Enzymatic Crosslinked Hydrogels for Biomedical Application

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Abstract—Self-assembled structures primarily arise through enzyme-regulated phenomena in nature under persistent conditions. Enzymatic reactions are one of the main biological processes constructing supramolecular hydrogel networks required for biomedical applications. Such enzymatic processes provide a unique opportunity to integrate hydrogel formation. In most cases, the structure and substrates of hydrogels are adjusted by enzyme catalysis due to enzymes’ chemo-, regio- and stereo-selectivity. Such hydrogels processed using various enzyme schemes showed remarkable characteristics as dynamic frames for cells, bioactive molecules, and drugs in tissue engineering, drug delivery, and regenerative medicine. The enzyme-mediated crosslinking hydrogels mimic the extracellular matrices by displaying unique physicochemical properties and functionalities such as water-retention capacity, biodegradability, biocompatibility, biostability, bioactivity, optoelectronic properties, self-healing ability, and shape memory ability. In recent years, many enzymatic systems investigated polymer crosslinking. Herein, we review efficient strategies for enzymatic hydrogelation, including hydrogel synthesis and chemistry, and demonstrate their applicability in biomedical systems. Furthermore, the advantages, challenges, and prospects of enzymatic-crosslinkable hydrogels are discussed. The results of biocompatible hydrogel products show that these crosslinking mechanisms can fulfill requirements for a variety of biomedical applications, including tissue engineering, wound healing, and drug delivery.

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INTRODUCTION

Most of the cells in the body are placed in a three-dimensional (3D) microenvironment [1‒3]. The organization and architecture of these 3D networks significantly affect cellular function, behavior, metabolism, and fate [4, 5]. Designing biomimetic 3D microenvironments in vitro using biocompatible reactions and biomaterials can provide insights into the complex mechanical and biochemical interactions responsible [6, 7]. These new developments in creating cultured systems are applicable in drug discovery, malignant or damaged tissue regeneration, and biological process analysis, as all fields of biomedicine and biotechnology are strongly influenced by these achievements [2, 6]. Moreover, tissue engineering (TE) aims to produce artificial tissues and organs demonstrating native processes and functions [8, 9]. Hence, fabricated scaffolds, with or without cells, are delivered to the injured or diseased body tissues and organs. Subsequently, implanted tissue construction regenerates tissue either by its degradation and new tissue synthesis from the embedded cells or by host cell stimulation [1, 2, 10]. However, designing and fabricating scaffold substrates is challenging due to the variation and complexity of the prospective applications [9, 11, 12]. Nevertheless, several essential requirements for fabricating all 3D architecture must be achieved. First, all incorporated substrates and applied treating methods must be biocompatible and tolerated under the mechanical loads [13, 14]. The second item in the design process is the integrity of the fabricated structures with sustained uniformity [15, 16]. Third, the hydrogel microstructure should facilitate cellular adhesion, and ideally, integrate into the surrounding biological microenvironment [17, 18]. Finally, highly porous systems are necessary to enable cellular migration and proliferation as well as the growth of surrounding tissue and angiogenesis [19, 20]. Other important considerations are the controlled stimulation of the predictable cellular responses, for example, proliferation, migration, organization, and differentiation [21–23]. In addition to the need for easy and convenient sterilization, handling, and processing of all included materials, these complex requirements further complicate the process [24, 25].
In recent years, numerous polymeric networks including synthetic, natural, inorganic, and their combinations have been used to develop scaffolds [26–28]. Hydrogels are crosslinked polymeric networks capable of retaining a high quantity of liquid. Both natural and synthetic polymers have been used to form hydrogels [26, 28]. Polymers are crosslinked through either physical interactions or chemical bonds to form a hydrogel, resulting in a network that swells in water while maintaining a defined 3D structure [4, 11]. Moreover, hydrogels display minor mechanical and frictional irritation to the surrounding tissues when implanted in the body since they have a similar architecture to the extracellular matrix and soft tissues due to their rubbery nature [29, 30]. Furthermore, 3D network hydrogels provide structural support and enable the transportation of oxygen, nutrients, and metabolites to and from the encapsulated cells [28, 31, 32]. Therefore, hydrogels have been further explored for in vivo applications; examples include a coating material on the inner surface of blood vessels to minimize thrombosis following surgery-induced tissue injuries [33, 34] or a barrier between tissues to prevent postoperative adhesion formation [35, 36]. Furthermore, hydrogel vehicles have been utilized for prolonged drug delivery. Biofabrication techniques and micrometer-sized cell- or drug-containing hydrogel vehicles generated via various methods such as wet spinning [37], emulsification methods [17, 38], microfluidic systems [17, 39] and bioprinters [40, 41]. If the precise site dimensions of a defect or injured tissue are not accessible or too complex to be fabricated, injectable hydrogels are the appropriate choice [42–44]. The injection of a hydrogel precursor solution and the subsequently triggered network formation are facilitated by the flexible nature of hydrogels, which can adapt to the given environmental conditions [45, 46]. Injured tissue sites can be filled rapidly and conveniently with minimal invasion from the host immune system [47]. Furthermore, the hydrogels’ chemical and physical properties are adjustable. For example, the network structure, physical and biochemical features of the hydrogel constructs can be tuned and customized for a given application by controlling parameters such as the chemical composition and crosslinking methodologies [48, 49].

Until now, many polymer crosslinking approaches, including physical and chemical, have been employed to form polymeric networks depending on the required structure and desired application [1, 2]. Physically crosslinked hydrogels are created by changes in environmental conditions whereby gelation often occurs under the influence of pH [28, 50], temperature [37, 49], polyelectrolyte complexation [4, 37], hydrogen bonding [51], crystallization, and hydrophobic association [37, 52]. Chemically crosslinked hydrogels comprising covalent bonds among the polymer chains are formed via radical polymerization and the non-enzymatic or enzymatic crosslinking of complementary groups [8, 9, 37]. Enzyme-mediated hydrogel formation is proven to be mild and effective when comparing existing physical and chemical approaches. Therefore, developing this approach with higher degrees of complexity is a current focus (Table 1) [1, 37]. The majority of enzymes involved in polymer crosslinking are common to enzymes that catalyze reactions in the body. Enzymatic reactions that occur at neutral pH, in aqueous solutions, and at physiological temperatures are highly desired for biomedical applications [9, 53].

Moreover, possible cytotoxic consequences and unexpected by-products that can arise from photoinitiator- or organic solvent-mediated reactions are avoided due to the substrate specificity of the enzyme [9, 53, 54]. Another unique advantage concerns the mildness of the enzymatic reaction under normal physiological conditions because this method for crosslinking natural polymers cannot withstand harsh chemical conditions, leading to the loss of bioactivities [9, 22]. Lastly, using enzymatic crosslinked hydrogels offers a perspective for employing various enzymatic systems to choose the desired hydrogel properties for different biomedical purposes [23, 53, 54]. This review selected the enzyme-mediated crosslinking method for hydrogel production as the template material for engineering tissue and regenerative medicine. This approach has emerged as a desirable alternative to overcome the difficulties associated with other chemical crosslinking methods or physical hydrogelation.

HYDROGELATION MECHANISMS

Transglutaminase

Transglutaminases (TGs) are a broad family of thiol enzymes that catalyze post-translational protein modification by inducing isopeptide bond configuration. The covalent conjugation of polyamines, lipid esterification, or the deamination of glutamine residues are also candidates for this purpose. Transglutaminases are a mild substitute for chemical crosslinking, which catalyzes the formation of covalent bonds between a free amine group from a protein or a peptide-bound lysine and the g-carboxamide group of proteins, or peptide-bound glutamine (Fig. 1a) [21, 54]. The TGs largely originate from both microbial [25] and mammalian cells [24].

