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Enzymatic crosslinked hydrogels for biomedical application

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Abstract

Self-assembled structures mostly arises through enzyme-regulated phenomena in nature under persistent conditions. Enzymatic reactions are one of main biological processes in fabrication and construction of supramolecular hydrogel networks required for biomedical applications. The enzymatic processes provide a unique opportunity to integrate hydrogel formation. In most of cases, structure and substrates of hydrogels are adjusted by enzyme catalysis due to the chemo-, regio- and stereo-selectivity of enzymes. Hydrogels processed by using various enzyme schemes showed remarkable characteristics as dynamic frames for cells, bioactive molecules and drugs in biomedical applications. A novel class of enzyme-mediated crosslinking hydrogels mimics the extracellular matrices by displaying unique physicochemical properties and functionalities like water-retention capacity, drug loading ability, biodegradability, biocompatibility, biostability, bioactivity, optoelectronic properties, self-healing ability, shape memory ability. In recent years, many enzymatic systems investigated hydrogel cross-linking. Results of biocompatible hydrogel products show that these mechanisms of crosslinking can fulfill requirements for variety of biomedical applications including tissue engineering, wound healing and drug delivery.

Keywords

Enzymatic reaction; crosslinking; Hydrogel; Biomedical application; Tissue engineering; Wound healing; Drug delivery

Introduction

Most of cells in the body are placed in a three-dimensional (3D) microenvironment (1, 2). The organization and geometry of these 3D structures have a great effect on the cellular

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charactristics incuding function,bahaviour, metabolism, and fate (3, 4). By designing biomimetic 3D microenvironement through biocompatible reactions and biomaterials can profound insights into the responsible complex, mechanical and biochemical interactions, and in following fundamental biological processes (5). These new developments in the creation of cultured systems are required in drug discovery, regeneration of malignant or damaged tissues and analy analysis of biological processes. As all fields of biomedicine and biotechnology are strongly influenced by these achievements (1, 2, 6). The purpose of tissue engineering (TE) is to produce artificial tissues and organs that can demonstrate native processes and functions (7, 8). To this end, fabricated scaffolds with or without cells are delivered to the injured or diseased body tissues and organs. Afterward, implanted tissue construct regenerates a new tissue either by its degradation and synthesis of new tissue from the embedded cells or by stimulating the host cells (2, 9). The design and fabrication of scaffold substrates is extremely complicated due to the variation and complexity of the prospective applications (2, 6, 10). Nevertheless, several essential requirements in fabrication of all 3D architecture need to achieve. First, all incorporated substrated and applied treating methods have to be biocompatible and . Endurement under the mechanical loads and sustaining mechanical uniformity and integrity of fabricated structures are second item in design process. Third, the microstructure of the hydrogel should facilitate cellular adhesion, and ideally, integrate into the surrounding biological microenvironment. Finally, highly porous systems are necessary to enable cellular migration and proliferation as well as the growth of surrounding tissue and angiogenesis(11-13). Other important considerations are the controlled stimulation of the predictable cellular responses, for example, proliferation, migration, organization, and differentiation (12-15). These complex requirements, in addition to the need for easy and convenient sterilization, handling, and

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processing procedures of all included materials, make the process further complicated (16-18). In recent years, numerous synthetic, natural, inorganic, and their combinations have been used to develop scaffolds (7, 8, 19). Hydrogels are crosslinked polymeric networks capable of retaining high quantity of water. Both natural and synthetic polymers have been used to form hydrogels. The polymers are crosslinked through either physical interactions or chemical bonds, resulting in a network that swells in the water while maintaining a defined 3D structure. (1, 3, 6). The high water content results in a transparent material, which is an important characteristic in the development of hydrogels for applications such as contact lens composed of crosslinked poly (hydroxyethyl methacrylate) (pHEMA) and polyvinyl alcohols (PVA) (3, 4). Because hydrogels have a similar architecture to the extracellular matrix and soft tissues in terms of their rubbery nature, they cause little mechanical and frictional irritation to the surrounding tissues when implanted in the body (2, 6, 20). Also, the 3D network hydrogels provide structural support and enable the oxygen, nutrients, and metabolites transportation to and from the encapsulated cells (21-23). Therefore, hydrogels were further explored for in vivo applications, and their examples are a coating material on the inner surface of blood vessels to minimize thrombosis following surgery-induced tissue injuries (5) or as a barrier between tissues to prevent postoperative adhesion formation (6, 24). Furthermore, the drug delivery can be magnetically triggered and actuated via the incorporation of magnetic moieties into the carrier system (23, 25). Biofabrication techniques and micrometer-sized cell-containing hydrogel vehicles generated via emulsification methods are both new uprising approaches in this field (26-28). Besides, these substrates are promising candidates for 3D printing and bioplotting processes because of their high viscosity of polymeric precursor solutions and their rapid gelation in the presence of appropriate cues (29). In the case in which the precise site's dimensions of a defect or injured

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tissue are not accessible or too complex to be fabricated, injectable hydrogels are the proper choice (2, 24, 30). The injection of a hydrogel precursor solution and the subsequently triggered network formation are the flexible nature of hydrogels, which can adapt hydrogels to the given environmental conditions. Injured tissue sites can be filled in a rapid and convenient way with minimal invasion from the host immune system (2, 24, 30). Furthermore, the chemical and physical properties of the hydrogels are adjustable, for example, the network structure and pore size of the hydrogel constructs can be tuned and customized for a given application by controlling parameters such as the chemical composition and crosslinking methodologies (2, 31).

Many hydrogel cross-linking approaches, including physical and chemical, have been employed to form polymer networks depending on the required structure and desired application (1, 2). Physically cross-linked hydrogels are formed through changes in environmental conditions whereby gelation often occurs under the influence of pH, temperature, polyelectrolyte complexation, hydrogen bonding, crystallization, and hydrophobic association (1-6). Chemically cross-linked hydrogels comprising covalent bonds among the polymer chains are formed via radical polymerization and non-enzymatic or enzymatic cross-linking of complementary groups (7-10). Comparing existing physical and chemical approaches, enzyme-mediated hydrogel formation is proven to be mild and effective, therefore development of this approach with higher degrees of complexity is a current focus (Table 1) (1, 9). The majority of enzymes involved in hydrogel cross-linking are common to enzymes that catalyze reactions in our body. Enzymatic reactions that occur at neutral pH, in aqueous solutions, and at physiological temperatures, are highly desired for biomedical applications. Moreover, possible cytotoxic consequences and unexpected by-products that can arise with the use of photo-initiator- or organic solvent-

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mediated reactions are avoided as a result of substrate specificity of the enzyme (8, 11, 12). Another more noteworthy advantage relates to mildness of the enzymatic reaction under normal physiological conditions, because this method for cross-linking natural polymers cannot withstand harsh chemical conditions leading to loss of bioactivities (8, 13). Lastly, the use of enzymatic cross-linked hydrogels offers prospective for employing a variety of enzymatic systems to choose the desired hydrogel properties for different biomedical purposes (11, 12, 14). This review selected the enzyme-mediated cross-linking method for hydrogel production as the template material for engineering tissue and regenerative medicine because this approach has emerged as a desirable alternative pathway to overcome the difficulties associated with chemical cross-linking methods.

Table 1. Advantages and drawbacks of different cross-linking methods for producing hydrogels (11, 12, 14).

	Advantages	Drawbacks
Physical cross-linking (e.g., ionic, electrostatic, protein and hydrophobic interactions, hydrogen bonding, crystallization, and thermo-responsive)	• Reversibility • Absence of potentially harmful chemical reactions • Homogeneous cross-linking	• No sufficient mechanical strength • Unstable • Lack of tailorability • Inflammatory due to changes in ion concentration and pH
Chemical cross-linking (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

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Enzyme-mediated cross-linking	• In situ gelation • Substrate specificity (chemo-selective) • No toxic by product • Neutral pH • Moderate temperature	• Substrate specificity (cross-linking reaction occurs only between the enzyme-specific polymers

Transglutaminase (TG)

Transglutaminases ***(TGs)*** are a broad family of thiol enzymes that catalyze post-translational protein modification mostly by inducing isopeptide bond configuration. The covalent conjugation of polyamines, lipid esterification, or the deamination of glutamine residues is also other candicates for this purpose. The TGs are a mild substitute for chemical crosslinking, which catalyze 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. 1**) (12, 15). The TGs mostly originate from both microbial (16) and mammalian cells (17). Davis et al. developed a protein-based hydrogel system that was cross-linkable by TG (18). This system consists of two de novo designed protein polymers named Kn and BQ, where the Kn block contains lysines, B is a random coil hydrophilic block, and Q block serves as a glutamine substrate (19). 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 any addition of co-factors. The biochemical function of TGs was revealed by discovering the role of isozyme factor XIII in blood coagulation for fibrin-stabilizing factor (20). These enzymes exist in a variety of tissues such as skin and brain tissues

