Natural and Synthetic Materials for the Regeneration of Osseous Tissue

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The undersigned, appointed by the dean of the Graduate School, have examined the

thesis entitled

Natural and Synthetic Materials for the Healing of Osseous Tissues

presented by Allison Josselet,

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and hereby certify that, in their opinion, it is worthy of acceptance.

Dr. Bret Ulery

Dr. Sheila Grant

Dr. Christina Goldstein

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ABSTRACT

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Bone is a complex tissue with many regulation mechanisms which can be taken advantage of through the release of simple signaling molecules delivered via a biodegradable material like a hydrogel. Hydrogels are water swollen networks that have similar properties to biological tissues. Synthetic hydrogels have problems with toxic leaching and commonly being non-biodegradable, however their properties are more easily controlled. Natural hydrogels tend to be weak and unpredictable under biological conditions, but are typically more biocompatible. This review compares three synthetic hydrogels to three natural hydrogels discussing each composition's strengths and weaknesses. Experimentally, our goal was to create a biocompatible hydrogel that can withstand the forces that would be placed on it if injected into osteoporotic bone.

1. Introduction

1.1. Bone Development and Dysregulation

Bone goes through two major phases during its lifetime: modeling, which occurs during development, and remodeling, which is the lifelong process of tissue renewal. Modeling is achieved through intramembranous and endochondral ossification during fetal development. Remodeling requires a delicate balance between osteoblast, bone depositing cells, and osteoclasts, bone resorbing cells.

While many signaling pathways are involved in osteogenic differentiation, Wnt signaling has be specifically shown to play an important role in increasing bone mass through several different mechanisms including the renewal of stem cells, pre-osteoblast replication, osteoblast differentiation, and the inhibition of osteoblast apoptosis.[2] Bone morphogenic proteins (BMPs) are a part of the transforming growth factor beta (TGFβ) superfamily (**Figure 1**), which consists of 30 different proteinaceous factors that control cellular functions from embryonic development to proliferation and differentiation.[1]

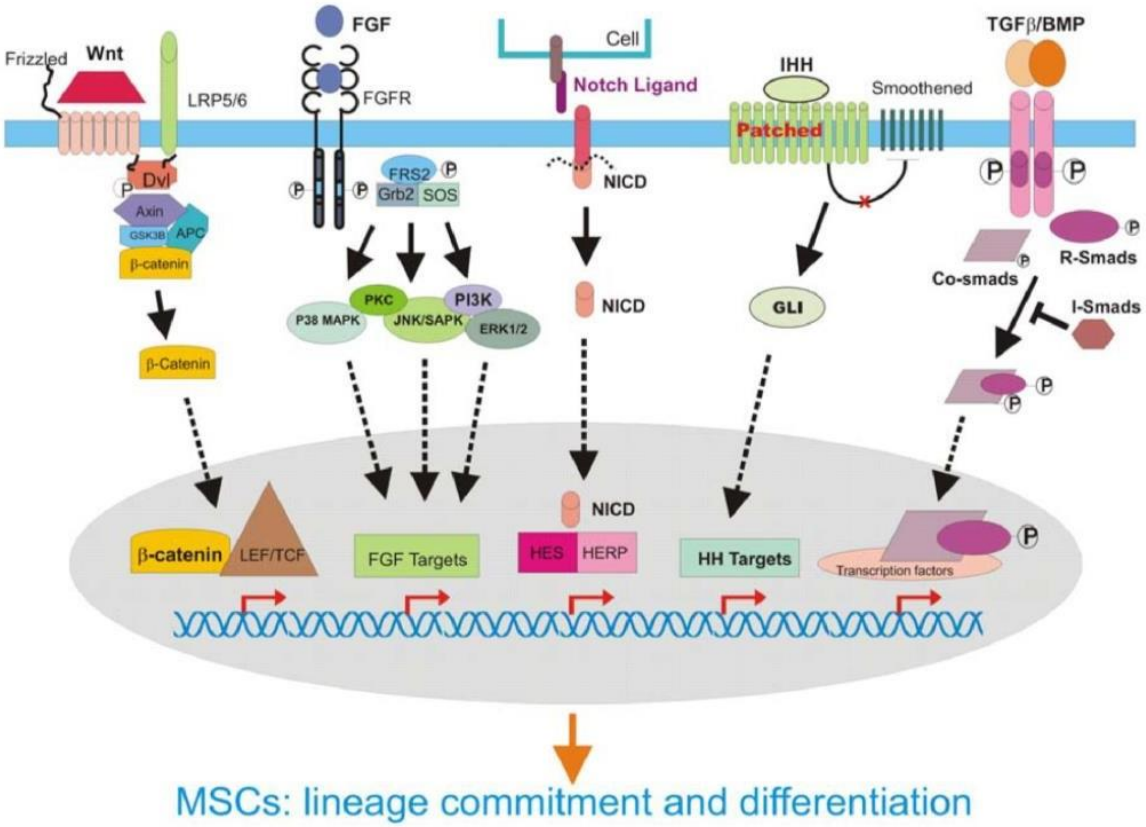

Figure 1. Signaling pathways for MSCs. Figure adopted from "Regulation of Osteogenic Differentiation During Skeletal Development" by Zhong-Liang Deng et. Al.[1]

There are several markers that can be used to identify which major stage of osteoblastogenesis a differentiation bone cell is in: proliferation, matrix maturation, and mineralization.[1,2,3] Runx2 expression can be detected in all three stages of osteoblastogenesis immature and mature osteoprogenitor cells as well as preosteoblasts, and mature osteoblasts.[1,2,3] Osteopontin (OPN) production can be detected in immature osteoprogenitor cells during proliferation.[3,4] Alkaline phosphate is expressed in all three stages of osteoblastogenesis by mature osteoprogenitor cells, preosteoblasts, and mature osteoblasts.[1,2,3] Preosteoblasts express greater quantities of alkaline phosphate and runx2 than as well as produce osterix.[2,3] A mature osteoblast expresses the highest level of

alkaline phosphate and runx2 as well as osterix, osteopontin, and osteocalcin.[2,3] **Table 1** shows the stage and degree to which the mentioned markers are expressed.

