Abstract

Bone tissue is one of the deepest tissue in the human body. Because of this, the cellular mechanisms occurring within the bone still remains a mystery. One way to better understand the inner workings of the bone is to perform studies *in vitro* through the use of bone tissue engineering. Although bone tissue engineering has aided in the uncovering of many mechanisms that are involved in bone remodeling, the current bone tissue scaffolds used still fails to encapsulate the complexity of the bone microenvironment. Since bone cells are known to be responsive to the surface morphology during the bone remodeling process, a scaffold that comprehensively captures the natural environment of the bone may give us a better insight as to how bone cells interact and react.

To achieve this, a bone tissue scaffold was fabricated that successfully captures the complexities of the bone. The scaffold was derived from demineralized bovine bone and sectioned into thin sheets. These sheets were then manipulated and weaved into a strip before osteoprogenitor cells were seeded. The strips were then rolled using an aluminum rod and cultured in osteogenic media. The resultant *in vitro* bone model is further tested to verify if the inner and outer space can be isolated. This will allow for a more comprehensive study that accounts for the diffusion of nutrients throughout the bone.

The successful fabrication of this *in vitro* bone model using demineralized bone sheets will allow for more in-depth studies on bone cells. The bioactive and translucent nature of this model will allow for more comprehensive studies and imaging of bone cells. Although the fabricated bone model shows promise as a potential candidate for bone tissue engineering, further studies must be conducted to verify the usability and to characterize this bone model.

Acknowledgements

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ECM	Extracellular Matrix
PEG	Poly(ethylene glycol)
DBM	Demineralized bone matrix
OPG	Osteoprotegerin
RANK-L	Receptor Activator of nuclear factor- κB ligand
RANK	Receptor Activator of nuclear factor-κB
DBS	Demineralized Bone Sheets
PVA	Polyvinyl Alcohol
PDMS	Polydimethylsiloxane
PBS	Phosphate Buffer Solution
ELISA	Enzyme-linked Immunosorbant Assay

Introduction

Today, there are over 55 million Americans over the age of 50 who suffer from osteoporosis or low bone density. It is predicted that 1 in 2 women and up to 1 in 4 men will suffer a fracture as a result of osteoporosis. With a disease that is so widespread, it is strange that the mechanisms as to why this happens is not fully understood. What is known is that the loss in bone mass occurs when bone resorption, caused by osteoclasts activity, outpaces bone replacement, a result of osteoblast activity. The selective lowering of osteoblast activity is not fully understood, let alone prevented.

There are many contributing factors that make the bone one of the hardest tissues on the body to study. For one, bone tissue is one of the deepest tissues in the body. This makes it hard to reach and even harder to study. Due to the limitations of current imaging technology, it is impossible for the bone to be imaged while in a live patient. Therefore, it is still not known how bone cells behave within the live bone. Another reason bone tissue is so hard to study is because of the complexity within the bone. The bone is made up of two main types of bone, cortical bone (hard bone) and trabecular bone (spongy bone). Trabecular bone is highly vascularized and has a mesh-like structure to provide structural support while leaving space for bone marrow. Cortical bone is the dense, opaque bone that provides the majority of the structural support of the skeletal system. However, despite its dense nature, the cortical bone is more vascularized than one might think. The structures within the trabecular bone include the Haversian Canals, Volksmann's Canals, and osteons (Figure 1). The combination of these two factors are the main reasons why bone cells are so difficult to study.

Due to this immense difficulty to study bone tissue, researchers have developed ways to study bone *in vitro*. This spurred a field known as bone tissue engineering where researchers mainly focused on developing methods to regrow bone tissue and to gain a better understanding of the bone in a controlled lab setting.

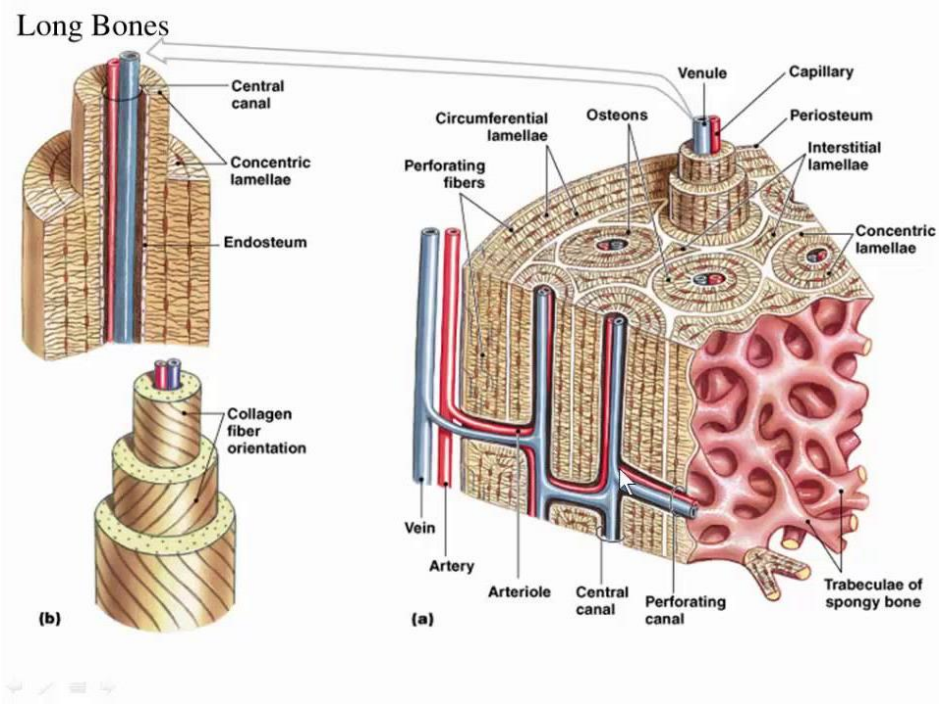

Figure 1. Anatomy of cortical bone. a) A detailed illustration that highlights the different structures within the cortical bone including the osteons, blood capillaries etc. b) An illustration focusing on the anatomy of the osteon. Picture Credit: anatomywiki101.com