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(21-23). The TGs are essential for formation of fibrin clots and cornified epidermis. Consequently, the lack of these enzymes strictly hampers wound healing (24). Davis et al. has reported a modular hydrogel with tunable characteristics that was formed within 2 min by using this system. Also, bioactive peptides allows customizable cell-signaling requirements when engrafted. Fibrin matrices are formed by factor XIII, which is the circulatory form of transglutaminases (18). Several applications have been studied both *in vitro* and *in vivo* for these matrices, including angiogenesis, nerve repair, and cartilage tissue engineering (25-28). Sala et al. used activated transglutaminase factor XIIIa which is 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 one for degradation process (29). 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 XIIIa to crosslink star-shaped PEG, functionalized by either a glutamine acceptor or donor, to bind growth factors to surfaces that were provided (29). Consecutive enzymatic reactions permit for site-specific immobilization of large quantities of biologically active substances. Tissue transglutaminase has a high degree of sequence similarity with other transglutaminases, such as factor XIII, however it doesn't require any proteolysis for activation. Furthermore, tissue TGs show stronger adhesiveness than fibrin-based glues and less vulnerability to physical parameters like humidity (30). Sperinde and Griffith described the combination between PEG and tissue TGs. In their models, the gelation time depended on polymer functionalities, primary stoichiometric ratios, and substrate kinetics (31, 32). Hu and Messersmith reported the high adhesive strength of the in

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situ formed peptide conjugated polymer hydrogels cross-linked by TG (33). The TG has also been used to create gelatin-based hydrogels. These gels can be used for the incorporation of the cells with excellent cytocompatibility, which is a superior characteristic for TE applications. Additionally, they show great transport properties, which assist in sustained drug delivery (34, 35). Genetically engineered elastin-like polypeptide hydrogels and peptide-PEG conjugates cross-linked by transglutaminases have shown hopeful features as injectable hydrogels or cartilage repair (36, 37). In the report published by Jones et al., reactive ECM components recognized that these could be allowed the coupling of peptide and peptide-polymer conjugates via tissue TG. The possibility to broaden the application of this strategy to a variety of tissue surfaces highlights the flexibility of this method for biomedical applications. Using this method, surfaces can be modified with molecules such as functional moieties, growth factors, or therapeutic drugs. The TGs are enzymes that depend on the presence of Ca^{2+} (37). The Ca^{2+} independent TG-catalyzed gel formation also represented the ability to entrap and release the cells. These gels come into view particularly practical for microfluidic biosensor systems (38).

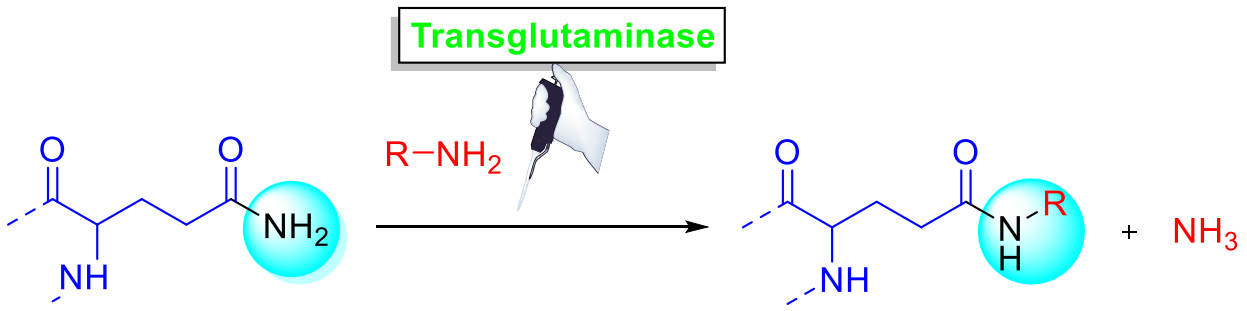

Fig. 1 Mechanism of the enzymatic reaction mediated by transglutaminase.

Tyrosinase

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Likewise transglutaminases, tyrosinases are also known as phenoloxidase and monophenol monooxygenase, and catalyze macromolecular network formation without co-factors (39). Tyrosinase is a copper-containing enzyme that catalyzes phenols oxidation into activated quinones in the presence of O_2, such as in dopamine and tyrosine residues (39). Activated quinones can react with an amino group or hydroxyl group, mostly via a michael-type addition reaction (**Fig. 2**) (40). Tyrosinases can be found in plants and animals and act in melanin formation, browning of food, and also cuticle hardening in insects (41). In most plants and animals, tyrosinases have rather a wide substrate specificity. Differently, substrate specificity is restricted to the L-form of tyrosine or DOPA in mammalian tyrosinases (41, 42). Chen et al. compared gels of gelatin and chitosan formed upon crosslinking using tyrosinase or transglutaminase and the results showed that tyrosinase induced faster gelation (57). Nonetheless, the hydrogels catalyzed by tyrosinase were fabricated in the presence of chitosan and were mechanically weaker. Gels formed by using tyrosinase are mainly appropriate as a glue (43) and wound dressings or could be used for protein immobilization (44), because of their quick degradation. Other applications of tyrosinase include the crosslinking of tyrosine residues in fibroin, sericin, and silk, yielding protein-polysaccharide conjugates (45, 46). Mishra et al used tyrosinase to provide enzymatically crosslinked carboxymethyl-chitosan/gelatin/nano-hydroxyapatite injectable gels at 37°C for in situ bone tissue engineering application (47). These hydrogels showed potential for biomedical applications because of their exclusive mechanical properties, adhesiveness, and non-toxicity (12).

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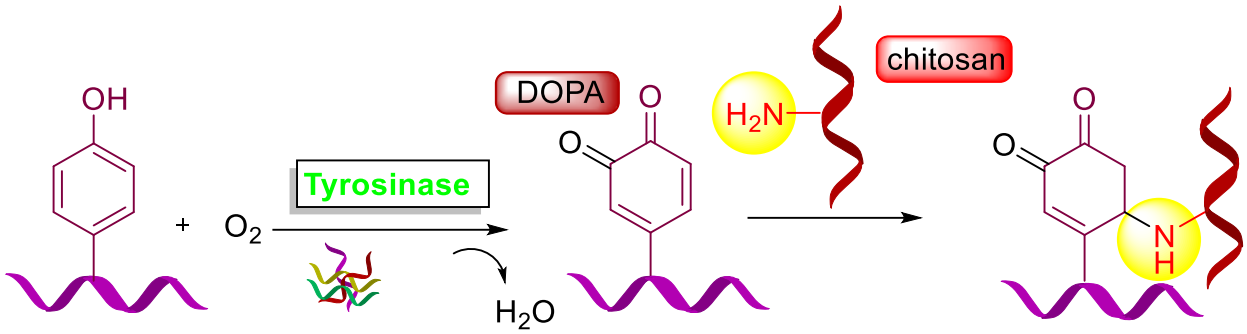

Fig. 2 Mechanism of the enzymatic reaction mediated by tyrosinase. Polymers conjugated with with phenol (Ph) moieties such as tyramine , phenylalanine, tyrosine, hydroxyphenyl propionic acid, and 4-hydroxyphenyl acetic acid in presence of oxygen and following amin groups of complementary polymer such as chitosan proceeded hydrogelation.

Phosphopantetheinyl transferase

Phosphopantetheinyl transferase is a small (16.2 kDa) enzyme that employs to catalyze covalent cross-linking of many polymers in high efficacy. Therefore, it is different from transglutaminases, which have inadequate recombinant production and are higher in natuare. Transferases, which are expressed generally in the cytosolic compartment in a wide range of tissues, both in yeast and animal cells, include large multifunctional polypeptides that contain all of the catalytic components essential for the synthesis of long-chain fatty acids (48). The main mechanism of transferase catalysis to create synthetic hydrogels occurs by transfer of a phosphopantetheine prosthetic group of coenzyme in A-functionalized PEG macromers to a serine residue of engineered carrier proteins (**Fig. 3**). Phosphopantetheinyl transferase was used by Mosiewicz et al. to create hydrogels (49). Hybrid hydrogels were constructed by mixing the precursors of 8-arm-PEG-coenzyme A at 37 °C and in the presence of Mg^{2+} and neutral pH. The gelation was rather slow and completed in nearly 15 min. The hydrogel reached an elastic

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modulus rate of 2.3 kPa. With this technique, selective covalent transferase-catalyzed hydrogels can be formed and modified with bioactive peptide ligands specifically the integrin receptor binding motifs, such as RGDs (Arg-Gly-Asp), which enable cell attachment and spreading (50). This type of reaction is greatly attractive for cell biology and tissue engineering applications.

