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PHYS 380: Molecular and Cellular Biophysics

Professor: Dr. Brenda Lee

Literature Review

Application of Hydrogels as a Drug Delivery System for Bone Regeneration

Richard (Zhi Fei) Dong 20775623

Justin (Runze) Li 20773362

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Abstract

Treating bone diseases such as bone fracture and bone tumour has become a major topic in biomedicine due to increasing demand. However, it is often accompanied with high prices because of the complexity associated with the process. To tackle this issue, one of the biopolymer materials called hydrogels has attracted significant attention in the field of bone repair and regeneration. The physical properties of hydrogel, which include high adhesion, high biocompatibility, high functionality, and high volume of water content, enable hydrogels to promote the interaction of cells by mimicking the extracellular environment of human tissues and use itself as a vector for bone regeneration drugs. To help future researchers better utilize the potential of hydrogels, this review aims to provide a comprehensive guide for developing hydrogels to be used as a bone regeneration drug delivery system through the discussion of hydrogel materials, techniques for modifying hydrogels, and drugs that can be loaded onto hydrogels. It is hoped that with the help of an effective hydrogel, the above-mentioned problems with bone regeneration can be addressed in order to promote the development of biomedicine and benefit patients worldwide.

Keywords: Hydrogels, Bone Regeneration, Tissue Engineering, Drug Delivery

1.0 Introduction

Bone fracture healing has become a very costly problem for the health care system around the globe. For instance, studies have shown that there are approximately 100,000 bone fracture cases in Canada each year, and the cost to treat an average Canadian patient can range anywhere from $5,000 to $50,000 a year for his or her bone fracture [1]. The primary reason of the high cost of bone fracture treatment lies in the complex nature of bone healing. Fracture healing emphasizes the continuous reformation of embryonic endochondral bone and includes two types: primary healing and secondary healing [2]. In most bone injuries, the body mainly utilizes secondary healing for bone fracture healing. This process requires four important elements to properly heal a damaged bone: cell populations, signalling molecules, osteoconductive materials, and mechanical stability. To start, energetic cell populations are considered as a major component of bone repair as it is critical for the quick regrowth of the bone tissues in the fracture site, whereas signaling molecules are critical to help increase the likelihood of healing occurring by recruiting the proper types of cells. In addition, osteoconductive material in the extracellular matrix is considered as an important scaffold for fracture healing and can have a significant importance on connecting fracture sites when it is combined with mechanical stability. Those four elements cooperate with each other to successfully and effectively achieve the process of fracture healing [2].

Although the current technology for bone regeneration in biomedicine has been well developed, there are still some drawbacks and limitations due to the relatively low accessibility, undesirable effects, and high costs of these techniques [3]. For example, the physical and mechanical properties of the current bone substitute cannot completely replace the bone and therefore often requires repeated interventions for proper bone healing [3]. Therefore, it has been a major goal in regenerative medicine to develop alternatives for assisting bone regeneration in recent decades.

As an attempt to achieve this goal, a new type of biomaterial named hydrogel has attracted major attentions lately and is believed to have the ability to serve as a potential new type of low cost, safe, and effective treatment for bone fracture healing [4]. Hydrogel is a polymeric material that maintains its structure by relying on physical and chemical interactions between multiple polymer chains [5]. Recently, clinical medicine and

biomedicine research in hydrogel has significantly advanced thanks to its diversity of physical properties that gives promising potentials. For instance, hydrogel has swelling properties in water and can store large amounts of water, which makes them flexible and have similar properties to those in natural tissues. In addition, some types of hydrogels such as gelatin and hyaluronic acid can be combined with various proteases to form new hydrogels, which provides high customizability for various situations [6]. Furthermore, hydrogels can also change their own volumes in response to some external physical and chemical stimuli such as temperature, light intensity, pressure, pH value, and certain chemicals, which makes them great candidates for bone fracture healings that often require different supports at different stages with different microenvironments [5, 6]. Last but not the least, hydrogels can serve as a great vehicle for drug delivery as most of them demonstrate natural biodegradability and therefore allowing a sustained drug release [7].

Because of the growth in interests of utilizing hydrogels as a drug delivery system, there are many published literatures that attempt to utilize the potential of such system to tackle the costly problem of bone regeneration in recent years. As a result, the goal of this review is to provide a comprehensive guide for researchers around the globe by summarizing the common techniques in choosing hydrogel materials, modifying hydrogel properties, and determining the desired drugs to be loaded onto the system for bone regeneration (**Figure 1**). It is hoped that with the help of this review, future researchers can better optimize their hydrogel systems in order to deliver drugs more efficiently to solve the costly problem of bone fracture healing.

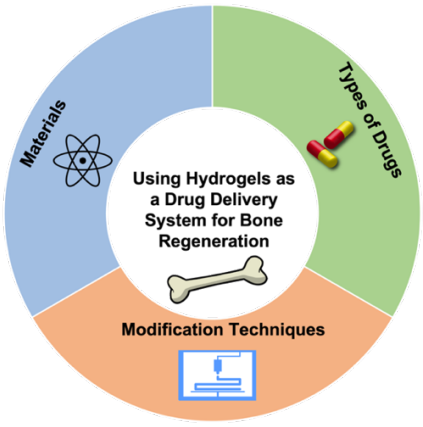

Figure 1: Graphical Abstract. This describes the main topics covered in this review.

2.0 Choosing Materials for Hydrogels

There are many different natural or synthetic materials for constructing a hydrogel for drug delivery in bone regeneration, each with their own advantages and disadvantages before any additional modifications (**Figure 2**). In this section, some of the popular materials amongst researchers will be discussed.