Table 1. Osteoblastic markers during osteogenesis. Adopted from "Signaling and transcriptional regulation in osteoblast commitment and differentiation" by Huang, Wei et. Al.

MSC	Immature Osteoprogenitor	Mature Osteoprogenitor	Preosteoblast	Differentiated osteoblast	Osteocyte
Alkaline Phosphatase (ALP)	−	+	++	+++	−
Phex	−	−	−	+++	+++
Osteocalcin (OCN)	−	−	−	−→+++	−
Osteopontin (OPN)	−/+	−/+	−→+	−→+++	−→+++
Runx2	+	+	++	+++	+++
Osterix	−	−	++	++	?
Colla1	−	++	++	++	−
Bone sialoprotein (BSP)	−↔++	++↔−	−→+++	−→+++	−→+++

Banse and colleagues studied 63 vertebral body samples from 27 different vertebral bodies and concluded an average stiffness of 352 MPa and an average strength 2.36 MPa for that tissue.[5] They also found that collagen contributes only approximately 10% (*i.e.* 0.25 MPa) to the strength of the vertebral bone.[5] There are many different causes of bone density loss, including disuse and disease. As bone density is lost the musculoskeletal system is weakened leading to a higher risk of fracture. Vertebral fractures only present clinically in roughly one third of patients, which can have debilitating effects later on.[6] Pressure has been shown to trigger some bone cell pathways. PGE_2 is involved in bone formation and healing, and also increases under continuous hydrostatic pressure.[7] MMP-13 and TIMP-1 decrease and increase respectively under cyclical hydrostatic pressure and vice-versa for cyclical hydrostatic tension.[4] One particularly damaging disease is osteoporosis, an imbalance in osteoblast and osteoclast activity where osteoblast activity

decreases and osteoclast activity increases leading to decreased bone mineral density. MMP-13 is a gene that codes for collagenase 3 to break down collagen matrices.[7] TIMP-1 on the other hand is a gene which has been linked to the inhibition of bone resorption.[7] Increased MMP-13 activity and decrease in TIMP-1 may lead to an increase risk of osteoporosis or increase in the degree to which osteoporosis affect the patient.

Many treatments for bone density loss are available, but oral bisphosphonates are by far the most popular treatment for homeostatis dyregulation-based bone density loss. They can increasing the mitogenic and metabolic activity of osteoblasts as well as decrease osteoclast activity.[8] This method of treatment has many disadvantages including adherence difficulty. It also cannot increase bone mineral density, it just slows the current loss and The drug must be taken on an empty stomach with nothing eaten for thirty minutes to an hour after to all for adequate absorption into the patient's system.[9] Even under these most ideal conditions only approximately 1% of the bisphosphonate is absorbed.[9]

Parathyroid hormone (PTH) and SST-VEDI/SSH-BMI have also been investigated for use in treating osteoporosis. PTH is normally secreted in the human body and helps to regulate blood calcium levels.[10] Synthetic PTH (PTH1-34) can be administered to increase osteoblast differentiation and inhibit osteoblast apoptosis, however it can have adverse side effects of nausea, vomiting, feelings of weakness, and other adverse events due to excess calcium in the blood.[10] SST-VEDI/SSH-BMI are two similar tryptophan derived compounds which decrease osteoblast apoptosis and promote calcification.[10] There is also evidence SST-VEDI/SSH-BMI may inhibit osteoclast activity and have fewer side effects

that PTH1-34.[10]

1.2 Synthetic bone solutions

Synthetic solutions have been investigated, however it is extremely difficult to replicate all, or even most, of the properties of bone. Poly(methyl)methacrylate is a popular injectable solution to bridge bone deformities through the use of vertebroplasty or kyphoplasty to repair vertebral compression fractures, which is often a result of a loss of bone density due to osteoporosis. Vertebroplasty involves injecting polymethylmethacrylate (PMMA) into the collapsed vertebrae to add structure and strength to the spine.[11] Kyphoplasty utilized a balloon placed into the collapsed vertebrae to realign the spine. Once the spine is aligned and the vertebral height has been restored the resulting cavity is filled with PMMA.[11] Both methods aid in alleviating pain and loss of mobility from these painful fractures. Unfortunately, this material is nondegradable and about twice as dense as native bone tissue and also possesses a compressive strength much higher than that of bone.[5,12] Due to the difference in biomechanics of PMMA versus native bone the patient may be at higher risk to future vertebral compression fractures.[13]

Calcium phosphate cements (CPCs) are biocompatible, bioactive, and osteoinductive materials making them an attractive alternative to other cements.[14] Once hardened they contain micropores which allow for either cell integration or bioactive solutions in the pores.[14] However, they have poor cohesive properties and are difficult to inject and thus have not been widely used in bone applications yet.[15] Hydrogels can be formed with a

wide variety of parameters making them an attractive solution to this problem. Hydrogels are generally very porous and can formed to be biocompatible, osteoconductive, as well as many other favorable properties.

1.2.1 PVA

Poly(vinyl alcohol) (PVA) has easily controllable hydrophilicity and solubility that can be achieved by altering the molecular weight and extent of hydrolysis.[16] Unfortunately, many of the crosslinkers used with PVA, like glutaraldehyde or epichlorohydrin, can be toxic and have problems with leaching that can be somewhat mitigated by freeze-thaw cycles.[17] An electron beam can also be used to crosslink PVA but result in a less stable gel than the gels that had gone through the freeze thaw cycles, which were reported as stable at room temperature.[17,18] PVA is not biodegradable and therefore is useful as a long- term permanent scaffold.[19]

1.2.2 PEO

Poly(ethyl oxide) (PEO) is currently FDA approved for a number of applications, such as biological conjugates, surface modifications, and the induction of cell membrane fusion, making it very attractive to use to form synthetic hydrogels.[20,21,22] It has very low toxicity and is very biocompatible. PEO can be synthesized by either anionic or cationic polymerization of ethylene oxide or through star-shaped monomers or oligomers and formed into hydrogels through UV photopolymerization.[23] Poly(propylene oxide) (PEO- PPO-PEO) possesses a hydrophonic block which allows for the formation of reversible gels without the need for permanent crosslinks.[24] This triblock have been used for drug delivery

applications as it has been known to enhance drug penetration.[25] However, PEG-based materials are not biodegradable potentially limiting their utility.[26] Poly(lactic acid) has been synthesized with PEO and shown to form temperature dependent reversible gel-sol transitions at body temperature for which the resulting hydrogel undergo a combination of hydrolytic degradation and dissociation.