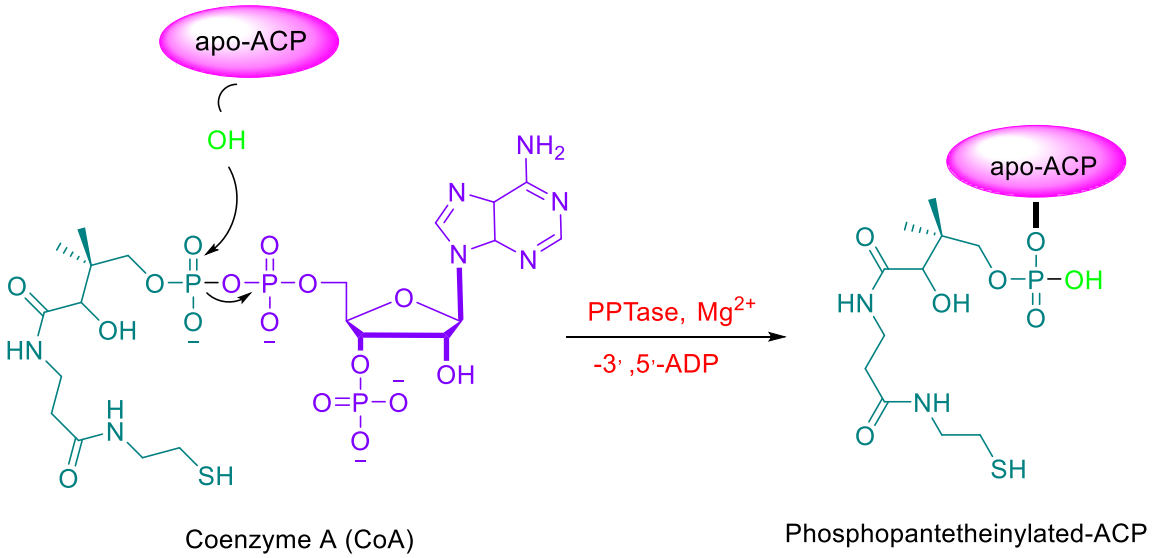

Fig. 3 Mechanism of lysyl phosphopantetheinyl transferase mediated reaction (49).

Sortase A (SrtA)

Sortase A (SrtA) categorizes among of a group of transpeptidases molecules with exceedingly specific activity for cross-linking which extracting from gram-positive bacteria. In this reaction, by cleavage of the bond between threonine and glycine in a *c*-terminal LPXTG recognition motif which proceed to form a thioester through conjugation of the amino group and carboxyl group from N-terminal glycine of complementary groups (51). Variaty of de novo designed peptides have been studied as feasible SrtA molecules (19, 52). Conjugation of human epidermal growth factor fused with a small soluble oligoglycine substrate, GGG motif (GGG-EGF) to poly

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(ethylene glycol) (PEG) hydrogels perfomed via sortase comprising LPRTG substrate. Increase of LPRTG in the hydrogel or GGG-EGF resulted raise of total tethered EGF. Results showed stimulation of DNA synthesis in human cells leads to activation of sortase-tethered EGF. The ease, and reversible sortase-mediated ligation and cleavage reactions demonesrated that as an desired methodology for adjustment of hydrogels (53).

Lysyl oxidase and plasma amine oxidase

Lysyl oxidase is one of important element in the constitution and regeneration of the original ECM. In the ECM in presence of lysyl oxidase, enzymatic reaction initirate through oxidation of primary amine of lysines to an aldehyde and the created reactive aldehydes spontaneously react with another amine to form a Schiff base or undergo an aldol condensation with another aldehyde (Fig. 4) (54). In fact, lysyl oxidase is acted to proceed covalent cross-linkage among the elastin and collagen fibrous derivatives and make them stable structure in ECM. Therefore, lysyl oxidase is resposible in the pattern formation of tissue, morphogenesis and repair ability of various connective tissues, comprising skeleton, cardiovascular, and respiratory tissues (55). Besides, plasma amine oxidase (PAO) is known as semicarbazide-sensitive amine oxidase which acts oxidation of primary amines, and it takes a crucial benefit of being accessible (54). Both above mentioned enzymes can be applied as a crosslinkers to develop biomaterial and tissue mechanical properties as wll as upregulation of ECM formation and tissue regeneration (56, 57). The lysyl oxidase was used by Bakota et al. to fabricate self anssembled nanofibrous from multi-domain peptides which proceed oxidative crosslinking of lysine residues. The most interesting feature of these hydrogels created by lysyl oxidase was their desirable mechincal properties including mechanical strength and handling of the hydrogel, as it becomes more

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robust with time rather than less so. This characteristic leads to a persistent upregulation in mechanical properties of networks made of rich lysine biopolymers. Furthermore, serum is contained lysyl oxidase. Therefore, in serum supplimented media, we can proceed crosslinking of lysine-containing polymers spontaneously without adding exogenous enzyme. Production of ECM by cells incorporated in the hydrogels enhanced by this enzyme. In summary, the lysyl oxidase have shown significance for a variety of applications including cell delivery, drug delivery and as scaffolds for tissue regeneration using multi-domain peptide hydrogels (58, 59). Besides, the inherent mechanical properties of tissue engineered constructs could be enhanced using lysyl oxidase by extention of culture time whereas facilites hydrogel integration with original tissue via covalent bond formation among lysine-rich molecules present in hydrogel and amines in tissue ECM.

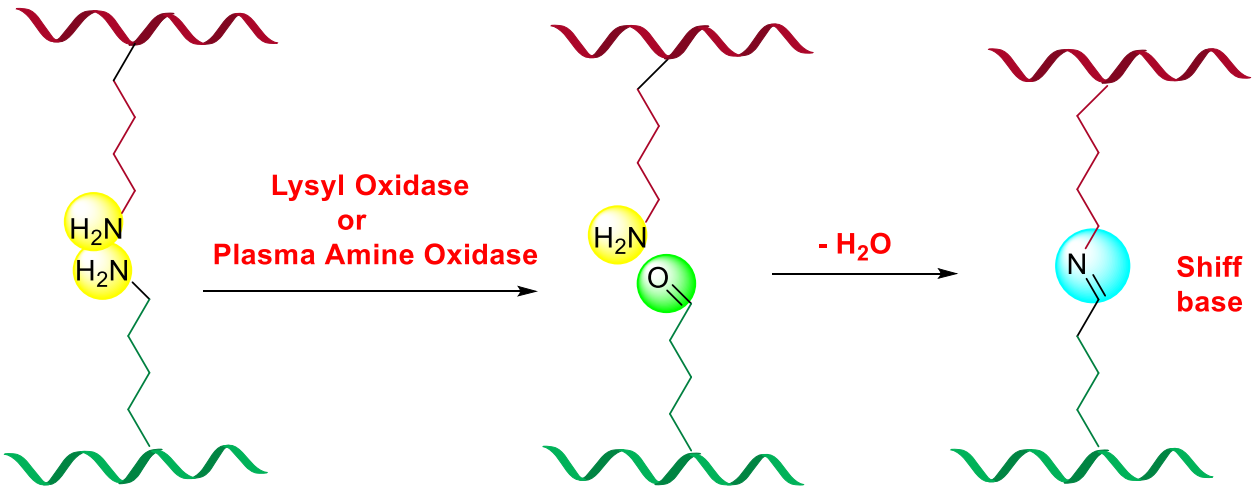

Fig. 4 Mechanism of lysyl oxidase or plasma amine oxidase-mediated reaction in fabrication of hydrogels (54).

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phosphatase/ kinase derivatives

Enzyme catalyzed or -regulated formation of hydrogels from small peptide derivatives can proceed through alteration of the amphiphilicity of phosphatase or kinase derivatives such as phosphatases, β-lactamase, thermolysin or phosphatase/kinase for instance, kinase or dephosphorylation interaction by a phosphatase. This alteration can, consequently, activate the physical interactions and self-assembly of the amphiphilic peptide moieties in hydrogel precursor solution, eventually leading hydrogelation, as shown in **Fig. 5** (60, 61). These small peptides which mostly possessed bioactive or organic molecules can endure the addition of extra bioactivesubstrates. For instances, Phosphatase mediated the exclusion of phosphate moities fron peptide, resulting hydrophobicity of substates which may self-assemble into fibous network by noncovalent midiations (for instance, charge interactions, p-p interactions, hydrogen bonding) that makes hydrogelation possible (62). The amphiphilicity of peptide reformed by alteration of peptide instead of breaking the covalent linkages between the phosphate group and the peptide using thermolysin. In detail, thermolysin mediates to form bonds between peptides by reverse hydrolysis. This enzyme can be used to combine two distinct peptide derivatives, reducing the solubility of one of the peptides. This blocking peptide can then self-assemble into a hydrogel by hydrophobic interactions. Thermolysin prefers aromatic, hydrophobic residues on the amino side of the peptide bond. This system was presented by Toledano et al. with potential applications in the construction of nanofibrous hydrogel scaffolds for cell culture. The β-Lactamases and esterases are other enzymes that can be used as catalysts for molecular self-assembly (63).

