CHAPTER 10

Enzymatic crosslinked hydrogels

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10.1 Introduction

Enzymatic crosslinking is a polymerization route that relies on enzymes as reagents to cleave or form covalent bonds. Enzymes are highly substrate-specific, possess short reaction times, and are used for catalytic crosslinking while suppressing potentially toxic side reactions, which makes these crosslinking methods more efficient than their chemical counterparts (Bae et al., 2015; Hu et al., 2019b). These reactions are also cytocompatible, noninvasive and provide good control over hydrogel formation by controlling the enzyme concentration (Sperinde & Griffith, 1997). Enzymatic crosslinking is an interesting approach for hydrogels used in tissue engineering and regenerative medicine because it can provide rapid gelation (generally under 10 minutes) under mild, physiological conditions, making them suitable for biological applications including in vivo forming hydrogels (Hu et al., 2019b; Mohammed & Murphy, 2009; Moreira Teixeira et al., 2012). Moreover, enzymatic activity can often be controlled by modifying external factors such as temperature, pH, or ionic strength (Claaßen et al., 2019; Heijnis et al., 2010).

Enzymes have been used for catalytic reactions since the 1950s when Kalckar et al. used xanthine oxidase to oxidate xanthopterin to leucopterin (Kalckar et al., 1950). However, one of the first described use of enzymes for hydrogel crosslinking applications dates from the late 1990s when Sperinde and Griffith used transglutaminase to form a hydrogel network by crosslinking functionalized poly-(ethylene glycol) (PEG) and a lysine-containing polypeptide (Sperinde & Griffith, 1997). Since then, transglutaminases have been some of the most extensively used enzymes in tissue engineering along with horseradish peroxidase (HRP). This later enzyme catalyzes the coupling of phenol or aniline derivatives by using hydrogen peroxide (H_2O_2) as an oxidant (Ren et al., 2017). This reaction allows for the easy tuning of the gelation time, mechanical strength, degradation kinetic and porous structure of subsequent hydrogels by control of the concentrations of the constituents (Bae et al., 2015; Cheng et al., 2018). The versatility and tuneability of enzymatically crosslinked hydrogels translate into their use

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in various biomedical applications including bone, cartilage and soft tissue engineering, drug and cell delivery, etc. via bio three-dimensional printed scaffolds or injectable hydrogels (Echave et al., 2019; Jin et al., 2010; Li et al., 2018b; Morelli et al., 2016; Niu et al., 2019; Zhu et al., 2018). However, enzymaticallycrosslinked hydrogels produced from natural polymers such as silk fibroin or hyaluronic acid may still display insufficient mechanical properties, degradation resistance, slow gelation rate and/or lack of bioactivity after enzymatic crosslinking, which limits their use (Hasturk et al., 2020; Raia et al., 2017). Recently, composite hydrogels have been produced using more advanced techniques such as bienzymatic- or dual-crosslinking methods which allowed for improved biocompatibility and tuneable mechanical characteristics, to overcome some of the limitations and making these hydrogels more suitable for tissue engineering, cell culture and cell encapsulation applications (Bae et al., 2015; Echave et al., 2019; Hasturk et al., 2020; Jin et al., 2010; Le Thi et al., 2017; Nezhad-Mokhtari et al., 2019; Raia et al., 2017; Wang et al., 2020b; Zhang et al., 2015).

Enzymatic crosslinking is a relatively new method which remains underexplored as compared to other techniques. These reactions first solicited a vivid interest in injectable hydrogels applications, which constitute a large proportion of the current literature on enzymatic crosslinking applications. Recently, these reactions also drew attention to their ability to form hydrogel scaffolds with high degrees of complexity similar to those of extracellular matrices, scaffolds with dynamic properties, and systems with controlled and tuneable releases of therapeutics (Mohammed & Murphy, 2009; Moreira Teixeira et al., 2012). Enzymatic crosslinking methodologies have been used as primary crosslinking mechanisms during the synthesis of the hydrogels, as well as secondary scaffold processing techniques. The state-of-the-art enzymatic crosslinking methodologies for the development of hydrogels are reviewed herein.

10.2 Enzymatic polymer crosslinking reactions

As mentioned before, enzymatic crosslinking methodologies can be used as primary or secondary polymerization methods. Secondary crosslinking is used to improve the mechanical properties of weak hydrogels synthesized by other techniques. For example, Kajave et al. recently used genipin as a secondary crosslinker to strengthen a mechanically weak photo-crosslinked methacrylated collagen (CMA) (Kajave et al., 2020). This dual-crosslinking strategy allowed them to improve some properties of their hydrogels, such as the compressive moduli and degradation times, while still allowing for high cell survival rates in low dually crosslinked CMA hydrogels. Primary and secondary enzymatic crosslinking rely on similar reactions to induce or increase crosslinking of hydrogels. However, it appears that in similar conditions, primary and secondary polymerization techniques do not result in similar degrees of crosslinking. Carnes et al. recently assessed the crosslinking degrees of fibrin microthreads scaffolds

crosslinked by HRP and H_2O_2 when these reagents were incorporated during extrusion and/or during postprocessing steps (Carnes et al., 2020). Their results showed that primary HRP-crosslinked scaffolds had the highest degree of tyrosine crosslinking and that secondary or primary-and-secondary HRP-crosslinked microthreads did not contain detectable levels of isodityrosine bonds. Clearly, the enzyme content of a hydrogel also influences its physical and mechanical properties. In 2019, Echave et al. assessed the effect of varying the proportion of microbial transglutaminase in gelatin-based three-dimensional scaffolds fabricated by freeze-drying (Echave et al., 2019). Their work concluded that the swelling capacity of their scaffold decreased with higher enzyme ratios, inversely to Young's modulus which increased.