Tissue engineering as a field of study has 3 main components: the cells, the growth factors, and the scaffold. All 3 of these factors can be independently studied to advance the field. In bone tissue engineering however, most work has gone into the development of a scaffold. The scaffold provides an initial structure to which the bone cells can reside in. This structure forms the foundation to which the cells, most likely stem cells, are grown in and the resultant tissue often takes up the shape of the initial scaffold. In bone tissue engineering however, the scaffolds used vary a significant amount as there is no one definitive scaffold that is used for all bone

tissue studies. The goal of the scaffold in bone tissue engineering is to mimic the extracellular matrix (ECM) as close as possible. The mimicry of the ECM allows for the attachment and differentiation of the cells within the scaffold (Wu et al., 2014). This is especially important in the study of bone cells as they are extremely sensitive and respond greatly to surface topology of a scaffold (Geblinger et al, 2010). Commonly used scaffolds for *in vitro* studies of bone tissue include hydrogel scaffolds using natural materials such as gelatin or fibrin, synthetic hydrogel scaffolds using polyethylene glycol or polyurethane or by using 3D printing (Scheinpflug et al., 2018). To further elevate the viability of the scaffolds, hydroxyapatite is used. The introduction of hydroxyapatite aids in cell adhesion and osteogenesis (Meskinfam et al., 2018).

The use of natural materials for the synthesis of a bone tissue engineering scaffold is a path that is often explored. By using natural materials, it is almost ensured that the scaffold produced will not be cytotoxic. With regards to bone tissue engineering however, it is paramount that the scaffold developed achieves a significant level of bioactivity. With the overarching goal of bone regeneration, it is important to highlight the inherent bioactivity when proposing the use of a bone tissue scaffold. One example of a natural material used is chitosan. This material is a sugar derived from the shells of lobsters, crabs, and shrimp. Chitosan is an extremely bioactive material and is commonly used in wound dressings. To create a viable scaffold for bone tissue engineering, chitosan can be made into a nanofiber via electrospinning (Mohsen et al., 2019). This nanofiber is then used to form biofilm where bone cells can be grown on. The porous nature of the resultant film coupled with the inherent bioactivity of chitosan promotes the differentiation and proliferation of bone cells *in vitro*. Another material that is highly explored for its use in bone tissue scaffolds is silk firoin (Nisal et al., 2018). Derived from the cocoon of *Bombyx mori*, this natural polymer is highly bioactive as has shown to promote regeneration in bones,

ligaments, and cartilage. Moreover, silk fibroin is a very durable material as it can withstand a large amount of stress and strain. Its thermo-mechanical stability coupled with the biocompatibility makes silk fibroin an extremely attractive material for bone tissue scaffolds. One way silk fibroin is made into a scaffold is via the production of silk fibroin spheroid nanoparticles. These nanoparticles are then held in place and fused using a dilute silk fibroin solution, resulting in a porous scaffold that can be further sterilized in an autoclave. These nanoparticles provide a porous yet stable structure for bone cells to adhere to. The spheroid structure also provides the bone cells with a suitable microenvironment to proliferate and differentiate. One final notable material that is commonly used for scaffold production is collagen. This macromolecule makes up approximately 60% of the bone itself and is responsible for the bone's flexibility. The long fibrous nature of collagen makes it extremely flexible and durable to mechanical stresses. Collagen scaffolds are most commonly fabricated by cross-linking collagen molecules to form a solid matrix. This provides sufficient surface area for bone sells to adhere to while maintaining a porous structure. Collagen scaffolds are bioactive and have shown to possess healing properties for spinal cord injuries (Deng et al., 2020).

An alternative material commonly used for bone tissue engineering scaffolds are polymer hydrogels. Made largely of hydrophilic cross-linked monomers, polymer hydrogels possess many characteristics that are not present when using natural materials. For one, synthetically manufactured hydrogels have improved consistency and has a lower variability when mass produced (Short et al., 2015). Furthermore, synthetic hydrogels give a higher degree of tunability in controlling characteristics such as pore size, degradation, and degree of cell binding (Scheinpflug, 2018). Poly(ethylene glycol) (PEG) is a polymer commonly used to make synthetic hydrogels. Besides providing a uniform and porous scaffold, hydrophilic polymer

scaffolds such as PEG allow for the incorporation of other substances ranging from small peptides to signal proteins (Shin et al., 2001). The incorporation of these substances allows for the increased cell adhesion to the scaffold without significantly altering the structure of the scaffold. With synthetic scaffolds, researchers are limited only by creativity as PEG is just one of many polymers or polymer combination that has been used. Different polymer combinations can be used to tune the properties of the scaffold such as thermal sensitivity, structural stability or, if implanted, time needed for degradation. One limitation to synthetic hydrogels, however, is the relatively lackluster bioactivity compared to their naturally derived counterparts.