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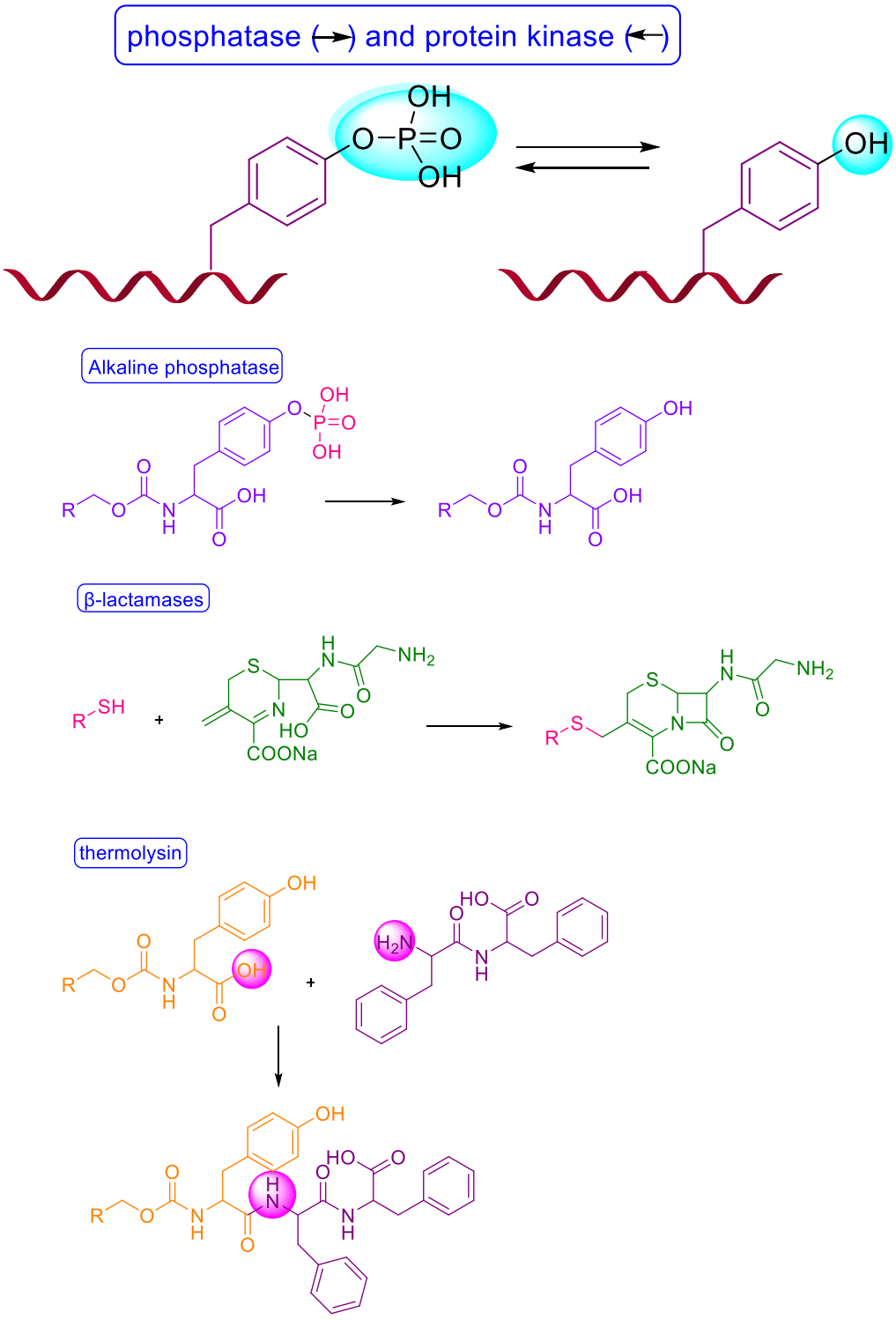

Fig. 5 Mechanism of the supermolecular hydrogel formation proceed different enzymes such as phosphatase/kinase, phosphatase, thermolysin, and b-lactamase (61).

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Both enzymes are involved in the hydrogel formation. The β-Lactamases are produced by Escherichia coli strains which are the reason of their resistance to β-lactam antibiotics, like streptomycin and penicillin. In this term, four-atom ring β-lactam molecular structure of antibiotics open by lactamase which this ring openting restict activation of antibacterial properties of the substate. In this reaction, the lactam ring of the hydrogel precursor compound opens by the lactamase and in following by release of hydrogelator, self-assembly convert precursors into hydrogel formation.Available lactamases in bacterial lysate enables them to act as a hydrogelator by converting the precursor molecule which leads hydrogel formation. These types of self-assembled hydrogels performed using β-lactamases or esterases can be used in bacterial analysises as well as trigger specific cellular cycle, since hydrogelation can take place within the cells (64, 65). Most of the enzymatic reactions in biological systems are irreversible and single enzyme hardly acts in reversible manner, which necessitate excessive modification of the peptide backbone of the crosslinked hydrogel to achieve reversible mechanism of reaction. Yang et al. synthesized a pentapeptidic hydrogelator forms hydrogels via the self-assembly using kinase/phosphatase which switch is used to control the phosphorylation and dephosphorylation of the hydrogelator and to regulate the formation of supramolecular hydrogels (66). This enzyme switch-regulated supramolecular hydrogels promises a new way to make and apply biomaterials because phosphorylation and dephosphorylation. These enzymes could regulate formation/dissociation of self-assembled hydrogel and the corresponding macroscopic transition in a supramolecular hydrogel. Using this enzymatic switch allows accurate control of biomaterial organization at a molecular level over time. hydrogel formation. These features of such enzymatic formed supramolecular hydrogels make them great promise for TE and other biomedical applications(67, 68).

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Alkaline phosphatase(ALP)

Alkaline phosphatases are especially remarkable source of the phosphatase to construct gels because of their participation in the mineralization of skeletal tissues (69). For this, self-assemble hydrogels fabricated by enzymatic dephosphorylation of N-fluorenylmethyloxycarbonyltyrosine-phosphate (FMOC: fluorenylmethyloxycarbonyl, compound I, are utilized to form supramolecular network when mineralization of calcium phosphate (hydroxyapatite) is occoured (**Fig. 6**). Dephosphorylation of compound I in water leads compound II that spontaneously assembles due to p-stacking of the fluorenyl end groups, which adopt a helical orientation because of super helical activities of the amino acid residues increasing from H-bonding interactions among the terminal tyrosine groups (69). Schnepp (69) fabricated materials by taking advantage of this property. These materials' viscoelasticity represents them appropriate substrates for application in rengerative medicine including tissue engineering, drug release, and wound healing purposes (12).

In addition, the core segment of yeast prion Sup35, a series of structurally related precursors have utilized for the alkaline phosphatase development of self-assembled hydrogels. Incorporation of an amyloid segment into a cytotoxic precursor could able to eliminate the cytotoxicity of the precursor solution and provides cytocompatible microenvironment (70). An enzyme-responsive hydrogel was designed by Toda et all by which elasticity could be changed by ALP in the cell culture. Consequently, the expression of Runx2, an osteogenic marker, was modified by ALP treatment for the deformed cells. This report demonstrated that the change of hydrogel's mechanical property could upregulate the expression of osteoblastic genes derived from human mesenchamal stem cells (MSCs) *in vitro* (71).

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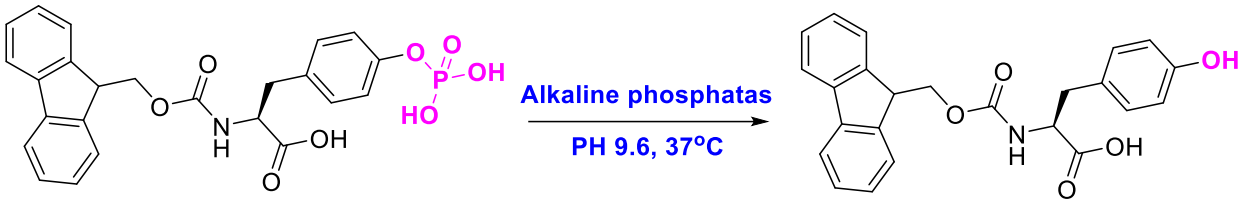

Fig. 6 Mechanism of hydrogel production by alkaline phosphatase-catalyzed dephosphorylation of FMOC-tyrosine-phosphate (I) which resulted formation of compound (II) with higher amphilicity and lower charge.