Moreover, optimal results from crosslinking reactions require the adequate selection of enzymes depending on the polymers to crosslink and the applications intended. Although different enzymes can be used to crosslink the same macromers at similar crosslinking conditions, they may certainly lead to different gelation rates, mechanical and biological properties as their reactions mechanisms, substrate specificities and biochemical properties will most likely differ (Heck et al., 2013; Roberts et al., 2016). For example, both Laccase and Tyrosinase (Tyr) can oxidize phenol moieties and induce crosslinking using molecular oxygen, but their crosslinking reactions are very different. Laccase reacts with molecular oxygen and hydrogen atoms from the hydroxyl groups from two phenols to produce H_2O_2, which is then used to form di-phenol bonds. However, Tyr reacts with molecular oxygen to convert phenols into catechols through the addition of hydroxyl groups on their aromatic ring, before that further oxidation generates reactive quinone, which then promptly crosslinks with other quinones through covalent bonds (Fig. 10.1) (Roberts et al., 2016). The Enzyme Commission classifies all enzymes into seven main classes according to their reactions, as depicted in Table 10.1 (Kobayashi & Uyama, 2011; McDonald et al., 2009). Each class is then subdivided into subclasses given further information about the reactions themselves, that is, what kind of bond is formed, the compounds implied, etc. The first group, oxidoreductases, regroups all the enzymes which can induce oxidoreduction reactions, similar to the reactions of Laccase and Tyr previously described. There are numerous available enzymes, which can also be modified to better comply with application-specific requirements (Li & Cirino, 2014). The cofactors associated with these enzymes must be carefully selected as well. For example, hydrogen peroxide is an oxidant used with various enzymes, including HRP, that has been linked with reduced cell viability in cell encapsulation applications (Roberts et al., 2016).

All biopolymers found within living cells are produced via enzymatically catalyzed reactions (Kobayashi & Uyama, 2011). These gelation chemistries are very suitable for crosslinking natural polymers because they allow avoiding harsh conditions usually associated with chemical crosslinking techniques, while still allowing for higher

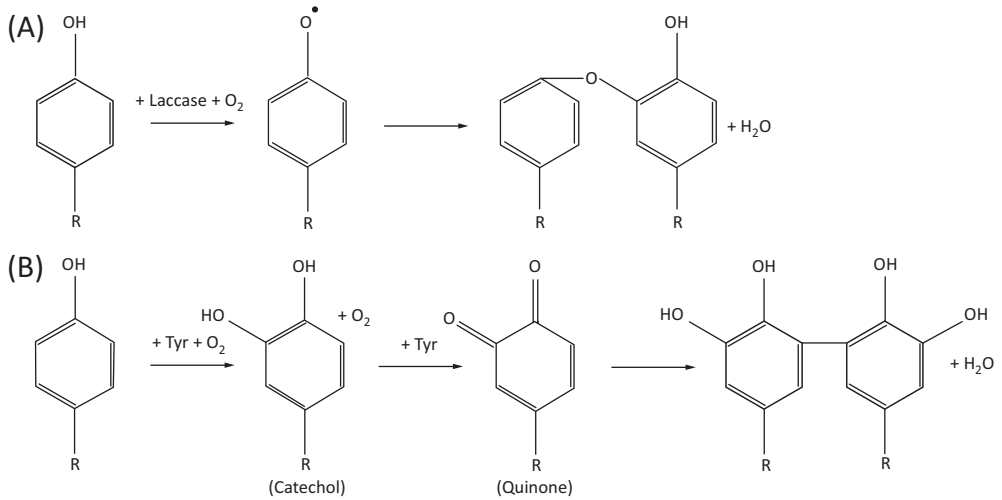

Figure 10.1 ***Polymerization reactions of phenol moieties by Laccase (A) and Tyrosinase (B) with molecular oxygen.*** Laccase (A) reacts with molecular oxygen and hydrogen atoms from two phenols to produce hydrogen peroxide, which is then used to form di-phenol bonds. Tyrosinase (B) reacts with molecular oxygen to convert phenols into catechols, before further oxidation generates reactive quinone, which finally crosslinks with other quinones. *Modified from Roberts, J. J., Naudiyal, P., Lim, K. S., Poole-Warren, L. A., & Martens, P. J. (2016). A comparative study of enzyme initiators for crosslinking phenol-functionalized hydrogels for cell encapsulation.* Biomaterials Research, 20. *https://doi.org/10.1186/s40824-016-0077-z; with permission of Springer Nature, Copyright © 2016, licensed under CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/).*

mechanical properties than physical crosslinking methodologies. Enzymatic methodologies are widely used for crosslinking proteins thanks to the broad variety of enzyme/cofactors combinations which allows to tailor the most suitable reaction for targeting specific functional groups present in amino acids (Heck et al., 2013; Wong & Jameson, 2011). Enzymatic methodologies have also been used to crosslink other natural polymers as well as their synthetic counterparts (Arkenberg & Lin, 2017; Ren et al., 2017; Wang et al., 2014). Although most of the literature references enzymatic crosslinking as a chemical crosslinking method, some articles differentiate these techniques into a third kind of polymerization route because of their similarity with both chemical and physical crosslinking methods (Lee et al., 2015; Parenteau-Bareil et al., 2010; Thakur et al., 2018; Yang et al., 2016). Namely, their mild reaction conditions resemble to those of physical crosslinking methods, whereas they produce covalent bonds similar to chemical routes.

Although enzymatic crosslinking appears to be an ideal polymerization methodology, several concerns have been raised regarding its safety. One of them regards enzyme retention within the hydrogels. Several enzymes including HRP are plant-derived proteins and as such, they can potentially reduce the immunological response

Table 10.1 Enzyme commission number (EC number) classification.

