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The Extraction of Type II Collagen and the Electrospinning of Nano-Fibrous Scaffolds

Danielle Careen Knapp
Virginia Commonwealth University

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THE EXTRACTION OF TYPE II COLLAGEN AND THE ELECTROSPINNING OF NANO-FIBROUS SCAFFOLDS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biomedical Engineering at Virginia Commonwealth University

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December 2005
Acknowledgement

I would like to especially thank Dr. Gary Bowlin for all of the guidance and assistance he has given me during both my undergraduate and graduate career at Virginia Commonwealth University. Many of the opportunities that I have been able to take part in as a biomedical engineering student would not have been possible without his help. I would also like to thank Scott Sell, Catherine Barnes, and Matthew Smith for their assistance in the laboratory.
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<tr>
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<td>autologous chondrocyte implantation</td>
</tr>
<tr>
<td>BME</td>
<td>β mercaptoethanol</td>
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<tr>
<td>C</td>
<td>Celsius</td>
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<tr>
<td>cm</td>
<td>centimeter</td>
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<tr>
<td>CPM</td>
<td>continuous passive motion</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>GAGs</td>
<td>glycosaminoglycans</td>
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<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>HAc</td>
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<td>HFP</td>
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<td>nm</td>
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<td>OATS</td>
<td>osteochondral autograft transplantation</td>
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<td>PDS</td>
<td>polydioxanone sutures</td>
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<tr>
<td>PGA</td>
<td>poly(glycolic acid)</td>
</tr>
<tr>
<td>PLA</td>
<td>poly(lactic acid)</td>
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<tr>
<td>RPM</td>
<td>rotations per minute</td>
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<tr>
<td>SDS-Page</td>
<td>sodium dodecysulfate polyamide gel electrophoresis</td>
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<td>SEM</td>
<td>scanning electron micrograph</td>
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<tr>
<td>TFE</td>
<td>2,2,2-trifluoroethanol</td>
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<td>UV</td>
<td>ultraviolet</td>
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Abstract

THE EXTRACTION OF TYPE II COLLAGEN AND THE ELECTROSPINNING OF NANO-FIBROUS SCAFFOLDS

By Danielle Careen Knapp, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biomedical Engineering at Virginia Commonwealth University.

Virginia Commonwealth University, 2005.

Major Director: Gary L. Bowlin, Ph.D., Associate Professor, Louis and Ruth Harris Exceptional Professorship, Biomedical Engineering

Articular cartilage lining joints, such as in the knee, functions to reduce friction and absorb shock. Collagen type II is the largest constituent in the extracellular matrix of articular cartilage and its restoration is of the highest interest to tissue engineers. Cartilage has little ability to naturally regenerate due to the absence of vascularity and the inability of the chondrocytes to proliferate at a high rate. It would be ideal to create a mimicking extracellular matrix/scaffold from type II collagen that could possibly be used to replace damaged articular cartilage that has the same function and morphology. Three different groups of cartilage chips were utilized to extract type II collagen. The yield of the three groups was compared. The extracted type II collagen from the three groups was electrospun at the concentrations of 0.06, 0.08, 0.10 and 0.12 g/mL. Both the pore size
and fiber diameter were analyzed. A SDS-Page was performed on the material to assure it was pure type II collagen and that no collagen type I contamination was present.
Introduction and Background

Researchers have utilized electrospinning over the years for materials that are both natural and synthetic in nature. Articular cartilage has proven to be a large problem located in the knees of aging individuals. This tissue is unable to easily regenerate naturally due to the absence of vascularity and the inability of chondrocytes to proliferate at a high rate. It is highly desirable to find a way to create a scaffold that could mimic the morphology and function of natural articular cartilage. Because articular cartilage is composed mainly of type II collagen, this material was electrospun into multiple scaffolds with varying fiber diameter and pore size in order to find an optimal extracellular matrix that could possibly be seeded with chondrocytes, potentially creating tissue engineered cartilage.

Anatomy of Articular Cartilage

Research over the years has proven that there are over 27 collagen types. Every collagen molecule is made up of three alpha chains wound in a triple helix configuration. Collagen type II consists of three specific alpha1 (II) chains that form its right-handed triple helix configuration. Type II collagen is of the highest concentration in articular cartilage. This tissue can be found at the ends of all articulating joints such as the tibia and femur. Articular cartilage is the thin layer of connective tissue that covers the surfaces of the bones in the synovial joints. This layer varies in thickness according to its location, ranging from one mm in the finger joint up to six mm in the knee joint (Hasler
et al. 416). Articular cartilage functions to transmit weight-bearing loads placed on the joints in the body from one bone to another. This allows the stresses on the subchondral bone to be correctly distributed and allows for low friction movement. There are high amounts of cyclic stresses that are induced on all the joints in the body (knee joint in particular), on a daily basis. The structure and composition of articular cartilage are what allows this tissue to properly function inside the body.

The composition of articular cartilage includes cells that are referred to as chondrocytes, which are imbedded in the extracellular matrix. The unique quality of articular cartilage is that it is completely aneural and avascular. Cartilage receives its nutrients via diffusion from the synovial fluid. In mature adult cartilage, chondrocytes make up only 10% of the total volume (Hasler et al. 417). The rest of the extracellular matrix is composed of water, electrolytes, collagens, proteoglycans, and glycoproteins.

Proteoglycans are large biomolecules composed of a long protein core and glycosaminoglycan chains that are attached along its length. The protein core only makes up 10% of the molecular weight of the proteoglycans, and the glycosaminoglycan chains make up the other 90%. The glycosaminoglycan chains hold a highly negative charge, which allows the proteoglycans to have a high charge density, giving rise to a large osmotic swelling pressure. There are two main types of proteoglycans present in cartilage. The first is a larger set that includes aggrecan and versican and a smaller set that includes biglycan, decorin, fibromodulin, and lumican. The most abundant proteoglycan overall is aggrecan, which is made up of a protein core with chondroitin sulfate and keratin sulfate chains attached along its length. An illustration of a proteoglycan aggregate can be seen below in Figure 1.
Because of the high weight-bearing load the articular cartilage must support, stiffness and durability are largely important. The strength of this tissue depends on the molecular structure and the size of the proteoglycan aggregates. As cartilage matures, a large problem exists due to the degeneration of the tissue. The constant large weight-bearing load that is placed on the knee causes a change to occur in the aggregan concentration. This concentration is decreased along with the length of glycosaminoglycan chains. With this large decrease in material inside the articular cartilage, the water content then increases causing a downward shift in the stiffness of the tissue. This can lead to mechanical damage because the articular cartilage no longer holds the same strength and pressure resistance as before.

Besides collagens and proteoglycans, the articular cartilage is composed of many noncollagenous proteins. These proteins include fibronectin, thrombospondin, chondroadherin, link protein, cartilage matrix oligomeric protein, and cartilage matrix proteins.
As mentioned before, the chondrocytes in articular cartilage receive their nutrients from the synovial fluid inside the tissue. The tissue fluid is made up of water with dissolved gas. Water is actually the main component of articular cartilage, making up 60-85% of its wet weight (Hasler et al. 423). The water is present in three areas of the cartilage: inside the chondrocytes, in the intrafibrillar space of collagen, and inside the domain of the proteoglycans. This high fluid content is important to the articular cartilage because the mobile cations of the water bind with the highly negative charge of the proteoglycans to produce a swelling pressure in the cartilage. This swelling pressure acts almost like a cushion that responds to the high stresses at the joints. The proteoglycans play a key role within the tissue because they are the main determinate of the swelling pressure inside the tissue, which dictates the strength, stiffness, and the ability to withstand pressure exerted on articular cartilage.

The chondrocytes are the only cell type that reside in the composition of articular cartilage. These cells synthesize the matrix components, organize the matrix composition, and control the eventual degradation of these components inside the extracellular matrix. There is no cell-to-cell communication between chondrocytes and, as previously addressed, cartilage lacks blood and lymphatic vessels, which is why these cells receive all nutrients via diffusion through the extracellular matrix. This means that any change in fluid flow due to any stress or strain induced on the tissue can affect the productivity of the chondrocytes. Changes in the chemical factors, such as taking various medications, or the matrix composition can also influence the normal function of the chondrocytes. If the cells aren’t in a normal or “happy” state, degradation of the articular
cartilage will occur because the cells aren't synthesizing the matrix components and properly diffusing nutrients in and out of the tissue.

Zones and Regions of Articular Cartilage

Articular cartilage is not a homogenous tissue; instead, it has four zones that are organized vertically which are distinctly different from one another. There are also three matrix regions that exist in layers around the chondrocytes. The superficial zone is the zone farthest from the actual subchondral bone. This zone is the thinnest of the four and functions to provide a gliding surface for the end of the joint. The collagen fibrils are arranged parallel to the articular surface. Because of the thinness of the zone, the fibrils have a small diameter and are packed in a highly dense manner. The chondrocytes are flat and inactive in this zone. Also, both the aggrecan content and the cell volume are decreased, while the cell density is increased. The proteoglycans and collagens are densely packed and closely interconnected in this zone, providing a strong structure to reduce the stresses produced at the surface of the bone due to motion.

Below the superficial zone is the transitional zone, which has a much greater thickness. The collagen fibrils are more loosely (randomly) arranged and have a larger diameter. The chondrocytes resume a more normal rounded shape, which allows them to be both larger and more active. This zone holds the highest proteoglycan content.

