Decellularized Cartilage as a Bioactive Biomaterial for Cartilage Tissue Engineering

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ABSTRACT

Damage to articular cartilage is a particularly perplexing clinical problem because cartilage has a limited ability to repair itself. Currently, clinical techniques to treat articular cartilage regeneration fail at regenerating fully functional articular cartilage and in extreme cases the cartilage regeneration may lead to the need for a total joint replacement. Recently, tissue engineering approaches to articular cartilage repair have explored the use of extracellular matrix (ECM) based materials for enhanced regeneration of cartilage. ECM-based materials are of interest in the tissue engineering field because in all tissues the ECM plays an important role in cell function by affecting cell communication, migration, and differentiation. Tissues can be decellularized to remove any immunogenic remains of cells to obtain an acellular ECM material to be used as a tissue engineering scaffold. The objective of this thesis was to evaluate a chemical and physical method for decellularizing articular cartilage and characterizing the cellular response to both the un-modified material and the material incorporated into microsphere-based scaffolds. Cells cultured in the presence of decellularized cartilage (DCC) alone exhibited increased expression of chondrogenic gene markers including collagen II, Sox9, and aggrecan relative to negative controls and TGF-β. Additionally, encapsulation of DCC in poly(lactic-co-glycolic acid) (PLGA) microspheres had comparable effect on cells *in vitro* compared to the encapsulation of TGF-β. These results indicate that DCC is a promising material for future use in cartilage tissue engineering and is worthy of additional future investigation.

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CHAPTER 1: Introduction

The overall objective of this thesis was to determine the cellular response to cartilage matrix *in vitro*. Cartilage regeneration remains a challenging problem for tissue engineers due to the reduced capacity for self-repair. Bioactive biomaterials may have the potential to aid in cartilage regeneration by recruiting and inducing differentiation of stem cells. To achieve the overall objective, first, porcine cartilage was fully characterized through a combined physical and chemical decellularization process. The cellular response to both decellularized cartilage (DCC) and devitalized cartilage (DVC) was also assessed *in vitro*. DCC is cartilage that has been fully decellularized via chemical and physical methods. The decellularization process removes a majority of the residual DNA and results in an acellular matrix. DVC is cartilage that has been devitalized by only physical methods and does not have a direct way of removing nucleic acids from the matrix. After fully characterizing the material and cellular response to the un-manipulated material (i.e., non-crosslinked, non-solubilized), the DCC was incorporated into microsphere-based tissue engineering scaffolds by one of two ways: encapsulation in poly(lactic-co-glycolic acid) (PLGA) microspheres and surface coating of PLGA microsphere-based scaffolds.

Development of a bioactive scaffold containing DCC for articular cartilage regeneration followed three specific aims: (1) chemical and physical decellularization of porcine articular cartilage, (2) assessing the *in vitro* BMSC response in the presence of DCC and DVC, and (3) incorporating DCC into microsphere-based scaffolds and assessing the response *in vitro*.

The first aim characterized cartilage tissue through a chemical and physical tissue decellularization protocol. Assessing the loss of DNA after each step during the decellularization process assessed the efficacy of the chemical and physical decellularization methods. The

biochemical content in the matrix was also measured following each tissue-processing step. The second aim used both DCC and DVC to assess the response of BMSCs cultured in pellets in the presence of the respective types of cartilage matrices. Cellular response was assessed using RT-qPCR and DNA content of the cell pellets. Aim 3 investigated the effect DCC had on BMSCs when incorporated into microsphere-based scaffolds. DCC was encapsulated in PLGA based microspheres and also used to coat the surface of PLGA microsphere-based scaffolds. The differences the methods to incorporate DCC (i.e., encapsulation vs. coating) had on cellular response were compared to each other as well as to positive and negative controls.

The organization of the thesis chapters is as follows:

Chapter 2 contains relevant background information about the bioactivity of cartilage matrix materials with respect to cartilage tissue engineering. The chapter outlines the differences between the source of cartilage matrices (tissue derived vs. *in vitro* derived) and the matrix processing (decellularization vs. devitalization). Additionally, it discusses the potential advantages and disadvantages to using cartilage matrix-based materials in tissue engineering applications. Chapter 2 provides insight into the motivation for the following chapters (3 and 4).

Chapter 3 addresses the first two aims: characterization of cartilage matrix through devitalization and decellularization and the cellular response *in vitro*.

Chapter 4 focuses on aim 3 involving DCC incorporation into microsphere-based scaffolds for cartilage tissue engineering.

The conclusion of the thesis is chapter 5, which summarizes findings from the two experimental chapters. The conclusions from this work are discussed from a global perspective with recommendations for future studies.

CHAPTER 2: The Bioactivity of Cartilage Extracellular Matrix in Articular Cartilage Regeneration

ABSTRACT

Cartilage matrix is a particularly promising acellular material for cartilage regeneration given the evidence supporting its chondroinductive character. The 'raw materials' of cartilage matrix can serve as building blocks and signals for enhanced tissue regeneration. These matrices can be created by chemical or physical methods: physical methods disrupt cellular membranes and nuclei but may not fully remove all cell components and DNA, whereas chemical methods when combined with physical methods are particularly effective in fully decellularizing such materials. Critical endpoints include no detectable residual DNA or immunogenic antigens. It is important to first delineate between the sources of the cartilage matrix, i.e., derived from matrix produced by cells *in vitro* or from native tissue, and then to further characterize the cartilage matrix based on the processing method, i.e., decellularization or devitalization. With these distinctions, four types of cartilage matrices exist: decellularized native cartilage (DCC), devitalized native cartilage (DVC), decellularized cell derived matrix (DCCM), and devitalized cell derived matrix (DVCM). Delivery of cartilage matrix may be a straightforward approach without the need for additional cells or growth factors. Without additional biological additives, cartilage matrix may be attractive from a regulatory and commercialization standpoint. Source and delivery method are important considerations for clinical translation. Only one currently marketed cartilage matrix medical device is decellularized, although trends in filed patents suggest additional decellularized products may be available in the future. To choose the most relevant source and processing for cartilage matrix, qualifying testing needs to include targeting

the desired application, optimizing delivery of the material, identify relevant FDA regulations, assess availability of raw materials, and immunogenic properties of the product.

INTRODUCTION

Articular cartilage injuries present a unique and challenging medical problem due to the tissue's lack of regenerative ability. The reduced vascularity, limited cell population, and dense extracellular matrix (ECM) inhibit cartilage regeneration. Untreated cartilage defects due to osteoarthritis or injury can lead to swelling, joint pain, and further degeneration of the tissue and eventually the need for a total joint replacement.[1]

The goal of cartilage regeneration and repair is to produce fully integrated tissue at both the articular surface and the subchondral bone that has mechanical and chemical properties similar to native cartilage.[2] Many current surgical cartilage defect treatments such as autologous chondrocyte implantation (ACI), microfracture, osteochondral transplantation (mosaicplasty), and current allograft implants usually do not produce fully integrated tissues, tissues with native mechanical strength, or tissues with the same composition as native articular cartilage.[1, 3, 4] Freezing allograft implants can decrease the cellularity of the grafts and can in turn cause the implant to have inferior clinical outcomes compared to fresh allograft tissues.[5] These treatment options may also be associated with additional surgical risks and time to regain joint function.

The tissue engineering field has recently seen an emerging trend toward acellular biomaterials as an alternative to cell-based therapies.[4, 6] In particular, the ECM in a variety of tissue types can be used as an acellular biomaterial through decellularization or devitalization processes. It is important to distinguish between the sources of the cartilage matrix (*in vitro* vs. tissue derived) and to further characterize cartilage matrix by either decellularization or

devitalization processing (Fig. 2.1). Decellularized native cartilage (DCC) can be obtained from human cadavers or xenogeneic sources and is typically decellularized via chemical processes, usually combined with physical methods to remove nearly all cells and residual cellular components. Native devitalized cartilage (DVC) can also be obtained from human or xenogeneic sources, but is subjected to only physical processing such as freeze-thaw cycles or freezer-milling without any chemical decellularization agents. Physical methods disrupt cellular membranes and nuclei but may not fully remove cellular DNA, cell associated proteins, and other cell remnants (e.g., phospholipids). Decellularized cell-derived matrices (DCCM), in contrast, are ECM materials secreted by cells *in vitro* that have been chemically decellularized. Devitalized cell-derived matrices (DVCM) are cell-derived matrices that have been devitalized via physical methods only. Cell derived matrices are generally less dense than native tissues and may not contain the same composition as native tissues.

Acellular tissues are promising biomaterials because they contain the materials found in native ECM, which can provide a unique microenvironment for cells that is dependent upon the tissue. These materials may provide both chemical and mechanical signals to aid in differentiation of stem cells and the regeneration of the tissue.[6] ECM materials can also be constructively remodeled and act as building blocks for the newly formed tissue instead of being degraded and removed. Many tissue types have been successfully decellularized including small intestinal submucosa (SIS), muscle, liver, kidney, adipose, tendon, colon, and heart valves.[7-11] Other tissues that have been decellularized have varying results with respect to their mechanical properties, biochemical content, and structure following decellularization. In general, less dense tissues are able to be decellularized more efficiently and can maintain their microstructure following decellularization. In all tissues, increasing exposure time to decellularization agents

decreases the mechanical integrity and structure of the tissue. Decellularized heart valves have been implanted in patients with little early success; however, SIS matrix has been successful in repair of numerous tissues.[12-14]

Hyaline cartilage is an ECM-rich tissue with approximately 95% of the dry weight being made up of ECM.[15] The primary components of hyaline cartilage ECM consist of the proteoglycan aggrecan, which itself is rich in glycosaminoglycans (GAGs), and collagen II. Because of the rich ECM nature of cartilage, decellularization of the material may result in high material yields of the native ECM components. The high density of articular cartilage, however, presents a challenge to effective decellularization.

The field of tissue decellularization is well developed in the cardiovascular field, but has only recently begun developing in the hyaline cartilage field. According to the Web of Science citation report on March 21, 2014, the decellularized cartilage topic has seen a marked increase in publications in the past decade, with over 20 publications reported in 2013 compared to only one in 2003. Previous reviews of acellular biomaterials and the use of ECM for osteochondral tissue engineering have covered these topics extensively with a broad overview.[1, 4] While these reviews have successfully introduced the field of acellular ECM materials for cartilage and bone tissue engineering, they have not clearly defined and delineated the differences between cartilage matrix sources or processing techniques, nor have implications of putative pathways for Food and Drug Administration (FDA) approval and commercialization been considered. Prior studies in the cartilage matrix field lack cohesiveness that follow similar methods to evaluate cartilage matrix materials including acellular controls to evaluate biochemical and DNA content of scaffolds and pre-implantation mechanical testing. This current review addresses the differences between decellularization and devitalization of native tissues and cell derived matrices, current

decellularization and devitalization methods used for cartilage matrices, the ways in which cartilage matrix has been incorporated into tissue engineering scaffolds, the immunogenicity of decellularized and devitalized tissues, and the future of cartilage matrix as a translational biomaterial for cartilage regeneration.

NATIVE CARTILAGE MATRIX

Native cartilage matrix is cartilage derived from articulating joints of either human or animal sources. The composition of native cartilage may vary depending on the donor organism's species, age, health status, and other genetic factors.[16-18] Certain disease states, particularly osteoarthritis, will produce articular cartilage with reduced amounts of GAG and collagen II.[18] Zonal variations (i.e., the depth at which it is collected) within the articular cartilage structure are also an important factor to consider with respect to the composition of the harvested material.

Decellularized Native Cartilage Matrix (DCC)

Chemical decellularization of cartilage is a method that primarily uses chemicals to lyse and remove the cells and their components from the surrounding ECM. Frequently used detergent decellularization chemicals include sodium dodecyl sulfate (SDS) or sodium lauryl sulfate (SLS), EDTA, Triton X-100, and Tris-HCl (Table 1).[19-22] Various formulations of DNases and RNases are also commonly used to remove nucleic acids from the material.[22, 23] Many chemical decellularization protocols encompass some combination of these chemicals. Because of the dense nature of articular cartilage, to improve the efficiency of chemical

decellularization the native macro-structure must often be disrupted, which allows the material to be more effectively exposed to the chemical decellularization agents for shorter amounts of time.[15] This may include mincing the cartilage into small particles or freezer-milling prior to the chemical decellularization process. Successful decellularization of intact cartilage slices has occurred, however, little GAG was retained within the matrix and the resulting material was primarily collagen.[24] The shorter exposure times to decellularizing agents that can be achieved by first mechanically processing the tissue is often beneficial for the retention of the microstructure including GAG and collagen II concentration while more effectively decreasing in double stranded DNA content. However, by sacrificing the macrostructure of the matrix, the mechanical integrity of the tissue is also compromised.[25]

The use of chemical detergents to decellularize cartilage results in a significant decrease in the amount of whole cells, cell nuclei, and DNA present in the tissue. Hematoxylin and eosin staining (H&E), immunohistochemistry (IHC), SEM imaging, mass spectrometry, ELISA, and quantitative DNA assays have confirmed the reduction of cells, cell fragments, cell associated proteins, and nucleic acids in chemically decellularized cartilage.[19, 21, 22, 24, 26, 27] Chemical decellularization methods such as 2% SDS treatment for 2 hours or tritonX-100, EDTA and nuclease treatment have also shown that the DCC can retain collagen II and GAGs in the material and has been confirmed by immunofluorescent staining, histological staining, 1,-9,-Dimethylmethylene Blue (DMMB) sulfated GAG assay, and chloramine-T hydroxyproline assay.[23, 28] The amount of GAG retained in DCC, however, significantly decreased with increasing chemical decellularization, while collagen II levels did not significantly decrease.[28] A reduction in GAG content may be undesirable based on previous studies that have shown that certain GAGs such as aggrecan are chondroinductive.[29, 30] Biomechanical properties such as the

aggregate modulus and linear modulus have also decreased following decellularization agent exposure.[24, 28, 31] The decellularization chemicals used in studies with decreased mechanical performance included: 2% SDS treatment, a non-enzymatic treatment with NaOH, ethanol, guanidine HCl-sodium acetate solution, H_2O_2, and NaCl, and another non-enzymatic method with washes of NaOH and H_2O_2. Although stiffness may be diminished following decellularization, Schwarz *et al.*[31] reported DCC regained as much as 77% of native cartilage stiffness after 42 days of *in vitro* culture with human chondrocytes after decellularization with NaOH, ethanol, guanidine HCl-sodium acetate solution, H_2O_2, and NaCl.

In vitro culture of canine bone marrow-derived mesenchymal stem cells (BMSCs) on a canine derived DCC scaffold have also differentiated into chondrocyte-like cells when cultured in chondrogenic differentiation media.[20] The BMSCs attached to the scaffold and exhibited a round or elliptical morphology confirmed by SEM.[20] Chondrogenically-induced canine MSCs also attached and proliferated on human derived DCC scaffolds after 21 days of culture confirmed by PKH26 imaging, SEM, IHC, and histology.[22]

In vivo implantation of DCC has been shown to enhance defect repair with implanted with pre-differentiated rabbit ASCs confirmed by histology, IHC, and biochemical quantification of GAG and collagen II content after 6 months implanted in rabbits (Table 2).[32] These scaffolds seeded with exogenous cells produced regenerated tissue with 83% of native cartilage stiffness after 6 months.[32] ASCs also attached to the DCC scaffold and exhibited a round morphology confirmed by SEM when seeded on DCC scaffolds.[32, 33] Other *in vivo* implantations of DCC scaffolds in canine knee osteochondral defects seeded with canine BMSCs showed that after 3 and 6 months, the defects were filled with higher quality and better integrated tissue than control groups implanted with scaffolds and without predifferentiated BMSCs.[20] At 6 months, the repair

cartilage exhibited 70% stiffness of native cartilage.[20] Comparisons by gross morphology, histological examination, and micro-CT analysis between experimental and control groups were all in agreement. Using a similar biphasic scaffold for femoral head osteochondral defects, however, resulted in failure of the implant leading to collapse of the femoral head and severe osteoarthritis.[34] Proposed mechanisms of failure included accelerated degradation of the cancellous bone region of the scaffold, ischemic conditions, and high load bearing conditions.[34] Implantation of DCC scaffolds seeded with ASCs also produced superior defect healing compared to groups without cells or no scaffold as confirmed by histological observation.[32]

If DCC is desired for a tissue engineering application, the best route of delivery to the defect site must be determined. DCC used in tissue engineering can be incorporated or made into some type of scaffold that has both form and mechanical function, but when used in joints, the scaffold must support relevant compressive and shear loads. A common scaffolding technique used with DCC following chemical decellularization is freeze-drying followed by crosslinking. The crosslinking may be achieved by various methods including genipin, ultraviolet radiation, carbodiimide chemistry, and dehydrothermal treatment.[20, 32, 33]

A sandwich model for tissue engineering DCC scaffolds has been reported that consists of layers of DCC sheets and chondrocytes.[19] This particular sandwich model used thin sections of porcine ear cartilage (10 or 30 μm) obtained through freeze sectioning of the tissue. The decellularization was carried out via 1% SDS after the freeze sectioning and the DCC sheets were then stacked alternatively with chondrocytes. This scaffold technique can be used to create different shapes and sizes of scaffolds.

DCC has also been combined with synthetic biomaterials such as poly(lactide-co-glycolide) (PLGA) to create scaffolds: porcine DCC was added to PLGA (70/30) dissolved in

dioxane at a concentration of 7% (w/w) and after using a temperature based phase separation, the scaffold was freeze-dried in a mold.[35] The DCC particles that were used were fibrous and aligned vertically in the scaffolds by a temperature guided phase separation. SEM confirmed the orientation of DCC fibers and uniform, interconnected pores in the scaffold.

Native articular DCC has also been digested by pepsin to create an injectable hydrogel for drug delivery purposes.[36] The DCC hydrogel was found to sustain release of a fluorescently labeled protein for up to 22 days *in vivo* in rats. This hydrogel could potentially also be used for tissue engineering applications that include release of bioactive proteins to aid in regeneration or disease mitigation.

In summary, chemical decellularization of cartilage tissue is an effective method for removing cells and their components from the surrounding ECM. This type of decellularization, however, may alter the biochemical composition of the ECM, including a reduction of GAG content. Chemical decellularization also may require complete destruction of the tissue macrostructure rendering the resulting material mechanically unstable. For delivery to defect sites, DCC that has been chemically and physically processed may require additional manipulation to fabricate mechanically functional scaffolds. *In vitro* and *in vivo* studies have shown favorable responses such as chondrogenic differentiation of stem cells and improved defect repair with chemically decellularized cartilage.

