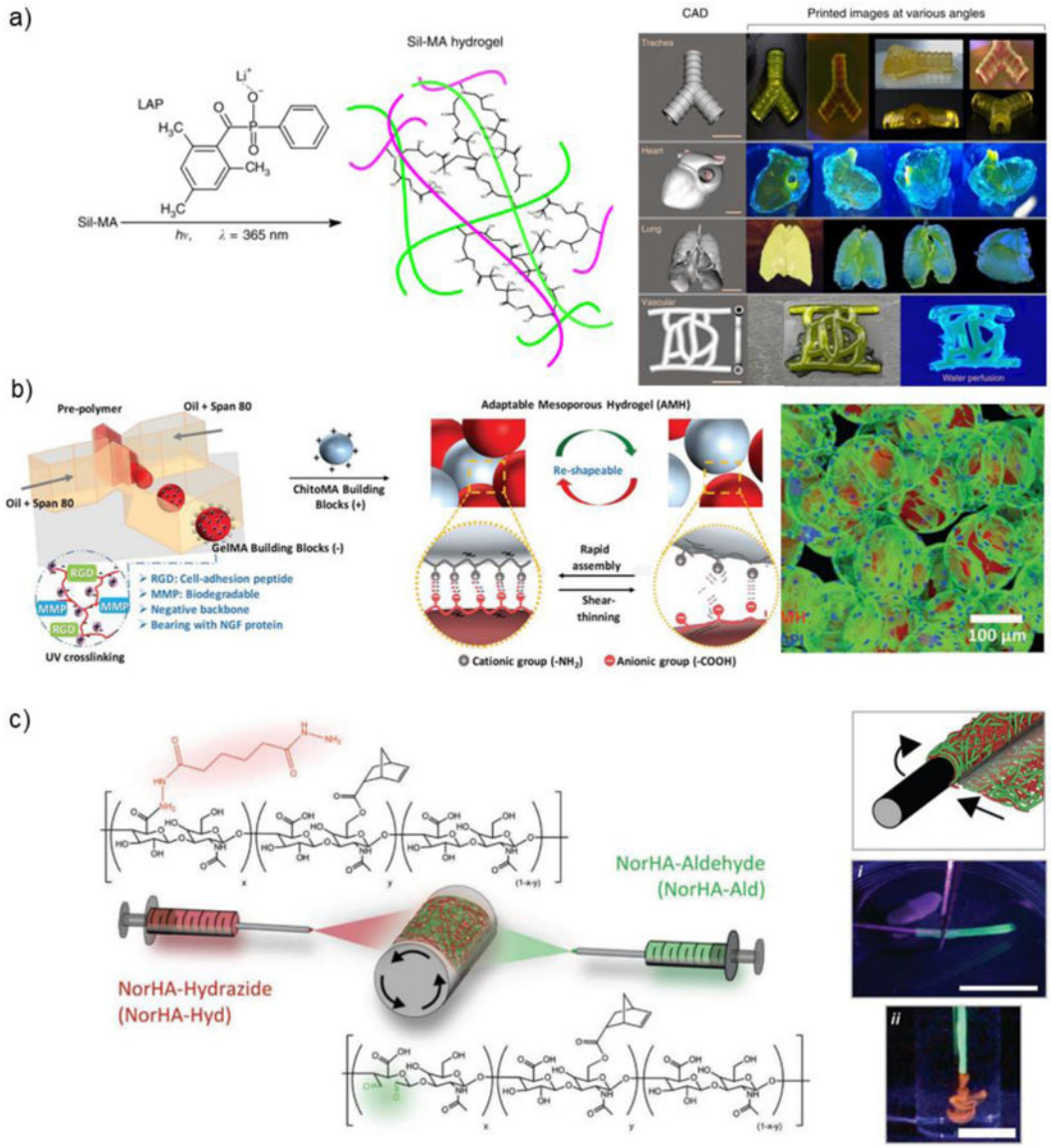


Figure 17: Representative examples of biofabricated hydrogel scaffolds made from chemically-modified biopolymers.

