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Review

Enzyme-catalyzed crosslinkable hydrogels: Emerging strategies for tissue engineering

Liliana S. Moreira Teixeira [a], Jan Feijen [b], Clemens A. van Blitterswijk [a], Pieter J. Dijkstra [b], Marcel Karperien [a,*]

[a] Department of Tissue Regeneration, MIRA Institute for Biomedical Technology and Technical Medicine, Faculty of Science and Technology, University of Twente, 7522 NB Enschede, The Netherlands
[b] Department of Polymer Chemistry and Biomaterials, MIRA Institute for Biomedical Technology and Technical Medicine, Faculty of Science and Technology, University of Twente, Enschede, The Netherlands

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ABSTRACT

State-of-the-art bioactive hydrogels can easily and efficiently be formed by enzyme-catalyzed mild-crosslinking reactions *in situ*. Yet this cell-friendly and substrate-specific method remains under explored. Hydrogels prepared by using enzyme systems like tyrosinases, transferases and lysyl oxidases show interesting characteristics as dynamic scaffolds and as systems for controlled release. Increased attention is currently paid to hydrogels obtained via crosslinking of precursors by transferases or peroxidases as catalysts. Enzyme-mediated crosslinking has proven its efficiency and attention has now shifted to the development of enzymatically crosslinked hydrogels with higher degrees of complexity, mimicking extracellular matrices. Moreover, bottom-up approaches combining biocatalysts and self-assembly are being explored for the development of complex nano-scale architectures. In this review, the use of enzymatic crosslinking for the preparation of hydrogels as an innovative alternative to other crosslinking methods, such as the commonly used UV-mediated photo-crosslinking or physical cross-linking, will be discussed. Photo-initiator-based crosslinking may induce cytotoxicity in the formed gels, whereas physical crosslinking may lead to gels which do not have sufficient mechanical strength and stability. These limitations can be overcome using enzymes to form covalently crosslinked hydrogels. Herewith, we report the mechanisms involved and current applications, focusing on emerging strategies for tissue engineering and regenerative medicine.

1. Introduction

Hydrogels are hydrophilic polymeric networks able to absorb and retain high quantities of water while retaining its shape [1,2]. Their three-dimensional (3D) structure is excellent to mimic cell and tissue culture environments and, consequently, they are frequently used to encapsulate cells in a 3D-microenvironment. Additionally, hydrogels have proven to be very efficient for the delivery of biologically active molecules, such as growth factors, as well as providing organization of cells and tissues, due to the possibility to create multilayered systems [3–6].

In the last few years, mild-crosslinking methods have been successfully developed to form artificial matrixes. Major advances have been achieved in both physically or chemically crosslinked gels [7]. In physically crosslinked gels, interactions between polymers chains in amphiphilic block and graft copolymers [8], are established by ionic and/or hydrophobic interactions [9], or crystallization [10]. On one hand, this type of crosslinking has the advantages of reversibility and absence of chemical reactions potentially harmful to the integrity of incorporated bioactive agents or cells. On the other hand, their stability *in vivo* might be severely affected by interactions with bodily functions, both physiological and mechanical. Examples of these functions include weight bearing actions, for example in the bone and joints, for which these gels might provide insufficient mechanical strength. Another example is the sudden change in ion concentration or changes in pH, occurring in a normal inflammatory response, which can ultimately lead to gel collapse. In chemically crosslinked gels, covalent bonds are formed between polymer chains. In contrast, chemical crosslinking allows the formation of gels with controllable mechanical strength and superior physiological stability. The crosslinks in these type of gels can be generated via e.g. radical polymerization, chemical reaction of complementary groups, by using high energy radiation or by mimicking of biological crosslinking methods using enzymes [11].

* Corresponding author. Tel.: +31 53 489 3400/3323; fax: +31 53 489 2150.
E-mail address: h.b.j.karperien@tnw.utwente.nl (M. Karperien).

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Recently, increasing interest has been devoted to enzymatically crosslinked hydrogels, mainly due to the mildness of this type of reaction. The majority of the enzymes involved in the crosslinking are common to the enzymes catalyzing reactions naturally occurring in our body [12–20]. Enzymatic reactions are catalyzed by most enzymes at neutral pH, in an aqueous milieu and at moderate temperatures implying that they also can be used to develop *in situ* forming hydrogels. Additionally, unwanted side reactions or toxicity, that can occur with photo-initiators or organic solvents mediated reactions, are avoided due to one of the best characteristics of this type of reaction: the substrate specificity of the enzyme. Another major advantage relates to the mildness of the enzymatic reactions at normal physiological conditions, which highlights the advantages of this method for the crosslinking of natural polymers that cannot withstand harsh chemical conditions. Possible loss of bioactivity is, therefore, avoided. The polymerization reaction can be directly controlled by modulation of the enzyme activity [21]. Smart enzyme-responsive systems can be designed not only to recreate native extracellular matrixes (ECM) [22], but also to form and degrade biomaterials. These events capture, in essence, the intricacy of one of the most important biological functionalities of ECM, which is remodeling. In addition to degradability, tailoring the gelation rate is fundamental for applications such as drug delivery and tissue regeneration strategies. A controlled gelation rate is essential to prevent diffusion of the precursors, to ensure localized drug delivery, to obtain a suitable cell distribution, and, finally to properly integrate the gel with the surrounding tissues (mainly for irregular-shape filling applications).

Minimally invasive procedures are highly advantageous in tissue engineering therapies, presenting an attractive alternative for the replacement of cartilage. *In situ* crosslinkable gels are based on aqueous mixtures of gel precursors with bioactive agents that can be administrated via a syringe [23,24]. Injectable matrices abolish the need of complicated surgical interventions and reduce both the discomfort and complications for the patient [25]. For this purpose, artificial matrices should be non-cytotoxic, non-inflammatory, easy to inject, stable after gelation, and, lastly, their resorption rate should match the rate of neo-tissue formation [26]. Moreover, injectable enzymatically crosslinked hydrogels offer a plausible solution for the generation of functional tissue substitutes due to the similarities in the mechanical and swelling properties of these gels and native tissue, thereby maintaining the cell phenotype. This feature is highly relevant for tissues such as cartilage, where cells tend to de-differentiate when placed in a 2D environment [27,28]. Importantly, integration within wounds and tissue defects, even oddly shaped, is permitted by *in situ* forming hydrogels. These hydrogels can be applied during endoscopic or arthroscopic procedures, due to the initial fluidity of the gel precursors prior to gelation.