Peroxidases

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

$$2RH_2 + \text{electron donor (2e)} \rightarrow 2\ RH^{\bullet} + 2H_2O$$

Most of the peroxidases use hydrogen peroxide as a electron donor. This family consists of up to 42 isozymes, which makes it challenging to define in vivo functions (8, 72). The most frequently used peroxidases in hydrogel formations are horseradish peroxidase (HRP) and soybean peroxidase. The most important advantage of this enzyme in comparison to the previously described enzymes is quick gelation, which can happen in seconds (12). They are plant enzymes and are known as useful tools for biotechnology and biosciences(8, 72). Horseradish peroxidase is a single-chain β-type hemoprotein responsible for the catalysis of the conjugation of phenol and aniline derivatives in the presence of hydrogen peroxide (11, 73, 74). Such groups are present, for instance, in tyramine, tyrosine, and 3-4-hydrophenyl propionic acid (75). In this reaction, the HRP rapidly combines with hydrogen peroxide, and the formed complex could oxidize hydroxyphenyl groups. In following the phenoxy radicals react through a radical coupling reaction generating covalent C–O and C–C bonds (**Fig. 7**). Soybean peroxidase is a potential substitute to the HRP, because of comparable mechanism of action and its superior

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stability. Human peroxidases have been widely studied, however usually, plant peroxidases have been utilized for enzymatic crosslinking to form hydrogels (76). This enzyme has advantages like non-cytotoxicity and the potential to crosslink in situ. An extensive range of natural and artificial polymers have been modified with tyramine or hydroxyphenyl propionic acid for the development of hydrogels by peroxidases. Examples of these hydrogels are hyaluronic acid (77, 78), gelatin (74, 79-81), dextran (82), chitosan (73, 83, 84), alginate (85, 86), xylans (87), carboxymethylcellulose (88), polyvinyl alcohol (PVA) (73), poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) block copolymer (89), and poly (amido amines) (90). Polymer-phenol molecules compared to thiol-modified polymers are not simply oxidized in the process of synthesis and are steady for extended time of maintenance. The HRP-mediated crosslinked reactions have been found significant attention and wide biomedical applications compared to other enzyme systems because of the easy possibility to regulate crosslinking and gelation rate by changing the amount of reactants including biopolymer, crosslinakble moieties, HRP and H_2O_2 (91).

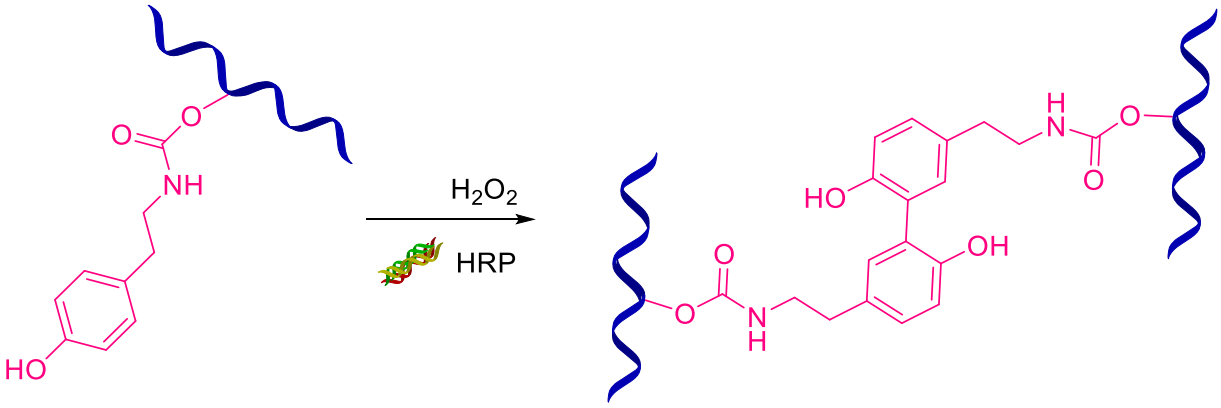

Fig. 7 Mechanism of the peroxidases reaction using hydrogen peroxide as an electron donor (14).

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Revealing the pathological and physiological manners attributed to extracellular reactive oxygen species (ROS) could facilitate hydrogelation without exogenous application of electron donor. Yang Liu et al. cytocompatible hydrogel membrane on indivitual cells produced via HRP-mediated reaction and endogenous secretion ROS as an elector donor (92). Sakai et al. created cytocompatible hydrogel bulks performed on the surfaces of individual HRP anchored cells membrane by cross-linking of biopolymer compound mediated by HRP in aqueous solution. To hold the cross-linking development only on the cell surface, cells were soaked in a Ph substituted polymer (polymer-Ph) solution containing the HRP conjugated with a biocompatible anchor molecule (BAM-HRP) for cell membrane and HRP was made to anchore cell membrane. The hydrogel sheath of about 1 μm thickness was attained by soaking the cells with the anchored HRP in an aqueous solution containing polymer-Ph and H_2O_2 within 10 min. The hydrogel sheaths could be created from a variety of polymer-Ph derivetives, such as derivatives of protein, polysaccharide, and synthetic polymer (8, 13). Cellular viability (>90%) and subsequent normal growth after removal of the hydrogel sheath confirmed the cytocompatibility of the on-cell surface hydrogel sheath formation (93). Advances in encapsulation technology is a great promise for fundamental studies in cell biology and biomedical applications.

HRP applications

Darr et al. evaluated HA-Ph hydrogels that showed *in vivo* biocompatibility and stability to dissociation for injected hydrogel in subcutaneous tissue while maintaining the main of carboxyl groups with negative charge, essential for the contribution of the physio-mechanical properties of tissue (94). The HA-Ph molecules conjugated by HRP are promising candidate for tissue engineering and other biomedicine applications (77, 95). Dextran-Ph hydrogel combined with

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HA-Ph and heparin-Ph via HRP-mediated reaction mimic the the ECM of native cartilage (14, 96, 97). Similarly, Kim et al. described injectable HA-Ph (98) as an efficient drug carrier for rheumatoid arthritis treatment. The supramolecular Ph-terminated PEG hydrogels are other polymer combinations using HRP to activate gelation (75, 89).

Catalytic mechanism of peroxidase

HRP is a heme-containing enzyme obtained from the root of horseradish (*Armoracia rusticana)*. Heme is a complex between protoporphyrin and Fe(III). In HRP, the heme iron makes a coordinated bond by the nearest His170 residue in core region. There are various HRP isoenzymes, and the majority of them are C isoenzyme (99, 100). Mostly the catalytic reaction peroxidase involves the heme group and the amino acid residues in the protein core, such as His42 and Arg38. After binding of H_2O_2 to Fe(III) as the heme in its resting state, a proton transfer from the α-oxygen of H_2O_2 to the His42 residue of HRP occurs. Afterward, the α-oxygen forms a single bond with Fe(III), which results in a transient Fe(III)-hydroperoxy intermediate that is defined as compound II (101). After that, the α-oxygen forms a double bond with the heme iron, a procedure that is facilitated by the cleavage of the peroxide O–O bond and an electron transfer from the porphyrin ring to the iron. This leads to a Fe(IV)=O ferryl group and a porphyrin cation radical, which altogether is called Compound I. The residual -OH group of H_2O_2 receives the proton from the His42 residue to form water moelcule. Compound I proceeded two oxidation states above the resting state, and thus two successive electron transfers are necessary to reduce it back to the resting state. Here, the Ph moieties of polymer-Ph conjugates as the reducing substrate comes into action (**Fig. 8**).

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In the primary reduction step, a phenolic hydrogen atom is donated to compound I, and the electron of the hydrogen atom is transferred to the porphyrin cation radical. At the same time, the proton goes to the His42 residue. The first reduction results in the formation of the Fe(IV)=O group and a phenolic radical. The second reduction step, which is a complex reaction involving the protonated His42 residue and an electron transfer from a phenol substrate, regenerates the Fe(III) and create a second phenolic radical with water as a leaving group. Thus, a catalytic cycle of HRP consumes one H_2O_2 molecule, converts two phenols into phenolic radicals and produces two water leaving groups. The phenol radicals pair with each other through either a C–C linkage between the *ortho*-carbons of the aromatic ring or a C–O linkage between the *ortho* carbon and the phenolic oxygen (102), and this leads to forming a cross-linkage among the polymer-Ph. Based on the catalytic mechanism, an increase in the H_2O_2 supply will generate more phenolic radicals, which in turn will form more phenol cross-linkages. In fact, when the concentrations of HRP and polymer-phenol conjugates are constant, increasing the H_2O_2 supply raises the shear modulus (G′) and decreases the swelling percentage of the consequential hydrogels, representing an increase in the crosslinking density (79, 103). Beyond an optimum range of H_2O_2 concentration, a further increase in H_2O_2 causes the decrease G′ and this is probably due to a surplus amount of H_2O_2 reacting with HRP which consequently resulting in formation of two different types of catalytically inactive, including compounds III and IV (104). The compound III is a reversible intermediate which will gradually turnover native enzyme, leading to the restoration of catalytic activity. On the other hand, Compound IV is an irreversible compound which cannot recover its catalytic activity. HRP may also be inactivated via an attack on the protoporphyrin by phenoxyl radicals, which leads to heme demolition (105). As a consequence of HRP inactivation, the crosslinking reaction is inhibited. Therefore, the optimum concentration