Davis et al. developed a protein-based hydrogel system that was crosslinkable using TG [27]. This system consists of two de novo designed protein polymers named Kn and BQ; the Kn block contains lysines, B is a random coil hydrophilic block, and the Q block serves as a glutamine substrate [26]. Residues are formed once isopeptide is bonded between glutamine and lysine. These bonds are highly resistant to proteolytic degradation. As a result, steady polymeric networks are assembled without additional co-factors. The biochemical function of TGs was revealed by dis-
ENZYMATIC CROSSLINKED HYDROGELS

Table 1. Advantages and drawbacks of different crosslinking methods for producing hydrogels [23, 53, 54]

| Crosslinking Method                                      | Advantages                                                      | Drawbacks                                                                 |
|---------------------------------------------------------|-----------------------------------------------------------------|--------------------------------------------------------------------------|
| Physical crosslinking (e.g., ionic, electrostatic, protein and hydrophobic interactions, hydrogen bonding, crystallization, and thermo-responsive) | • Reversibility • Absence of potentially harmful chemical reactions • Homogeneous crosslinking | • Insufficient mechanical strength • Unstable • Lack of ability to tailor • Inflammatory due to changes in ion concentration and pH |
| Chemical crosslinking (e.g., radical, polymerization, photo-crosslinking, organic solvents-mediated reactions) | • Covalent bonds • Controllable mechanical strength • Superior physiologic stability | • Undesired side reactions • Toxic reagents • Harsh conditions • Cluster formation |
| Radiation crosslinking (e.g., Gamma-ray, ionizing radiation) | • No need for any catalyst or chemicals • Does not leave toxic residues • Simultaneous crosslinking and sterilization | • Remaining crosslinking agents hinder biomedical applications • Restriction for cell encapsulation • Limited number of polymers |
| Enzyme-mediated crosslinking                            | • In-situ gelation • Substrate specificity (chemo-selective) • No toxic by-product • Neutral pH • Moderate temperature | • Substrate specificity (crosslinking reaction occurs only between enzyme-specific polymers) |

covering the role of isozyme factor XIII in blood coagulation for fibrin-stabilizing factors [30]. These enzymes exist in various tissues, such as the skin and brain [31, 32]. The TGs are essential for fibrin clot and cornified epidermis formation. Consequently, the lack of these enzymes strictly hampers wound healing [36]. Davis et al. reported a modular hydrogel with tunable characteristics formed within 2 min using this system. Furthermore, bioactive peptides allow customizable cell-signaling requirements when engrafted. Fibrin matrices are formed by factor XIII, which is the circulatory form of TG [27].

Several applications have been studied in vitro and in vivo regarding these matrices, including angiogene-

![Fig. 1. (a) Mechanism of the enzymatic reaction mediated by transglutaminase. Adapted with permission from [54]. By courtesy of Elsevier. (b) Mechanism of the enzymatic reaction mediated by tyrosinase. Polymers conjugated with phenol (Ph) moieties such as tyramine, phenylalanine, tyrosine, hydroxypHENYL propionic acid, and 4-hydroxyphenyl acetic acid in presence of oxygen and following amine groups of complementary polymer such as chitosan proceed hydrogelation. Adapted with permission from [54]. By courtesy of Elsevier. (c) Mechanism of lysyl phosphopantetheinyl transferase mediated reaction. Adapted with permission from [54]. By courtesy of Elsevier. (d) Mechanism of lysyl oxidase or plasma amine oxidase-mediated reaction in fabrication of hydrogels. Adapted with permission from [54]. By courtesy of Elsevier.](image)
sis, nerve repair, and cartilage TE [55–57]. Sala et al. used activated T factor XIII, a fibrin-stabilizing factor, to simultaneously couple site-specific cell adhesion ligands and crosslink modified multi-arm poly(ethylene glycol) (PEG) precursors. In their system, the material building blocks are responsive to two enzymatic systems, one responsible for matrix formation and the other for the degradation process [15]. The enzyme-mediated site-specific coupling of ligands allowed enhanced cell spreading, proliferation, and migration, as well as proteolytic matrix degradation by cell-derived matrix metalloproteinases (MMPs). Well-designed strategies also used factor XIIIs to crosslink star-shaped PEG, functionalized using either a glutamine acceptor or donor, to bind growth factors to provided surfaces [15]. Consecutive enzymatic reactions permit the site-specific immobilization of large quantities of biologically active substances in the proposed site. Tissue TG has a high degree of sequence similarity with other TGs; however, it does not require any proteolysis for activation. Furthermore, tissue TGs demonstrate stronger adhesiveness than fibrin-based glues and less vulnerability to physical parameters such as humidity [45]. Spindle and Griffith described the combination of PEG and tissue TGs. In their models, the gelation time depended on polymer functionalities, primary stoichiometric ratios, and substrate kinetics [47, 58]. Hu and Messersmith reported high adhesive strength in peptide-conjugated polymer hydrogels formed in situ and crosslinked using TG [59].

Transglutaminase has also been used to create gelatin-based hydrogels. These gels have excellent cytocompatibility, which is a crucial characteristic of TE applications. They also demonstrate superior transport properties, which assist in sustained drug delivery [60, 61]. Genetically engineered elastin-like polypeptide hydrogels and peptide-PEG conjugates crosslinked using TGs have shown promising features such as injectable hydrogels or cartilage repair [62, 63]. A report published by Jones et al. revealed that reactive ECM components could allow the coupling of peptide and peptide-polymer conjugates via tissue TG [63]. The possibility of applying this strategy to a variety of tissue surfaces highlights the flexibility of this method in biomedical applications. Moreover, surfaces can be modified using functional moieties, growth factors, or therapeutic drugs [1, 2]. Transglutaminases are enzymes that depend on the presence of Ca\(^{2+}\) [63]. The Ca\(^{2+}\)-independent TG-catalyzed gel formation also demonstrates the ability to entrap and release cells. These gels are seen as particularly practical in microfluidic biosensor systems [64].

**Tyrosinase**

Tyrosinases are also known as phenoloxidase and monophenol monoxygenase, and they catalyze macromolecular network formation without co-factors [65]. Tyrosinase is a copper-containing enzyme that catalyzes phenol oxidation into activated quinones in the presence of O\(_2\), as in dopamine and tyrosine residues [65]. Activated quinones can react with an amino or hydroxyl group, primarily via a Michael-type addition reaction (Fig. 1b) [66]. Tyrosinases can be found in plants and animals and act in melanin formation, food browning, and cuticle hardening in insects [67]. In most plants and animals, tyrosinases have interacted with wide range of substrates. Conversely, substrate specificity is restricted to the L-form of tyrosine or DOPA in mammalian tyrosinases [67, 68]. Chen et al. compared gelatin and chitosan gels formed by crosslinking using either tyrosinase or TGs; the results showed that tyrosinase induced faster gelation [67]. Nonetheless, the hydrogels catalyzed using tyrosinase were fabricated in the presence of chitosan and were mechanically weaker. Gels formed using tyrosinase are primarily appropriate for glue and wound dressings or can be used for protein immobilization due to their rapid degradation [69, 70]. Additional tyrosinase applications include crosslinking tyrosine residues in fibroin, sericin, and silk, yielding protein-polysaccharide conjugates [71, 72]. Mishra et al. used tyrosinase to provide enzymatically crosslinked carboxymethyl-chitosan/gelatin/nano-hydroxyapatite injectable gels at 37°C for in-situ bone TE [73]. These hydrogels showed potential in biomedical applications because of their exclusive mechanical properties, adhesiveness, and non-toxicity [54].

**Phosphopantetheiny Transferase**

Phosphopantetheinyl transferase is a small (16.2 kDa) enzyme that catalyzes covalent crosslinking in many polymers with high efficacy. Therefore, it differs from transglutaminases, which demonstrate inadequate recombinant production and are more abundant in nature. Transferases are expressed in the cytosolic compartment in a wide range of yeast and animal cell tissues and include large multifunctional polypeptides that contain all of the catalytic components essential for the synthesis of long-chain fatty acids [74]. The primary mechanism of transferase catalysis is to create synthetic hydrogels by shifting a phosphopantetheine prosthetic group of coenzymes in A-functionalized PEG macromers to a serine residue of engineered carrier proteins (Fig. 1c). Mosiewicz et al. used phosphopantetheinyl transferase to create hydrogels [75]. The authors constructed hybrid hydrogels by mixing eight-arm PEG-coenzyme A precursors at 37°C in the presence of Mg\(^{2+}\) and a neutral pH. The gelation was relatively slow and completed in nearly 15 min. The hydrogel reached an elastic modulus rate of 2.3 kPa. This technique allows selective covalent transferase-catalyzed hydrogels to form and modify bioactive peptide ligands, specifically the integrin receptor binding motifs, such as RGDs (Arg–Gly–Asp), which enable cell attachment and spread-
ing [76]. This type of reaction is attractive for cell biology and TE applications.