In this contribution, enzymatically crosslinked gels will be reviewed, focusing on their application in regenerative strategies. The purpose of this review is to provide examples of combinations of enzymes and materials that offer innovative avenues for further exploration in tissue engineering applications.

2. Enzymatically crosslinked hydrogels

2.1. Transglutaminase

Transglutaminases are a wide family of thiol enzymes that catalyze post-translational protein modification mainly by inducing isopeptide bond formation, but also through the covalent conjugation of polyamines, lipid esterification, or the deamination of glutamine residues. Transglutaminases are a mild alternative to chemical crosslinking, catalyzing the formation of covalent bonds between a free amine group from a protein or peptide-bound lysine and the γ-carboxamide group of a protein or peptide-bound glutamine (Fig. 1). Once formed, these bonds are highly resistant to proteolytic degradation. Consequently, stable polymeric networks are assembled, without addition of co-factors. The biochemical role of transglutaminases was discovered in 1968,

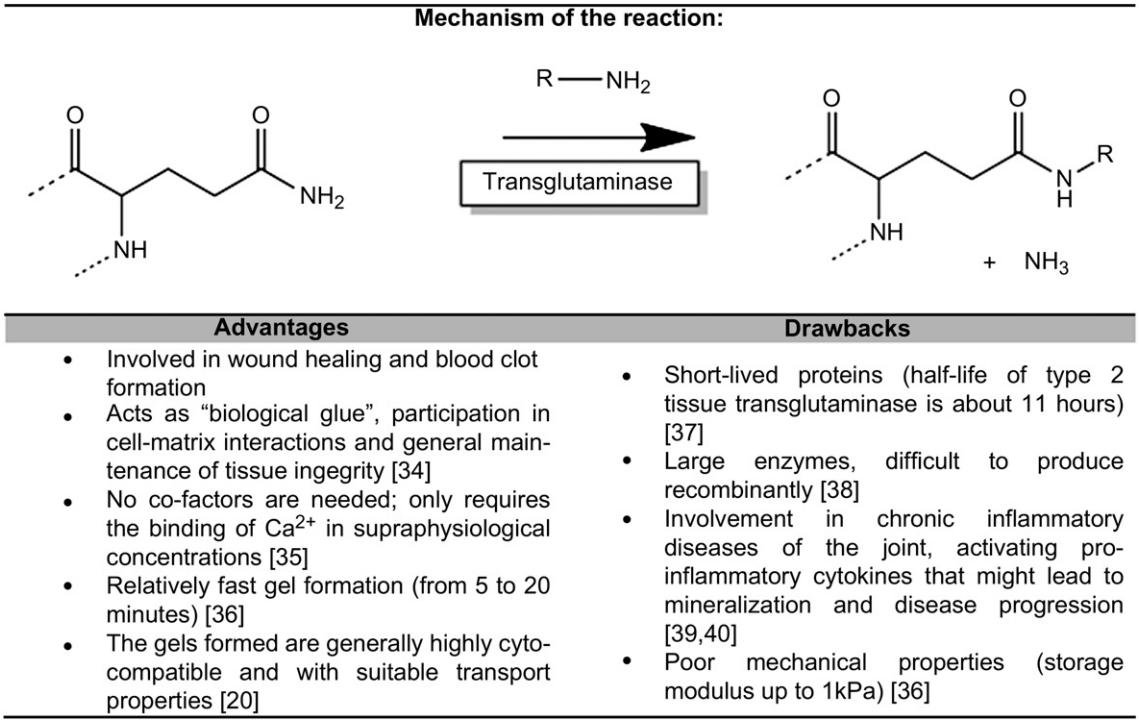


Fig. 1. Mechanism of the enzymatic reaction mediated by transglutaminase, advantages and drawbacks [34,35,37,39,40].

when the function of isozyme factor XIII in blood coagulation was revealed as a fibrin-stabilizing factor [29]. These enzymes are found in a variety of tissues, such as skin and the brain [30–32]. Transglutaminases are responsible for the formation of fibrin clots and cornified epidermis. Consequently, the absence of these enzymes severely hampers wound healing [33].

Using this system, Davis [12] has reported a modular hydrogel with tunable characteristics that is formed within 2 min. Additionally, bioactive peptides could be engrafted, allowing customizable cell-signaling requirements. Fibrin matrices are formed by factor XIII, which is the circulatory form of transglutaminases. These matrices have been studied both *in vitro* and *in vivo* for several applications, including angiogenesis, nerve repair and cartilage tissue engineering [41–44]. Ehrbar [36] used activated transglutaminase factor XIIIa to simultaneously couple site-specific cell-adhesion ligands and crosslink modified multi-arm poly(-ethylene glycol) (PEG) precursors. Interestingly, in their system, the material building blocks are responsive to two enzymatic systems, one responsible for matrix formation and the other one for degradation. The enzyme-mediated site-specific coupling of ligands allowed extensive cell spreading, proliferation and migration, as well as proteolytic matrix degradation by cell-derived matrix metalloproteinase's (MMPs). Elegant strategies also reporting the use of factor XIIIa to crosslink star-shaped PEG, functionalized by either a glutamine acceptor or donor, to tether growth factors to surfaces were provided [45]. In this study, consecutive enzymatic reactions allowed for site-specific immobilization of large quantities of biologically active substances. This system highlights the advantages of the use of enzymatic crosslinking, as their mild conditions and high specificity do not jeopardize protein's bioactivity, providing the cells simultaneously with adhesive sites and morphogens. Tissue transglutaminase shows a high degree of sequence similarity with other transglutaminases, such as factor XIII, however, requiring no proteolysis for activation. Moreover, tissue transglutaminases present stronger adhesiveness than fibrin-based glues and less susceptibility to physical parameters like humidity [46]. The combination between PEG and tissue transglutaminases has been described by Sperinde and Griffith [47,48]. In their model, the gelation time was dependent on polymer functionalities, initial stoichiometric ratios and substrate kinetics. Hu and Messersmith [49] reported the high adhesive strength of the *in situ* forming peptide conjugated polymer hydrogels crosslinked by transglutaminase. Transglutaminase has also been used to prepare gelatin-based hydrogels. These gels can be used for incorporation of cells showing excellent cytocompatibility and promising features for TE applications. In addition, they show excellent transport properties, which facilitate sustained drug delivery [20,50]. Genetically engineered elastin-like polypeptide hydrogels and peptide-PEG conjugates crosslinked by transglutaminases have shown promising features as injectable hydrogels for cartilage repair [51,52]. In the study published by Jones [52], reactive ECM components have been identified, that allowed the coupling of peptide and peptide-polymer conjugates via tissue transglutaminase. The possibility to broaden the application of this strategy to a variety of tissue surfaces highlights the versatility of this method. Using this method, surfaces can be modified with molecules such as growth factors, therapeutic drugs or functional moieties. Transglutaminases are enzymes that rely on the presence of Ca^{2+}. Interestingly, Ca^{2+} independent transglutaminase-catalyzed gel formation has also been described with the ability to entrap and release cells. These gels appear especially useful for micro-fluidic biosensor systems [53].

