Formulation of a Bovine Gelatin Cross-Linked Scaffold for Potential Human Tissue Applications: A Patient Specific, Cost-Effective Alternative Treatment of Acetabular Labral Tears of the Hip

Introduction

Acetabular labral tear injuries are attributed to 22% of the human population reporting groin pain. The acetabular labrum lines the rim of the hip socket acting as a cushion for the joint as seen in ***Figure 1.*** It is a ring of cartilage composed of fibrocartilage and dense connective tissue that allows for a wide range of motion in this ball and socket joint, providing strength. The injury can occur through an acute trauma such as a one time violent motion, however it generally occurs as a long term wear and tear injury.

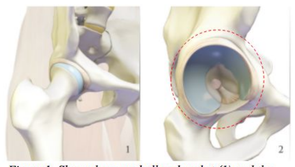


Figure 1: Shown here are ball and socket (1) and the inside of the acetabular cup highlighting the acetabular labrum (2)
Image taken from www.dailybadha.com

Symptoms include pain in the groin, instability in the hip joint, catching, locking or an audible click. Current treatment generally involves a surgical repair, though physical therapy treatment is always attempted first to exhaust all options. When a surgical repair is required, the surgeon performs a "minimally invasive" arthroscopic procedure. The torn labrum of an arthroscopic labral repair is shown in ***Figure 2***. In this case, it is minimally invasive due to the small incisions, however while in the hip two things can be done: refixation using anchors to stitch the torn tissue back to the bone or debridement, or simply just removing the torn tissue. In both cases, the body isn't being given a chance to heal naturally, and with debridement the support of the labrum is just being taken away. Secondly, there has been a direct correlation between current treatments of labral repairs and osteoarthritis, a degenerative joint disease. Recovery time can be extensive, up to six months until the patient is back to normal activities and athletics.

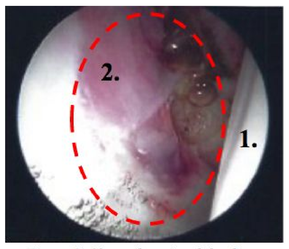


Figure 2: Shown here is a labral tear from inside the hip. The labrum has pulled away from the bone. 1.)Acetabular Cup 2.) Inflamed Torn Labrum
Student Researcher's Arthroscopic Imaging

Research and Justification

Over the last couple of years, a group of orthopedic surgeons lead by Martha Murray M.D. at Boston Children's Hospital have developed a sponge based scaffold as a more proactive treatment for Anterior Cruciate Ligament (ACL) tears. The standard ACL repair is performed on those with the injury by harvesting a graft from elsewhere in the knee and using it to replace the torn ACL. The patient then has to recover from both where the ACL has been repaired and where the graft was taken from. The team wanted to eliminate the common development of osteoarthritis in the knee and timely recovery, so they developed the sponge based scaffold as seen in ***Figure 3***.

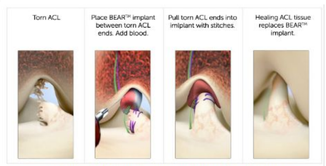


Figure 3: Shown here is the method in which the Bridge-Enhanced ACL Repair works, devising a regenerative technique almost identical for the proposed acetabular labral scaffold being designed through the described research.
Image taken from www.childrenshospital.org

The sponge was first tested in various animals, and then studies on ten human patients were completed. The ACL is a tendon that doesn't mend itself back together like some other tendons. The same goes for the acetabular labrum due to the lack of blood supply in the area. The sponge scaffold is placed between the two torn ends of the ACL and lightly set into place with stitches. Blood is then drawn from the patient and injected into the site, causing a blood clot to form and natural healing to occur. This method has cut the recovery time in half, going from 10-12 months to 5-6 months. Being said, this would do the same for the scaffold being developed for the acetabular labrum. **Recovery time could go from 6 months to 3.** The Bridge Enhanced ACL treatment scaffold is produced with collagen, a higher grade of gelatin, making it not the most reasonably priced treatment, given that 5 mL of collagen can cost upwards of $263. **Using bovine gelatin for the acetabular scaffold** designed through this research **makes it much more cost-effective.**

