Enzyme Crosslinking Approaches for Bone Tissue Engineering

Subjects: Materials Science, Biomaterials

Contributor: Ankur Sood, Seong Min Ji, Anuj Kumar, Sung Soo Han

Crosslinking strategies have been extensively explored in order to design novel hydrogels for bone tissue engineering. Lately, the fabrication of hydrogels with the help of enzyme-mediated crosslinking approaches has been extensively explored. This approach has resulted in promising outcomes with convincing prospects. Enzymes are required in minimal quantity and are very efficient in their actions, as they increase the reaction rate without being expended during the course of the reaction process. The efficiency of an enzyme is defined by the number of substrate molecules converted into products per unit of enzyme, which is also known as turnover number (k cat). The high efficiency of enzyme-based reactions comes from the precise specificity, which ensures the conversion of a particular type of substrate to products. Many enzymes have been explored in order to prepare biomimetic hydrogels for bone tissue engineering. The details of every enzyme-based crosslinking approach are discussed in the following sections.

Keywords: crosslinking ; enzyme ; hydrogels ; polymers

1. Tyrosinase

Of all the other enzymes, tyrosinase has been most often explored for various tissue engineering applications, including bone tissue engineering. In the case of mammals, the tyrosinase is located in melanosome, which manufactures melanin [1]. The mechanism of the action of tyrosinase is based on the oxidation of the phenolic groups (tyrosine, catechol, and polyphenols) present in their active sites, without the prerequisite of any cofactors. Due to the abundance of these phenolic groups in human proteins, they can be easily conjugated to hydrogels, making them efficient players in crosslinking strategies [2][3] (**Figure 1**). Moreover, the mechanism of the oxidation reaction of tyrosinase strongly resembles the cascade of events in the 3,4-dihydroxy-l-alanine (DOPA) induced mussel adhesion on sea rocks in mussel foot protein (Mfp), which has been extensively explored in tissue engineering and regenerative medicines [4].

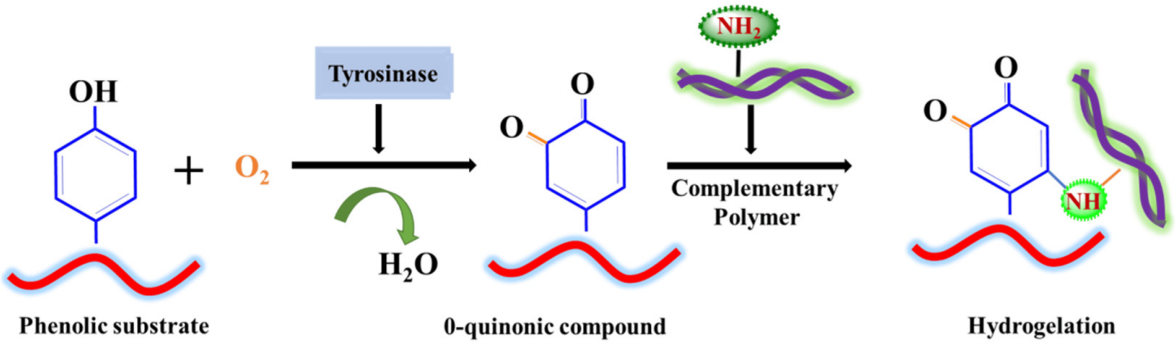

Figure 1. Mechanism of action of tyrosinase.

Inspired by mussels, Sousa et al. fabricated a dopamine moiety on the surface of hyaluronic acid to develop freestanding multilayer membranes using layer by layer technology to enhance the cell adhesion and interaction between the construct and cells [5]. The mechanical attributes and enhanced adhesion were examined and found to be optimal for bone tissue engineering. Further, Mishra et al. demonstrated the manufacturing of injectable ink, comprising of carboxymethyl-chitosan (CMC)/gelatin/nano-hydroxyapatite (nHAp) via tyrosinase/p-cresol-mediated in situ crosslinking [6]. The study highlighted the degree of crosslinking as a prime factor to uncover the differentiation and proliferation of osteoblast cells along with the stability of in situ formed gels in vivo. In another study, Sharma et al. fabricated tyrosinase-crosslinked silk fibroin and gelatin-mixed hydrogel doped with calcium ions. The study aimed to investigate the rheological and calcium releasing behavior of the fabricated hydrogel in bone tissue regeneration. The work also highlighted the ability of the designed hydrogels in augmenting the osteogenic differentiation of human bone marrow-derived mesenchymal stromal