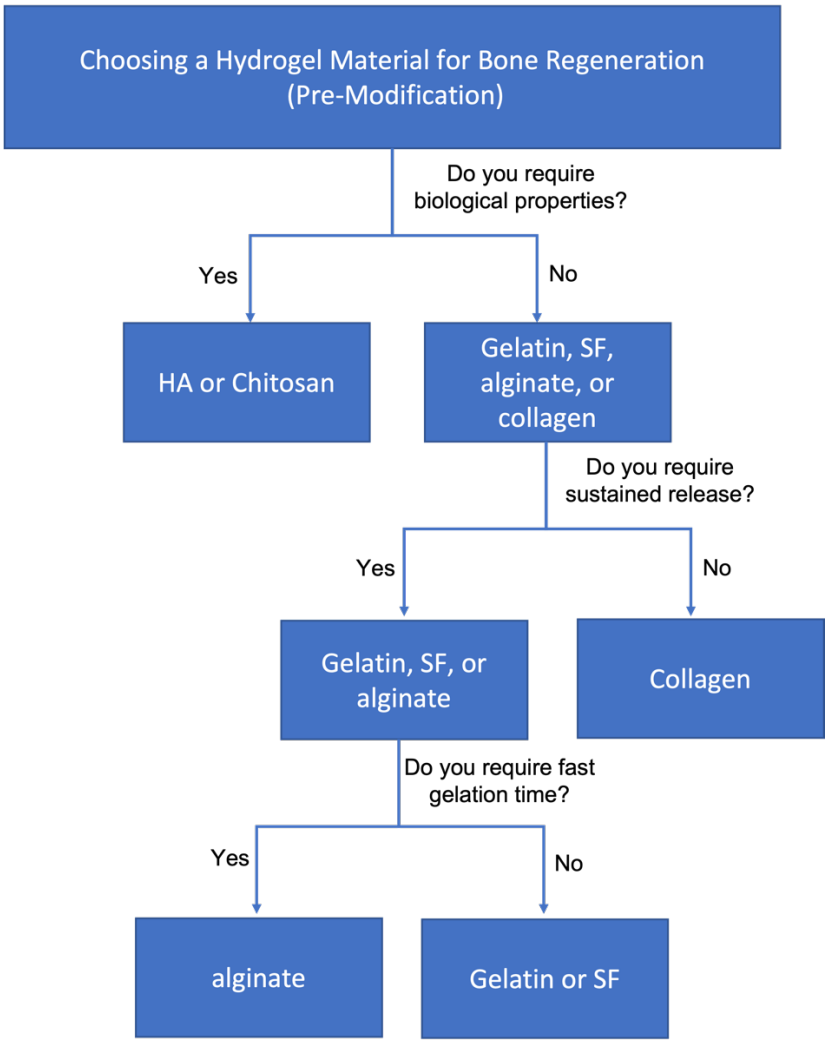

Figure 2: A Decision Diagram for Choosing the Most Appropriate Material for Manufacturing Hydrogels for Bone Regeneration. This flow chart provides a step-by-step protocol to determining whether HA, chitosan, collagen, alginate, gelatin, or SF should be used for different bone regeneration processes that require different properties.

2.1 Gelatin and Hyaluronic Acid (HA)

Gelatin is a collagen derived from animal collagen products and has been commonly used in the process for bone regeneration [8]. It has attracted wide attentions in the field due to its high biocompatibility and low immunogenicity and cell toxicity [9]. In addition, one key physical property of gelatin is that it has the ability to form a thermally reversible network in water. At a low temperature (below 30 degree Celsius), gelatin forms a gel network in the presence of water, which is stabilized by hydrogen bonding. This stability is disrupted when the temperature is raised and causes the content in the gel to be released [10], which makes gelatin a great candidate for being a drug delivery vehicle at body temperature. To obtain gelatin, porcine and bovine skins and bones can be used as sources for extraction, yet the product obtained this way is often not suited for the purpose of tissue regeneration and engineering as they have undesired biomechanical proprieties and could be a potential source for pathogenetic transmission [11]. Instead, more commonly, gelatins derived from fish are used as they possess a greater thermal stability and lower melting temperature as well as higher viscosity and due to different amino acid compositions than those of porcine and bovine skins and bones [12]. More recently, recombinant gelatins have also been developed to improve quality and reproducibility [8].

On the other hand, hyaluronic acid (HA) is found naturally within the connect tissue and extracellular matrix (ECM) of the human body. It is a hydrophilic polysaccharide that binds water tightly to serve as a moisturizing agent as well as play a critical role in maintaining cellular structure [13]. In addition, HA has been demonstrated to directly promote the process of bone regeneration due to its ability to activate the cellular surface marker CD44 to recruit mesenchymal stem cells (MSCs) and guide osteoprogenitor cell migrations [14]. HA can easily be synthesized *in vitro* and be chemically modified with a variety of chemical groups (such as hydrazide, thiol, and tyramine groups) to achieve great non-immunogenicity, biocompatibility, and non-immunogenicity [15]. HA also comes with great biodegradability as it is found naturally within the body, but this also means that HA-based hydrogels would be endogenously degraded by hyaluronidase, which limits its lifetime *in vivo [14]*.

2.2 Silk Fibroin (SF) and

Silkworm silk is the primary source for silk fibroin (SF) as it is the main component of silkworm silk and provides mechanical strength for the structure [16]. Due to its unique hierarchical protein structure, SF is incredibly strong mechanically while also very light weighted to have a very high strength-to-density ratio. In addition, SF is naturally insoluble in water and has a very high thermal stability. This makes SF to have the desired property of controllable biodegradation, as it is very stable naturally and its degradation rate can be precisely optimized by tuning the content of the crystalline phase during the manufacturing process [17]. As a result, SF has been shown to be a great candidate for controlled drug delivery in a variety of biomedical applications for bone regeneration [16].