Parts of research were constructed through interviews with Jerffrey M. Klauser M.D.,Timothy King DPT., and Young-Jo Kim M.D.- Boston Children's Hospital*

Engineering Goal

This research seeks to produce a cost-effective and patient specific gelled sponged based scaffold as an alternative treatment for acetabular labral tears of the hip. This device is produced with a cross-linked gelatin/hyaluronic acid blend rather than collagen due to its high cost. By cross-linking the bovine gelatin/hyaluronic blend, the properties are being altered, making the protein optimal for its proposed placement in the body. The method of implementing the device at the location of the tear and then injecting blood to form a clot to encourage regeneration requires no removal of tissues and allows for the body to heal on its own, which is always the preferred method. The scaffold can also be soaked in the patient's blood and then implemented. The same process is ultimately being performed, keeping the blood and cells in one place to encourage faster regeneration, however this would just be a different approach.
Being that this device is being used instead of complete removal or anchors, the recovery would be less painful and faster for the patient, Secondly, because the body is being allowed to do what it does best, heal, the patient is less likely to develop forms of arthritis, specifically osteoarthritis in the long run.

Formulation of Scaffold Material: Gelatin Base

Bovine gelatin, a polymer, comprised of peptides and proteins in a long chain, is just a lower grade of collagen. The chain is made up of about 18 amino acids that eventually form a triple helix, giving the polymer the ability to gel. It is obtained from partial hydrolysis of the collagen from various animals skin, bones, and connective tissues, commonly those of a cow. Bovine gelatin is rich in proteins and there is no correlation between development of diseases such as Mad Cow Disease with implementation of such structures devices in the body according to a review done by the University of Maribor (2011).

Bovine gelatin bases were first initially tested for relative strength. To be optimal for testing, the gelatin base had to be weaker than the desired outcome knowing that the crosslinkers would make the structure stronger. Gelatin solutions at 1.5,2, 3, 5, and 10% (w/v) were made. For 1.5%, 1.5 grams (2.35 mL) of bovine gelatin powder was weighed using a precise balance scale. Based on a 100 mL solution, the gelatin powder was mixed with 97.65 mL of H_2O, and then placed on a hot plate while being constantly mixed. Once the homogenous mixture was formed,

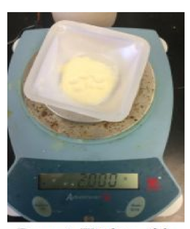


Figure 4: Weighing of the bovine gelatin powder for the 2% gel base.

10ml of the solution was poured into a 100 x 15 mm petri dish, forming a thin layer. The petri dish was placed in a 36℉ sterile refrigerator. Cover the petri dish with a lid to prevent the water from evaporating and drying out the gel. These steps were repeated for the 2, 3, 5, and 10% gels as well, while altering the amounts to fit the percentages. The 2% was 2 grams gelatin (3.133 mL) mixed with 96.87 mL H_2O, the 3% being 3 grams gelatin (4.7 mL) and 95.3 mL H_2O, the 5% being 5 grams (7.83 mL) and 92.17 mL H_2O, and lastly the 10% being 10 grams gelatin (15.66 mL) with 84.34 mL H_2O. Final result shown in ***Figure 5.***

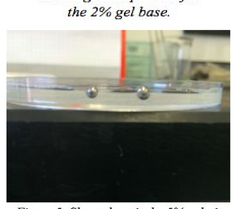


Figure 5: Shown here is the 5% gelatin base in petri dish, un-crosslinked.

Based on physical properties using the eye and common knowledge of proposed outcome, the 10% was far too strong, the 1.5% was too weak, and the 5% and 3% would be too strong after crosslinking. as crosslinking alters the properties and makes the polymer stronger. The 2% base would then be crosslinked with two different crosslinkers, glutaraldehyde ($C_5H_8O_2$) and D,L-glyceraldehyde ($C_3H_6O_3$), to then determine which crosslinker is optimal and by what concentration.