cells [7]. A similar strategy was explored by Chameettachal et al. to fabricate tyrosinase-crosslinked silk fibroin and gelatin hydrogels to explore the chondrogenic differentiation and suppression of hypertrophic differentiation across different cell modalities for chondrocytes and mesenchymal progenitor cells (hMSCs) [8]. The combination of silk fibroin and gelatin has been reported by other research group for bone tissue engineering [9]. In another study, tyrosinase-mediated chitosan/gelatin and chitosan/gelatin/nanohydroxyapatite hydrogels were synthesized for bone tissue engineering [10]. Rapid and permanent gelation was the hallmark of this work. Here, derivatized tyrosinase (mTyr-CNK)) was engineered to exhibit a high catalytic efficiency for tyrosine/DOPA-tethered hydrogels across a broad pH range. The obtained hydrogels exhibited good porosity with high swelling ratios. Further, in order to enhance the interfacial adhesion of an osteo-mucosal construct, a study reported tyrosinase-based adhesion approach was presented that resulted in healthy tissue regeneration along with the enhanced adhesion for the soft/hard components of designed constructs [11]. The data obtained from the study confirmed the enhanced proliferation of osteoblast cells via aminolysis and improved osteoblast cells differentiation via tyrosinase present in collagen. Furthermore, the evidence of multilayered epithelium on the osteo-mucosal model with viable fibroblasts and osteoblasts was also demonstrated. The fabricated construct could find clinical implications as a graft material in surgeries and can serve as an in vitro model to investigate the applicability of dental materials. All these studies reflect the elevated adhesion capacity of tyrosinase. The gentle and fast crosslinking process of tyrosinase-based crosslinking in an aqueous environment further aids in its widespread utility in bone tissue engineering.

2. Lysozyme

Lysozyme is a naturally occurring protein belonging to the class of glycoside hydrolase and unanimously appears in diverse human tissues, including tears, saliva, and other body secretions [12]. Lysozyme plays a significant role in the innate protection system [13] and also exhibits antiviral, antiseptic, and anti-inflammatory features, making it a key aspirant in the pharmaceutical sector [14][15]. It is stable across various pH ranging from 5–9 and demonstrates a stable three-dimensional (3D) structure, make it a suitable candidate remodeling tissue microenvironment [16][17]. The approach in case of lysozyme crosslinking is presented in **Figure 2**. Lysozyme and its mutants have been used as crosslinking agents to synthesize different hydrogels for bone tissue engineering. In a study by Chen et al., a chitosan oligosaccharide-based hydrogel was prepared by crosslinking it with the help of T4 lysozyme mutant (T4M) [4]. The surface of T4M is rich in free amine groups, which function as efficient covalent crosslinkers, imparting good strength to the hydrogel network along with high specificity towards the binding of multivalent cations. This property was explored to exhibit localized delivery and the synergistic release of Mg^{2+} and Zn^{2+} for bone tissue repairment. Further, in order to improve the cellular affinity and rapid tissue regeneration, the integrin receptor-binding Arg-Gly-Asp (RGD) sequence was attached to the C-terminus of T4M. In another study, lysozyme-crosslinked 4-arm-PEG hydrogel was developed as a surgical sealant for tissue engineering [18]. The developed hydrogel could provide adequate mechanical stability responsible to endure high pressure. The in-situ formation of sealant to avoid fluid leakage is very important in medical sciences and can be utilized across many biomedical applications. The formation of hydrogels via lysozyme-based crosslinking is still under active exploration and has great potential. Rationally engineered lysozyme mutants have been reported to have enormous effect on hydrogel strength along with their enhanced cellular responses. The development of these variants could enhance the stability across a wide range of pH.

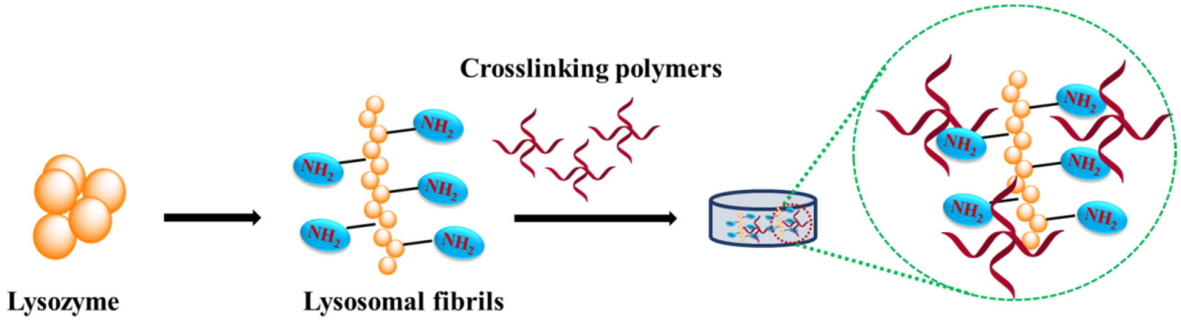

Figure 2. Approach towards lysozyme crosslinking for hydrogel formation.