Devitalized Native Cartilage (DVC)

Physical devitalization of tissue uses physical methods to disrupt cellular functions or lyse cells within a tissue. One example is freezer-milling followed by heat-inactivation to inactivate the cells found in the tissue without removing cells and all cellular components.[37]

Freezer-milling pulverizes the tissue into particles at low temperatures and the tissue is then heat-inactivated in a gravity oven. Devitalization can also be accomplished through hydrated tissue homogenization followed by retrieval of tissue particles, freezing, and lyophilization.[38, 39] Freeze thaw cycles followed by sonication has also been used to devitalize CDM constructs and could conceivably be used to devitalize native cartilage.[40]

Physical devitalization of articular cartilage does not have direct means to remove cellular components after deactivation of the tissue. The effect of physical devitalization on the ECM composition has not been widely reported in tissue engineering applications. Yang *et al.*[39] found following devitalization via tissue homogenization and centrifugation that GAG and collagen II remained in the DVC matrix confirmed by histology and IHC. Most studies reporting the use of physical devitalization to process DVC have not confirmed loss of DNA or retention of GAG or collagen content. Studies exploring the freezing and thawing of tissues for cryopreservation have shown that freezing cartilage induces apoptosis and necrosis in chondrocytes.[41] Freezing the tissue causes extracellular ice crystals to form that may cause an osmotic imbalance within the tissue creating acidic conditions, which activate degradation enzymes that degrade collagen fibers. This degradation of ECM components, however, may be minimal, as Szarko *et al.*[42] found no detectable change in collagen and GAG content following one freeze-thaw cycle. The rate of freezing and thawing can be controlled to attempt to preserve the ECM. One study has suggested that fast thawing conditions can help maintain the mechanical integrity of the tissue and that the temperature at which the tissue is frozen does not affect the cartilage stiffness.[42] Protease inhibitors may also be used to increase the preservation of the ECM during freeze-thaw cycles.[43] For a complete review of cryopreservation induced stresses in articular cartilage, the reader is directed to Kaur *et al.*[41]

Comparing human devitalized cartilage particles via heat inactivation and native cartilage particles shows that heat-inactivated cartilage particles exhibited a reduced formation of neocartilage compared to cartilage particles that were not devitalized when implanted in a critical-sized chondral defect in immunocompromised rats for 28 days.[37] Cartilage particles alone, heat inactivated or not, did not induce high quality chondrogenesis without the addition of exogenous growth factors. Peretti *et al.*[44] reported that when comparing porcine live and devitalized cartilage implants' formation of neocartilage when implanted subcutaneously in nude mice, porcine chondrocytes suspended in fibrin glue sandwiched between devitalized constructs saw a delay in neocartilage formation over an 8 week period. Analysis was performed using confocal microscopy and histological staining and compared to acellular fibrin glue and cartilage controls. The acellular control groups without cells in the fibrin glue did not produce cartilage-like matrix. This suggests that although devitalized cartilage may have a delayed effect compared to live cartilage on neocartilage formation, neocartilage formation could still be possible with the use of devitalized cartilage when seeded with exogenous cells.[44]

Cartilage that had been devitalized by homogenization of the tissue was molded into scaffolds and seeded with ASCs. The constructs were cultured in chondrogenic differentiation medium without exogenous growth factors and showed significant up regulation aggrecan and collagen II gene expression after 14 days.[38] Biochemical analysis also revealed statistically significant decreasing GAG content after 42 days.

DVC has been incorporated into electrospun scaffolds by combining solubilized DVC with poly(ε-caprolactone) (PCL). The mixture was electrospun into single and multiple layer porous constructs.[45] When seeded with P4 human ASCs the DVC-PCL constructs showed

increased GAG and dsDNA content at times 0, 14, and 28 compared to PCL only constructs *in vitro.*

In vitro studies with DVC followed similar trends as DCC with respect to mechanical performance. Cheng *et al.*[38] reported that the aggregate modulus of frozen and lyophilized DVC increased over 28 days when cultured with human ASCs in incomplete chondrogenic differentiation medium. DVC has also been incorporated into scaffolds with synthetic polymer components such as PCL. Woven PCL-DVC constructs had a lower aggregate modulus than PCL constructs alone but a greater aggregate modulus than DVC constructs alone.[46] The aggregate modulus of DVC scaffolds can also be increased by increasing the crosslinking percentage as reported by Cheng *et al.*[33]

In summary, physical devitalization of cartilage deactivates the tissue without removing the cells and their components; however, the effect on the tissue's ECM is unknown when devitalizing native articular cartilage. When cultured in the presence of devitalized cartilage, ASCs have undergone chondrogenic differentiation.[38] Devitalized cartilage has been shown to have the ability to induce neocartilage formation when implanted subcutaneously.[44]

CELL DERIVED MATRIX (CDM)

CDM is derived from cells grown *in vitro*, whether in monolayer or 3D culture. CDM can be obtained from mesenchymal stem cells (MSCs), fibroblasts, chondrocytes, preosteoblasts, or any other cell type that can be induced to excrete cartilage-like matrix.[47, 48] Different cell types can be mixed or the resulting ECM materials can be combined to create mixed or gradient tissue scaffolds. CDM cannot be obtained until the cells have been cultured long enough to secrete

ECM materials, which can require up to 3 weeks.[49] Perfusion bioreactors have been used to encourage cell deposition of ECM *in vitro* and may be used for increased ECM production.[50, 51]

The effect that the CDM has on tissue regeneration and cellular response has been shown to be dependent on the age of the cells secreting the matrix. CDM from fetal human synovium-derived stem cells (SDSCs) had greater positive effects on stem cell proliferation, differentiation, and mechanical functionality compared to CDM from adult SDSCs.[52]

Cells can be seeded into 3D scaffolding materials such as open-cell foams to create CDM constructs with tunable 3D geometry and composition, after which the synthetic foam can be removed.[53] This process creates a porous ECM-derived scaffold that may then be decellularized without additional manipulation of the matrix such as crosslinking. The synthetic portion of the 3D constructs may alternatively remain as part of the tissue engineering scaffold with the CDM that has been deposited onto the surface of the synthetic material.[40, 50]

A benefit of CDM is that it may be created from an autologous or allogeneic source to reduce the possibility of a negative immunological response. If autologous CDM is attainable, decellularization or devitalization may not be necessary but the product would need to be specially produced for each patient and therefore there would be no off the shelf product. The procedure to create autologous CDM must overcome many of the same challenges associated with the current ACI treatment including the need for two surgeries, the time between surgeries required to wait for the cells to expand *in vitro*, good manufacturing practice (GMP) facilities to culture the cells and associated costs, and thus also health insurance reimbursement. Another challenge with CDM is that the cells must remain in the differentiated state to excrete the correct type of ECM material. CDM is also obtained in smaller quantities in a greater amount of time than native cartilage tissue. To observe enhanced chondrogenesis, *in vitro* exogenous growth

factors may be necessary.[51, 54] The composition of CDM may also vary from that found in typical native tissue, and reproducibility may also become a concern.

Decellularized Cell-Derived Cartilage Matrix (DCCM)

DCCM is CDM that has been fully decellularized. Because CDM is less dense than native cartilage, the decellularization process is generally shorter, less abrasive to the matrix materials, and more efficient at cell removal.[1, 40] Mechanical methods that are usually paired with chemical decellularization for efficient decellularization of DCC are usually not necessary. DCCM was shown to be susceptible to decreasing aggregate modulus with long decellularization protocols.[25] The type of chemical used to decellularize DCCM also affects the aggregate modulus. A greater aggregate modulus can be maintained for chondrocyte derived DCCM with 1% SDS, 2% SDS, and 2% TnPB treatments for 1 hour.[25] Increasing exposure to these methods for 8 hours significantly reduces the aggregate modulus. Decellularization treatments of 2% Triton-X or osmotic shock both significantly decrease the aggregate modulus of DCCM constructs after 1 hour of exposure.[25]

Devitalized Cell-Derived Matrix (DVCM)

DVCM is derived from cells *in vitro* just as DCCM. However, DVCM is devitalized via physical processes instead of chemical methods. As with DVC, there is no means to fully remove the DNA from the matrix. When using cell derived matrix, however, Levorson *et al.*[40] has shown a large decrease in DNA, GAG, and collagen content following freeze thaw cycles and sonification of DVCM constructs. This may be due to the large differences between native and cell derived matrices in their density and composition. DVCM has the same benefits as DCCM

such as the easy manipulation of the matrix orientation and the ability to coat synthetic surfaces. The physical methods to devitalize the construct must be considered if the material is also combined with synthetic materials as the methods may alter the synthetic material composition or mechanical properties.

In summary, CDM is derived from *in vitro* cell culture and can be easily decellularized and devitalized largely due to the low density of the matrices. DCCM and DVCM can be created from many different cell types and can further be incorporated into tissue engineering scaffolds by either coating synthetic materials or crosslinking constructs.

CLINICAL TRANSLATION

Decellularized materials are attractive options from a commercialization and regulatory approval standpoint. Because the tissue can be processed in a way that removes cellular components and ECM antigens, the decellularized tissues may be negligibly immunogenic and conducive to FDA approval. Operational costs of maintaining viable tissue could also be decreased because the decellularized tissue may be stored for longer periods of time before use than cellular allografts.

Only one currently marketed cartilage repair technique employs the use of cartilage matrix that has been decellularized. Zimmer markets a product called the Chondrofix® Osteochondral Allograft, which is a decellularized allograft plug designed for osteochondral regeneration therapy.[55] The osteochondral device is treated to remove potentially harmful viruses and lipids and to sterilize the tissue and contains distinct cartilage and bone regions. There are no available clinical results describing the efficacy of the decellularized allograft.

Zimmer also markets the DeNovo^{TM} NT Natural Tissue Graft, which is particulated juvenile human cartilage allograft for osteochondral defect repair.[56] The juvenile cartilage allograft has a greater chondrocyte density and greater regenerative potential and has been shown to create hyaline-like cartilage *in vivo* in goats.[57] The resulting neocartilage from juvenile human chondrocytes did not elicit a T-cell-mediated immune response in goats.[57] This treatment option is primarily a cell-based approach as it relies on the potential of the juvenile chondrocytes. This product is only available for special orders and is not readily available off the shelf.

Arthrex markets BioCartilage®, a one step treatment that contains a combination of micronized human cartilage particles and autologous platelet rich plasma (PRP) to be used with microfracture techniques.[58] In a study with baboons, BioCartilage® was shown to promote chondral lesion regeneration without any adverse immunological reactions.[58]

Although the medical device field has only one decellularized tissue option for chondral or osteochondral regeneration, numerous patents describe cartilage decellularization and scaffolding techniques (Table 3).[55, 56, 59-68] These patents may indicate an increasing trend in products that have been decellularized to treat chondral and osteochondral defects. The FDA first approved a decellularized xenograft surgical mesh in 1998. A decellularized heart valve allograft and a decellularized pulmonary artery patch were also approved in 2008 via the 510(k) route. Shortly after, the Zimmer Chondrofix® implant was put on the market in 2011. The Chondrofix decellularized allograft is classified by the FDA as a human cell or tissue product (HCT/P) and therefore does not require investigational new drug or device exemption approval. With this precedent, this route may be likely for subsequent decellularized cartilage options as long as there is no additional cellular component or engineering to attract enhanced homing of stem cells *in vivo*. The material would likely need to be fully decellularized and, if xenogeneic, free of all

antigens. Lastly, the device would benefit from comparable mechanical properties to currently used allograft implants.

To summarize, there is only one truly decellularized cartilage matrix product currently on the market, however, without available clinical results the efficacy of the implant cannot be assessed. Patents suggest that decellularized cartilage matrix products may become increasingly available in the future. Limitations to currently marketed strategies such as reliance on cell viability and special orders further highlights the potential desirability of acellular cartilage matrices for therapeutic treatments as the viability of cells does not need to be considered and the final products may be available as off the shelf products.

IMMUNOGENICITY OF CARTILAGE MATRIX

Host immune response to tissue grafts can arise from cell surface markers, ECM epitopes, and residual DNA. Little work has been done to determine the immunogenicity of chondral and osteochondral xenograft implants. Cartilage-only repair treatments are somewhat immuno-privileged as compared to osteochondral approaches that expose the scaffold construct to the subchondral bone. The majority of research to determine the immune response due to decellularized xenograft implants has been assessed in cardiovascular implants, however, these findings may be valuable for osteochondral and cartilage only decellularized implants.[69-71]

Residual DNA Response

Studies have assessed the effect of differing decellularization levels of porcine small intestinal ECM (SIS) on macrophage phenotypes *in vitro* and *in vivo*.[72] Macrophages are

important for immune defense and normal tissue remodeling. Generally, for tissue engineering, M2 macrophage populations mark a repair and remodeling response whereas M1 macrophages represent a destruction and elimination response. Keane *et al.*[72] found that the more aggressive decellularization technique that resulted in the greatest reduction in DNA with only a small amount of short fragments remaining in the tissue helped to promote the macrophage phenotype to M2 *in vitro*.[72] However, in some cases a construct with greater amount of DNA in the tissue had a smaller M2 population than tissue with slightly less residual DNA. These opposing results may suggest that residual DNA within the tissue is not the only determinant of host immunological response.

Another study exploring the role of residual DNA in the immunogenicity of decellularized heart valves found that even with complete cell removal that human monocytic cells were attracted to the matrix *in vitro*.[73] This study also found that residual DNA was not the only factor in eliciting an adverse immunological response and that antigenic epitopes found in xenogeneic tissues may also play a role.

Alpha-Gal Epitope Response

Other causes of immunological responses may be due to the disaccharide galactose (α1,3)galactose (alpha-Gal epitope) found commonly in xenogeneic tissues.[74] The alpha-Gal epitope is commonly found in xenograft materials originating from nonprimate animals and is a carbohydrate found within the ECM.[75] The removal of alpha-Gal is important because it does not follow previous assumptions that the immunogenicity of xenograft materials arises solely from residual cells. One case in which the alpha-Gal epitope was not been fully removed was reported with a decellularized heart valve, Synergraft.[76] Due to both the presence of the alpha-Gal

epitope, incomplete decellularization, and inferior mechanical properties, clinical implantation of these decellularized grafts failed early when implanted in pediatric patients.[12] Methods to reduce the antigenicity of xenogeneic materials have been studied including the solubilization of proteins to enhance antigen removal from decellularized bovine pericardium.[77] In the bovine pericardium example, the solubilization of proteins during the decellularization process enhanced the removal of xenogeneic antigens and exhibited reduced antigen levels compared to decellularization protocols alone.

Some currently employed decellularization protocols, however, have reported effectively removing the alpha-Gal epitope during the decellularization process without additional enzymatic treatment such as alpha-galactosidase in bovine ligament and sheep artery tissues.[71, 75] The sheep tissue was tested for immune responses as an allograft material *in vivo*. Bovine tissue was tested *in vitro* using human peripheral blood mononuclear cells (PBMCs). Both of these studies found that the removal of the antigens in the decellularized tissue resulted in reduced immunogenicity of the tissue.

Physically devitalized human cartilage fragments (DVC) have been evaluated for host immune responses in immunocompromised rats.[37] Histological examination showed that there was no significant inflammation in the rats. In a separate study, porcine DCC was created from both physical processing and chemical decellularization and was devoid of the alpha-Gal epitope following decellularization as confirmed by immunohistochemistry.[26] The staining of fresh cartilage showed there was no expression of the alpha-Gal epitope in the tissue and the epitope was primarily found in the subchondral bone region. The decellularized porcine tissue was implanted subcutaneously in GTKO mice and showed a reduced fibrous capsule thickness and greater cell infiltration.[26] Although antigen removal from decellularized tissue may negatively

impact the material's mechanical performance or biochemical content, the removal of the antigen is important for successful xenotransplantation.[24]

Human Leukocyte Antigen (HLA) Response

HLA genes encode for cell surface antigens expressed by host cells. Normal HLA function is essential for disease defense and recognizing "non-self" antigens. Decellularization of human heart valves has shown that decellularized grafts elicited reduced anti-HLA antibody formation than implants that were not decellularized.[70] Fresh allografts are particularly difficult to reduce HLA antigenicity because the HLA antigens remain in the matrix. Since osteochondral allografts are composites of two tissue types, the immunological response may be varied. It has been hypothesized that much of the immune response to osteochondral allografts are due to the bone region of the implant.[78, 79] The dense nature of articular cartilage may help reduce immune response to the cartilage region because the cells are deeply embedded within the matrix and not easily assessable to immune cells. By matching HLA antibodies between host and donor tissues, the success and integration of allografts has been increased.[79] Hunt *et al.*[79] showed that increased HLA antibody formation was correlated with a greater diameter of the implanted osteochondral graft.

It has been shown that residual DNA is not the only cause of an unfavorable immune response. Although the most exhaustive immunological testing of decellularized tissues has been performed on cardiovascular implants, efforts have been made to ensure complete removal of the alpha-Gal epitope and HLA antigens from decellularized cartilage matrices. The removal of xenogeneic and cell surface antigens is important for successful implantation of xenogeneic or allogeneic material to ensure cell infiltration into the implanted material and successful repair of

the tissue, however, if using human tissue, alpha-Gal antigen removal is not needed, but careful attention to HLA type may be necessary.

RECELLULARIZATION AND HOST INTEGRATION

Recellularization of decellularized or devitalized cartilage matrices that have not been mechanically processed may be difficult because of the dense ECM. A proposed solution to the difficulty of repopulating cartilage ECM is to use microscopic units of cartilage matrix.[15] For an in depth review of the rationale for using microscopic donor units, the reader is directed to Ghanavi *et al*.[15] Using microscopic cartilage matrix units greatly decreases the distances required to travel by cells and allows greater infiltration into the tissue. Cartilage matrix materials that have been mechanically disrupted into small particles have then been crosslinked or freeze-dried to create scaffolds with large macroscopic pores that have shown to have successful cell infiltration and attachment in the scaffold.[20, 38] Another method that has been used to encourage cell infiltration into the dense cartilage matrix is to use thin sections of cartilage matrix (10 or 30 μ m) as used by Gong *et al*.[19] The constructs with 10 μ m thick cartilage matrix had a greater amount of penetrating lacuna that allowed for successful cell infiltration into the matrix. CDM coatings on synthetic biomaterial scaffolds also allow for constructs with greater porosity such as CDM coating on PCL electrospun scaffolds or other polymer-based scaffolds.[51, 80] CDM constructs fabricated using open-cell foams also result in scaffolds with high porosity to allow for cell migration and infiltration.[53] Decellularization of intact cartilage (no mechanical processing) may benefit from a significant decrease in GAG content to increase the porosity of

the matrix.[24] While this decrease in GAG content may aid in successful recellularization, the mechanical properties are greatly decreased with an approximately 70% reduction in stiffness.

Graft incorporation into host tissue is vital to successful long-term implantation of cartilage matrices for cartilage defect repair. The dense nature of articular cartilage makes host cell infiltration within the cartilage difficult and limited. However, it has been suggested that when implanting fresh osteochondral allografts with viable donor cells, host infiltration is not desired as host remodeling will promote formation of fibrocartilage and destroy the intact articular cartilage.[78] Studies exploring the use of DCC for cartilage regeneration have seen successful host integration when exogenous cells were added prior to implantation (Table 1.2). When compared to acellular scaffold controls, groups containing exogenous cells generally showed greater repair and regeneration including at the defect boundaries.