Formulation of Scaffold Material: Crosslinked Gelatin

Using formulae 2% (w/v) bovine gelatin base, solutions of .1, 1, and 10% glutaraldehyde and D,L-glyceraldehyde were developed. For the D,L-glyceraldehyde, being that is came in a powdered form, 3 mL vials were used to prepare 3 10% D,L-glyceraldehyde solutions. Under a chemical fume hood, .283 grams of the D,L-glyceraldehyde powder shown in ***Figure 6*** were weighed via a balance scale. 2.83 mL of a 70% ethanol solution was then added to the vial. To prepare the 1 and .1% solutions of D,L-glyceraldehyde, vial #2 was diluted with 70% ethanol solution 10 times (28.3 mL),and vial #3 was diluted with 70% ethanol solution 100 times (283 mL). Prepared solutions shown in ***Figure 7.***
Being that the glutaraldehyde solution came in the liquid form, 25% in H_2O, no solution was required to be made. However, the initial concentration always had to be multiplied by 4, to make it an 100% solution. 8 mL of the 2% bovine gelatin base solution was poured into 6 different 100 x 15 mm petri dishes. Let the gelatin set for 2 hours in a 36℉ sterile refrigerator , and cover them while they are setting to avoid evaporation. At concentrations of .1, 1, and 10% the crosslinkers were poured individually on each of the set gels. The different scaffolds were prepared as follows:

- .1% Glutaraldehyde: Under the chemical fume hood, 32 µL was extracted from the bottle and mixed with 1-2 mL of H_2O. The solution was then piped evenly across the gel to form a very thin layer. This was done by using a pipette pen as seen in ***Figure 8***.
- .1% D,L-Glyceraldehyde: Under the chemical fume hood, 8 µL was extracted from the bottle of .1% (w/v) D,L-Glyceraldehyde solution and evenly piped across the gel to form a very thin layer. Pipette pen was once again used.
- 1% Glutaraldehyde: Under the chemical fume hood, .32 mL was extracted from the bottle and mixed with 1-2 mL of H_2O. The solution was then piped evenly across the gel to form a very thin layer.
- 1% D,L-Glyceraldehyde: With use of the chemical fume hood, .08 mL of the 1% (w/v) was extracted from the bottle and piped on the gel to form a very thin layer across the gel with a 5 mL pipette and pipette pump.
- 10% Glutaraldehyde: With the use of the chemical fume hood, 3.2 mL was extracted from the bottle of glutaraldehyde solution and mixed with 1-2 mL of H_2O. The solution was then evenly piped across the gel to form a thin layer of solution with the use of a 5 mL pipette and pipette pump.
- 10% D,L-Glyceraldehyde: Under the chemical fume hood, 0.8 mL of 10% (w/v) D,L-Glyceraldehyde solution was extracted from the bottle via a 5 mL pipette and pipette pump and piped onto the gel to form a very thin layer.

Figure 6: Shown here are the two different crosslinkers (Sigma-Aldrich). Large Bottle: 25% Glutaraldehyde in H_2O. Small Bottle: D-L Glyceraldehyde Powder.

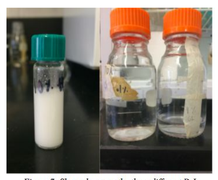


Figure 7: Shown here are the three different D-L Glyceraldehyde solutions. L-R: 10%, .1%, 1%

Figure 8: Pipette pen used to extract and measure the crosslinkers in microliters.

Figure 9: Chemical crosslinking of gels by student researcher under chemical fume hood.

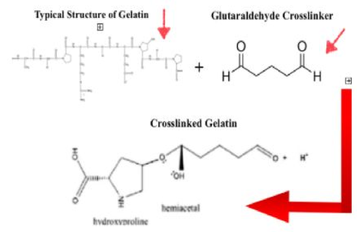


Figure 10: The chemical process of the bovine gelatin crosslinking via glyceraldehyde.

Images courtesy of researchgate.net

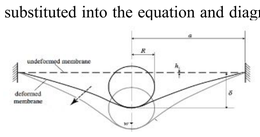
The 6 petri dishes were covered with petri dish covers and placed in a 36℉ sterile refrigerator and left to crosslink for 24 hours. By pouring the crosslinker solutions on top of the gels in an even thin layer, the solutions were able to diffuse into the gelatin polymer and link the chains of one polymer to another as seen in ***Figure 10***.