2.2. Tyrosinase

Similarly to transglutaminases, tyrosinases, also known as phenoloxidase and monophenol monooxygenase, catalyze macromolecular network formation in the absence of co-factors. Tyrosinase is a copper-containing enzyme that catalyses the oxidation of phenols, such as in tyrosine residues and dopamine, into activated quinones [54], in the presence of O_2. Activated quinones can react with a hydroxyl group or amino group mainly via a Michael-type addition reaction [55]. Tyrosinases are present in plants and animals. These enzymes are involved in melanin production, browning of food and also cuticle hardening in insects [56]. In most plants and animals, tyrosinases have rather broad substrate specificity. In contrast, substrate specificity is restricted to the L-form of tyrosine or DOPA in mammalian tyrosinases.

Chen [56,58] compared gels of gelatin and chitosan formed upon crosslinking using tyrosinase (Fig. 2) or transglutaminase and concluded that tyrosinase induced faster gelation. However, the hydrogels catalyzed by tyrosinase only formed in the presence of chitosan and were mechanically weaker or unstable. This and similar studies, suggested that the gels formed by tyrosinase were

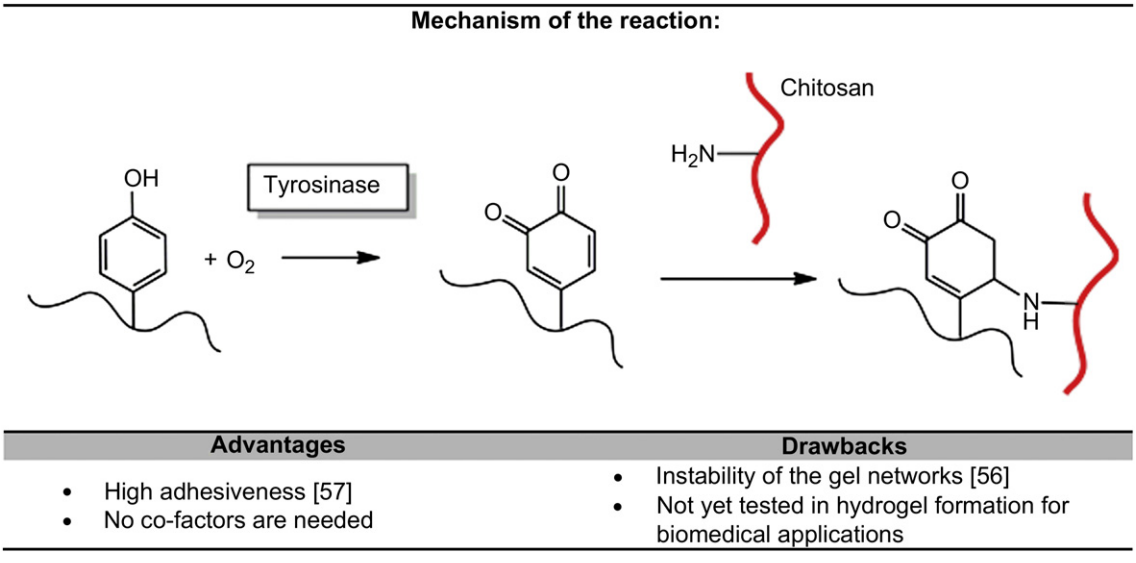


Fig. 2. Mechanism of the enzymatic reaction mediated by tyrosinase, advantages and drawbacks.

mainly suitable as glue [59] and wound dressings or could be used for protein immobilization [60], due to their fast degradation. Kang [57] reported the efficacy of tyrosinase crosslinking of silk fibroin and chitosan conjugates. Other applications of tyrosinase involve the crosslinking of tyrosine residues in silk, fibroin and sericin, yielding protein-polysaccharide conjugates [61,62]. These hydrogels showed potential for biomedical applications, due to their unique mechanical properties, adhesiveness and non-toxicity. However, no specific descriptions as TE approaches were reported.

