Chapter 2
Crosslinking Strategies to Develop Hydrogels for Biomedical Applications

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Abstract Hydrogels can be defined as the networks of either chemically or physically crosslinked hydrophilic polymers containing large amounts of water when hydrated. They are usually used as biomaterials for various applications in the biomedical field. These applications vary from 3D cell culture and drug delivery to tissue engineering and regenerative medicine. The most important step in the development of hydrogel-based biomaterials is to make them stable under application conditions. Crosslinking of polymer chains using various approaches is utilized to stabilize the hydrogels and make them appropriate biomaterials. Mechanical and swelling characteristics of the developed materials mainly depend on the crosslinking density. Depending upon the specific requirements, different crosslinking strategies can be adopted. This chapter covers the available methods of crosslinking of polymers and preparing hydrogels. It also provides some of the advantages and disadvantages of each approach along with potential applications.

1 Introduction

Hydrogels are networks of crosslinked hydrophilic polymers with high water content when hydrated [1, 2]. They are extensively used in industrial applications including fast moving consumer goods (FMCG), cosmetics and biomaterials [3]. Due to their versatile properties and their high water-absorbing capacity, biocompatibility, temperature resistance, and sensitivity, they are used in various biomedical applications [4, 5] such as drug delivery [6, 7], wound dressings [8, 9], regenerative medicine and tissue engineering [10, 11].

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Hydrogels are formed when the hydrophilic groups or segments in the polymeric network are hydrated in aqueous solutions [2]. In order to prevent the dissolution of the hydrophilic group in water or body fluid, crosslinking must be introduced between the polymer chains. Many different methods have been developed to achieve the effective crosslinking of polymers and generate hydrogels, which include physical, chemical, and natural methods. The degree of the crosslinking plays a key role in determining the physical properties of the developed hydrogel. A non-crosslinked hydrophilic polymer dissolved in solution will show a low viscosity; a limited crosslinking will result in an elastomer, whereas highly crosslinked polymers will be much more rigid [12, 13].

Biomedical applications usually require materials with sufficient mechanical properties and adequate stability in aqueous and physiological environments. Achieving such mechanical robustness while being biocompatible and biodegradable (in most cases such as tissue engineering) remains as one of the major challenges in the use of biopolymeric materials [14]. Crosslinking became the main solution to overcome this issue, as it improves the mechanical properties as well as the stability of the polymeric network by interconnecting individual macromolecular chains, and increasing the molecular weight [15]. Biodegradability is a key property that determines the application potential of many hydrogels used for therapeutic and biomedical applications [16]. To achieve this, often a labile bond which can be broken under physiological condition by enzymatic or chemical means is introduced between the polymer side chains or in the backbone of the polymer itself. The degradation products and their properties can be modulated to some extent if the hydrogel building blocks and crosslinking methods are selected properly.

In addition to the benefits, crosslinking may change the viscosity of the polymers, and the processing parameters will be different from non-crosslinked counterparts. Moreover, the crosslinked hydrogels show decreased degradability because of the reduced availability of functional groups to react with water and undergo hydrolysis [17–19]. Thus, crosslinking may sometimes lead to an increase in cytotoxicity and increased difficulties with the subsequent processing of the material [20].

The structure and properties of the hydrogel can vary depending upon crosslinking methods, duration of the crosslinking, and the conditions of crosslinking. As mentioned already, hydrogels can be crosslinked by different methods, such as physical, chemical, or biological. In the case of physical crosslinking, one or more physical interactions may exist between the polymer chains, preventing the polymeric network from dissembling in the aqueous environments. However, covalent bonds are generally formed between the different polymer chains in chemical crosslinking. Commonly used crosslinking methods for the preparation of hydrogels are described in the following sections.

2 Crosslinking by Physical Methods

Physically, crosslinked hydrogels do not require toxic chemical crosslinking agents. Such crosslinking agents can be harmful and cause several issues unless they are completely extracted from the hydrogel prior to use the use. In addition to that, chemical crosslinking agents can also affect integrity and stability of the entrapped bio-substance within the hydrogel. Due to all these disadvantages of chemical crosslinkers, physically crosslinked hydrogels received an increasing attention over the recent years.

2.1 Crosslinking by Ionic Interactions

Crosslinking of hydrogels with ionic interaction can be achieved under physiological or mild conditions at room temperature. A commonly used example of crosslinking polymers by ionic interaction is the crosslinking of polyuronates such as alginate and pectin [21]. Because of the biocompatibility and ease of gelation [22], calcium ions can be used to crosslink alginate [23]. The common biomedical applications of alginate hydrogel includes drug delivery, wound healing, tissue engineering, and it is often used as matrix for living cell encapsulation [24], and protein release [22, 23, 25]. The major advantage of this system is the possibility to generate stable hydrogels at physiological temperature and pH [26]. In this method, binding of bivalent cations such as Ca-ions to α-L-guluronic acid residues generate dimerizing junctions with other polymer chains that result in the formation of insoluble hydrogel networks. The resulting structure can be represented by the so-called eggbox model (Fig. 1). The gelation or crosslinking results in the stacking of the guluronic acid blocks of alginate chains. Further, the gel microparticles encapsulated with active agents can be stabilized by dropping a solution of sodium alginate and the protein/drug into an aqueous solution of calcium chloride. Controlled protein or drug release from the hydrogel microparticles can be achieved by coating the particles with cationic polymers such as polylysine [27] and chitosan [28, 29]. Costa et al. showed that crosslinking influences the structure and properties of alginate in terms of moisture content, solubility, mechanical properties, and water vapor permeability [30]. Crosslinking with high $CaCl_2$ concentrations resulted in the considerable increase in tensile strength.

Pectin is another natural polymer with similar chelating properties like alginate. Pectin can also be crosslinked using bivalent cations such as Ca^{2+}, Zn^{2+}, Mg^{2+}, etc. [32–35]. The interaction of ions and the carboxylate groups in pectin involves intermolecular chelate bonding of the cations, leading to the formation of macromolecular assemblies.

Plantago ovata husk mucilage (PHM)-blended Zn^{2+}-crosslinked low methoxy (LM) pectinate composite encapsulated with aceclofenac (ACF) was prepared by Guru et al. [36]. They successfully used this system for the controlled release of ACF in patients with rheumatoid arthritis and zinc deficiency. Hwang and Shin used

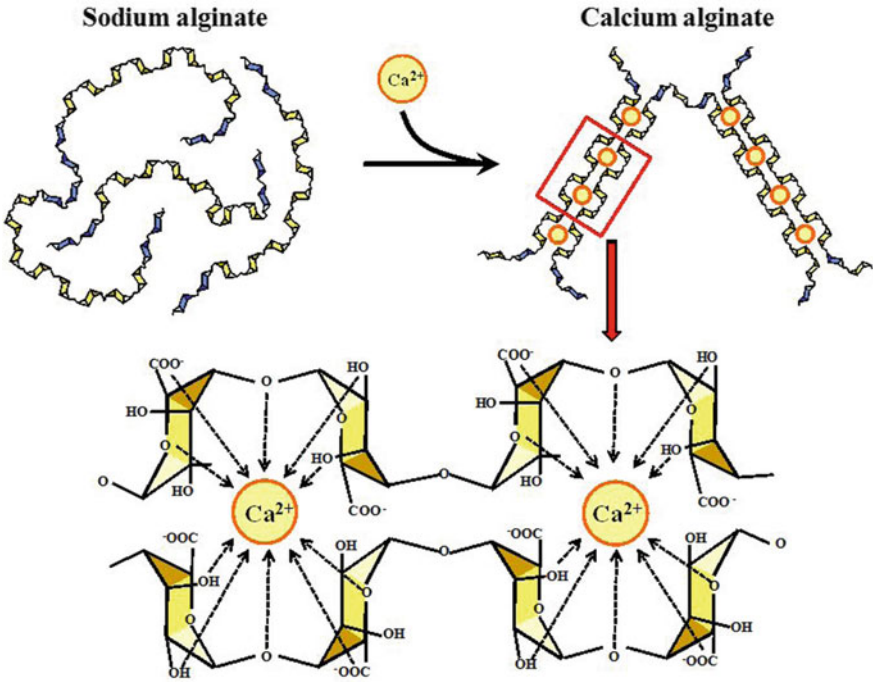

Fig. 1 Egg-box model of gelation of homopolymeric blocks of α-L-guluronic acid junction with calcium ions. Reproduced with permission from [31]

Mg^{2+} for the crosslinking of pectin; they prepared curcumin-loaded chitosan–pectin microparticles for delayed drug release [37].