In summary, cartilage matrices have recently been manipulated to make the products more clinically relevant by creating scaffolds with larger pore sizes than found in native cartilage. This allows for greater cell infiltration and migration within the scaffolds. *In vivo* studies have reported successful integration of cartilage matrix based scaffolds in both osteochondral and cartilage only defects.

CHONDROINDUCTIVE NATURE OF CARTILAGE MATRIX

Cartilage matrix has been shown to have chondroinductive effects on cells *in vitro.* Human ASCs have differentiated into chondrocyte-like cells when cultured *in vitro* in the presence of porcine DCC.[33, 38] Both aggrecan and collagen II gene expression were increased over a 2-3 week period. Porcine DCC has also been shown to influence P1 human chondrocyte gene expression by

increasing both collagen II and aggrecan expression levels *in vitro*.[31] GAG production *in vitro* was also shown to increase when human chondrocytes were cultured in the presence of porcine DCC. Upregulation of aggrecan and collagen II gene expression has also been reported in dedifferentiated rat chondrocytes when cultured on fibroblast-, preosteoblast-, and chondrocyte-CDM.[47]

Human and porcine chondrocytes seeded on DVC constructs were both shown to proliferate and secrete GAG based on PicoGreen and DMMB assays without any added growth factors.[81] Histology and IHC showed the presence of collagen II and GAG in both human and porcine cell seeded constructs. In a separate study, human MSCs seeded on DVC constructs subjected to different crosslinking methods (UV, carbodiimide, and dehydrothermal) reported that crosslinking of DVC may help chondrogenesis.[54] Increases in DNA, GAG, and collagen content were reported in crosslinked groups compared to a noncrosslinked control without additional growth factors.

When solubilized DVC was incorporated into poly(ε-caprolactone) (PCL) electrospun scaffolds, chondrogenic differentiation of human P4 ASCs was also seen when compared to control PCL only constructs. Aggrecan gene expression was increased after 7 days *in vitro*.[45] Both collagen content and GAG content significantly increased between control PCL constructs and DVC-PCL constructs at 0, 14, and 28 days.

Little is currently known about the mechanism by which cartilage matrix promotes chondrogenesis. Proposed hypotheses include: ECM influence on cell shape, ECM stiffness, residual bound growth factors, ECM structure, or ECM biochemical content (GAG and collagen).[47, 81, 82] The decellularization or devitalization protocol used may affect either the biochemical content or the residual growth factors within the matrix. Wong *et al*.[77] demonstrated

that by altering existing decellularization protocols to keep proteins solubilized, they were able to effectively remove protein antigens from the tissue. Altering decellularization conditions to remove small antigenic proteins may also remove growth factors. Devitalization techniques such as freeze-thaw cycles may also increase the degradation or denaturation of latent growth factors within the ECM by activating degradation enzymes or pH changes due to physiochemical chemical stress within the tissue.[41] Additional studies are needed to determine the mechanism of chondrogenesis seen in cartilage matrix-based scaffolds.

The ability of cartilage matrix to influence the differentiation or re-differentiation of cells *in vitro* is promising, as these acellular materials may not need additional exogenous cells or growth factors when implanted *in vivo.* If this simplistic approach is viable, it is attractive because it could reduce costs and potentially gain FDA approval more quickly.

DISCUSSION

The use of ECM and other raw materials are gaining popularity in the regenerative medicine field as an alternative to synthetic materials due to their two main advantages of becoming integrated (rather than degraded and removed) and providing bioactive cues to autologous cells. ECM plays an important role in native tissue function by providing mechanical stability to the tissue and signals, both mechanical and chemical, to the cells contained within the ECM. Because the ECM plays a large role in cell signaling and differentiation, in the case of cartilage ECM, it has the potential to act as a *chondroinductive* material. Decellularized tissues largely retain the native composition of the ECM and therefore have similar signaling effects on cells. Cartilage must be able to support high compressive loads and therefore the repaired tissue must have high compressive strength. The chemical composition of the regenerated cartilage is

extremely important in the compressive strength of the tissue as many of the ECM components such as the GAGs and collagen II recruit and trap water within the tissue. Future work is necessary to determine whether the structure or composition of the matrix is more important for chondroinductive effects.

Cartilage matrix in hyaline cartilage tissue engineering has shown that it has the capacity to help differentiate both ASCs and BMSCs into chondrocyte-like cells, as well as dedifferentiate dedifferentiated chondrocytes. Although cartilage matrix can promote a favorable response *in vitro* and no negative responses *in vivo* on tissue repair, future work needs to evaluate whether the DCC has chondroinductive effects on cartilage healing *in vivo*. Induction of tissue repair is particularly important in cartilage regeneration because of the tissue's decreased ability to regenerate and repair itself, which is why successful cartilage regeneration has been cited as the most vexing problem in musculoskeletal medicine.[83]

Delivery of cartilage ECM materials must be considered to achieve the desired mechanical performance. Crosslinking cartilage matrix is a common method for creating scaffolds, but the effects of crosslinking on the chemical composition of the material have not yet been determined. The crosslinking creates a porous 3D scaffold that resists cell-mediated contraction. However, crosslinking may alter the matrix in ways that may slow or prevent its incorporation into the neocartilage, which may ultimately delay or prevent healing. Other scaffolding techniques such as combining with synthetic materials may be a promising avenue for delivery of cartilage matrix because the mechanical properties and 3D structure can be controlled.

We reiterate that it is important to distinguish between matrix derived from cell culture and matrix derived from native tissue, and to further categorize cartilage matrix into

decellularized and devitalized matrix. Both CDM and native cartilage matrix have appealing qualities, however, tradeoffs when considering whether to use native cartilage or CDM should also be evaluated to direct the field. Using CDM may result in a greater ability to manipulate the structure of the scaffold by controlling the shape the matrix forms without additional crosslinking or other chemical modifications. On the other hand, native cartilage materials can be produced in much greater quantities in shorter amounts of time.

Additional work should explore the immunogenicity of cartilage only and osteochondral xenograft implants. Most immunogenicity research regarding decellularized tissue is focused on cardiovascular tissue implants. While the cardiovascular implant studies may help inform and direct the cartilage matrix field, more information about how the xenograft material acts in the osteochondral environment is needed. A few studies have reported successful removal of xenogeneic antigens following chemical decellularization, however, this critical decellularization endpoint is not considered globally in the field. The removal of this antigen has only been considered with chemically decellularized matrices and has not been explored in devitalized tissues.

Currently, the ability to obtain human tissues is more established and easier than obtaining animal tissues. The cost of the tissue retrieval process as well as the tissue processing after harvest must be considered. The FDA approval process must also be considered; using allogeneic tissue may be more successful than xenogeneic tissue and approved more quickly because human tissue does not contain the alpha-Gal epitope and decellularization of the tissue would further decrease the immunogenicity by removing the donor cells. Native cartilage matrix and CDM may also suffer from insufficient supply for obtaining autologous or allograft tissue. Creating a viable business model and insurance reimbursement due to the need for multiple

surgical procedures may further complicate the use of autologous tissue for creation of CDM constructs. Currently, only DCC materials have been delivered to cartilage defect sites and are available on the market. Little is known about the FDA regulatory pathways CDM materials would follow and their efficacy *in vivo*.

In summary, cartilage matrix appears to be a promising material for hyaline cartilage tissue engineering applications. Native cartilage matrix and CDM are both ECM materials with established decellularization or devitalization techniques; however, at this time native cartilage matrix can be made in larger quantities in a short amount of time. To choose the most successful type of cartilage matrix for a particular application, we must decide if full decellularization is desired or if devitalization is acceptable. The source of the matrix, native or cell derived, must also be considered when designing the delivery construct as the chemical composition and mechanical properties of each type may differ greatly. FDA regulatory approval may affect the decision to use native or cell derived matrices as well as the type of processing the matrix undergoes. Most likely, for quicker approval, a full chemical decellularization of allogeneic matrix may be more successful because of the reduced antigenicity of the material due to both the removal of cells and no cross-species interactions. Insurance reimbursements are also an important consideration because route of delivery of the matrix must be designed in a way that reduces costs and performs as well as or better than current treatments. Cartilage matrix has been incorporated into different types of scaffolds including crosslinked, layered, and combination with other biomaterials, however; only one commercially available product consisting of decellularized cartilage exists at this time. Until clinical results are available, the success of this product is unknown. Other currently marketed products for cartilage repair have limitations such as cell viability and storage considerations that may be overcome through the use of acellular

matrices. The future of cartilage matrix must identify the effects of scaffold incorporation on the chemical composition of the matrix and define clear guidelines outlining the definition and limits of decellularization. Because previous work has identified cartilage matrix as being potentially chondroinductive, cartilage matrix may replace the need for more invasive surgical techniques to treat cartilage defects and arthritis.

CHAPTER 3: Decellularized Cartilage as a Chondroinductive Material for Osteochondral Tissue Engineering

ABSTRACT

Extracellular matrix (ECM)-based materials are attractive for regenerative medicine in their ability to potentially aid in stem cell recruitment, infiltration, and differentiation without added biological factors. In musculoskeletal tissue engineering, demineralized bone matrix is widely used, but recently cartilage matrix has been attracting attention as a potentially chondroinductive material. The aim of this study was thus to establish a chemical decellularization method for use with articular cartilage to quantify removal of cells and analyze the cartilage biochemical content at various stages during the decellularization process, which included a physically devitalization step. To study the cellular response to the cartilage matrix, rat bone marrow-derived mesenchymal stem cells (rBMSCs) were cultured in cell pellets containing cells only (control), chondrogenic differentiation medium (TGF-β), chemically decellularized cartilage particles (DCC), or physically devitalized cartilage particles (DVC). The chemical decellularization process removed the vast majority of DNA and about half of the glycosaminoglycans (GAG) within the matrix, but had no significant effect on the amount of hydroxyproline. Most notably, the DCC group significantly outperformed TGF-β in chondroinduction of rBMSCs, with collagen II gene expression an order of magnitude or more higher. While DVC did not exhibit a chondrogenic response to the extent that DCC did, DVC had a greater down regulation of collagen I, collagen X and Runx2. A new protocol has been introduced for cartilage devitalization and decellularization in the current study, with evidence of chondroinductivity. Such bioactivity along with providing the 'raw material' building blocks of

regenerating cartilage may suggest a promising role for DCC in biomaterials that rely on recruiting endogenous cell recruitment and differentiation for cartilage regeneration.

INTRODUCTION

Degeneration of articular cartilage can be caused by traumatic injury or arthritis. Articular cartilage regeneration is a particularly difficult problem because cartilage has a limited capacity for self-repair and low vascularity.[18, 83] Current clinical treatments for cartilage degeneration include autologous chondrocyte implantation (ACI), mosaicplasty, microfracture, and allograft implants. These treatments have limited success in fully regenerating functional articular cartilage. The repair tissue is often fibrous with inferior mechanical performance compared to native cartilage.

In the past, tissue engineering has aimed to regenerate articular cartilage utilizing synthetic biomaterials due to the ability to alter and control synthetic materials' mechanical properties.[84-86] These synthetic materials, however, have limited ability to recruit and differentiate stem cells without added biological components such as peptide sequences or growth factors. Recently, acellular extracellular matrix (ECM) materials have become popular because the matrices retain the native structure of cartilage, which provides cells with both mechanical and chemical signals to aid in stem cell differentiation and recruitment, and ultimately in tissue regeneration.[1, 4, 6] ECM materials can induce differentiation and regeneration without additional biologic additives, which may be an attractive alternative from both cost and regulatory standpoints.[31]

ECM materials can be obtained from either cell-derived matrices secreted during *in vitro* culture (CDM) or from native tissue.[1, 21, 24, 33, 47, 80] Both types of matrices have been either

decellularized to fully remove all cellular components and nucleic acids or devitalized to kill all remaining cells within the matrix without completely removing them. In contrast, fully decellularized native cartilage (DCC) tissue presents a unique challenge because the dense ECM makes full decellularization difficult due to diffusion limitations.[15, 24] The tissue is often mechanically disrupted to increase the efficacy of chemical decellularization but destroys the mechanical properties of the matrix.[21-23] The dense nature of native articular cartilage also restricts cell migration into the matrix.[24] Successful decellularization results in an acellular matrix that has low immunogenicity with the same biochemical make-up as native cartilage.[20, 24, 26] Devitalized cartilage (DVC), on the other hand, may still contain antigenic cell surface markers. Both types of cartilage matrix can additionally be combined with synthetic biomaterials or crosslinked to achieve the desired mechanical properties or shape.[45, 46]

Previous studies have investigated the use of DCC and DVC as chondroinductive materials, but have not fully characterized the materials through the decellularization processes.[20, 23, 33, 46, 54] Other studies have also chemically or physically crosslinked the DCC or incorporated the DCC into synthetic material scaffolds prior to *in vitro* cell culture. The cellular response to native, non-crosslinked DCC has not been investigated. Additionally, DCC has not been directly compared to devitalized native cartilage (DVC) *in vitro*.

The primary goal of the current study was to assess changes to native cartilage matrix throughout both decellularization and devitalization protocols and to further investigate the chondroinductive potential of cartilage ECM materials. The current study investigated the combined physical and chemical decellularization and the physical devitalization of native porcine cartilage processing effects on the biochemical and DNA content of the material at different steps of the decellularization process. The chondroinductive nature of non-crosslinked

DCC were also examined for future use in cartilage tissue engineering by assessing the differentiation of rat bone marrow derived mesenchymal stem cells (rBMSCs) in 3D pellet culture without added growth factors.

MATERIALS AND METHODS

Tissue Retrieval, Decellularization, and Devitalization

Ten porcine knee and hip joints were purchased from a local abattoir following sacrifice (120 kg, mixed breed, mixed gender) (Bichelmeyer Meats, Kansas City, KS). Articular cartilage from both the knee and hip joints was carefully removed and collected using scalpels. The cartilage was rinsed in phosphate buffered saline (PBS) and stored at -20°C. Following freezing, the cartilage was coarsely ground using a cryogenic tissue grinder (BioSpec Products, Bartlesville, OK). The coarsely ground tissue was packaged into dialysis tubing (3500 MWCO) packets for decellularization.

Cartilage was devitalized following tissue harvest by immediately freezing at -20°C and then lyophililzing the tissue. The lyophilized tissue was then processed in a freezer-mill and frozen again at -20°C.

The cartilage was decellularized using an adapted version of our previously established method using reciprocating osmotic shock, detergent, and enzymatic washes.[87] Reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. All steps of decellularization were carried out under agitation (200 rpm) at 21°C unless otherwise noted. First, the cartilage packets were placed in hypertonic salt solution (HSS) overnight to disrupt membranes and lyse the cells. Following HSS treatment, the tissue was subjected to 2 cycles of reciprocating triton-X 100 (0.05% v/v) and HSS treatments to further breakdown cellular

membranes. The tissue was then treated with benzonase (0.0625 KU ml^{-1}) overnight at 37°C to fragment nucleic acids. Sodium-lauroyl sarcosine (NLS, 1% v/v) was then used overnight to further solubilize and remove cells. Next, the tissue was washed with 40% ethanol, followed with organic exchange resins to remove all organic solvents. Lastly, the tissue was removed from the dialysis tubing packages and rinsed with deionized water before freezing.

After decellularization, the tissue was lyophilized for 48 hours and cryo-ground into a fine powder with a freezer-mill (SPEX SamplePrep, Metuchen, NJ). Following cryo-grinding, the cartilage particles were sifted through size-specific meshes (350, 100, and 45 μm) to obtain a more homogenous particle size range.

Tissue Decellularization and Devitalization Characterization

Decellularization analyses including quantification of remaining DNA, GAG, and hydroxyproline within the matrix, were performed on native hydrated, native frozen, native lyophilized, native cryo-ground (DVC), decellularized coarse ground, and decellularized cryo-ground cartilage (DCC) (n=6).

Scanning Electron Microscopy

The size and morphology of DCC particles were observed using LEO 1550 field emission scanning electron microscopy (SEM). Prior to imaging, DCC particles were lyophilized and sputter-coated with gold.

BMSC Harvest and Cell Seeding

Rat bone marrow mesenchymal stem cells (rBMSC) were harvested from the femurs of 4 male Sprague-Dawley rats (200-250 g) following a University of Kansas approved IACUC protocol. The BMSCs were cultured in minimum essential medium (MEM) α culture medium with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (anti-anti) during expansion. At passage 4, the cells were suspended in MEM α culture media at $1x10^6$ cells/mL. 1 mL of cell suspension was added to 25 mg of both DCC and DVC (no size exclusion) and centrifuged for 5 minutes (n=5). The TGF-β and negative control groups contained 10^6 cells without additional material. Experimental and negative control groups were cultured in 1 mL of the medium used during expansion. The TGF-β control group was cultured in chondrogenic differentiation medium containing 10 ng/mL human recombinant TGF-$β_3$ (PeproTech, Rocky Hill, NJ), 50 µg/mL ascorbic acid, 1% Penicillin Streptomycin, 40 µg/mL L-proline, 100 µmol sodium pyruvate, 0.1 µm dexamethasone, 1% insulin-transferrin-selenium 100X (ITS), and 1% non-essential amino acids (NEAA). The medium was changed every 48 hours.

Biochemical Analysis

The biochemical content of the cartilage was assessed after each processing step: native cartilage harvest, coarse grinding, decellularization, and cryo-grinding (n=6). The DNA content was also assessed at day 1 and 7 of pellet culture (n=5). Prior to biochemical analysis, all tissue samples were digested in a papain solution for 24-48 hours.

Biochemical content was measured as previously reported.[29, 88, 89] Briefly, glycosaminoglycan (GAG) content was measured with a dimethylmethylene blue (DMMB) assay kit (Blyscan, Westbury, NY). Total hydroxyproline content was measured using a commercially available hydroxyproline detection kit (Sigma, St. Louis, MO). Double-stranded

DNA was detected using a high sensitivity PicoGreen assay kit (Molecular Probes, Eugene, OR). All assay kits were used in accordance with each manufacturer's guidelines.

Gene Expression

RNA was isolated and purified from cells using the Qiagen RNeasy mini kit (Valencia, CA). All RNA samples were reverse transcribed using a high capacity cDNA reverse transcription kit (Invitrogen, Carlsbad, CA). Real-time quantitative polymerase chain reaction (qPCR) was performed using a RealPlex MasterCycler (Eppendorf, Hauppauge, NY) and TaqMan gene expression assays using equal concentrations of DNA for each sample. Rat specific Col2A1, Col1A1, Runx2, Sox9, Col10A1, Acan, and GAPDH commercially available primers were used (Invitrogen, Carlsbad, CA). The $2^{-\Delta\Delta Ct}$ method was used to determine the relative expression of each gene with GAPDH used as an endogenous control.[90, 91]

Statistical Analysis

Results are reported as a mean ± standard deviation. SPSS statistical software was used to construct boxplots to remove outliers prior to performing statistical analyses. All statistical analyses were performed using a one way analysis of variance (ANOVA) and Tukey's *post-hoc* tests. Significance was determined for $p < 0.05$.