From a manufacturing perspective, 3D printing has been widely explored for use in bone tissue engineering. The most notable impact of 3D printing is on the treatment of bone defects. Current treatment of bone defects involves the introduction of a calcium phosphate-based ceramic. The stiffness and inherent bioactivity make this material suitable to both provide structural support as well as encourage bone regeneration. The biggest drawbacks to current clinical methods, however, lie in the scalability and flexibility of such scaffolds and grafts. To combat this, researchers have taken advantage of 3D printing to manufacture these bone grafts. This method of graft fabrication addresses the issue of scalability and flexibility of traditional ceramic grafts. Moreover, the use of 3D printing allows for more robust designs and will increase the overall treatable population. One example of this was a group of researchers who developed a form of "ink" that is largely made of hydroxyapatite and supplemented with polycaprolactone to allow for the rapid hardening of the material (Jakus et al., 2016). The result of this was the successful application of 3D printing to create bone scaffolds and grafts with a high structural integrity as well as excellent bioactivity. Moreover, the material possessed the flexibility that was missing from the current ceramic grafts used today. Although 3D printing is

less widely explored to synthesize lab scale *in vitro* tissue scaffolds, it is definitely a versatile and viable method to cater to more sophisticated and precise scaffold designs.

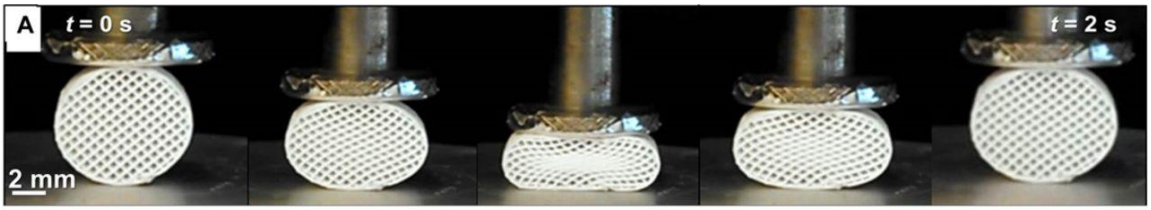

Figure 2. Demonstration of structural stability of 3D printed "hyperelastic" bone. A) Sample of 3D printed "hyperelastic" bone scaffold undergoing mechanical compression and returning to its original shape, thus showcasing the structural flexibility of 3D printed bone grafts. (Jakus et al.,2016)

As stated earlier, one of the main issues with synthetic polymer hydrogels is the relatively lower bioactivity compared to their naturally derived counterparts. To combat this limitation, hydroxyapatite is incorporated into the hydrogel scaffold to increase the bioactivity of the scaffold. Derived from natural bone, hydroxyapatite is responsible for the structural integrity and strength of the skeletal system. Hydroxyapatite incorporated into a hydrogel scaffold is shown to increase bone cell adhesion, differentiation, and osteogenesis as a whole (Fu et al., 2012). There are many ways for hydroxyapatite to be incorporated into the scaffold. One way is via mixing hydroxyapatite powder into the polymer solution before fabrication of the scaffold (Fu et al., 2012). Doing this will cause hydroxyapatite particles to be embedded into the polymer matrix and thus give the scaffold more structural strength as well as a higher bioactivity. Another way to incorporate hydroxyapatite is via chemical means. To do this, a fully synthesized scaffold is submerged in solutions containing calcium ions followed by solutions containing phosphate ions (Meskinfam et al., 2018). Instead of incorporating hydroxyapatite within the polymer matrix, this chemical method aims to coat the scaffold in a thin layer of hydroxyapatite, thus ensuring a more

even distribution. Though it is not determined which method is best to achieve a desired amount of bioactivity, both methods do amount to a significant nominal increase in osteogenesis of cultured bone cells.

Similar to hydroxyapatite, other calcium compounds also have the ability to promote and accelerate osteogenesis. One such example is the use of calcium titanate micro sheets as a base for a scaffold (Cheng et al., 2020). One key difference with this scaffold compared to the previous scaffolds discussed is that calcium micro-sheets do not form a matrix. Instead, this scaffold relies on the formation of titanium micro-sheets via treatment with sodium hydroxide. This is followed by submerging the micro-sheets in saturated calcium hydroxide to attain calcium titanate. One advantage of this method is the ability to precisely control the amount of calcium added by adjusting the concentration of calcium hydroxide used.