Poly-[di(carboxylatophenoxy)phosphazene] (PCPP), is a synthetic polymer which is degradable under physiological condition when prepared as an ionotropic hydrogel. Similar to alginate, it can also be crosslinked with calcium ions. The degradation process can be tailored by introducing hydrolysis labile functional groups such as glycinato groups to the hydrogel [38]. The major advantage of PCPP in drug delivery is that the drug encapsulating efficiency of this polymer can reach upto 95% [39].

Chitosan is a natural biopolymer which can be obtained by chitin deacetylation [40]. It is widely used in biomedical applications such as wound dressings [41, 42], tissue engineering scaffolds [43], and drug delivery systems [44]. Crosslinking chitosan with glycerol-phosphate disodium salt is a biofriendly method to develop chitosan hydrogels. Below room temperature, chitosan solution remains aqueous in the presence of glycerol-phosphate disodium salt; however under physiological temperature (37 °C and above), it will quickly transform to a gel [45]. This transition from solution to a gel can be tuned by controlling the degree of deacetylation, which has an inverse relationship with the temperature required for sol–gel transition. The

gelation time of chitosan/β-sodium glycerophosphate can be decreased, and the thermostability can be improved by introducing sodium bicarbonate to the hydrogel [46]. The major advantage of this hydrogel is its applicability as injectable thermogelling solutions containing proteins and cells which can solidify at the body temperature (37 °C). Mechanical properties of chitosan hydrogel can be further be improved by different modifications such as physical blending, chemical modification, and by using different crosslinking techniques [47]. Depending upon the crosslinking method, various viscoelastic properties such as the storage and loss modulus (G' and G'') of chitosan hydrogel may vary. Rheological study demonstrated that the gelation process appears to be governed by delicate interplay between the pH and the temperature. Rheological properties measured at low temperature (~10 °C), after the incorporation of β-GP reduced both G' and G'' compared to chitosan alone [48]. This might be due to the charge neutralization and increased flexibility of the polymer. Upon heating, from 5 to 70 °C, a rapid increase of G' indicated the gelation near 37 °C. After incubation at 37 °C for at least 60 min, rheological measurements indicated a nearly frequency independent G', while G'' increased slightly with the frequency as the general trend for hydrogel materials. Chitosan can also be crosslinked by oppositely charged low-molecular-weight anionic crosslinkers such as tripolyphosphate (TPP) [49].

Carrageenan is a natural seaweed polysaccharide which is composed of α(1–4) and β(1–3)d-galactose and different amounts of sulfate groups. This polymer can form gels in both salts containing (e.g., potassium ions) and salt-free conditions. However, gels formed in the presence of metallic ions were found to be stronger than those prepared under salt-free conditions [50]. Iota carrageenan under identical conditions of concentration and ionic strength can form gel with metal ions (K^+, Rb^+, Cs^+), and salt (NH_4^+) [51]. Swelling capacity of such hydrogels decreases with the increase of ionic salt solution in crosslinking solution [52].

Interestingly, the occurrence of cationic or anionic groups in the polymer chains is not a necessity for achieving crosslinking by ionic interaction. For instance, dextran, a polymer with no ionic binding sites for cations, can crosslink in the presence of potassium ions and form hydrogels. Watanabe et al. [53] showed that this is achieved by the formation of a cage-like structure by the oxygen atoms of glucose units of different dextran chains, and the perfect fitting of ionic radius of potassium inside this cage. However, this gel is not suitable for biomedical applications as it is relatively unstable under biological conditions.

Crosslinking anionic polymers using metallic ions is not the only way to prepare a hydrogel. Crosslinking of polyanions with polycations is another way to obtain a stable hydrogel. Chitosan-based biomaterials that are crosslinked ionically by the complex formation between chitosan and polyanions like polyphosphoric acid or dextran sulfate showed good stability under physiological conditions [54].

2.2 *Crosslinking by Crystallization*

2.2.1 Crosslinking by Crystallization in Homopolymer Systems

Polyvinyl alcohol (PVA) is a water-soluble synthetic polymer which can form hydrogels if crosslinked properly [55]. A mechanically weak gel will be gradually developed when aqueous solution of PVA is stored at room temperature. However, if the same aqueous solution underwent a freeze-thawing cycle, a strong and highly elastic gel could be formed [56]. Stability and rheological properties of PVA gel can be tuned by changing the temperature and time of the freeze-thawing cycle. Moreover, the number of freeze-thawing cycles, polymer molecular weight, and its concentration in the water influence the properties of the hydrogel. PVA crystallites formed during the freeze-thawing cycle are believed to be the reason for the gel formation by acting as a physical crosslinking centers in the polymeric network [57]. When prepared under optimum conditions, PVA hydrogel can be stable for about 6 months at 37 °C [58]. Polyvinyl alcohol/cellulose nanocrystals (PVA/CNC) prepared by freeze-thawing cycle showed an increased swelling, re-swelling, and adsorption properties which can be promising for water or fluid absorbing applications [59]. Formed protein crystals can be further crosslinked with agents such as glutaraldehyde [60]. A schematic representation of such crosslinking is given in Fig. 2.

PVA hydrogels prepared by crystallization also have important application in the biomedical field due to the lack of toxicity associated with crosslinking agents. PVA hydrogel encapsulated with bovine serum albumin (BSA) can be prepared by freeze-thawing crystallization. The protein was released by Fickian diffusion with its structure preserved [61]. Adding polymers such as alginate to the PVA solution before the freeze-thawing cycles enables the modulation of the hydrogel properties such as mechanical strength. Mechanical strength of the PVA hydrogel can be increased by increasing alginate concentration, this strengthening was associated with decreased release of drugs [62].

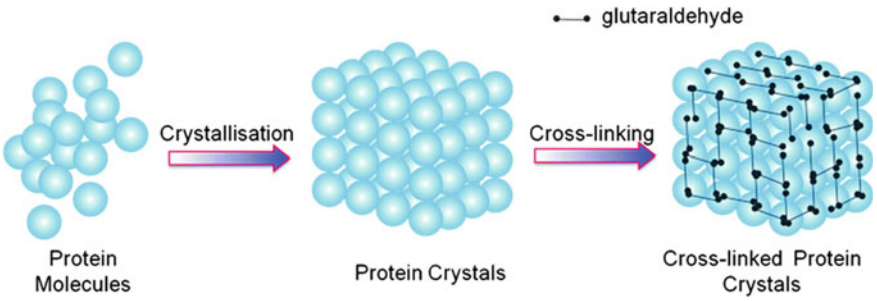

Fig. 2 A schematic illustration of the preparation process for crosslinked protein crystals. Reproduced from [60] with permission from The Royal Society of Chemistry

2.2.2 Crosslinking by Stereocomplex Formation

Stereocomplex is an intermolecular complex formed by macromolecules that share an identical chemical composition, but different configuration of repeating units [63]. Classical example of crosslinking by stereocomplex formation is the stereocomplex formation between enantiomeric PLA; poly(L-lactide) [i.e., poly(L-lactic acid) (PLLA)] and poly(D-lactide) [i.e., poly(D-lactic acid) (PDLA)]. PLLA and PDLA are semicrystalline homopolymer stereoisomers of polylactic acid. Both high molecular weight PLLA and PDLA have a melting temperature around 170 °C; however, blends of high molecular weight of the two stereoisomers have a melting temperature of 230 °C. This increase in melting temperature is ascribed to stereocomplex formation. Ikada and coworkers [64] were the first to report this ability of PLA to form stereocomplexes. Figure 3 shows the general mechanism of stereocomplex crosslinking of hydrogels.

Bare PLLA/PDLA stereocomplexes cannot be considered as hydrogels due to the low swelling behavior. However, stereocomplex formation can be established by the blends of PLLA-PEG-PLLA and PDLA-PEG-PDLA triblock copolymers which may enhance the swelling behavior. Lim and Park [65] studied BSA protein release from such triblock copolymers. They have compared the release of BSA from the microspheres of the triblock copolymers with BSA release from microspheres prepared with only one of the enantiomeric from of the triblock copolymers and with PLLA microspheres. A slightly larger burst release was observed in the stereocomplex triblock copolymer group in comparison to PLLA microsphere group. The higher water absorption capacity of the microspheres containing PEG might be the cause of the observed burst release. Lim et al. also developed another stereocomplex-based hydrogel system by grafting enantiomeric oligo(lactic acid) side chains on pHEMA (polyHEMA-g-oligo(1)lactate) [66].