The radial zone lies beneath the transitional zone and is divided into two separate areas—the upper and lower radial zone. The collagen fibrils are loosely organized in a perpendicular fashion with respect to the subchondral bone. They have their largest diameter in this zone. The water content is very low in this zone and the proteoglycan content is high. The metabolic activity of the chondrocytes is at its highest.
The deepest zone is the calcified zone, which lies between the deep zone and the subchondral bone plate. There is a separation that exists between the deep zone and the calcified zone called a tidemark. This zone is a bit stiffer than the others because the cartilage is mineralized with crystals of calcium salts. The proteoglycan content is low and the collagen fibrils are arranged starting from the deep zone and insert into the calcified zone acting as an anchor for articular cartilage to be attached to the underlying subchondral bone. This zone serves as a structural integration mechanism between the articular cartilage and the underlying subchondral bone (Hasler et al. 426).

Below, in Figure 2, is a diagram illustrating the four zones and three regions existing in articular cartilage.

Figure 2: Four Zones and Three Regions of Articular Cartilage (Hasler et al. 424)
There are three matrix regions surrounding the chondrocytes. The pericellular matrix is the layer immediately surrounding the cells. The chondron is a term that refers to the chondrocyte and the pericellular matrix, which acts as the structural and functional unit of the cartilage. There is a rapid rate of turnover for the matrix components in this region due to the high presence of proteoglycans. Collagen filaments are thin in this matrix region, ranging from 10 to 15 nm in diameter (Hasler et al. 426). The pericellular matrix acts as a protective barrier for the chondrocytes against the high mechanical pressures that are exerted on this tissue.

The territorial matrix is a woven network of fine cartilage fibrils that are located around the pericellular matrix. Within the radial zone, the territorial matrix is organized in a way that it actually surrounds columns of chondrocytes. The chondrocytes have cytoplasmic projections that allow them to come into contact with the collagen fibrils located within this zone.

Lastly, the interterritorial matrix of articular cartilage is both the outermost layer and the largest of the three in size. As the distance at which the collagen fibrils are located is increased from the chondrocyte they surround, the collagen fibrils increase in size. Therefore, collagen fibrils will be larger the farther away they are located from the chondrocyte it is surrounding. The proteoglycan content is decreased in this area.

Functions of Articular Cartilage

As previously mentioned, the largest task of articular cartilage is the ability to resist compression. The idea of cartilage swelling due to a pressure gradient is a large contributing factor to helping with this task. There are negatively charged groups connected to the glycosaminoglycan chains, giving cartilage a highly negative charge.
density. In the synovial fluid there are positively charged sodium and calcium ions floating around that are attracted to the GAGs, which will allow for the existence of electroneutrality. When there is an imbalance of ions inside of the tissue that is higher than that of the external solution, an osmotic pressure is created. The negative charges on the GAGs produces a strong repelling force, which causes the extracellular matrix within the articular cartilage to swell. The collagen mesh has the ability to balance the swelling of the proteoglycans and return the tissue to its normal state. As weight is put onto the joint, the proteoglycans are pushed out of the cartilage (the negative charge is pushed out of the tissue as well), which increases the charge density. Both the osmotic and interstitial fluid pressure within the matrix are now increased. The ideal situation is for the interstitial fluid pressure to rise above the osmotic pressure, allowing the interstitial fluid to exude and increase the proteoglycan content. The abundance of proteoglycans leads to an increase in the repulsive forces until an equilibrium is formed that has the ability to withstand the applied load. This is how the articular cartilage has the ability to resist compressive forces in the joints of the body.

Articular Cartilage Limitations

As humans mature, a major problem in articular cartilage is degeneration of the tissue. There is a limited potential to restore cartilage located at the joint surfaces. The chondrocytes have a low level of mitotic activity and the tissue is completely avascular. An injury to the cartilage can occur in any joint and is referred to as a lesion or defect. The most common area of degeneration is the knee, and treatment of this joint is studied most often because of its importance for daily human activity. There are two main
categories of articular cartilage damage: partial thickness defects and full thickness defects. An illustration of the two defect types can be seen below in Figure 3.

![Image of partial thickness and full thickness defects]

**Figure 3: Partial Thickness and Full Thickness Defects (Redman et al. 23)**

A defect is defined as partial or full thickness according to the depth it penetrates into the marrow spaces of the subchondral bone. Partial thickness defects are analogous to the clefts and fissures that are seen in the early stages of osteoarthritis (Hunziker et al. 721). As the stages of osteoarthritis progress, the fibrillated lesions grow larger and deeper. These lesions do not heal spontaneously. Many researchers have made the assumption that the failure to regenerate is due to the fact that the defect does not penetrate the subchondral bone (bone marrow); therefore, there is no access to the progenitor cells in the bone marrow space. In opposition to this idea, it has been proven that spontaneous repair of a superficial defect does occur with no fibrous scar and restoration of the zonal organization of articular cartilage. Whether or not this repair is "true" repair or void filling is still a question that remains unanswered (Redman et al. 23).
Often, in mature cartilage, a limited amount of repair does occur within the tissue immediately adjacent to the site of the lesion. This process begins with the occurrence of cell death in the areas adjacent to the wound. After twenty-four hours have passed an increase in cell proliferation and matrix synthesis at this area occurs, but it is short lived and there is no actual repair of the defect. Growth factors signals additionally make an attempt to heal the defect by inducing cells to migrate across the articular surface from the synovium to the lesion, but again fail to do so due to the anti-adhesive properties of the proteoglycans (Redman et al. 23). These processes suggest it is not just the absence of bone marrow cells that causes incomplete healing of partial thickness defects; instead, there are other mechanisms involved that play a role in the inability of the partial thickness defects to heal correctly.

Full thickness defects fully penetrate the subchondral bone and are able to access the cells in the bone marrow. The repair response that occurs due to this defect results in the formation of a fibrocartilaginous tissue at the site of the defect. In most cases, once the subchondral bone is penetrated by the in a full thickness defect, there is immediate formation of a hyaline-like articular cartilage. This tissue is an undesirable replacement for cartilage and degeneration of both the repair tissue and the native tissue occurs. It has been noted in some cases that during this process, the tissue adjacent to the wound becomes necrotic and almost no remodeling occurs (Redman et al. 24). Upon examination of the full thickness tissue defect, there were many areas of continuity as the tissue changed from native to repair tissue, but no true integration was present. Because the outcome of both partial and full thickness defects have a lifelong negative effect on
the joints, there is a great challenge for tissue engineers to discover a successful way to heal articular cartilage defects.

Osteoarthritis is the number one disease causing articular cartilage damage for adults. Avascular necrosis is another condition that can increase the risk of articular cartilage degeneration. This disorder involves the death of the bone tissue that lies beneath the cartilage as a result of insufficient blood supply. Also, osteochondritis dissecans is a condition when the blood supply to a discrete area of the bone is disrupted and there is breaking away of fragments of bone and cartilage in the knee. Traumatic injury due to sports or accidents of any type can also cause articular cartilage injury. Lastly, inflammatory disease, such as rheumatoid arthritis can cause articular damage.

Current Methods For Treating Articular Cartilage

There are many methods currently available that attempt to both heal and initiate the regeneration of articular cartilage. For minor articular cartilage defects, the abrasion technique is utilized to try and rid a small area of loose cartilage pieces. A technique called arthroscopy is used, which is when an incision is made at the site of the injury and both fiber optics and small instruments are used to clean out any loose particles located in the joint. Bleeding is also induced at the bone surface at the site of the wound. Fibrocartilage growth is then induced at that area as the body attempts to heal itself at the site. The positive aspect of this procedure is that it is brief and does not require the patient to be hospitalized. The negative aspect of the procedure is that it does not have a very high success rate since the fibrocartilage that is produced at the area of the defect may not fully fill the gap and become a durable tissue replacement. Fibrocartilage will never be able to function in the same way as native articular cartilage.
The next treatment option available for damaged articular cartilage is microfracture, which is highly similar to the abrasion method, but with an added step. Debridement, which is the act of provoking bleeding, is done at the site of the wound. Fibrocartilage is formed, but in addition to clearing away the damaged area, the surgeon uses a sharp instrument to make perforations in the bone. The bone marrow contains pluripotent cells that have the ability to differentiate into cartilage in this technique, under the influence of motion. A continuous passive motion (CPM) machine is often utilized by the patient, which allows the knee to repeatedly follow a circular range of motion without putting any pressure on the joint. This machine is used following the operation for several hours a day. The positive aspect of microfracture is the cheap cost and simple procedural steps. Microfracture serves as the best treatment for well-contained lesions (Novick Cartilage Regeneration-An Overview). Once again the ability for the fibrocartilage to fill the entire gap is questionable and the patient must refrain from placing weight on the affected joint for a specified period of time. If fibrocartilage is formed at the affected area, the durability of the new tissue will not be that of the native tissue.