Similarly, chitosan is also found in animals. It is a biomolecule that is rich in amino polysaccharides and is often obtained by the deacetylation of chitin found in crustacean shells [18]. Similar to other materials discussed above, chitosan is proven to have great biocompatibility, low cell toxicity, and low immunogenicity [19]. However, what makes chitosan more interesting from the other materials is that chitosan has been shown to be able to improve cell adhesion, proliferation, and differentiation. In addition, the polycation polymers of chitosan can possess good affinities for active biomolecules such as bone morphogenetic protein (BMP)-2 and BMP-7 cytokines [20]. As a result, both of those properties make chitosan a good candidate to be attached with bioactive drug and serve as a drug delivery vehicle for bone regeneration.

2.3 Alginate and Collagen

Alginates are found in the cell walls of some brown micro-algae and bacteria and serve as a primary scaffolding polysaccharide [21]. Depending on the source that the alginate is obtained, different types of alginates can exhibit very different properties due to different chemical structures. For instance, different alginates can have different physical properties such as rate of gel formation and drug release [22]. Furthermore, different alginates can also have different biocompatibilities and immunogenicity depending on the degree of purity and the amount of G- and M-blocks (two subunits of alginates) in the compound [23]. As a result, this makes the quality control of the material a key component in the success of utilizing such material as a hydrogel. Despite this,

when tuned correctly, alginate-based hydrogels can have exceptional stretchability and toughness and can form gels very rapidly, making them great candidates to delivery drugs for bone healing [24].

Last but not the least, collagen is a very abundant protein in bone and especially cartilage tissues and therefore has great safety and biocompatibility. This great biochemical property allows it to attract major attentions in the field of bone regeneration [25]. In addition, since cells in collagen can migrate directly, collagen-based hydrogels can significantly promote osteoconduction and bone cell differentiation. However, collagen degrades very rapidly *in vivo* due to its weak mechanical property, which can lead to unsustainable repairing [26]. Therefore, collagen-based hydrogels have primarily been used to support short-term healing on bone defective sites.

3.0 Special Modifications of Hydrogels

As evident from *Section 2.0: Choosing Materials for Hydrogels*, no material is perfect, and all the above-mentioned common materials have their own distinctive advantages and disadvantages. Therefore, to better utilize the potential of hydrogels as a delivery system, much research has been done to investigate some potential modifications that will improve the overall biochemical and/or biophysical properties of hydrogels. In this short section, two common types of modifications, which are photo-crosslinking and photothermal, will be briefly discussed, before diving into their applications in *Section 3.0*.

To start, hydrogels can be cross-linked in several ways, with the most effective of which being photo-crosslinking due to its physical properties including low toxicity, high biocompatibility, and high *in situ* gelation efficiency [27]. Photo-crosslinking is the technique where photons are used to excite the photoinitiator, which will then generate free radicals to cause the formation of chemical crosslinks between polymers [28]. The degree of cross-linking can be modified by controlling the light intensity and duration to effectively promote bone regeneration [7, 29, 30]. This technique can be applied in a very general sense, as many hydrogel materials that contain different functional groups can be modified using photo-crosslinking.

One of the most commonly manufactured photo-crosslinkable hydrogel is HAMA, which is an HA-based hydrogels [31]. In particular, HAMA hydrogel is obtained by adding a photopolymerizable group called methacrylate (MA) to the hydroxyl or carboxyl group of hyaluronic acid at a suitable temperature. This modification results in the improved elasticity and degradability of HAMA hydrogels, and the degree of those two properties can be tuned by changing the polymer concentration [29].

Other than HA-based hydrogels, gelatin-based hydrogel is also a popular candidate for photo-crosslinking modification. The preparation process of photo-crosslinkable gelatins can be achieved by modifying primary amines with thiol groups to obtain thiolated gelatins. This modified material can then be photo-crosslinked using the photo-click method. The degree of photo-crosslinking can be modified through changing the concentration of thiol groups [29]. Another type of photo-crosslinkable gelatin is called GelMA, where similar to HAMA, the major material (gelatin) is functionalized with MA

groups to achieve an improved mechanical property. Similar to HAMA, the rate of degradation and viscosity of the hydrogel system can be optimized by effectively modifying the concentration of MA groups [32].

Moreover, as mentioned, chitosan naturally exhibits desired biological properties for bone regeneration. As a result, scientists have tried many modifications to obtain a photo-crosslinkable chitosan, but its low mechanical properties and low biocompatibility render the results unusable. At present, the best method for preparing photo-crosslinkable chitosan is also to modify it with methacrylate to improve its corresponding physical properties [33].

In addition to photo-crosslinkable hydrogels for the treatment of bone regeneration, photothermal hydrogels used in photothermal therapy (PTT) has also attracted significant interests. PTT is an effective technique for eradicating bone tumour diseases while also effectively reducing damage to the human body. During the treatment, photothermal converters destroy tumor cells, proteins, and cell membranes by converting the energy of absorbed near-infrared (NIR) light into heat. This technique is also known to promote bone regeneration as minor hyperthermia (around 40 to 42 degree Celsius) induced by the heat has been shown to be beneficial for bone healing [34]. The most popular photothermal converter to date is gold nanorods (GNRs) due to their high biocompatibility and stability and therefore are widely used in clinical medicine [35]. Furthermore, another very popular photothermal converter is polydopamine (PDA) due to its high effectiveness in energy conversion and high biodegradability [36].