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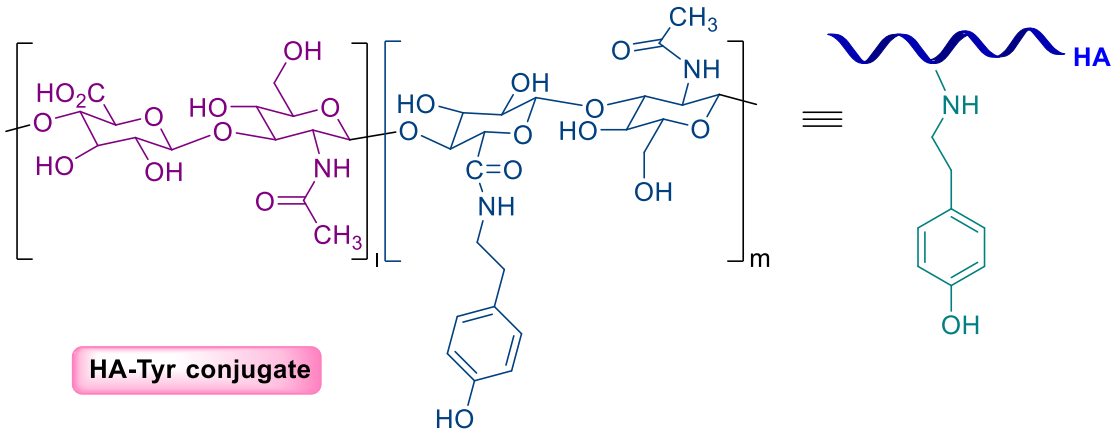
of H_2O_2 should be determined experimentally in order to avoid excessive use of H_2O_2. Anja Schmidt et al. suggested that an H2O2: HRP molar ratio of higher than 100 would lead to a reduction in G′, which corresponds to the reported value at which Compound IV is formed (104). The gelation rate of the HRP-mediated crosslinking reaction is greatly dependent on the concentration of HRP (79, 103). It is anticipated that further enzymes would allow a faster rate of phenolic radical formation, conducting to a faster velocity of crosslinking. Hydrogels are produced ina range of time of a few seconds to several minutes, depending on the HRP concentration.



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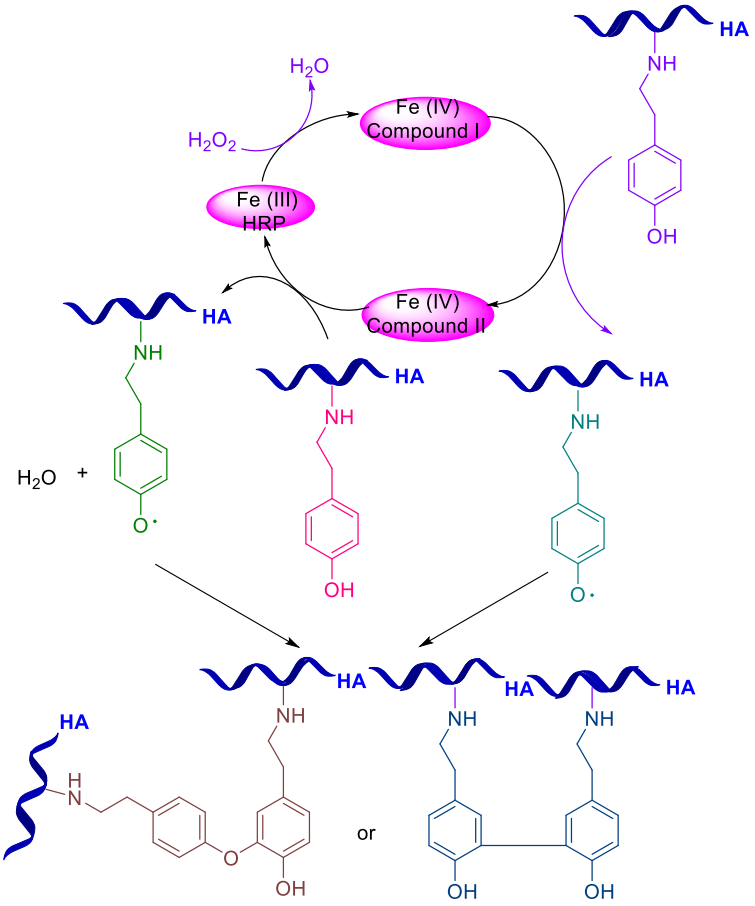

Fig. 8 Crosslinking mechanism of HA-Tyr conjugates by the HRP-mediated crosslinking reaction(106).

Applications of hydrogels formed by enzymatic reactions

Drug Delivery

In order 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 like an abrupt leakage of gel precursors and protein drugs to the surrounding tissues, caused by slow gelation after injection (106). The crosslinking density of hydrogels is a crucial factor in leading their drug release properties (107). 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 (108, 109). The HRP catalyzed crosslinking method allows independent regulation of

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the gelation rate and crosslinking density through a variation of HRP and H_2O_2 concentrations (108, 109). This is very advantageous for *in situ*-forming hydrogels for the delivery of protein drugs and growth factors (95). An injectable hyaluronic acid-tyramine (HA-Tyr) hydrogel was formed using this system for protein drug delivery by van de Wetering P et al. (95). 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) (110), which is an antineoplastic cytokine used in the treatment of various types of human cancers. The IFN-incorporated HA-Tyr hydrogels demonstrated greater antitumor efficiency compared to the IFN solution injected at the same dose. Improved anticancer result was related to the inhibition of tumor angiogenesis with the treatment of IFN-incorporated HA-Tyr hydrogels. Lutolf et al have design oligopeptide building blocks through dual enzyme-responsive poly(ethylene glycol) based hydrogel able to undergo formation and degradation in response to cell-secreted matrix metalloproteinases.

Paeonol as hydrophobic drug model release behaviour from poly(vinyl alcohol) (PVA)/silk fibroin hydrogels fabricated through the HRP crosslinking method were invistaged. The pheonol from the hydrogels showed an initial burst release and slow sustained release in longer period of time which proved these hydrogels are attractive candidates for drug delivery and engineering applications. Self-assembled heparin-pluronic (HP) nanogels encapsulating bFGF were dispersed in gelatin-poly(ethylene glycol)-tyramine (GPT) polymer solution and used as a protein drug carrier. The macro/nanogel composite hydrogels were produced by the HRP-catalyzed oxidative coupling of GPT conjugates (111, 112). The GPT/HP composite hydrogels presented a considerably higher density of urethral muscles nearby the urethral wall compared to the GPT hydrogels. Devolder et al. showed an improvement in protein release kinetics from composite

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hydrogel systems through the HRP-mediated crosslinking of pyrrole groups (113). An 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. In spite of 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 bipyrrole 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 both the number and size of blood vessels at the injection site, as equated to the Ca^{2+} crosslinked hydrogels (91). Dehghan-Niri et al. represented injectable enzymatically crosslinked hydrogels based on Gum tragacanth (GT)-tyramine that were prepared by oxidative coupling reaction in the presence of HRP and H_2O_2. 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 lower molecular size and weight. TGA–TA/Gtn–TA hydrogels can be a promising candidate for in situ-forming systems for delivery of therapeutic proteins(114).

Tissue engineering and cell therapy

The mechanical characteristics are critical in designing injectable hydrogels for tissue engineering not only to have sufficient durability under dynamic *in vivo* conditions, but also to provide encapsulated cells with an appropriate mechanical environment that leads to proper cell

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proliferation, differentiation, and tissue regeneration (115, 116). The mechanical property of hydrogels requires to be adjusted based on the mechanical microenvironment of the aimednative tissue. In this regard, the HRP-catalyzed crosslinking method is favorable because it offers advanced control over the mechanical strength of hydrogels through changing the H2O2 concentrations (117). Wang *et al.* created gelatin-hydroxyphenyl propionic acid (Gtn-HPA) hydrogels as an injectable scaffold for tissue engineering (116, 118). The storage moduli (G′) of Gtn-HPA hydrogels was readily adjusted from 280 to 12800 Pa through a variation of H_2O_2 concentrations. The hydrogel hardness resulted the focal adhesion, proliferation, and differentiation of human mesenchymal stem cells (hMSCs) cultured in 2D and 3D environments. The cells cultured in a softer hydrogel expressed much more neurogenic protein markers, compared to 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 (ANSCs) (119). The G′ of Gtn-HPA hydrogels was adjusted (449-1717 Pa) to be similar to adult brain tissue. Remarkably, the ANSCs encapsulated in Gtn-HPA hydrogels demonstrated a greater resistance to H_2O_2–induced oxidative stress, whereas those encapsulated in collagen and alginate hydrogels had harsh necrosis (120). This property would be beneficial for the regeneration of injured brain tissue since ANSCs transplanted at damaged sites in the brain have poorly survived probability because of exposure to oxidative stress and other uncongenial factors (121). The Gtn-HPA hydrogels displayed an improved differentiation of ANSCs towards neuronal lineage, compared to polyornithine/laminin coated polystyrene substrates in mixed conditions for both neuronal and astrocytic differentiation. Gtn-HPA hydrogels were used for cartilage repair in an *in vivo* osteochondral defect model (122). Gtn-HPA hydrogels with different hardness (G′ = 570, 1000, and 2750 Pa) were mixed with rabbit