Mosaicplasty is a highly talked about technique, which is also referred to as osteochondral autograft transplantation (OATS). Both fresh and cadaveric donors are used for this method, but research has shown that the fresh tissue is more successful in terms of preventing cell death and the ability to achieve mechanical stability (Redman et al. 25). Osteochondral transfer is a repair method that is used in the same way as the periosteum and perichondrium grafts that will next be described, but in the next method both allogenic and autogenic grafts are used to repair the defective tissue area.
Autologous osteochondral grafts involve the removal of plugs of osteochondral tissue from a low weight bearing area in the body that is transferred to the debrided defective area. Depending on the size of the damaged area, one plug or multiple plugs are utilized. This method is usually chosen for small to medium size defects because the larger the defect, the larger the risk for rejection (immunologic response). This method is also better for patients under the age of 45 with stable joint mechanical alignment (Boland, Arthur et al.). An advantage of this technique is the high survival rate of the chondrocytes and the ability of the plugs to maintain the original hyaline cartilage characteristics. The disadvantages are morbidity at the site where the plugs are removed, the limited supply of tissue available to use as plugs (must be located at a low weight bearing area), and the long rehabilitation time that is required (Boland, Arthur et al.). Also, it is hard to find any area in the joint that is not bearing any weight, so the smaller the defect this technique is used for, the better. There is possibility of chondrocyte death around the edges of the plugs that have been implanted, which causes a space to form between the graft and the native surrounding tissue. This discontinuity can lead to degeneration of the graft over time if cartilaginous tissue (or fibrocartilage) is not formed to fill in the gaps. Multiple studies have showed that patients undergoing this treatment do show a decrease in pain and an improvement in joint function.

A technique that is fairly simple and not often used is the removal of a piece of the periosteum or perichondrium, which is the connective tissue that covers all bones, from a low weight bearing area on the shin. The periosteum/perichondrium is then transplanted into the site of the defect. The idea is that there are pluripotent cells located in this tissue that lead to cartilage regeneration. This method is used to repair full
thickness defects. In experimental trials, a defect is created at the damaged site that is four mm in diameter. One to two mm of thickness of subchondral bone is removed to make room for the insertion of the healthy tissue. The periosteal or perichondrial graft is then implanted into the defective cartilage and fibrin glue is used for adhesion. When this method was used in a rabbit study, 33% of the cases showed the formation of repair tissue that was derived solely from the transplanted periosteum. The other 67% had repair tissue that was derived from both the transplanted tissue and the bone marrow mesenchymal stem cells (Redman et al. 25). Continuous passive motion (CPM) is an important factor in the successful healing of the damaged joint when utilizing this technique. This procedure does require an overnight visit to the hospital and the result can be one of mixed normal and fibrous tissue at the damaged site.

A highly popular method for natural regeneration of articular cartilage is autologous chondrocyte implantation (ACI). Chondrocytes are taken from a healthy area of the knee that is not involved in the injury and are implanted at the area of damage. Chondrocyte precursor cells from the grafted periosteum and mesenchymal stem cells derived from the subchondral bone marrow space are cell sources that can also be utilized. The cells are cultured for approximately 14-21 days and then injected into the affected area. The entire area is then covered with a sutured periosteal flap that is taken from the proximal medial tibia. Brittberg et al. performed a study that involved 16 patients that were treated with ACI at the femoral joint and seven at the patellar joint and followed them for a mean of 39 months. The transplants initially eliminated pain, swelling, and locking of the knee for all patients. At the three month mark, all of the patients showed that the transplant area was level with the surrounding healthy tissue.
The tissue also appeared to have the same spongy (resistant) characteristics as the healthy surrounding tissue. After two years, 14 of the 16 femoral ACI patients had good to excellent results. The other two patients had to undergo a second operation. The results in the patients that underwent patellar ACI were good to excellent in only two of the seven patients, fair for three, and poor for two. The two that were rated as poor underwent a second operation. There were signs of hyaline cartilage in one of the seven patellar ACI patients and eleven of the sixteen femoral ACI patients (Brittberg et al. 892). The major advantage of this technique is the possibility of normal hyaline cartilage restoration. The disadvantages include the high price, requirement of two separate procedures (cell insertion and the adhesion of the flap to cover affected site), and the unpredictability of the results for the operation. It has been proven that implanted chondrocytes do have the ability to participate in the formation of repair tissue denoted by the formation of type II collagen and can persist in helping to heal the defect for up to 14 weeks.

A method that is a continuation of ACI is the use of a specific chondrocyte population taken from an identified area within the articular cartilage that may have better replication and integration characteristics in comparison to chondrocytes taken from another area. Waldman et al. performed a study which took cells from the full thickness, mid and deep zone, and deep zone of articular cartilage. The cells were seeded onto calcium phosphate substrates and cultured in vitro. The full thickness chondrocytes showed the highest amount of collagen synthesis, but the mid and deep zone chondrocytes showed the best mechanical properties and accumulation of proteoglycans.
If the cells from the mid and deep zone were used to as the implanted cells for ACI, there would be an improved rate of articular cartilage tissue repair (Redman 28).

Another method in repairing articular cartilage is the use of a tissue engineered scaffold as a tissue substitute to treat the damaged cartilage. When chondrocytes are implanted without a scaffold (such as in the ACI technique), they only have the ability to synthesize their native ECM proteins, but when the cells are first cultured onto an actual scaffold and implanted, the proteins are deposited within the engineered matrix and thus have the ability to organize and remodel this structure. When only a cell culture method is used, the ECM proteins will just diffuse out into the culture media. The major requirements the engineered scaffold must meet are structural integrity, biocompatibility, and the correct mechanical stability. The scaffold must be strong enough to support the high loads that are placed on the knee and be porous enough to allow synovial fluid to flow in and out of the matrix for proper infiltration of the structural molecules. There are two major scaffold types: those made from synthetic materials such as polymers and those made from natural materials. There are positives and negatives to choosing either scaffold material. Scaffolds are three-dimensional and can be formed into a desired shape depending on the method of its construction.

Two highly popular synthetic materials used to create a three-dimensional scaffold for the replacement of articular cartilage are poly(glycolic acid) (PGA) and poly(lactic acid) (PLA) polymers. Tissue engineers often use the materials individually or combine them into various combinations such as a copolymer made up of 50% PLA and 50%PGA to form a scaffold that mimics their mechanical requirements. These scaffolds are naturally biodegradable and, depending on the method used for the
fabrication of the construct, pore size and the fiber diameter can be manipulated. Polymer scaffolds have been shown to equally promote proteoglycan regeneration and chondrocyte proliferation, differentiation and maturation in comparison to collagen-based scaffolds (Grande et al. 213). When utilizing synthetic scaffolds, the need for any donor tissue is eliminated and the shape of the scaffold can be completely formed to perfectly fit the defective site. The downfall is the risk of rejection from the body.

There are various methods used to create the synthetic scaffolds for articular cartilage replacement. Freed et al. had Acufex Inc. form PGA scaffolds that contained 12-14 µm diameter fibers forming a non-woven mesh that is 0.1 to 0.2 cm thick with a bulk density of 55-65 mg/cm³. The mesh was then cut into 1 cm diameter discs that were sterilized and seeded with cells. Freed et al. found that chondrocytes were able to proliferate on the PGA scaffold and regeneration of the cartilaginous matrix occurred. This study also compared the difference between seeding the scaffolds with primary chondrocytes, unpassaged chondrocytes and passaged chondrocytes. Defects that were filled with all three cell types did have signs of a white colored repair tissue (similar to the blush white appearance of native articular cartilage) after one month of in vitro implantation. The passaged chondrocytes showed 1.7 times as much GAG production and 2.6 times as much collagen production when compared to the scaffolds seeded with primary chondrocytes (Freed et al. 891).

Grande et al. compared the pros and cons of using synthetic and natural scaffolds to create tissue engineered articular cartilage. They used PGA to make two mm thick non-woven fiber mats with a pore size of 200 µm; a PGA-PLA copolymer to make a flat woven mesh mat with 210 µm pores; and nylon to make flat, square, knitted mesh with
90 µm pores. A comparison of the rates of synthesis of proteoglycans and collagen were examined for all of the scaffold types. Also, whether or not a bioreactor closed loop recirculation system could increase the synthesis of molecular proteins was studied. There was a greater rate of proteoglycan synthesis on the polymer scaffolds compared to collagen production. The use of the closed loop bioreactor system increased synthesis of all molecular proteins for all three scaffolds in comparison to its absence. This increase only occurred initially and did not sustain a difference over a time period of three weeks (Grande et al. 211). Nylon was the least efficient of the three polymer types. For the PLA-PGA blend, the chondrocytes easily attached and replicated in the scaffold, but did not transverse into the large pores. Grande et al. stated that when chondrocytes have a spindle shape they are not as happy on the scaffold as when they have a spherical morphology. For all three polymer scaffolds the cells had a spindle shape, which indicates they were not perfectly “happy.”

Grande et al. also compared these three polymer scaffolds to a collagen type I bovine fibrillar matrix with a two mm thickness and pore size ranging from 200 to 300 microns that were also seeded with chondrocytes. The proteoglycan synthesis was limited compared to PGA for this scaffold, but the collagen production was higher than that of the polymer scaffolds. The cells on the natural scaffold showed a spherical morphology, suggesting the scaffold could induce the proper gross phenotypic characteristics in the cells, allowing them to attain their natural shape. Grande et al. discovered that the proteoglycan synthesis increased at a continuous rate when the collagen type I scaffold was cultured in a closed culture system at all the time points under observation.
In 1997, Nehrer et al. compared sponge-like scaffolds made from type I and type II collagen for the synthesis of chondrocytes. The type I collagen scaffold was constructed from bovine hide using an acetic acid extraction followed by freeze-drying to form a porous membrane. The type II collagen scaffold was produced by treating porcine cartilage to form a three-dimensional sponge-like material with pores that were interconnected. The type II sponges underwent UV irradiation to cross-link and allow for an increase in strength. Both matrices were seeded with chondrocytes in vitro for three hours, seven days, and fourteen days. There was a higher percentage of chondrocytes with a normal spherical shape for the type II matrix at all three time periods. The GAG and DNA content increased for all three time points in a larger amount for the type II collagen scaffold compared to the type I collagen scaffold. The type II matrix allowed for a better site for chondrocyte implantation because it had a higher percentage of spherical chondrocytes and there was greater molecular activity for this scaffold as was illustrated by the increased rate of GAG and DNA production. This assumption was reaffirmed by Pieper et al. in 2001 and Gigante et al. in 2003.