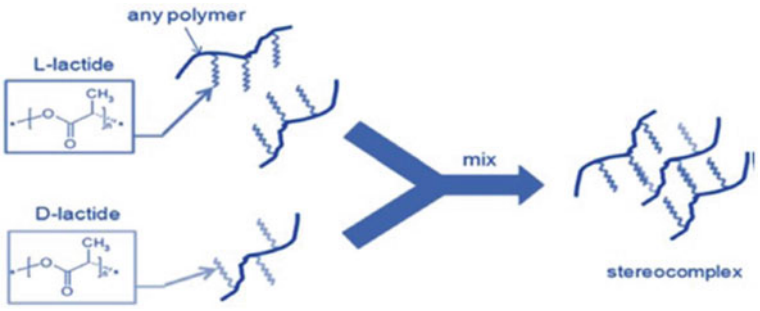

Fig. 3 Crosslinking of hydrogels by stereocomplex formation. Reproduced with permission from Taylor & Francis [67]

2.3 *Physical Crosslinking of Amphiphilic Block and Graft Copolymers*

In general, physically crosslinked hydrogels are assembled by graft or multi-block copolymers. Physically crosslinked thermo-responsive hydrogels are assembled via the entanglement of the polymer micelles. Such systems show higher biodegradability compared to chemically crosslinked hydrogels. Some copolymers such as graft copolymers and amphiphilic block copolymers have the ability to self-assemble in aqueous solutions to form hydrogels and other types of organized structures where the hydrophobic parts of the polymer are aggregated in the center [68]. Poly(N-isopropylacrylamide) (PNIPAM) and poly(p-phenylene oxide) (PPO) are thermoreversible polymers that are commonly used in such systems [69]. Such materials have the characteristic ability to crosslink physically and form gels near physiological temperature while maintaining low viscosity at low temperatures. PNIPAM which exhibits a lower critical solution temperature (LCST) around 33 °C remains as a transparent solution below 33 °C. They show low viscosity liquid behavior at room temperatures (below LCST); however, they can form a reversible hydrogel at body temperature. This makes them as excellent candidates for drug delivery systems [70]. PEG-PNIPAM is an example of thermosensitive physically crosslinked hydrogel based on block copolymers [71]. Linear and multi-arm PEG is the water-soluble central block whereas the thermosensitive terminal block is PNIPAM. In comparison with saline and single network delivery systems, PEG-PNIPAM double-network hydrogel showed significantly enhanced in vivo cell retention when used as the carriers of stem cells [72].

Using poly(lactic acid), glycolic acid and poly(ethylene glycol), several biodegradable block copolymers can be prepared. These copolymers can be used for drug delivery applications where the drug will be released either by passive diffusion or by degradation phenomena.

By combining two PEG–PLGA diblock copolymers, a triblock polymer can be prepared with PLGA segment which is the hydrophobic part being in the middle [70, 73, 74]. In this system, different outcomes can be achieved by varying the copolymer concentrations. They form micelles at low concentrations in water; however at higher concentrations, thermoreversible gels are formed. Upper critical solution temperature (UCST) and the critical gel concentration depend strongly on the composition of the blocks and the molecular weights.

By polycondensation of dicarboxylated PLA and PEG, multiblock copolymers of PEG and PLGA can be prepared [75, 76]. The temperature of phase transition depends on the molecular weight of PLA; polymers containing small PLA blocks show LCST behavior and are soluble in water. Some preliminary results indicated the preservation of basic fibroblast growth factor bioactivity in dried films of the multiblock copolymer as it improved wound healing in rats [76].

Feijen and team investigated multiblock copolymers of PEG and poly(butylene terephthalate) (PBT) which is another hydrophobic polyester [77–81]. These hydrogels are prepared by melt polycondensation of PEG, butanediol and dimethyl terephthalate where PBT hard domains form thermally reversible physical crosslinks. Lysozyme was loaded in the polymer as a model protein. The polymer solutions were prepared in a mixture of chloroform and hexafluoro isopropanol, followed by water-in-oil emulsion containing the protein in the aqueous phase [78]. It takes 3 days for the swelling of PEG/PBT films in water to reach equilibrium [77, 78]. Control over the release rate of the protein can be achieved by controlling the copolymer composition. Increasing molecular weight of PEG and increasing PEG/PBT weight ratio resulted in the increase of release rates. Other researchers loaded vitamin B12 (1335 Da) in multiblock copolymers composed of hydrophilic poly(ethylene glycol)-terephthalate (PEGT) blocks and hydrophobic PBT blocks [82]. The release can last from one day up to 12 weeks with a relatively constant release according to the copolymer composition. Increasing PBT content or increasing PEG molecular weight resulted in enhanced phase separation which influences the mechanical properties, degradation rates and swelling properties of the copolymers. Moreover, copolymer composition shows considerable effect on the physical properties and degradation behavior of poly(ethylene oxide) (PEO)-PBT copolymers [83].

2.4 Crosslinking of Polysaccharides by Hydrophobic Interactions

Chitosan, pullulan, carboxymethyl curdlan and dextran are some examples of polysaccharides used for assembling physically crosslinked hydrogels using hydrophobic modification approaches. Sunamoto and his group focused on cholesterol-bearing pullulan-based hydrophobized hydrogels [84–89]. Chitosan solutions containing glycerol-2-phosphate (β-GP), which undergoes temperature-controlled pH-dependent sol–gel transition at a temperature close to 37 °C, have recently been proposed by this approach [90].

For gene delivery, a modified hydrophobized glycol chitosan (HGC) was prepared by modifying a primary amine of glycol chitosan with 5β-cholanic acid [91]. DNA nanoparticles were formed spontaneously by hydrophobic interaction between HGC and hydrophobized DNA. In COS-1 cells, endocytic uptake of HGC nanoparticles was enhanced by increasing HGC content. In genetic engineering applications, HGC showed enhanced and superior transfection efficiencies both in vitro and in vivo. Another example of hydrophobic modified polysaccharide is glycol chitosan substituted with palmitoyl chains. In the presence of cholesterol, they form unilamellar polymeric vesicles [92] that are not only biocompatible, but also can entrap water-soluble drugs [93]. Upon freeze drying, a solid and highly porous material that can hydrate without swelling upto 20 times its dry weight in alkaline buffer was formed [94].

Qu et al. grafted chitosan with PLGA where the hydrophobic interactions in water resulted from the hydrophobic polyester side chains [95]. Changing the pH between 2.2 and 7.4 showed reversible water uptake. The highest swelling of this hydrogel was obtained with the lowest pH which is caused by the charge repulsion due to the protonation of the free amine groups in the polymer. Other examples of chitosan hydrogels which can respond to external conditions such as temperature and pH are poly(acrylic acid) (PAAc) [96] and poly(*N*-isopropylacrylamide) (PNIPAAm) [97]. Poly(*N*-vinylpyrrolidinone-*g*-styrene) hydrogels [98] and PMMA microemulsion particles [99] can also be developed by hydrophobic interactions.

2.5 Crosslinking by Hydrogen Bond Formation

Hydrogels physical crosslinking can also be achieved by hydrogen bonding. In this approach, by mixing two or more natural polymers, a gel-like structure can be prepared. Poly(acrylic acid) and poly(methacrylic acid) can form complexes with poly(ethylene glycol) by hydrogen bond formation between the oxygen of poly(ethylene glycol) and the carboxylic group of poly((meth)acrylic acid) [100]. Hydrogen bonding has also been observed in poly(methacrylic acid-*g*-ethylene glycol) [101, 102].

Injectable physically crosslinked hydrogels based on polymer systems such as gelatin–agar, starch–carboxymethyl cellulose and hyaluronic acid–methylcellulose can also be prepared by hydrogen bond formation. In such cases, the hydrogen bonds form only after protonation of the carboxylic acid groups which suggest a strong dependency of the swelling based on the pH. The issue with hydrogen-bonded gel-like structures is their fast collapse of gel structure which restricts their use to only relatively short acting drug release systems. Nagahara et al. made a DNA hydrogen bonding mimicking hydrogel in which crosslinking was established by hybridization [103]. To achieve this, they coupled oligodeoxyribonucleotides to a water-soluble polymer (poly(*N*, *N*-dimethylacrylamide-co-*N*-acryolyloxysuccinimide)). Xue et al. developed tissue mimicking composite hydrogels based on poly(acrylic acid)/surface-modified boron nitride nanosheets (PAA/BNNS-NH2) through molecular-scale metal coordination interaction between –COOH of PAA and Fe^{3+} and H-bond between –COOH of PAA and $–NH_2$ of BNNS-NH_2 (Fig. 4).