4.0 Drug Delivery Using Hydrogels in Bone Regeneration

All the above preparations are beneficial for achieving a hydrogel with desired biophysical and biochemical property, yet most of those hydrogels lack the ability to promote bone regeneration naturally. Therefore, proper drugs must be delivered by the hydrogel system to achieve the best bone regeneration outcome. In this section, recent advancements (discoveries since 2021) of using hydrogels to deliver a variety of substances for bone regeneration will be discussed.

4.1 Growth Factors

Growth factors are a group of polypeptides that promote cell proliferation and growth [37]. Because they could interact with cellular signalling pathways directly and promote regeneration, they have attracted major interests to be loaded onto hydrogels for bone healing.

Recently, there are several common growth factors that have been experimented with many different hydrogel designs, with the most popular one being bone morphogenetic protein 2 (BMP-2). BMP-2 is a critical protein involved in the development of bone and cartilage and has shown to induce the differentiation of osteoblasts [38]. Yet, BMP-2 is structurally unstable and therefore cannot be delivered alone for clinical applications. To overcome this issue, Chen et al. developed a transglutaminase (TG) and tannic acid (TA) crosslinked gelatin-based hydrogel for the long-term deployment of BMP-2 [39]. This hydrogel had shown to improve the gelation time, mechanical properties, and adhesiveness due to the smaller pore sizes caused by the additional hydrogen bonds introduced from the crosslink (**Figure 3A-i** and **Figure 3A-ii**). This novel hydrogel was used as a delivery vehicle for a modified BMP-2 compound (named MPs-His6-T4L-BMP2) and has demonstrated to significantly enhance the ALP activity of C2C12 cells, thereby proving an improved viability and proliferation (**Figure 3A-iii**). Last but not the least, this system had also been shown to be able to accelerate the bone formation of rats' skulls *in vivo* by showing a significantly improved bone mineral density value (BMD) and bone volume vs. total defect volume ratio (BV/TV) (**Figure 3A-iv**). Similarly, Datta et al. also attempted to deliver BMP-2 for bone regeneration, but they used a chitosan-based hydrogel instead [40]. They loaded the hydrogel with both alendronate in a gelatin

microsphere (ALN-GM) and BMP-2 directly in the hydrogel to result in a sustained release. This synergistically improved the bone regeneration outcomes of both *in vitro* with human amniotic mesenchymal stromal cells (HAMSC) and *in vivo* with rabbit tibial bone.

Another popular growth factor used by researchers to be loaded onto hydrogels is basic fibroblast growth factor (bFGF or FGF-2). This growth factor has been shown to promote the osteogenic and chondrogenic differentiation of stem cells and therefore is critical for bone fracture repair [41]. To unleash the potential of this protein, He et al. incorporated bFGF into a newly synthesized RADA16/$CaSO_4$/HA hydrogel [42]. The result was a delicate system with a well-controlled release of bFGF for more than 32 days. Both the *in vitro* osteogenic differentiation results and *in vivo* bone formation results on the femoral condyle defects of rats had demonstrated that this system could promote the osteogenic differentiation and osteogenesis on the bone defect for an extended period of time, thus proving its great potential for clinical application. Furthermore, Yu et al. developed a chitosan-glutamate (Cs-Glu) based self-healing hydrogel system (DFH) loaded with transforming growth factor β3 (TGFβ3) in chitosan microspheres (CMs) and bFGF (**Figure 3B-i**) [43]. This self-healing hydrogel mimiced the tissue injury microenvironment in alveolar bone defect by recruiting different endogenous cells at different stages of bone healing. This was accomplished by releasing bFGF and TFGβ3 in a controlled and sequential manner through the degradation of the DFH (**Figure 3B-ii**). During *in vitro* testing on human periodontal ligament stem cells (hPDLSCs), DFH were able to recruit MSCs and induce cell migration (**Figure 3B-iii**), which had been shown to be beneficial for in later *in vivo* tests on the alveolar bones of rats. All the above examples have demonstrated that well-developed hydrogel systems can be great vehicles for delivering growth factors into the defect bone sites to promote bone regeneration.