3. Horseradish Peroxidase

Horseradish peroxidase (HRP) is a member of the peroxidase family and is known to be oxidoreductase with hydroperoxide as electron acceptor. HRP is a heme containing enzymes derived from horseradish roots and has a molecular weight of 40,000. HRP are commercially available in high purity form making it a representative system to investigate the structure, dynamic, and thermodynamic properties of peroxidases [19]. Several HRP isoenzymes have

been reported with the most abundant and widely characterized C isoenzyme [20]. HRP crosslinked hydrogel have been widely reported as suitable for tissue engineering applications. HRP are capable of catalyzing the oxidative coupling of polymers-phenols in the company of hydrogen peroxide (H_2O_2), resulting in the formation of hydrogels with the tunable rate of gelation and crosslinking density [21] (**Figure 3**). The application of HRP has also been studied in tissue engineering; for the first time, Carnes et al. examined the HRP-catalyzed dityrosine-crosslinking of a fibrin scaffold for tissue engineering applications [22]. The work reported the effect of the concentration of HRP and H_2O_2 on the crosslinking density of fibrin microthreads, targeting its utility across a broad range of tissues for structural stability and mechanical strength. Further, Shoji et al. reported the in-situ formation of hyaluronic acid and tyramine-based hydrogel (HA-T) via taking the advantage of HRP in presence of H_2O_2 [23]. The fabricated hydrogel was loaded with bone morphogenetic protein (BMP)-2 and was examined for their ability to promote osteogenesis in a in vivo model. The study reported considerably greater bone volume, bone mineral content, and bone union at the fracture sites upon introduction with BMP-2-loaded HA-T hydrogel compared with the fractured sites with no treatment received.

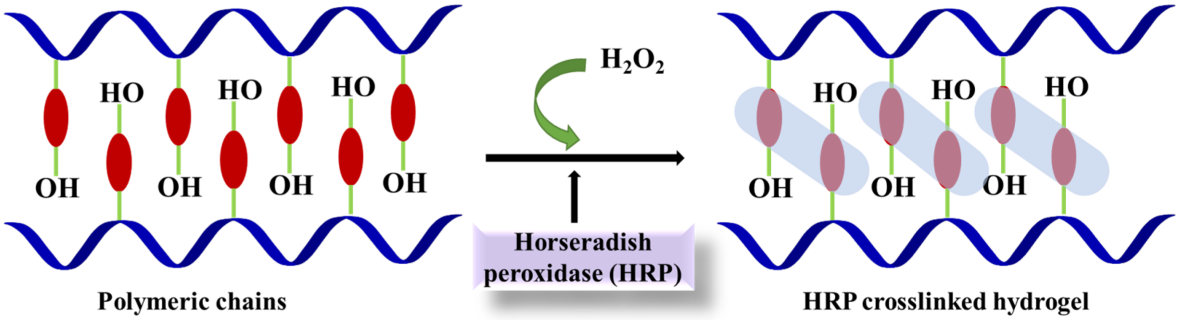

Figure 3. Mechanism of crosslinking via horseradish peroxidase for hydrogel formation.

The loading and controlled release of bioactive agents, such as proteins and peptides, into hydrogels has been popular approaches in order to progress in the regenerative capacity of bone. In this regard, the research group of Park et al. synthesized bioactive calcium-accumulating peptide (CAP) comprising a collagen-binding design to accelerate osteogenic differentiation. Here, a gelatin-based hydroxyphenyl propionic acid hydrogel was fabricated with the help of an HRP-crosslinking approach in the presence of H_2O_2. Finally, CAP was chemically conjugated to the surface of the hydrogel. It was demonstrated that the presence of CAP-tagged hydrogel loaded with human periodontal ligament stem cells (PDLSCs) induced bone mineralization around the PDLSCs and also increased the expressions of the osteogenic markers at the in vitro level. The fabricated hydrogel system was able to recover a bone layer in a calvarial defect four weeks post-implantation [24]. Further, silk fibroin (SF)-based hydrogel has gained the considerable interest of researchers globally as a material of choice for tissue engineering applications. In this regard, Hasturk et al. fabricated SF which was enzymatically crosslinked with tyramine-substituted silk fibroin (SF-TA) or gelatin (G-TA)-hybrid hydrogels with the help of HRP and H_2O_2 [25]. Here, the manufactured hydrogel was chemically conjugated with RGD peptides. This approach gives precise control over the gelation of hydrogel along with adequate mechanical properties and bioactivity. In another study, the hydrogels of tyramine-modified gellan gum (Ty-GG) was manufactured by exploring both the physical and chemical method of crosslinking in the presence of HRP and H_2O_2 [26]. Here, the fabricated hydrogel was loaded with betamethasone, a potent drug to treat patients with rheumatoid arthritis. The sustained release of betamethasone in vitro, along with proliferation and inflammatory activity was also studied on chondrogenic primary cells and THP-1 cells. All these studies have presented a valuable approach for using HRP-based enzymatic crosslinking in the presence of H_2O_2 in order to synthesize in situ injectable hydrogels. A fast gelation rate along with control over the crosslinking density are some keynote features making HRP/H_2O_2 an attractive method for hydrogel crosslinking.