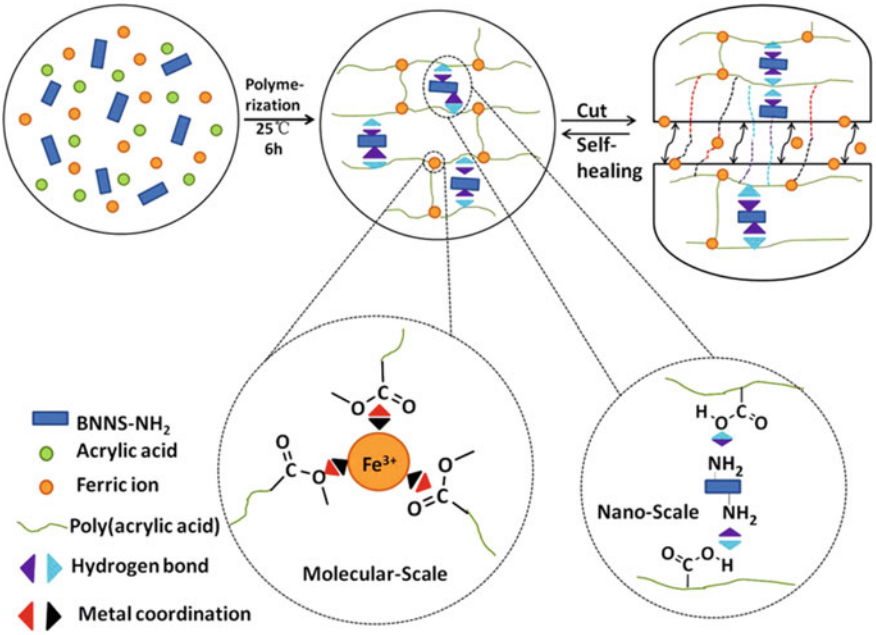

Fig. 4 Scheme illustrating the formation of a poly(acrylic acid)/surface-modified boron nitride nanosheets. Reproduced with permission from [104]

3 Crosslinking of Hydrogels by Chemical Methods

3.1 Crosslinking by Radical Polymerization

Radical polymerization method is used in the presence of suitable crosslinking agents to chemically crosslink low-molecular weight polymers like poly(2-hydroxyethyl methacrylate) (pHEMA). pHEMA was first described by Wicheterle and is a frequently studied hydrogel system in biomedical applications [105]. It can be fabricated by the polymerization of HEMA with a crosslinking agent (e.g., ethylene glycol dimethacrylate). Also, many other hydrogel system have been synthesized using this procedure [106]. Furthermore, by the addition of *N*-isopropylacrylamide (temperature-sensitive gels) [107] or methacrylic acid (pH-sensitive gels) [108], stimuli-sensitive materials can be obtained [109]. In addition to this, radical polymerization of vinyl monomers mixture, hydrogel can also be obtained by chemically crosslinking the water-soluble polymers by radical polymerization. For the design of hydrogel via this route, water soluble polymers like natural, synthetic and semi-synthetic polymers have been used. Particularly, dextran is a polysaccharide and is being used as a building block of degradable hydrogels. It consists of α-1,6 linked d-glucopyranose residues. Dextran (molecular weight between 40 and 100 kDa) has been used as a plasma expander and examined for the delivery of imaging agents,

proteins and drugs. Furthermore, dextran-based gels are under investigation as a colon delivery system due to the presence of dextranase in the colon [110]. Edman et al. [111] have pioneered in the production of polymerizable dextran by reacting glycidyl acrylate with dextran dissolved in water to form a hydrogel. An initiator system consisting of ammonium peroxydisulfate and *N*, *N*, *N′N′*-tetramethylene-diamine was added to aqueous solution of the acryl dextran which contains *N*, *N*, -methylenebisacrylamide. By employing an emulsion polymerization technique, enzymes were immobilized with almost full retention of their activity in micro-spheres of polyacryldextran [112]. Using the method developed by Edman et al., some water-soluble polymers other than dextran were also functionalized with (meth)acrylic groups, e.g., hyaluronic acid [113], polyvinyl alcohol [114], poly-aspartamide [115–117], (hydroxyethyl) starch [118] and albumin [119]. Because of very low degree of substitution due to the reaction in an aqueous solution, it is difficult to control the degree of substitution due to the hydrolysis of glycidyl(meth)acrylate with water-soluble polymer before and after the reaction. Therefore, an alterna-tive method was used for the synthesis of methacylated dextran [10]. The glycidyl methacrylate functionalized dextran (Dex-GMA) were prepared by crosslinking in the presence of a crosslinker: N,N-methylene-bisacrylamide (NMBA), and a photoinitiator: 2,2-dimethoxy-2-phenyl acetophenone (DMPA) (Fig. 5) [120]. In addition to superior mechanical strength, developed Dex-GMA hydrogel exhibited good biodegradability.

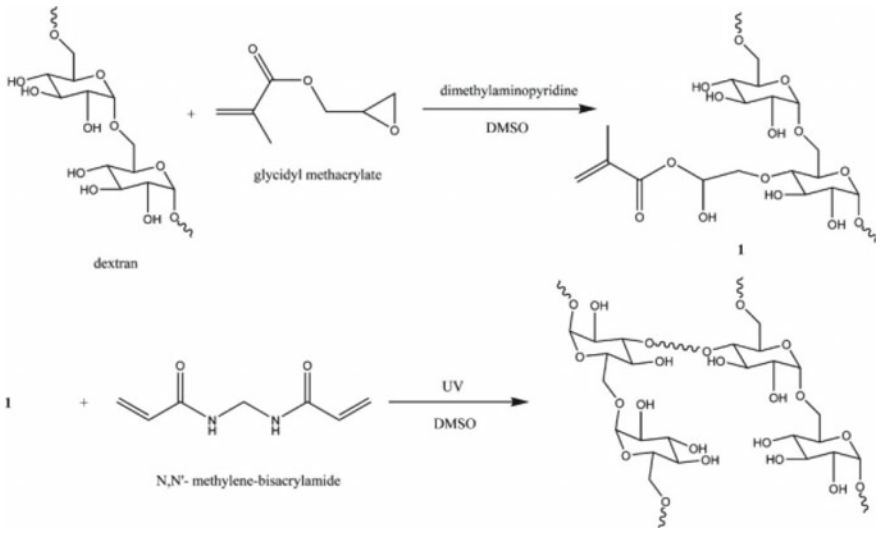

Fig. 5 Synthesis route of Dex-GMA hydrogel. Reproduced with permission from [120]

3.1.1 Free Radical Polymerization (FPR)

Free radical polymerization (FPR) is one of the most suitable polymer synthesis methods which require relatively moderate conditions and can be applied to a large number of monomers. In addition to its simple experimental setup, it has tolerance toward impurities, solvents and functional groups, and their purifying agents are easy to prepare and inexpensive. However, it is not possible to obtain polymer with narrow distribution of molecular weights and accurately defined end groups. For free radical polymerization of vinyl monomers on carbohydrate polymers, various initiation methods including thermal and photolysis, γ-radiation, Fenton's reagent, ceric ion and persulfate were examined. Ceric ion and persulfate with various organic compounds through a redox reaction create free radicals capable of initiating radical polymerization in tetravalent state (Ce^{4+}) [121–123]. γ-radiation produces macro-radicals on carbohydrate polymers using its high energy radiation [124]. In addition, Fenton's reagent generates hydroxy radicals by the redox reaction of ferrous ion (Fe^{2+}) with hydrogen peroxide [125]. Free radical polymerization of vinyl monomers can be initiated by macro-radicals on carbohydrate polymers produced by these initiation methods. However, there are some limitations associated with free radical polymerization method. FRP produces homo-polymer as a side product with attached copolymer and provides insufficient control over molecular weight distribution and molecular weight (Mw/Mn > 2.0) of attached vinyl polymers. Therefore, it is essential to limit those undesirable radical reactions that do not contribute in polymer chain growth in order to control the molecular structure of polymer chains. However, to control the radical polymerization, several procedures have been developed during the last 10–15 years [126].

Gelatin methacryloyl (GelMA) undergoes photoinitiated radical polymerization (i.e., under light exposure with the presence of a photoinitiator) to form covalently crosslinked hydrogels. Gelatin is the hydrolysis product of collagen and contains the key components of natural extracellular matrix like RGD (arginine-glycine-aspartic acid) peptides that enhance cell attachment [19]. GelMA can be synthesized by the substitution of the free amine groups of gelatin with methacrylate anhydride without losing the RGD sequences. Photocrosslinking of GelMA hydrogel can be done under UV light using a photoinitiator. A generalized scheme of the crosslinking process is given in Fig. 6. Hyaluronic acid methacrylate (HAMA) can be used as an optional component to make the gel more cell friendly. For crosslinking, most commonly used water-soluble initiators are lithium acylphosphinate salt (LAP) [127] and 2-hydroxy-1-[4-(2-hydroxyethoxy) phenyl]-2-methyl-1-propanone (Irgacure 2959) [128, 129]. Particularly, for photopolymerization in aqueous environments, Irgacure 2959 is being used, having higher solubility in water (up to 8.5 wt%) which is more than sufficient for the photopolymerization in aqueous environment. In addition, a water-soluble photoinitiator LAP has newly developed, which has higher molar extinction coefficient than Irgacure 2959 at 365 nm and comparable water solubility with Irgacure 2959 [28]. GelMA hydrogel has highly tunable physical properties. Some major parameters like UV exposure time, initiator concentration, degree of substitution and GelMA concentration can be changed for required physical properties