RESULTS

Tissue Decellularization and Processing

Following coarse grinding, chemical decellularization, and cryo-grinding there was an 86% reduction in DNA content ($p < 0.01$) (Fig. 3.1) and a 55% reduction in GAG content

(p<0.01) (Fig. 3.2). However, there was no significant difference in hydroxyproline content during any steps of the tissue processing (Fig. 3.3). Freezing, lyophilization, and cryo-grinding had no significant effect on DNA or GAG content in the tissue.

SEM Imaging

SEM imaging revealed that size exclusion filtering was successful at achieving a more homogenous cartilage particle size population (Fig. 3.5).

Cell Viability

DNA was quantified in cell pellets at 0 and 7 days to determine cell proliferation on devitalized and decellularized cartilage. All cell pellets showed a significant increase in DNA amount over 7 days (p<0.05) (Fig. 3.4). At 7 days, cell pellets with DCC had approximately 40% more DNA than the DVC cell pellets at the same time (p<0.01) (Fig. 3.4). DCC pellets also had approximately 30% more DNA than the negative control group (p<0.01) (Fig. 3.4).

Gene Expression

qPCR was used to determine the expression of both chondrogenic and osteogenic genes. DCC pellet group gene expression showed over a 90% increase in collagen II expression compared to both the TGF-β and negative control groups at day 1 (p<0.01) (Fig. 3.6). Collagen II expression remained greater than both the TGF-β and negative control groups at day 3 (p<0.01). The DCC pellet group also showed 75% greater upregulation of Sox9 compared to the TGF-β group at day 0 (p<0.01) (Fig. 3.6). A 60% increase in aggrecan expression was observed in the DCC group compared to the negative control at day 1 (p<0.01) (Fig. 3.6). The osteogenic marker collagen X was expressed 5 times greater in the DCC group compared to the negative

control group at day 7 (p<0.01) (Fig. 3.6). Collagen X was also expressed 45 times greater in DCC than in the TGF-β group (p<0.01). Runx2 expression in the DCC group was also upregulated compared to the negative control group by 75% at day 7 (p<0.05) (Fig. 3.6). Collagen I expression in the DCC group was also over 50% greater than both the negative control and TGF-β groups at day 1 (p<0.01), but significantly decreased at both days 3 and 7 compared to day 1 (p<0.05) (Fig. 3.6).

The DVC group saw no significant difference in collagen II expression from both the negative control and TGF-β groups at days 1 and 3 (Fig. 3.6). Aggrecan expression in the DVC group was over 40% greater than both the TGF-β and negative control groups at day 7 (p<0.05), however, it was not statistically significant from the DCC group at the same time (Fig 3.6). The DVC group Sox9 expression increased approximately 30% between days 3 and 7 (p<0.05) but was not significantly greater than either control group or the DCC group (Fig. 3.6). Expression of osteogenic marker collagen X was 100% less in the DVC group compared to the DCC group at day 7 (p<0.01) (Fig. 3.6). Runx2 expression was also 30% less in the DVC group compared to the DCC group at day 7 (p<0.01) (Fig. 3.6). Collagen I expression in the DVC group was 60% less than the DCC group at day 1 (p<0.01) and 3 times less than the TGF-β group at day 7 (p<0.05) (Fig. 3.6).

DISCUSSION

Synthetic biomaterials have achieved clinically relevant mechanical performance for osteochondral implantation; however, they have not been successful at fully regenerating functional articular cartilage. The use of ECM-based materials for osteochondral tissue engineering is a promising avenue because of the ECM materials' ability to mimic the native

cartilage environment by providing cells with adhesion sites and biochemical signals that aid in recruiting and differentiating stem cells for tissue regeneration. ECM-based materials may be able to provide these signals to cells without manipulation of the material with added biological factors (e.g., growth factors or adhesion peptides).

Successful decellularization of articular cartilage has previously been accomplished using different methods with differing results with respect to the remaining biochemical content, cell removal, and mechanical performance.[19, 20, 31, 32] Cartilage ECM has also been used as a scaffolding material that has only been physically devitalized as opposed to being decellularized. The different effects that devitalization vs. decellularization have on the cartilage matrix until now have not been fully characterized throughout each respective process. Moreover, DVC and DCC have not been directly compared *in vitro* or to a positive control such as TGF-β. The current study has shown that DCC may outperform both DVC and TGF-β at inducing chondrogensis in BMSCs *in vitro*.

The current combined physical and chemical decellularization method was successful at reducing the amount of detectable dsDNA within the cartilage matrix by 86% ($p<0.01$). Freezing, lyophilization, and cryo-grinding, all common devitalization techniques, as expected were not effective at removing any dsDNA from the matrix. Although the DNA content of the DCC was significantly reduced, the GAG content was also significantly reduced by 55% (Fig. 3.2).

Currently, little is understood about the mechanism by which cartilage matrix materials induce chondrogenesis *in vitro*. Retention of GAG within the matrix may be beneficial for chondroinduction based on previous studies citing that GAGs such as chondroitin sulfate and aggrecan may have chondroinductive effects *in vitro*.[29, 30, 92] Although GAG retention may be

beneficial for cell signaling, a partial reduction in GAG content may be beneficial to create a less dense matrix that allows for cell infiltration and migration.[24]

In the current study, BMSCs cultured with DCC showed increased expression of both chondrogenic and osteogenic gene markers compared to the negative control group. The DVC group showed lower expression of the osteogenic markers collagen X and Runx2 than the DCC group. Although the DVC seemed to limit osteoinduction in the BMSCs, the chondrogenic gene markers collagen II, aggrecan, and Sox9 were significantly upregulated in the DCC group compared to the DVC group. This suggests that chondroinduction by DCC is not affected by the decellularization method. Additionally, comparison between chondroinduction via TGF-β and DCC showed that DCC chondroinduction was not statistically significant with respect to expression of aggrecan and Runx2 at all time points. Chondroinductive markers including collagen II and Sox9 were expressed 20 and 4 times higher respectively in the DCC group compared to the TGF-β group at day 1. Similar expression of collagen I between the DCC group and the TGF-β group was also seen at 3 and 7 days. These results suggest that DCC may outperform TGF-β at inducing chondrogenesis but does not confirm that latent TGF-β within the DCC matrix is responsible for the observed chondrogenesis.

Physical size exclusion filtering was also successful at achieving a more homogenous size distribution of cartilage particles compared to the unfiltered DCC particles (Fig. 3.5). The ability to control the size distribution of the cartilage particles may be beneficial when considering incorporation of cartilage particles into scaffolds either via crosslinking or combination with synthetic materials in the future, however, size distribution was not specifically investigated in the current study.

It is still unclear whether full decellularization of articular cartilage is necessary when delivering cartilage matrix materials to osteochondral defects *in vivo*. Decellularization may reduce the antigenicity of the matrix by removing cellular materials that have been previously shown to elicit immune responses such as human leukocyte antigens (HLA) and the alpha-Gal epitope. Previous studies have shown successful removal of the alpha-Gal epitope through chemical decellularization but not physical devitalization alone.[24]

Although not specifically explored in the current study, a delivery method of this material must also be considered to create a tissue engineering scaffold with that contains both the benefits of cartilage matrix materials with enhanced mechanical performance. The development of an acellular, non-biologically modified biomaterial that has the ability to induce chondrogenesis is of particular importance to the tissue engineering field because of it may have the ability to replace current surgical techniques with more positive outcomes. The ECM material approach is also highly attractive from both a regulatory and commercialization standpoint because of the cost of materials and no added biologic factors.

This is the first study to fully characterize both DCC and DVC through the respective decellularization and devitalization processes. Addtionally, this is the first study to directly compare the bioactivity of non-crosslinked DCC, DVC, and TGF-β *in vitro*. DCC was found to have superior effects compared to both DVC and TGF-β at inducing chondrogensis and supporting cell proliferation. The ability to influence cell differentiation without additional biological manipulation makes DCC a promising biomaterial for use in future cartilage tissue engineering applications.

CHAPTER 4: Bioactive Microsphere Based Scaffolds Containing Decellularized Cartilage

ABSTRACT

The aim of this study was to fabricate mechanically functional microsphere-based scaffolds containing decellularized cartilage (DCC), with the hypothesis that this approach would induce chondrogenesis of rat bone marrow derived mesenchymal stem cells (rBMSCs) *in vitro*. The DCC was derived from porcine articular cartilage and fully decellularized using a combination of physical and chemical methods. Four types of scaffolds were fabricated: Poly(D,L-lactic-co-glycolic acid) (PLGA) only (negative control), TGF-β encapsulated (positive control), PLGA surface coated with DCC, and DCC-encapsulated. These scaffolds were seeded with rBMSCs and cultured up to 6 weeks. The compressive modulus of the DCC-coated scaffolds prior to cell seeding was significantly lower than all other scaffold types. Gene expression was comparable between DCC-encapsulated and TGF-β encapsulated groups. Notably, DCC-encapsulated scaffolds contained 70% greater amount of GAG and 85% more hydroxyproline compared to the TGF group at week 3 (with baseline levels subtracted out from acellular DCC scaffolds). Certainly bioactivity was demonstrated in eliciting a biosynthetic response from the cells with DCC, although true demonstration of chondrogenesis remained elusive under the prescribed conditions. Encapsulation of DCC appeared to lead to improved cell performance relative to coating with DCC, although this finding may be a dose-dependent observation. Overall, DCC introduced via microsphere-based scaffolds appears to be promising as a bioactive approach to cartilage regeneration, although additional studies will be required to conclusively demonstrate chondroinductivity.

INTRODUCTION

Articular cartilage has limited capability for self repair after traumatic injury or osteoarthritis. Self-repair is limited in part because of the dense extracellular matrix (ECM), sparse chondrocyte population, and reduced access to systemic circulation. Current clinical treatments include osteochondral transplantation (mosaicplasty), autologous chondrocyte implantation (ACI), and microfracture.[3, 18] These current treatment options may produce inferior repair cartilage with respect to mechanical performance, tissue reintegration, and composition.[26, 33] They also have additional associated risks such as donor site morbidity and the need for multiple surgical procedures. Recently, acellular biomaterials have gained much popularity in the tissue engineering field due to the potential to create an off the shelf product with characteristics that aid in repairing cartilage tissue by enhancing stem cell recruitment, infiltration, and differentiation.[1, 4] One such material in particular, decellularized native cartilage (DCC) may be beneficial as it contains similar biochemical content as native cartilage and current problems associated with allograft implants (i.e., long term storage and immunogenicity) are mitigated.[24, 71, 72, 93] Previous studies have reported adipose derived stem cell (ASC) and bone marrow-derived mesenchymal stem cell (BMSC) differentiation in the presence of DCC.[23, 33, 38]

One drawback of DCC-based scaffolds is the mechanical function of the scaffolds is often compromised during the decellularization process.[24, 26, 31] To help fabricate a material with desired compressive strength as needed for articular cartilage repair, combining DCC with a polymeric scaffold has previously been shown to achieve greater mechanical performance than DCC scaffolds alone.[46] Using cartilage matrix to coat polymeric based scaffolds has also been

investigated previously with electrospun scaffolds but instead of using native cartilage, cell-derived cartilage matrix (CDM) secreted *in vitro* was used.[40]

The difference between native derived (DCC) and CDM must be made, as the matrices may vary in both composition and mechanical performance. Decellularization efficiency may be greater in CDM because the matrix is less dense, but the material may not contain the same composition as native cartilage ECM. DCC was chosen for use in this study for the ease of acquiring the material and the ability to fully decellularize the tissue while maintaining biochemical content similar to native cartilage ECM.

Microsphere-based scaffolds for osteochondral tissue engineering are an attractive delivery vehicle for DCC due to the ability to control the morphology of the microspheres and, in turn, the properties of the bulk scaffold.[92, 94] The polymeric material can also be selected for desired degradation and release rates of a wide variety of encapsulated materials.[89, 95-97] Previously, "raw materials" have been encapsulated in Poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres that aided in BMSC differentiation and proliferation.[92] DCC, however, has never previously been incorporated into microsphere-based scaffolds.

In the present study, we investigated the encapsulation of DCC in PLGA microsphere based scaffolds and the coating of the surface of PLGA microsphere scaffolds. Our hypothesis was that the DCC material would aid in chondrogenic differentiation of BMSCs *in vitro*.

MATERIALS AND METHODS

Materials

All reagents for decellularization were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted. PLGA (50:50 D, L-PLGA with acid end group, intrinsic viscosity 0.40-

0.50 dL/g) was purchased from Lakeshore Biomaterials (Birmingham, AL). Human recombinant TGF-β_3 was purchased from PeproTech (Rocky Hill, NJ). 10 porcine knee and hip joints were obtained from crossbreed hogs (Cheshire White, Yorkshire, Berkshire, Duroc, Landrace, and Hampshire) with an approximate average mass of 120 kg. The joints were purchased from a local abattoir following sacrifice (Bichelmeyer Meats, Kansas City, KS).

Tissue Harvest and Decellularization

Articular cartilage was collected from joint surfaces using scalpels and immediately rinsed in phosphate buffered saline (PBS). PBS was drained from the material and cartilage was stored at -20°C until further use. Decellularization of the cartilage was performed using a protocol adapted from Converse *et al.*[87] The cartilage was first coarsely ground using a cryogenic tissue grinder (BioSpec Products, Bartlesville, OK). Following additional freezing at -20°C, the cartilage particles were packaged into dialysis tubing (3500 MWCO) and stored in hypertonic salt solution (HSS) overnight at 21°C with gentle shaking (70 rpm). All subsequent steps were performed at 21°C under agitation (200 rpm) unless otherwise noted. The tissue was then subject to two reciprocating washes of triton X-100 (0.01% v/v) followed with HSS. Overnight, the tissue was treated with benzonase (0.0625 KU ml^{-1}) to fragment nucleic acids at 37°C. Next, the tissue was treated with sodium-lauroylsarcosine (NLS, 1% v/v) overnight. Following NLS, the tissue was washed with ethanol (40% v/v). The tissue was then subjected to organic exchange resins to extract the organic solvents. Lastly, the tissue was washed in saline-mannitol solution (SMS). Following decellularization, the cartilage tissue was rinsed with deionized water and stored at -20°C. After freezing, the tissue was lyophilized and cryo-ground in a freezer-mill (SPEX Sample Prep, Metuchen, NJ).

Microsphere and Scaffold Fabrication

Three types of microspheres were produced (1) PLGA only (PLGA) (negative control), (2) TGF-β_3 encapsulated (TGF) (positive control), and (3) solubilized DCC encapsulated (DCC-encapsulated). All microspheres were fabricated using the patented precision particle formation method.[17, 21, 23, 24] Microspheres were approximately 350-400 μm in diameter.

PLGA microspheres were fabricated at 20% w/v. DCC microspheres were fabricated by first solubilizing the DCC in an acid-pepsin solution for 24 hours. The acid pepsin solution contained 0.1 M HCl, 20 mg DCC / 1 mL HCl, and 1 mg pepsin / 1 mL HCl (pH = 5) (Sigma Aldrich, St. Louis, MO). After the solubilization period, the pH of the solution was raised by adding one-tenth the solubilized solution volume of 1 M NaOH and one-tenth the final solution volume of 10x PBS (pH = 8).[25] The solubilized solution was then frozen and lyophilized. The solubilized DCC was added to a PLGA solution at 10% w/w.

TGF-β was reconstituted in 10 mM citric acid TGF microspheres were created with a concentration of 30 μg TGF-β_3 / g PLGA.[17, 23]

Scaffolds were fabricated as previously described.[17, 21, 26] Briefly, microspheres were first loaded in a glass cylindrical mold. A 10 μm filter was used at the bottom of the mold and DI H$_2$O was pulled through the mold by a vacuum pump. The microspheres were sintered together in a 95% v/v ethanol-acetone solution for 45 minutes. DCC-coated PLGA scaffolds were created using PLGA scaffolds (from PLGA-only microspheres). The PLGA scaffolds were placed in the DCC acid-pepsin solution for 3 minutes and the pH was adjusted to neutral by adding 1 M NaOH. The scaffolds were then lyophilized. Following sintering, the scaffolds were lyophilized. All scaffolds were sterilized with ethylene oxide prior to cell seeding. Additionally, acid treated

scaffolds were created for mechanical testing purposes. PLGA scaffolds were exposed to an acidic HCl solution (pH = 5) for 3 minutes and then lyophilized to mimic the effects of the acid-pepsin solution the scaffolds were exposed to during the DCC-coating process.

Cell Isolation and Culture

Following a University of Kansas approved IACUC protocol, BMSCs were isolated from the femurs of 4 male Sprague-Dawley rats (200-250 g). Cells were isolated from the femurs by flushing the bones with cell culture media and immediately transferring the isolated bone marrow to tissue culture flasks. During expansion, the BMSCs were cultured in minimum essential media (MEM) alpha with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (anti-anti). The BMSCs were expanded to passage 4 and suspended in cell culture media at a concentration of 12×10^5 cells/mL and 40 μL of cell suspension was pipetted onto constructs at 1×10^7 cells/cm^3 (500,000 cells/scaffold). After cells were seeded on scaffolds, the scaffolds were placed in the incubator for 1 hour to allow the cells to attach before adding culture media. The cell seeded scaffolds were cultured in medium containing high glucose DMEM, 1% insulin-transferrin-selenium 100X (ITS), 50 μg/mL ascorbic acid, 40 μg/mL L-proline, 1% penicillin-streptomycin, 0.1 μM dexamethasone, 25 mM HEPES buffer, 1% non-essential amino acids (NEAA), and 100 μM sodium pyruvate.

SEM Imaging and Energy Dispersion Spectroscopy

Microspheres were imaged with a LEO 1550 scanning electron microscope (SEM) to observe the morphology of the microsphere surfaces. All microspheres were sputter-coated with

15 nm gold. The presence of nitrogen on the surface of the DCC-encapsulated microspheres was detected by SEM using energy dispersive spectroscopy (EDS) at 10 kV.

Biochemical Content Analysis

Biochemical content analysis was performed on solubilized DCC, acellular scaffolds, and cell seeded scaffolds (n=5). For all analyses day 0 samples were collected at 24 hours. The biochemical content of the cell-seeded scaffolds was measured at 0, 3, and 6 weeks. Acellular scaffolds matched each time point and were used to subtract base values for all biochemical content. All samples were digested in 1 mL of papain solution containing 125 µg/mL papain, 5mM N-acetyl cysteine, 5mM ethylenediaminetetraacetic acid (EDTA), and 100 mM PBS.[29, 88, 89]

Biochemical content was measured as previously described.[29, 88, 89] Briefly, double stranded DNA content was measured with the PicoGreen assay (Molecular Probes, Eugene, OR). The assay was performed according to the manufacture's instructions. Sulfated glycosaminoglycan (GAG) content was measured with the dimethylmethylene blue (DMMB) assay (Blyscan, Westbury, NY) according to the supplier's protocol. Total hydroxyproline content was measured with the Sigma Aldrich commercially available hydroxyproline assay kit (St. Louis, MO).