4. Transglutaminase (TG)

Transglutaminases, Ca^{2+}-dependent acyl transferases, belong to the family of enzymes (EC 2.3.2.13) and are regarded as mild alternatives for chemical crosslinking strategies that catalyze the establishment of an amide bond between the γ-carbonyl and ε-amino groups of glutamine and lysine residues, respectively [27] (**Figure 4**). These enzymes have also been reported to contain an active site thiol group within a cysteine/histidine/aspartic acid (Cys-His-Asp) catalytic triad [28]. TG has been widely explored in enzyme-based crosslinkers for a wide range of polymers (such as gelatin, collagen and so on) and proteins [29][30][31]. In fact, transglutaminase is emphasized as the best studied enzyme for protein-based hydrogel formation via enzymatic crosslinking for tissue engineering applications [32]. The adhesion supremacy and strong integration between the TG driven, in situ-formed hydrogel and the host tissue architect makes it a potent choice in for tissue regeneration process.

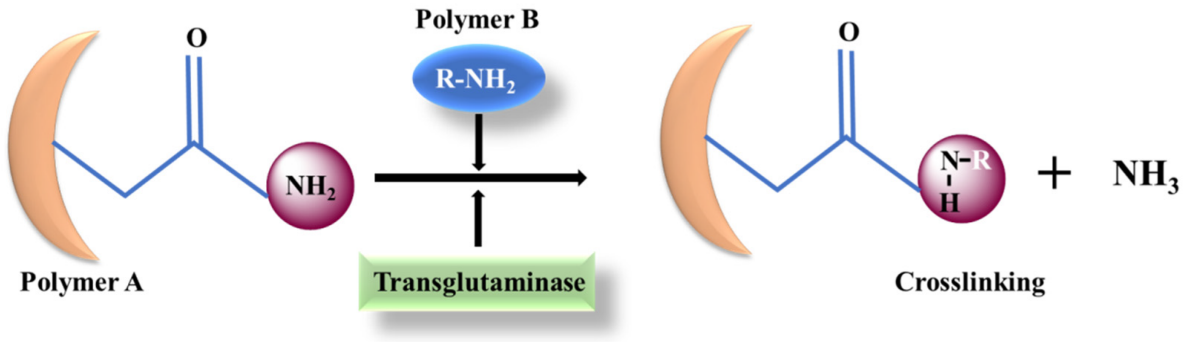

Figure 4. Mechanism of action of transglutaminase-mediated crosslinking strategy for hydrogel formation.

TG-crosslinked hydrogels have also been explored as implant coatings for the slow releasing of pharmaceutical agents. In this regard, Sun et al. fabricated TG-crosslinked gelatin-alginate hydrogel loaded with antibiotics (vancomycin) to prevent bacterial infection via the sustained release of vancomycin in tissue implants [33]. The study emphasized the release kinetics of loaded molecules as a function of the concentration of the enzymes used, as it is directly related to the degree of crosslinking. Further, the osteogenic potential of TG-crosslinked gelatin/hyaluronan in the presence of biotechnological chondroitin was explored by La Gatta et al. [34]. Herein, a semi-interpenetrating gel was formed with high stability, improved stiffness, and lower swelling extent compared to native gelatin hydrogel. The effect of formed hydrogel on bone regeneration was evaluated via the assessment of the osteogenic differentiation by seeding the hydrogel with human dental pulp stem cells. Over a period of 30 days, the upregulation of the expression of both osteocalcin and osteopontin at gene and protein level was observed.