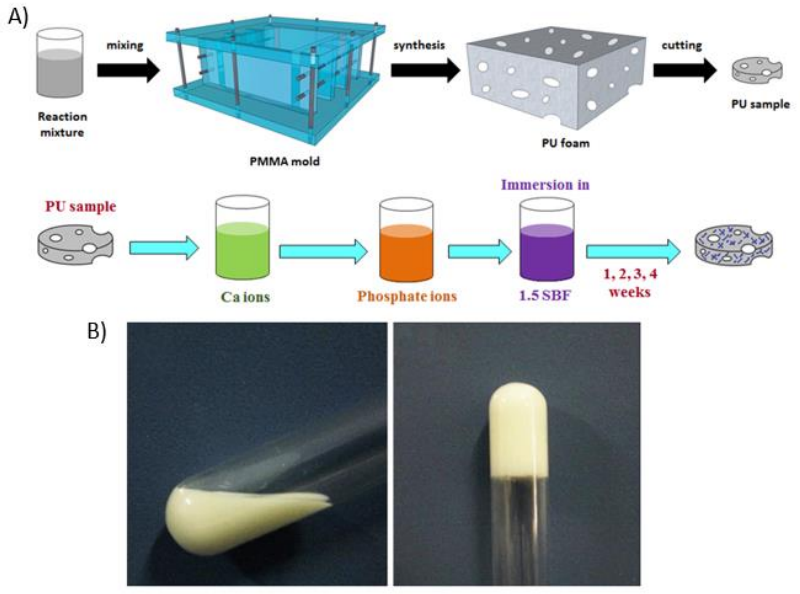

Figure 3. Various methods hydroxyapatite can be incorporated into bone tissue scaffolds. A) Chemically incorporating hydroxyapatite into the scaffold by submerging polyurethane scaffold in separate solutions of calcium and phosphate ions to create a layer of hydroxyapatite (Meskinfam et al.,2018). B) Physically mixing powdered hydroxyapatite into the polymer solution before scaffold fabrication (Fu et al., 2012).

Besides calcium compounds, another substance that increases the rate of osteogenesis is demineralized bone matrix (DBM). DBM is produced by removing the calcium compounds from the bone. This leaves the collagen matrix behind as well as releasing the bone morphogenic proteins found within the bone (Blum et al., 2004). This increases the rate of differentiation of osteogenic progenitor cells and leads to a greater rate of bone repair. This property of DBM has led to its common use in the medical field as an additive to promote faster healing of bone tissue post operation.

At a glance, the ideal scaffold for bone tissue engineering must sustain bioactivity, have non-cytotoxic properties, be structurally stable, and promote osteogenesis. Although scaffolds developed thus far do find success following the aforementioned criteria, current scaffolds still fail to capture the complexity of the bone. The presence of blood capillaries, osteons and the uneven surface topology is an aspect of scaffold design that is overlooked in current bone tissue engineering. If the complexity of the bone is well encapsulated and included in the scaffold, it is possible that bone cells and the bone remodeling process can be studied in greater detail as the natural bone microenvironment is captured in a controlled lab environment.

Bone remodeling is the result of the constant breaking down and rebuilding of the bone. For bone tissue to be regenerated, the bone tissue must first be broken down. This ensures that the bone tissue is regenerated evenly and efficiently. The two main cell populations responsible for this process are osteoblasts (bone building cells) and osteoclasts (bone eating cells). The two cell populations are constantly working in tandem to remodel and strengthen the bone (Kapinas et al.).

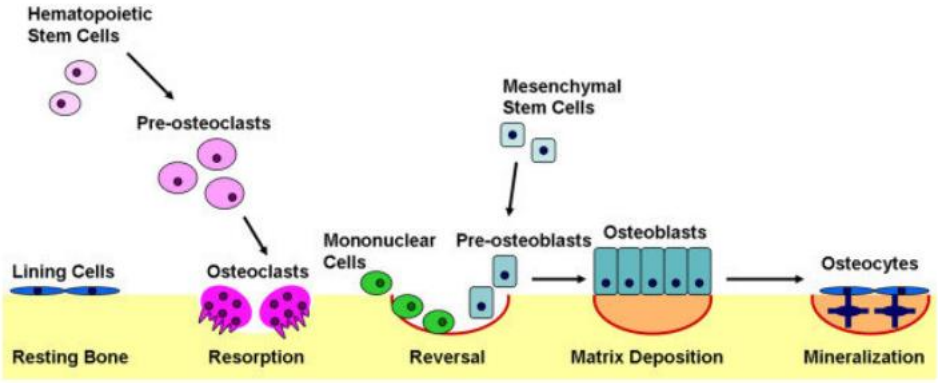

Figure 4. Schematic of bone remodeling process. Simplified schematic that briefly shows the maturation of osteoclasts and how they are responsible for bone resorption followed by osteoblast activity to rebuild the bone. (Kapinas et al, 2011)

Later in life however, osteoblast activity decreases, throwing off the delicate balance of the bone remodeling process, resulting in low bone density and osteoporosis. This makes osteoblasts and osteoclasts the predominant focus in bone tissue engineering studies. To determine osteoblast and osteoclast activity in this study, two secretory molecules in OPG and RANK-L are measured.

RANK-L is a transmembrane protein that is expressed as both a transmembrane protein as well as a secreted protein (Boyce et al., 2007). When secreted, RANK-L binds to RANK found on pre-osteoclasts, triggering osteoclast maturation. Mature osteoclasts with RANK-L bound then proceeds to demineralize and dissolve bone matrix. On the other hand, osteoblasts secrete OPG to counteract osteoclast activity (Rutkovskiy et al., 2016). OPG is an inhibitory molecule secreted by osteoblasts. When secreted, OPG binds to RANK-L, this makes RANK-L unable to bind to RANK and thus lowering osteoclast activity relative to osteoblast activity. The relative ratio of OPG and RANK-L can help determine if the bone tissue is currently undergoing

bone destruction or bone resorption. More specifically, these molecules can serve as an indicator of osteoclast and osteoblast activity.

Considering the strict criteria for successful bone tissue engineering scaffolds as well as the failure of current scaffolds in capturing the complexity of the bone microenvironment, I have successfully developed a novel scaffold in the form of demineralized bone sheets (DBS). This study will focus on the fabrication of DBS as well as its application in fabricating an *in vitro* bone model.