Major Classes	Reaction catalyzed	Typical Reaction	Examples
EC 1. Oxidoreductases	Oxidoreduction, i.e., transfer of H and O atoms or electrons from one substance to another	$AH + B \rightarrow A + BH$ (reduction) $A + O \rightarrow AO$ (oxidation)	Peroxidase, Tyrosinase
EC 2. Transferases	Transfer of a functional group from one substance to another	$AB + C \rightarrow A + BC$	Transglutaminase, Acyltransferase
EC 3. Hydrolases	Bond cleavages by hydrolysis	$A\text{-}B + H_2O \rightarrow A\text{-}OH + B\text{-}H$	Lipase, Glycosidase
EC 4. Lyases	Nonhydrolytic addition or removal of a group to a substrate	$X\text{-}A\text{-}B\text{-}Y \rightleftarrows A = B + X\text{-}Y$	Decarboxylase, Dehydratase
EC 5. Isomerases	Intramolecular rearrangement	$ABC \rightarrow BCA$	Racemase, Epimerase
EC 6. Ligases	Joining of two large molecules or two parts of a molecule with concomitant hydrolysis of the diphosphate bond in ATP or a similar triphosphate.	$X + Y + ATP \rightarrow XY + ADP + P_i$	DNA Ligase, Synthetase
EC 7. Translocases	Catalyze the movement of ions or molecules across membranes or their separation within membranes		Ornithine translocase

of hydrogels when implanted in the body (Lee et al., 2015). In 2002, Bardor et al. demonstrated that the protein core and the N-glycans of HRP could induce the production of antibodies in rodents (Bardor et al., 2003). Using a human peroxidase could offer a safer alternative to HRP, but no such enzyme has been found to efficiently catalyze the oxidative coupling of phenols yet, probably due to the high cost of human proteins and the poor activity of human peroxidase in catalyzing polymer-phenol conjugates crosslinking (Lee et al., 2015). Some enzymes may also display cytotoxicity at specific concentrations. Recently, Kajave et al. demonstrated that direct cell exposure to genipin is cytotoxic at concentrations greater than 1 mM (Kajave et al., 2020). Such issues cannot be overlooked, especially in therapies implying repeated exposure to these enzymes. Several enzymatic crosslinking methodologies have been developed to reduce or eliminate enzyme retention within the hydrogels.

By using enzyme-immobilized microparticles, researchers have been able to produce in situ forming hydrogel solutions without subsequent enzyme retention (Bae et al., 2014; Li et al., 2018a). By varying the contact time between the polymer solutions and the enzyme-loaded particles, they were also able to tune different hydrogel properties, including the gelation rate and the storage modulus. Another concern of enzymatic crosslinking is the toxicity related to the cofactors used. For example, H_2O_2 is used with many enzymes, but studies have proved that micromolar concentrations of H_2O_2 prevent cell growth in a culture medium while higher concentrations (micro to millimolar) lead to apoptosis and necrosis (Gardner et al., 1997). The incubation period also greatly affects this toxicity, which suggests that faster reaction times and quick consumption of H_2O_2 could help to minimalize it (Gülden et al., 2010; Wang et al., 2014). Several studies demonstrated that brief exposures to H_2O_2 during HRP-catalyzed reactions cause minimal toxicity along with inducing resistance to oxidative stress, following a high embedded cell survival rate ($>90\%$), especially if cells are exposed a second time to H_2O_2 (Jin et al., 2009; Lim et al., 2012; Toh et al., 2012). Novel enzymatic crosslinking methodologies continue to be developed to allow new processing possibilities while becoming more efficient and safer.

10.3 Advanced crosslinking methodologies

Enzymatically crosslinked hydrogels have been successfully used for numerous biomedical applications. Table 10.2 present some studies published in the last three years about polymer−enzyme combinations and their subsequent applications, partially demonstrating the broad diversity of the research area (Arkenberg & Lin, 2017; Broguiere et al., 2018; Carnes et al., 2020; Ching-Cheng et al., 2020; Echave et al., 2019; Hou et al., 2018; Hu et al., 2019a; Khanmohammadi et al., 2019; Kim et al., 2018; Le Thi et al., 2017; Ren et al., 2017; Ribeiro et al., 2019; Wang et al., 2020a; Ztürk et al., 2020). Such a variety of combinations and approaches will continue to grow since researchers continue to develop new crosslinking methodologies to overcome the challenges they face.

In recent years, crosslinking methodologies based on several complementary enzymes have been developed to overcome the limitations previously encountered. Le Thi et al. developed a dual-enzymatic crosslinking system based on HRP and Tyr to prepare in situ forming bio-adhesive hydrogels with strong adhesive properties (Le Thi et al., 2017). Concentration tuning of the enzymes provided control over gelation time and mechanical properties of the hydrogels. Moreover, Tyr allowed to double the adhesive strength of HRP-crosslinked hydrogels while still being biocompatible and biodegradable. Dual-enzymatic crosslinking methodologies can rely on the simultaneous action of both enzymes as previously presented, or on two-step processes where one enzyme relies on the reaction of the second one to induce crosslinking.

Table 10.2 Applications of enzymatically crosslinked hydrogels.