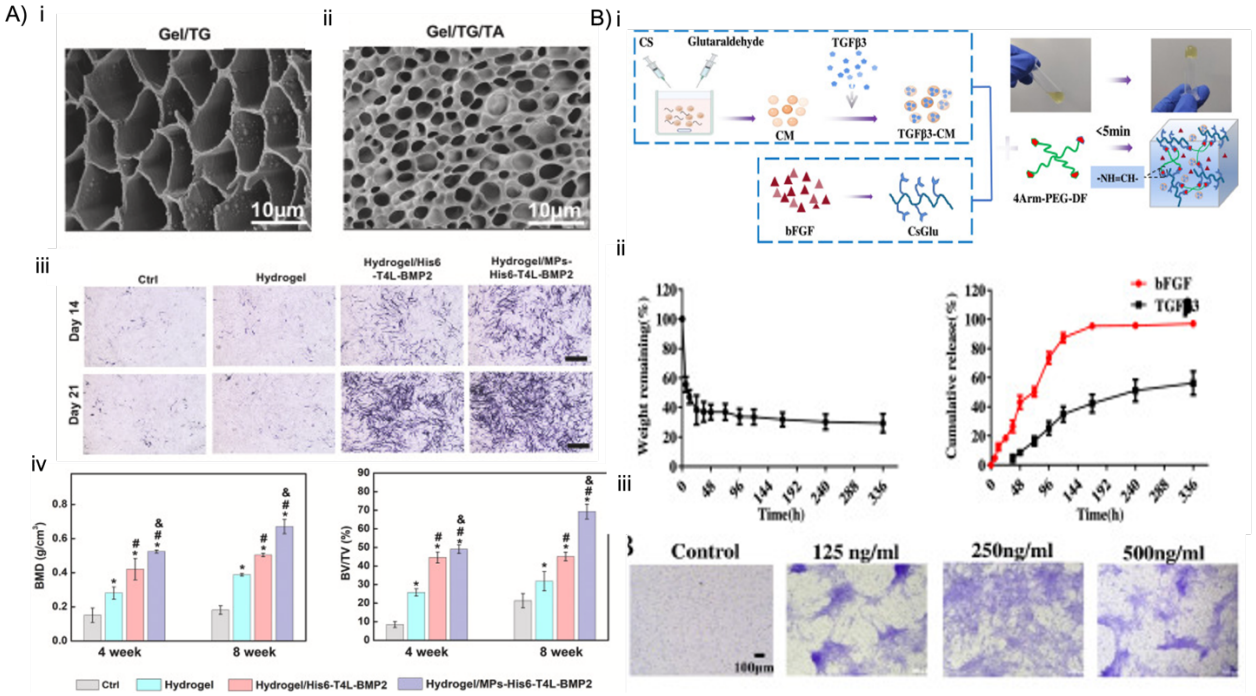

Figure 3: Using Hydrogels as a Delivery System for Growth Factors. A) TG and TA crosslinked gelatin hydrogel for the delivery of MPs-His6-T4L-BMP2. i) The SEM of gelatin + TG hydrogel. ii) The SEM image of gelatin + TG + TA crosslinked hydrogel with improved physical properties. iii) The 14-day and 21-day ALP activity of C212 cells with control, hydrogel, hydrogel + His6-T4L-BMP2, and hydrogel + MPs-His6-T4L-BMP2. iv) Quantitative analysis of bone mineral density and bone volume vs. total defect volume ratio of control, hydrogel, hydrogel + His6-T4L-BMP2, and hydrogel + MPs-His6-T4L-BMP2 at week 4 and week 8. Figures are adapted from ref. [39] with permission from Elsevier. B) A self-healing chitosan-glutamate hydrogel loaded with bFGF and TFGβ3-CM. i) A schematic diagram for the manufacturing of the hydrogel system. ii) Controlled release curve of bFGF and TFGβ3 *in vitro*. iii) *In vitro* tests of MSC migration, with different concentrations of TFGβ3. Figures are adapted from ref. [43] with permission from Elsevier.

4.2 Nanoparticles

Recently, more and more interest has been developed on utilizing nanoparticles for local bone regeneration. This is because that those molecules have sizes that are similar to the integral parts of the natural bone tissues and therefore can elicit many different cellular responses to promote the regeneration process [44]. Out of the many potential candidates, one of the most popular nanoparticles used today is silica due to its desirable properties. Silica nanoparticles have great biocompatibility, but what really makes them to be the star of bone regeneration is their large surface area, large pore volume, and controllable sizes. All of those interesting properties make them very easy to be used for drug delivery [45]. As an attempt to utilize the potential of silica nanoparticles, Yuan et al. developed a dynamic gelatin-based hydrogel by photo-crosslinking MA groups and silica nanoparticles (**Figure 4A-i**) [46]. The resulted hydrogel was not only locally stiff with a high Young's modulus for great mechanical support (**Figure 4A-ii**), but also had a great cell adaptability and viability of human stem cells (**Figure 4A-iii**). Further *in vivo* experiments in rat calvaria defects also proved that the system could effectively promote bone regeneration, making it a great candidate for delivering silica nanoparticles for bone regeneration. Similarly, Perez-Moreno et al. developed a bioactive chitosan-based silica containing hydrogel for bone regeneration [47]. This system had demonstrated an *in vitro* release of silica nanoparticles when soaked in phosphate-buffered saline (PBS). This delivery of silica had successfully promoted the cell attachment, focal adhesion development, and maturation and growth during *in vitro* experiments using osteoblasts, suggesting that this hydrogel system could be used for future tests of *in vivo* bone regeneration experiments.

Other than silica nanoparticles, gold nanoparticles have also attracted much attention lately due to its desirable biological properties arose from its size. In particular, studies have found that gold nanoparticles can modulate immune responses by regulating the polarization states of macrophages in order to act as an anti-inflammatory factor to promote bone tissue healing [48]. As a result, Zhang et al. developed several different polyethylene glycol (PEG) hydrogels containing gold nanoparticles with different pore sizes and investigated effect of the systems on osteogenic differentiation [49]. For *in vitro* tests on MC3T3-E1 cell lines, after the application of the hydrogel systems, both the

viability of the cells (**Figure 4B-i**) and the osteogenic differentiation (measured by ALP activities, **Figure 4B-ii**) increased significantly. Because of the most promising results from the pore size of 45 nm, this system had been selected to proceed to be tested *in vivo*, where the researchers implemented this system into the femur condyle defects of rabbits. At week 4 and week 8, the 3D micro-CT result demonstrated significant bone growth on the defect for the nanoparticle containing hydrogel group (**Figure 4B-iii**), thereby further proving the potential of this hydrogel system to deliver gold nanoparticles for bone regeneration. Last but not the least, Huang et al. also developed a hydrogel system for delivering gold nanoparticles but with a very interesting material called bacterial cellulose. This material has demonstrated great mechanical strength, yet naturally it lacks osteogenic activities. To overcome this problem, the bacterial cellulose-based hydrogel was combined with gold nanoparticle in order to promote bone tissue regeneration. *In vitro* tests of this system demonstrated a sustained release of gold nanoparticles, which led to the significant promotion of the osteogenic differentiation of human bone marrow derived MSCs (hBMSCs). During *in vivo* tests on rabbit femoral defect, this hydrogel system had also demonstrated a significant increase in new bone formation. As a result, all the above examples shows that there are many interesting studies recently that utilizes hydrogel systems to deliver nanoparticles for bone regeneration.