**Sortase A**

Sortase A (SrtA) categorizes transpeptidase molecules with exceedingly specific activity for crosslinking, extracting from gram-positive bacteria. In this reaction, cleaving the bond between threonine and glycine in a c-terminal LPXTG recognition motif forms a thioester by conjugating the amino and carboxyl groups from the N-terminal glycine of complementary groups [77]. Many peptides designed de novo have been studied as feasible SrtA molecules [26, 78]. Conjugating human epidermal growth factor with a small soluble oligoglycine substrate, GGG motif (GGG-EGF) to PEG hydrogels, was demonstrated to be the desired methodology for adjusting cell-interactive hydrogels [79].

**Lysyl Oxidase and Plasma Amine Oxidase**

Lysyl oxidase is one of the critical elements in constituting and regenerating the original ECM. In the ECM and the presence of lysyl oxidase, an enzymatic reaction initiates through the oxidation of the primary amine of lysine to an aldehyde [80, 81]. The resulting reactive aldehydes spontaneously react with another amine to form a Schiff base or undergo an aldol condensation with another aldehyde (Fig. 1d) [82]. Lysyl oxidase is activated to proceed with covalent crosslinking between the elastin and collagen fibrous derivatives to make them stable structures in ECM. Therefore, lysyl oxidase is responsible for tissue pattern formation, morphogenesis, and the repairability of various connective tissues, including skeletal, cardiovascular, and respiratory tissues [83]. Moreover, plasma amine oxidase (PAO) is an accessible semicarbazide-sensitive amine oxidase that acts as an oxidation of primary amines [82]. As previously discussed, both enzymes can be applied as crosslinkers to develop bio-material and tissue mechanical properties and upregulate ECM formation and tissue regeneration [80, 84]. Bakota et al. used lysyl oxidase to fabricate self-assembled fibrous scaffold from multi-domain peptides, which advanced the oxidative crosslinking of lysine residues. The most remarkable feature of these hydrogels created using lysyl oxidase was their desirable mechanical properties, including mechanical strength and handling of the hydrogel, which becomes more robust with time. This characteristic of rich lysine polymers leads to persistent upregulation in the networks’ mechanical properties.

Furthermore, as the serum is contained in lysyl oxidase, in serum-supplemented media, we can proceed with crosslinking the lysine-containing polymers spontaneously without adding an exogenous enzyme. Producing ECM using cells in hydrogels could enhance this enzyme’s use. In summary, lysyl oxidase has shown significant applicability in multiple usages, including cell or drug delivery, and as scaffolds for tissue regeneration using multi-domain peptide hydrogels [85, 86]. Moreover, the inherent mechanical properties of tissue-engineered constructs could be enhanced using lysyl oxidase by extending culture time, which facilitates hydrogel integration with original tissue via covalent bond formation among lysine-rich molecules present in hydrogel and amines in tissue ECM [81, 85].

**Phosphatase or Kinase Derivatives**

The enzyme-catalyzed or -regulated formation of hydrogels from small peptide derivatives can proceed by altering the amphiphilicity of phosphatase or kinase derivatives such as phosphatases, β-lactamase, thermolysin, or phosphatase/kinase [87, 88]. An example is kinase or dephosphorylation interaction by a phosphatase. This alteration can activate the physical interactions and self-assembly of the amphiphilic peptide moieties and eventually lead to hydrogelation, as shown in Fig. 2a [87, 88]. These small peptides, which primarily possess bioactive or organic molecules, can endure the addition of specific bioactive substrates. For example, the phosphatase-mediated exclusion of phosphate moieties from peptides, resulting in the hydrophobicity of substrates that may self-assemble into a fibrous network by noncovalent mediations (such as charge interactions, p—p interactions, hydrogen bonding), makes hydrogelation possible [89]. The peptide’s amphiphilicity is reformed by altering the peptide instead of breaking the covalent linkages between the phosphate group and the peptide using thermolysin. Specifically, thermolysin mediates to form bonds between peptides by reverse hydrolysis. This enzyme can combine two distinct peptide derivatives while reducing the solubility of one of the peptides. The blocking peptide can then self-assemble into a hydrogel via hydrophobic interactions.

Thermolysin prefers aromatic, hydrophobic residues on the amino side of the peptide bond. This method was presented by Toledano et al. with potential applications in the construction of nanofibrous hydrogel scaffolds for cell culture systems. Beta-lactamases and esterases are other enzymes in this category and can be used as catalysts for molecular self-assembly systems in hydrogel formation [90]. Beta-lactamases are produced from Escherichia coli strains, which is why they are resistant to β-lactam antibiotics, such as streptomycin and penicillin. Hence, the four-atom ring β-lactam molecular structure of antibiotics opened by lactamase restricts the activation of the substrate’s antibacterial properties. In this reaction, lactamase opens the lactam ring of the hydrogel precursor compound. Following the release of a hydrogelator, self-assembly converts precursors into hydrogel formation.
Available lactamases enable bacterial lysate to act as a hydrogelator by converting the precursor molecule, leading to hydrogel formation. The self-assembled hydrogels can be used in bacterial analyses and trigger specific cellular cycles since hydrogelation can occur within the cells [91, 92]. Most of the enzymatic reactions in biological systems are irreversible, and a single enzyme scarcely acts in a reversible manner. Hence, excessive modification of the crosslinked hydrogel peptide is necessary to achieve a reversible reaction mechanism. Yang et al. synthesized a pentapeptide hydrogelator, forming hydrogels via self-assembly using kinase/phosphatase, which switch to control hydrogelator phosphorylation and dephospho-

Fig. 2. (a) Mechanism of the supermolecular hydrogel formation proceed different enzymes such as phosphatase/kinase, phosphatase, thermolysin, and β-lactamase. Adapted with permission from [88]. By courtesy of American Chemical Society. (b) Mechanism of hydrogel production by alkaline phosphatase-catalyzed dephosphorylation of FMOC-tyrosine-phosphate (I) which resulted formation of compound (II) with higher amphiphilicity and lower charge. Adapted with permission from [96]. By courtesy of John Willey and Sons.
phorylation and regulate supramolecular hydrogel formation [93]. This enzyme switch-regulated supramolecular hydrogel promises a new way to apply biomaterials for biomedical use due to phosphorylation/dephosphorylation phenomena. These enzymes could regulate the formation or dissociation of self-assembled hydrogel and the corresponding macroscopic transition in a supramolecular hydrogel. Using this enzymatic switch allows the accurate control of biomaterial organization at a molecular level over time. These features of enzymatic-formed supramolecular hydrogels are very promising for TE and other biomedical applications [94, 95].

**Alkaline Phosphatase**

Alkaline phosphatases (ALPs) are an excellent resource for constructing gels because they participate in the mineralization of skeletal tissues [96]. The enzymatic dephosphorylation of N-fluorenylmethoxycarbonyl tyrosine–phosphate (FMOC: fluorenylmethoxycarbonyl) fabricates the self-assembled hydrogels while Compound I forms the supramolecular network.

The mineralization of calcium phosphate (hydroxyapatite) is described as follows (Fig. 2b). The dephosphorylation of Compound I in water produces Compound II, which spontaneously assembles due to the p-stacking of the fluorenyl end groups. These adopt a helical orientation due to the superhelical activities of the amino acid residues, increasing from H-bonding interactions among the terminal tyrosine groups [96]. Schnepf fabricated materials by taking advantage of this property [96].