The bone microenvironment consists of natural structures such as osteons and blood capillaries. The randomness yet structured nature of this makes it near impossible to synthetically recreate. Therefore, to capture this complexity, DBS is derived from actual bovine bone. Bovine bone sourced from the nearby slaughterhouse undergoes an acid treatment. This process, known as demineralization, strips the bone off the calcium compounds and leaves behind a predominantly collagen structure. This method of demineralization is similar to how DBM is made for clinical use. Once all the calcium minerals are removed, the resultant demineralized bone is sectioned into thin sheets, hence creating DBS.

DBS is an adequate and robust scaffold for bone tissue engineering. Since DBS is derived from a natural source, cytotoxicity should not be a concern. Moreover, DBS is extremely durable and can withstand mechanical a high degree of mechanical stress. Furthermore, the entire sheet is made of demineralized bone, hence it possesses all the osteogenesis promoting properties of DBM. Lastly, during the demineralization process, the natural topography of the bone, hence successfully capturing the complexity that is missing from all the previously developed scaffolds thus far. Overall, DBS satisfies all the criteria to be used as a bone tissue scaffold while capturing the complexity of the bone microenvironment.

One possible application of DBS proposed here is to create an *in vitro* bone model. Leveraging the complexity that is successfully captured by DBS, it is possible to create a bone model that most accurately mimics the natural bone microenvironment. To do this, DBS are weaved into a long strip. This strip is then seeded with osteogenic progenitor cells (sourced from mice femur) and rolled. The cylindrical shape resembles the structure of the cortical bone and the presence of an inner space allows for the further culture of bone marrow. The semi-transparent nature of DBS also makes for easier imaging of the cultured bone cells. This will hopefully shed light as to how bone cells grow and communicate within the cortical bone.

Methods

Bone Preparation

Bovine femur was sourced from a local slaughterhouse and cut into smaller chunks of approximately 3 inches in height using a hand saw. Excess bone was stored in -80C for future use. The bone chunks were cleaned off as much muscle and fat tissue as possible using a razor blade. The marrow from the bone was also removed and discarded. The bone chunks were treated with a 1:1 mixture of chloroform and methanol for 24 hours to remove excess fat and lipids from the bone.

Bone Demineralization

Once excess lipids were removed from the bone chunks, the bone chunks were further reduced to a smaller size using a hand saw. The bone pieces of approximately 2in x 2in in size was then placed in a container with 300mL of 1.2M hydrochloric acid. The container was then placed into a pressure chamber where the bones in the acid will be subjected to a constant cyclic pressurizing

followed by depressurizing of the system. The system will be pressurized to four bar (+atmospheric pressure) for 10 seconds followed by a depressurization for another 10 seconds. This cycle of constant pressure and depressurization was carried out for 24h before the acid was replaced. The initial layer of demineralized bone was removed using a razor blade to ensure maximum surface area of cortical bone was exposed for the following acid treatments. The bone pieces were then placed back into a fresh batch of acid solution, the container was placed back into the pressure chamber and run for 48h. The acid solution was replaced every 48h until bone pieces were fully demineralized. To assess the extent of bone demineralization, the bone pieces were placed in a beaker of distilled water for up to 12h. This will aid in the visualization of the demineralized layer. A typical batch of bone pieces take approximately seven days (one cycle of 24h followed by three cycles of 48h) of pressurized acid treatment to fully demineralize.

Preparation of DBS

To fabricate DBS, demineralized bone pieces were embedded in optimal cutting temperature medium and frozen at -20C. Once solid, the bone was then cut into thin sheets using a Cryostat (Cryostar NX70, Thermo Scientific). In this step, bone sheets of varying thickness could be produced. For this study, bone sheets of 20um were made. The DBS were then stored in distilled water in 4C for later use.

Manipulation of DBS

To manipulate the DBS such that it is possible to make a long strip for rolling, the DBS must be fixed in place to allow for the DBS to be cut. This was done by arranging the DBS in a flat tray and submerging them in a 1% solution of PVA and leaving it out for 24h to dry. Once the PVA is dry, the DBS can be cut using a plot cutter (Graphtec-2000 CE). A specific pattern is cut from

the DBS to allow weaving of the DBS. For this study, a strip of 15 DBS was made, and 6 strips were fabricated and rolled.

Fabrication of *in vitro* Bone Model

Fabrication of the bone model was done in a biosafety cabinet under room temperature. The DBS strips were laid out in a sterile tray and held in place using PDMS molds which prevented them from moving about in the tray. An acrylic comb was placed on top of the strips to further hold them in place. The PVA was then dissolved off the strips by adding sterile water to the DBS strips using a pipette. The strips were submerged in water for 12h to ensure the PVA is fully dissolved. The acrylic combs were then removed, and the strips were further washed with sterile water to ensure as much PVA was removed as possible. The DBS strips were then submerged in a 95% solution of ethanol for 12h to sterilize the strips. The sterilized DBS strips were then washed with PBS to remove any excess ethanol. Osteogenic progenitor cells were then seeded onto the DBS strips at a density of approximately 1.5mil cells per strip. The strips were incubated for 24h in osteogenic differentiation media (a-MEM supplemented with 1% penicillin-streptomycin, 10% fetal bovine serum, 10 mM b-glycerophosphate, and 200 µM L-ascorbic acid) to allow for cell adhesion to the DBS. The DBS strips were then rolled using sterile aluminum rods (10mm x 5mm) and cultured for 2 weeks in a 24 well-plate. Osteogenic media was changed every 3 days to ensure sufficient nutrients are present for cell growth and differentiation. After 2 weeks of culture, the rod is removed. Should the bone model fabrication be successful, the resultant structure should be able to remain upright and show structural integrity.