DCC Release Analysis

Acellular DCC-encapsulated and DCC-coated were cultured in the same conditions as cell seeded scaffolds. The scaffolds were used to determine the remaining amount of GAG and hydroxyproline as described above. The amount of GAG and hydroxyproline content in acellular scaffolds was used to approximate the release of the DCC from the scaffolds.

Mechanical Testing

Uniaxial unconfined compression testing was performed on acellular scaffolds (n=4-5) with a custom-built compression-bath assembly in an Instron 5848 microtester (Norwood, MA).[28] PLGA, TGF, DCC-coated, DCC-encapsulated, and acid treated scaffolds were tested. Following a tare load of 0.01 N samples were compressed at a strain rate of 10%/min in PBS at 37 °C. The compressive modulus was calculated from the linear region of the stress-strain curve.

Gene Expression Analysis

Gene expression analysis was performed at 0, 1.5, 3, and 6 weeks (n=5). Day 0 was defined as 24 hours following cell seeding. RNA was isolated and purified with the Qiagen RNeasy kit following provider recommendations (Valencia, CA). RNA was reverse-transcribed using a high capacity reverse transcriptase kit (Invitrogen, Carlsbad, CA). RT-PCR was performed with a RealPlex thermocycler (Eppendorf, Hauppauge, NY) and TaqMan gene expression assays (Invitrogen, Carlsbad, CA). All primers were commercially available and purchased from Invitrogen. Gene expression analysis was performed for both chondrogenic and osteogenic gene markers including Coll1A1, Coll2A1, Aggrecan, Sox9, and Runx2. GAPDH was used as an endogenous control. All results are reported as relative expression to GAPDH using the $2^{-\Delta\Delta Ct}$ method.[88, 102]

Immunohistochemistry Staining

Immunohistochemistry (IHC) was performed on week 3 DCC-encapsulated scaffolds. The scaffolds were fixed in 10% formalin and embedded in optimal cutting temperature (OCT)

medium (Tissue-Tek, Torrance, CA). 10 μm thick sections were cut using a cryostat (Micron Hm-500 OMP, Vista, CA). Primary antibodies for collagen I, collagen II, and aggrecan were obtained from Abcam (Cambridge, MA). Following the primary antibodies, biolynated secondary antibodies were used followed with the ABC complex (Vector Labratories, Burlingame, CA). The antibodies were visualized with the DAB substrate per the manufacturer's protocol. Both cell-seeded and acellular scaffolds were stained.

Statistical Analysis

All results are reported as mean ± standard deviation. Boxplots were created to remove all statistical outliers. Statistical analyses were performed using one way analysis of variance (ANOVA) and Tukey's *post-hoc* tests. Significance was reported for $p<0.05$. SPSS statistical software was used for all analyses (Armonk, NY).

RESULTS

Microsphere Morphology

PLGA microspheres had a smooth, even surface without any pores (Fig. 4.1). TGF microspheres showed small pores on the surfaces. The DCC microspheres also had numerous small pores on the surface. The presence of nitrogen was used to indicate the presence of amino acids in the material. EDS showed that nitrogen was present in low amounts on the surface of the DCC-encapsulated microspheres (Fig. 4.2) revealing that the DCC was dispersed throughout the microspheres and near the surface.

Biochemical Analysis

Dry SDCC contained 42.6±2.6 μg GAG/mg prior to incorporation into scaffolds. Acellular scaffolds were used to determine the remaining GAG and hydroxyproline content in the DCC and coated scaffolds. At time 0, the acellular DCC scaffolds contained nearly 4 times as much GAG as the coated scaffolds (p<0.001) (Fig. 4.3). The DCC group showed an approximately 50% decrease in GAG content from day 0 to week 3 (p<0.001) (Fig. 4.3). After week 3, the GAG content remained roughly equal through week 6 in DCC-encapsulated scaffolds (no significant difference). The hydroxyproline content in the DCC-encapsulated scaffolds was approximately 10 times greater in the DCC-encapsulated group than in the DCC-coated group at day 0 (p<0.001). The DCC-encapsulated scaffold hydroxyproline content remained similar between weeks 3 and 6. Relative GAG loss in both DCC-encapsulated and DCC-coated scaffolds was similar by week 6 (Fig. 4.4).

At day 0, the cell seeded DCC-coated scaffold group had approximately 30% more DNA than all of the other groups at all times (p<0.001) (Fig. 4.5). By week 3, all of the groups had similar amounts of DNA and remained constant through week 6 at approximately 3.5 μg DNA/scaffold. Since there were no statistically significant differences in DNA content other than the day 0 DCC-coated group, the HYP and GAG totals were reported on a basis of total content per scaffold.

The DCC-encapsulated scaffolds contained more hydroxyproline than both the blank and TGF scaffolds at day 0 and week 3, even with baseline values subtracted out from acellular scaffolds (p<0.001) (Fig. 4.6). The DCC-encapsulated group had nearly 7 times as much hydroxyproline compared to the blank group at day 0 (p<0.001). Additionally, the DCC-encapsulated group had almost 10 times as much hydroxyproline than the TGF group at day 0 (p<0.001). At week 3, DCC-encapsulated scaffold group contained approximately 8 times the

amount of hydroxyproline compared to TGF scaffolds and 40 times the amount of hydroxyproline compared to PLGA scaffolds ($p<0.001$). Following week 3, DCC-encapsulated scaffolds exhibited a significant reduction in the amount of hydroxyproline/scaffold ($p<0.005$). There was no significant change in hydroxyproline content in the DCC-coated scaffolds. At week 6 the DCC-encapsulated group had over 10 times as much hydroxyproline as the blank group ($p<0.05$).

In blank, DCC-encapsulated, and DCC-coated cell seeded scaffolds the GAG content significantly decreased between weeks 3 and 6 ($p<0.001$) (Fig. 4.6). Similar trends were seen between GAG and hydroxyproline content, i.e. decreased amount of biochemical content at week 6 compared to week 3. DCC-encapsulated scaffolds had significantly greater GAG and hydroxyproline content at 3 weeks compared to day 0 values and TGF group at the same times ($p<0.001$). However, at week 6, the hydroxyproline content of the DCC-encapsulated scaffolds significantly decreased by 70% and the GAG content decreased by 65% from the week 3 values ($p<0.005$).

Mechanical Testing

The blank, TGF, and DCC-encapsulated scaffolds groups at week 0 had compressive elastic moduli around 80 kPa that were not significantly different from one another. However, the DCC-coated group compressive elastic modulus was approximately 75% less than all other groups ($p<0.05$) (Fig. 4.7). Additionally, the acid treated scaffolds were not statistically significant from the blank scaffolds or the DCC-coated scaffolds.

Gene Expression

At day 0, the TGF group had over 3 times greater expression of collagen II than the DCC group, however, there was no statistical significance (Fig. 4.8). The TGF group had nearly 16 times the expression of collagen II compared to the blank group at day 0 ($p<0.001$). There was no statistically significant difference between collagen II expression in the DCC-encapsulated group and TGF group. The DCC-coated groupo had significantly less expression than the TGF group at day 0 ($p<0.001$). After day 0, no group showed expression of collagen II.

At day 0, day 10, and week 3, the DCC-encapsulated group had similar expression of Sox9 compared to the blank group. Additionally, the DCC-encapsulated group had similar expression of Sox9 as the TGF group at day 10 and week 3. At day 0, the TGF group had nearly 3 times the expression of Sox9 compared to the DCC-coated group ($p<0.001$).

There were no statistically significant differences in aggrecan expression among groups at any time point. However, it was worth noting that the aggrecan expression in the blank group decreased 87% at week 3 compared to days 0 and 10 ($p<0.01$).

Both TGF and DCC-encapsulated groups had nearly 80% less expression of Collagen I at day 0 compared to the blank and DCC-coated groups ($p<0.001$). At day 0, the blank group had 2.5 times greater expression of Runx2 compared to the TGF group ($p<0.001$). There was no significance among the TGF, DCC-coated, and DCC-encapsulated groups with respect to Runx2 expression.

Immunohistochemistry Staining

IHC staining of week 3 DCC-encapsulated scaffolds was positive for collagen II, collagen I, and aggrecan (Fig. 4.9). Collagen I staining was more intense in the cell-seeded DCC-

encapsulated scaffolds compared to the acellular DCC-encapsulated scaffolds. Collagen II and aggrecan staining were nearly equal between cell-seeded and acellular scaffolds at week 3.

DISCUSSION

In the current study, DCC was incorporated into microsphere-based scaffolds either by coating PLGA microsphere scaffold surfaces or by encapsulating the DCC within PLGA microspheres. Although cellular response characterization to the DCC in the microsphere-based scaffolds did not overwhelmingly indicate chondrogenesis in BMSCs, the DCC material did induce some bioactivity to the cells. The difference between the loading amounts achieved by different DCC incorporation methods (coating vs. encapsulating) may have contributed to the difference in cellular responses to the respective scaffold types as the acellular DCC-encapsulated scaffolds contained significantly greater amounts of GAG and hydroxyproline than the acellular DCC-coated scaffolds. The processing of the DCC material in both DCC-coated and DCC-encapsulated groups was the same, however, the presentation of the material to the BMSCs was different and may have also contributed to differences between the coated and encapsulated groups. The DCC-encapsulated group relied more greatly on diffusion of the DCC out of PLGA microspheres even though there was a small amount of nitrogen present on the surface of the DCC-encapsulated microspheres. The DCC-coated group was available for cells on the surface of the scaffolds and did not rely on diffusion out of porous microspheres.

A benefit to the microsphere-based scaffolds and encapsulation technique used in the study was that the DCC-encapsulated scaffolds had comparable mechanical properties to the PLGA scaffolds. The DCC-coated scaffolds, however, had a significantly reduced compressive modulus compared to all other scaffold types. The decrease in mechanical performance of the

DCC-coated scaffolds may have been partially due to the coating procedure and exposure to acid as the acidic conditions could increase the rate of degradation of the PLGA. The acid treated scaffolds were not statistically significant from any other type of scaffold. Previous studies have reported decreased mechanical properties in cartilage matrix-polymer constructs compared to polymer only constructs.[14, 30]

The porous morphology of the microspheres allowed for diffusion of both TGF-β and DCC out of the respective scaffold types. Based on quantification of remaining hydroxyproline and GAG content in acellular scaffolds, by week 6, the remaining GAG content reduced by 50% and the remaining hydroxyproline content reduced by 75% in the DCC-encapsulated scaffolds. At week 6, the total hydroxyproline and GAG content in cell-seeded DCC-encapsulated scaffolds also significantly decreased compared to previous time points. Additionally, at week 3, chondrogenic gene markers Sox9, aggrecan, and collagen II all decreased in the DCC-encapsulated group. The decrease in chondrogenic gene markers at week 3 in the DCC-encapsulated scaffold group was consistent with the more intense staining of collagen I at week 3 in the DCC-encapsulated scaffolds. However, although chondrogenic gene markers decreased at week 3, collagen I and the osteogenic marker Runx2 remained low and not statistically significant from that of the TGF group. At all gene expression time points, the DCC-encapsulated scaffolds did not differ significantly from the TGF group. Hydroxyproline content was greater in DCC-encapsulated constructs at all time points compared to the TGF scaffolds. The GAG content was also greater in DCC-encapsulated scaffolds than in TGF scaffolds at day 0 and week 3. These higher biochemical contents in the DCC-encapsulated group may suggest that the encapsulated DCC was as effective as TGF-β at inducing chondrogenesis in BMSCs. Once less than half of the originally encapsulated DCC remained in the scaffolds, a decrease in GAG

and hydroxyproline production rate was observed as well as a decrease in chondrogenic gene markers. The significant reduction in remaining DCC encapsulated within the scaffoldssuggests that the bioactivity that was observed through week 3 was due to the encapsulated DCC.

Prior to cell seeding, the DCC-encapsulated scaffolds contained nearly 5 times as much GAG and 10 times as much hydroxyproline as the DCC-coated scaffolds. Both types of scaffolds in the absence of cells saw a significant reduction in both GAG and hydroxyproline content at week 3. Although the rate of biochemical content loss was nearly equal, at weeks 3 and 6 the DCC-encapsulated scaffolds still contained 10 times as much hydroxyproline and 5 times as much GAG as the DCC-coated scaffolds. The differences in the amount of DCC material did not have an effect on the amount of cells within the scaffolds except at day 0, when the DCC-coated scaffolds had a significantly greater amount of DNA than all other scaffolds. The greater amount of DNA may be due to the immediate exposure to DCC, which may contain cellular adhesion sites to aid in cell attachment and migration. The difference in amount of DCC material between the DCC-coated and DCC-encapsulated scaffolds also affected the total amount of GAG and hydroxyproline in cell-seeded scaffolds. At all time points, DCC-encapsualted scaffolds contained significantly greater amounts of hydroxyproline and GAG compared to the DCC-coated scaffolds. The DCC-coated scaffolds exhibited no clear trend of chondrogenesis or osteogensis compared to TGF scaffolds at day 0. However, by week 6, DCC-coated, DCC-encapsulated, and TGF groups had equal expression of all genes (chondrogenic and osteogenic). Most significant differences among groups with respect to gene expression were observed at days 0 and 10.

The mechanism by which DCC induces chondrogenesis is still unclear at this time. In the current study, we showed that encapsulated DCC and TGF-β had similar effects on BMSC gene

expression *in vitro*, but encapsulated DCC had a greater effect on BMSC production of GAG and collagen than TGF-β. The encapsulation of DCC compromised the macro-structure of the matrix during solubilization and the DCC effect remained positive on the cells, this may indicate that the structure of the matrix is not vital to bioactivity induced by DCC. Additionally, the difference between the amounts of DCC material each scaffold type contained (coated vs. encapsulated) may have had an effect on the differences seen in cellular response. The response to DCC may be dose dependent. Additional work to identify an ideal loading dose may be beneficial for future work.

The decrease of encapsulated factors in PLGA microsphere-based scaffolds limits the duration of cellular response effects. The amount of DCC in both DCC-coated and DCC-encapsulated groups was significantly decreased between day 0 and week 3. This trend matched the decrease in all gene expression at week 3 and subsequently the loss of biochemical content by week 6 in all cell seeded scaffolds. Although not specifically explored in the current study, previous studies have reported a burst release of encapsulated TGF-β resulting in a nearly 80% decrease of remaining TGF within the scaffolds at 3 weeks. The lack of sustained release of TGF would also contribute to the reduced chondrogenesis seen in TGF scaffolds by week 3.[23] Using polymers with greater degradation times or fabricating microspheres to reduce the porosity on the surfaces may enhance extended release of encapsulated materials *in vitro*.

Microsphere-based scaffolds are a promising alternative to current cartilage repair techniques due to the ability to control both their mechanical properties and to encapsulate a wide variety of materials.[17, 18, 21, 23] Encapsulated materials can be selected to aid in stem cell differentiation and cartilage tissue repair.[17] DCC was chosen for encapsulation and coating because of previous studies citing cartilage matrix as a potentially chondroinductive material.[12,

[14, 15, 30, 31] The use of decellularized cartilage instead of native or devitalized cartilage is advantageous from clinical and commercial standpoints because of decreased immunogenicity and long term storage of the material. Successful decellularization of tissues, i.e., complete removal of residual DNA and immunogenic antigens, may also eventually lead to safer xenogeneic tissue implants for all tissue types.[10, 32, 33]

In summary, DCC microsphere-based scaffolds led to gene expression and mechanical performance comparable to that of TGF-β, while outperforming both the TGF-β and control groups in biosynthesis, suggesting that DCC in a microsphere-based scaffold may indeed be bioactive, but additional work remains in terms of method of incorporation (i.e., coated vs. encapsulated) and dose to determine whether indeed a chondroinductive approach is achievable. In terms of the method of incorporation, the DCC-encapsulated group generally outperformed the DCC-coated group with the techniques presented. Overall, using microsphere-based scaffolds as a means to incorporate and deliver DCC to regenerating cartilage may be a powerful tool in the future for treatment of cartilage defects.

CHAPTER 5: DISCUSSION

The overall goal of this thesis was to develop an ECM-based material for exploration and use in tissue engineered scaffolds for cartilage tissue engineering. Previous studies on the use of cartilage ECM for cartilage tissue engineering have reported numerous methods to obtain the matrix (tissue vs. *in vitro* derived) and process the matrix (decellularization vs. devitalization). Additionally, numerous methods have also been reported for manipulating the cartilage matrix material into a structurally functional scaffold. This thesis represents the first clear distinction between types of cartilage matrix and the processing methods. The studies reported in this thesis were the first to fully characterize cartilage matrix through both devitalization and decellularization processes. They were also the first report describing incorporation of DCC into microsphere-based scaffolds.

Cells cultured in pellets in the presence of DCC exhibited upregulation of chondrogenic gene markers including collagen II, Sox9 and aggrecan. Additionally, the cells cultured with DCC had greater amounts of DNA after 7 days than cells cultured with DVC. These findings indicated that the significant loss of GAG from the DCC material did not eliminate the effect the cartilage matrix had on the cells, although it is unknown if the loss of GAG content reduced the effect on cells or not. It is still unclear whether or not the differences seen between DCC and DVC *in vitro* would remain true in different culture conditions or *in vivo* with more homogenous nutrient access compared to cell pellets. The pellet culture results also showed that DCC had superior effects on cells compared to cells cultured in the presence of TGF-β. The simplicity, ease, and reduced need for biological manipulation when using DCC materials may contribute to the attraction and motivation for use of DCC with respect to FDA regulation and clinical delivery. This thesis only explored the changes in biochemical and DNA content through a

particular decellularization protocol. Additional work may be helpful in characterizing the potential immunogenicity of the material including determining if the alpha-Gal epitope (in xenogeneic materials) is removed during decellularization. Prior to delivery to a cartilage defect site, it may also be beneficial to determine if any other inflammatory responses due to the material exist *in vivo*.

DCC was successfully incorporated into microsphere-based scaffolds. The encapsulation of DCC in microspheres did not reduce mechanical performance of the scaffolds, however, the DCC surface coating process used did significantly reduce the mechanical properties of the scaffolds. If surface coating of microsphere scaffolds is explored in the future, a different technique that does not subject the scaffolds to acidic conditions may be favorable. However, when characterizing the cellular response to both encapsulated and coated scaffolds, coating scaffolds may not be necessary as DCC is released from microspheres quickly due to the high porosity of the surface. The cellular response seen in the microsphere scaffolds with DCC was limited by the amount of DCC remaining in the scaffolds. DCC-encapsulated scaffolds were able to incorporate greater amounts of DCC than the coated scaffolds. To improve the long-term release of materials from these scaffolds, a higher molecular weight polymer or greater loading concentration of DCC may be necessary.