In addition, the core segment of yeast prion sup35, a series of structurally related precursors, has been utilized for the alkaline phosphatase development of self-assembled hydrogels. Incorporating an amyloid segment into a cytotoxic precursor could eliminate the cytotoxicity of the precursor solution and provide a cytocompatible microenvironment [97]. Toda et al. designed an enzyme-responsive hydrogel enabling ALP to adjust elasticity in the cell culture. Consequently, ALP treatment of the deformed cells upregulated Runx2 expression, an osteogenic marker. This report demonstrates that the hydrogel’s mechanical property switch could upregulate the expression of osteoblastic genes derived from human mesenchymal stem cells (MSCs) in vitro [98]. These materials’ viscoelasticity makes them appropriate substrates for application in regenerative medicine, including TE, drug release, and wound healing [54].

**Peroxidases**

Peroxidases are a broad family of enzymes that particularly catalyze the following reaction:

\[ 2\text{RH}_2 + \text{electron donor (2e)} \rightarrow 2\text{RH}^- + 2\text{H}_2\text{O}. \]

Most of the peroxidases use hydrogen peroxide as an electron donor. This family consists of 42 isozymes, making it challenging to define in vivo functions [9, 99]. The most frequently used in hydrogel formations are horseradish peroxidase (HRP) and soybean peroxidase. The most significant advantage of this enzyme compared to other described enzymes is rapid gelation, which can occur in seconds [54]. As plant enzymes, peroxidases are useful tools for biotechnology and biosciences [9, 99]. Horseradish peroxidase is a single-chain β-type hemoprotein responsible for conjugating phenol and aniline derivatives in the presence of hydrogen peroxide [38, 53, 100]. Such groups are present in different moieties, such as tyramine, tyrosine, phenylalanine, and hydroxyphenyl propionic acid or acetic acid [101]. Horseradish peroxidase rapidly combines with hydrogen peroxide in this reaction, and the formed complex can oxidize hydroxyphenyl groups. Phenoxy radicals generate covalent C–O and C bonds through a radical coupling reaction (Fig. 3a) [102].

Soybean peroxidase is a potential substitute for HRP because of its similar mode of action and superior stability. Human peroxidases have been widely studied, and plant peroxidases have typically been utilized in enzymatic crosslinking to form hydrogels [102]. This enzyme has advantages such as non-cytotoxicity and the potential to crosslink in situ. An extensive range of natural and artificial polymers has been modified using tyramine or hydroxyphenyl propionic acid to develop hydrogels from peroxidases. Examples of these hydrogels are hyaluronic acid [103, 104], gelatin [14, 105, 106], dextran [107], chitosan [100, 108, 109], alginate [17, 110], xylans [111], carboxymethylcellulose [112], polyvinyl alcohol (PVA) [100], poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) block copolymer [113], and poly(ami no) amines) [114]. Moreover, HRP is composed of cysteine residues, and its thiol moieties can bind oxidase, whereas glucose has a reduced disulfide bond to thiol moieties. Therefore, the oxidation and subsequent reduction cycle of sulfate derivatives advanced the hydrogelation of polymer-Ph and could consider polymer-Ph hydrogelation in H₂O₂ excluding reaction by mixing polymer derivatives with HRP in higher concentrations. These findings demonstrate that the HRP-mediated crosslinking reaction is appropriate for fabricating cell-laden hydrogel constructs [115]. Compared to thiol-modified polymers, polymer-Ph molecules are not simply oxidized in the synthesis process and remain stable for an extended incubation period. Compared to other enzyme systems, HRP-mediated crosslinked reactions have attracted significant attention and extensive biomedical applications due to the potential ease of regulating the crosslinking and gelation rates by changing the number of reagents, including biopolymer, crosslinkable moieties, HRP, and H₂O₂ [115].
Fig. 3. (a) Mechanism of the peroxidases reaction using hydrogen peroxide as an electron donor. Adapted with permission from [102]. By courtesy of Royal Society of Chemistry. (b) Crosslinking mechanism of HA-Tyr conjugates by the HRP-mediated crosslinking reaction. Adapted with permission from [9]. By courtesy of Royal Society of Chemistry.
Revealing the pathological and physiological manners of extracellular reactive oxygen species (ROS) could facilitate hydrogelation without the exogenous application of electron donors. Yang Liu et al. reported development of cytotemplates in hydrogel membrane on surface of individual cells produced via HRP-mediated reaction and endogenous secretion of ROS as an electron donor by cells [116]. Sakai et al. created cytotemplates by hydrogel bulks on the surfaces of individual HRP-anchored cell membranes by crosslinking biopolymer compounds mediated by HRP in an aqueous solution. To develop crosslinking on the cell surface only, cells were soaked in a phosphorus-substituted polymer (polymer-Ph) solution containing HRP conjugated with a biocompatible anchor molecule (BAM-HRP) for the cell membrane, and the HRP was made to anchor the cell membrane. The hydrogel sheath thickness was attained by soaking the cells with the anchored HRP in an aqueous solution containing polymer-Ph and H₂O₂ in under 10 min. The hydrogel sheaths can be created from various polymer-Ph derivatives, such as protein, polysaccharide, and synthetic polymers [9, 22]. Cellular viability (>90%) and subsequent normal growth after hydrogel sheath removal confirmed the gels’ cytocompatibility [117]. Advances in encapsulation technology demonstrate great promise in fundamental studies of cell biology and biomedical applications.

**Horseradish Peroxidase Applications**

Darr et al. evaluated HA-Ph hydrogels that showed in vivo biocompatibility and stability in dissociation with injected hydrogel in subcutaneous tissue while maintaining the main of negative charge carboxyl groups, essential for the contribution of tissue physio-mechanical properties [118]. The HA-Ph molecules conjugated by HRP are promising candidates for TE and other biomedical applications [103, 119]. Dextran-Ph hydrogel combined with HA-Ph and heparin-Ph via HRP-mediated reaction mimics the ECM of native cartilage [20, 23, 120]. Similarly, Kim et al. described injectable HA-Ph [121] as an efficient drug carrier for rheumatoid arthritis treatment. The supermolecular Ph-terminated PEG hydrogels are other polymer combinations that use HRP to activate gelation which due to super viscoelastic properties resulted better cartilage regeneration [101, 113].

**Catalytic Mechanism of Peroxidase**

Horseradish peroxidase (HRP) is a metalloenzyme of heme obtained from the root of the horseradish plant (*Armoracia rusticana*). Heme has a planar structure held by an iron atom held firmly in the center of a porphyrin ring with four pyrrole molecules. The heme group forms a coordinated bond with the adjacent histidine residue in the central region. The HRP has various isoenzymes in the C isoenzyme form [13, 122]. Catalytic reaction peroxidase primarily involves the heme group and, in the protein core, histidine 42 and argenin 38 amino acid residues. After binding H₂O₂ to Fe(III) as the heme in its resting state, the α-oxygen of H₂O₂ transfers its proton to the histidine 42 residue. Afterward, the single α-oxygen bond forms with Fe(III), resulting in transient intermediate Compound II formation [9, 54]. Next, a double bond forms from α-oxygen with the heme, assisted by peroxide O–O bond cleavage, and the iron subsequently receives the electron from the porphyrin ring, leading to a ferryl porphyrin π-cation radical called Compound I. The water molecule forms via interaction between the residual –OH group of H₂O₂ and H from histidine 42. After proceeding with two oxidation states of Compound I, and thus two sequential electron transfers to reduce it back to the resting state, the Ph moieties of polymer-Ph conjugates as the reducing substrate comes into action (Fig. 3b). Specifically, through the catalytic cycle of HRP, the H₂O₂ molecule consumes and transforms two Phs into phenolic radicals and subsequently leaving H₂O molecules. The available Ph radicals bond through C–C or C–O linkages between the ortho carbons of the aromatic ring or the ortho carbon and the phenolic oxygen, which leads to the formation of a crosslinkage among the polymer-Ph substrates [123]. Theoretically, the increased H₂O₂ supply implies that more phenolic radicals are created, resulting in further crosslinkage among Phs. Moreover, the constant concentration of polymer-Ph conjugates and HRP raising the H₂O₂ supply increases the shear modulus (G̃) and decreases the swelling percentage of the resulting hydrogels, representing crosslinking intensification [14, 124]. Beyond an optimum range of H₂O₂ concentration, a further increase in H₂O₂ decreases G̃, which is probably due to surplus H₂O₂ reacting with HRP, resulting in the formation of two different types of catalytically inactive compounds, including compounds III and IV [125]. Compound III is a reversible intermediate that steadily turns over its native structure and restores its catalytic activity. Conversely, Compound IV, as an irreversible structure, does not indicate any catalytic activity. However, HRP inactivation is possible via an attack on the protoporphyrin by phenoxyl radicals, which causes heme demolition [126]. Consequently, HRP inactivation inhibits crosslinking and hydrogelation. Therefore, to avoid excessive use of H₂O₂, its implied optimum concentrations must be evaluated experimentally.