Results

The key factor that separates DBS from other scaffolds is the ability to capture the complexity of the bone. To show the successful preservation of the surface topology of the bone, DBS was imaged under a microscope. To highlight the blood capillaries within the scaffold, the sheets were submerged in a dilute solution of red food coloring for a short period of time. This procedure highlighted the blood capillaries as well as the osteon where osteocytes would reside. As shown in the Figure 5, DBS is significantly vascularized and highly complicated. Moreover, this shows that the demineralization process along with the DBS fabrication procedure did not have a significant impact on the surface topology of the bone, hence achieving the aim of preserving the innate complexity of the bone.

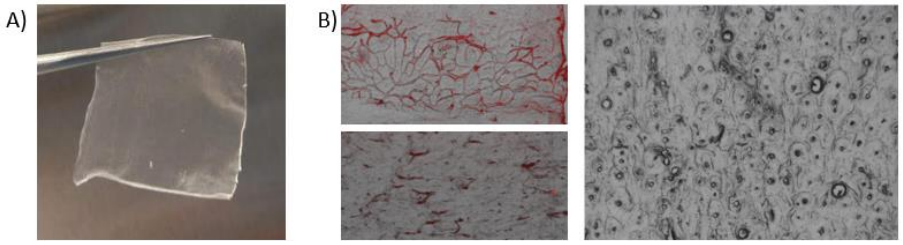

Figure 5. Synthesized demineralized bone sheet scaffold and surface topology of the scaffold. A) Picture of the fully fabricated DBS. The sheet was approximately 20μm and is mostly transparent. B) DBS was imaged using a microscope. The red coloration highlighting the structures on the surface of the DBS was the result of submerging the DBS in a dilute solution of red food coloring.

When treated with PVA, the DBS are easily manipulated and can be cut into intricate shapes and sizes to suit the needs of the experiment. For this study, DBS was to be weaved into a long strip followed by rolling to create the *in vitro* bone model. Although DBS is stable when coated with PVA, DBS becomes extremely hard to work with when submerged in water. It is because of this, that DBS must be coated with PVA before it can be cut. Therefore, the proposed design for a single DBS block is as shown in Figure 6. This design secures the DBS to each other by weaving a 'T' shape into a slit of the next sheet. However, there was an issue with the DBS as

the two sides of the 'T' would fold on itself and slip out of the slit. This would often happen when the DBS strip was submerged in water. To combat this, two additional slits were made towards the side. This secures the two opposing ends of the 'T' shape, thus minimizing the chances of the strip falling apart.

One possible improvement to this design would be to increase the scale of each block. The initial size of the DBS block (approx. 10mm x 15mm) was determined for a small-scale experiment where strips of six would be weaved. However, the scale of the experiment was increased, and this resulted needing six strips of 15 pieces of DBS. This made the weaving step of the DBS strips the most time consuming and tedious. Therefore, one possible optimization for future experiments would be to increase the size of the DBS block. This will aid in the total time needed to prepare DBS strips and thus increasing the efficiency of the experiments down the road.

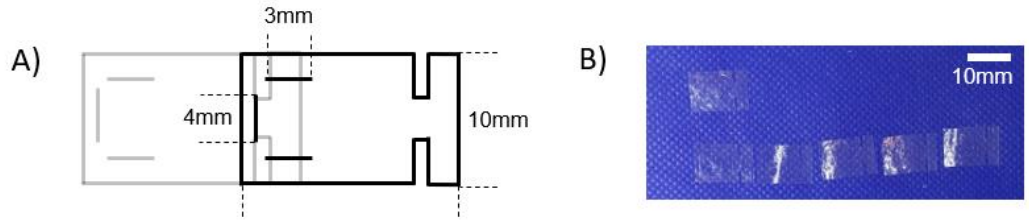

Figure 6. Demineralized bone sheets weaving design. A) Design and dimensions used for each sheet of DBS and how the DBS will be weaved to form a strip. B) How the design looks when applied to DBS coated in a layer of PVA.

As a proof-of-concept, the *in vitro* bone model was first tested in a small scale. To do this, six strips of six DBS were prepared. The strips were placed in a square well-plate and washed with sterile water to dissolve the layer of PVA. The strips were then submerged in 95% ethanol overnight (approximately 10 hours) to sterilize. The strips were then further washed with PBS to eliminate any traces of ethanol left on the strip. Before cell seeding, the strips were laid out to dry. This would prevent the DBS sheets from moving around during the cell seeding

process. The strips were then seeded with osteogenic progenitor cells from DsRed mice femur at a density of approximately 750,000 cells per strip. Osteogenic media was added, and the seeded strips were then incubated for 24h to allow for cell adhesion. The strips were then rolled using a sterile acrylic rod (15mm x 5mm x 5mm). The rolled structure was left to incubate for two weeks with the replacement of osteogenic media every three days. The resultant structure showed some structural stability and was able to maintain its structure even after the acrylic rod was removed (Figure 7A). This demonstration concluded the proof-of-concept experiment as it proved that the rolled structure could be made using the following protocol.

The following full-scale experiment followed the methods of the proof-of-concept test but with a few changes. Firstly, the length of the strip was increased from six to 15 (this was the maximum length that could fit into the sterile tray that will be used for the experiment). Secondly, PDMS molds and acrylic combs were used to prevent the strips from moving about during the PVA dissolving and sterilization step. The PDMS molds also aid in the cell seeding step by containing the growth media within the strip itself. Third, an aluminum rod (15mm x 4mm) was used to roll the strips (Figure 7B). This ensured a more evenly rolled structure as well as making the rolling process easier. Lastly, the rolled structure was initially cultured in a 12 well plate as it is wide enough to accommodate for the length of the rolled DBS (Figure 7C). After two weeks of culture, the rolled DBS was transferred into a 24 well-plate where it was further cultured for two weeks vertically (Figure 7D). After a total of approximately four weeks of

culture, the rod was removed, and the free-standing rolled structure was further cultured for a week free standing.