The mechanism by which DCC influences cellular response remains unclear. Even though DCC has superior effects compared to TGF-β *in vitro*, it does not imply that residual TGF-β (if any) within the matrix is responsible for the observed cellular response. Additionally, the pellet study used DCC with the microstructure still intact and the DCC-encapsulation sacrificed much of the microstructure of the tissue. The intact DCC used in the pellet study had a greater cellular response, which may suggest the microstructure of the DCC aided in enhanced

cellular response. The effect DCC had on cells seemed to have possibly been dose dependent in the microsphere scaffolds, however, dosage was not explored in the cell pellet characterization. Also, the cell pellets were in direct contact with the DCC and did not depend on the release of the material from a synthetic polymer, this direct exposure may have been beneficial for cell signaling. It must be noted that since the delivery mechanism of DCC to cells was different between the cell pellet and microsphere scaffolds comparison between the studies is limited. Future work should aim to understand the mechanism behind the bioactivity of DCC and effects of DCC dosage. The understanding of the mechanism may help aid in the design of even more simplistic acellular biomaterial approaches for cartilage regeneration.

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APPENDIX A: Figures
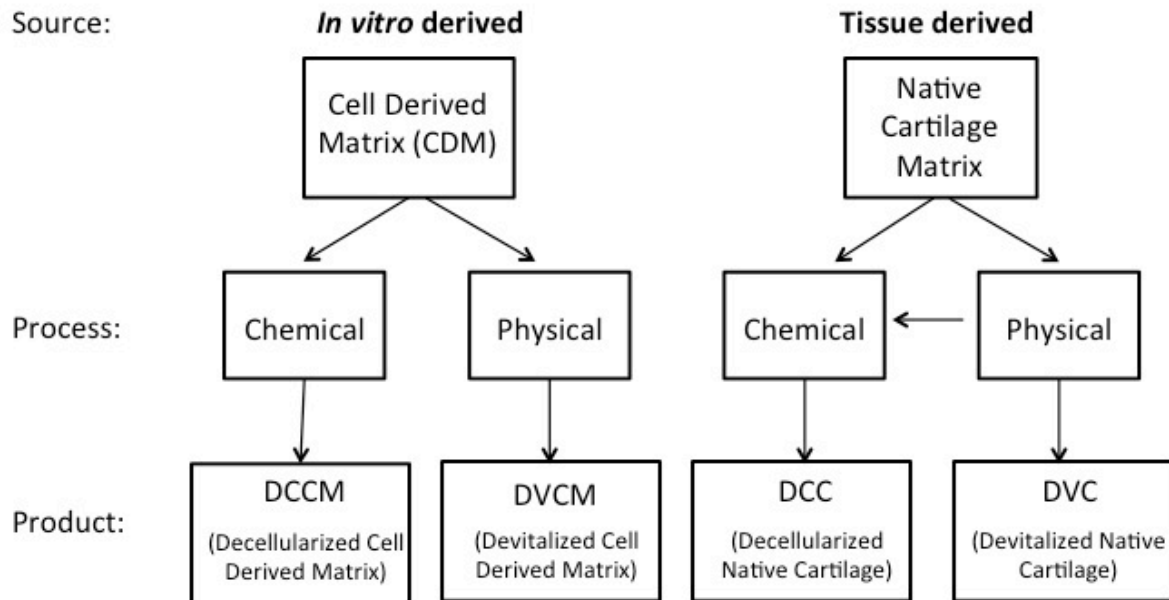
Figure 2.1: Cartilage matrix classifications.

A schematic depicting the distinctions between cartilage matrix final products dependent on the source and processing.
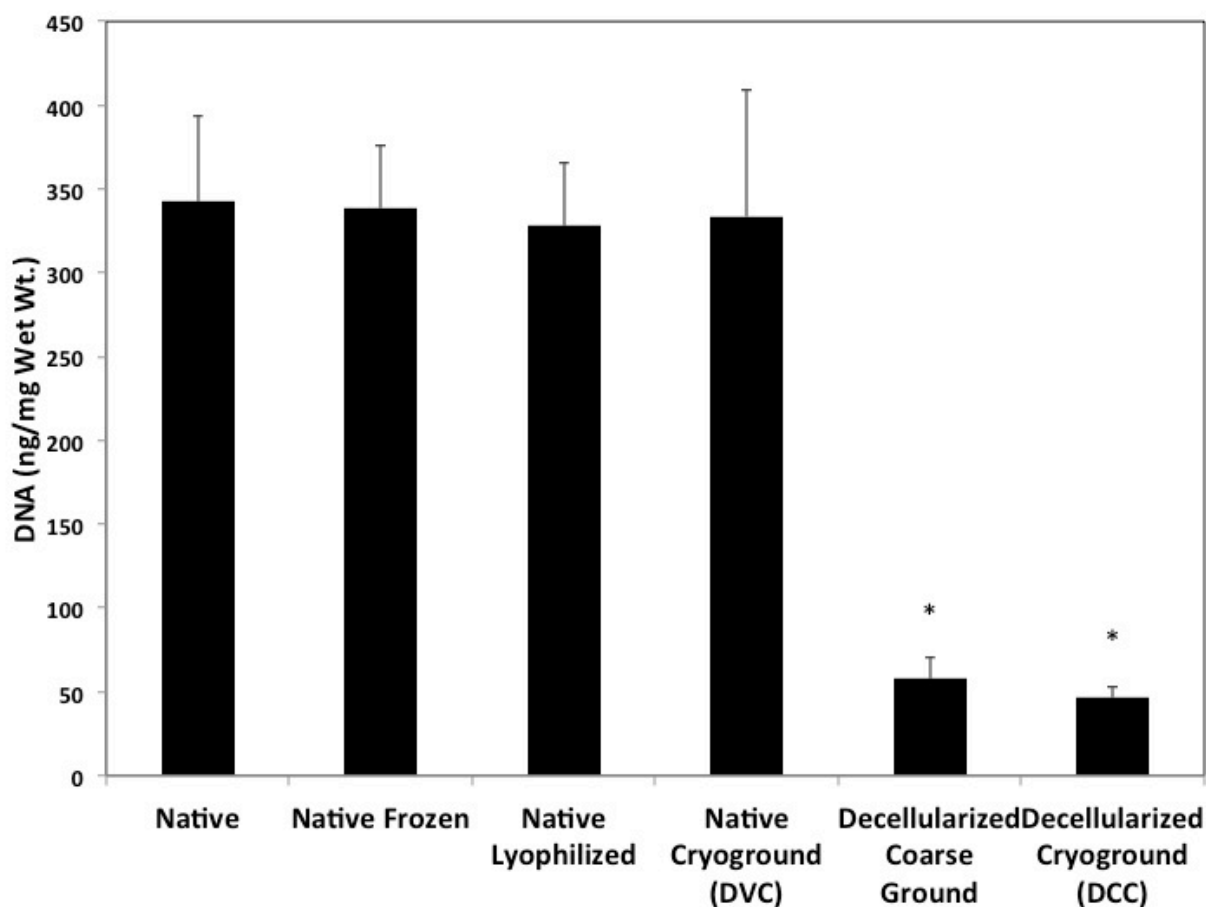
Figure 3.1: Quantification of DNA loss during decellularization and devitalization.

PicoGreen results depicting changes in double stranded (ds)DNA amounts in articular cartilage throughout the decellularization process. Processing the cartilage with both physical and chemical methods significantly reduced the amount of dsDNA in the matrix by 86%. * denotes $p<0.01$ (n=6). All results are reported as mean ± standard deviation.
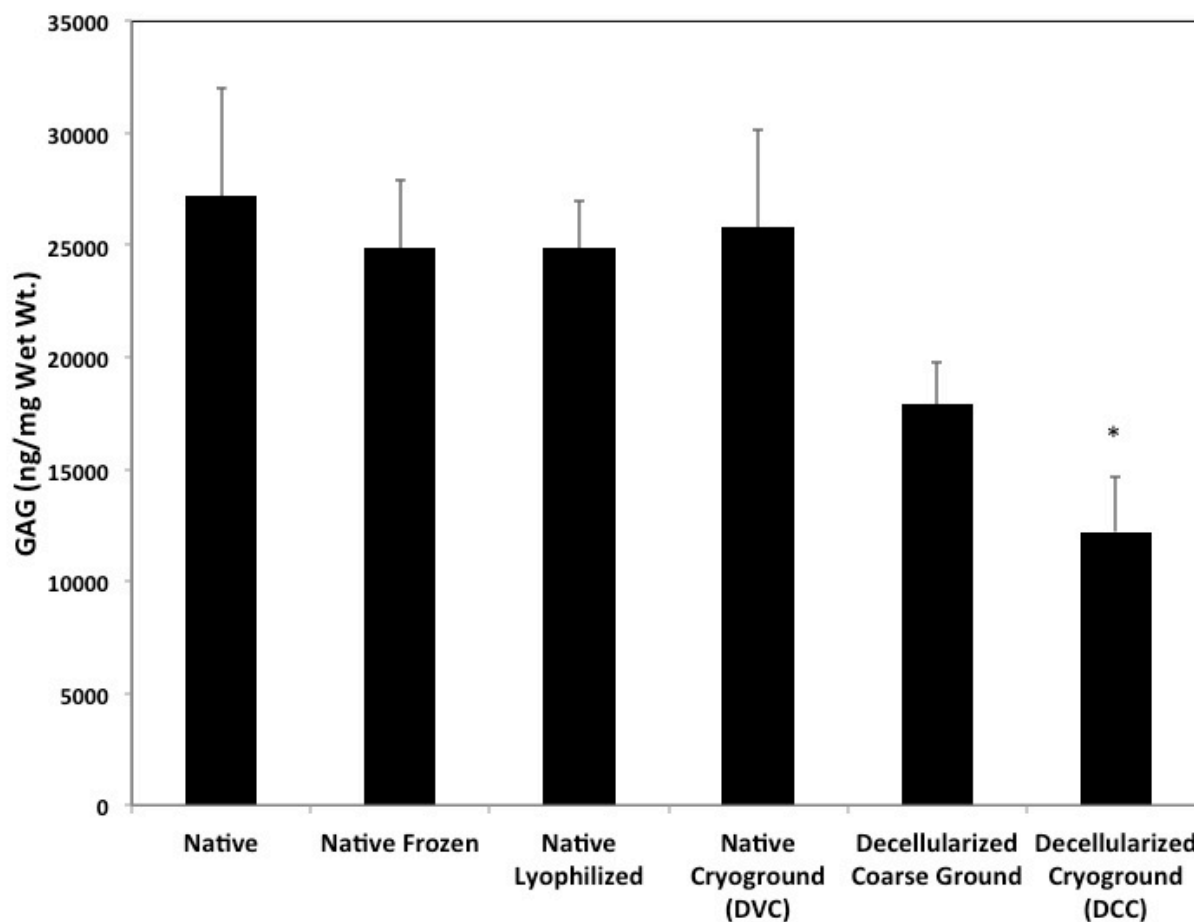
Figure 3.2: GAG quantification during decellularization and devitalization.

DMMB assay results depicting change in glycosaminoglycan (GAG) content of cartilage matrix during physical and chemical decellularization. Only DCC (both physical and chemical methods) significantly reduced the GAG content in the cartilage matrix by 55%. * denotes $p < 0.01$ (n=6) All results are reported as mean ± standard deviation.
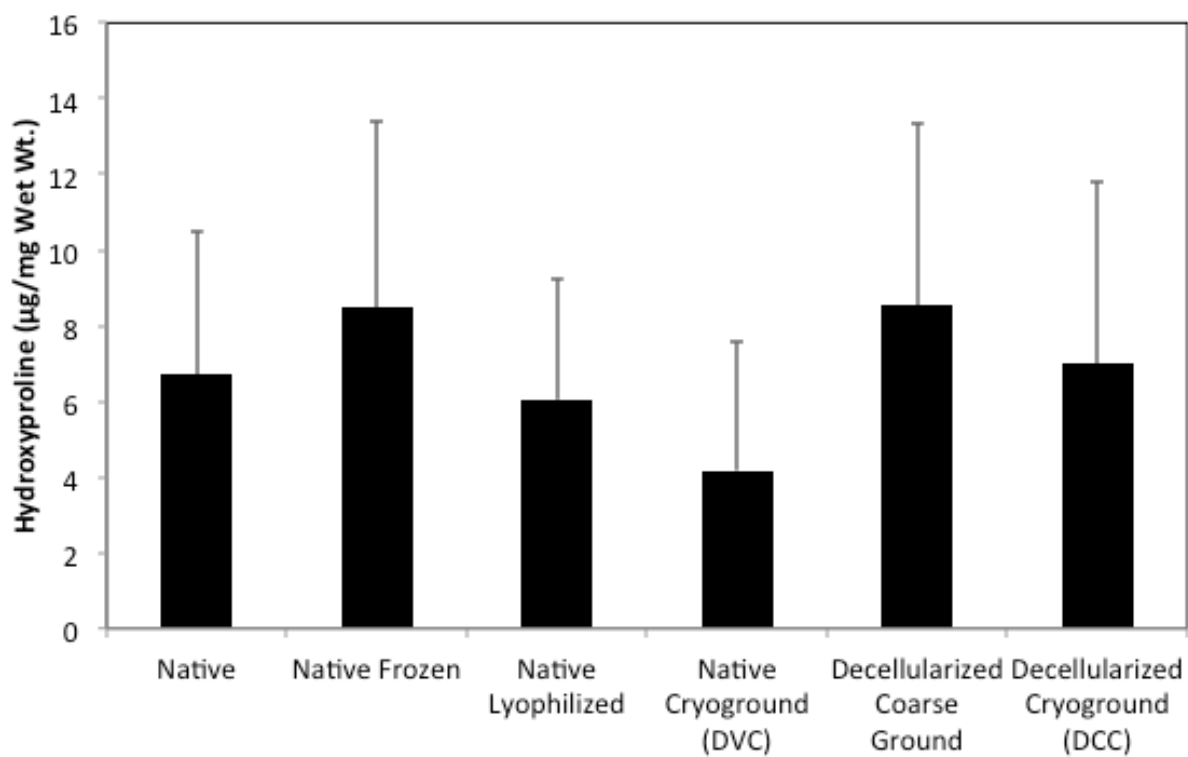
Figure 3.3: Hydroxyproline quantification during decellularization and devitalization.

Hydroxyproline content of cartilage matrix during physical and chemical decellularization process. No statically significant differences were observed during the processing (n=6). All results are reported as mean ± standard deviation.
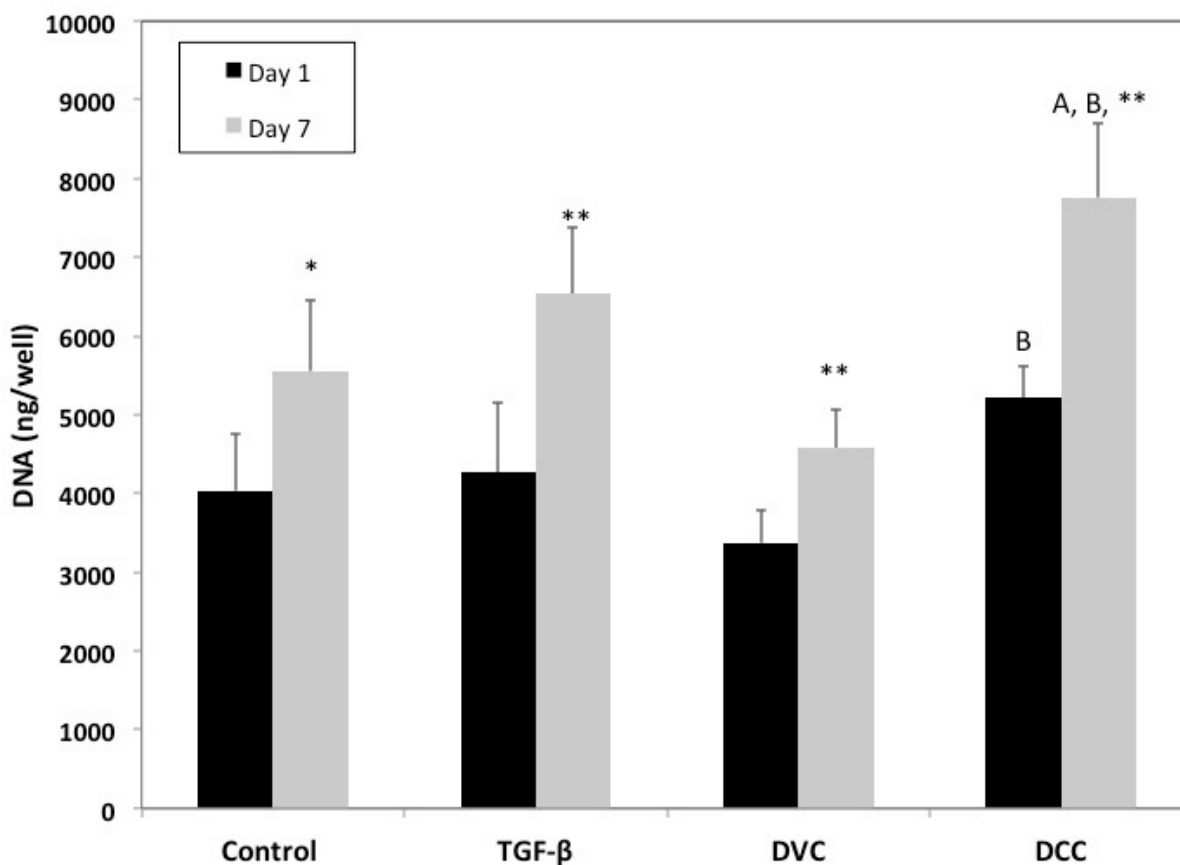
Figure 3.4: DNA quantification of cell pellets.