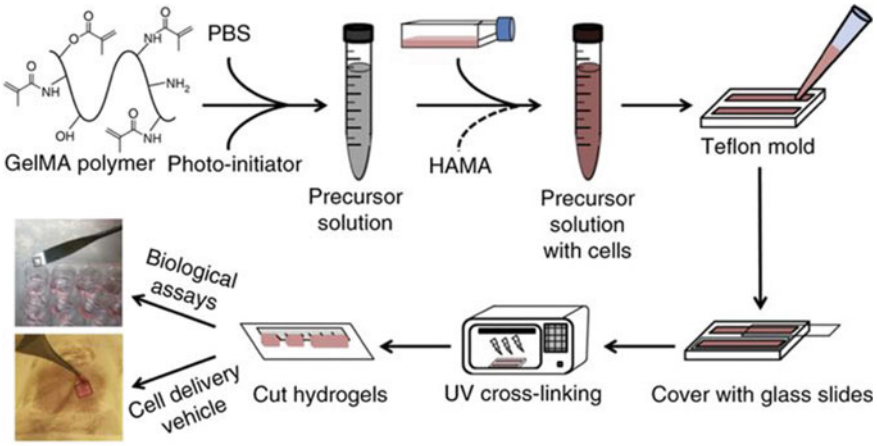

Fig. 6 Overview of the GelMA-based hydrogel preparation protocol [134]

of GelMA hydrogels. High crosslinking degree of polymer can be obtained at low concentration of photoinitiator within a minute or even second, which minimizes cytotoxicity. The proliferation and attachment of different cells in GelMA hydrogels have been widely characterized and established. GelMA hydrogels can be used in tissue engineering due to the acceptable mechanical properties, existence of bioactive peptide sequences and adequate biocompatibility [130]. For example, for the synthesis of cell-laden 3D hydrogels, cells can be suspended in GelMA prepolymer solutions and crosslinked upon exposure to UV light. Generally, in photocrosslinked cell-laden GelMA hydrogels, higher cell viability (upto 80%) was observed [131]. In a relatively similar approach, alginate hydrogels can be synthesized by the in situ photo-crosslinking of alginate polymers. Here also, polymers can be modified with functional groups (i.e., methacrylates) and then crosslinked with free radical polymerization under UV light in the presence of photoinitiator. This polymerization reaction provides an ideal environment for in situ encapsulation of cells under physiological conditions [132]. In contrast to the synthetic ethylene glycol derivatives, methacrylated alginate is more similar to the negatively charged mucopolysaccharides in cartilaginous tissues and has also been studied as platforms for tissue engineering applications [133]. Compared to ionically crosslinked alginate constructs, photocrosslinked alginate hydrogels have enhanced mechanical properties, ECM accumulation and structural integrity [132].

3.1.2 Controlled/Living Radical Polymerization

Controlled/living radical polymerization (CLRP) is a highly useful method for producing controlled molecular weight, chain architecture, polydispersity, composition and site-specific functionalities in hydrogels which cannot be generated by

conventional free radical chemistries [135, 136]. All the steps in free radical polymerization reaction are also applied in controlled/living radical polymerization. However, to control the polymerization, a mediating species can be employed which in turn can aid in the formation of block copolymers, polymers with narrow molecular weight distributions and very short oligomers. Despite the successful implementation of controlled/living polymerization technique in bulk or solution polymerization, successful transmission of these polymerization reactions into aqueous dispersed phase system such as micro-emulsion, mini-emulsion and emulsion system is essential in order to produce these hydrogels at an industrially feasible scale. By employing CLRP approaches, hydrogel can be synthesized at much higher monomer and crosslinker concentrations. Due to the living nature of CRP, it is possible to achieve chain extensions after the addition of a second monomer batch. By this approach, the structural arrangement and features of the hydrogels can be controlled, and different forms of gels can be developed [137]. Moreover, the use of functional initiators facilitates the integration of functionalities in the core or at the surface of hydrogels which is not possible by conventional radical crosslinking polymerization (Fig. 7). This is particularly very useful when nano- or microgels for drug delivery applications are produced. Such surface functional groups can be used for the conjugation of biomolecules.

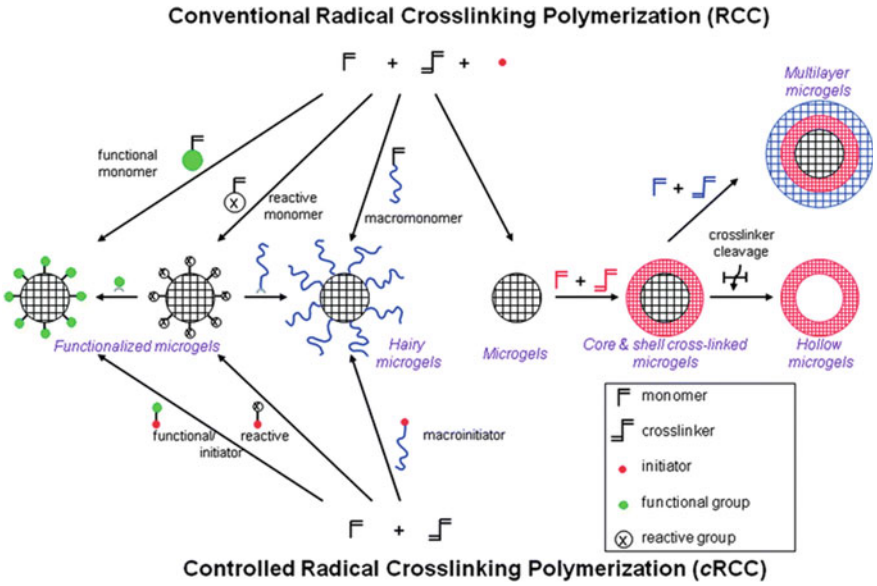

Fig. 7 Scheme showing the synthesis approaches for the preparation of nano- or microgels of different morphologies and functionalities by conventional and controlled radical crosslinking. Reproduced with permission from [137]

3.2 *Crosslinking by Chemical Reaction of Complementary Groups*

Several polymers show their aqueous solubility due to the presence of functional groups such as COOH, OH and NH_2. Covalent bonding between different polymer chains can be established by the reaction between such functional groups with complementary reactivity.

3.2.1 Crosslinking with Aldehydes

Crosslinking with aldehyde groups is a commonly used technique for the crosslinking of polymeric systems such as chitosan-PVA hydrogels. In the range of different aldehyde crosslinkers, glutaraldehyde is mostly used because it can attach with different functional groups in both proteins and carbohydrates. Glutaraldehyde crosslinking of hydrogels considerably increases the tensile strength. One of the drawbacks of glutaraldehyde crosslinked hydrogel is their higher cytotoxicity on mammalian cells. However, it can be reduced by optimizing crosslinking conditions such as pH and temperature.

Hydrogel can be prepared by the crosslinking of gelatin with polyaldehydes such as dextran dialdehydes [138]. Per-iodate oxidation of dextran (Dex) was used for the preparation of polyaldehyde derivatives. Since in dextran, the structural units contain three vicinal hydroxyl groups, the oxidation can lead to various types of aldehydes (Fig. 8a). The crosslinking was mainly due to Schiff base formation between amino groups of lysine and hydroxylysine residues of gelatin and the aldehyde (Fig. 8b). The fabricated gelatin hydrogel film was used in wound treatment where epidermal growth factor (EFG) was encapsulated to enhance wound healing. The dextran dialdehyde crosslinked hydrogels showed acceptable biocompatibility under both in vitro and in vivo conditions [139].