Pieper et al. followed in Nehrer et al.’s footsteps and compared collagen type I and type II scaffolds as a site for the implantation of chondrocytes. At first, both the bovine Achilles tendon to make the type I scaffold and the bovine tracheal cartilage to make the type II scaffold underwent an acetic acid extraction. The type II collagen did not swell properly in the acetic acid; therefore, a pepsin digestion method was utilized. Porous type I and type II collagen matrices were produced via freezing and lyophilization. Pieper et al. also treated some of both scaffolds types with chondroitin sulfate to see if cell integration was improved. The matrices were cross-linked and the
chondrocytes were seeded in vitro. The matrices were evaluated according to the ability of the chondrocytes to proliferate and differentiate. The type II collagen scaffold provided a better distribution of cells throughout the matrix. Both the type I and type II collagen scaffolds did show a cartilaginous-like layer at the surface and the presence of chondrocytes throughout the matrix. The type II collagen showed an increase in these characteristics in comparison to the type I scaffold, but the addition of chondroitin sulfate had no affect on the scaffold quality (Pieper et al. 3191).

After both Nehrer et al. and Pieper et al., Gigante et al. compared scaffolds made from type I and type II collagen for use as a cartilage replacement. The type I collagen scaffold was made from equine tendons that were put through an acid extraction process. After stringent purification techniques were performed and the acetic acid was removed, the material was lyophilized and formed as films in dies that were contained in an electrostatic field. The collagen type I scaffold was made from porcine samples that were processed the same exact way as those made from the equine tendon, except HCl was used for the acid extraction instead of acetic acid. The scaffolds were seeded with chondrocytes and the cell morphology was studied in order to decide which scaffold allowed for a conducive environment. On the type I collagen matrix, the cells held an elongated shape and formed a layer of only one to two cells thick on the membrane surface. The cells on the collagen type II matrix were spherical in shape that were several cells thick on both the membrane surface and penetrating through the scaffold (Gigante et al. 714).

The latest technology that is under investigation is the use of stem cells that are derived from tissue sources other than articular cartilage. Stem cells have the ability to
self-renew and morphologically change according the environment into which they are implanted. Stem cells will spark both regeneration and repair in a desirable manner that is required by the tissue at the site they are implanted when damage occurs.

Mesenchymal stem cells taken from bone marrow are currently being considered. These cells can then be used in the ACI method previously described or grown on a scaffold and inserted into the body.

Overall, the use of a scaffold, whether natural or synthetic, has proven to give the best results when implanted into the body to encourage cartilage regeneration. If the scaffold is composed of collagen type II, which is the material making up the majority of the articular cartilage tissue, a high degree of reproducibility is not guaranteed, but has shown better results in comparison to scaffolds made from type I collagen. Polymers are usually manufactured with high reproducibility and can be molded into nearly any shape. The degradation rate of the polymer can be easily controlled, but this scaffold type has an increased risk of rejection from the body. It is common sense to assume that the body would be more accepting of a natural material. The cells would more easily adapt and regenerate in its natural environment. A blend of natural and synthetic materials to make a scaffold would be potentially advantageous because the body would easily accept the scaffold because of the natural material, but the polymer material would increase the strength, integrity, and reproducibility of the scaffold. An improvement for the actual construction method of the scaffold is the major area that needs improvement.

Electrospinning Background

Electrospinning is a method of creating a micro to nano-fibrous non-woven mat that was first utilized in 1934 by Anton Formhals (Formhals 1) who was granted a patent
to use this idea to spin a cellulose ester solution. Next, William Huebner (Huebner 1) received a patent that allowed him to utilize the method of electrospinning to spin sheets or webs. Both patents utilized this technology to form continuous sheets of material for commercial use.

A high voltage power supply, ground and source electrodes, a dissolved solution to be spun, and a grounded collection surface are the required equipment for electrospinning. The electrospinning system requires a charged solution that can be either a natural or synthetic material that is dissolved in a solvent. Electrospinning requires that a fluid jet be subjected to a highly charged electric field with little or no current present. The jet is drawn out of the syringe at a set speed, which is dependant upon the material that is being electrospun. As the fluid jet leaves the needle tip, the solvent evaporates and a dry single fiber is formed that whips around in the air between the needle tip and the collecting plate (mandrel). A grounded surface, referred to as a mandrel, is located at a distance from the charged solvent creating an electric field. The fibers wrap around this surface to form a porous sheet of fibers. The shape and size of the collection plate is completely dependant on the desired use of the mat. A diagram of the electrospinning process can be seen below in Figure 4.

![Figure 4: Diagram of Electrospinning Apparatus](image)
Collagen Type II Extraction

Type II collagen is the main constituent that makes up cartilage ECM, so this material was chosen for use in this experiment. There are various sources that can be used to extract type II collagen. Pieper et al. extracted type II collagen from bovine tracheal cartilage via pepsin digestion. Various dialyses were performed on the material to assure purity and the final form of the material was reached after lyophilization. Herbage et al. used the same extraction method but instead of using bovine tracheal cartilage, bovine patellar articular cartilage was used. Gigante et al. extracted type II collagen from porcine trachea using HCl for the extraction procedure. Films were then formed in dies from this material with the use of electrostatic charge. Nehrer et al. also extracted type II collagen from porcine cartilage, but the end product was a sponge-like material with the use of lyophilization. Three groups of researchers: Edward Miller, Trentham et al. and Von Der Mark et al. utilized chick cartilage to extract type II collagen. A SDS-Page was used to identify the level of purity of the type II collagen and to check for any type I collagen contamination. Because bovine samples are the most readily available, this was the material utilized for the extraction process in our experiment. The largest amount of type II collagen is found in the patellas of bovine specimens in comparison to other test animals including rats, chicks, or porcine specimens. The age of the cartilage chips that will yield the greatest amount of material is also in question. Our study compared the type II collagen found in fetal cartilage chips and two groups of patellas from bovine samples that are 140-180 days old.

The electrospinning of type II collagen is novel, and will produce a material that has fibers which resemble that of the native tissue fibers. The material will be natural
and completely composed of type II collagen, which makes up 90% of the total collagen content in cartilage. This material should be easily accepted by the body without the risk of rejection and can be formed into any shape for implantation. The yield of the extraction for the three groups of cartilage chips will be examined along with the characteristics of the electrospun scaffolds.
Materials and Methods

This project was undertaken to discover the different end amounts of type II collagen that can be extracted from the cartilage chips of different age bovine specimens. It will also be determined whether or not the purity of the end product of collagen type II is affected according to whether or not there are remnants of blood vessels or bone marrow present in the chips. If the extraction of collagen type II from the cartilage chips is successful, it is desirable to discover if collagen type II can be electrospun into scaffolds that have the ability to be tailored to the use of interest. The properties of the electrospun scaffolds should be evaluated according to pore size and fiber diameter at the concentrations of 0.06, 0.08, 0.10, and 0.12 g collagen type II/mL HFP for the three bovine groups.

The protocol utilized for the collagen type II extraction was donated by Dr. David Brand. He also donated the fetal cartilage chips for the study. The cartilage from all of the fetal bovine joints were utilized for the extraction of the type II collagen. Cartilage chips were purchased from Lampire Biologics (Pipersville, PA), which contained fragments of bone marrow and blood vessels. The age of the cows utilized for these chips was 140-180 days old and only the patellar joint was utilized. Whole patellas were purchased from Spear Products (Quakertown, PA) within the same age groups as the chips from Spear Products. The articular cartilage was manually scraped from the Spear Products patellar surfaces with the use of a scalpel. Only the clear hyaline surface was
used to create the chips, leaving no blood vessel or marrow contamination when the type II collagen was extracted. The following section describes the steps followed to extract collagen type II from the cartilage groups.

**Extraction of Type II Collagen**

1. Cartilage chips from the ends of all joints of fetal bovine specimens were obtained from Dr. David Brand. Bluntly pre-cut cartilage chips from bovine patellas were obtained from Lampire Biologics that were slightly contaminated with fragments of bone marrow and blood vessels (140-180 days old). Whole patellas from calves (140-180 days old) were obtained from Spear Products and the hyaline cartilage surface was scraped off creating cartilage chips with no contamination (completely clean chips). Below is a picture of each of the three cartilage chip groups used for the experiment.

*Figure 5: Lampire Cartilage Chips*
2. The weight of the cartilage chips was obtained for each batch, and the chips were cut into smaller pieces with a razor blade. The cutting tray was set over a bucket filled with ice in order to keep the cartilage from reaching a temperature over 4°C. A Waring CB15 three horsepower blender was used to blend the chips with
deionized water (Barnstead International combination organic removal/ultrapure filter #D8922) and ice to macerate them into the smallest size possible. For every 100 grams of material that was put into the blender, a combination of approximately one liter of ice (equal to 500 mL of water) and 500 mL of deionized water was added. The blender underwent three pulses at the maximum setting for the duration of one minute each. Between each pulse, the lid was removed and the material was wiped down from the sides to assure all the material was blended.