Anja Schmidt et al. suggested that H₂O₂ is used in a >100 fold excess, which lessens the shear modulus of the sample as the heme group is irreversibly destroyed, and the characteristic bond of Compound III is absent [125]. Moreover, the gelation rate of the HRP-catalyzed reaction is greatly dependent on the concentration of HRP and the quantity of Ph moieties [14, 124]. It is anticipated that increasing these factors would
result in a rapid phenolic radical formation and conduct higher covalent bonds. Hydrogels production ranges from a few seconds to several minutes, depending on the number of Ph moieties, H₂O₂ and HRP.

**Laccase**

Laccases are multicopper oxidases available in natural resources, including insects, fungi, bacteria, and plants [127, 128]. During hydrogelation in the presence of O₂, the laccases oxidize tyrosine residues substrates, including diphenols, methoxy-substituted monophenols, aromatic amines, aliphatic amines, while oxidized Ph molecules form various resonance structures (Figs. 4a, 4b) [127, 128]. Corresponding radical species while concomitantly transferring four electrons to dioxygen results in the formation of two water molecules and a crosslinking reaction (Fig. 4c) [33, 127]. The application of laccase-mediated hydrogels developed via the oxidation of catechol moieties and their reactions with free amino groups of chitosan forms a C–N bond via a radical coupling or Michael addition [129]. Wang et al. prepared a kind of composite hydrogel of HA-Ph and silk derivative via laccase-mediated crosslinking. They found that compared to the pure silk-based hydrogels, the obtained composite hydrogels demonstrated excellent shape-formation and swelling ability, which could be due to building intermolecular bonds via the laccase reaction [128]. Park et al. developed hypoxia-induced hydrogels by conjugating ferulic acid on polymer backbone and consuming O₂ in laccase-mediated reactions (Fig. 4c) [130]. The fabricated O₂ controllable biomaterials guided vascular morphogenesis via the hypoxia-inducible factor activation of matrix metalloproteinases and promoted fast neovascularization from the host tissue during subcutaneous wound healing [130].

**Fig. 4.** (a) Schematic illustrations of laccase mediated hydrogelation. In presence of O₂, hydrogels are made via laccase-catalase dimerization of grafted ferulic acid (DiFA). Adapted with permission from [130]. By courtesy of Springer Nature. (b) Laccase oxidizes Ph moieties in various resonance structures. Adapted with permission from [127]. By courtesy of Elsevier. (c) Chemical structures of DiFA could crosslink polymer chains. Adapted with permission from [130]. By courtesy of Springer Nature.
APPLICATIONS OF HYDROGELS FORMED BY ENZYMATIC REACTIONS

Drug Delivery

To use hydrogels as injectable protein delivery systems, it is essential to adjust the gelation rate and crosslinking density of gel networks to avoid encapsulation inefficiencies such as an abrupt leakage of gel precursors and protein drugs to the surrounding tissues caused by slow gelation after injection [131]. The crosslinking density of hydrogels is a crucial factor for having a sustained drug release [132]. An increase in the degree of crosslinking causes a reduction in the porosity and permeability of gel networks. Therefore, the rate of drug release typically decreases [133, 134].

The HRP-catalyzed crosslinking method allows independent regulation of the gelation rate and crosslinking density through variations in HRP and H$_2$O$_2$ concentrations [133, 134]. This process is highly advantageous for in situ-forming hydrogels to deliver protein drugs and growth factors [119].

P. Van de Wetering et al. used this system to form an injectable hyaluronic acid-tyramine (HA-Tyr) hydrogel for protein drug delivery [119]. The release rate of α-amylase and lysozyme could be regulated by manipulating the crosslinking density of HA-Tyr hydrogels. HA-Tyr hydrogels were applied for the in vivo delivery of interferon-α2a (IFN) [135], an antineoplastic cytokine used to treat various human cancers. The IFN-incorporated HA-Tyr hydrogels demonstrated greater antitumor efficiency compared to the IFN solution injected at the same dose. The improved anticancer result was related to inhibiting tumor angiogenesis with the treatment of IFN-incorporated HA-Tyr hydrogels. Lutolf et al. designed oligopeptide building blocks using a dual enzyme-responsive PEG-based hydrogel able to undergo formation and degradation in response to cell-secreted matrix metalloproteinases.

Self-assembled heparin-pluronic (HP) nanogels encapsulating bFGF were dispersed in gelatin-PEG-tyramine (GPT) polymer solution and used as a protein drug carrier. For this, macro/nanogel composite hydrogels produced by the HRP-catalyzed oxidative coupling of GPT conjugates. The GPT/HP composite hydrogels presented a considerably higher density of urethral muscles near the urethral wall than the GPT hydrogels [136, 137]. Devolder et al. showed an improvement in protein release kinetics from composite hydrogel systems through the HRP-mediated crosslinking of pyrrole groups [138]. Alginate-g-pyrrole hydrogel, including PLGA microspheres loaded with vascular endothelial growth factor (VEGF), was developed by the HRP-catalyzed crosslinking of pyrrole groups that were conjugated to an alginate backbone. Despite a lower crosslinking density, the enzymatically crosslinked alginate-g-pyrrole hydrogels released VEGF in a more sustained manner than Ca$^{2+}$ crosslinked alginate-g-pyrrole hydrogels. The pyrrole moieties produced following the enzymatic crosslinking contributed to a more sustained VEGF release by increasing the affinity of the growth factor and the gel matrix. This characteristic was advantageous to enhancing neovascularization in vivo. The enzymatically crosslinked alginate-g-pyrrole hydrogels notably improved the number and size of blood vessels at the injection site, as equated to the Ca$^{2+}$ crosslinked hydrogels [115]. Dehghan-Niri et al. presented injectable enzymatically crosslinked hydrogels based on Gum tragacanth (GT)-tyramine prepared by the oxidative coupling reaction in the presence of HRP and H$_2$O$_2$. The incorporation of tyramine-conjugated gelatin (Gtn-TA) into the hydrogels reduced the hydrogel swelling degree and the burst release of BSA. The release of BSA and insulin from tragacanthin (TGA)-TA/Gtn-TA hydrogel showed that the release of insulin was faster due to its smaller molecular size and weight. These hydrogels can be a promising candidate for in situ-forming systems for the delivery of therapeutic proteins [139].

Tissue Engineering (TE) and Cell Therapy

Mechanical characteristics are critical in designing injectable hydrogels for TE that have sufficient durability under dynamic in vivo conditions and provide encapsulated cells with an appropriate mechanical environment that leads to proper cell proliferation, differentiation, and tissue regeneration [140, 141]. The mechanical property of hydrogels must be adjusted based on the mechanical microenvironment of the targeted native tissue. Hence, the HRP-catalyzed crosslinking method is favorable because it offers advanced control over the mechanical strength of hydrogels by changing the H$_2$O$_2$ concentrations [142].