The understanding of the interplay between the bone marrow microenvironment and resident cells is very important and has been explored as a vital tool with substantial clinical value [35][36][37]. Fundamentally, the biophysical and biochemical aspects across the bone marrow niche play a profound role in the complete functioning of the organ [38]. In this regard, Vallmajo-Martin et al. fabricated a TG-crosslinked hybrid hydrogel system comprising poly (ethylene glycol) (PEG) and hyaluronic acid (HA) for the formation of bone marrow analogues [39]. Herein, it was demonstrated that the fabricated hybrid hydrogel was able to maintain, inflate, and differentiate human bone marrow-derived stromal cells and human hematopoietic stem and progenitor cells in vitro. This hydrogel could serve as an ideal scaffold for various tissue engineering applications. For an ideal hydrogel, the concentration of the initial molecules and the enzyme is very crucial, and its ratio will decide the degree of crosslinking and the crosslinking density, as both factors are very critical in the formation of scaffolds for tissue engineering applications.

5. Alkaline Phosphatase (ALP)

ALP plays a crucial role in the mineralization of bone and works by cleaving phosphate from organic phosphate [40]. **Figure 5** represents its mechanism of action, where the hydrolysis of phosphate occurs upon the action of ALP in the presence of organic phosphate group.

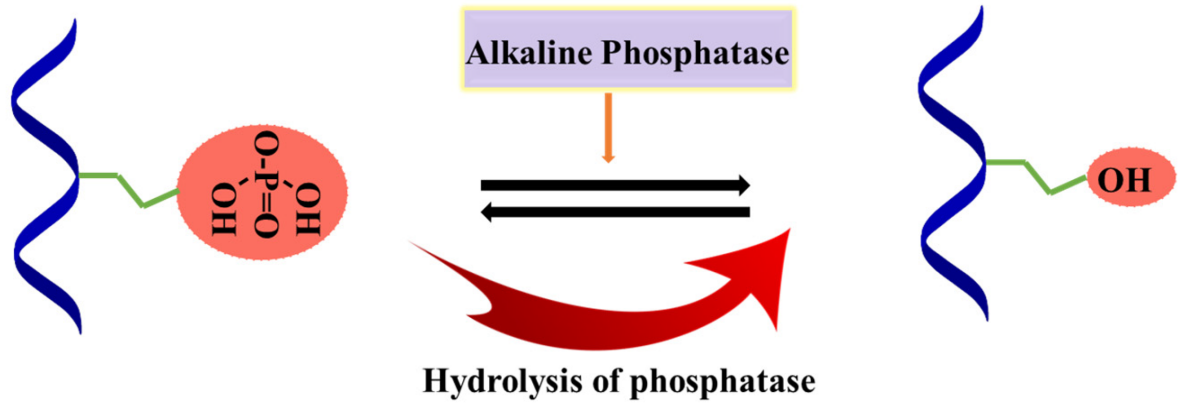

Figure 5. Cleavage of phosphate group from organic phosphate in presence of ALP.

Owing to their participation in the mineralization of skeletal tissues, ALP has been explored as an outstanding supplier of the phosphatase for hydrogel formation [41]. In the field of bone tissue engineering, the utility of ALP-crosslinked hydrogels is to encourage homogeneity in the mineralization process of hydrogels, which in turn is crucial for imparting the mechanical strength or bestow them more suitability in the area of bone replacements. In this direction, Douglas et al.

fabricated three ALP-crosslinked hydrogel systems, namely, mussel protein-inspired catechol-PEG (cPEG), type I collagen, and oligo PEG fumarate (OPF), for bone tissue replacements and to induce mineralization via calcium phosphate (CaP) [42]. Herein, it was to inspect the retention power of ALP in these hydrogels along with the initiation of its mineralization. Additionally, the nature and amount of the mineral formed in the hydrogel systems along with the effect of mineralization on the morphology and mechanical stability of the synthesized hydrogels were also investigated.

Up recently, supramolecular hydrogels have gained substantial attraction in the field of tissue engineering, cancer therapy, and drug delivery [43]. In this line, Yuan et al. fabricated an ALP-crosslinked hydrogel series using the self-assembly capability of the core segment (GNNQQNY) of the yeast prion Sup35 [44]. The study highlighted the ability of ALP to promote the functioning of precursors, such as hydrogelator, which then self-assembles in an aqueous environment to form nanofibers (width ≤ 10 nm). It could be implemented in bone tissue engineering to promote self-assemblies-based hybrid hydrogels. In order to fabricate and efficient hybrid hydrogel system using ALP-crosslinking, the ratio of the formed minerals and the utilized polymer networks is extremely critical, along with the ALP concentration-dependent mineralization rate. Optimal conditions, together with the thorough regulation of governing parameters, could provide a hybrid hydrogel to induce and accelerate bone mineralization, which serves as an alternative to including calcium phosphate in the designed system.