3. A volume of cold (4°C) 5 M guanidine (Sigma Aldrich #G4505) that was made to have a pH of 7.4 with the addition of 0.05 M Tris (VWR buffer Tris pH 7.50 #VWR8731-1) was added in an equal volume amount to the blended mixture. The guanidine removed the proteoglycans. For example, if the batch were 100 grams, one liter of ice and 500 mL of deionized water would be added to the blender. The guanidine solution, with a pH of 7.4, would be added to this solution in the amount of 750 mL. The addition of these solutions to the blended cartilage pieces was done in a seven liter bucket (Nalgene #7012-0800) for a starting solution that was smaller than two liters or a 13 liter bucket (Nalgene #7012-0140) for a starting solution that was over two liters. The bucket sat on a stirring plate (Corning heavy duty stirrer #PC-611) with a magnetic stir bar (VWR polygon magnetic stir bar 70 mm x 10 mm #VW8731-1) at the bottom of the bucket stirring at the setting of seven. The solution was then placed in a refrigerator (4°C) or cold room (4°C) to stir overnight.
4. The mixture was then put into a Beckman Coulter Avanti J-20 XP centrifuge (JLA 8.100 rotor, 6 slots with a volume of 1000 mL each, 20° angle) at 8336 g (6000 RPM) for 30 minutes. The centrifuge was set to a temperature of 4°C. The supernatant was discarded and the deionized water was added to the cartilage pellet in a volume equal to the starting volume from step #2 (1500 mL for the example explained in step #3). The cartilage chips and deionized water stirred in the cold room for one hour to wash the pellet. The addition of the deionized water to the cartilage and the stirring of the material were done in the same manner as the stirring set up that was described in step #3.

5. The cartilage pellet was then again centrifuged in the Beckman Coulter Avanti J-20 XP centrifuge at 8336 g at 4°C for 30 minutes. The pellet was washed and centrifuged a minimum of three times. The reason for so many repeated washings of the pellet was to assure all of the guanidine was completely removed before moving on to the next step.

6. After the last wash was completed, the pellet was suspended in a volume of 0.5 M acetic acid (HAc) (Fisher Scientific #UN2789) that was equal to the amount of deionized water that was added for washing the pellet (1500 mL for the example batch). This was done by removing the pellet from the centrifuge tubes with a spatula and adding it to the 0.5 M HAc and stirring the solution in a manner identical to that described in step #3. The pH of the stirring solution was then adjusted to 2.8 with the addition of formic acid (Sigma Aldrich #F0507).

7. Pepsin (Sigma Aldrich #P-6887) was then added to the solution in an amount of one gram per one liter of solution. The needed amount pepsin was first dissolved
in a small amount of cold distilled 0.5 M HAc (approximately 3 mL 0.5 M HAc per gram of pepsin). This was then immediately added to the stirring (stirring set-up described in step #3) cartilage homogenate. If the pepsin solution was not added quickly, it would autolyze in the acetic acid solution.

8. The solution was then mixed with tall electric stirrer that entered into the liquid from the top of the bucket (Arrow Engineering Company, Inc #850) via a ring stand (Caframo #A210) that could be adjusted according to the height of the bucket at 4°C in the cold room or refrigerator overnight. The homogenate was viscous with minimal cartilage pieces the within 24 hours.

9. The homogenate was then centrifuged in a Beckman Coulter Avanti J-20 XP at 8336 g for 60 minutes at 4°C. The supernatant then contained the solubilized type II collagen and the pellet could either be discarded or another pepsin extraction could have been performed at this point.

10. Ten milliliters of 1 M Tris was added for every liter of supernatant collected. The pH of the solution was then brought to 7.4 with the use of 10 M NaOH (VWR #VW3247-1). An equal volume of 5 M NaCl (Fisher Scientific #S640-500) was then added to the solution at a very slow rate while the solution was constantly stirred. The addition of both the 10 M NaOH and the 5 M NaCl was done while the solution stirred in the manner described above in step #3. The solution was then left out at room temperature for 24 hours. A cloudiness was observed in the solution after the 24 hours passed.
11. The collagen precipitate was then collected by centrifugation in the Beckman Coulter Avanti J-20 XP at 8336 g for 60 minutes at 4°C. The pellet was collected and the supernatant was discarded.

12. The pellet was put into a solution of 0.05 M Tris that was equal to the beginning volume from step #2 (water and ice volume combined). 0.2 M NaCl was then added to adjust the pH to 7.4. The pellet was dissolved (stirred as described in step #3) in this solution for 24 hours at 4°C in the cold room or refrigerator. A dialysis (dialysis membrane tubing, MWCO 12,000 to 14,000 Daltons, Fisher Scientific #08-667E) was then performed on the material against the same buffer the pellet was dissolved in for two total changes, once every 24 hours, at 10 times its volume. The dialysis tubing utilized had a flat width of 45 mm, diameter of 29 mm, and a volume/length of 6.4 mL/cm. The tubing was cut into lengths of two feet and filled with the material and knotted at the ends. A maximum of three tubes were placed in each of the 13 liter buckets.

13. The dialyzed material was centrifuged in the Avanti J-20 XP at 8336 g for 60 minutes. The supernatant was collected and the pellet was discarded.

14. 500 g of DE 52 (Whatman #4057200) was added to the supernatant and was stirred (described in step #3) at 4°C for 24 hours.

15. The solution was centrifuged in the Avanti J-20 XP at 8336 g for 30 minutes at 4°C, and the supernatant was kept.

16. The correct volume of 5 M NaCl was added to the supernatant to make the solution have a final concentration of 0.8 M NaCl. Also, the correct volume of HAc was added to the solution to make the final concentration 0.1 M HAc. The
solution was then stirred (described in step #3) at 4°C for 24 hours to precipitate the collagen.

17. The solution was centrifuged in the Avanti J-20 XP at 8336 g for 60 minutes at 4°C and the supernatant was discarded.

18. The collagen pellet was then dissolved in a volume of 0.1 M acetic acid that was equal to the original volume in step #2. The solution was stirred (described in step #3) at 4°C for 24 hours.

19. The solution was centrifuged in the Avanti J-20 XP at 8336 g for 60 minutes at 4°C and the supernatant was collected. The supernatant was then placed in dialysis tubing (Fisher Scientific #08-667E) and dialyzed against 0.01 M Na₂HPO₄ (Sigma Aldrich #S-9638) at a volume of ten times that of the solution in the tubing (described in step #12). The dialysis stirred (described in step #3) at 4°C and the buffer was changed a total of four times (once every 24 hours). The collagen precipitated in this step.

20. The solution was centrifuged in the Avanti J-20 XP at 8336 g for 60 minutes at 4°C the pellet was dissolved (discard the supernatant) in a volume of 0.1 M HAc that equal to the volume used in step #18. The solution was stirred (described in step #3) at 4°C for 24 hours to assure that the pellet was completely dissolved.

21. The solution was centrifuged in the Avanti J-20 XP at 8336 g for 60 minutes at 4°C and the supernatant was collected. The supernatant was then placed in dialysis tubing (described in step #12) and dialyzed against 0.01 M HAc at a volume of ten times that of the solution in the tubing. The dialysis was stirred
(described in step #3) at 4°C and the buffer was changed a minimum of six times (once every 24 hours).

22. This material was then taken out of the dialysis tubing and poured into a traditional ice cube trays (16 cubes per tray with the dimensions of 4 cm x 3 cm x 3 cm) and frozen in a Forma Scientific –20°C freezer for 24 hours.

23. The frozen cubes were then put into 200 mL lyophilization jars, at a maximum of four cubes per jar, and the material was lyophilized using a Labconco Freezone 4.5 freeze drying machine until it reached a fluffy white cotton ball consistency.

24. This freeze dried material was weighed and stored at -20°C

25. The end weight of the lyophilized type II collagen for all three groups was then compared to the beginning weight of the cartilage chips in order to calculate the actual yield.

**SDS Page of Type II Collagen to Check for Purity**

Once the collagen was extracted for the three cartilage chip groups, a purity test is required to assure the end product is actually clean collagen type II without other contaminants. A SDS-Page was chosen to check the quality of the extracted material against a collagen type I and type II standard that was obtained from Dr. David Brand. The following steps describe the protocol that was utilized.

1. To check for purity, the extracted collagen type II was dissolved at a concentration of four μg/μL in 0.01 HAc. A 10% Pierce Endogen 12 well gel was utilized (Pierce Endogen product # 25221) for the following procedure.

2. Twenty microliters of the dissolved collagen solution was placed into a 1.5 mL microcentrifuge tube (Fisher Scientific #05-406-16). An equal part of the
denaturing buffer was added. The buffer chosen was composed of 50 μL of β-mercaptoethanol (BME) (BioRad #161-0710) and 950 μL Laemmli sample buffer (BioRad product # 161-0737).

3. Step #2 was repeated for both the collagen type I and type II standard that had the same concentration of four μg/μL.

4. All samples were then boiled for five minutes on a Thermolyne Nuova II stir plate at a heat setting of 10.

5. All samples were placed in a microcentrifuge (Fisher Scientific Micro 16) at 100 g for five minutes.

6. The gel was then placed in the gel holder (BioRad Mini-Protean II Cell model) and properly aligned by loosening all the screws and assuring the gel was in place. The screws were tightened and this set-up was referred to as a rig. Two gels must be run at once, forming an inner chamber between the two.