Type of enzyme(s)	Polymer(s)	Application(s)	Ref.
Horseradish peroxidase	Silk fibroin	Tissue engineering	Ribeiro et al. (2019)
Horseradish peroxidase	Fibrin	Tissue engineering	Carnes et al. (2020)
Horseradish peroxidase	Tyramine-conjugated poly(ethylene glycol)	Injectable hydrogels	Ren et al. (2017)
Horseradish peroxidase	Hyaluronic acid	Cell encapsulation	Khanmohammadi, Sakai and Taya (2019)
Horseradish peroxidase & tyrosinase	Gelatin	Cell & drug delivery	Le Thi et al. (2017)
Tyrosinase	Tyramine- and sulfate-conjugated alginate	Tissue engineering	Ztürk et al. (2020)
Tyrosinase	Tyramine-conjugated hyaluronic acid and gelatin	Tissue engineering	Kim et al. (2018)
Microbial transglutaminase	Gelatin	Wound healing	Hou et al. (2018)
Microbial transglutaminase	Gelatin	Bone tissue engineering	Echave et al. (2019)
Microbial transglutaminase	Gelatin	Cartilage tissue engineering	Tsai et al. (2020)
Microbial transglutaminase	Chitosan & collagen peptides	Wound dressing	Hu et al. (2019a)
Glucose oxidase, acetylcholine esterase and urease	Polyacrylamide	Drug delivery	Wang, Fischer, Ehrlich, Nahmias, and Willner (2020a)
Sortase A	Hyaluronic acid	Tissue engineering	Broguiere, Formica, Barreto, and Zenobi-Wong (2018)
Sortase A	Poly(ethylene glycol)-peptides	In situ cell encapsulation	Arkenberg and Lin (2017)

Recently, Shen and colleagues used HRP and glucose oxidase in a similar process (Shen et al., 2020). Their polymer was first oxidized by glucose oxidase to form H_2O_2 which then reacted with HRP to form a mechanically weak but printable polysaccharide hydrogel. This hydrogel was then further crosslinked by a gradual process to adjust its Young's modulus from 3.29 to 86.73 kPa.

The development of enzymatic crosslinking methodologies has also led to the emergence of controlled polymerization of hydrogels by external stimuli. In 2013, Milleret et al. were able to electrochemically control the polymerization of PEG hydrogels using Factor XIII-mediated transglutamination reactions (Milleret et al., 2014). Their work also demonstrated the inhibitive potential of this electrochemical control technique as the hydrogels displayed decreased adhesion to the electrodes, an interesting property to improve the reproducibility of template-removal-based techniques. More recently, Wang et al. modified DNA-based polyacrylamide hydrogels with glucose oxidase (GO*x*), acetylcholine esterase and urease to cooperatively yield stable two pH-responsive crosslinking motifs (Fig. 10.2) (Wang et al., 2020a). This technique led to pH-induced reversible biocatalytic control over the stiffness of the fabricated matrices, which allowed for the controlled release of loaded drugs through substrate-triggered chemistries. Specifically, their GOx/insulin-loaded hydrogel showed great potential as its stiffness was controlled by glucose-triggered chemistries, and the release of insulin was controlled by the dose of glucose. Altogether, this hydrogel displays promising results to create systems for the autonomous control of glucose levels through the release of insulin. The different hydrogels developed in this study also show great potential for the development of innovative approaches to enzyme-guided shape-retaining and self-healing matrices.

Enzymatic crosslinking methodologies also allow for novel approaches to mix reagents together, especially when using emulsions. In 2017, Kamperman et al. developed an

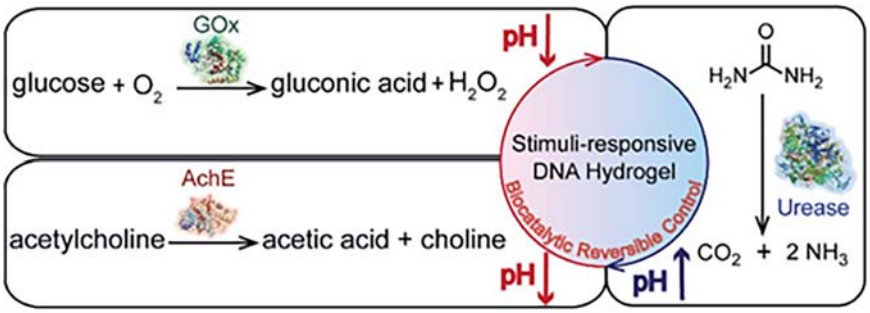

Figure 10.2 ***Schematic reversible biocatalytic control of pH in stimulus responsive DNA-based hydrogels using three biocatalysts (GOx, AchE, and urease) and two different motifs as reconfigurable cross-linkers.*** This multienzymatic system allows for a two pH-responsive crosslinking motif, creating a reversible switchable control over the stiffness of the matrix. In the application presented, the hydrogel mimics the natural function of the pancreas, releasing insulin in response to the environmental concentration of glucose. *With permission from Wang, C., Fischer, A., Ehrlich, A., Nahmias, Y., & Willner, I. (2020). Biocatalytic reversible control of the stiffness of DNA-modified responsive hydrogels: applications in shape-memory, self-healing and autonomous controlled release of insulin.* Chemical Science, 11*(17), 4516–4524. https://doi.org/10.1039/d0sc01319f; with permission of the Royal Society of Chemistry, Copyright © 2020, licensed under CC BY 3.0 (http://creativecommons.org/licenses/by/3.0/).*

in-emulsion enzymatic crosslinking technique for tyramine-functionalized polymers reacting with HRP and a H_2O_2/oil nanoemulsion (Kamperman, Henke, Zoetebier, et al., 2017). This technique supported the formation of monodisperse hydrogel particles ranging from the nano- to the millimeter-scale, and overcame a lot of limitations previously encountered by other crosslinking methodologies. Most polymerization techniques require crosslinker molecules such as ions or radicals (Hennink & Van Nostrum, 2002), which is difficult to obtain in emulsions due to the immiscibility of these multiphase systems as oil tend to disturb the direct mixing of hydrogels and their precursors (Kamperman, Henke, Zoetebier, et al., 2017). Moreover, typical polymerization techniques used with emulsions can result in inhomogeneous polymeric networks, device clogging and off-center cell encapsulation, which is detrimental to the long-term application of cell-laden hydrogel particles (Kamperman, Henke, Visser, et al., 2017). More recently, researchers from the same group developed a single-step outside-in crosslinking method to produce hollow single-core hydrogel-based microcapsules using the same reagents (van Loo et al., 2020). Polymer solution microdroplets were exposed to an outside-in diffusion of low amounts of H_2O_2, which were consumed by the crosslinking reaction before reaching the microdroplets' core, and which could be controlled by adjusting different processing parameters. That method also overcome many of the limitations previously encountered by other production techniques as it is a cleaner, more chemically defined process which uses no inhibitors while allowing for faster production rates with lower chances of microdevice failures, and that does not require dedicated infrastructures (van Loo et al., 2020).