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chondrocytes, and injected into full-thickness osteochondral defects of the femur. The results suggested that the higher 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 polymerphenol hydrogels have been broadly used for tissue engineering applications. The HA-Tyr hydrogels were investigated as biomimetic matrices for the chondrogenic differentiation of caprine MSCs (123). Recombinant human lactoferrin (rhLF) hydrogels were also created for bone tissue engineering due to the anti-apoptotic and osteogenic activity of rhLF towards pre-osteoblast cells (124). Jin *et al.* demonstrated injectable dextran-tyramine hydrogels as 3D scaffolds for cartilage regeneration (14, 91). Chondrocytes incorporated in the hydrogels kept their phenotype and formed a cartilaginous-specific matrix following *in vitro* culture. Teixeira *et al.* represented that the HRP-mediated crosslinking reaction induced strong adhesion between cartilage and dextran tyramine hydrogels (125). Histological and morphological assay 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. The incorporation of heparin-tyramine conjugates made the homing of human chondrocytes and chondrocyte progenitor cells easier using the dextran-tyramine hydrogels. The cell-attracting effect was probably related to the high affinity of heparin with growth factors and cytokines (91, 111). Kuo et al prepared autologous extracellular matrix scaffolds, murine collagen-Ph hydrogels, and demonstrated the appropriateness of injectable horseradish peroxidase cross-linked collagen-Ph hydrogels for human progenitor cell-based development of 3D vascular networks *in vitro* and *in vivo*. They also confirmed that the biodegradability, swelling properties and stiffness of the collagen-Ph hydrogels regulated by changing the amount of crosslinking

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could be used to adjust not only the extent of the vascular network but also adipose and 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 to engineer cell based 3D tissue constructs (126). 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 a cross-linking reaction, catalyzed by an HRP-conjugated antibody immobilized on the cell surface. This method was achieved by using two different systems including using primary antibody conjugated with HRP, and using a primary antibody with a secondary antibody conjugated with HRP. This method has great potential of cell-to-cell communication, regenerative medicine, and cell therapies (127). Morelli et al. reported the conversion of ulvan, a sulfated polysaccharide of algal origin, into an in situ gelling material suitable for biomedical applications. Ulvan was successfully modified with tyramine units in order to be susceptible to crosslinking reactions catalyzed by horseradish peroxidase enzymatic action in the presence of H_2O_2. The amount of used enzyme and H_2O_2 were optimized in order to induce times of gelation appropriate for the development of injectable hydrogels. Also, the preliminary biological investigations indicate the suitability of the enzymatically crosslinked ulvan hydrogels to function as a vehicle for viable cells supporting the possibility of their application as injectable cell delivery systems (128). Khanmohammadi et al. introduced the preparation of cell-enclosing hyaluronic acid (HA) microparticles with either solid or liquid cores through cell-friendly horseradish peroxidase (HRP)-catalyzed hydrogelation using a microfluidic system. The spherical tissues covered with a heterogeneous cell layer would be useful for biomedical and pharmaceutical applications (129-131). Liu et al. assembled small tissue enclosing hydrogel

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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 together in the collagen gel. Alginate-based hydrogel constructs degraded by alginate lyase and perfusable vascular network-like structure was formed. Confirmation was done by introducing solution including tracer particles of about 3 μm in diameter into the lumen model by the alginate hydrogel fiber. The introduced solution moved into the created capillary branches and distributed to the individual spherical tissues. The method is a great promise to develop the technique for constructing an internal perfusable vascular network for the fabrication of dense 3D tissues *in vitro* for biomedical applications (132). Tomita et al. produced HRP fabricated hyaluronan (HA) microcapsules feasible for use in the cryopreservation of a small number of sperms for clinical applications (133).

Wound healing

Tissue adhesives are useful for sealing defects in tissues, stopping bleeding, and promoting wound healing (134). Fibrin glue and cyanoacrylates are two clinically available adhesives. Fibrin glue has been regularly used as an adjunct to hemostasis in a surgical procedure. Nevertheless, fibrin glue has been used only to stop low-pressure bleeding because of its comparatively low adhesive power (135). Cyanoacrylates revealed comparatively strong adhesion to tissues but released toxic substances like cyanoacetate and formaldehyde during degradation (136). 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.

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Lih et al. created injectable chitosan poly (ethylene glycol)-tyramine (CPT) hydrogels as fast biocompatible and durable tissue adhesives (137). 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 seconds throughout the HRP-catalyzed coupling of tyramine moieties. These hydrogels provided great tissue adhesion with an adhesive power of almost 20 times higher than that of fibrin glue. CPT hydrogels rapidly stopped the bleeding from injured livers through the combined effect of the adhesiveness of these hydrogels and the hemostatic characteristic of chitosan (138). CPT hydrogels efficiently closed the skin injuries and showed greater wound healing property in comparison to suture, fibrin glue, and cyanoacrylate. Tran et al. described rutin-conjugated CPT hydrogels for dermal wound healing (139). Rutin is a flavonol glycoside that exhibits antioxidative, cytoprotective, and wound healing properties (140). Rutin was conjugated to a chitosan backbone of CPT polymer by an ester linkage. *In vitro* study revealed that the conjugated rutin was gradually released from CPT hydrogels through hydrolysis of the ester linkage. The rutin-conjugated hydrogels considerably enhanced wound healing compared to the hydrogels without rutin. These results imply that the combination of bioactive molecules and injectable hydrogels can produce a novel type of tissue adhesives with improved hemostatic and wound healing properties (91). Zavada et al. reported more developed enzyme-mediated hydrogel for wound dressing, fabricated from a phenol functional poly(vinyl alcohol) and the glucose oxidase (GOx)/HRP combination (141). In order to proceed polymerization, this method needed both glucose and oxygen. In vivo application, the mixture of polymer, HRP and GOx would only solidify once it gets in touch with a glucose and oxygen supply which is avaialbe in blood flow. Therefore, the constructed hydrogel could be practical when the formulation is utilized for a bleeding wound. Gelation times of less than 10 s

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were readily achievable and the hydrogels demonstrated no marks of bio-incompatibility from any of the residual enzymes. Another advancement would be containing glucose in the formulation and using molecular oxygen, everywhere in the atmosphere, as the environmentally-borne stimulus. This approach has been represented for GOx/HRP-mediated thiol–ene polymerization (142). A formulation of thiol-ene monomers with glucose, GOx and HRP could be useful via spray, where the promptly accessible surfaces of atomized droplets would allow quick oxygen diffusion, leading to the initiation of the polymerization. An advantage of this method over the formulations based on blood glucose is that the risk of left unpolymerized material is reduced. In addition to the GOx/HRP pair, there are probably other enzymatic systems of inducing oxygen-mediated in situ polymerization, particularly laccase as it is able to create radicals straight from molecular oxygen (143). Sakai et al. fabricated an on-tissue surface hyaluronic acid-based hydrogel by administration of a pre-hydrogel aqueous solution with the purpose of preventing postoperative peritoneal adhesion which is a serious surgical complication. The hydrogelation is initiated when hydrogel precursor solution contact with the body fluid containing glucose on tissue surfaces, and it is accomplished in 5s. In hydrogelation process, a hyaluronic acid derivative possessing phenolic hydroxyl moieties cross-link by a cascade reaction of GOx and HRP. A significant reduction in peritoneal adhesions was found in animal models (144).

Safety of HRP hydrogelating reactions

One of the worries related to the enzyme mediated crosslinking reaction is the maintenance of the enzyme in the hydrogel. Because HRP is a plant-derived protein, it may provoke an immunological response when inserted into the human body. Researchers have revealed that the

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protein core, as well as the N-glycans of HRP, could evoke the production of antibodies in rodents (145). Even though the rodents were immunized under the stimulation of Freund's adjuvants to intensify the immune response, the immunogenicity of HRP in the human body, especially with frequent exposures, cannot be ignored. Therefore, the safety of HRP in humans must be confirmed before the clinical application of hydrogel formulations containing HRP. On the other hand, if a human peroxidase, which can competently catalyze the oxidative coupling of phenols, is recognized, then it can act as a safer alternative to HRP (12). But the high price of human proteins and the deficiency of the activity of the human peroxidase in catalyzing the crosslinking of polymer-phenol conjugates still hinder their application. For example, human myeloperoxidase has been revealed to catalyze the oxidative crosslinking of free tyrosine residues in the presence of H_2O_2. Still, it wasn't able to crosslink tyrosine-containing polypeptides because of probable steric interference towards larger substrates (146). consequently, it stays to be studied whether human peroxidases like myeloperoxidase are able to catalyze the crosslinking of polymer-phenol conjugates and the creation of hydrogels.