7. The rig is then placed into the bucket and the inner chamber of the rig was filled with running buffer, which is composed of one packet of BupH Tris-HEPES-SDS running buffer (BioRad #161-0790) dissolved in 500 mL of distilled water. It is important that there were no leaks present.

8. Each well in the gel was then flushed with a 20 μL of the running buffer. A long 0.1 to 10 μL long reach pipette tip (VWR #82028-534) was used to push the buffer into each well and then to remove the buffer.

9. The outside of the chamber (area between the outer rig area and the bucket) was then filled with the running buffer at a height of approximately two inches.

10. The samples were loaded into each well at a volume of 40 μL.
11. The correct lid was placed on the SDS-gel apparatus (BioRad Mini Proten II Cell model) and the gel was run at an amplitude of 230 and a voltage of 140 (BioRad power supply 1000/500 model). The gel was run until the dye ran off of the plate, which took approximately 60 minutes.

12. The gel was then taken off of the gel plate and the teeth of the wells where the material was pipetted into the gel was removed in order to form a perfect rectangular gel.

13. The gel was then placed in a box with the dimensions of 22.5 cm x 22.5 cm x 5 cm (Nalgene #5705-2020) allowing it to be covered with coomassie blue (BioRad #161-0435) and shaken on an orbital shaker for one hour.

14. The gel was then rinsed with water multiple times in a highly gentle fashion or the gel will tear.

15. The gel shook in deionized water for a few hours up to 24 hours. The gel was stored in this fashion until an image was taken of the gel.

16. A picture was taken of the gel within two days using a BioRad Gel Doc 2000.

17. The extracted type II collagen from the three groups was compared to the collagen type I and type II standards. This allowed for the discovery of any type I collagen contamination and for the comparison between the type II standard and the extracted type II collagen banding pattern.

**Electrospinning of Type II Collagen**

Once the extracted type II collagen was proven to have a desirable level of purity, the material was electrospun into scaffolds from solutions with the desired concentrations
of 0.06, 0.08, 0.10, and 0.12 g/mL HFP. The following steps describe the procedure that was followed when forming the various scaffolds.

1. If there was no collagen type I contamination and the collagen type II standard matched the extracted type II collagen banding pattern, the lyophilized extracted type II collagen was then dissolved in HFP (1,1,1,3,3,3, hexafluoro-2-propanol Sigma #105228) at the concentrations of 0.06, 0.08, 0.10, and 0.12 g/mL HFP. Because the process during the first collagen type II extraction had many errors, the yields of the groups were very small. The concentrations utilized to electrospin the extracted material from the three groups was quite different. For this group, the fetal cartilage chip group was electrospun at the concentrations of 0.05, 0.06, 0.07, 0.08, 0.09, 0.10, 0.11, 0.12, 0.13, 0.14, and 0.15 g/mL HFP; the Spear material was spun at 0.04 and 0.06 g/mL HFP; and the Lampire group was spun at 0.08, 0.10, and 0.12 g/mL HFP.

2. The SEM pellet was utilized as the grounded mandrel. The pellet was taped onto a rectangular (3 cm x 1 cm) mandrel that was set at a distance of 3.5 in. from the needle tip. The RPM at which the mandrel spun was approximately 800 and the applied voltage was 20 kV with minimal current present. The viscosity and appearance of the solution should be noted.

3. The dissolving of the collagen type II into the HFP at the proper concentration was done in a vial (VWR #15-425). The solution was drawn out of the vial with a five mL syringe (Becton Dikinson #309603) and a blunt needle tip (Becton Dikinson 18 gauge 1.5 short bevel #305199) was attached to the end of the syringe.
4. The syringe with the needle tip was then placed on a syringe pump (KD Scientific #100) that was set to dispense the fluid at the speed of 2 mL/hr and a banana clip attached to the 20 kV power source (Spellman #CZEOPN100) was connected to the needle tip.

5. The solution was then drawn out of the syringe as a single fiber that collected onto the grounded spinning mandrel/SEM pellet. Below is a picture of the actual apparatus that was used for this experiment.

![Electrospinning Apparatus](image)

**Figure 8:** Electrospinning Apparatus

6. The SEM pellet was removed from the mandrel and kept in order to take SEMs of the material.

7. The SEM pellets were dry gold coated with a sputter coater from Electron Microscopy Sciences.

8. SEMs were taken of each of these scaffolds at the four concentrations (or more for the first extraction) for the three different patella groups using a Joel 820
Scanning Electron Microscope (Jeol LTD, Tokyo, Japan) at a magnification ranging from 500X to 4000X.

9. ImageTool 3.0 (University of Texas Health Science Center, San Antonio, TX) is an image processing and analysis program for displaying, editing, and analyzing grey scale images that was used to measure the fiber diameter and pore size of each concentration for each of the patella groups at all concentrations.

10. Using ImageTool 3.0, the fiber diameter of each picture was measured using a point-to-point method at 60 measurements per picture. One point was started on a side of the fiber that measured the perpendicular distance to the other side of the fiber. For pore size, the same program and method was used, but the distance that existed between the fibers was measured from all angles at a total of 60 measurements per picture.

Statistical Analysis

The statistical analysis of the results for the comparison of the percent yield of the three cartilage groups and both the fiber diameter and pore size for the various concentrations of the electrospun mats was done utilizing Tukey’s method. JMP Version 5.0.1.2 is statistical discovery software designed by SAS Institute, Inc. that was used to determine the comparison of the significant difference(s) of the results that were under analysis.
Results

Yield for the First Extraction

Below, in Figure 9, the percent yield results for the first extraction can be seen. The fetal group had the highest percent yield of 0.67%; the Lampire group had a percent yield of 0.08%; the Spear group had a percent yield of 0.06%.

Figure 9: Percent Yield of Extracted Collagen Type II for Fetal, Lampire, and Spear Cartilage Chip Groups
Purity Results for the First Extraction

Below, in Figure 10, a SDS-Page was performed for all three groups and both a collagen type I and type II standard. It is visually apparent that the banding pattern for the fetal and Lampire cartilage chip groups match that of the collagen type II standard and not that of the collagen type I standard suggesting the presence of type II collagen. The Spear cartilage group does show a lighter banding pattern that is similar to that of the collagen type II standard, but it is much lighter in color and a bit harder to read suggesting there may have been an issue of concentration/contamination.

Figure 10: SDS-Page for Fetal Bovine Patella Chips, Spear Bovine Patella Chips, and Lampire Bovine Patella Chips
Electrospinning Results for the First Extraction

When electrospinning the scaffolds for the first extraction, an 18 gauge syringe needle was used, the voltage was set to 20 kV with minimal current, the mandrel spun at approximately 800 RPM, and the needle was 3.5 inches away from the mandrel. Due to lack of material, the fibers were directly spun onto a SEM pellet that was attached to the spinning mandrel (same electrospinning parameters/method was used for the electrospinning in extraction two and three). The concentrations chosen for the electrospinning of the scaffolds for the three groups was inconsistent for the initial run because it was the first time the material had been extracted and dissolved in HFP. The fetal cartilage group had the highest yield and it also was easily electrospun at low and high concentrations. Because of this, the material was electrospun at the following eleven concentrations: 0.05, 0.06, 0.07, 0.08, 0.09, 0.10, 0.11, 0.12, 0.13, 0.14, and 0.15 g/mL. The Spear material electrospun at the lower concentrations of 0.04 and 0.06 g/mL, but was too thick to be electrospun when the concentration was increased. The Lampire material was electrospun at 0.08, 0.10, and 0.12 g/mL. This material was not viscous enough to be electrospun when dissolved at the lower concentrations. This was the first time the material was manipulated for electrospinning, so it was a matter of trial and error to figure out the best concentrations for spinning of the material. The fact that there was such a small amount of material made the task even more difficult.
Figure 11: SEM of 0.05 g/mL Collagen Type II (Fetal Bovine Cartilage Chip)

Figure 12: SEM of 0.06 g/mL Collagen Type II (Fetal Bovine Cartilage Chip)
Figure 13:  SEM of 0.07 g/mL Collagen Type II (Fetal Bovine Patella Chip)

Figure 14:  SEM 0.08 g/mL Collagen Type II (Fetal Bovine Patella Chip)
Figure 15: SEM 0.09 g/mL Collagen Type II (Fetal Bovine Cartilage Chip)

Figure 16: SEM 0.10 g/mL Collagen Type II (Fetal Bovine Cartilage Chips)
Figure 17: SEM 0.11 g/mL Collagen Type II (Fetal Bovine Cartilage Chip)

Figure 18: SEM 0.12 g/mL Collagen Type II (Fetal Bovine Cartilage Chip)
**Figure 19:** SEM 0.13 g/mL Collagen Type II (Fetal Cartilage Patella Chip)

**Figure 20:** SEM 0.14 g/mL Collagen Type II (Fetal Bovine Cartilage Chip)
Figure 21: SEM 0.15 g/mL Collagen Type II (Fetal Bovine Cartilage Chip)

Figure 22: SEM 0.04 g/mL Collagen Type II (Spear Bovine Patella Chip)
**Figure 23:** SEM 0.06 g/mL Collagen Type II (Spear Bovine Patella Chip)

**Figure 24:** SEM 0.08 g/mL Collagen Type II (Lampire Bovine Patella Chip)
**Figure 25:** SEM 0.10 g/mL Collagen Type II (Lampire Bovine Patella Chip)

**Figure 26:** SEM 0.12 g/mL Collagen Type II (Lampire Bovine Patella Chip)
Fiber and Pore Measurement for the First Extraction

The data that was utilized to create the graphs in the figures 27 and 28 seen below, the statistics for both the fiber diameter and pore size can be calculated. Using the Tukey’s W procedure, the values for the mean fiber diameter are significantly different between all concentrations for the fetal cartilage chip group except 0.05 and 0.06, 0.08 and 0.06, 0.07 and 0.08, 0.14 and 0.11 and 0.12 g/mL. The values for fiber diameter were all significantly different for the Lampire and Spear group. When analyzing pore size, the fetal cartilage chip group utilizing Tukey’s W procedure the values were all significantly different except 0.06 and 0.07, 0.06 and 0.08, 0.10 and 0.11 and 0.12 g/mL. For the Lampire and Spear groups, the pore size values were all significantly different. These values were all calculated with $\alpha = 0.05$.