2.3. Phosphopantetheinyl transferase

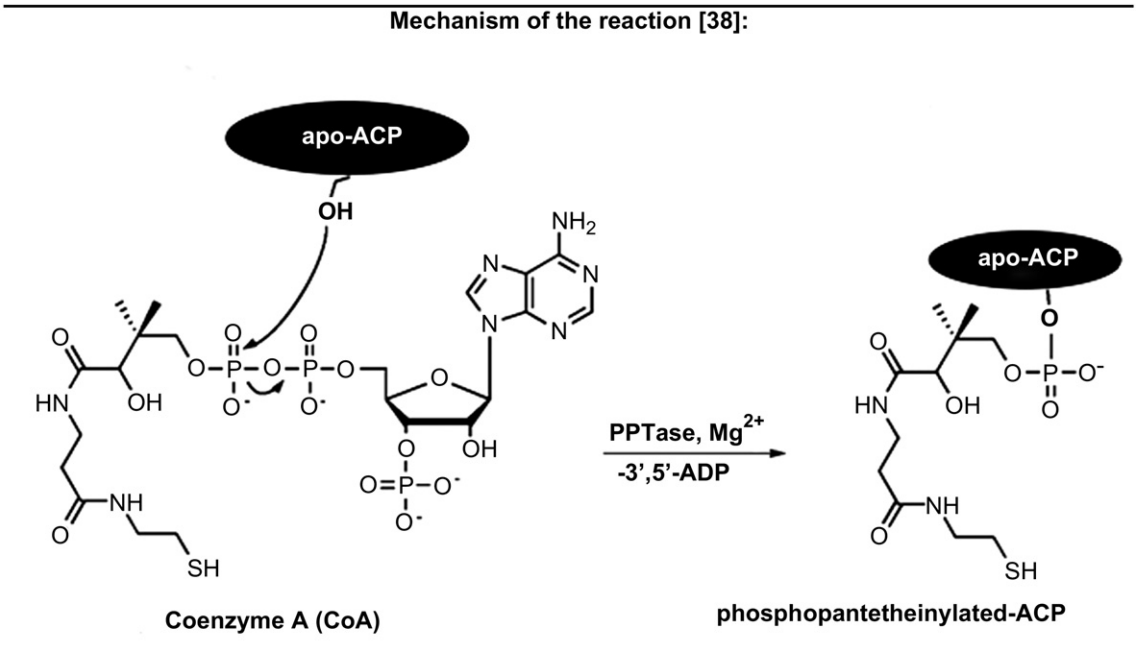
Phosphopantetheinyl transferase is a small enzyme that can be obtained with high expression yields, thus, offering an alternative to transglutaminases, which are larger and have limited recombinant production. Transferases, which are expressed mainly in the cytosolic compartment in a wide range of tissues, both in yeast and animal cells, comprise large multifunctional polypeptides that contain all of the catalytic components required for the synthesis of long-chain fatty acids [63]. The general mechanism of transferase catalysis to form synthetic hydrogels occurs by transfer of a phosphopantethein prostetic group of coenzyme A-functionalized PEG macromers to a serine residue of engineered carrier proteins.

The use of phosphopantetheinyl transferase-catalyzed formation of polymer hydrogels (Fig. 3) was recently reported by Mosiewicz [38]. Hybrid hydrogels were formed by mixing the precursors of 8-arm-PEG-coenzymeA, at 37 °C, neutral pH and in the presence of Mg^{2+}. The gelation was rather slow and occurred in approximately 15 min. The hydrogel reached an elastic modulus value of 2.3 kPa. Furthermore, in this study, they also explored the potential to incorporate bioactive peptides, more specifically the integrin receptor binding motifs, such as RGDs (Arg-Gly-Asp), which enable cell attachment and spreading [64]. With this method, selective covalent formation and modification of these transferase-catalyzed hydrogels with bioactive peptide ligands occurred simultaneously. This type of reaction is, on one hand highly attractive for cell biology and tissue engineering applications, but, on the other hand, still under explored.

2.4. Lysyl oxidase and plasma amine oxidase

Lysyl oxidase is a key component in the formation and repair of the native extracellular matrix. This ubiquitous enzyme oxidizes primary amines of lysines to aldehydes (Fig. 4). The formed reactive aldehydes react further to crosslink the extracellular matrix [65]. Lysyl oxidase is responsible for the covalent crosslinkages which stabilize collagen and elastin fibrous proteins. Consequently, lysyl oxidase is involved in the morphogenesis and regeneration potential of many connective tissues including skeleton, respiratory tract and cardiovascular tissue [66]. Plasma amine oxidase also functions by oxidation of primary amines and has the major advantage that it is commercially available [65]. Interestingly, both these enzymes can be used as matrix crosslinkers, not only to improve tissue or biomaterial strength over time, but also to enhance matrix formation [67,68].

Bakota [65] used lysyl oxidase to fabricate nanofibers of multidomain peptides, by oxidative crosslinking of lysine residues. Interestingly, unlike other hydrogel systems that degrade overtime,



Advantages	Drawbacks
• Small size enzyme with very high specificity • Efficient recombinant production with high expression yields and easy purification	• Slow crosslinking • Soft gels • Requirement of coenzyme A

Fig. 3. Mechanism of the enzymatic reaction mediated by lysyl phosphopantetheinyl transferase, advantages and drawbacks.