10.4 Conclusion and outlook

Hydrogels performances greatly rely on their ability to yield stable biocompatible structures with adequate mechanical strength and in suitable timeframes. These properties are strongly influenced by material choice and processing parameters, but they are also heavily affected by crosslinking mechanisms. Physical and chemical polymerization methodologies both present their own characteristics along with their strengths and weaknesses. Namely, physical polymerization techniques produce mechanically weak structures under mild conditions whereas chemical crosslinking yield strong covalently bonded hydrogels which can potentially display cytotoxicity. Enzymatic crosslinking methodologies are relatively new compared to their chemical and physical counterparts, but they show great potential as they appear to be an ideal in-between which can rapidly produce strong covalent intermolecular bonds under mild conditions. Enzymatic crosslinking mechanisms are abundant in nature and provide an excellent source of polymerization routes for hydrogels. Their mild characteristics are very attractive for the development of strong biocompatible structures and in situ forming hydrogels. Numerous enzymes can be modified,

combined with other polymerization mechanisms or with other enzymes to better comply with the material- and application-specific requirements. However, several safety concerns related to the generally plant-derived origins of enzymes, and to the potential toxicity of the cofactors used, raise the need to further develop these techniques to become more suitable for biomedical applications. The development of innovative biocompatible covalent crosslinking methods brings the biomedical engineering community closer to the clinical expectations of hydrogels of all-encompassing ideal properties, more suitable to biomedical applications, whilst further enhancing regeneration and therapeutic outcomes.

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References

Arkenberg, M. R., & Lin, C. C. (2017). Orthogonal enzymatic reactions for rapid crosslinking and dynamic tuning of PEG-peptide hydrogels. *Biomaterials Science*, *5*(11), 2231–2240. Available from https://doi.org/10.1039/c7bm00691h.

Bae, J. W., Choi, J. H., Lee, Y., & Park, K. D. (2015). Horseradish peroxidase-catalysed in situ-forming hydrogels for tissue-engineering applications. *Journal of Tissue Engineering and Regenerative Medicine*, *9*(11), 1225–1232. Available from https://doi.org/10.1002/term.1917.

Bae, J. W., Kim, B. Y., Lih, E., Choi, J. H., Lee, Y., & Park, K. D. (2014). In situ formation of enzyme-free hydrogels via ferromagnetic microbead-assisted enzymatic cross-linking. *Chemical Communications*, *50*(89), 13710–13713. Available from https://doi.org/10.1039/c4cc04436c.

Bardor, M., Faveeuw, C., Fitchette, A. C., Gilbert, D., Galas, L., Trottein, F., Faye, L., & Lerouge, P. (2003). Immunoreactivity in mammals of two typical plant glyco-epitopes, core α(1,3)-fucose and core xylose. *Glycobiology*, *13*(6), 427–434. Available from https://doi.org/10.1093/glycob/cwg024.

Broguiere, N., Formica, F., Barreto, G., & Zenobi-Wong, M. (2018). Sortase A as a cross-linking enzyme in tissue engineering. *Acta Biomaterialia*, 77, 182–190. Available from https://doi.org/10.1016/j.actbio.2018.07.020.

Carnes, M. E., Gonyea, C. R., Mooney, R. G., Njihia, J. W., Coburn, J. M., & Pins, G. D. (2020). Horseradish peroxidase-catalyzed crosslinking of fibrin microthread scaffolds. *Tissue Engineering - Part C: Methods*, *26*(6), 317–331. Available from https://doi.org/10.1089/ten.tec.2020.0083.

Cheng, Y., He, C., Ren, K., Rong, Y., Xiao, C., Ding, J., Zhuang, X., & Chen, X. (2018). Injectable enzymatically cross-linked hydrogels with light-controlled degradation profile. *Macromolecular Rapid Communications*, *39*(18). Available from https://doi.org/10.1002/marc.201800272.

Ching-Cheng, T., Shuo-Hsiu, K., Ting-Yu, L., Nai-Chen, C., Ming-You, S., & Jiashing, Y. (2020). Enzyme-cross-linked gelatin hydrogel enriched with an articular cartilage extracellular matrix and human adipose-derived stem cells for hyaline cartilage regeneration of rabbits. *ACS Biomaterials Science & Engineering*, 5110–5119. Available from https://doi.org/10.1021/acsbiomaterials.9b01756.

Claaßen, C., Gerlach, T., & Rother, D. (2019). Stimulus-responsive regulation of enzyme activity for one-step and multi-step syntheses. *Advanced Synthesis and Catalysis*, *361*(11), 2387–2401. Available from https://doi.org/10.1002/adsc.201900169.

Echave, M. C., Pimenta-Lopes, C., Pedraz, J. L., Mehrali, M., Dolatshahi-Pirouz, A., Ventura, F., & Orive, G. (2019). Enzymatic crosslinked gelatin 3D scaffolds for bone tissue engineering. *International Journal of Pharmaceutics*, *562*, 151–161. Available from https://doi.org/10.1016/j.ijpharm.2019.02.043.

Gardner, A. M., Xu, F. H., Fady, C., Jacoby, F. J., Duffey, D. C., Tu, Y., & Lichtenstein, A. (1997). Apoptotic vs. nonapoptotic cytotoxicity induced by hydrogen peroxide. *Free Radical Biology and Medicine*, *22*(1–2), 73–83. Available from https://doi.org/10.1016/S0891-5849(96)00235-3.