1.2.3 PAA

Poly(acrylic acid) (PAA) and its many derivatives can form a variety of hydrogels. Poly(2-hydroxyethyl methacrylate) or poly(HEMA) has been used for a variety of applications including as contact lenses and in drug delivery.[27,28] Macroporous gels can be formed using freeze-thaw cycling. Poly(HEMA) gels are non-degradable under normal physiological conditions.[29] Dextran has been used to modify poly(HEMA) to allow for the resulting hydrogels to be degradable by enzymes.[30] Poly(N-isopropylacrylamide) or PNIPAM solidifies at temperatures above 32°C making it attractive for injectable applications.[31] PNIPAM can be copolymerized with acrylic acid to alter the gel for different applications, however these gels form non-degradable crosslinks and include vinyl monomers which can be toxic.

1.3. Natural Hydrogel Bone Solutions

Hydrogels are hydrophilic networks of polymers capable of holding large amounts of water.[33] The human body is mostly water, so hydrogels tend to be highly compatible with natural tissues. Natural and synthetic hydrogels are attractive as an injectable solution in bone defects which allow for fewer surgeries to be performed in many cases. Unfortunately hydrogels tend to be mechanically weak preventing them

from being readily used for load bearing tissues like bone.

Hydrogels formed from natural components are attractive options due to their abundancy and biocompatibility. They can form crosslinks through physical or chemical bonds shown in **Figure 2**. Physical bonds are held together by tangled molecular chains, which are less predictable than chemical bonds and are non-homogenous and reversible.[34] Chemical hydrogels are formed through the ionic, covalent, or hydrogen bonding of a crosslinker to a polymer, like a protein or carbohydrate.[34] However, both physical and chemical hydrogels tend to be very mechanically weak.

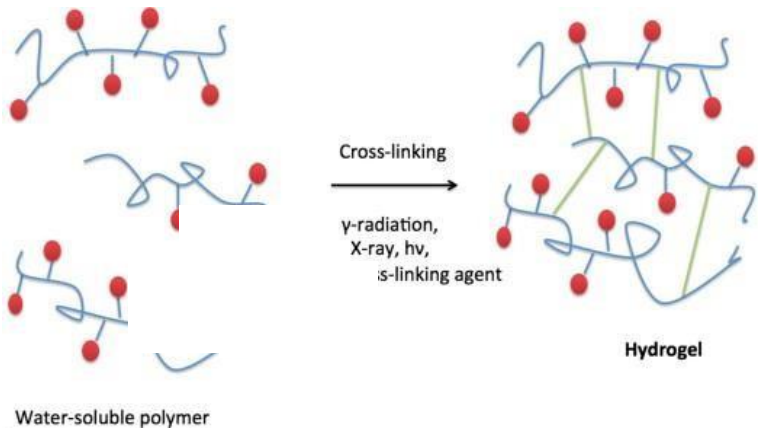

Figure 2. Hydrogels are formed by linking polymer strands through chemical, thermal, radiation, or other methods to form a hydrophilic material with a relatively high water swelling capacity. Adopted from Enrica Caló et. Al.[32]

1.3.1. Collagen

Collagen is the main component of the extracellular matrices found in mammalian tissues including bone.[26] It is composed of three polypeptide chains wrapped around one another to form a braid-like structure held together by hydrogen and covalent

bonds.[35] Collagen readily attaches to many different cell types, can form a unique gel that is not easily replicated by synthetic materials, and is readily degraded by cell-based proteases.[36] Collagen can also be chemically modified to alter cell attachment as well as improve its mechanical properties by grafting using one of the aforementioned synthetic polymers.[35]

Gelatin is a collagen derivative that has also been studied for its capacity to be used for the fabrication of hydrogels. The triple helix structure of collagen is first broken down into single strand molecules, which easily form gels through thermal changes.[37] Gelatin hydrogels are favorable for tissue engineering because of their biocompatibility.[26] Gelatin can also be modified with styrene to enable it to be photocrosslinking.[36] Gelatin gels have previously been used for the delivery of growth factors.[38]

1.3.2 Alginate

Alginate has been used as an injectable material for cell delivery and wound dressings.[26] It is a readily available resource as it is derived from algae and seaweed and it can gel under very gentle conditions. Alginate gel beads have been investigated as a delivery vehicle for islets of Langerhans cells for the treatment of diabetes.[39] Alginate hydrogels have been studied as potential scaffolds for cartilage engineering as they can be mixed with chondrocytes and injected into the site of interest to form an *in situ* construct.

The strength of alginate gels is directly dependent on the type of crosslinker used. If

the ionic crosslinking used has a high affinity for alginate, high concentration solutions will gel into stronger materials.[35] The enzymatic degradation of alginate is generally slow, unpredictable, and uncontrollable, therefore mass loss is typically through the dissociation of individual chains. This lack of control makes it an imperfect system for many applications.[26] Alginate is hydrophilic, which causes it to discourage protein adsorption and thus minimally interacts with mammalian cells.[35] Alsberg and colleagues demonstrated that alginate can be assigned precise adhesive characteristics by the covalent coupling of specific adhesion ligands.[40]

1.3.3 Chitosan

Chitosan is a derivative of chitin, a carboyhydrate found in the shells of many shellfish and some varieties of grasses and mushrooms, which is formed through deacetylation. It is biocompatible, non-toxic, and can be readily degraded by enzymes such as glycosyl hydrolases and lysosomes.[41] It is insoluble in neutral pH conditions so the materials must be added to a slightly acidic solution to be dissolved. Some chitosan derivatives have been reported to have increased solubility compared to other derivatives of chitosan.[42] This increased solubility could strengthen the mechanical properties of a hydrogel by increasing the density of the gel and the crosslinks. RGD modified chitosan promotes cell adhesion to allow for cell association.[43]

Chitosan can form hydrogels through ionic or covalent bonding. When high crosslinker concentrations are used the molecule diffusion rate through the gel decreases.[35] If cells are not able to diffuse through the gel the tissue won't heal. Chitosan derivatives and blends have been gelled through UV irradiation and thermal changes.[35] Once a gel is formed, it

is degradable by enzymes *in vivo* for which degradation kinetics are inversely related to the scaffold's degree of crystallinity.