Mechanical Property Testing via Ball Bearing Assay & ImageJ

Using a known mass ball bearing of 0.689 grams, each of the crosslinked scaffolds were tested for their elastic modulus (*E*). Each labeled petri dish containing the cross-linked gel was placed parallel to an upright piece of graph paper (1cm squares). Using sterile tweezers, the ball bearing was placed gently on top of the scaffold.
Using a camera, a picture was taken. This was repeated for each of the six gels as seen in ***Figure 11***.
Using ImageJ, a image processing program developed by the National Institute of Health, each image was imported to the software, and analyzed. To do so, the scale must be removed in "set scale" on the toolbar. Using the line tool, the numbers of pixels in one cm was recorded. The number of pixels of the thickness of the gel and the central displacement were recorded as seen in ***Figure 11***. Using those values, the values needed for the equation below were converted.
To calculate Young's Modulus (E), the values shown in Figure 12 were substituted into the equation and diagram below:

$$\frac{6w}{EhR} = 0.075\left(\frac{\delta}{R}\right)^2 + 0.78\left(\frac{\delta}{R}\right),$$

* Equation and diagram courtesy of Institute for Science and Technology in Medicine, School of Medicine, Keele University, Stoke-on-Trent ST4 7QB, UK (Ju & Liu 2001)*



In this equation *w* is the weight of the ball bearing, *h* is the membrane or gel thickness, *R* is the radius of the ball bearing, δ is the central displacement of the ball bearing into the gel, *0.075* and *0.78* are constants, and Young's Modulus *(E)* is what is being solved for. Data is shown in ***Figures 12-13***.

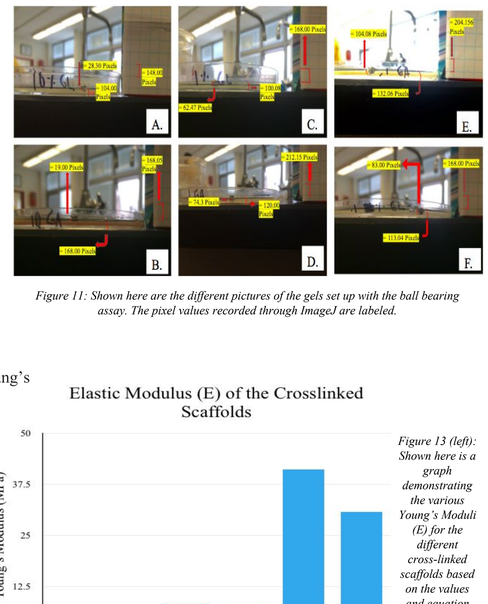


Figure 11: Shown here are the different pictures of the gels set up with the ball bearing assay. The pixel values recorded through ImageJ are labeled.

Scaffold Type	.1% Glutaraldehyde	1% Glutaraldehyde	10% Glutaraldehyde	.1% D,L-Glyceraldehyde	1% D,L-Glyceraldehyde	10% D,L-Glyceraldehyde
1 cm in Pixels	204.156	212.15	168.05	168.00	168.00	148.00
Thickness of Membrane in Pixels	132.06	120.00	168.00	113.04	100.08	104.00
Depth of Ball Bearing in Pixels	104.08	74.3	19.00	83.00	62.47	28.30
Ball Bearing Mass (grams) (w-equation)	0.689	0.689	0.689	0.689	0.689	0.689
Thickness of Membrane in cm (h-equation)	0.509	0.566	0.999	0.672	0.596	0.700
Central Displacment in cm (δ-equation)	0.788	0.612	0.113	0.734	0.624	0.190
Radius of Ball Bearing in inches (R-equation)	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625
E- Young's Modulus (MPa)	5.975	7.897	41.216	5.047	7.271	30.837

Figure 12 (left): Shown here are the values for each of the variables in the equation. These values were recorded from the ImageJ software analysis and then some manipulated (divided) to be substituted for values w, h, R, and δ. The final Young's Modulus (E) was recorded in row 9 based on the values listed above it and the equation (Ju & Liu 2001).