a) Silk modified with GMA (Sil-MA) is photocrosslinked using LAP as a photoinitiator (left). Scaffolds that mimic the shape of trachea, heart, lung, and vessel are printed using dynamic light processing (DLP) (right). Adapted with permission from Kim et al.[389] Copyright, 2018 Springer Nature. **b)** GelMA (red) and ChitoMA (gray) microgels are fabricated using a microfluidic device and then mixed to form a self-healing granular hydrogel scaffold with ionic interparticle interactions. The scaffold is combined with human adipose-derived stem cells (hADSCs) to form a cell-laden network. Adapted with permission from Hsu, et al.[401] Copyright 2019, Wiley. **c)** HA modified with norbornene (NorHA) and either hydrazides (NorHA-Hyd, red) or aldehydes (NorHA-Ald, green) are electrospun to create a multifiber network with dynamic covalent inter-fiber crosslinks (left). Luminal scaffolds are created by wrapping the multifiber network around a needle and visualized i) while removing the scaffold from the needle and ii) while extruding rhodamine-

labeled dextran dye through the lumen. Adapted with permission from Davidson et al.[415] Copyright, 2020 Wiley.

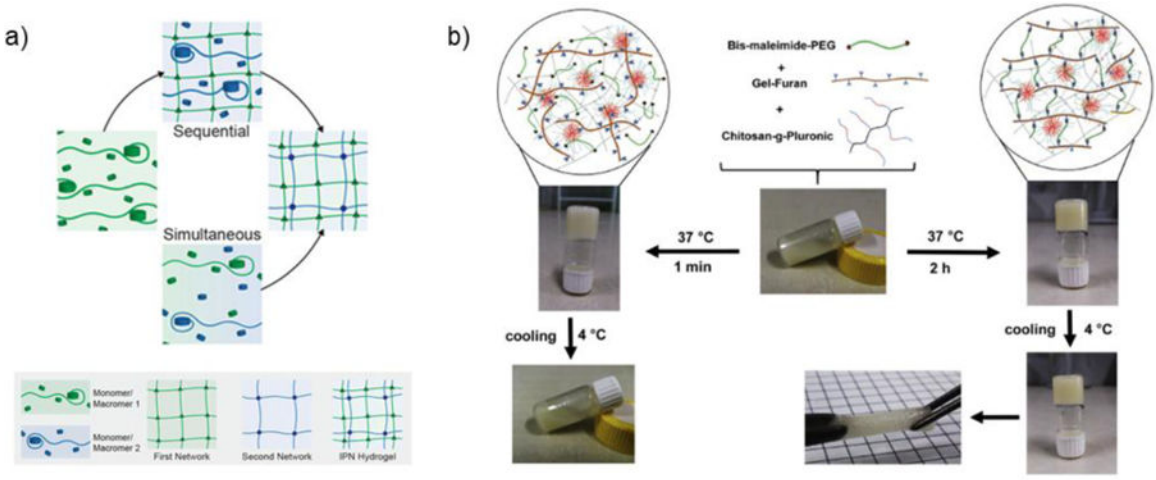


Figure 18. Interpenetrating network (IPN) biopolymer hydrogels.

a) IPN hydrogels are formed through various synthesis techniques, including the sequential (swelling of first network in a secondary monomer/macromer) or simultaneous (orthogonal crosslinking of both first and second networks) introduction of networks. Adapted with permission from Dhand, et al.[439] Copyright 2020, Elsevier. b) An IPN is formed by combining bis-maleimide-PEG, furan-modified gelatin (Gel-Furan), and chitosan grafted with Pluronic F127 (Chitosan-g-Pluronic). Initially, Chitosan-g-Pluronic formed a physically crosslinked, thermosensitive hydrogel network. After 2 h, Diels-Alder crosslinks between bis-maleimide-PEG and Gel-Furan covalently stabilize the hydrogel. Adapted with permission from Abandansari, et al.[442] Copyright 2018, Elsevier.

Table 1.