Wang and colleagues formed a collagen/chitosan composite gel crosslinked with β-glycerophosphate. The gels only form when they are elevated to body temperature, meaning they have the potential to be used as an injectable scaffold.[44] The composite gel demonstrated gel remodeling and promoted cell survival with the collagen component showed an osteogenic effect.[44] Human bone marrow stem cells in the chitosan only model remained spherical for three weeks indicating they maintained their stem phenotype without the added collagen component.[44] This material could be used as an injectable in situ forming gel for tissue repair or even as a drug delivery system.[44]

For our experiment we chose to use a chitosan solution crosslinked with both ionic and covalent bonds. Covalent bonding helps to strengthen the gel mechanically through irreversible bonds. These bonds don't break down *in situ* so without the balance of weaker ionic bonds the gel would be a permanent implant. Ionic bonding allows the gel to break down and release bioactive ions to induce bone healing. We chose to use sodium phosphate as a bioactive ionic crosslinker because it aids in bone mineralization.[45] Sodium carbonate is a non-bioactive ionic crosslinker that we chose as our control because it formed a gel mechanically similar to the phosphate gels. Genipin was used as a non-bioactive covalent crosslinker to enhance the mechanical strength of both gels.

2. Materials and Methods

2.1 Materials

Low molecular weight chitosan, disodium phosphate, genipin, alizarin red, and

cetylpyridinium chloride (CPC) were purchased from Sigma Aldrich. Sodium carbonate was obtained from the Resource Recovery Center at the University of Missouri. Mesenchymal stem cells were purchased from ATCC. The picogreen assay was acquired from Thermo Fisher Scientific. MTS and alkaline phosphatase assays were secured from Abcam.

2.2 Gel Formation

Chitosan powder was dissolved in water at 1.4% wt/v in a 0.5% acetic acid solution. Once dissolved the mixture was vacuum filtered through a grade 1 filter. Disodium phosphate, genipin, and sodium carbonate were all dissolved in distilled deionized water (ddH_2O). The solutions were made so the final solution for each crosslinker was in a ratio either 15 or 5 disodium phosphate or sodium carbonate molecules and 0.327 or 0.082 of genipin per chitosan site. The crosslinking solutions were made separately and then the phosphate or carbonate solution were combined with the genipin before being added to the chitosan. The resulting four chitosan hydrogel formulations are shown in **Table 2**.

Table 2. Ratios of sodium phosphate or sodium carbonate and genipin to chitosan crosslinking sites

	Phosphate or Carbonate 15	Phosphate or Carbonate 5
Genipin 0.327	15:0.327:1	5:0.327:1
Genipin 0.082	15:0.082:1	5:0.082:1

Once all solutions were prepared the appropriate amounts of crosslinking solutions were placed in a microcentrifuge tube before being 0.2 mL or 200 μL were pipetted into 1

mL of the chitosan solution. The gel was then mixed for 15-20 seconds with a vortex at its highest setting. This solution was allowed to sit over night before being used. The resulting gels can be seen in **Figure 3**.

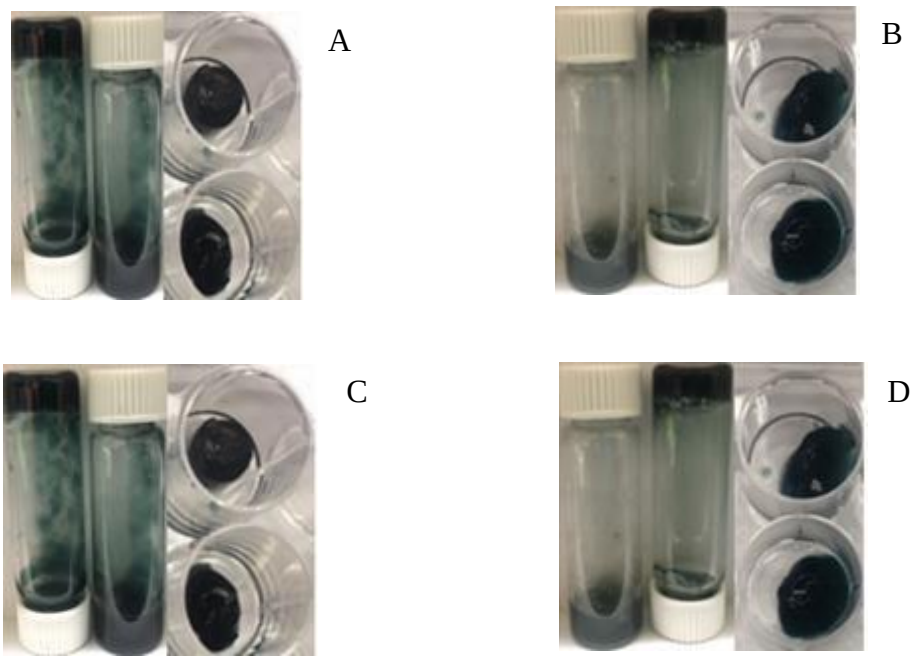

Figure 3. Phosphate and Genipin crosslinked chitosan hydrogels. (A) 15:0.327:1 (B) 15:0.082:1 (C) 5:0.327:1 (D) 5:0.082:1

2.3 Hydrogel Compressive Strength Assessment

Chitosan gels were prepared as stated above except in straight necked vials to allow for easier removal. The gels were isolated by a flexible plastic spatula to keep them intact and were placed in the center of the bottom plate of a parallel flat plate compression testing machine. The top plate was lowered a total of 8 cm over about 5 seconds taking measurements every 5 milliseconds until the top plate met the bottom.