The development and clinical translational aspect of bone tissue engineering requires the careful administration of encapsulated biomolecules/pharmaceutical excipients inside the hydrogel scaffolds. In order to achieve this, regulation in the pore size, pore arrangement and pore capacity of the designed hydrogel plays a substantial role. The design of hydrogel plays a vital role in regulating the cell attachment and its proliferation. Researchers have also emphasized the accountability of the functionally graded scaffold (FGS) and non-functionally graded scaffold (NFGS) as functions of computational dynamics for permeability analysis [45][46]. A crosstalk between different fields, including but not limited to computational science, materials sciences, nanotechnology, tissue engineering, etc., provides an innovative and comprehensive approach to envisage our vision and understanding in the vast field of science.

References

1. Choi, S.; Ahn, H.; Kim, S.-H. Tyrosinase-mediated hydrogel crosslinking for tissue engineering. J. Appl. Polym. Sci. 2022, 139, 51887.
2. Song, W.; Ko, J.; Choi, Y.H.; Hwang, N.S. Recent advancements in enzyme-mediated crosslinkable hydrogels: In vivo-mimicking strategies. APL Bioeng. 2021, 5, 021502.
3. Chen, T.; Embree, H.D.; Brown, E.M.; Taylor, M.M.; Payne, G.F. Enzyme-catalyzed gel formation of gelatin and chitosan: Potential for in situ applications. Biomaterials 2003, 24, 2831–2841.
4. Chen, S.; Gil, C.J.; Ning, L.; Jin, L.; Perez, L.; Kabboul, G.; Tomov, M.L.; Serpooshan, V. Adhesive Tissue Engineered Scaffolds: Mechanisms and Applications. Front. Bioeng. Biotechnol. 2021, 9, 683079.
5. Sousa, M.P.; Mano, J.F. Cell-Adhesive Bioinspired and Catechol-Based Multilayer Freestanding Membranes for Bone Tissue Engineering. Biomimetics 2017, 2, 19.
6. Mishra, D.; Bhunia, B.; Banerjee, I.; Datta, P.; Dhara, S.; Maiti, T.K. Enzymatically crosslinked carboxymethyl–chitosan/gelatin/nano-hydroxyapatite injectable gels for in situ bone tissue engineering application. Mater. Sci. Eng. C 2011, 31, 1295–1304.
7. Sharma, A.; Desando, G.; Petretta, M.; Chawla, S.; Bartolotti, I.; Manferdini, C.; Paolella, F.; Gabusi, E.; Trucco, D.; Ghosh, S.; et al. Investigating the Role of Sustained Calcium Release in Silk-Gelatin-Based Three-Dimensional Bioprinted Constructs for Enhancing the Osteogenic Differentiation of Human Bone Marrow Derived Mesenchymal Stromal Cells. ACS Biomater. Sci. Eng. 2019, 5, 1518–1533.
8. Chameettachal, S.; Midha, S.; Ghosh, S. Regulation of Chondrogenesis and Hypertrophy in Silk Fibroin-Gelatin-Based 3D Bioprinted Constructs. ACS Biomater. Sci. Eng. 2016, 2, 1450–1463.
9. Das, S.; Pati, F.; Chameettachal, S.; Pahwa, S.; Ray, A.R.; Dhara, S.; Ghosh, S. Enhanced Redifferentiation of Chondrocytes on Microperiodic Silk/Gelatin Scaffolds: Toward Tailor-Made Tissue Engineering. Biomacromolecules 2013, 14, 311–321.
10. Lim, S.; Jeong, D.; Ki, M.-R.; Pack, S.P.; Choi, Y.S. Tyrosinase-mediated rapid and permanent chitosan/gelatin and chitosan/gelatin/nanohydroxyapatite hydrogel. Korean J. Chem. Eng. 2021, 38, 98–103.
11. Tabatabaei, F.; Rasoulianboroujeni, M.; Yadegari, A.; Tajik, S.; Moharamzadeh, K.; Tayebi, L. Osteo-mucosal engineered construct: In situ adhesion of hard-soft tissues. Mater. Sci. Eng. C 2021, 128, 112255.

12. Pangburn, S.H.; Trescony, P.V.; Heller, J. Lysozyme degradation of partially deacetylated chitin, its films and hydrogels. Biomaterials 1982, 3, 105–108.