Gülden, M., Jess, A., Kammann, J., Maser, E., & Seibert, H. (2010). Cytotoxic potency of H_2O_2 in cell cultures: Impact of cell concentration and exposure time. *Free Radical Biology and Medicine*, *49*(8), 1298–1305. Available from https://doi.org/10.1016/j.freeradbiomed.2010.07.015.

Hasturk, O., Jordan, K. E., Choi, J., & Kaplan, D. L. (2020). Enzymatically crosslinked silk and silk-gelatin hydrogels with tunable gelation kinetics, mechanical properties and bioactivity for cell culture and encapsulation. *Biomaterials*, 232. Available from https://doi.org/10.1016/j.biomaterials.2019.119720.

Heck, T., Faccio, G., Richter, M., & Thöny-Meyer, L. (2013). Enzyme-catalyzed protein crosslinking. *Applied Microbiology and Biotechnology*, *97*(2), 461–475. Available from https://doi.org/10.1007/s00253-012-4569-z.

Heijnis, W. H., Wierenga, P. A., Van Berkel, W. J. H., & Gruppen, H. (2010). Directing the oligomer size distribution of peroxidase-mediated cross-linked bovine α-lactalbumin. *Journal of Agricultural and Food Chemistry*, *58*(9), 5692–5697. Available from https://doi.org/10.1021/jf100168x.

Hennink, W. E., & Van Nostrum, C. F. (2002). Novel crosslinking methods to design hydrogels. *Advanced Drug Delivery Reviews*, *54*(1), 13–36. Available from https://doi.org/10.1016/S0169-409X(01)00240-X.

Hou, S., Lake, R., Park, S., Edwards, S., Jones, C., & Jeong, K. J. (2018). Injectable macroporous hydrogel formed by enzymatic cross-linking of gelatin microgels. *ACS Applied Bio Materials*, *1*(5), 1430–1439. Available from https://doi.org/10.1021/acsabm.8b00380.

Hu, W., Liu, M., Yang, X., Zhang, C., Zhou, H., Xie, W., Fan, L., & Nie, M. (2019a). Modification of chitosan grafted with collagen peptide by enzyme crosslinking. *Carbohydrate Polymers*, *206*, 468–475. Available from https://doi.org/10.1016/j.carbpol.2018.09.023.

Hu, W., Wang, Z., Xiao, Y., Zhang, S., & Wang, J. (2019b). Advances in crosslinking strategies of biomedical hydrogels. *Biomaterials Science*, 7(3), 843–855. Available from https://doi.org/10.1039/c8bm01246f.

Jin, R., Moreira Teixeira, L. S., Dijkstra, P. J., Karperien, M., van Blitterswijk, C. A., Zhong, Z. Y., & Feijen, J. (2009). Injectable chitosan-based hydrogels for cartilage tissue engineering. *Biomaterials*, *30*(13), 2544–2551. Available from https://doi.org/10.1016/j.biomaterials.2009.01.020.

Jin, R., Moreira Teixeira, L. S., Dijkstra, P. J., van Blitterswijk, C. A., Karperien, M., & Feijen, J. (2010). Enzymatically-crosslinked injectable hydrogels based on biomimetic dextran-hyaluronic acid conjugates for cartilage tissue engineering. *Biomaterials*, *31*(11), 3103–3113. Available from https://doi.org/10.1016/j.biomaterials.2010.01.013.

Kajave, N. S., Schmitt, T., Nguyen, T. U., & Kishore, V. (2020). Dual crosslinking strategy to generate mechanically viable cell-laden printable constructs using methacrylated collagen bioinks. *Materials Science and Engineering C*, 107. Available from https://doi.org/10.1016/j.msec.2019.110290.

Kalckar, H. M., Kjeldgaard, N. O., & Klenow, H. (1950). Xanthopterin oxidase. *BBA - Biochimica et Biophysica Acta*, *5*(C), 575–585. Available from https://doi.org/10.1016/0006-3002(50)90203-4.

Kamperman, T., Henke, S., Visser, C. W., Karperien, M., & Leijten, J. (2017). Centering single cells in microgels via delayed crosslinking supports long-term 3D culture by preventing cell escape. *Small*, *13*(22). Available from https://doi.org/10.1002/smll.201603711.

Kamperman, T., Henke, S., Zoetebier, B., Ruiterkamp, N., Wang, R., Pouran, B., Weinans, H., Karperien, M., & Leijten, J. (2017). Nanoemulsion-induced enzymatic crosslinking of tyramine-functionalized polymer droplets. *Journal of Materials Chemistry B*, *5*(25), 4835–4844. Available from https://doi.org/10.1039/c7tb00686a.

Khanmohammadi, M., Sakai, S., & Taya, M. (2019). Characterization of encapsulated cells within hyaluronic acid and alginate microcapsules produced via horseradish peroxidase-catalyzed crosslinking. *Journal of Biomaterials Science, Polymer Edition*, *30*(4), 295–307. Available from https://doi.org/10.1080/09205063.2018.1562637.

Kim, S. H., An, Y. H., Kim, H. D., Kim, K., Lee, S. H., Yim, H. G., Kim, B. G., & Hwang, N. S. (2018). Enzyme-mediated tissue adhesive hydrogels for meniscus repair. *International Journal of Biological Macromolecules*, *110*, 479–487. Available from https://doi.org/10.1016/j.ijbiomac.2017.12.053.

Kobayashi, S., & Uyama, H. (2011). Enzymatic polymerization, In *Encyclopedia of polymer science and technology*, *Vol. 2*, 328–364. Available from https://doi.org/10.1002/0471440264.pst118.pub2.