2.4 Hydrogel Rheological Property Evaluation

Gels were formed in a 56.9 mm x 91.5 mm x 2 mm rectangle and stamped out to be 2 cm diameter circle, the exact dimensions of the rheometer plates. A serrated plate,

shown in **Figure 4** was used to ensure the gel would not slide against the plate. A frequency sweep was conducted to find the storage and loss moduli as well as the contact angle of the plate.

Figure 4. Haake serrated rheometer parallel plates

2.5 Hydrogel Morphology

The gels were prepared as described above, then frozen at -80°C, and lyophilized for 48 hours to remove water with minimal disruption to the gel structure. The gels were then cut into four pieces to fit on the mounting stubs used in for scanning electron microscopy. Images were taken at two different magnifications to visualize structures on the gel surface.

2.6 Mesenchymal Stem Cell Culture and Seeding

Mesenchymal stem cells (MSCs) from Cyagen were cultured initially in T-75 cell culture flasks from Corning in a solution of Dulbecco's modified Eagle's medium (DMEM) from Invitrogen supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-Strep) both from Invitrogen. The flasks were place in a humidified incubator at 37°C with 5% CO_2 with media changes every 48 hours until the cells were 80% or more confluent. At this point the cells were dissociated from the cell culture plastic using 0.05% trypsin solution from Invitrogen and counted using a hemocytometer. The cells were then either diluted and seeded in another T-75 flask or, after the 5^{th} generation, used for *in vitro* gelation studies.

The solutions were prepared with the same procedure above with the added step of sterilization. The crosslinking solutions were pushed through a 0.22μm syringe filter unit. The chitosan solution was too viscous for this method, so it was sterilized through dry heat sterilization at 170°C for 2 hours then rehydrated to its starting volume using sterile filtered ddH_2O. The gels once mixed were pipetted into a 24 well plate to gel. 0.5 mL of media containing 30,000 mesenchymal stem cells (MSCs) was added to the preformed gels. The study was conducted with time points at 1, 3, 7, and 14 days after which cell health and osteoinduction were evaluated using PicoGreen, MTS, Alkaline Phosphatase, and Alizarin Red.

2.6.1 Cell Proliferation

The Quanti-iT PicoGreen dsDNA assay from Thermo Fisher Scientific was used to determine cellular proliferation. At each timepoint the remaining media was removed and replaced with a 1% solution of Triton X-100 from Sigma-Aldrich followed by three freeze-thaw cycles to lyse the cells. The lysate was dilute using TE buffer and mixed with the PicoGreen reagent according to the manufacturer's protocol. A plate reader was used to excite each sample at 480 nm and the fluorescence emission was measured at 520 nm then compared to a MSC standard curve (0-200,000 cells).

2.6.2 Cell Viability

The MTS Cell Proliferation Colorimetric Assay from Abcam was used to evaluate cell viability at each time point. The MTS reagent was added to the media (30 μL per 0.5 mL) then incubated for 4 hours in a humidified incubator at 37°C with 5% CO_2. At the end of 4 hours the absorbance of each sample was measured at 490 nm.

2.6.3 Alkaline Phosphatase Activity

Alkaline phosphatase activity (ALP) of the cells was quantified using the Alkaline Phosphatase Assay from Abcam. 20 µL of the same lysate from above was combined with 50 µL of p-nitrophenyl phosphate (pNPP) in assay buffer. The mixture was allowed to sit away from for 1 hour at room temperature. 20 µL of stop solution was added at the end of 1 hour and the absorbance was measured at 405 nm. 1% Triton X-100 was incubated with pNPP along with the other samples and stop solution was added at the end of 1 hour. The absorbance of the triton solution was deducted from the sample's absorbance to eliminate and background effect. The absorbance was then converted to dephosphorylated p-nitrophenyl (pNP) using a pNP standard curve (0 - 20 nmol) which was dephosphorylated using excess ALP Enzyme. The ALP activity was then reported as the pNP content after normalizing the data for cell count.

2.6.4 Mineralization Assay

At each time point the mineral deposition was measured using an alizarin red assay. Media was removed, and the sample was fixed with 70% ethanol for 24 hours. The ethanol was then removed and replaced with 1 mL of an alizarin red solution (Sigma-Aldrich) for 10 minutes. The samples were then washed with ddH_2O 5 times to ensure all non-absorbed stain was removed. The stain that had been absorbed was then desorbed using 1 mL of 10% cetylpyridinium chloride (CPC) solution from Sigma-Aldrich. The absorbance of this solution was then measured at 550 nm using a plate reader. A standard curve linear range (0-0.274 mg/mL) was used to convert the absorbance of each sample to the concentration of alizarin red. The samples were diluted with CPC if they fell above the linear range. The mineral deposition was

calculated by subtracting the absorbance of acellular gels from the cellular gels exposed to the same conditions, then the results were normalized by cell count.

3. Results

3.1. Hydrogel Compressive Strength Assessment

Over all the results (shown in **Figure 5**) were very consistent. The phosphate crosslinked gels were found to possess lower ultimate strength than the carbonate crosslinked gels. The difference was 4 - 5 times between the comparable gels, however the ultimate strength at the point of failure for the strongest carbonate gel and phosphate gel were found to be 1.509 kPa and 0.495 kPa, respectively.