13. Chen, X.; Niyonsaba, F.; Ushio, H.; Okuda, D.; Nagaoka, I.; Ikeda, S.; Okumura, K.; Ogawa, H. Synergistic effect of antibacterial agents human beta-defensins, cathelicidin LL-37 and lysozyme against Staphylococcus aureus and Escherichia coli. J. Dermatol. Sci. 2005, 40, 123–132.

14. Wu, T.; Wu, C.; Fu, S.; Wang, L.; Yuan, C.; Chen, S.; Hu, Y. Integration of lysozyme into chitosan nanoparticles for improving antibacterial activity. Carbohydr. Polym. 2017, 155, 192–200.

15. Kim, S.; Fan, J.; Lee, C.S.; Lee, M. Dual Functional Lysozyme-Chitosan Conjugate for Tunable Degradation and Antibacterial Activity. ACS Appl. Bio. Mater. 2020, 3, 2334–2343.

16. Kim, S.; Cui, Z.K.; Koo, B.; Zheng, J.; Aghaloo, T.; Lee, M. Chitosan-Lysozyme Conjugates for Enzyme-Triggered Hydrogel Degradation in Tissue Engineering Applications. ACS Appl. Mater. Interfaces 2018, 10, 41138–41145.

17. Proctor, V.A.; Cunningham, F.E. The chemistry of lysozyme and its use as a food preservative and a pharmaceutical. Crit. Rev. Food Sci. Nutr. 1988, 26, 359–395.

18. Tan, H.; Jin, D.; Qu, X.; Liu, H.; Chen, X.; Yin, M.; Liu, C. A PEG-Lysozyme hydrogel harvests multiple functions as a fit-to-shape tissue sealant for internal-use of body. Biomaterials 2019, 192, 392–404.

19. Hu, S.; Lu, Q.; Xu, Y. Biosensors based on direct electron transfer of protein. In Electrochemical Sensors, Biosensors and Their Biomedical Applications; Zhang, X., Ju, H., Wang, J., Eds.; Academic Press: San Diego, CA, USA, 2008; pp. 531–581.

20. Veitch, N.C. Horseradish peroxidase: A modern view of a classic enzyme. Phytochemistry 2004, 65, 249–259.

21. Lee, F.; Bae, K.H.; Kurisawa, M. Injectable hydrogel systems crosslinked by horseradish peroxidase. Biomed. Mater. 2015, 11, 014101.

22. Carnes, M.E.; Gonyea, C.R.; Mooney, R.G.; Njihia, J.W.; Coburn, J.M.; Pins, G.D. Horseradish Peroxidase-Catalyzed Crosslinking of Fibrin Microthread Scaffolds. Tissue Eng. Part C Methods 2020, 26, 317–331.

23. Shoji, S.; Uchida, K.; Satio, W.; Sekiguchi, H.; Inoue, G.; Miyagi, M.; Takata, K.; Yokozeki, Y.; Takaso, M. Acceleration of bone union by in situ-formed hydrogel containing bone morphogenetic protein-2 in a mouse refractory fracture model. J. Orthop. Surg. Res 2020, 15, 426.

24. Jo, B.S.; Lee, Y.; Suh, J.S.; Park, Y.S.; Lee, H.J.; Lee, J.-Y.; Cho, J.; Lee, G.; Chung, C.P.; Park, K.D.; et al. A novel calcium-accumulating peptide/gelatin in situ forming hydrogel for enhanced bone regeneration. J. Biomed. Mater. Res. Part A 2018, 106, 531–542.

25. Hasturk, O.; Jordan, K.E.; Choi, J.; Kaplan, D.L. Enzymatically crosslinked silk and silk-gelatin hydrogels with tunable gelation kinetics, mechanical properties and bioactivity for cell culture and encapsulation. Biomaterials 2020, 232, 119720.

26. Oliveira, I.M.; Gonçalves, C.; Shin, M.E.; Lee, S.; Reis, R.L.; Khang, G.; Oliveira, J.M. Enzymatically crosslinked tyramine-gellan gum hydrogels as drug delivery system for rheumatoid arthritis treatment. Drug Deliv. Transl. Res. 2021, 11, 1288–1300.

27. Yokoyama, K.; Nio, N.; Kikuchi, Y. Properties and applications of microbial transglutaminase. Appl. Microbiol. Biotechnol. 2004, 64, 447–454.

28. Savoca, M.P.; Tonoli, E.; Atobatele, A.G.; Verderio, E.A.M. Biocatalysis by Transglutaminases: A Review of Biotechnological Applications. Micromachines 2018, 9, 562.