Partially depolymerized alginate produced by oxidation with poly(aldehyde guluronate) can be transformed into a hydrogel by crosslinking with adipic acid dihydrazide. Crosslinking with this crosslinker has improved the swelling and degradation rate of the gel [140]. Daunomycin, a cancer drug used for the chemotherapy, was incorporated in the hydrogel through covalent linkage. Because of the hydrolysis of this linkage, the drug was released in the time period between 2 days and 6 weeks [141]. Hyaluronic acid hydrogel can also be prepared by the crosslinking of hyaluronic acid with adipic dihydrazide. This reaction further proceeded with the crosslinking with a macromolecular crosslinker (poly(ethylene glycol)-propionaldehyde). The obtained hydrogels were enzymatically degradable and have shown anti-bacterial activity and manifested the controlled release of therapeutic drugs [142]. Wang developed an injectable hydrogel using a combination of hydrazine-modified elastin-like protein (ELP) and aldehyde-modified hyaluronic acid by dynamic covalent hydrazone bond formation (Fig. 9a, b) which can be performed at room temperature [143]. This hydrogel facilitated the successful

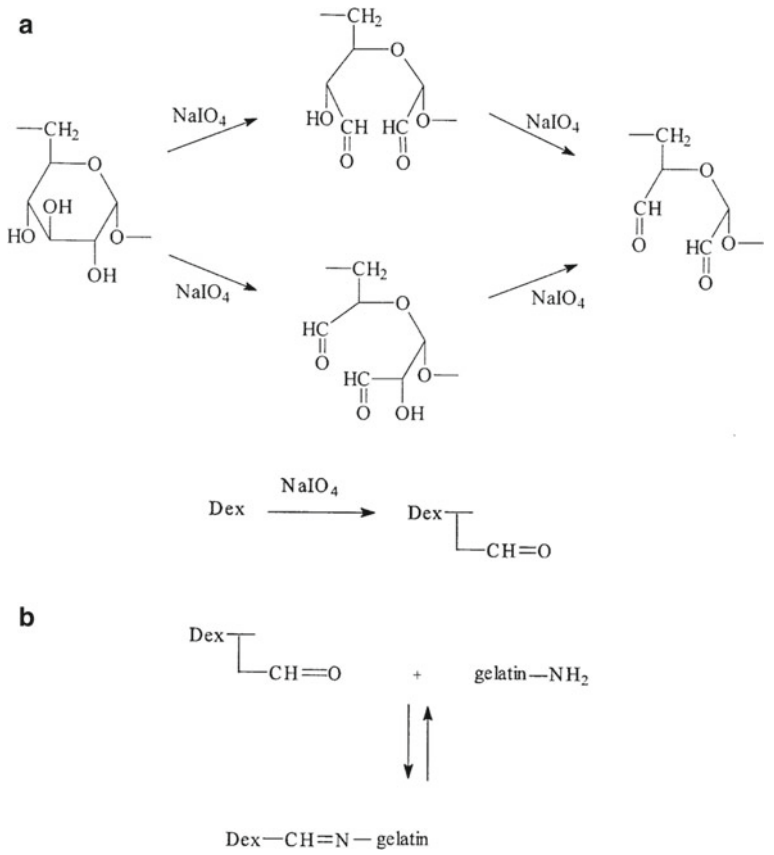

Fig. 8 Crosslinking of polymers containing gelatin dextran aldehyde. **a** Partial oxidation of dextran (Dex) for synthesizing dextran dialdehyde. **b** Crosslinking of gelatin. Reproduced with permission from [138]

injectability of stem cells (Fig. 5c). Developed hydrogels were able to support cell proliferation for three weeks post injection, and encapsulated cells maintained their ability to differentiate into multiple lineages.

3.2.2 Crosslinking by Addition Reactions

Hydrophilic polymers can be converted into hydrogels by using highly reactive crosslinking agents which react with the functional groups of polymers via addition reactions. Different crosslinkers have been used for crosslinking with polysaccharides such as 1,6-hexamethylene diisocyanate [144], divinyl sulfone [145], or 1,6-hexane dibromide [146] and many other chemicals. By using the addition reactions, the functional properties can easily be tailored by changing the concentration

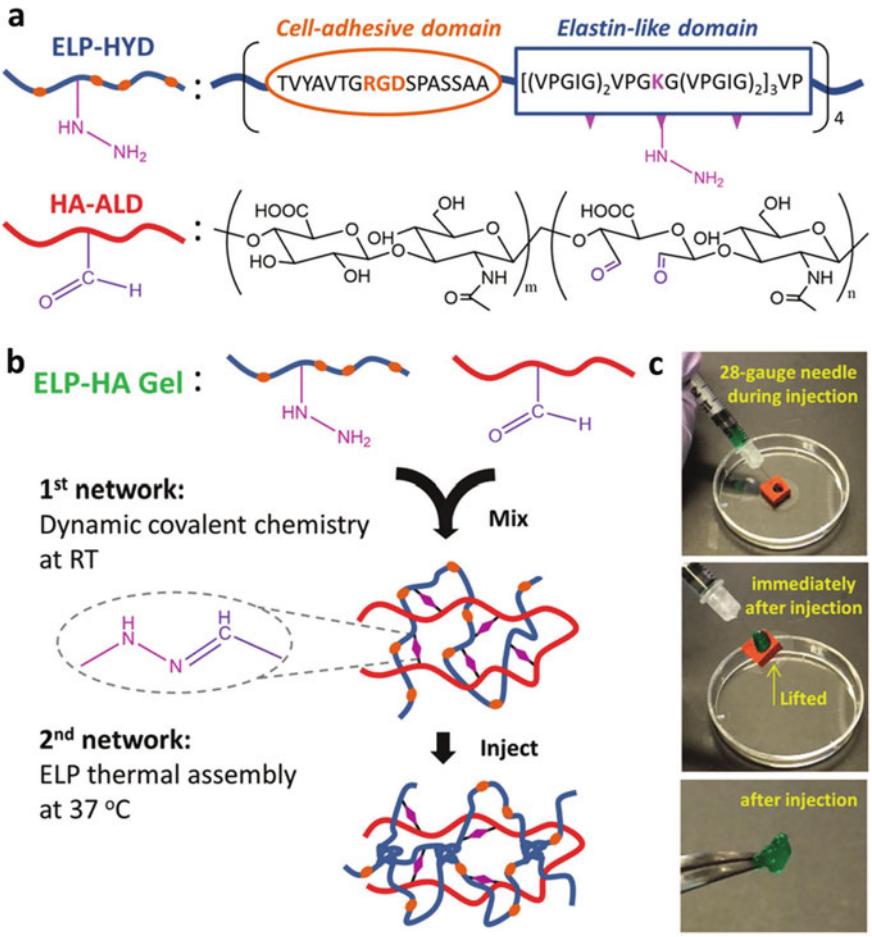

Fig. 9 **a** Elastin-like protein (ELP–HA) is composed of hydrazine-modified elastin-like protein (ELP-HYD) and aldehyde-modified hyaluronic acid (HA-ALD). **b** Schematic of ELP–HA hydrogel formation. **c** Photographs demonstrating the injectability and rapid self-healing of ELP–HA hydrogels. Reproduced with permission from [143]

of dissolved polymer and the quantity of crosslinker. Organic solvents are preferred over aqueous solutions for crosslinking to avoid the unfavorable reactions between water and crosslinker which affects polymer crosslinking. However, these organic solvents sometimes can leave their traces after the crosslinking. Therefore, it must be washed extensively to remove the unreacted traces of the crosslinker.

3.2.3 Crosslinking by Condensation Reactions

Crosslinking by condensation reactions is useful when polymer chains with hydroxyl, amines or carboxylic acid groups were used. They are commonly used for the synthesis of polymers to produce polyesters and polyamides. N,N-(3-dimethylaminopropyl)-N-ethyl carbodiimide (EDC) is one of the most frequently used crosslinkers to crosslink water-soluble polymers with amide bonds. Feijen et al. used this EDC crosslinker for the fabrication of gelatin hydrogels [147]. While the process of crosslinking incorporated *N*-hydroxysuccinimide (NHS) to reduce the possible side reactions which could give better crosslink density to the gels. This hydrogel was formulated as a drug delivery medium to provide the release of antibacterial proteins and later utilized in Dacron prosthetic valves. Moreover, the same EDC/NHS crosslinker can be used to crosslink collagen films where several fold increase in tensile strength and modulus can be achieved in crosslinked films [148]. In addition, swelling of the films was decreased significantly. In another research, Kuijpers et al. [149] have used a negatively charged polysaccharide, chondroitin sulfate to enhance the loading capacity. In earlier studies, researchers have used the ionic crosslinking approach to crosslink alginate gels to obtain better mechanical properties; however, the degree of crosslinking was limited. Then, Mooney et al. used EDC chemistry to covalently crosslink alginate and PEG diamines where the mechanical properties could be controlled by changing the quantity of PEG diamines in the gel [150].

Hydrolysable polyrotaxane have been used to crosslink PEG hydrogels where α-cyclodextrins (α-CD) were joined by a PEG chain and capped with bulky and degradable ester end groups [151]. After that, hydroxyl groups of the cyclodextrins can be activated via carbonyldiimidazole, and subsequently, PEG bisamines can be crosslinked with it. Because of the hydrolysis of the ester groups, the prepared gel was degraded steadily. However, the degradation period of the gel can be controlled by its composition. Figure 10 shows the preparation of cationic PEG hydrogels crosslinked by the hydrolysable polyrotaxane. The developed gel was used as a scaffold for soft tissue regeneration.