DNA content of cell pellets at days 1 and 7 (n=5). All groups significantly increased DNA content between 1 and 7 days. * $p<0.05$ between day 1 and 7, ** $p<0.01$ between day 1 and 7, A = $p<0.05$ between DCC and control, and B = $p<0.01$ between DCC and DVC. All results are reported as mean ± standard deviation.
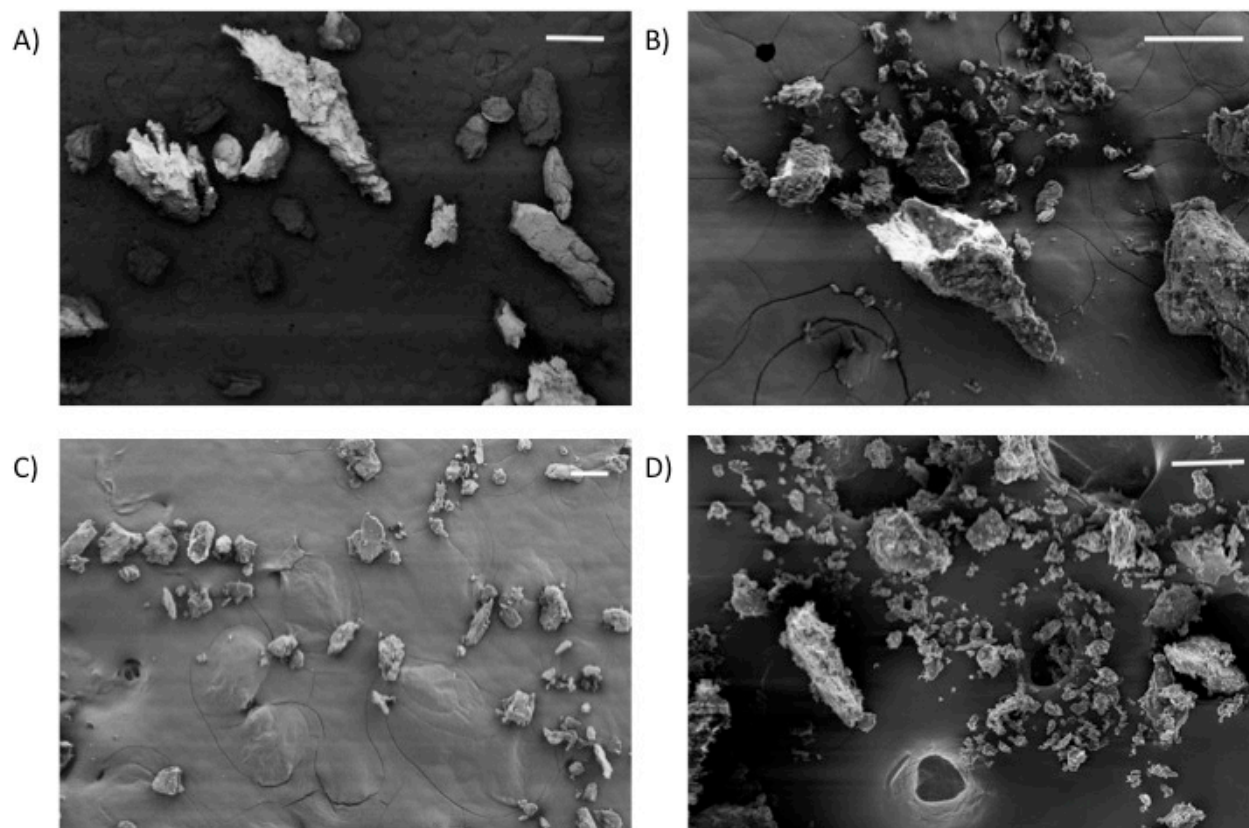
Figure 3.5: SEM images of DCC.

SEM images of DCC following size exclusion sieving. (A) Cryo-ground DCC prior to separation, scale bar 1 mm (B) 350 μm, scale bar 200 μm (C) 100 μm, scale bar 100 μm (D) 45 μm meshes, scale bar 100 μm.
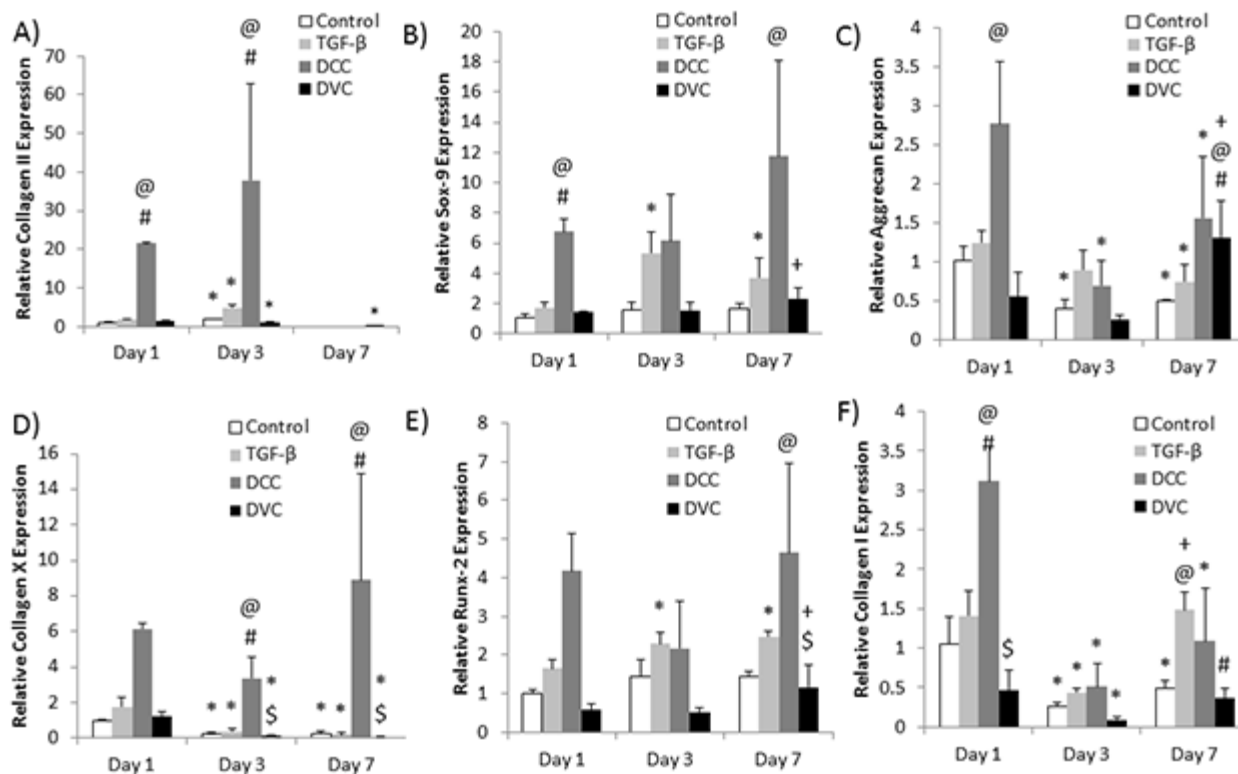
Figure 3.6: qPCR results.

Relative expression of chondrogenic and osteogenic gene markers (n=5). A) collagen II, B) Sox-9, C) aggrecan, D) collagen X, E) Runx-2, and F) collagen I. @ denotes significant difference from control group at same time point, # denotes significant difference from TGF-β group at same time point, * denotes significant difference between day 1 value, + denotes significant difference from previous time point, $ denotes significant difference from DCC group at same time point.
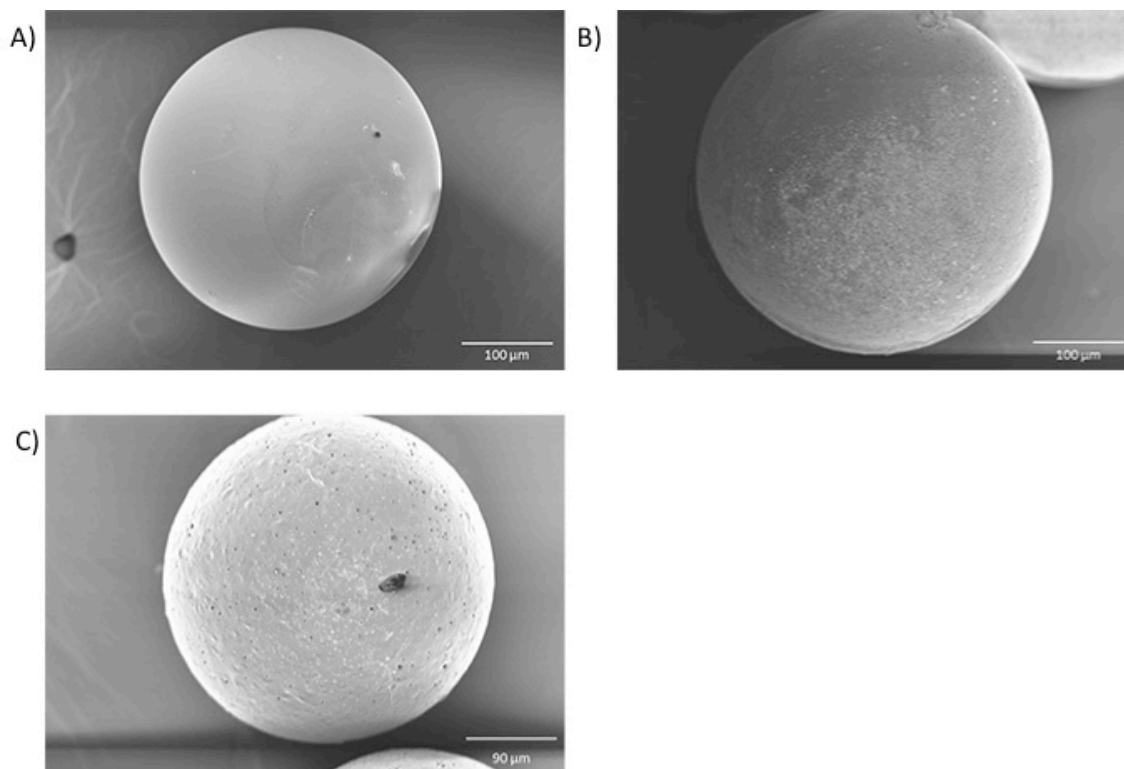
Figure 4.1: Microsphere morphology**.**

SEM images of microsphere morphology. Both TGF and DCC-encapsulated microspheres have porous surfaces compared to PLGA microspheres that have a smooth surface. A) PLGA microsphere, B) DCC-encapsulated microsphere, and C) TGF microsphere.
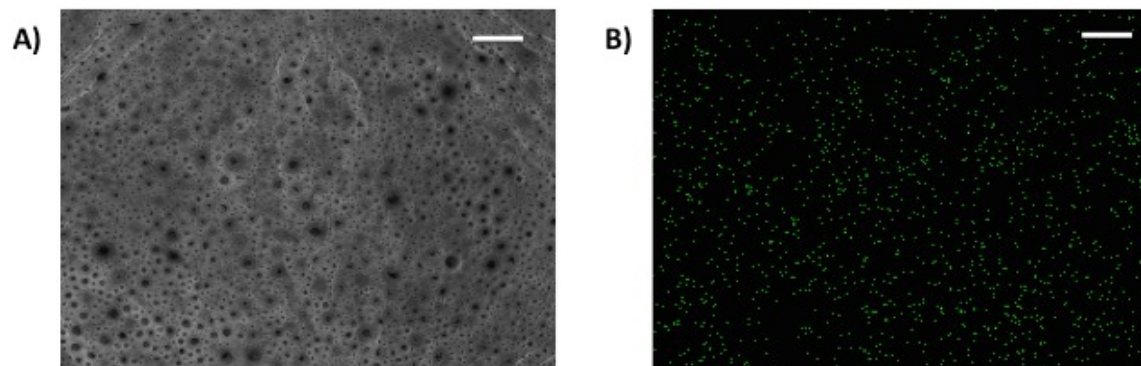
Figure 4.2: SEM and EDS of DCC-encapsulated microspheres**.**

A) SEM image of DCC-encapsulated microsphere B) EDS pixel map depicting location of nitrogen on the surface of the DCC-encapsulated microsphere. Scale bars are 10 μm.
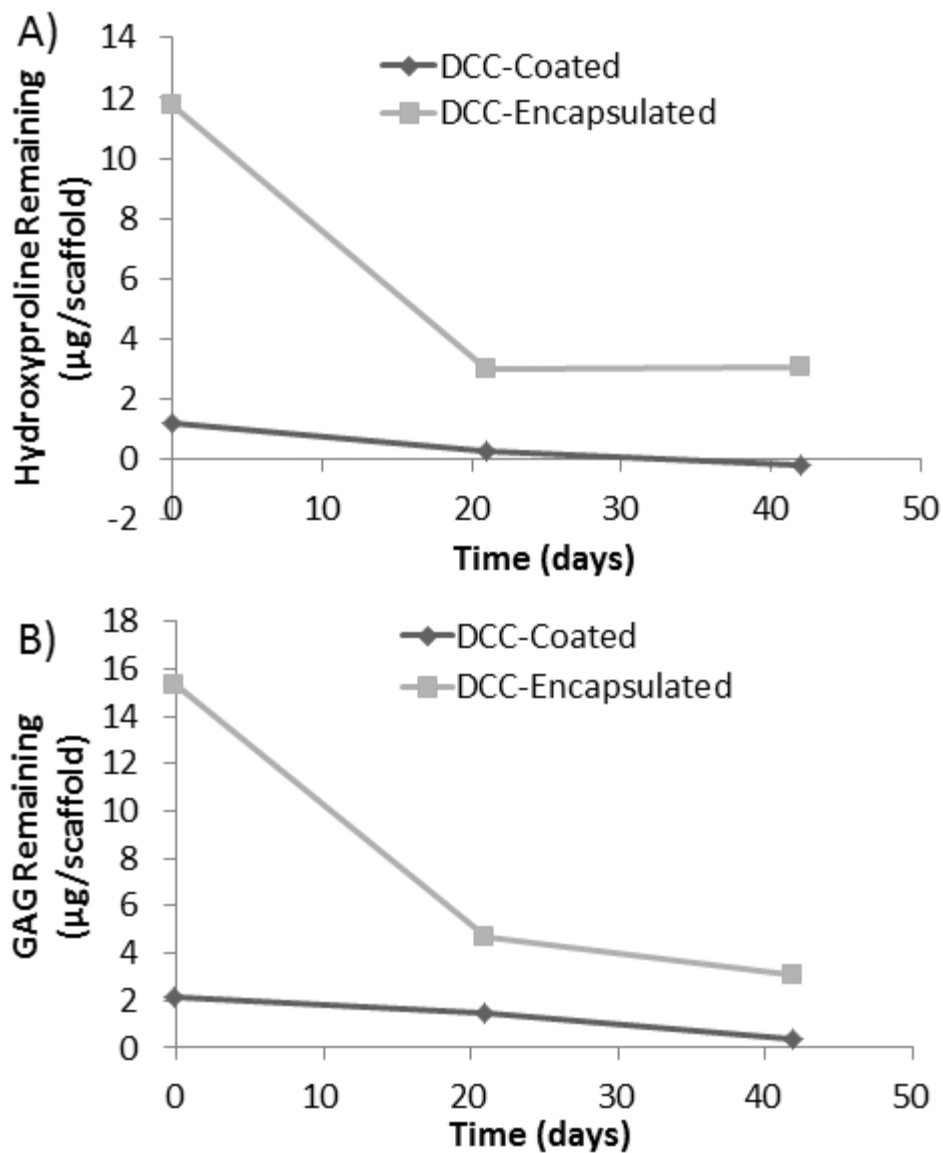
Figure 4.3: Hydroxyproline and GAG content.

Remaining GAG and hydroxyproline in DCC-encapsulated and DCC-coated scaffolds at day 0, week 3 and week 6. All scaffolds exhibited a decrease in biochemical content by week 3 ($p < 0.05$).
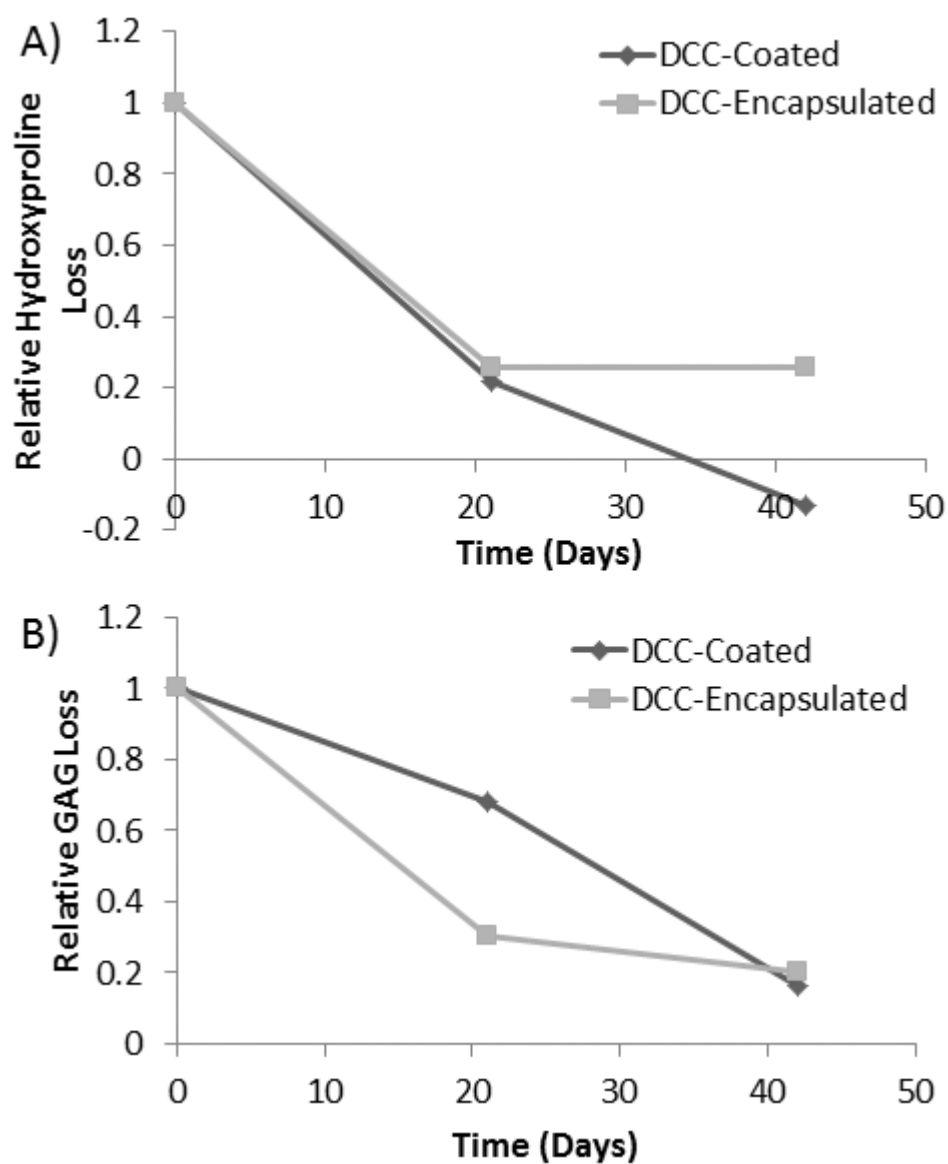
Figure 4.4: Relative loss of hydroxyproline and GAG content.