Polysaccharide-based biopolymers.

Representative chemical structures, function, and sources of various polysaccharide biopolymers that have been modified to form biomedical hydrogels.

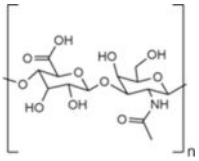
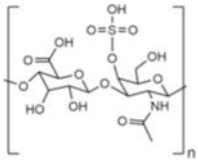
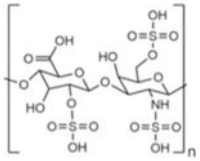
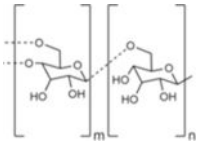
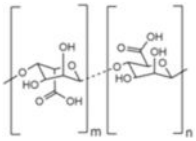
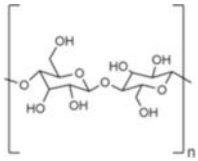
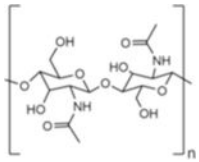
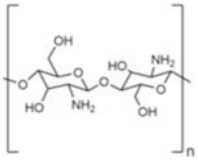
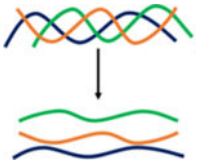
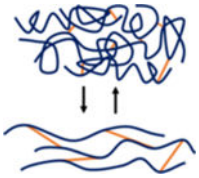
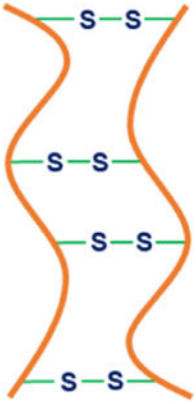
	Polysaccharides			
Name	Hyaluronic Acid (HA)	Chondroitin Sulfate (CS)	Heparin Sulfate (HS)	Dextran
Chemical structure				
Type	Linear GAG	Linear GAG	Linear GAG	Branched
Native function	Major component of ECM in human connective tissue	Major component of ECM in human connective tissue	Stored in mast cells in humans and secreted into vasculature upon injury	Component of some bacterial ECM
Source for biomedical applications	Bacterial culture; rooster comb[19]	Animal sources (e.g., bovine trachea, porcine nasal septa)[29]	Animal sources (e.g., porcine intestines)[34]	Bacterial culture[39]
Name	Alginate	Cellulose	Chitin	Chitosan
Chemical structure				
Type	Linear	Linear	Linear	Linear
Native function	Structural support in seaweed	Structural support in plant cell walls; component in some bacterial ECM	Structural support in insect and crustacean exoskeletons	n/a
Source for biomedical applications	Brown algae[42]	Bacterial culture[49]	Shrimp and crab shells from food industry waste products[56]	Deacetylated derivative of chitin[57]

Table 2.

Polypeptide-based biopolymers.

Representative structures, function, and sources of various polypeptide biopolymers that have been modified to form biomedical hydrogels.

	Polypeptides				
Name	Gelatin	Silk Fibroin	Albumin	Elastin	Keratin
Structure					
	Contains RGD motif for cell adhesion	Contains sequences that form crystalline β-sheets	Heart shaped, globular protein	Contains hydrophobic domains (i.e., VPGXG) for mechanical resilience	Contains high cysteine content for disulfide bond formation
Native function	Denatured derivative of collagen (structural component of mammalian ECM)	Structural component of silk fibers produced by some arthropods	Modulates fluid distribution and provides oncotic pressure in blood plasma	Responsible for recoiling response when stress is applied in vertebrate connective tissue	Structural and protective component of hard tissues such as skin, hair, nails, horns, wool, feathers, and hooves
Source for biomedical applications	Animal sources (e.g., porcine skin)[63]	*Bombyx mori* (silkworm)[73]	Human (HSA) or bovine (BSA) blood plasma[82]	Human donors or animal sources (e.g., bovine, murine)[91]	Human hair,[98] animal sources (e.g., feathers, wool)[99,100]