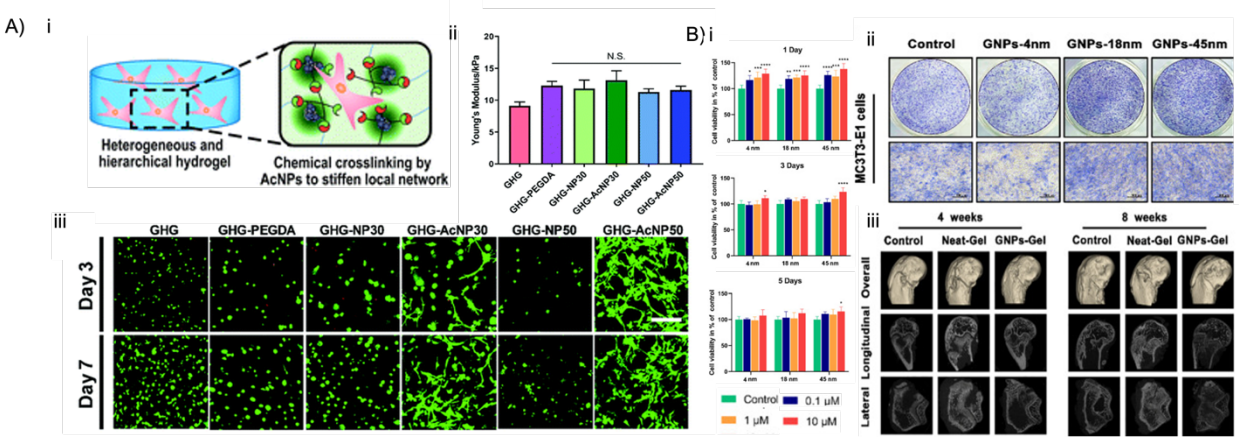

Figure 4: Using Hydrogels as a Drug Delivery System for Nanoparticles. A) Delivering silica nanoparticles using a gelatin-based hydrogel. i) The schematic design of the hydrogel system. ii) Measurements of Young's modulus of different experimental hydrogel groups. iii) Live (green) and dead (red) staining of human stem cells of different experimental hydrogel groups. Figures are adapted from ref. [46] with permission from The Royal Society of Chemistry. B) Delivering gold nanoparticles using a PEG-based hydrogel. i) The cell viability of various concentration of MC3T3-E1 cells after treated with the hydrogel system of different pore diameters. ii) ALP staining of MC3T3-E1 cells after treated with the hydrogel system of different diameters. iii) 3D micro-CT images of the femoral condyle at week 4 and 8 for the control, blank (Neat-Gel), and 45 nm gold-nanoparticle containing PEG hydrogel (GNPs-Gel). Figures are adapted from ref. [49] with permission from Elsevier.

4.3 Artificial Drugs

So far, this review has covered delivering naturally occurring elements in hydrogels systems for bone regeneration. Another significant type of molecules that can be loaded onto hydrogels is artificial drugs that are designed and manufactured by humans thanks to the advancements in medicinal chemistry. For instance, the use of simvastatin for bone regeneration has attracted much attention lately [50]. Simvastatin is a derivative of lovastatin and has been shown to be anti-inflammatory, promote osteoblastic activity, and inhibit osteoclastic activity. However, only less than 5% of this drug is absorbed when taken orally, which means that an efficient delivery vehicle must be found for this drug in order to use it effectively [51]. To this end, Zhang et al. developed a supramolecular LAPONITE® based hydrogel to deliver simvastatin for osteogenesis [52]. This hydrogel material had been demonstrated to have great osteoinductivity and could be easily used as a platform to load drugs due to its negatively charged surface. The resulting hydrogel had great injectability and could slowly release the loaded simvastatin during the entire experimental period (**Figure 5A-i**). In addition, the system also significantly enhanced osteoinduction *in vitro* by stimulating ALP expression. During *in vivo* tests for osteogenesis using calvaria defect model of mice, the hydrogel system performed well as it was proven to be able to stimulate the growth of bone tissues, which was evident from 3D micro-CT measurements (**Figure 5A-ii**) and histological staining (**Figure 5A-iii**). Similarly, Ossipov et al. developed a HA-based hydrogel system for delivering simvastatin [53]. This hydrogel system was capable of automatically releasing simvastatin under acidic environments, which usually occurs at the sites of bone resorption. Therefore, this system had been envisioned to be a candidate for the treatment of osteoporosis as it could deliver the drug as a response to disease microenvironments.

Other than simvastatin, another promising drug for bone regeneration is resveratrol. Studies have found that resveratrol can promote the differentiation of osteoblasts and inhibit the differentiation of osteoclasts, therefore making it a great candidate for the treatment of bone defects [54]. To this extent, Wei et al. developed a gelatin-based hydrogel as a delivery system for this drug [55]. The researchers embedded resveratrol into a phospholipid before loaded it into the GelMA hydrogel (**Figure 5B-i** and **Figure 5B-ii**), which enhanced the duration of the sustained release of the drug (**Figure 5B-iii**). This

prolonged release was proven to significantly enhance not only cell viability and osteogenic differentiation of BMSCs *in vitro*, but also new bone tissue formation *in vivo*. Furthermore, Fan et al. loaded resveratrol into a PEG-based hydrogel to be used as a targeted therapy for hypoxic bone defects [56]. During *in vitro* tests on BMSCs with hypoxic microenvironment, the researchers found that the application of the hydrogel system can efficiently deliver resveratrol to improve cell survival by activating the autophagy pathway. In addition, based on the histological staining results from *in vivo* tests on tibial defect, it was also evident that the hydrogel system can successfully promote new bone formation, and therefore making this device a promising candidate in targeted bone therapy.