Wang et al. created gelatin-hydroxyphenyl propionic acid (Gtn-HPA) hydrogels as an injectable scaffold for TE [141, 143]. The storage moduli (G') of Gtn-HPA hydrogels was readily adjusted from 280 to 12,800 Pa through variations in H$_2$O$_2$ concentrations. The hydrogel rigidity resulted in the focal adhesion, proliferation, and differentiation of human mesenchymal stem cells (hMSCs) cultured in 2D and 3D environments. Cells cultured in a softer hydrogel expressed more neurogenic protein markers than those cultured in a harder hydrogel. Lim et al. studied the influence of Gtn–HPA hydrogel matrices on the oxidative stress resistance, migration, and differentiation of adult neural stem cells (ADSCs) [144]. The G' of Gtn–HPA hydrogels was adjusted (449–1717 Pa) to resemble adult brain tissue. Remarkably, the ANSCs encapsulated in Gtn–HPA hydrogels demonstrated greater resistance to H$_2$O$_2$-induced oxidative stress, whereas those encapsulated in collagen and alginate hydrogels exhibited severe necrosis [145]. This resistance would be beneficial for brain tissue regeneration since ANSCs transplanted at damaged sites in the brain
have a poor probability of survival due to exposure to oxidative stress and other uncongenial factors [146]. The Gtn-HPA hydrogels improved ADSC’s differentiation toward neuronal lineage compared to polyornithine/laminin-coated polystyrene substrates in mixed conditions for neuronal and astrocytic differentiation.

Gtn-HPA hydrogels have been used for cartilage repair in an in vivo osteochondral defect model [147]. Gtn-HPA hydrogels with different hardness (G' = 570, 1000, and 2750 Pa) were mixed with rabbit chondrocytes and injected into full-thickness osteochondral defects of the femur. The results suggested that the increased hardness of Gtn-HPA hydrogels stimulated the biosynthesis of type II collagen and sulfated glycosaminoglycans (sGAG) from the encapsulated chondrocytes and consequently promoted cartilage tissue repair in vivo. In addition to Gtn-HPA hydrogels, various polymer-phenol hydrogels have been broadly used for TE applications [147]. HA-Tyr hydrogels have been investigated as biomimetic matrices for the chondrogenic differentiation of caprine MSCs [148]. Recombinant human lactoferrin (rHLF) hydrogels have also been created for bone TE due to the anti-apoptotic and osteogenic activity of rHLF toward pre-osteoblast cells [149].

Jin et al. demonstrated injectable dextran-tyramine hydrogels as 3D scaffolds for cartilage regeneration [23, 115]. Chondrocytes incorporated in these hydrogels kept their phenotype and formed a cartilaginous-specific matrix following in vitro culture. Teixeira et al. postulated that the HRP-mediated crosslinking reaction induced strong adhesion between cartilage and dextran-tyramine hydrogels [16]. Histological and morphological assays of the cartilage-hydrogel interface indicated that dextran-tyramine conjugates were chemically bound to collagen molecules present in the cartilage ECM by a tyramine-tyrosine linkage. Incorporating heparin-tyramine conjugates made the homing of human chondrocytes and chondrocyte progenitor cells easier using dextran-tyramine hydrogels. The cell-attracting effect was probably related to the high affinity of heparin with growth factors and cytokines [115, 136]. Kuo et al. prepared an autologous extracellular matrix scaffold, murine collagen–Ph hydrogels. They demonstrated the appropriateness of injectable HRP-mediated collagen–Ph hydrogels for the human progenitor cell-based development of 3D vascular networks in vitro and in vivo. The authors also confirmed that the biodegradability, swelling properties, and stiffness of the collagen–Ph hydrogels, regulated by changing the amount of crosslinking, could be used to adjust the extent of the vascular network and adipose mineralized tissue configuration in vivo. They emphasized the significance of ECM in providing proper signals for endothelial-mediated vascular development, which is important for the initial stages of organogenesis in engineering cell-based 3D tissue constructs [150].

Sakai et al. reported the first method of cell-selective encapsulation in hydrogel sheaths. Cell selectivity was accomplished through an antigen-antibody reaction. Biocompatible encapsulation was achieved throughout the crosslinking reaction, catalyzed by an HRP-conjugated antibody immobilized on the cell surface. This method was achieved using two different systems: a primary antibody conjugated with HRP and a primary antibody with a secondary antibody conjugated with HRP. This method has significant potential for cell-to-cell communication, regenerative medicine, and cell therapies [151].

Morelli et al. reported the conversion of ulvan, a sulfated polysaccharide of algal origin, into an in situ-gelling material suitable for biomedical applications [152]. Ulvan was successfully modified with tyramine units to be susceptible to crosslinking reactions catalyzed by HRP enzymatic action in the presence of H$_2$O$_2$. The amount of used enzyme and H$_2$O$_2$ were optimized to induce gelation times appropriate for developing injectable hydrogels [152]. Furthermore, preliminary biological investigations indicate the suitability of enzymatically crosslinked ulvan hydrogels to function as a vehicle for viable cells, supporting their potential application as injectable cell delivery systems.

Khanmohammadi et al. introduced the preparation of cell-enclosing hyaluronic acid (HA) microparticles with either solid or liquid cores through cell-friendly HRP-catalyzed hydrogelation using a microfluidic system. The spherical tissues covered with a heterogeneous cell layer would be useful for biomedical and pharmaceutical applications [153–155]. Liu et al. assembled small tissue enclosing hydrogel microcapsules (about 200 μm in diameter) and a single hydrogel fiber, both covered with human vascular endothelial cells in a collagen gel. The fiber and microcapsules were made from gelatin and alginate derivatives and had cell adhesive surfaces. The endothelial cells on the hydrogel constructs sprouted and spontaneously produced a network linking the hydrogel constructs in the collagen gel. Alginate-based hydrogel constructs degraded by alginate lyase and perfusable vascular network-like structure were formed. Confirmation was achieved by introducing solution, including tracer particles of 3 μm in diameter, into the lumen model via the alginate hydrogel fiber. The introduced solution moved into the created capillary branches and dispersed to the individual spherical tissues. This method has great promise in developing the technique for constructing an internal perfusable vascular network to fabricate dense 3D tissues in vitro for biomedical applications [39]. Tomita et al. produced HRP-fabricated hyaluronan (HA) microcapsules feasible for use in the cryopreservation of a small amount of sperm for clinical applications [8].


**Wound Healing**

Tissue adhesives are useful for sealing defects in tissues, stopping bleeding, and promoting wound healing [156]. Fibrin glue and cyanoacrylates are two clinically available adhesives. Fibrin glue has been regularly used as an adjunct to hemostasis in surgical processes. Nevertheless, fibrin glue has been used only to stop low-pressure bleeding because of its comparatively low adhesive power [55, 157]. Cyanoacrylates revealed comparatively strong adhesion to tissues but released toxic substances like cyanoacetate and formaldehyde during degradation [158]. The HRP-catalyzed crosslinking technique provided a new prospect because this technique enabled fast gelation ranging from seconds to a few minutes in mild conditions.

Lih et al. created injectable chitosan poly (ethylene glycol)-tyramine (CPT) hydrogels as rapid biocompatible and durable tissue adhesives [159]. Tyramine-modified PEG chains were engrafted into a chitosan backbone to raise its solubility at physiological pH. Upon application, CPT hydrogels were produced in less than 5 s during the HRP-catalyzed coupling of tyramine moieties. These hydrogels provided great tissue adhesion with an adhesive power almost 20 times higher than fibrin glue. Chitosan poly (ethylene glycol)-tyramine hydrogels rapidly stopped bleeding from injured livers through the combined effects of the hydrogels’ adhesiveness and the hemostatic characteristic of chitosan [160]. The chitosan poly (ethylene glycol)-tyramine hydrogels efficiently closed the skin injuries and showed greater wound healing properties than suture, fibrin glue, and cyanoacrylate. Tran et al. described rutin-conjugated CPT hydrogels for dermal wound healing [161].