Figure 13 (left): Shown here is a graph demonstrating the various Young's Moduli (E) for the different cross-linked scaffolds based on the values and equation found in Figure 12 to the left.

Development of Interface (Layered Scaffold)

When implemented in the body, the scaffold takes the position of the entire acetabular labrum or the small fragment that is torn. As seen in ***Figure 3***, the BEAR sponge is sewn in place via stitches. The acetabular labrum scaffold would be sewn in place via stitches as well, however to maximize regenerative success a layering technique was used to produce an interface. The interface allows for a 10%, stiff, bovine gelatin base to be placed below the candidate 2% gel as seen in the diagram in ***Figure 14a***. Normally the two layers would separate once taken out of their mold, however with the candidate 10% glutaraldehyde crosslinker, the two layers are bound together through the linking of one polymer chain to another. This improves the targeted regenerative aspect of the devices being that cells prefer to colonize on rigid or firm surfaces. The 10% base would be the adhering surface, making proposed regeneration and natural healing ideally more successful.
A 10% (w/v) bovine gelatin solution was made using 10 grams gelatin (15.66 mL) with 84.34 mL H_2O as seen in ***Figure 15***.
5 mL of the 10% bovine gelatin solution was piped into a 1.5 x 1.5 x .75 in. deep silicone mold using a 5 mL pipette and pipette pump. The first layer was left to set for three hours in a 36℉ sterile refrigerator . The candidate 2% bovine gelatin was produced using 2 grams gelatin (3.133 mL) mixed with 96.87 mL H_2O. 16 mL of the 2% bovine gelatin solution was piped on top of the set 10% base and put back in the refrigerator to let the new layer set for 7 more hours. Once completely set, 8.4 mL of glutaraldehyde crosslinker mixed with 1-2 mL of H_2O was piped on top of the layered scaffold to form a 10% GA crosslinked scaffold with interface. The crosslinker solution was left to diffuse in the sterile refrigerator for 24 hours. The final product is shown in ***Figure 14b***.

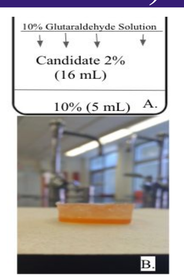


Figure 14A-B: Shown here is the interface schematic (A) and the final crosslinked interface product (B).

Figure 15: Formulating of the 10% bovine gelatin base with use of a hot place to help dissolve gelatin powder faster.

Producing the Crosslinked, Bovine Gelatin Scaffold Prototype with 3D Printed Hip Mold

Development of 3D Printed Hip Mold

In order to make a patient specific scaffold, the student researcher's CT scan files were converted into the stl. format and imported into Materialise 3-Matic, a computer aided design (CAD) software. In 3-Matic, the hip was cut down to just the acetabular cup and femoral head needed for the mold, and cleaned up as the transition to the stl. format made the files very pixelated as shown in Figure 16. The files were sent to Oxford Performance Materials, and 3D printed with instructions from the student researcher. The parts were printed with the material Polyetherketoneketone (PEKK) as it is the closest material to cortical bone, but more importantly isn't porous, which was necessary to prevent leakage when setting the candidate interface scaffold in the mold for the final prototype. The hip mold was printed to scale.