Hematin-based HRP biomimetic

Catalytically useful HRP mimetics are prospective options that can avoid the safety issues of HRP. One example is hematin, which is a complex between protoporphyrin IX and ferric ion (Fe^{3+}), with a hydroxyl group adjacent to the iron atom. Like HRP, hematin is initially oxidized by H_2O_2 and subsequently experiences a range of electron transfers with the substrates previous to finally returning to its original state (147). Sakai et al. reported that hematin could catalyze the crosslinking of gelatin-phenol and carboxymethyl cellulose-phenol conjugates, producing hydrogels *in situ (148)*. Despite that the catalytic activity of hematin is lower than HRP in terms

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of per unit weight of the catalyst, the most important advantage of utilizing hematin as a substitute catalyst in hydrogel construction is its safety and lower potential side effects in human (149, 150). However, one disadvantage of hematin is its low solubility in water. The dissolution of hematin demands alkaline solution before neutralization, and even with this dissolution protocol, the constitution of hematin aggregates was still detected (150). To enhance the solubility of hematin, pegylated hematin (151) and chitosan-*g*-hematin (150) have been combined, which facilitated the gelation of catechol-containing polymers. The potential cytotoxicity of hematin at high concentrations is another issue (152). Even though 95% cell viability was seen when incorporated in gelatin-phenol hydrogels crosslinked by 0.05% (w/v) hematin and 4 mM H_2O_2 (148), more detailed studies are needed to establish the effect of hematin for tissue engineering applications.

Enzyme/catalyst/ -free hydrogels

Hematin has also been immobilized on the inner surface of passing channel including needle surface to produce catalyst-free hydrogels (152). To immobilize hematin, primary amine was inserted to the inner surface of a syringe by coating with poly(dopamine) (pDA), which next reacted with the carboxyl group of hematin throughout NHS/EDC chemistry. Hydrogels were effectively created by passing a solution of catechol-tethered chitosan and H_2O_2 through the syringe. The oxidation of catechols occurred in contact with the immobilized hematin, creating quinone radicals which react with catechols or amine groups of chitosan to construct cross linkages. It was revealed that the G′ being increased 1 h after the gel precursors were rinsed out of the syringe, representing that when the catechols oxidized during contact with hematin, they stayed active for crosslinking formation. Although this method is performed widely for catechol-

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containing polymers, still more experiments are needed for the formation of tyramine-based hydrogels. Bae et al. represented a new perspective to producing enzyme-free hydrogels using ferromagnetic microbeads conjugated with HRP (153). In this system, a combination of gelatin-poly(ethylene glycol)-tyramine and H_2O_2 were loaded into a syringe filled with HRP-beads. As the mixture was moved through and rinsed out of the syringe, the tyramine moieties were oxidized whereas the HRP-beads remained. It was determined that the contact time regulated the hardness of the consequential hydrogel. Increasing the contact time from 5 to 30 s raised the storage modulus from 10 to 2200 Pa. The reason is that the HRP-mediated oxidative reaction could just be happened while the polymer and H_2O_2 mixture were in interaction with the HRP-beads. Therefore, increasing the contact time would cause the creation of more tyramine radicals, which could contribute to the crosslinking reaction. Although this system forms hydrogels that are free of HRP, injectability may become an issue if stiffer hydrogels are produced during longer contact time with the HRP-beads in the syringe. Interstingly, catalyst/enzyme-free hydrogels prospect the most worthwhile alternative for biomedical applications of injectable hydrogels crosslinked by phenol coupling.

Cytotoxicity of H_2O_2

The utilizing H_2O_2 is a further concern related to the enzyme mediated crosslinking reactions. *In vitro* models have revealed that low concentrations of H_2O_2 (micromolar) in the culture medium cease the cell growth whereas high concentrations (micro to millimolar) cause apoptosis and necrosis (154). The H_2O_2-induced cytotoxicity also relies on the incubation time. In one experiment using C6 glioma cells, it was dosclosed that the average cytotoxic concentration of H_2O_2 was reduced from 500-30 μM as the incubation time was raised from 1 to 24 h (155). This

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dependance indicates that the cytotoxic consequences of H_2O_2 could be less significant if H_2O_2 is quickly used by the HRP mediated oxidative reaction (119, 123, 156). Also, it was shown that the transient contact to H_2O_2 through the crosslinking procedure could help rat aNSCs to endure oxidative stress, showing a higher survival rate in comparison to the cells without pre-exposure when exposed to the second addition of H_2O_2 (119). Moreover, because HRP inactivation may result in slow or not complete use of H_2O_2, which can make the residual to cause toxicity to the embedded cells, it is essential to avoid using higher concentraions of H_2O_2 in order to avoid HRP inactivation. It is also suggested to combine the polymer-phenol conjugates, HRP, and cells before the addition of H_2O_2 to lessen the exposure time of the cells to H_2O_2. Moreover, the HRP is composed cysteine residues and its thiol moites can oxidase whereas glucose is reduced disulfide bond to thiol moites. Therefore, the oxidation and in following reduction cycle of sulfate derivatives was proceeded hydrogelation of polymer-Ph and even could consider polymer-Ph hydrogelation in H_2O_2 exlude reaction with mixing polymer derivative with HRP in higher concentrations. These findings have shown that the HRP-mediated crosslinking reaction is an appropriate choice to form cell-laden hydrogels (91).

HRP enzymatic reaction in 3D printing

3D printing provides the possibility to simulate complex 3D tissues and biomedical products by precise deposition of gel precursors and cells through printing techniques, like microextrusion or inkjet printing (157). Rapid gelation rate, which facilitates the fabrication of 3D structures, is crucial for crosslinking reaction in bioprinting. HRP has suitable properties to use in this approach. Murphy et al. evaluated commercially available tyramine-substituted HA. Since the high viscosity of the gel precursor solution is another crucial issue in bioprinting they used

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compressed air to drive the gel precursors to the printing-head nozzle (158). Hu et al. revealed that cell-seeded hollow fibers could be fabricated through enzymatically crosslinking the Gtn-HPA hydrogel in solutions flowing laminarly inside a triple-orifice extruder (159). Different diameters of fibers could be formed by changing the input flow rate (160). Additionally, it is possible to create enzyme-free 3D tissue constructs by driving the gel precursors through HRP-beads or hematin-coated tubing before exiting from the printing-head nozzle. The printing of hydrogels crosslinked by the enzyme-mediated reactions like HRP has a great potential in tissue engineering and other biomedical applications.

A-chymotrypsin

A-chymotrypsin was found to promote the self-assembly of amino acid derivatives for the production of supramolecular hydrogels (161, 162). It was observed that a-chymotrypsin considerably reduces the gelation time from 8 days (or no gelation happened in 2 weeks) to 10 min- 4 h depending on the structure of amino acid derivatives . Nanofibers are produced by Fmoc-amino acid and amino acid, which were 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. The results reveal that the transparent hydrogels comprised of long nanofibers with about 20 nm in diameter.

Xie et al. reported an a-chymotrypsin promoted self-assembly method through the enzyme–substrate interactions for the synthesis of supramolecular hydrogels. Mainly, some easily accessible amino acid derivatives, like Fmoc-F, Fmoc-W, F-OMe, F-OEt andY-OMe were selected as building blocks to produce well-defined nanofibers as the matrices of hydrogels (163). Furthermore, they revealed that instead of the enzymatic synthesis of hydrogelators, the

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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 (161). However, it is considerably different from that in terms of the enzyme-assisted self-assembly, which engages the enzymatic synthesis or the hydrolysis procedure (68, 164, 165). Also, they showed that co-assembly most probably occurred in this system proved by the compositional analysis of the supramolecular scaffolds.

Conclusions and future outlook

Enzymatic crosslinking reactions are a mild and useful method to form hydrogels *in situ*. Characteristics like gelation rate, hydrogel stiffness, and cytocompatibility are essential factors to consider when choosing a suitable enzymatic system for any biomedical application. Rapid gelation ensures a localized gel formation and it is suitable for drug delivery and tissue adhesives. Slow gelation permits the filling of irregularly shaped wounds with gel precursors, resulting in cohesion between the hydrogel and the surrounding native tissue. Changing the crosslinking density can also modify the release rate of the encapsulated proteins. Transglutaminase, tyrosinase, sortase A, phosphopantetheinyl transferase, lysyl oxidase, plasma amine oxidase, phosphatases, thermolysin, b-lactamase, phosphatase/ kinase, peroxidases, and a-chymotrypsin have been studied, and each one has potential properties to perform crosslink reaction to form suitable hydrogels. The discriped enzymatic hydrogelations indicate promising approaches for clinical applications of hydrogels crosslinked by enzyme-mediated oxidative reaction. Enzyme-mediated crosslinking reaction coupled with bioprinting technologies is an innovative prospective in tissue engineering and other biomedical applications, which provides the ability to produce much more precise biomedical products in a short time. The versatile

enzymatic hydrogel properties can guide us to investigate better novel approaches for biomedical purposes.

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