Rutin is a flavonol glycoside that exhibits antioxidative, cytoprotective, and wound healing properties [162]. Rutin was conjugated to a chitosan backbone of CPT polymer using an ester linkage. In vitro study revealed that the conjugated rutin was gradually released from CPT hydrogels through the hydrolysis of the ester linkage. The rutin-conjugated hydrogels considerably enhanced wound healing compared to hydrogels without rutin. These results imply that the combination of bioactive molecules and injectable hydrogels can produce a novel type of tissue adhesive with improved hemostatic and wound healing properties. Zavada et al. reported a more developed enzymemediated hydrogel for wound dressing fabricated from a combination of phenol functional poly(vinyl alcohol) and the glucose oxidase (GOx)/HRP [34]. To proceed with 3D polymerization, this method required both glucose and oxygen. Regarding in vivo application, the mixture of polymer, HRP, and GOx would only solidify once in contact with a glucose and oxygen supply available in the blood flow. Therefore, the constructed hydrogel could be practical when the formulation is utilized for a bleeding wound. Gelation times of less than 10 sec were readily achievable, and the hydrogels demonstrated no bio-incompatibility markers from any of the residual enzymes.

Another advancement would be containing glucose in the formulation and using molecular oxygen in the atmosphere as an environmentally-borne stimulus. This approach has been represented for GOx/HRP-mediated thiol-ene crosslinking [163]. A formulation of thiol-ene monomers with glucose, GOx, and HRP could be applied via spray, where the promptly accessible surfaces of atomized droplets would allow rapid oxygen diffusion, leading to the initiation of the 3D polymerization. An advantage of this method over formulations based on blood glucose is that the risk of remaining un-polymerized material is reduced. In addition to the GOx/HRP pair, there are probably other enzymatic systems for inducing oxygen-mediated in situ 3D polymerization, particularly laccase, which can create radicals from molecular oxygen [164]. Sakai et al. fabricated an on-tissue surface hyaluronic acid-based hydrogel by administering a prehydrogel aqueous solution to prevent postoperative peritoneal adhesion, which is a serious surgical complication. Hydrogelation is initiated when hydrogel precursor solution makes contact with the body fluid containing glucose on tissue surfaces, and it is accomplished in 5 s. In the hydrogelation process, an HA derivative possessing phenolic hydroxyl moieties crosslinks via a cascade reaction of GOx and HRP. A significant reduction in peritoneal adhesions has been found in animal models [29]. Like peroxidases, laccases are oxidases that catalyze the oxidation of various aromatic compounds and inorganic substrates, resulting in the generation of reactive species for wound healing. Huber and et al. proved the applicability of different laccases that oxidized phenolic moieties as crosslinking agents to fabricate biocompatible hydrogels even for vied substrates such as collagen and HA, chitosan, silk, and CMC derivatives [33, 128]. Wei et al. focused on the fabrication of antioxidant hydrogel using a laccase-mediated crosslinking reaction to combine a feruloyl-modified peptide with glycol chitosan [165]. Feruloyl-glycol chitosan promoted the mechanical properties of peptide hydrogel, and laccase could oxidize phenolic groups of feruloyl, followed by covalent crosslinking of the gel matrix. The inherent antioxidant property of the designed gel demonstrated free radical scavenging benefits for the elevated ROS level in the wound site, and feruloyl-containing gel accelerated wound closure processed and promoted the formation of mature skin tissue. Overall, these biocompatible antioxidants or functionalyzed hydrogels could be applied as promising wound dressing substrates in cutaneous wound healing.

**Hematin-Based Hydrogels**

Hematin is an alternative enzyme catalyst to HRP due to its iron-containing activity center and can avoid the safety issues of HRP. Hematin possesses an equi-
site HRP-mimicking activity in the hydrogelation of polymers conjugated with Ph moieties under mild physiological conditions when initially oxidized by H₂O₂ and subsequently through sequential electron transfers, finally returning to its original state (Fig. 5a) [166]. Sakai et al. reported that this enzyme could mediate the crosslinking of carboxymethyl cellulose and gelatin derivatives for producing gels, resulting in a biocompatible cell microenvironment (Fig. 5b) [167]. The most significant benefit of utilizing hematin catalyst in gel construction is its safety and reduced potential side effects in the human body [168, 169]. However, a disadvantage of hematin is its low solubility. It is suggested that it primarily dissolves in an alkaline solution before neutralization [169].

Chitosan-grafted hematin (Fig. 5c) [170] and polyethylene glycol-grafted hematin (Fig. 5d) [170] have been synthesized to develop hematin solubility, facilitating the gelation of catechol-containing polymers. Nagarajan et al. reported the design and synthesis of hematin tethered with methoxy PEG amine chains as a novel stable biomimetic catalyst. The authors applied one-step amidation performed under solventless conditions and catalyzed by a hydrolase. Through this process, amidation extensively upgraded the stability of hematin even at low pH. Moreover, this catalyst was soluble in water and catalyzed aniline-based monomers’ 3D polymerization [171]. Another issue regarding hematin is possible cytotoxic impact when used in high concentrations [172]. Even though more than 90% cell viability was seen in fabricated relative derivative gels using 0.05% (w/v) hematin and 4 mM H₂O₂ [167], more detailed studies are required to evaluate hematin performance in biomedical applications.

**Enzyme/Catalyst-Free Hydrogels**

Catalyst-free hydrogels can be produced through enzyme immobilization on the surface of the passing channel, including the needle or nozzle [172]. For example, hematin has been immobilized on the inner surface of the tube using a coating of primary amine of poly(dopamine) (PDA) and following exposure with the carboxyl group of hematin, which was reacted throughout NHS/EDC chemistry. The enzyme-free hydrogel is effectively designed through the catechol-
because the HRP-mediated oxidative reaction could alter the contact time from a few seconds to a minute on hydrogels. The storage modulus of these hydrogels decreased from 10 to 2200 Pa by increasing the concentration of H₂O₂. Indicated that the contact time between the polymeric solution and HRP-beads regulated the hardness of the resulting hydrogel. The storage modulus of these hydrogels was passed among the HRP-beads and rinsed out of the tube. Conjugated catechol moieties oxidized upon contact with the available hematin and created quinone radicals, which react with catechols or amine groups of polymer to construct cross-linkages (Figs. 6a, 6b). The young modulus of hydrogel increased during media extrusion from the tube and even until 1 h, indicating that the catechol moieties oxidized by enzyme and remained active for further cross-linking (Figs. 6c, 6d) [172]. Although this method is widely performed using catechol-containing polymers, more experiments are required to form other crosslinkable moieties in enzyme-free hydrogel fabrication. Bae et al. presented a new perspective on producing enzyme-free hydrogels using ferromagnetic beads conjugated with HRP (Figs. 6e–6g) [173]. In this system, a combination of gelatin-PEG-Ph precursor solution and H₂O₂ flowed into a syringe filled with a similar HRP-beads packing column. As the mixture was passed among the HRP-beads and rinsed out of the syringe, the Ph moieties were oxidized. The results indicated that the contact time between the polymeric solution and HRP-beads regulated the hardness of the resulting hydrogel. The storage modulus of these hydrogels could alter from 10 to 2200 Pa by increasing the contact time from a few seconds to a minute because the HRP-mediated oxidative reaction could occur while the polymer and H₂O₂ mixture was interacting with the applied catalyst. Therefore, a longer contact time between reactants would create a higher number of Ph radicals, which could contribute to the crosslinking reaction. However, the injectability of these types of hydrogels may become a concern during the production of stiff gels through increasing contact time with the activating surface in the syringe and a higher number of oxidative moieties. Moreover, several researchers speculate the potential for some enzymes in hydrogel fabrication to trigger immune responses, and immunogenicity is noteworthy for the clinical application of enzyme-catalyzed hydrogel formation in situ. The developed techniques can overcome the safety concerns associated with injecting enzyme-free hydrogels [19]. Interestingly, enzyme-free hydrogels offer a practical alternative for the biomedical applications of injectable hydrogels.