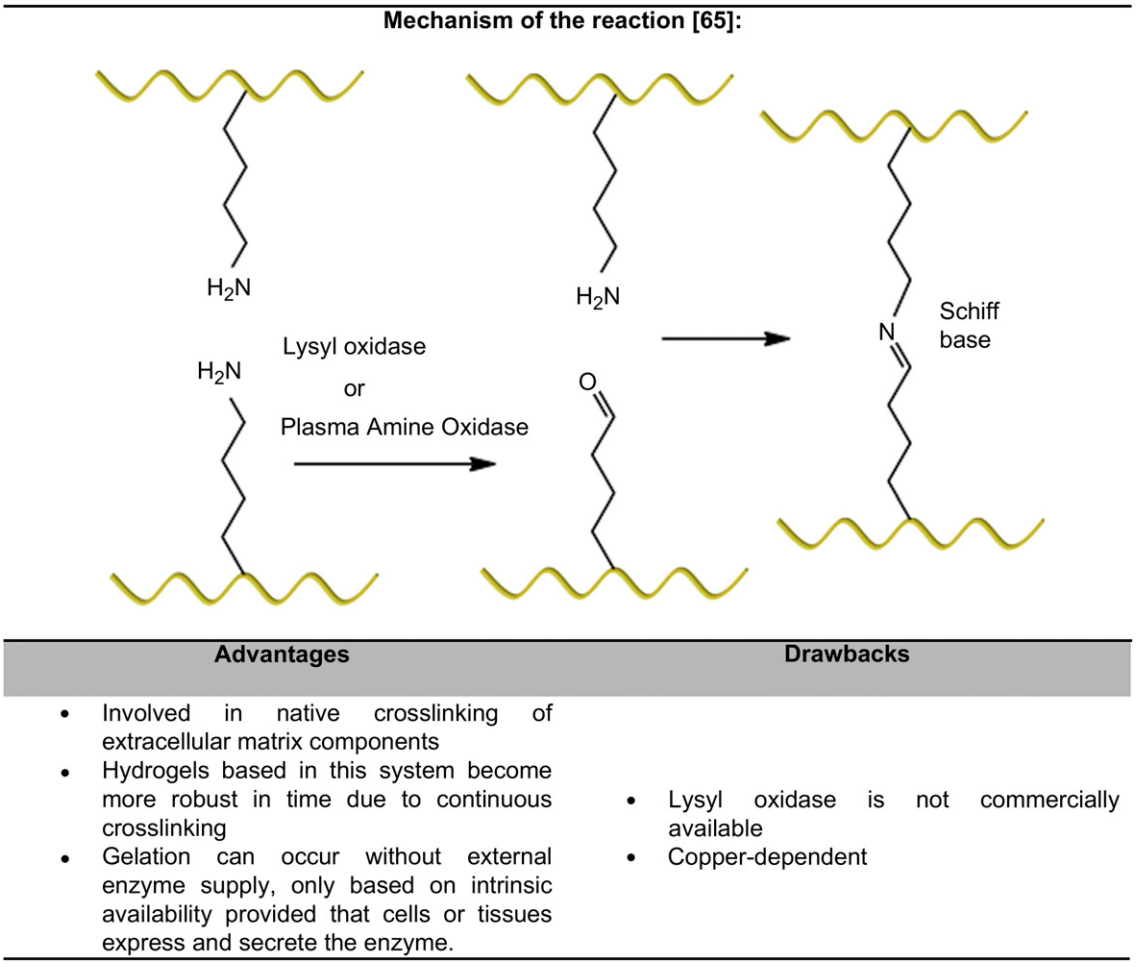


Fig. 4. Mechanism of the enzymatic reaction mediated by lysyl oxidase and plasma amine oxidase, advantages and drawbacks.

the hydrogels formed by this enzyme family become more robust due to the continuous activity of lysyl oxidase. This interesting feature leads to continuous increase in the mechanical stability of hydrogels composed of biopolymers rich in lysine. Lysyl oxidase is abundantly present in serum. Thus, in serum containing conditions, crosslinking of lysine containing polymers spontaneously occurs without addition of an exogenous enzyme source. Lysyl oxidase shows great value in tissue engineering using lysine-rich peptide-based hydrogels [69,70]. It might be explored to enhance extracellular matrix production by cells incorporated in the hydrogel. Moreover, it may also improve the intrinsic mechanical properties of tissue engineered constructs over time and allow hydrogel fixation with native tissue by covalent bond formation between the lysine-rich polymers of the hydrogel and primary amines in native tissue proteins.

2.5. Phosphatases, thermolysin, β-lactamase and phosphatase/kinase

Enzyme catalysis mediated by phosphatases, thermolysin, β-lactamase or phosphatase/kinase can change the amphiphilicity of small peptide derivatives, for example, by phosphorylation mediated by a kinase or dephosphorylation mediated by a phosphatase. This change can, subsequently, trigger the self-assembly and non-covalent interactions of the amphiphilic peptides in nanofibers, ultimately resulting in hydrogel formation, as represented in Fig. 5 [71,72]. These small peptide derivatives are usually organic or bioactive molecules, which tolerate the addition of bioactive components.

Phosphatases catalyze the removal of phosphate groups from a substrate, which becomes more hydrophobic. In an aqueous milieu, these hydrophobic substrates may self-assemble into a 3D nanofiber network by non-covalent interactions (for example, π-π interactions, hydrogen bonding, charge interactions) that allow gel formation [75]. In this respect, alkaline phosphatases are particularly interesting classes of enzymes to form hydrogels due to their involvement in mineralization of skeletal tissues. Schnepp [76] exploited this property of alkaline phosphatase by fabricating materials with a range of mineral loadings. Interestingly, these materials maintained their viscoelasticity rendering them suitable as biomaterials for application in tissue engineering, wound healing, and drug release purposes. Instead of breaking the covalent bonds between the peptide and the phosphate group, as it occurs with phosphatases, thermolysin exploits another way of changing the amphiphilicity of a peptide. Thermolysin catalyzes the formation of bonds between peptides by reverse hydrolysis. This enzyme can be used to couple two distinct peptide derivatives, reducing the solubility of one of the peptides. This block peptide can then self-assemble into a hydrogel by hydrophobic interactions. Thermolysin favors hydrophobic, aromatic residues on the amine side of the peptide bond. This system has been reported by Toledano [77] with possible applications in the production of nanofibrous hydrogel scaffolds for cell culture. β-Lactamases and esterases are two other enzymes that can be used as catalysts for molecular self-assembly.