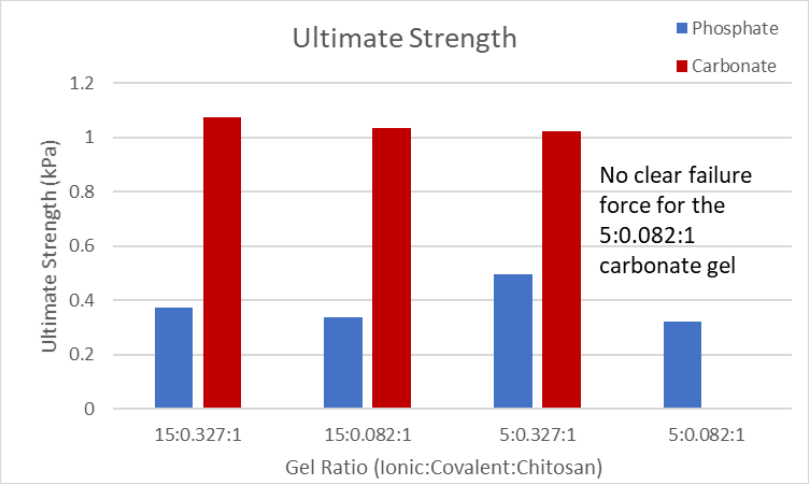

Figure 5. Ultimate strength of phosphate crosslinked gels compared to equivalent carbonate crosslinked gels

3.2. Hydrogel Rheological Property Evaluation

The data shown in **Figure 6** shows that the less crosslinker used in the solution correlated to the gel possessing greater liquid-like behavior.[26] The storage modulus was quite similar for all ratios, however the loss modulus and the phase angle varied through the frequency sweep. They appear parallel in all cases but shift from linear to curved as the frequency increases, which is where we can see more of the liquid-like behavior. The storage modulus (G') is seen to be greater than the loss modulus (G"). This shows that the material overall behaves as a solid.

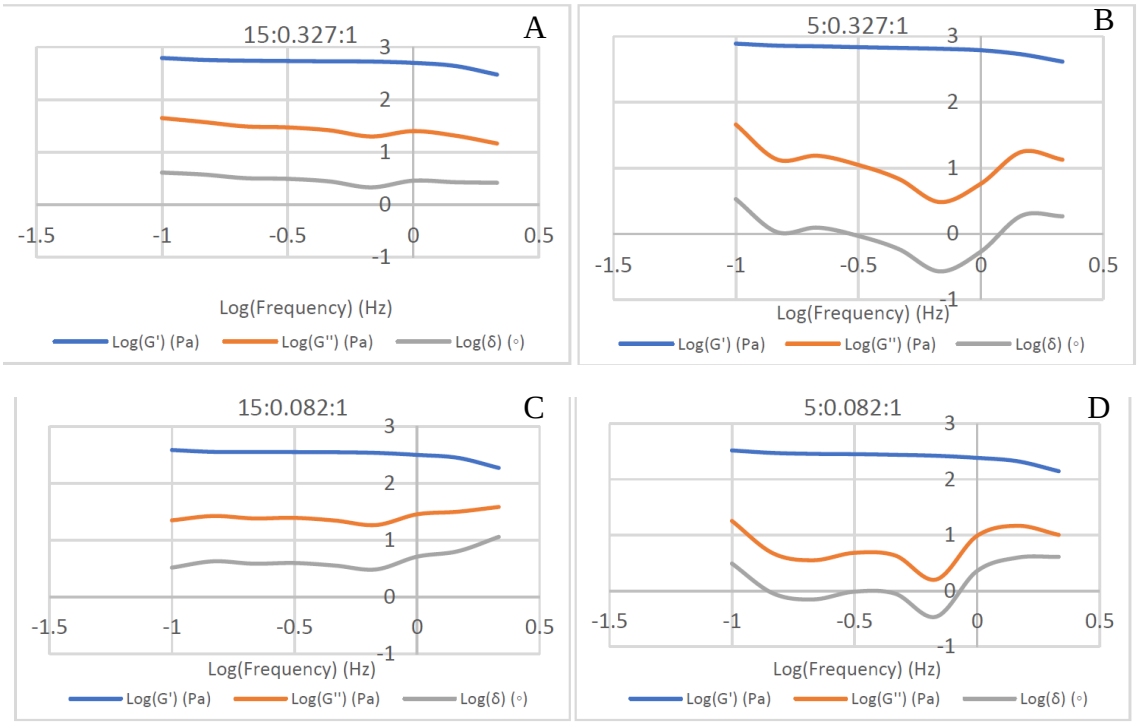

Figure 6. Storage moduli (G'), loss moduli (G"), and contact angle of four phosphate/genipin ratios collected using a frequency sweep (A) 15:0.327:1 (B) 5:0.327:1 (C) 15:0.082:1 (D) 5:0.082:1

3.3. Hydrogel Morphology

The phosphate gels appear to have a ribbed surface texture with limited microscopic pores on their surface. It is unclear if these features are characteristic of the gel or from the sample preparation. The white substance on the surface was found to be composed mostly of sodium deposits found through completing EDS while taking SEM images. These images can be seen in **Figure 7**.