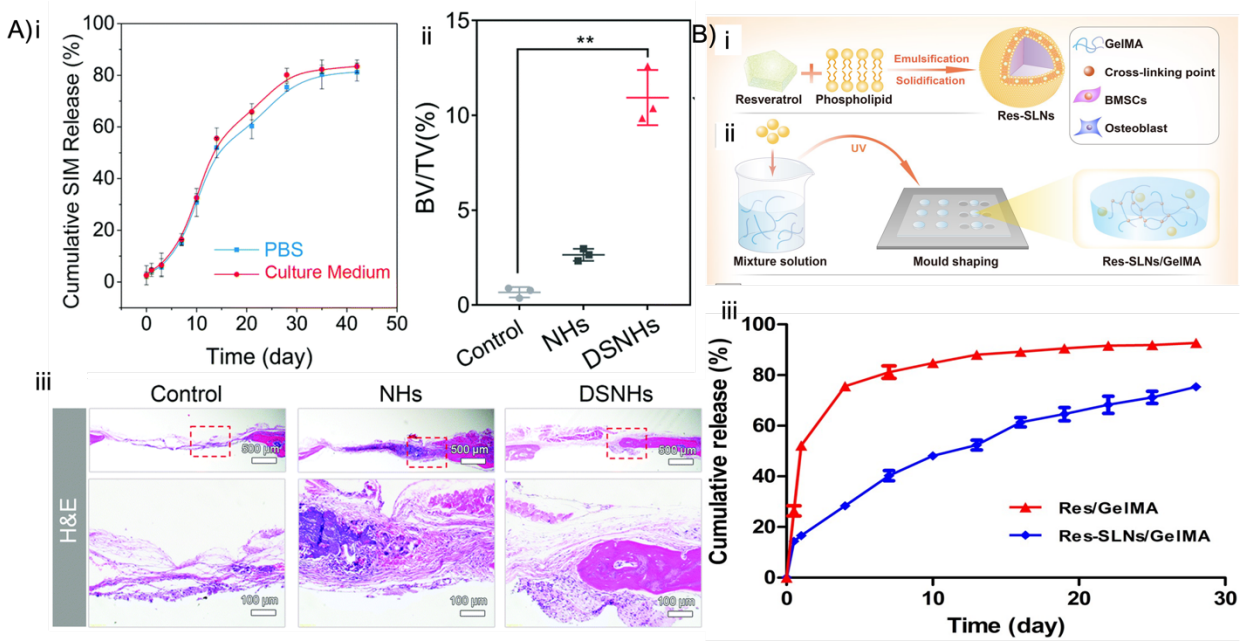

Figure 5: Using Hydrogels as a Drug Delivery System for Artificial Drugs. A) Delivering simvastatin using a LAPONITE® based hydrogel. i) The cumulative simvastatin release curve *in vitro*. ii) Quantitative analysis of bone volume vs. total defect volume ratio of control, blank (NHs), and experimental groups (DSNHs). iii) H&E staining of control, blank (NHs), and experimental groups (DSNHs). Figures are adapted from ref. [52] with permission from The Royal Society of Chemistry. B) Delivering resveratrol using a gelatin-based hydrogel. i) The schematic diagram of the synthesis of resveratrol loaded lipid. ii) The schematic diagram of the synthesis of the drug-lipid loaded hydrogel. iii) The cumulative release of Res/GelMa (no lipid) and Res-SLNs/GelMa (with lipid). Figures are adapted from ref. [55] with permission from Oxford Academic.

4.4 Bone Grafts and Stem Cells

Last but not the least, there has been some novel attempts to deliver bone grafts and stem cells in hydrogels for bone regeneration. Bone grafts are bone tissues that are implanted into the bone defect site to promote bone healing responses [57]. There are several types of bone grafts, yet one of the most promising one is xenograft due to its high availability when compared to autographs. Bone xenograft is the transplant of the bone tissue from another species and can be tuned to have great biocompatibility, porosities, and osteoconductive properties [58]. However, because xenografts come from another species, xenograft rejection often occurs through the activation of innate immune cells and results in the destroy of the grafts [59]. To overcome this issue, Kim et al. applied hydrogels to bone xenografts due to hydrogels' anti-inflammatory properties [60]. During *in vitro* experiment from culturing human MSCs, the group containing both 20% and 50% hydrogel-xenograft extracts (named S1-XB) demonstrated improvements for cell proliferations (**Figure 6A-i**). In addition, the S1-XB group had also shown significantly higher ALP activity (**Figure 6A-ii**) as a result of the increased osteogenic differentiation evident from elevated expression of osteogenesis-related genes such as OPN (**Figure 6A-iii**) at day 12. As a result, the researchers concluded that when combined with hydrogels, the xenograft could achieve a much better osteogenic differentiation result, which makes this system a promising candidate for future clinical application to improve the healing outcome of xenograft.