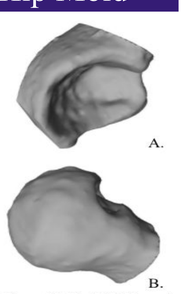


Figure 16: The CAD files sent to OPM.
A= Acetabular Cup
B= Femoral Head

3D Printing Process

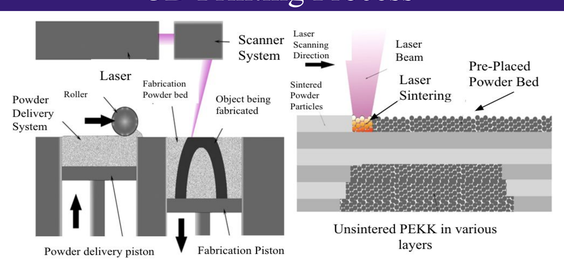


Figure 17 : Shown here is the 3D printing, laser sintering method used to manufacture the hip mold by Oxford Performance Materials. As seen, the laser and scanner system basically regurgitate the stl. file created by student researcher. PEKK powder is laid, the laser then engraves the design, more powder is laid. The process is repeated until implant is fully developed. It is produced as a block of PEKK. That block is then blasted with glass microbeads because they break before PEKK powder, but after hardened PEKK. Image courtesy of wikipedia.

Producing the Crosslinked Bovine Gelatin Scaffold Prototype with 3D Printed Hip Mold Cont.

To produce the final prototype, the 10% (w/v) bovine gelatin solution was prepared with 10 grams gelatin powder (15.66 mL) with 84.34 mL H_2O. 8 mL of this solution was piped into the base of the mold (acetabular cup) via a 10 mL pipette and pipette pump. The base of the mold was set flat while the femoral head was suspended from a metal rack leaving just enough room for the first layer of solution between the acetabular cup and femoral head as seen in ***Figure 18***. The first layer was left in a sterile refrigerator at 36℉ to set for 6 hours. While the base layer was setting, a 2% bovine gelatin solution was prepared using 2 grams gelatin (3.133 mL) mixed with 96.87 mL H_2O. .312 grams (.5 mL) hyaluronic acid sodium salt (.5% solution) was added to the 2% solution and mixed to form a homogeneous mixture. Hyaluronic acid, a Glycosaminoglycan (GAG), was added to the formulation as it has the capability of retaining water molecules, meaning that it makes the scaffold much more "cushiony."The compound used is shown in ***Figure 19***. One of the acetabular labrum key roles is to cushion the joint. Adding this aspect provides yet another positive property to the developed scaffold. 24 mL of the 2% bovine gelatin/hyaluronic acid solution was piped on top of the base layer with a pipette and pipette pump and put back into the refrigerator to set for 24 hours. Once set, 12.8 mL of the glutaraldehyde crosslinker was mixed with 1-2 mL of H_2O and piped onto the set layered scaffold. The scaffold was left to crosslink for 24 hours. Using a scalpel, a 24.26 mm in diameter circle was cut out of the middle of the scaffold to replicate the human acetabular labrum, as it is shaped like a ring. The final prototype is shown in ***Figure 20***. The scaffold was flash frozen in liquid nitrogen and lyophilized (freeze dried) to test for long-term shelf use as seen in ***Figure 21***. The scaffold must be thoroughly rinsed before implementation in the body.

Figure 18: Setting of the scaffold in the 3D printed hip mold to make scaffold patient specific.

Figure 19: Hyaluronic acid compound used in formulation of scaffold (Sigma-Aldrich)

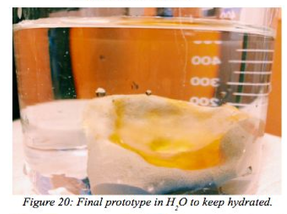


Figure 20: Final prototype in H_2O to keep hydrated.

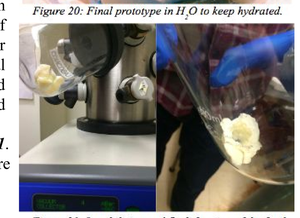


Figure 21: Lyophilizing and flash freezing of the final scaffold prototype for long-term shelf use.