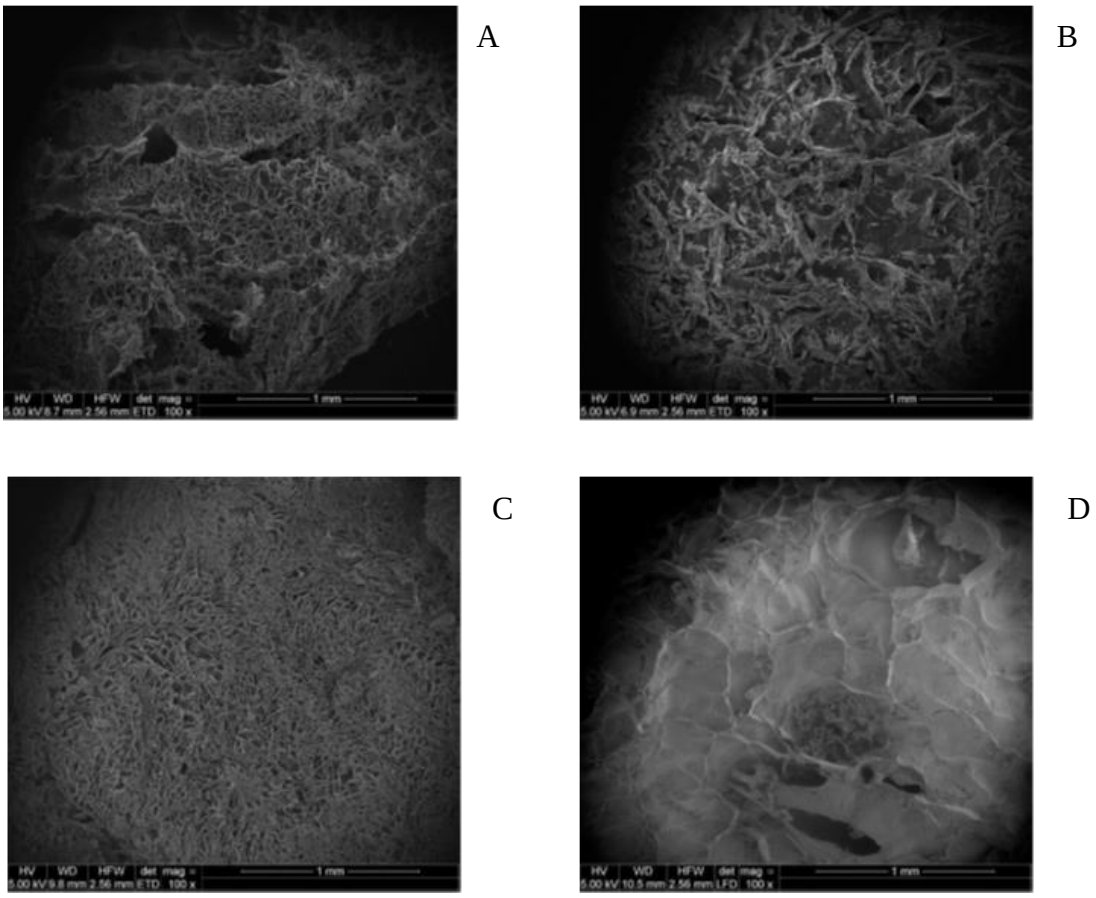

Figure 7. SEM images of phosphate and genipin crosslinked chitosan hydrogels. (A) 15:0.327:1 (B) 15:0.082:1 (C) 5:0.327:1 (D) 5:0.082:1

The carbonate gels appear more flakey and delicate in nature than the phosphate gels. Macroscopically the carbonate-crosslinked gels appeared much more sponge-like than

the phosphate-crosslinked gels which seemed to better hold their shape. The carbonate gels were also prone to falling apart when handled after lyophilization.

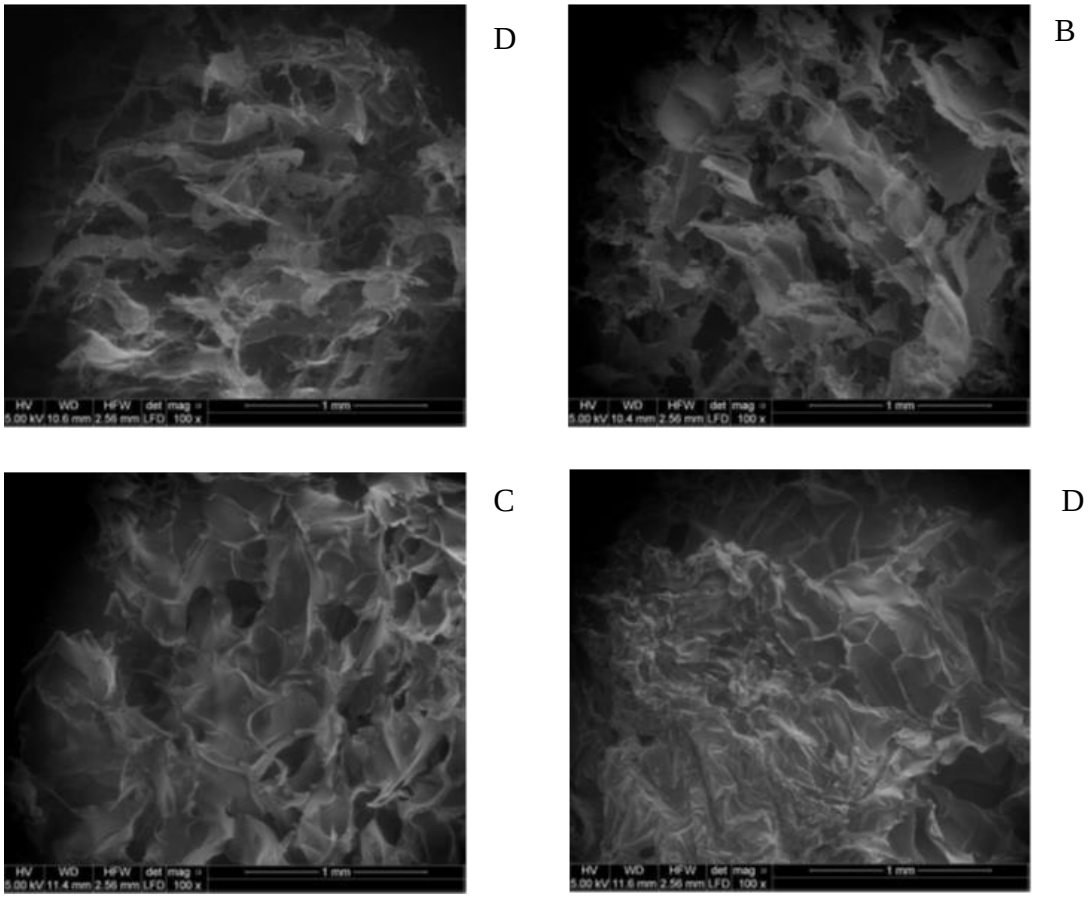

Figure 8. SEM images of carbonate and genipin crosslinked chitosan hydrogels. (A) 15:0.327:1 (B) 15:0.082:1 (C) 5:0.327:1 (D) 5:0.082:1

3.4. Mesenchymal Stem Cell Viability and Differentiation

Picogreen, MTS, alkaline phosphatase, and alizarin red were run for each time point. These assays revealed no usable cell data. When analyzing the picogreen background values were very close to the experimental values for every time point which likely indicates the cells had not able to adhered to the gels. Because of this the MTS, alkaline phosphatase, and alizarin red experiments could not provide useful data.