Le Thi, P., Lee, Y., Nguyen, D. H., & Park, K. D. (2017). In situ forming gelatin hydrogels by dual-enzymatic cross-linking for enhanced tissue adhesiveness. *Journal of Materials Chemistry B*, *5*(4), 757–764. Available from https://doi.org/10.1039/c6tb02179d.

Lee, F., Bae, K. H., & Kurisawa, M. (2015). Injectable hydrogel systems crosslinked by horseradish peroxidase. *Biomedical Materials (Bristol)*, *11*(1). Available from https://doi.org/10.1088/1748-6041/11/1/014101.

Li, L., Bae, K. H., Ng, S., Yamashita, A., & Kurisawa, M. (2018a). Peroxidase-immobilized porous silica particles for in situ formation of peroxidase-free hydrogels with attenuated immune responses. *Acta Biomaterialia*, *81*, 103–114. Available from https://doi.org/10.1016/j.actbio.2018.09.054.

Li, T., Song, X., Weng, C., Wang, X., Wu, J., Sun, L., Gong, X., Zeng, W. N., Yang, L., & Chen, C. (2018b). Enzymatically crosslinked and mechanically tunable silk fibroin/pullulan hydrogels for mesenchymal stem cells delivery. *International Journal of Biological Macromolecules*, *115*, 300–307. Available from https://doi.org/10.1016/j.ijbiomac.2018.04.046.

Li, Y., & Cirino, P. C. (2014). Recent advances in engineering proteins for biocatalysis. *Biotechnology and Bioengineering*, *111*(7), 1273–1287. Available from https://doi.org/10.1002/bit.25240.

Lim, T. C., Toh, W. S., Wang, L. S., Kurisawa, M., & Spector, M. (2012). The effect of injectable gelatin-hydroxyphenylpropionic acid hydrogel matrices on the proliferation, migration, differentiation and oxidative stress resistance of adult neural stem cells. *Biomaterials*, *33*(12), 3446–3455. Available from https://doi.org/10.1016/j.biomaterials.2012.01.037.

McDonald, A. G., Boyce, S., & Tipton, K. F. (2009). ExplorEnz: the primary source of the IUBMB enzyme list. *Nucleic Acids Research*, *37*(1), D593–D597. Available from https://doi.org/10.1093/nar/gkn582.

Milleret, V., Simona, B. R., Lienemann, P. S., Vörös, J., & Ehrbar, M. (2014). Electrochemical control of the enzymatic polymerization of PEG hydrogels: Formation of spatially controlled biological microenvironments. *Advanced Healthcare Materials*, *3*(4), 508–514. Available from https://doi.org/10.1002/adhm.201300479.

Mohammed, J. S., & Murphy, W. L. (2009). Bioinspired design of dynamic materials. *Advanced Materials*, *21*(23), 2361–2374. Available from https://doi.org/10.1002/adma.200803785.

Moreira Teixeira, L. S., Feijen, J., van Blitterswijk, C. A., Dijkstra, P. J., & Karperien, M. (2012). Enzyme-catalyzed crosslinkable hydrogels: Emerging strategies for tissue engineering. *Biomaterials*, *33*(5), 1281–1290. Available from https://doi.org/10.1016/j.biomaterials.2011.10.067.

Morelli, A., Betti, M., Puppi, D., Bartoli, C., Gazzarri, M., & Chiellini, F. (2016). Enzymatically crosslinked ulvan hydrogels as injectable systems for cell delivery. *Macromolecular Chemistry and Physics*, *217*(4), 581–590. Available from https://doi.org/10.1002/macp.201500353.

Nezhad-Mokhtari, P., Ghorbani, M., Roshangar, L., & Soleimani Rad, J. (2019). Chemical gelling of hydrogels-based biological macromolecules for tissue engineering: Photo- and enzymatic-crosslinking methods. *International Journal of Biological Macromolecules*, *139*, 760–772. Available from https://doi.org/10.1016/j.ijbiomac.2019.08.047.

Niu, C., Li, X., Wang, Y., Liu, X., Shi, J., & Wang, X. (2019). Design and performance of a poly (vinyl alcohol)/silk fibroin enzymatically crosslinked semi-interpenetrating hydrogel for a potential hydrophobic drug delivery. *RSC Advances*, *9*(70), 41074–41082. Available from https://doi.org/10.1039/c9ra09344c.

Parenteau-Bareil, R., Gauvin, R., & Berthod, F. (2010). Collagen-based biomaterials for tissue engineering applications. *Materials*, *3*(3), 1863–1887. Available from https://doi.org/10.3390/ma3031863.

Raia, N. R., Partlow, B. P., McGill, M., Kimmerling, E. P., Ghezzi, C. E., & Kaplan, D. L. (2017). Enzymatically crosslinked silk-hyaluronic acid hydrogels. *Biomaterials*, *131*, 58–67. Available from https://doi.org/10.1016/j.biomaterials.2017.03.046.

Ren, K., Li, B., Xu, Q., Xiao, C., He, C., Li, G., & Chen, X. (2017). Enzymatically crosslinked hydrogels based on linear poly(ethylene glycol) polymer: Performance and mechanism. *Polymer Chemistry*, *8*(45), 7017–7024. Available from https://doi.org/10.1039/c7py01597f.

Ribeiro, V. P., Pina, S., Costa, J. B., Cengiz, I. F., García-Fernández, L., Fernández-Gutiérrez, M. D. M., Paiva, O. C., Oliveira, A. L., San-Román, J., Oliveira, J. M., & Reis, R. L. (2019). Enzymatically cross-linked silk fibroin-based hierarchical scaffolds for osteochondral regeneration. *ACS Applied Materials and Interfaces*, *11*(4), 3781–3799. Available from https://doi.org/10.1021/acsami.8b21259.