Discussion of Results

A patient specific, cost-effective, and on it's way to being regenerative scaffold as an alternative treatment for acetabular labral tears of the hip has been developed. Through the formulation of 6 different 2% bovine gelatin scaffolds crosslinked at .1,1, and 10% (w/v) concentrations with D,L-glyceraldehyde and glutaraldehyde it has been realized realized that the 2% bovine gelatin scaffold crosslinked with a 10% glutaraldehyde solution is the candidate for this research as the scaffold holds a Young's Modulus of 41.216 MPa, the closest to that of an actual acetabular labrum being given between 42 and 44 MPa. Since this value has a variance range, the candidate scaffold confirms to be successful replacement or treatment of the torn labrum within the body. The glutaraldehyde had a stronger effect on the gelatin than the D,L-glyceraldehyde in the sense that it produced much stronger scaffolds as seen by the ball bearing indentations in ***Figure 11a-f*** and the data in ***Figures 12-13***. Additional research has confirmed that bovine gelatin glutaraldehyde crosslinked scaffolds have been accepted in the body (Drexel University). Out of all the -yde crosslinking agents, glutaraldehyde is the least toxic, producing a better application for the designed scaffold in the body. On the lower end of the spectrum of crosslinker concentrations (.1 and 1%), the fluctuations between the two different crosslinkers weren't great, however as seen through the results of the 10% (w/v) crosslinked scaffolds, the glutaraldehyde scaffold was approximately 10 MPa stronger than the D,L-glyceraldehyde crosslinked scaffold.

When the prototype scaffold was flash frozen in liquid nitrogen no obstacles were met, however when lyophilized (freeze dried), the scaffold became very brittle. This obstacle can be defined as a structural issue, on a micro/nanometer scale as seen in ***Figure 21***. Collagen is a fibrous material, whereas bovine gelatin forms more of a solid block of material. Chemically, gelatin and collagen are very similar, however structurally they are very different. The lyophilized scaffold is shown in ***Figure 22***. The awkward shape of the scaffold can cause local increases in stress. Due to this fault, until freeze drying is optimized, it is suggested that the scaffold be supplied in a hydrated sterile pack.

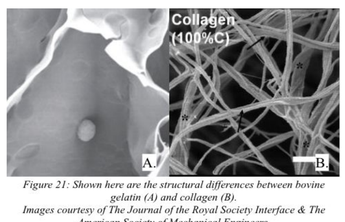


Figure 21: Shown here are the structural differences between bovine gelatin (A) and collagen (B).
Images courtesy of The Journal of the Royal Society Interface & The American Society of Mechanical Engineers.

Figure 22: The brittle, cracked prototype scaffold after being lyophilized.

Conclusion

The 2% (w/v) bovine gelatin/hyaluronic acid scaffold crosslinked with 10% (w/v) glutaraldehyde solution was successfully fabricated and analyzed, devising a product that almost identically matches the known values of a human acetabular labrum. All proposed targets were met, as the scaffold was set in a 3D printed mold of the student researcher's hip, making it patient specific. The scaffold promotes natural healing/regeneration through it's interface feature. The hyaluronic acid added to the scaffold formulae closely resembles the "cushioning" aspect of the acetabular labrum. This scaffold has future capability to confirm natural healing, leading to shorter post-surgical healing time, and isn't likely to promote development of osteoarthritis. Since bovine gelatin, denatured collagen, was used over pure collagen the scaffold is cost-effective. Based on the materials used, it was determined through cost analysis that this product can be formulated for a cost of approximately $22.00 per unit.

Future Research

Future research would seek to confirm the regenerative aspect of the device through the seeding of cells. MC3t3-EI (bone formulating) and/or NIH3t3 cells will be seeded on the gel to test for survival. These osteoblast cell lines are derived from a mouse. Using this component it can be determined if, when implemented in the body, the implant will knit together naturally with the bone and tissues in order to heal properly. An in-vivo approach would be completed as well in an animal such as a chimpanzee or canine as they have very similar anatomy to a human, specifically the hip joint. In those instances, the scaffold would be placed in the ordinary location of the acetabular labrum and the regenerative processes would be carried out. Blood would be drawn from the animal, injected into the site of the new labrum allowing for a blood clot to form, and the applied tissue to knit to either the bone or other surrounding tissues depending on the case. Either an entire labral shaped crosslinked gel is implemented or just a fragment of the crosslinked gel depending on the severity of the inflamed labrum. Optimizing the lyophilization process would also be carried out. Lastly, this formulation process and results can be used as a baseline for development of engineered tissue for other tendons within the body, such as making the current ACL method cost-effective or the rotator cuff of the shoulder.

All graphs and images were student developed unless otherwise cited.