3.3 Crosslinking by High Energy Irradiation

High energy irradiation technique has been used widely to crosslink different polymers. Recently, collagen films with the formulation of glucose have successfully been crosslinked with UV irradiation. The idea is based on the fact that when UV irradiation hits the target sample, it can generate free radicals. This free radicals react with linear glucose molecules which could facilitate the crosslinking [148]. UV crosslinking has improved the mechanical properties and reduced enzymatic degradation of collagen [148]. Usually, high energy radiation, such as gamma and electron beam, has been used to polymerize unsaturated compounds. Furthermore, high energy irradiation was used to crosslink water-soluble polymers synthesized

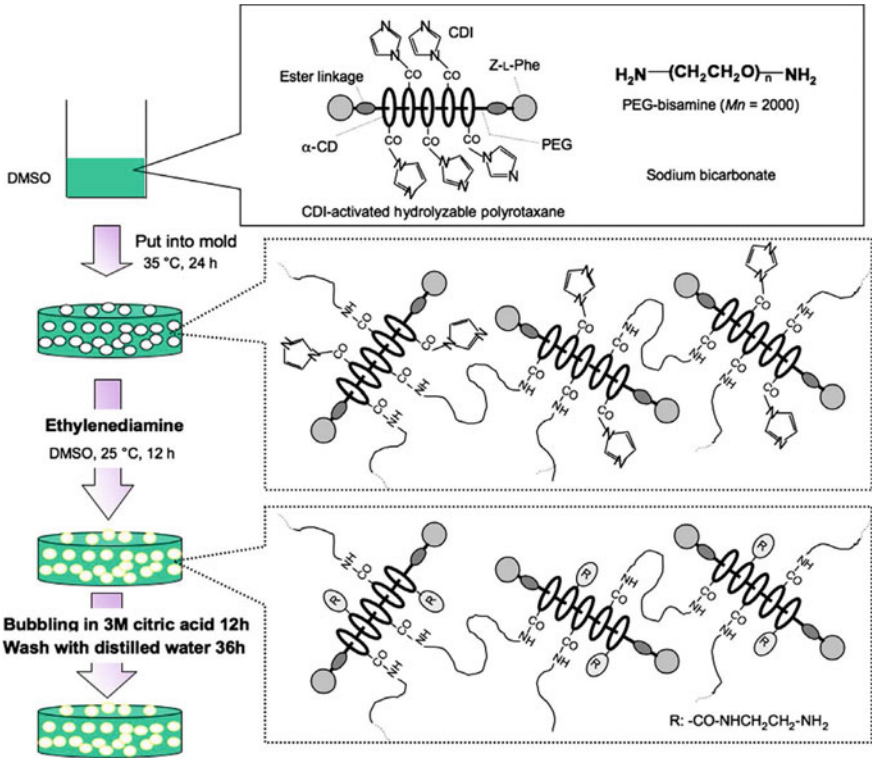

Fig. 10 Preparation of cationic PEG hydrogels crosslinked by the hydrolysable polyrotaxane. Reproduced with permission from [152]

from vinyl groups to form hydrogels [106]. It can also crosslink the water-soluble polymers in the absence of vinyl groups. PVA [153], GelMA [130], poly(acrylic acid) [154] and PEG [155–157] can be crosslinked with high energy irradiation. The swelling, degradation and other properties of the formed hydrogel are dependent on the concentration of polymer and the radiation time. In an interesting study, poly(amino acid)-based hydrogel was developed by crosslinking polypentapeptides with gamma irradiation [158]. High energy irradiation-based crosslinking can be performed in aqueous conditions at specific temperature and/or pH. In addition, the toxicity associated with chemical crosslinking agents can be avoided. However, biologically active drugs/materials can be incorporated only after the irradiation because the radicals generated during the exposure may damage the biologically active compounds.

4 Crosslinking Using Enzymes

Crosslinking of hydrogels by proteins especially by enzymes has been emerged as one of the most useful technique for developing hydrogels for biological applications. Hydrogels crosslinked with chemical agents show toxicity to the cells due to the presence of reactive free functional groups and unreacted chemicals. Another advantage of enzymatic crosslinking is that this can be performed at mild biological conditions. Crosslinking of hydrogels by enzymes such as transferases, tyrosinases and lysyl oxidases makes them excellent vehicles for controlled drug release [159]. In the presence of calcium, transglutaminase enzyme catalyzes covalent bond formation between lysine and glutamine residues in in vivo conditions during wound healing as well as extracellular matrix stabilization and organization [160]. This crosslinking potential of transglutaminase family was later employed for the synthesis of poly(ethylene glycol) hydrogel [161] and elastin-like protein polymers [162]. Transglutaminase crosslinked carboxymethyl chitosan/carboxymethyl cellulose/collagen composite membranes have shown enhanced mechanical properties and improved biodegradability [163]. Westhaus and Messersmith designed a hydrogel system [164] based on a mixture of fibrinogen and Ca-loaded liposomes which was crosslinked with Ca^{2+}-dependent transglutaminase enzyme. This remained as fluid at room temperature, but as soon as the mixture was warmed to higher temperature (37 °C only), gelling process started leading to the formation of a hydrogel. Such hydrogels may find application in thermoresponsive drug delivery systems. Sperinde and Griffith reported that addition of transglutaminase enzyme in lysine end-functionalized PEG polymers can result in the formation of hydrogels [165]. In another study, a two step crosslinking method composed of the enzymatic crosslinking and Diels–Alder (DA) click chemistry was adopted to prepare injectable hyaluronic acid/PEG (HA/PEG) hydrogel system [166]. The enzymatic crosslinking resulted in the formation of HA/PEG injectable hydrogel within short time due to faster gelation of polymers. Furthermore, use of DA click reaction attributed remarkable anti-fatigue and shape retaining properties. In addition to the improvement in mechanical strength and modulus, hydrogels displayed desirable compressive strain recovery properties.

Horseradish peroxidase (HRP) is commonly used as di-tyrosine crosslinker between silk fibroin proteins [167]. Similar method of crosslinking was used to develop tyramine-substituted hyaluronic acid (HA) bioactive hydrogels, but it possesses poor mechanical properties and stability leading to rapid degradation. Therefore, HA was covalently crosslinked with silk fibers resulting in the formations of composite hydrogels that possessed improved mechanical stability and good hydrophilicity [168]. In these HA silk fiber hydrogel assemblies, increase in HA concentration resulted in decreased gelation time and increased degradation rate. This offers controllable stiffening and elasticity characteristics which could be highly advantageous in tissue engineering applications. Yang et al. prepared gelatin hydrogels using multiple crosslinking agents comprising of genipin (GP), glutaraldehyde (GTA), 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and

microbial transglutaminase (mTG) [169]. Hydrogels developed using GTA and GP as crosslinking agents showed very high compressive moduli, whereas EDC crosslinked sponges (hydrogels) displayed fast degradation rate. In addition, GTA and GP crosslinked sponges showed immediate rejection during in vivo trials, whereas GP crosslinked sponges showed poor growth of adipose derived stromal cells during in vitro experiments. mTG–sponge displayed desirable properties with enough porosity, durability, improved compressive modulus and good biocompatibility. In another study, transglutaminase crosslinked collagen hydrogels were fabricated to mimic native extracellular matrix architecture [170]. HRP crosslinkable injectable hydrogel based on poly(L-glutamic acid)-graft-tyramine (PLG-g-TA) was developed to explore the behaviors of BMSCs during three-dimensional (3D) culture. A fast geleation was observed after subcutaneous injection of PLG-g-TA, HRP and H_2O_2-based hydrogel. The histological analysis of the tissues at the application site after different time points demonstrated its biocompatibility [171]. Such systems may also find applications in cardiac tissue repair [172]. It can also be used as a cell carrier [173], drug delivery system and wound healing patches [174–176]. The biocompatibility of this hydrogel system is a great advantage and major factor for its potential use in biomedical applications [177, 178].

Tyrosinase (Tyr) is a copper-containing enzyme present in both plants and animal tissues that catalyze the production of pigments from tyrosine by oxidation. Tyrosinase catalyzes oxidation of phenols into activated quinones [179] in the presence of copper co-factor and O_2. These activated quinones can react with hydroxyl group or amino group of polymers by Michael-type addition reaction and form hydrogels [180]. Chen et al. [181] compared the effects of tyrosinase or transglutaminase enzymes on the formation of gelatin and chitosan hydrogels and concluded that tyrosinase induced faster gelation as compared to transglutaminase.

In another work, gelatin-based tissue adhesive hydrogels were prepared by dual-enzymatic crosslinking using HRP and Tyr (Fig. 11) [182]. Here, Tyr convert phenol groups of gelatin derivatives into *o*-quinone, which can react with amines or thiols on tissue surfaces and facilitate tissue adhesion. Incorporating tyrosinase did not affect the gelation rate or mechanical strength of HRP-crosslinked hydrogels. Importantly, the dual-enzymatically crosslinked hydrogels (GH/HRP/Tyr) exhibited significantly improved adhesive strength (34 kPa), which was superior to single HRP-crosslinked hydrogels (GH/HRP; 19 kPa) and commercially available fibrin glues (7 kPa). Thus, dual-enzymatic crosslinking of gelatin-based hydrogels could be a promising approach to develop bio-adhesives for tissue engineering or surgical applications.