The cell culture was completed through three varying methods. With all methods there was significant cell death within the first 24 hours. The first method attempted involved seeding the cells in a three-dimensional scaffold within the gel and placing media on the gel (**Figure 9A**). We believe the cell death occurred because the media had to be removed from the cells and replaced with ddH_2O to ensure the gel properties remained as similar to acellular gels as possible. The gels would not only be in unfavorable conditions from the removal of media, but also because of the lack of ions in the ddH_2O. Second, we attempted to seed the gels on the tissue culture plastic before the gels were added. However, this also required the removal of media from the cells while the gels formed within the wells. We believe this was the initial cause of cell death. Once the gels were formed media was placed on the top (**Figure 9B**). The media may not have been able to reach the cells due to being absorbed by the gel causing a nutrient deficiency. If no solution was reaching the cells, then that leads us to believe no waste was being removed from the cells either. Third, we attempted to seed the cells on top of formed gels (**Figure 9C**). We believe the gels were absorbing the media and since gels do not tend to adhere to non-modified chitosan the cells would lack nutrition and a place to adhere, so any surviving cells may have been removed in the first media change.

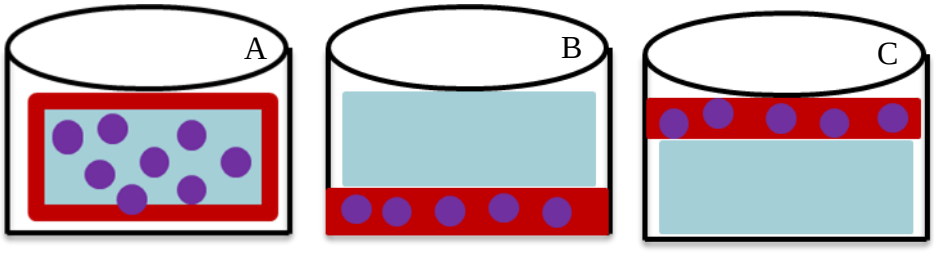

Figure 9. Mesenchymal stem cell seeding methods. (A) 3-D scaffold (B) Cells on tissue culture plastic and gel formed on top (C) Cells seeded on top of formed gel.

4. Discussion

Synthetic and natural polymers have been investigated for a variety of biological applications. Many of the synthetic hydrogels do not readily degrade through natural biological means and cannot be replaced with regenerating tissues. This makes them more ideal for permanent scaffolds and slow release drug delivery systems. Natural polymers tend to form weaker gels that do not hold up well to physical forces within the body. This can be advantageous for fast release of drugs and ions, but in most cases they do not hold their shape well enough to be used for load-bearing applications.

Phosphate can be used as an ionic crosslinker with chitosan, a biocompatible, non-toxic, and FDA approved polymer. These ionic links are very weak, which can be partially mitigated through the addition of a covalent crosslinker like genipin. Even with the addition of a covalent crosslinker, however, the hydrogels formed are still very weak and cannot withstand significant force and were several thousand times weaker than what is needed for bone applications. Rheological data showed as the crosslinking ratios decrease the strength of the gel decreases as well. The resulting gels were also found to have minimal cell compatibility requiring the need for further modification to be used for biological applications.

Gao and colleagues found that a gel with 2% wt/v chitosan and 5.5 mM genipin produced a gel which allowed for the greatest cell adhesion and spreading.[46] By comparison, gels with other concentrations of chitosan and crosslinked caused some level of biological incompatibility.[46] An increase in the hydrophobicity of the gel caused by an increase in genipin could lead to better cell adhesion by promoting

interactions between the scaffold and the cells.[46]

4.1. Implications and Suggestions for Future Research

Cellular nanocrystals have recently been integrated into chitosan/phosphate/genipin gels to try to increase gel mechanical stability. CNC surface charge can be modulated to either allow or prevent participation in electrostatic crosslinking. The cell adhesion properties may be mitigated by utilizing a more hydrophobic filler or through the inclusion of cell adhesion peptide sequences like RGD.

With such a significant difference in mechanical stability between the chitosan hydrogels created and natural bone tissue, (about 5,000 times difference in ultimate strength) it may be most effective to explore alternative materials for this application. There are several polymers that have been synthesized that may be able to withstand the mechanical forces of vertebral bone, but be altered to minimize their toxicity or uncontrollable degradation. A natural and synthetic polymeric composite material has the potential to balance the biological and mechanical properties of a hydrogel. Nano-fibers can also be used to strengthen a hydrogel by acting as pillars to hold the polymers in place. A challenge with the nano-fibers is synthesizing consistently sized fibers with ends that will bond with the polymer. There is also the challenge of balancing the degradation of the gel with the regeneration of tissues. In order to improve the mechanical properties of chitosan hydrogels, Wang and colleagues added chitin nano-whiskers to their chitosan/β- glycerophosphate hydrogel. They also were able to achieve a more gradual drug release rate due to the material's higher crosslinking density. The nano-whiskers had minimal effect on cell viability

indicating that adding gel-stiffening fillers are a good solution for enhancing the mechanical properties of hydrogels.[47]

5. Conclusion

Hydrogels have great potential to be utilized in biological applications. However, their weak mechanical stability leads to few practical applications. Some synthetic hydrogels have a high ultimate strengths, but either do not degrade naturally or are toxic when they degrade. We attempted to use covalent crosslinking paired with ionic crosslinking to create a biocompatible chitosan hydrogel with the ability to withstand the forces experienced by vertebral bone to be utilized to repair vertebral compression fractures. While the ultimate strength could be enhanced by changing gel variables, it did not increase enough to keep its structure when put under significant stress. Also, the gels did not show the biocompatibility or osteogenic differentiation behavior we had hoped.

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