**Cytotoxicity of H₂O₂**

Utilizing H₂O₂ is a further concern regarding enzyme-mediated crosslinking reactions. Low concentrations of H₂O₂ (micromolar) during the cell encapsulation process and available in culture media did not affect cell viability and growth. Conversely, high concentrations (several millimolar) caused cellu-
lar death through apoptosis and necrosis. The H$_2$O$_2$-induced cytotoxicity also depends on the incubation time [174]. Gülden et al. recommend expressing the toxic potency of H$_2$O$_2$ in vitro via the incipient toxic time [174]. This dependence indicates that the cytotoxic consequences of H$_2$O$_2$ could be less significant if H$_2$O$_2$ is quickly used by the HRP-mediated oxidative reaction [144, 148]. Furthermore, it was demonstrated that the rapid contact with H$_2$O$_2$ through the crosslinking procedure could help rat ANSCs endure oxidative stress, demonstrating superior cell viability [144]. Moreover, because inactivation of the enzyme could cause deceleration or the incomplete use of H$_2$O$_2$, which can cause residual toxicity in the surrounding cells, it is essential to avoid using higher concentrations of H$_2$O$_2$ to avoid HRP inactivation. Hence, combining the biopolymer precursor solution, enzyme, and cells before adding H$_2$O$_2$ can lessen cell exposure time to H$_2$O$_2$.

**Enzymatic Reaction in 3D Printing**

Three-dimensional printing provides the opportunity to simulate complex 3D tissues and biomedical products by accurately placing hydrogel precursor solution, extracellular matrix components, cells, and biochemical factors to create defined structures using several types of materials and printing techniques, including microextrusion, laser-assisted printing, inkjet printing and 4D bioprinting (Fig. 7) [42]. Rapid gelation rate, which facilitates the fabrication of 3D structures, is crucial for crosslinking reactions in bioprinting [40]. The HRP has suitable properties for use in this approach. Murphy et al. evaluated the potential for naturally derived hydrogels using bioprinting. Since the hydrogel precursors’ fluidity was manipulated through its viscosity as a crucial issue in bioprinting, the authors used compressed air to drive the gel precursors to the printing-head nozzle [43]. Sakai et al. proved the feasibility of 3D cell-laden hydrogel fabrication by sequentially dropping a bioink containing polymers crosslinkable through the enzymatic reaction and H$_2$O$_2$ onto droplets of another bioink containing the polymer, HRP, and cells [176]. Gantumur et al. printed a cell-laden hydrogel construct successfully without collapse. The authors demonstrated the feasibility of utilizing glucose-mediated enzymatic hydrogelation through an HRP-catalyzed reaction that consumes H$_2$O$_2$ generated by HRP and glucose, enabling them to print a 3D cell-laden construct with good shape fidelity [40]. Double-network hydrogels created from poly(2-ethyl-2-oxazoline) (PEOXA)-peptide conjugates, which can be crosslinked in the presence of SrtA, and physical networks of alginate yielded structures with improved mechanical properties and excellent laden chondrocytes viability [44]. Moreover, incorporating cellulose nanofibrils within this hydrogel formulation provides the required rheological properties for extrusion-based 3D printing with good shape fidelity [44]. Irvine et al. demonstrated the use of gelatin, crosslinked with microbial TG, as a substrate to print cell-laden hydrogels for both 2D precision patterns and 3D constructs [41]. However, this system includes the TG in the printing bio-ink, leading to clogging of the printing tip. It has been discovered that sustained gelatin solution printing is possible without TG, followed by crosslinking by soaking in TG media. The printed cells were intact and spread, forming pseudopodia-like extensions. Overall, printing substrates in the form of hydrogel through enzyme-mediated reactions like HRP, SrtA, hematin, and TG has excellent potential in TE and other biomedical applications.

**a-Chymotrypsin**

a-Chymotrypsin was found to promote the self-assembly of amino acid derivatives to design supramolecular hydrogels [177, 178]. a-Chymotrypsin was observed to reduce the gelation time considerably, from eight days (or no gelation occurred in two weeks) to 10 min to 4 h, depending on the structure of amino acid derivatives. Nanofibers are produced by Fmoc-amino acid and amino acid, created by the hydrolysis of the amino acid ester. The outcomes showed that the enzyme-substrate interactions are essential for promoting the supramolecular self-assembly of these amino acid derivatives into fibrous hydrogels 20 nm in diameter (Fig. 8) [179]. Xie et al. reported an a-chymotrypsin promoted self-assembly method through the enzyme-substrate interactions to synthesize supramolecular hydrogels. Several easily accessible amino acid derivatives, such as Fmoc-F, Fmoc-W, F-OMe, F-OEt, and Y-OMe, were selected as building blocks to produce well-defined nanofibers as the matrices of hydrogels [179]. Furthermore, they revealed that instead of the enzymatic synthesis of hydrogelators, the enzyme-substrate interactions promoted the self-assembly of amino acid derivatives into supramolecular hydrogels. This mechanism is similar to the ligand-receptor interaction that catalyzed aggregation [177]. However, it is distinctive from enzyme-assisted self-assembly, which engages the enzymatic synthesis or the hydrolysis procedure [95, 180, 181]. The authors also demonstrated that co-assembly most likely occurred in this system, proved by the compositional analysis of the supramolecular scaffolds.
**Fig. 7.** (a) Schematic illustration of drop-on-drop 3D hydrogel construct fabrication by ejecting bioinks and photographs of the inkjet bioprinting system as well as inkjetting heads indicated by the dashed rectangle in panel. Reprinted with permission from [176]. By courtesy of John Willey and Sons. (b) Schematic of extrusion–based bioprinting through glucose-mediated enzymatic hydrogelation and printed human nose construct based on blueprint before and after post crosslinking. Reprinted with permission from [40]. By courtesy of Whoice Publishing. (c) Schematic illustration of (1) hydrogel precursor solution contains PEOXA-LPETG and PEOXA-GGGG and alginate in Tris buffer for printing. (2) Bioink used for printing the featured DN-CN mixed with hACs. (3) Fabrication of 3D-printed hydrogel grids was carried out by first extruding the prehydrogel mixture with hACs and subsequently crosslinking the printed bioink via incubation with SA and Ca\textsuperscript{2+} solutions. Reprinted with permission from [44]. By courtesy of American Chemical Society. (d) Free form fabrication apparatus controlled by a computer and a pressure driven syringe dispenser to dispense the ink, 3D printed gelatin gel woodpile structure viability and cell spreading within the 3D printed hydrogel. Reprinted with permission from [41]. By courtesy of Springer Nature.
CONCLUSIONS AND OUTLOOK

Enzymatic crosslinking reactions are a gentle and appropriate approach to fabricating biocompatible hydrogels required in biomedical applications. Gelation rate, hydrogel stiffness, and cytocompatibility are essential factors to consider when choosing a suitable enzymatic system for biomedical applications. The rapid 3D polymerization of substrates in the presence of bioactive molecules leads to localized 3D hydrogels applicable in drug delivery, TE, and regenerative medicine. Conversely, the long gelation time of polymers has certified their application as fillers of irregular-shaped wounds, ensuring the cohesion of surrounding tissue and fabricated hydrogel and facilitating the injectability of the precursor solution. Moreover, changing the density of crosslinking can also modify the release rate of the encapsulated proteins and other essential molecules. Transglutaminase, tyrosinase, SrtA, phosphopantetheinyl transferase, lysyl oxidase, PAO, phosphatases, thermolysin, b-lactamase, phosphatase/kinase, peroxidases, and a-chymotrypsin have been studied, and each one has potential properties to perform crosslink reactions to form suitable hydrogels. The described enzymatic hydrogelation indicates promising approaches for the biomedical applications of hydrogels crosslinked by enzymatic reactions. Interestingly, developing the enzyme-mediated coupling of biopolymers through bioprinting technologies is an innovative prospective in TE and other biomedical applications, providing the opportunity to produce more specific biomedical products in a short time. The versatile enzymatic hydrogel properties can guide us to investigate novel approaches for biomedical purposes.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and or analyzed in the current study are available from the corresponding author upon reasonable request.

AUTHORS CONTRIBUTIONS

E.B., M.M., M.H., and S.S. contributed to the reports' primary draft preparation and review. E.B. and S.H. worked on the schematic figures of reactions. M.H., J.H., and M.Kh. committed to data analysis and editing the manuscript. All authors read and approved the final manuscript.

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