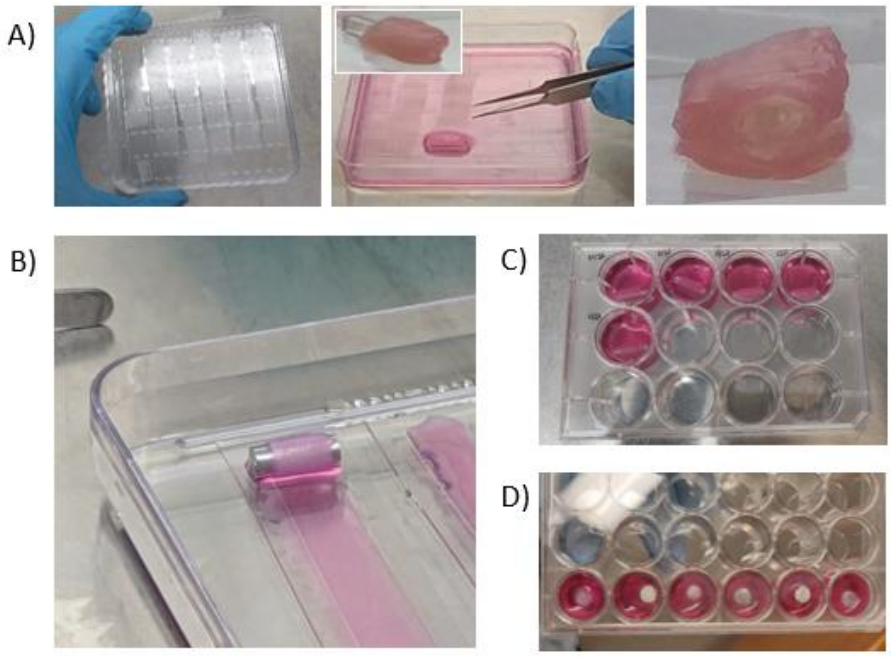

Figure 7. *In vitro* bone model fabrication. A) Proof-of-concept experiment where it was shown that a rolled structure can be successfully fabricated and produces a free-standing structure. B) Using aluminum rod to roll DBS strips post cell seeding. The use of a rod made rolling more even and easier to perform on a longer strip. C) Initial 2-week incubation post rolling. The rolled DBS were first cultured horizontally to prevent the structure from unwinding and collapsing. D) Subsequent vertical incubation prior to rod removal. The rolled DBS were transferred to a 48 well plate and cultured for a further 2 weeks.

One key consideration when proposing the rolled design for the bone model was that it would leave a void inner space that can be isolated from the outer area. This opens up opportunities to create an isolated system on the inside of the one model. To test this, a small amount of beta glycerol phosphate and ascorbic acid was added to the inner space of the rolled DBS structure. Since beta glycerol phosphate and ascorbic acid are key components osteogenic media, the addition of these compounds will lead to an accelerated rate of bone cell

differentiation. Therefore, there will be a disproportionate amount of osteogenesis in the inner space of the bone model compared to the outer layers. To measure this, beta glycerol phosphate and ascorbic acid was added to the middle of the free standing rolled DBS structure. The rolled DBS was incubated for 48 hours and an enzyme-linked immunosorbant assay (ELISA) was performed on the inner and outer space of the rolled DBS. In conjunction with this experiment, aluminum rods of different diameters were used to create rolled DBS structures of differing thickness. Using ELISA, the samples taken from the inner and outer spaces were tested for the presence of RANK-L and OPG. Since beta glycerol phosphate and ascorbic acid was only added to the inner space, it is expected that the inner space would show a higher concentration of OPG and RANK-L.

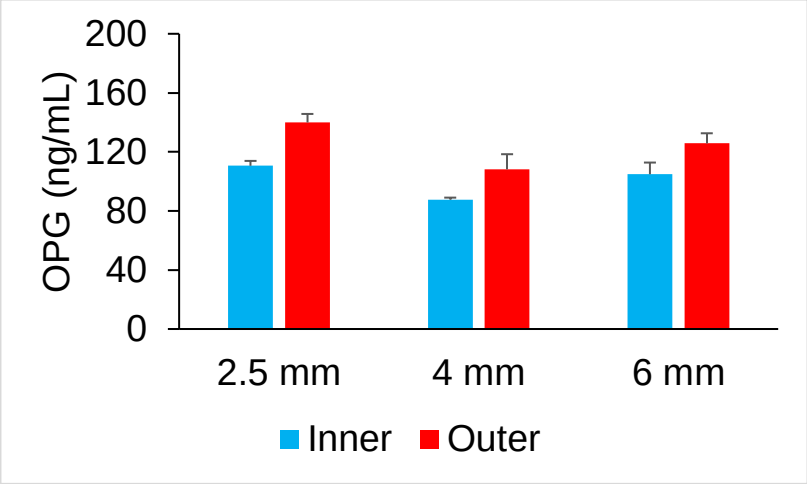

Figure 8. Relative levels of OPG between inner and outer space of rolled demineralized bone sheets. A graph showing the relative concentrations of OPG between the inner and outer spaces of the rolled DBS structure. In this experiment, aluminum rods of different diameters were used to influence the thickness of the resultant rolled structure.