Other than bone grafts, bone marrow-derived mesenchymal stem cells (BMSCs) have also been used clinically to treat bone defects due to their natural ability to differentiate and promote bone healing. Yet, like the case with simvastatin discussed in *Section 4.3*, an efficient delivery vehicle must be found to directly apply those cells to the site where they are needed. To this end, Yuan et al. developed an injectable gelatin-based crosslinkable hydrogel to be loaded with BMSCs as a potential treatment for osteonecrosis [61]. A cell viability test was performed with mouse fibroblast L929, with the BMSC-containing hydrogel demonstrating a statistically improved cell viability. During *in vivo* tests with osteonecrosis rat model, this hydrogel system was found to be able to prevent bone loss significantly, which is key for the treatment of early osteonecrosis patients. Therefore, the researchers believed that this system could be applied clinically

to improve the outcome of BMSC-based tissue therapy. Furthermore, Li et al. also developed a self-healing injectable gelatin-based hydrogel to delivery BMSC [62]. However, the researchers pre-treated the BMSC to be hypoxic as an attempt to activate more growth factors associated with bone regeneration. During *in vitro* cell assay, this hydrogel system with hypoxia BMSC was proven to increase the expression of osteogenesis protein (OCN, **Figure 5B-i**) and angiogenesis factor (VEGF, **Figure 5B-ii**) when compared to the system load with normoxic BMSC. This promising result demonstrated the potential of this creative method, thereby providing a new direction for using both the hydrogels and hypoxic BMSCs in a clinical setting for bone regeneration.

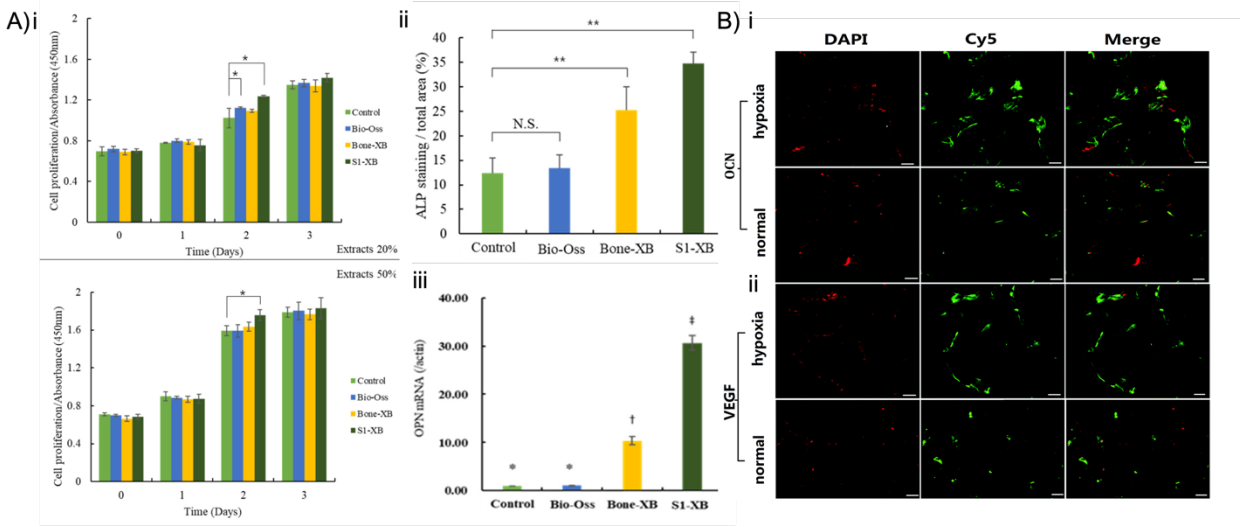

Figure 6: Delivery Bone Grafts and Stem Cells Using a Hydrogel System. A) Delivering xenografts using hydrogels. i) Cell proliferation results of culturing human MSCs on 20% and 50% extracts of control, two non-hydrogel xenograft groups (Biol-Oss and Bone-XB), and hydrogel xenograft groups (S1-XB). ii) ALP activities of four groups at Day 12. iii) Expression of OPN, a key osteogenesis-related gene, of four groups at Day 12. Figures are adapted from ref. [60] with permission from MDPI. B) Delivering hypoxic BMSCs using hydrogels. The figure shows the cell immunofluorescence results of OCN (i) and VEGF (ii) in both hypoxic and normoxic BMSCs. Figures are adapted from ref. [62] with permission from The Royal Society of Chemistry.

5.0 Conclusion and Future Perspectives

In this review, we summarized the recent advancements of hydrogels as a drug delivery system for bone regeneration, which includes different types and materials, modifications, and drugs for achieving an effective drug delivery to skeletal tissue. In particular, hydrogels can be made from different materials such as gelatin, HA, SF, chitosan, alginate, and collagen. Each of those material has its own unique structures, properties, preparation methods, as well as unique advantages and disadvantages. Those hydrogels can also be modified to be better suitable for bone regeneration through methods such as photo-crosslinking and photothermal modifications, which typically enhances the mechanical stabilities and creates a better environment to house bone cells. Those modified hydrogels systems can then be used to deliver molecules such as growth factors, nanoparticles, artificial drugs, and bone grafts and stem cells, and many recent *in vitro* and *in vivo* studies have demonstrated promising results for utilizing those systems for bone fracture healing.

Despite many optimistic findings recently, the deployment of hydrogels for clinical application still has a long way to come. Although the application of hydrogels in the treatment of bone repair problems has demonstrated its potential by having low toxicity and high biocompatibility, the introduction of such polymer materials into the market still faces a major challenge. This is primarily due to the unique structure of hydrogels, which makes it difficult to be prepared. In addition, there is a lack of clinical trials of utilizing hydrogels for bone regeneration, and therefore the safety and effectiveness of such systems on humans still need to be tested before broader application clinically. As a result, future research should focus on translating the potential of hydrogel systems to clinical studies in order to prepare them for further adoption as a novel method of treatment. It is hoped that with more research in the field of regenerative medicine, costly and painful bone fracture healing will become a problem of the past, and more patients around the globe will benefit from using hydrogels to deliver drugs for effective bone regeneration.

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