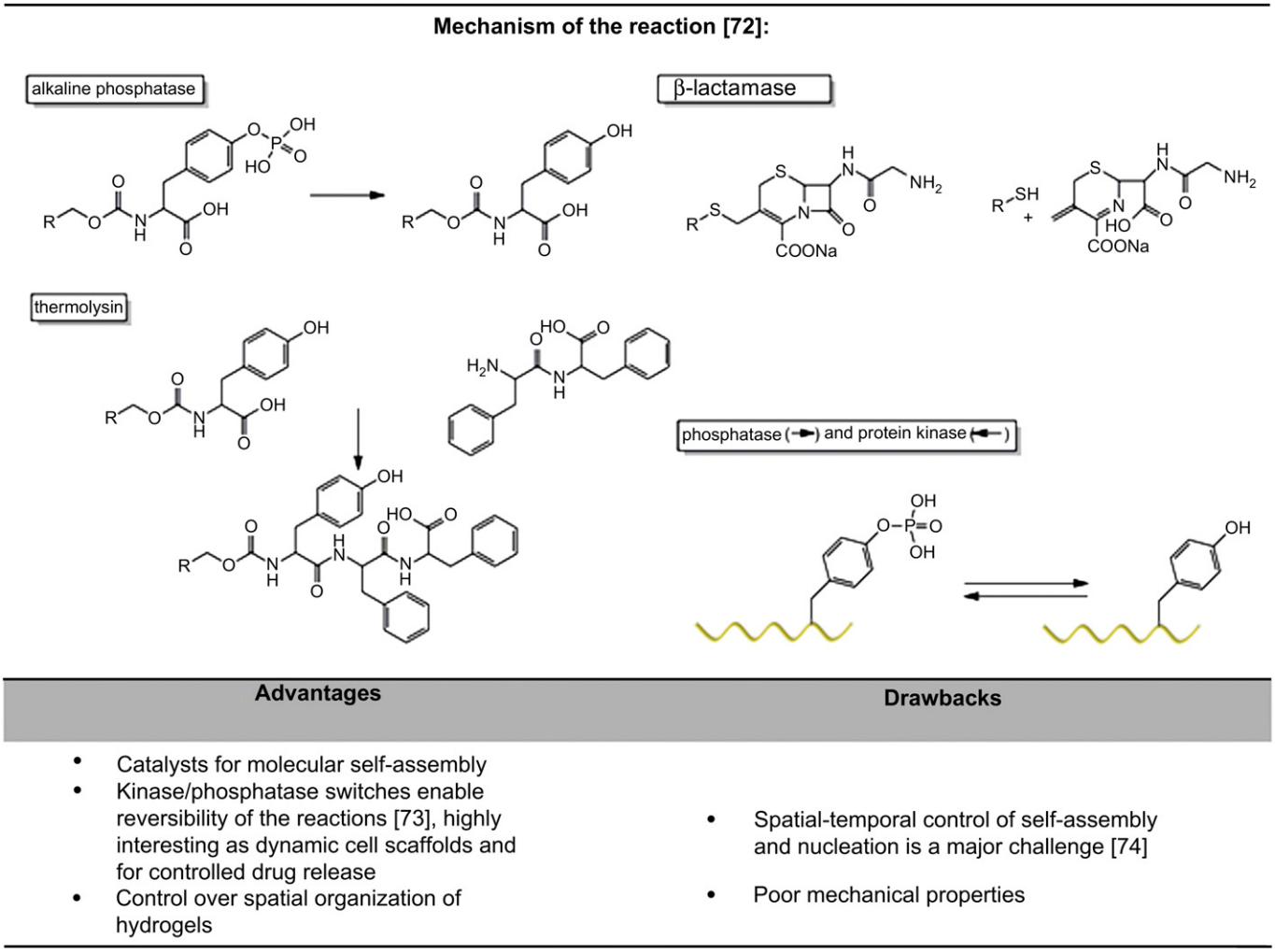


Fig. 5. Mechanism of the enzymatic reaction mediated by phosphatases, thermolysin, β-lactamase and phosphatase/kinase, advantages and drawbacks.

If the self-assembly occurs in an aqueous medium, the gels are referred to as a supramolecular hydrogels, and the small molecules are referred to as supramolecular hydrogelators. Both enzymes couple with the formation of hydrogelators. β-Lactamases are produced by some bacteria and are responsible for their resistance to β-lactam antibiotics, such as penicillin. The lactamase breaks a four-atom ring present in the molecular structure of antibiotics, known as β-lactam. As this ring opens, the molecule's antibacterial properties are deactivated. Upon the action of a lactamase, the lactam ring of the hydrogel precursor molecules opens and the hydrogelator is released. This release results in their self-assembly, subsequent nanofiber assembly and hydrogel formation. The presence of lactamases in bacterial lysate are able to convert the precursor to an hydrogelator, which triggers supramolecular hydrogel formation The intracellular self-assembly of a hydrogels mediated by β-lactamases or esterases can potentially be used as in bacterial assays or to trigger specific cell death, as the formation of these supramolecular hydrogels can occur inside the cells [78,79].

Most enzymatic reactions are irreversible, thus, leading to irreversible modification of the peptide backbone of the crosslinked hydrogel. To allow reversibility of the enzymatic reactions, Yang [73] proposed a kinase/phosphatase switch to control supramolecular hydrogels. This enzyme switch regulates the phase transition of the peptide backbone of the hydrogel. This occurs by either adding or removing a hydrophilic phosphate group from the peptide backbone, thereby, controlling both dissociation and formation of the self-assembled nanostructures. Exploiting this enzymatic switch allows precise control of biomaterial organization at the molecular level over time. This may have a wide range of applications in tissue engineering. In contrast to the random formation of polymer chains obtained by other ways of enzymatic crosslinking of polymeric hydrogels, the self-assembly of supramolecular hydrogels allows an ordered molecular arrangement within the nanofibers (hierarchical nanostructures) that ultimately leads to hydrogel formation. These are unique features of enzymatically formed supramolecular hydrogels [74,80].

2.6. Peroxidases

Peroxidases are a wide family of enzymes that typically catalyze the following reaction: ROOR' + electron donor ($2\ e^-$) + $2H^+$ → ROH + R'OH. The majority of the peroxidases use hydrogen peroxide as substrate. This family consists up to 42 isozymes, which becomes a challenge when defining the *in vivo* function [81]. The most commonly used peroxidases in hydrogel formation are horseradish peroxidase and soy bean peroxidase. Both are plant enzymes and are explored as useful tools for biosciences and biotechnology, even though soy bean peroxidase has only become known in the last 20 years. 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 [82]. In this reaction the horseradish peroxidase promptly combines with hydrogen peroxide and the formed complex can oxidize hydroxyphenyl groups. Such groups are present for instance in tyramine, tyrosine and 4-hydrophenyl acetic acid [83]. Soybean peroxidase is a suitable alternative to horseradish peroxidase, due to comparable stability and mechanism of action. The family of human peroxidases includes myeloperoxidase, lactoperoxidase, eosinophil peroxidase, thyroid peroxidase and prostaglandin H synthases. Mammalian enzymes contribute mainly in host defense against infection, hormone synthesis and pathogenesis. Plant peroxidases differ from human peroxidases in size and how the heme-group is bound. Plant peroxidases consist of approximately 300 amino acids and the heme-domain is not covalently bound, whereas mammalian peroxidases are larger, ranging from 576 to 738 amino acids, with heme covalently bound [84,85]. Although human peroxidases have been widely investigated, to our knowledge, only plant peroxidases have been explored for enzymatic crosslinking to form hydrogels.