![Graph](image)

**Figure 27:** Fiber Diameter Comparison Results for Collagen Type II Extraction #1

**Pore Size Results for Collagen Type II Extraction #1**
Figure 28: Pore Size Results for Collagen Type II Extraction #1
Yield Results for the Second Extraction

Below, in Figure 29, the percent yield results for the second extraction can be seen. The Spear group had the highest percent yield of 0.045%; the Lampire group had a percent yield of 0.031%; the fetal group had a percent yield of 0.014%.

<table>
<thead>
<tr>
<th>Cartilage Chip Age (Source) vs. % Yield Type II Collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Yield Type II Collagen</td>
</tr>
<tr>
<td>Fetal (Dr. Brand)</td>
</tr>
<tr>
<td>140-180 Days Old (Lampire)</td>
</tr>
<tr>
<td>140-180 Days Old (Spear)</td>
</tr>
</tbody>
</table>

Figure 29: Percent Yield of Extracted Type II Collagen for Fetal, Lampire, and Spear Cartilage Chip Groups

Purity Results for the Second Extraction

There are no purity results for the second run. The liquid material from the last dialysis did not have a collagen concentration high enough to show a banding pattern when the SDS-Page was performed. There was not enough material to make an attempt to spin the material and to dissolve material for an SDS-Page. Because actually electrospinning the material is the first priority, the small amount of lyophilized end material was used for the attempt to spin the material from the three batches.
Electrospinning Results for the Second Extraction

Again, for this run, the electrospinning results were inconsistent due to the small amounts of material that were extracted from the three groups. The ideal concentrations were electrospun (0.06, 0.08, 0.10, and 0.12 g/mL), but only the Lampire bovine cartilage chip group gave a yield of lyophilized collagen type II material in an amount large enough for the electrospinning procedure. There were still errors present in the procedure.

Figure 30: SEM 0.06 g/mL Collagen Type II (Lampire Bovine Patella Chip)
Figure 31: SEM 0.08 g/mL Collagen Type II (Lampire Bovine Patella Chip)

Figure 32: SEM 0.10 g/mL Collagen Type II (Lampire Bovine Patella Chip)
Figure 33: SEM 0.12 g/mL Collagen Type II (Lampire Bovine Patella Chip)
Fiber and Pore Measurement for the Second Extraction

Utilizing the values used to create the graphs below in Figures 34 and 35, the statistics for fiber diameter and pore size can be calculated. Using the Tukey’s W procedure with a $\alpha = 0.05$, the values for the mean of all concentrations for the fiber diameter and pore size for the second run of the collagen type II extraction were significantly different.

![Fiber Diameter for Collagen Type II Extraction #2](image)

**Figure 34**: Fiber Diameter Results for Collagen Type II Extraction #2
Figure 35: Pore Size Results for Collagen Type II Extraction #2
Yield Results for the Third Extraction

Below, in Figure 36, the percent yield results for the third extraction can be seen. The Spear group had the highest percent yield of 0.64%; the Lampire group had a percent yield of 0.18%; the fetal group had a percent yield of 0.14%.

Figure 36: Percent Yield of Extracted Collagen Type II for Fetal, Lampire, and Spear Cartilage Chip Groups
Purity Results for the Third Extraction

When analyzing the SDS-Page below in Figure 37, it is visibly apparent that the banding pattern for all three of the cartilage chip groups matches that of the collagen type II standard. This allows us to conclude that the extracted type II collagen has a high level of purity. The banding pattern for the three groups does not match that of the type I collagen standard, suggesting that there is little type I collagen contamination.

Figure 37: SDS-Page for Fetal Bovine Patella Chips, Spear Bovine Patella Chips, and Lampire Bovine Patella Chips
Electrospinning Results for the Third Extraction

For this final run, all three groups were electrospun at the four desired concentrations of 0.06, 0.08, 0.10, and 0.12 g/mL HFP and a comparison of the scaffold characteristics was possible.

Figure 38: SEM 0.06 g/mL Collagen Type II (Fetal Bovine Cartilage Chip)
Figure 39: SEM 0.08 g/mL Collagen Type II (Fetal Bovine Cartilage Chips)

Figure 40: SEM 0.10 g/mL Collagen Type II (Fetal Bovine Cartilage Chip)
Figure 41: SEM 0.12 g/mL Collagen Type II (Fetal Bovine Cartilage Chip)

Figure 42: SEM 0.06 g/mL Collagen Type II (Spear Bovine Patella Chip)
Figure 43: SEM 0.08 g/mL Collagen Type II (Spear Bovine Patella Chip)

Figure 44: SEM 0.10 g/mL Collagen Type II (Spear Bovine Patella Chip)
**Figure 45:** SEM 0.12 g/mL Collagen Type II (Spear Bovine Patella Chip)

**Figure 46:** SEM 0.06 g/mL Collagen Type II (Lampire Bovine Patella Chip)
Figure 47: SEM 0.08 g/mL Collagen Type II (Lampire Bovine Patella Chip)

Figure 48: SEM 0.10 g/mL Collagen Type II (Lampire Bovine Patella Chip)
Figure 49: SEM 0.12 g/mL Collagen Type II (Lampire Bovine Patella Chip)
Fiber and Pore Measurement for the Third Extraction

Utilizing the data used to create the graphs below in Figures 50 and 51, the statistics for the fiber diameter and pore size can be calculated. Using the Tukey’s W procedure with an $\alpha = 0.05$, the values for the fiber diameter for the Lampire, Spear and fetal cartilage chip groups at all concentrations are all significantly different from one another within each group. When analyzing the pore size for each of the groups in the same manner, the values for the Lampire and fetal cartilage chip groups are all significantly different from one another at all concentrations. For the Spear group, the values are all significantly different from one another except for the concentrations of 0.06 and 0.08 g/mL, which are not significantly different.

![Fiber Diameter for Collagen Type II Extraction #3](image)

**Figure 50:** Fiber Diameter Results for Collagen Type II Extraction #3
Figure 51: Pore Size Results for Collagen Type II Extraction #3
Overall comparison for all extractions for fiber diameter and pore size

The following graphs in Figures 52 thru 57 visually show how the fiber diameter and pore size compare to one another for the three extractions. For the fetal and Spear groups, there will only be two extraction groups for comparison of both fiber diameter and pore size because the second extraction only yielded material for the Lampire group.

**Figure 52:** Fiber Diameter Comparison for Fetal Cartilage Chips
Figure 53: Fiber Diameter Comparison for Lampire Cartilage Chips

Figure 54: Fiber Diameter Comparison for Spear Cartilage Chips
Figure 55: Pore Size Comparison for Fetal Cartilage Chips

Figure 56: Pore Size Comparison for Lampire Cartilage Chips
Figure 57: Pore Size Comparison for Spear Cartilage Chips
Statistical Analysis

Below, in Figure 58, the Tukey procedure with an $\alpha = 0.05$ is used to assess whether the Lampire, Spear, and fetal cartilage chip groups are significantly different from one another according to fiber diameter. It was determined that the Spear group is significantly different from the Lampire and fetal groups in terms of fiber diameter. The Lampire and fetal cartilage chip groups are not significantly different from one another.

Below, in Figure 59, the Tukey procedure with an $\alpha = 0.05$ is used to assess whether the Lampire, Spear, and fetal cartilage chip groups are significantly different from one another according to pore size. The same relationship exists for the pore size as the fiber diameter. The Spear group is significantly different from both the fetal and Lampire groups. The fetal and Lampire cartilage chips groups are not significantly different from one another in terms of pore size.