Relative loss of A) hydroxyproline and B) GAG from DCC-Coated and DCC-Encapsulated scaffolds. By week 6 a greater proportion of hydroxyproline was lost from the DCC-Coated scaffolds and an equal proportion of GAG was lost from each type of scaffold.
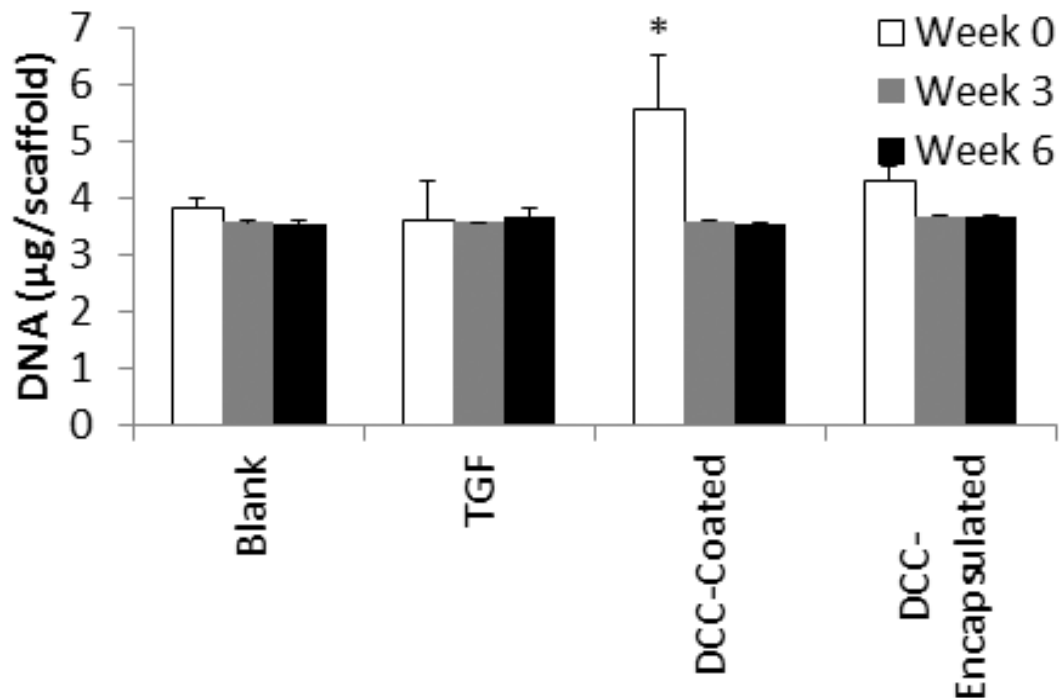
Figure 4.5: DNA content of all cell seeded scaffolds.

PicoGreen results depicting greater DNA content on DCC-coated scaffolds at day 0. *
denotes statistically significant difference from all other groups at same time and subsequent
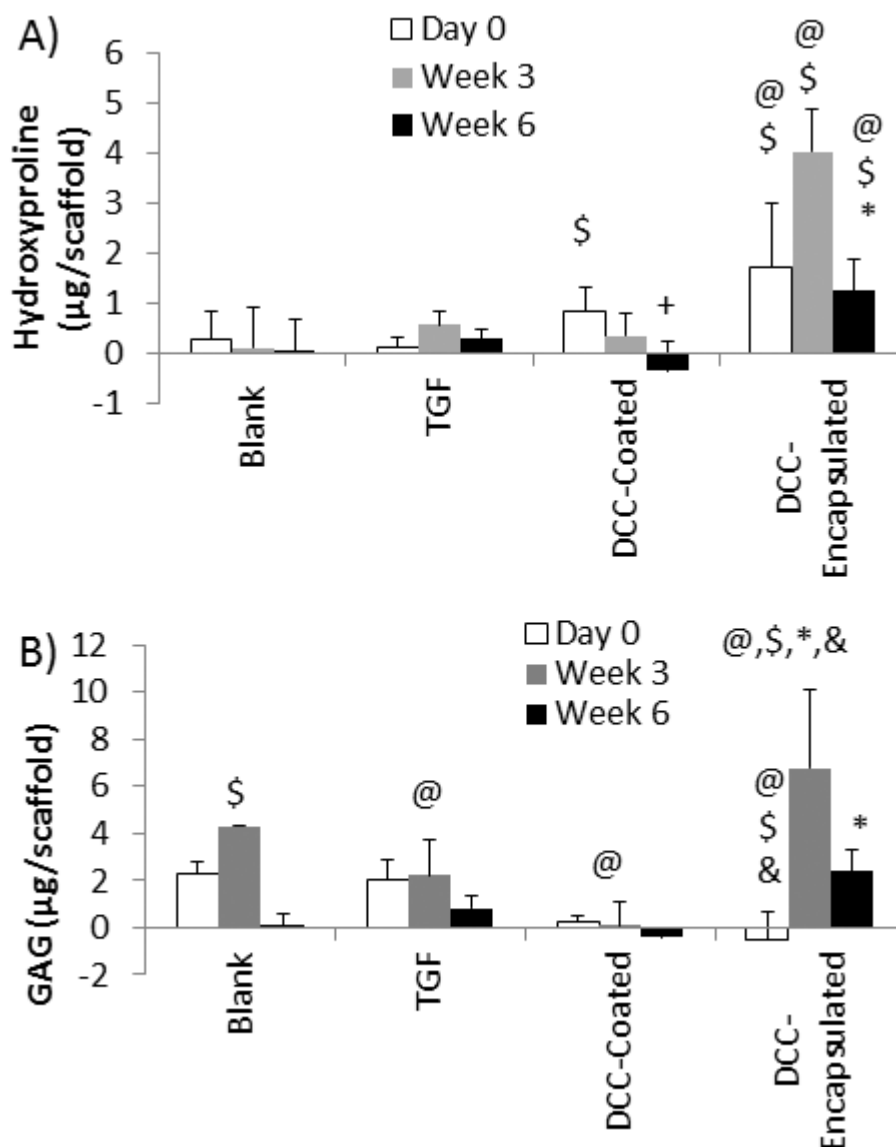times of same group (p<0.05). Data is reported as mean ± standard deviation.

Figure 4.6: Biochemical content of all cell seeded scaffolds.

Biochemical content of engineered constructs (n=5). Note that HYP and GAG contents were measured for acellular scaffolds containing DCC and subtracted out as a baseline value. A) Hydroxyproline content on all scaffolds, a decrease in hydroxyproline is observed following week 3 on all scaffolds. B) GAG content on all scaffolds, also with a decrease in content following week 3.* denotes statistically significant difference from day 0 value, @ denotes statistically significant difference from blank group at same time point, $ denotes statistically significant difference from TGF group at same time point, and + denotes statistically significant difference from DCC at same time point. All significance reported for ($p<0.05$). Data is reported as mean ± standard deviation.
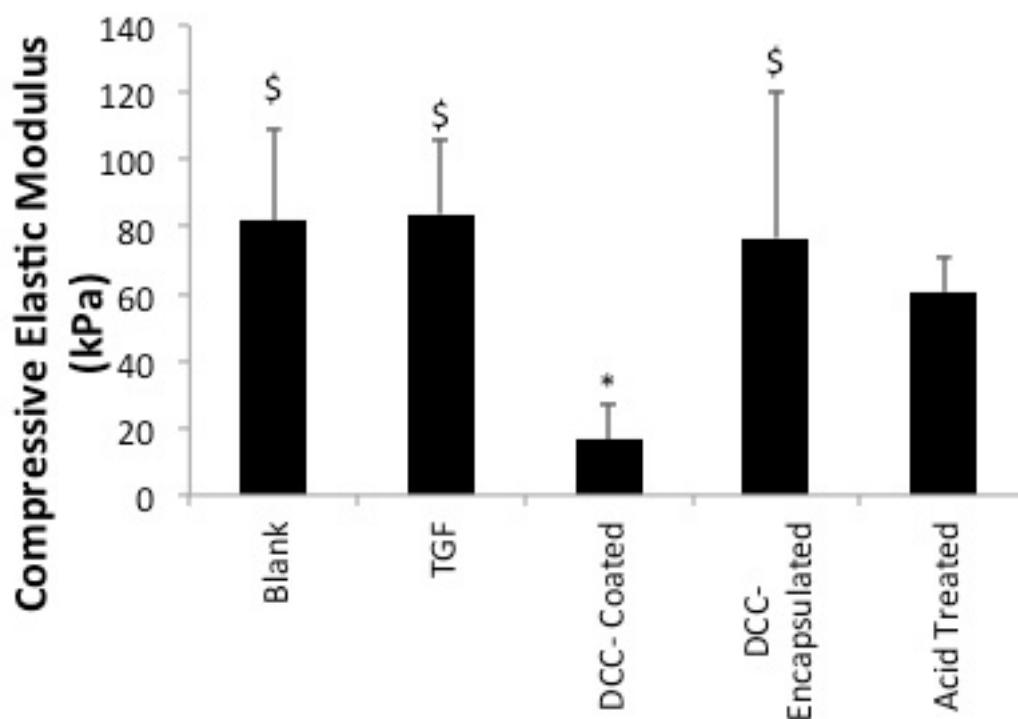
Figure 4.7: Compressive elastic moduli of all acellular scaffolds.

The compressive elastic moduli of engineered scaffolds prior to cell seeding (n=4-5). The DCC-coated scaffolds had significantly lower compressive moduli than the blank, TGF, and DCC-encapsulated scaffolds. *denotes significance from blank scaffolds (p<0.05) and $ denotes significance from DCC-coated scaffolds. Data is reported as mean ± standard deviation.
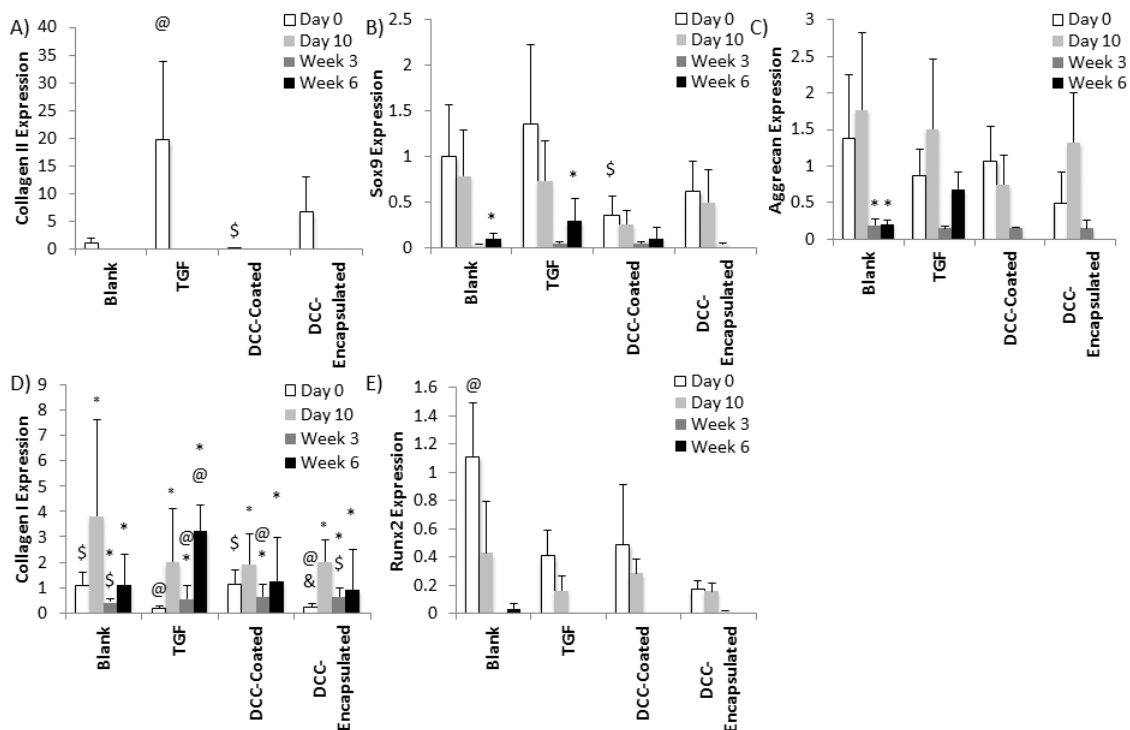
Figure 4.8: RT-qPCR results.

RT-qPCR results for all scaffolds and time points (n=5). A) Collagen II expression was significantly greater in TGF scaffolds compared to all others. B) Sox9 expression was significantly lower in DCC-coated scaffolds at day 0 compared to all other scaffolds. C) Aggrecan expression was nearly equal among all scaffold types during culture. D) Collagen I expression was lower in both TGF and DCC-encapsulated scaffolds at day 0. E) Runx2 expression was greatest in blank scaffolds at day 0. * denotes significant from time 0 value (same group), @ denotes significant from blank at same time point, $ denotes significant from TGF at same time point, & denotes significant from DCC-coated at same time point. For all significance noted (p<0.05). Data is reported as mean ± standard deviation.
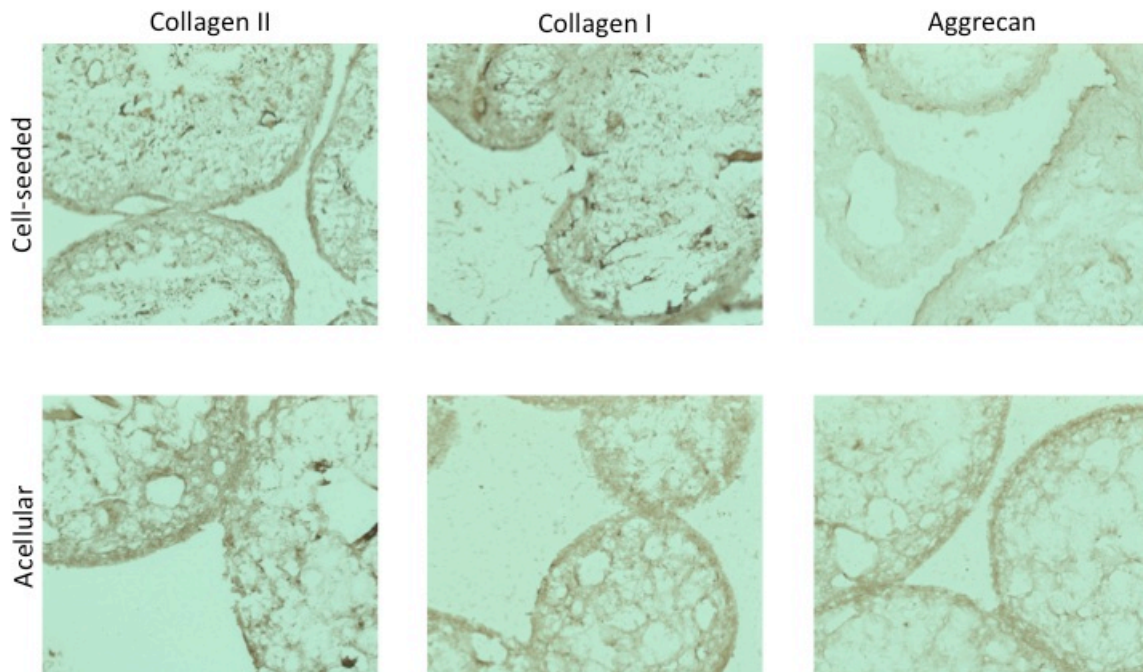
Figure 4.9: Immunohistochemistry images.

IHC staining of DCC-encapsulated scaffolds at week 3. Staining was positive for collagen II, collagen I, and aggrecan. Collagen I staining was more intense in the cell-seeded group compared to the acellular group.

APPENDIX B: Tables

Table 2.1: Common decellularization reagents.

A general list of common chemicals used in decellularizing cartilage with the chemical description and the purpose of the chemical for decellularization.

Reagent	Description	Purpose in Decellularization
Triton X-100	Non-ionic surfactant	Permeabilize cellular membranes, solubilize membrane proteins, and extract DNA
Sodium dodecyl sufate (SDS) or Sodium lauryl sulfate (SLS)	Anionic surfactant	Lyse cells and denature proteins by disrupting non-covalent bonds
EDTA	Mineral and metal chelator	Deactivate metal dependent enzymes and prevent cell to cell attachment
Nucleases (DNase, RNase)	Nucleic acid degradation enzyme	Cleave phosphodiester bonds between nucleotides
Tris-HCl	Buffer component	Increase cell membrane permeability and acts as a buffer component in DNA and RNA phenol extraction
Sodium Deoxycholate	Anionic surfactant	Lyse cells, dissolve lipid bilayer
Trypsin	Digestive enzyme	Disruption of ECM to improve reagent penetration
Sodium Lauroyl Sarcosine	Anionic surfactant	Non-denaturing, solubilize and remove cells

Table 2.2: Key *in vivo* studies.

A summary of key *in vivo* studies that have delivered cartilage matrix derived scaffolds to cartilage defect sites. Defect sites are all in the femoral condyle unless otherwise noted. Abbreviations: DCC = Decellularized native cartilage (Fig. 2.1), BMSC = bone-marrow derived mesenchymal stem cell, ASC = adipose derived stem cell, MSC = mesenchymal stem cell

Matrix Type	Matrix Species	Implant Species	Defect Type	Exogenous Cells	Time	Results	Reference
DCC	Canine	Canine	Osteochondral	Canine BMSC	6 months	Unclear defect borders, stiffess was 70% of normal cartilage	Yang *et al.*
DCC	Human	Rabbit	Chondral	Rabbit ASC	6 months	No defect boundary, basal integration, 83% stiffness of normal cartilage	Kang *et al.*
DCC	Bovine	Rabbit	Osteochondral	Rabbit MSC	3 months	Greater histological scoring and macrographic examnication in cell seeded group than group without exongenous cells	Z. Yang *et al.*
DCC	Canine	Canine	Osteochondral (femoral head)	Canine BMSC	6 months	Severe collapse of femoral head and osteoarthritis, fibrous tissue formation, uneven articular surface and no scaffold integration	Yang *et al.*

Table 2.3: Summary of patents describing cartilage matrix materials.

Summary of current patent applications and awarded patents describing formulations of cartilage matrices as of May 25, 2014. Abbreviations: DVC = devitalized native cartilage, DCC = decellularized native cartilage, DCCM = decellularized cell derived matrix, DVCM = devitalized cell derived matrix, CDM = cell derived matrix (Fig. 2.1).

Cartilage Matrix	Applicant	Year Filed	Country	Status	Patent Number
DVC	Theodore I. Malinin	2013	US	Application	US20130330391
DVC	Katherine G. Truncale, Moon Hae Sunwoo, Arthur A. Gertzman, William W. Tomford	2010	US	Application	US20110070271
DCC	Musculoskeletal Transplant Foundation	2009	US	Application	US20090291112
DCC	People's Liberation Army General Hospital (China)	2008	China		CN101574540
DCC	William Marsh Rice University	2009	World	Application	WO2010022074
DCC	Wake Forest University Health Sciences	2010	US	Awarded	US20110046732
DCCM	Byung Hune Choi, Filmiagen Co Ltd, Byoung-Hyun Min, So Ra Park	2007	US	Application	US20100137203
DVCM	Cheng Zhe Jin, Byoung-Hyun Min, Kwideok Park, So Ra Park	2007	US	Application	US20100136645
CDM	Regents of University of California	2012	US	Application	US20140023723