5 Crosslinking by Natural Crosslinking Agents

Natural crosslinking agents not only improve the thermal stability of hydrogels but also enhance their biocompatibility with biological systems. Crosslinking of collagen films by proanthocyanidin (PA), a polyphenol found in grape seeds, not only

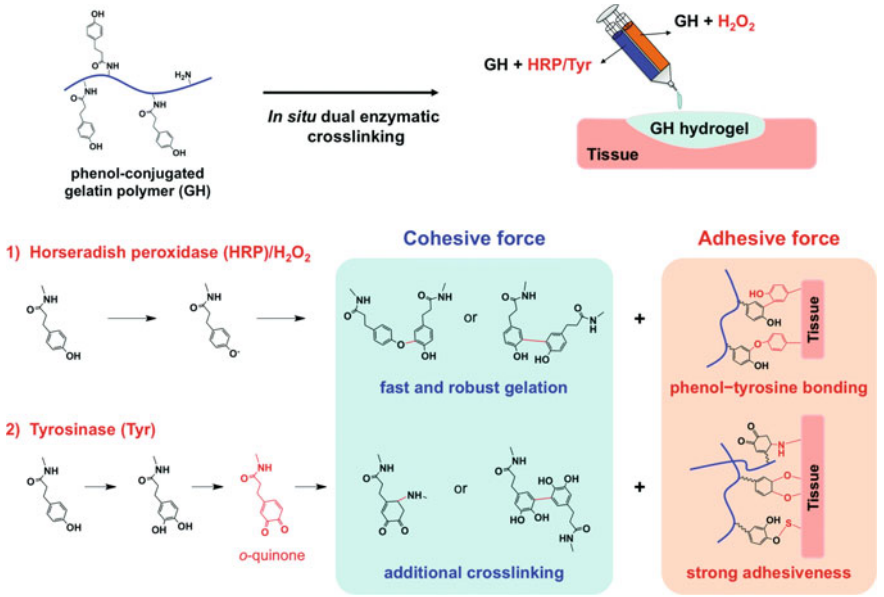

Fig. 11 Schematic showing the development of GH hydrogels by dual-enzymatic crosslinking using HRP and Tyr. Reproduced with permission from [182]

improves the thermal resistance but also enhances its resistance to enzymatic degradation without disturbing their cytocompatibility [183]. PA-crosslinked collagen membranes after several weeks of subcutaneous implantation displayed significantly greater penetration of fibroblasts without causing any damage to nearby native tissues.

Genipin can also be used for crosslinking hydrogels and making stable biomaterials. For example, genipin was used to crosslink chitosan, bovine serum albumin (BSA), and gelatin [184]. The results obtained through several spectroscopic techniques demonstrated that primary amine groups were crosslinked with two types of chemical reactions. The first reaction was a nucleophilic attack on genipin by a primary amine group of chitosan. This resulted in the formation of a heterocyclic compound of genipin that was bonded to the glucosamine residue in chitosan as well as the basic residues of gelatin and BSA. The second reaction involved a nucleophilic substitution of ester group of genipin leading to the formation of a secondary amide linkage among gelatin, chitosan and BSA. The rheological behavior of chitosan solutions changes with amount of genipin used [185]. The stress and frequency sweeps were used to find out G' of the crosslinked hydrogels. Results of this study shows that solutions of chitosan crosslinked with genipin could form strong and stable flexible gels when compared to those of pure chitosan.

Additionally, proanthocyanidin (PA) was selected as a natural crosslinking agent to crosslink biopolymers in biological tissues [183]. The evaluation of crosslinking and degradation rate besides cytotoxicity testing of PA on fixed tissues showed very interesting results. The cytotoxicity studies showed that PA crosslinked tissues are

~120 times less toxic compared to the tissues where glutaraldehyde (GA) was used as the crosslinking agent. The fixed tissues displayed marked resistance to bacterial collagenase digestion during in vitro studies. Unlike fresh tissues, PA crosslinked tissues showed a comparable stability with that of GA crosslinked tissues after subcutaneous implantation in animal models. Unlike GA counterparts, PA fixed implants started to degrade after six weeks of implantation which led to the migration and subsequent proliferation of fibroblasts into the PA fixed implants. Therefore, PA crosslinked collagen matrices could be very useful for designing tissue engineering scaffolds which will enable better cell proliferation and encourage cell ingrowth. In another study, PA crosslinked gelatin (PCG) conduit was developed and used for the peripheral nerve regeneration [186]. Crosslinking of gelatin conduit with PA improved the resistance to enzymatic degradation. In addition, use of PA as a crosslinking agent has been proved as beneficial for the enhanced cell adhesion, cell viability and growth of Schwann cells. Furthermore, the application of PA crosslinked gelatin conduit on a sciatic nerve wound (10 mm) in rat resulted in a complete recovery of damaged nerve tissues within 8 weeks. PA crosslinked gelatin nanofibrous membranes showed a twofold increase of L929 fibroblast cell adhesion compared to non-crosslinked fibers [187]. Gelatin crosslinked with PA can be used in drug delivery applications also [188].

6 Challenges in Hydrogel Crosslinking

Hydrogels provides a vast variety of biomedical applications ranging from drug delivery to tissue engineering. There are several methods used for the crosslinking of hydrogels to make them suitable for biomedical applications. However, there are many disadvantages and limitations for such methods too. The major disadvantage is that several crosslinking methods are not adequate to provide enough mechanical properties and stability under physiological conditions. For examples, they start breaking up immediately, especially when they are placed in the aqueous medium due to the collapsing of gel-like structure. This is particularly more evident in hydrogels that are crosslinked by physical methods. This is because, in physically crosslinked gels, interactions between polymers chains in amphiphilic block and graft copolymers are established by ionic and/or hydrophobic interactions, or crystallization which can be weakened under physiological conditions. In order to improve their stability and mechanical properties, crosslinking with a chemical agent is preferred to enhance their lifetime. However, cytotoxicity associated with the chemical crosslinking agents is a major disadvantage of chemically crosslinked hydrogels. Cytotoxicity of chemical crosslinkers such as glutaraldehyde is dependent on the concentration of crosslinking agents used [189]. Apart from glutaraldehyde, several other agents including epichlorohydrin, carbodiimide and sodium metaphosphate have also been used for the crosslinking of biopolymers, but they show limited improvement in properties owing to their low crosslinking efficiency. There are other methods of crosslinking of hydrogels such as those using ionic radiations. On one

hand, these types of crosslinking methods have the advantage of reversibility and lack of potentially harmful chemical reactions that affect the incorporated bioactive agents or cells. On the other hand, their stability in vivo might be severely affected by biochemical as well as mechanochemical conditions. For instance, conditions including application in weight bearing regions, for example in the bone and joints, for which these gels might provide insufficient mechanical strength.

For the successful encapsulation of human cells in hydrogels, they should be completely biocompatible. Moreover, the crosslinking agents and crosslinking conditions should be cell friendly. But, most of the crosslinking agents and conditions currently used are not favorable to maintain the cells at a viable state. In addition, a quick gelation of the polymer is very important, especially when techniques like bioprinting are used [190]. Another important issue is the lack of enough porosity under swollen state where porosity is very important for the cell migration and to facilitate fluid transport.

Crosslinked hydrogels are extensively used in drug delivery applications. However, when the delivery of small hydrophilic molecules is concerned, hydrogels show a burst release of drugs which may create transient higher plasma drug concentration and results in adverse effects. So, future research should focus on the development alternative approaches to encapsulate small molecule in hydrogel network such as increasing the crosslinking density.

7 Conclusions

Hydrogels are synthesized either from natural or synthetic polymers by crosslinking with various methods such as physical, chemical and biological approaches. The physical hydrogels are developed by reversible crosslinking while chemical hydrogels are made by irreversible covalent bond formation. For the encapsulation of biomolecules such as growth factors and living cells, physically crosslinked gels are popular since it can be performed under simulated physiological conditions. However, for fabricating highly stable and rigid hydrogels for applications such as bone tissue engineering, chemical crosslinking approaches are inevitable. In order to avoid the disadvantages of chemical crosslinking agents, relatively nontoxic crosslinking agents and processes were developed. Enzymatic crosslinking uses various enzymes that can facilitate the interaction between end functional groups in polymer chains and can be performed under mild conditions. Also, natural agents might contribute to the development of nontoxic hydrogel systems and thus provide the full advantage of their application potential in health care. Finally, it can be expected that smart hydrogels which will be developed in near future in which triggered gelation and gel collapsing will find great applications in controlled drug delivery and tissue engineering.

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