Sofia [21] reported the use of peroxidases to catalyze the crosslinking of functionalized polyaspartic acid-based hydrogels. This study is one of the first comparing the activity of several peroxidases and their efficiency to form hydrogel networks. The use of this enzyme was further explored due to its non-cytotoxicity and potential to crosslink *in situ*. Recently, multiple biomaterials have been developed taking advantage of this system for the crosslinking of tyramine conjugated polymers. Darr [87] has characterized tyramine-based hyaluronan hydrogels that have shown *in vivo* non-cytotoxicity and resistance to degradation after subcutaneous injection, while preserving most of the negative charge from the carboxyl groups in hyaluronic acid, essential for the contribution of the physio-mechanical properties of tissue. Other studies have reported the use of hyaluronic acid-tyramine conjugates crosslinked by horseradish peroxidase, which possess promising features for controlled drug delivery and for application as injectable *in situ* forming gels [88,89]. The success of crosslinking system using peroxidases was extended to the use of other polysaccharide-derived polymers such as chitosan [90,91], alginate [18], carboxymethylcellulose [16] and dextran [86]. Horseradish crosslinkable dextran-tyramine hydrogels (Fig. 6) also in combination with hyaluronic acid and heparin have recently shown high potential as artificial extracellular matrixes for cartilage tissue engineering [13–15]. These polysaccharide hybrids were designed to mimic the molecular structure of the extracellular matrix of native cartilage. Similarly, injectable hyaluronic acid-tyramine has been described by Kim [92] as an effective drug carrier for the treatment of rheumatoid arthritis. Other polymer combinations using horseradish peroxidase to induce gelation include tetronic-tyramine conjugates and supramolecular hydrogels based on tyramine-terminated PEG [83,93]. The major advantage of this enzyme in comparison to the above mentioned enzymes, such as transglutaminase, is the fast gelation that can occur within seconds.

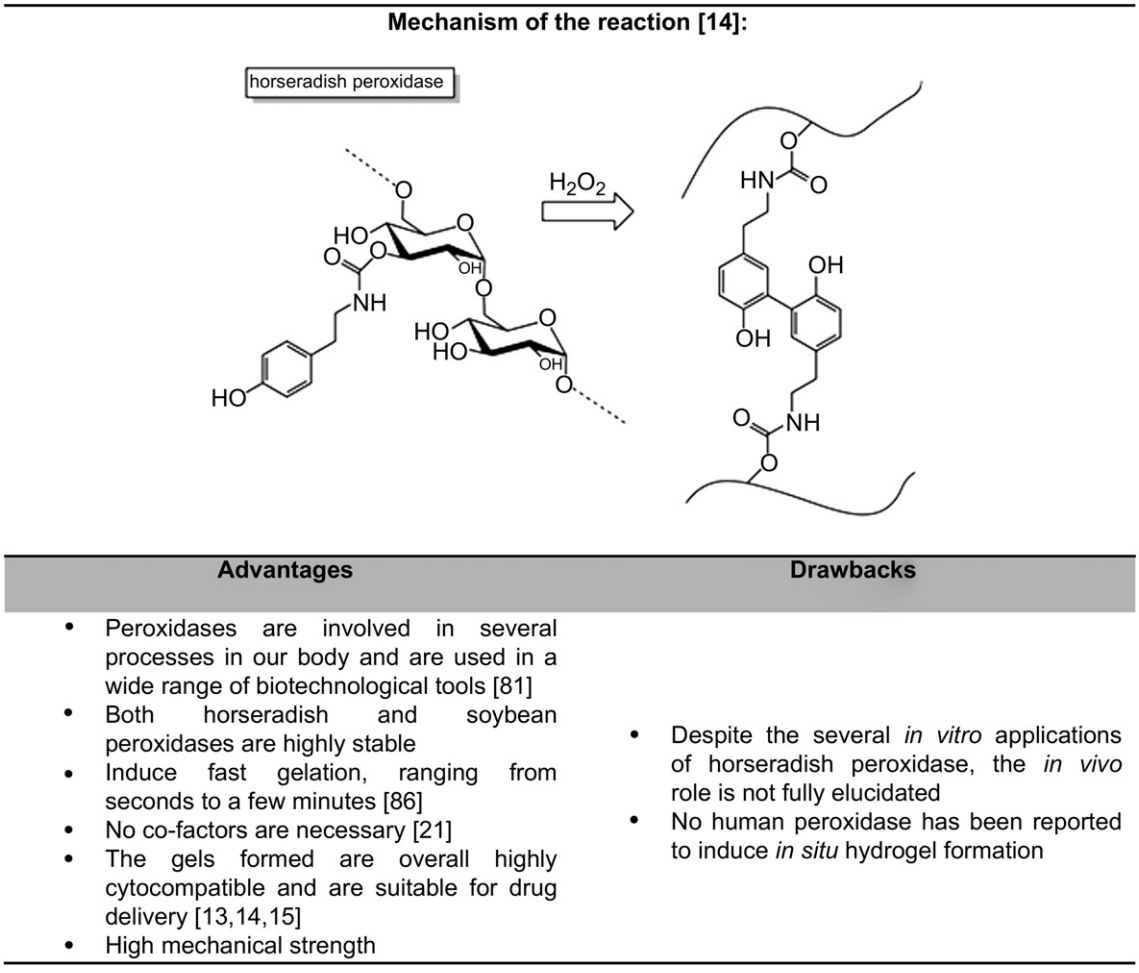


Fig. 6. Mechanism of the enzymatic reaction mediated by peroxidases, advantages and drawbacks.

Table 1
Enzyme-catalyzed crosslinkable hydrogels and potential applications.