When analyzing the different concentrations for all three groups combined according to the fiber diameter using the Tukey's procedure with an $\alpha = 0.05$, all concentrations (0.06, 0.08, 0.10, and 0.12 g/mL) are significantly different from one another (Figure 60). In Figure 61, the different concentrations for all three of the cartilage chip groups combined are analyzed according to pore size in the same manner as was done for fiber diameter in Figure 60. For the pore size of all three groups, the concentrations of 0.06 and 0.08 are not significantly different. The concentrations of 0.10 and 0.12 are also not significantly different. The two sets of concentrations are significantly different from each other in terms of pore size when all three of the cartilage chips groups are combined.
Figure 58: Tukey’s One Way Analysis of the Lampire, Spear, and Fetal Cartilage Chip Groups According to Fiber Diameter

Figure 59: Tukey’s One Way Analysis of the Lampire, Spear, and Fetal Cartilage Chip Groups According to Pore Size
Figure 60: Tukey's One Way Analysis of Fiber Diameter for Lampire, Spear, and Fetal Cartilage Chips According to Concentration

Figure 61: Tukey's One Way Analysis of Pore Size for Lampire, Spear, and Fetal Cartilage Chips According to Concentration
Discussion

For the first run of the collagen type II extraction, there were three major mistakes that were made, which led to much smaller end yields than were expected. The first mistake was the blender used to grind the cartilage chips in step #2. Only a Hamilton Beach 14 speed blender (model #54252B) was used with 400 Watts of power (0.5 horsepower), but a Waring blender with a minimum of three horsepower was necessary for the proper maceration of the cartilage chips. The type II collagen cannot be fully extracted if the pieces of cartilage are too large. The blender utilized barely made the pieces any smaller for this first extraction then when the pieces were cut by hand. The second mistake was made in step #10, which was that the pepsin extracted collagen type II supernatant was not left out at room temperature overnight after the 5 M NaCl was added. Instead, the solution was placed in the refrigerator because of the possibility of denaturalization of the material. Leaving this solution out at room temperature actually precipitates the collagen. Lastly, when preparing the sample solution for the SDS-Page, the material used was the actual dialyzed collagen solution from step #22. When the final material (liquid form before freezing and lyophilization) from step #22 was used, there was no way to measure the concentration of type II collagen in the dilute acetic acid because it was so low (difficult to detect). If the lyophilized collagen was used (as was done in the third extraction), the dissolved concentration is known and can be made equal to that of the standards.
When comparing the SEM pictures for the fetal cartilage chip group from the first extraction, Figures 19, 20 and 21 (0.13, 0.14, 0.15 g/mL) are a bit different from the rest. There is a combination of smaller and larger fibers. This could be due to the fact that these are the higher concentrations and the dissolved material was very thick and was sputtering out of the needle. It is also possible that the material may not have been mixed properly due to the presence of a high level of viscosity or there may have been a contamination issue.

For the second extraction, the correct blender was utilized for the second step of the collagen type II extraction. A Waring CB15 three horsepower blender was used to blend the chips into a fine milkshake consistency. There was still a problem with getting an acceptable yield for the three batches and that was due to the addition of salt to begin the precipitation in step #10 of the collagen type II extraction process. The speed of the addition of the 5M salt to the solution is critical. It must be added slowly and not create any disturbance in the solution (bubbles). During this extraction, the carboy of 5M NaCl was placed above the container of the stirring solution and to allow the salt to drip into the container below in a stream-like fashion (not a slow drip). The speed of the addition was too fast, which can cause an improper precipitation of the collagen type II. The solution was left out at room temperature and there was an actual cloudiness in the solution, which was a visible sign that the collagen type II had precipitated. This was not seen in the first extraction. For the first extraction the solution was completely clear when taken out of the refrigerator after the salt precipitation. This proved that leaving the solution at room temperature overnight does play a vital role in the collagen type II precipitation. In the second extraction, a SDS Page was performed, but absolutely
nothing showed up on the gel. This was due to the mistake of not actually taking the
lyophilized collagen and dissolving it in dilute acetic acid to be used in the SDS Page.
Instead, we used the liquid material from step #22 which will have a very small collagen
concentration (also unknown concentration). The concentration was even smaller for this
processing run as compared to the first as is shown by the smaller calculated percent
yield for the three batches. Once this was realized, it was too late to repeat the SDS Page
because all of the lyophilized collagen type II was used to make an attempt to spin the
type II collagen from the three groups of cartilage chips. Again, for this run, the
electrospinning results were inconsistent due to the small amounts of material that were
extracted from the three groups. The ideal concentrations were electrospun (0.06, 0.08,
0.10, and 0.12 g/mL), but only the Lampire bovine cartilage chip group gave a yield of
lyophilized collagen type II material in an amount large enough for the electrospinning
procedure.

After two trials, we were able to detect and correct many errors. For the third
extraction, the correct blender was used, the salt was added at a drip by drip speed when
precipitating the collagen type II, and the material was left out overnight at room
temperature after the salt precipitation. There was enough end product from all three
cartilage chip groups to perform both electrospinning at all four concentrations and a
SDS-Page. The end product was greatest for this extraction for all three cartilage chip
types. This extraction allowed us to create much more comparable results because the
same four concentrations were electrospun for all three groups and the same
concentration of type II collagen actual lyophilized end product dissolved in dilute acetic
acid was used to run the SDS-Page. In the first trial, only the fetal bovine chips spun well
while the Lampire and Spear chips could only be spun at certain concentrations. In the second trial, only the Lampire chips produced material that could be electrospun and the material from the other two groups were wasted during the attempt to spin scaffolds, leaving no material for purity testing. This final third extraction yielded enough material to both run a SDS page to assure the material was pure collagen type II without any type I contamination and to electrospin each of the three cartilage chip groups at the desired concentrations of 0.06, 0.08, 0.10, and 0.12 g/mL HFP for the analysis of fiber diameter and pore size.

The main goal of this experiment was the extraction and electrospinning of type II collagen from the cartilage of bovine specimens. This was achieved for bovine specimens ranging from fetal to six months old. It was proven that through the manipulation of concentration of the dissolved collagen type II in HFP, the fiber diameter and pore size can be increased or decreased according to the desired size. This allows the collagen type II scaffold to have the ability to suit any need according to the size of the cell that will need to infiltrate into the scaffold. An optimal scaffold can be spun once the proper parameters are determined. Electrospinning is a method of creating scaffolds that has many variables including voltage, mandrel speed, distance between the mandrel and needle, and pump speed. The machine used to create the scaffolds in this experiment has room for error when adjusting the mandrel speed. There is no precise way to assure that the mandrel is spinning at the exact same RPM for the duration of the time the scaffold is constructed and there may be a difference when electrospinning one scaffold as compared to another at the same RPM setting. It was proven in the experiment that for all three cartilage groups, both fiber diameter and pore size increase as concentration increases.
As concentration decreases the opposite occurs. This is a highly important factor in tailoring an ideal matrix.

Both temperature and humidity may also affect the electrospinning of the scaffolds. Sometimes there may also be a charge build-up at the site where the material leaves the end of the syringe that is to be deposited onto the mandrel. A system design that is more highly enclosed with a highly precise way to measure the consistency at which the mandrel rotates may give the ability to have a much more regular fiber diameter and pore size. There is still quite a bit of variation in the scaffolds that were electrospun for this project.

The ability to electrospin an entire scaffold and not just onto a SEM pellet is highly important. Due to the small yields that were extracted during this experiment, we were unable to do so, but the processing technique has now been refined. Once the material is electrospun into mats, the material properties can be analyzed according to the varying concentration utilized to electrospin the mats.

The yield results for the three processing runs were inconsistent. This was due to the fact that there were many processing errors that were worked out along the way. For the first run, the fetal group had the highest percent yield, but for both the second and third run, the Spear cartilage chip group had the highest yield. This could be due to the possibility that the chips were manually scraped and had no contamination of blood vessels or bone marrow. For the second and third processing runs, the fetal chip group had the lowest yield and the Lampire chip group was in the middle.
Future Work

There is a great deal of work that needs to be done now that it has been proven that type II collagen can be extracted and electrospun. Articular cartilage poses a great problem to the aging society. The idea that collagen type II can be electrospun into any desired shape and size and manipulated to have the desirable pore size and fiber diameter for the seeding of chondrocytes gives great possibility for a tissue engineered product. The testing of the mechanical properties is highly important because it must be durable enough to resist high loads, yet porous enough to allow for the synovial fluid to flow in and out of the tissue to deliver the needed nutrients to the chondrocytes. It is likely that the collagen type II scaffold will need to be blended with a polymer to increase the strength. There are many FDA friendly polymers that are available that have minimal rejection from the body, such as PDS. Once the mechanical properties for tensile strength, modulus of elasticity, peak load, peak stress, and strain at break are determined to be the best match to that of the articular cartilage tissue inside the body, then this material will be chosen for the seeding of chondrocytes. Next, the concentration of this material must be chosen. The desired pore size and fiber diameter for optimal infiltration and regeneration of chondrocytes must be determined and the scaffold can be manipulated by varying the concentration to fit within these parameters. The scaffold should then be seeded with chondrocytes to see how the cells interact with the material.

The use of a combination of a natural and synthetic material would be optimal because the natural material will help with the possibility of rejection and will also be recognizable as "home" for the cells. Cells will be able to identify this familiar material as being similar to that of their natural environment. The addition of the synthetic
material will allow for a better determination of degradation rate inside the body. It will also increase the consistency of the material. Synthetic materials are produced in a highly regulated fashion, whereas the extraction of natural materials can often times be inconsistent from batch to batch.

The addition of growth factors or the use of stem cells is also an additional possibility once the optimal scaffold has been chosen. Once the scaffold has proven to allow chondrocytes to infiltrate, proliferate and regenerate, the scaffold will be ready for testing in vivo. The idea of a tissue engineered product made predominately of collagen type II could give many possibilities for the remedy of osteoarthritis or any other injuries to the joints of aging individuals.
References


Danielle Careen Knapp was born on February 16, 1980, in Visalia, California. In 1998, she graduated from Brookville High School in Forest, Virginia. She received her Bachelor of Science degree in Biomedical Engineering from Virginia Commonwealth University in May of 2002. Danielle will receive her Master of Science degree in Biomedical Engineering at Virginia Commonwealth University in December of 2005.