Roberts, J. J., Naudiyal, P., Lim, K. S., Poole-Warren, L. A., & Martens, P. J. (2016). A comparative study of enzyme initiators for crosslinking phenol-functionalized hydrogels for cell encapsulation. *Biomaterials Research*, 20. Available from https://doi.org/10.1186/s40824-016-0077-z.

Shen, S., Shen, J., Shen, H., Wu, C., Chen, P., & Wang, Q. (2020). Dual-enzyme crosslinking and post-polymerization for printing of polysaccharide-polymer hydrogel. *Frontiers in Chemistry*, 8. Available from https://doi.org/10.3389/fchem.2020.00036.

Sperinde, J. J., & Griffith, L. G. (1997). Synthesis and characterization of enzymatically-cross-linked poly (ethylene glycol) hydrogels. *Macromolecules*, *30*(18), 5255–5264. Available from https://doi.org/10.1021/ma970345a.

Thakur, G., Rodrigues, F. C., & Singh, K. (2018). Crosslinking biopolymers for advanced drug delivery and tissue engineering applications. *Advances in experimental medicine and biology* (1078, pp. 213–231). New York LLC: Springer. Available from https://doi.org/10.1007/978-981-13-0950-2_11.

Toh, W. S., Lim, T. C., Kurisawa, M., & Spector, M. (2012). Modulation of mesenchymal stem cell chondrogenesis in a tunable hyaluronic acid hydrogel microenvironment. *Biomaterials*, *33*(15), 3835–3845. Available from https://doi.org/10.1016/j.biomaterials.2012.01.065.

Tsai, C.-C., Kuo, S.-H., Lu, T.-Y., Cheng, N.-C., Shie, M.-Y., & Yu, J. (2020). Enzyme-Cross-linked Gelatin Hydrogel Enriched with an Articular Cartilage Extracellular Matrix and Human Adipose-Derived Stem Cells for Hyaline Cartilage Regeneration of Rabbits. *ACS Biomaterials Science and Engineering*, *6*(9), 5110–5119. Available from https://doi.org/10.1021/acsbiomaterials.9b01756.

van Loo, B., Salehi, S. S., Henke, S., Shamloo, A., Kamperman, T., Karperien, M., & Leijten, J. (2020). Enzymatic outside-in cross-linking enables single-step microcapsule production for high-throughput three-dimensional cell microaggregate formation. *Materials Today Bio*, 6. Available from https://doi.org/10.1016/j.mtbio.2020.100047.

Wang, C., Fischer, A., Ehrlich, A., Nahmias, Y., & Willner, I. (2020a). Biocatalytic reversible control of the stiffness of DNA-modified responsive hydrogels: Applications in shape-memory, self-healing and autonomous controlled release of insulin. *Chemical Science*, *11*(17), 4516–4524. Available from https://doi.org/10.1039/d0sc01319f.

Wang, R., Leber, N., Buhl, C., Verdonschot, N., Dijkstra, P. J., & Karperien, M. (2014). Cartilage adhesive and mechanical properties of enzymatically crosslinked polysaccharide tyramine conjugate hydrogels. *Polymers for Advanced Technologies*, 568–574. Available from https://doi.org/10.1002/pat.3286.

Wang, L. S., Lee, F., Lim, J., Du, C., Wan, A. C. A., Lee, S. S., & Kurisawa, M. (2014). Enzymatic conjugation of a bioactive peptide into an injectable hyaluronic acid-tyramine hydrogel system to promote the formation of functional vasculature. *Acta Biomaterialia*, *10*(6), 2539–2550. Available from https://doi.org/10.1016/j.actbio.2014.02.022.

Wang, L., Li, J., Zhang, D., Ma, S., Zhang, J., Gao, F., Guan, F., & Yao, M. (2020b). Dual-enzymatically crosslinked and injectable hyaluronic acid hydrogels for potential application in tissue engineering. *RSC Advances*, *10*(5), 2870–2876. Available from https://doi.org/10.1039/c9ra09531d.

Wong, S. S., & Jameson, D. M. (2011). Chemistry of protein and nucleic acid cross-linking and conjugation. *Chemistry of protein and nucleic acid cross-linking and conjugation* (second edition, pp. 1–586). CRC Press. Available from https://www.crcpress.com/Chemistry-of-Protein-and-Nucleic-Acid-Cross-Linking-and-Conjugation-Second/Wong-Jameson/p/book/9780849374913.

Yang, G., Xiao, Z., Ren, X., Long, H., Qian, H., Ma, K., & Guo, Y. (2016). Enzymatically crosslinked gelatin hydrogel promotes the proliferation of adipose tissue-derived stromal cells. *PeerJ*, *2016*(9). Available from https://doi.org/10.7717/peerj.2497.

Zhang, Y., Fan, Z., Xu, C., Fang, S., Liu, X., & Li, X. (2015). Tough biohydrogels with interpenetrating network structure by bienzymatic crosslinking approach. *European Polymer Journal*, *72*, 717–725. Available from https://doi.org/10.1016/j.eurpolymj.2014.12.038.

Zhu, C., Lei, H., Fan, D., Duan, Z., Li, X., Li, Y., Cao, J., Wang, S., & Yu, Y. (2018). Novel enzymatic crosslinked hydrogels that mimic extracellular matrix for skin wound healing. *Journal of Materials Science*, *53*(8), 5909–5928. Available from https://doi.org/10.1007/s10853-017-1956-y.

Ztürk, E., Stauber, T., Levinson, C., Cavalli, E., Arlov, O., & Zenobi-Wong, M. (2020). Tyrosinase-crosslinked, tissue adhesive and biomimetic alginate sulfate hydrogels for cartilage repair. *Biomedical Materials (Bristol)*, *15*(4). Available from https://doi.org/10.1088/1748-605X/ab8318.