Gel type/composition	Enzyme type	Potential applications	References
Modular protein hydrogel of lysine and glutamine	Animal derived tissue transglutaminase Recombinant human tranglutaminase	Non-specific TE applications	[12]
Fibrin gel	Factor Xllla (transglutaminase isoenzyme)	Angiogenesis Neurite extension Bone and cartilage tissue repair	[41–44]
8-arm PEG-peptide conjugates	Factor Xllla (transglutaminase isoenzyme)	Drug delivery systems Smart implants for *in situ* TE	[36,45]
Multi-arm comb PEG	Tissue transglutaminase	Gelation model	[47]
Elastin-like polypeptide gels	Tissue transglutaminase	Cartilage tissue repair	[51]
PEG-peptide conjugates	Tissue transglutaminase	Surgical tissue adhesives Cartilage tissue repair	[49,52]
Gelatin	Microbial transglutaminase	Scaffolds for TE Sustained drug release devices	[20,50,99,100]
Gelatin	Calcium-independent microbial transglutaminase	Microfluidic biosensor systems	[53]
Casein	Microbial transglutaminase	Sustained drug release	[101]
Gelatin-chitosan conjugates	Tyrosinase	Tissue glue Wound dressings	[56,59]
Silk fibroin/chitosan conjugates	Tyrosinase	Scaffolds for TE	[57]
Gelatin-chitosan conjugates	Tyrosinase Microbial transglutaminase	Film biofabrication Scaffolds for TE	[54,58]
Coil-chitosan bioconjugate	Tyrosinase	Protein immobilization	[60]
Coenzyme A-functionalized PEG	Phosphopantetheinyl transferase (surfactin synthetase)	Cell biology and TE	[38]
Nanofibrous lysine-rich peptide hydrogel	Lysyl oxidase Plasma amine oxidase	No application described	[65]
Supramolecular tyrosine-based hydrogel	Alkaline phosphatase	Assay platform for enzyme inhibitors	[75]
Supramolecular tyrosine- phosphate-based hydrogel	Alkaline phosphatase	Scaffolds to assist biomineralization	[76]
Fmoc-$(Phe)_3$ hydrogel	Thermolysin	Nanofibrous scaffolds for cell culture	[77]
Pentapeptidic hydrogelator (Nap-FFGEY)	Kinase/phosphatase	Non-specific TE applications	[73]
Functionalized polyaspartic acid	Peroxidases	Drug delivery, wound healing and TE	[21]
Chitosan derivative	Horseradish peroxidase	Drug delivery and TE	[90]
Chitosan-glycolic acid conjugates modified with phloretic acid	Horseradish peroxidase	Cartilage tissue repair	[91]
Hyaluronic acid-tyramine	Horseradish peroxidase	Protein delivery Non-specific TE applications Cartilage tissue repair	[87–89,92]
Alginate-phenol tyramine conjugates	Horseradish peroxidase	Multicellular spheroids for TE	[18]
Carboxymethylcellulose	Horseradish peroxidase	Biomedical applications	[16]
Dextran-tyramine conjugates	Horseradish peroxidase	Protein delivery and TE Cartilage tissue repair	[14,86]
Dextran-hyaluronic acid conjugates	Horseradish peroxidase	Cartilage tissue repair	[15]
Dextran-heparin	Horseradish peroxidase	Cartilage tissue repair	[13]
Tetronic-tyramine conjugates (propylene oxide and ethylene oxide)	Horseradish peroxidase	Drug delivery and TE	[93]
Tyramine-terminated PEG	Horseradish peroxidase	Drug delivery and TE	[83]

2.6.1. Horseradish peroxidase mimetic enzymes

Even though natural enzymes are remarkably specific, in general, these biomolecules are expensive, unstable and prone to deactivation when in solution. Thus, artificial enzymes with similar selectivity and catalytic activity have been developed, with superior stability compared to the natural enzymes [94–97]. Chen [94] has described a catalytic system, which is a water dispersible imprinted hydrogel based on a tetrapolymer of 4-vinylpyridine, hemin, acrylamide, and N-isopropylacrylamide. Hemin, also named chloro [3,7,12,17-tetramethyl-8,13-divinylporphyrin-2,18-dipropanoato(2-)]-iron(III) or Fe(III)protoporphyrin(IX) chloride, are co-functional monomers that act as the catalytic centers. This tertrapolymer was crosslinked by ethylene glycol dimethacrylate with homovanillic acid as template molecule, designed as an enzyme mimic of horseradish peroxidase. Wang [96,97] has previously reported a supramolecular hydrogel with encapsulated hemin as an artificial enzyme to mimic peroxidases, reaching approximately 90% of the activity of horseradish peroxidase. This artificial enzyme allowed catalysis with operational stability and reusability. Additionally, poly(NIPAAm/MBA/hemin) has been reported by Li [95] as a substitute for peroxidase. In addition to hemin, two other biocatalysts, microperoxidase-11 and cytocrome c, display peroxidase activity when activated by an electron receptor. These alternatives based on hemin, microperoxidases, or cytochrome *c* to mimic natural peroxidases have shown great promise for industrial purposes [98], although not yet explored for biomedical applications.

3. Conclusions and future perspectives

As outlined, enzyme-mediated systems are a relatively recent concept, pointing towards promising directions in hydrogel design. Despite the major advances and advantages of using biocatalysts, there are still challenges to overcome. These relate mainly to the slender amount of studies *in vivo,* instability of some of the enzyme types, such as transglutaminases and tyrosinases, and limited mechanical properties of the gels formed. The reduced *in vivo* applications are essentially due to the recent establishment of these systems. Additionally, the stability and availability of enzymes can be enhanced by the development of more recombinant enzyme types. Lastly, the poor or limited mechanical properties of some hydrogels can, in principle, be improved by combining enzyme types, after adjusting the material design.

Predominantly, transglutaminases and horseradish peroxidases can be highlighted as the best studied enzyme systems involved in hydrogel crosslinking for tissue engineering approaches (Table 1). Transglutaminases are highly interesting since they offer intimate

integration between the *in situ* formed gel and the native host tissue. The gels formed act as biological glues, due to the ubiquitous bodily distribution and equal supply of natural substrates [48]. Additionally, these enzymes have proven to successfully catalyze the crosslinking reaction of very different types of materials, such as PEG, elastin and gelatin. Horseradish peroxidases are likewise attractive due to their high stability, easy purification and availability mainly directly from the horseradish but also of recombinant forms [102]. Engineered peroxidases with even higher stability and catalytic efficiency are currently being developed, which is indicative that, in the near future, further applications using this enzyme type will be developed and continue to prosper in the tissue engineering field.

Overall, enzyme catalysis allows exceptional control over hydrogel formation, providing a step forward regarding higher complexity, non-cytotoxicity and non-invasiveness, vitally desired for the next generation of biomaterials for tissue engineering and regenerative medicine.

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