29. Kuwahara, K.; Yang, Z.; Slack, G.C.; Nimni, M.E.; Han, B. Cell delivery using an injectable and adhesive transglutaminase-gelatin gel. Tissue Eng. Part C Methods 2010, 16, 609–618.

30. Spurlin, T.A.; Bhadriraju, K.; Chung, K.H.; Tona, A.; Plant, A.L. The treatment of collagen fibrils by tissue transglutaminase to promote vascular smooth muscle cell contractile signaling. Biomaterials 2009, 30, 5486–5496.

31. Yang, G.; Xiao, Z.; Ren, X.; Long, H.; Qian, H.; Ma, K.; Guo, Y. Enzymatically crosslinked gelatin hydrogel promotes the proliferation of adipose tissue-derived stromal cells. PeerJ 2016, 4, e2497.

32. Teixeira, L.S.; Feijen, J.; van Blitterswijk, C.A.; Dijkstra, P.J.; Karperien, M. Enzyme-catalyzed crosslinkable hydrogels: Emerging strategies for tissue engineering. Biomaterials 2012, 33, 1281–1290.

33. Sun, C.-K.; Ke, C.-J.; Lin, Y.-W.; Lin, F.-H.; Tsai, T.-H.; Sun, J.-S. Transglutaminase Cross-Linked Gelatin-Alginate-Antibacterial Hydrogel as the Drug Delivery-Coatings for Implant-Related Infections. Polymers 2021, 13, 414.

34. La Gatta, A.; Tirino, V.; Cammarota, M.; La Noce, M.; Stellavato, A.; Pirozzi, A.V.A.; Portaccio, M.; Diano, N.; Laino, L.; Papaccio, G.; et al. Gelatin-biofermentative unsulfated glycosaminoglycans semi-interpenetrating hydrogels via

microbial-transglutaminase crosslinking enhance osteogenic potential of dental pulp stem cells. Regen. Biomater. 2021, 8, rbaa052.

35. Dennis, J.E.; Esterly, K.; Awadallah, A.; Parrish, C.R.; Poynter, G.M.; Goltry, K.L. Clinical-scale expansion of a mixed population of bone-marrow-derived stem and progenitor cells for potential use in bone-tissue regeneration. Stem Cells 2007, 25, 2575–2582.
36. Keating, A. Mesenchymal stromal cells: New directions. Cell Stem Cell 2012, 10, 709–716.
37. Horwitz, E.M. MSC: A coming of age in regenerative medicine. Cytotherapy 2006, 8, 194–195.
38. Morrison, S.J.; Scadden, D.T. The bone marrow niche for haematopoietic stem cells. Nature 2014, 505, 327–334.
39. Vallmajo-Martin, Q.; Broguiere, N.; Millan, C.; Zenobi-Wong, M.; Ehrbar, M. PEG/HA Hybrid Hydrogels for Biologically and Mechanically Tailorable Bone Marrow Organoids. Adv. Funct. Mater. 2020, 30, 1910282.
40. Orimo, H. The mechanism of mineralization and the role of alkaline phosphatase in health and disease. J. Nippon Med. Sch. 2010, 77, 4–12.
41. Schnepp, Z.A.C.; Gonzalez-McQuire, R.; Mann, S. Hybrid Biocomposites Based on Calcium Phosphate Mineralization of Self-Assembled Supramolecular Hydrogels. Adv. Mater. 2006, 18, 1869–1872.
42. Douglas, T.E.; Messersmith, P.B.; Chasan, S.; Mikos, A.G.; de Mulder, E.L.; Dickson, G.; Schaubroeck, D.; Balcaen, L.; Vanhaecke, F.; Dubruel, P.; et al. Enzymatic mineralization of hydrogels for bone tissue engineering by incorporation of alkaline phosphatase. Macromol. Biosci. 2012, 12, 1077–1089.
43. Su, H.; Koo, J.M.; Cui, H. One-component nanomedicine. J. Control. Release 2015, 219, 383–395.
44. Yuan, D.; Shi, J.; Du, X.; Huang, Y.; Gao, Y.; Baoum, A.A.; Xu, B. The enzyme-instructed assembly of the core of yeast prion Sup35 to form supramolecular hydrogels. J. Mater. Chem. B 2016, 4, 1318–1323.
45. Dias, M.R.; Fernandes, P.R.; Guedes, J.M.; Hollister, S.J. Permeability analysis of scaffolds for bone tissue engineering. J. Biomech. 2012, 45, 938–944.
46. Akbar, I. Permeability study of functionally graded scaffold based on morphology of cancellous bone. Malays. J. Med. Health Sci. 2021, 17, 60–66.