As seen in the Figure 8, there is no observable difference the inner and outer space of the rolled bone structure. Moreover, the difference in thickness did not seem to affect the rate of

diffusion of OPG in any way as the levels of OPG was basically equal throughout the entire rolled DBS structure. It should also be noted that RANK-L was not detected. This makes sense as DBS at its core is a demineralized collagen matrix. Therefore, osteogenesis is expected to lean towards a higher degree of osteoblast bone building activity compared to osteoclast activity.

Originally, it was assumed that a total of three weeks of vertical culture could successfully create a closed system that allows for the isolation of the inner and outer space. However, it seems as though most of the diffusion of material occurs through the bottom of the rolled DBS structure. Thus, it is paramount that the bottom of the rolled DBS be sealed somehow before a true *in vitro* model is created.

Discussion and Future Directions

Given the potential of DBS as a scaffold for bone tissue engineering, DBS could have a wide range of possible applications. This study showed the steps in which DBS is fabricated and loosely applied. This proof-of-concept study showed that DBS can be efficiently fabricated and the rolling application to create an *in vitro* bone model could be further studied. Due to current circumstances being the global pandemic of COVID-19, this study could not be completed as the rolled bone model proposed here was unable to be validated.

During the demineralization step where calcium is removed from the initial bone pieces, the system is constantly pressurized and depressurized, causing the bone piece to experience a constant cyclic hydrostatic pressure. Initial protocols for DBM fabrication did not call for the use of hydrostatic pressure. When hydrostatic pressure was not involved however, the time taken to demineralize an entire piece of bone would range from four to eight weeks. This called for the

need to better optimize the DMB fabrication process. When cyclic hydrostatic pressure was introduced, the time taken to fully demineralize a whole piece of bone was shortened to just five to 6 days. This dramatic improvement allowed for the more efficient production of DBS, which ultimately allowed for the extensive studies performed in the lab. The mechanisms of how cyclic pressure influences the rate of reaction between acid and a solid was not studied but proved to be the most effective and efficient method to demineralize bone pieces.

The rolling of a long strip of DBS was originally proposed as it most closely mimicked the overall structure of the bone. A cylindrical shape is observed as the shape of the osteon as well as the shape of compact bone itself. Therefore, the rolled structure was proposed in favor of the traditional scaffold structure which would involve the stacking of DBS in a well-plate. Though the end goal of this study was to create an *in vitro* bone model, the study could not be completed due to the global pandemic. The inner and outer spaces of the rolled bone could not be isolated, thus invalidating the rolled DBS structure as an accurate bone model. One possible method to isolate the inner and outer space is to seal the bottom of the bone model. It was suggested that majority of the diffusion of substances occurred through the bottom of the rolled structure as it was not deliberately sealed. Therefore, one way to seal the bottom and isolate the inner space is to use agar. The rolled bone structure could be vertically inserted into cooled agar and once the agar is solidified, it will create a seal at the bottom of the rolled DBS structure. Since agar is not cytotoxic, the addition of it would not negatively harm the cells. One possible concern of this method is the temperature as agar has to be heated to a relatively high temperature to get it into a liquid state. Should the rolled DBS structure be put in too early, it could result in a significant amount of cell death. However, this method could be further worked on and optimized to prevent this from happening. Once the bottom of the rolled DBS structure is

sealed, most if not all the diffusion would occur through the DBS layers. Once a significant difference in secretory molecules can be observed, the *in vitro* bone model is successfully fabricated.

With the potential of the rolled DBS idea, it is possible to further expand this model to include other tissues with the aim of creating an even more comprehensive *in vitro* model. For one, a cylindrical piece of trabecular bone could be inserted into the inner space of the rolled model. This will allow for the culture of bone marrow cells and adipocytes. Should the rolled DBS bone model be verified, it would be possible to isolate bone marrow and hence create a separate bone marrow microenvironment. This will allow for the more extensive study of bone marrow and the marrow-cortical bone interface. Another possible direction is to apply the DBS fabrication method to cartilage. With sheets of cartilage, similar to DBS, it would be possible to layer the rolled bone structure with cartilage. This will allow for the further study of bone-cartilage interaction and may give greater insights as to how bone cells interact with the highly porous yet decellularized cartilage. Lastly, the rolled DBS model provides a unique opportunity to study material transport into the bone. Should this bone model be verified, it would be interesting to study the rate of diffusion of nutrients, growth factors and small molecules into the bone. With the translucent nature of DBS and the fact that the rolled model has the ability to be unrolled, imaging and staining protocols can be easily done. This will allow for the determination of the diffusion rate and diffusion patterns of molecules through each layer of DBS. Having an accurate establishment of the diffusion rate through cortical bone could aid in the development of a potential future drug that can be administered locally to repair fractured bone.

To conclude, DBS is a viable and robust material for the synthesis of bone tissue scaffolds. The bioactive and structurally stable nature of DBS makes it suitable as a bone tissue scaffold. Moreover, the fact that it also comprehensively captures the complexity of cortical bone differentiates it from other bone tissue scaffolds that are used today. Though it is unfortunate that the *in vitro* bone model could not be validated this semester, the potential for this bone model is extremely high and thus makes it worth pursuing further in the near future. With further experience and optimizations to the rolling protocol, it is possible to achieve greater success with this model in the future.

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