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The Bioactivity of Cartilage Extracellular Matrix in Articular Cartilage Regeneration

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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.

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1. 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] 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.[4] 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.[3b, 5] 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 (Figure 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.[5] 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.[6] 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.[7]

Hyaline cartilage is an ECM-rich tissue with approximately 95% of the dry weight being made up of ECM.[8] 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, 3b] 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.

2. 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.[9] Certain disease states, particularly osteoarthritis, will produce articular cartilage with reduced amounts of GAG and collagen II.[9c] 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.

2a. Decellularized Native Cartilage (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).[10] Various formulations of DNases and RNases are also commonly used to remove nucleic acids from the material.[10d, 11] 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.[8] 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.[12] 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.[13]

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.[10a, 10c, 10d, 12, 14] 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.[11, 15] The amount of GAG retained in DCC, however, significantly decreased with increasing chemical decellularization, while collagen II levels did not significantly decrease.[15] A reduction in GAG content may be undesirable based on previous studies that have shown that certain GAGs such as aggrecan are chondroinductive.[16] Biomechanical properties such as the aggregate modulus and linear modulus have also decreased following decellularization agent exposure.[12, 15, 17] 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.*[17] 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.[10b] The BMSCs attached to the scaffold and exhibited a round or elliptical morphology confirmed by SEM.[10b] 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.[10d]

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).[18] These scaffolds seeded with exogenous cells produced regenerated tissue with 83% of native cartilage stiffness after 6 months.[18] ASCs also attached to the DCC scaffold and exhibited a round morphology confirmed by SEM when seeded on DCC scaffolds.[18-19] 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.[10b] At 6 months, the repair cartilage exhibited 70% stiffness of native cartilage.[10b] 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.[20] Proposed mechanisms of failure included accelerated degradation of the cancellous bone region of the scaffold, ischemic conditions, and high load bearing conditions.[20] 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.[18]

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.[10b, 18-19]

A sandwich model for tissue engineering DCC scaffolds has been reported that consists of layers of DCC sheets and chondrocytes.[10a] 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.[21] 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.[22] 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.

2b. 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.[23] 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.[24] Freeze thaw cycles followed by sonication has also been used to devitalize CDM constructs and could conceivably be used to devitalize native cartilage.[25]

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*.[24b] 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.[26] 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*.[27] 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.[27] Protease

inhibitors may also be used to increase the preservation of the ECM during freeze-thaw cycles.[28] For a complete review of cryopreservation induced stresses in articular cartilage, the reader is directed to Kaur *et al*.[26]

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.[23] Cartilage particles alone, heat inactivated or not, did not induce high quality chondrogenesis without the addition of exogenous growth factors. Peretti *et al*.[29] 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.[29]

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.[24a] 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.[30] 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*.[24a] 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.[31] The aggregate modulus of DVC scaffolds can also be increased by increasing the crosslinking percentage as reported by Cheng *et al*.[19]

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.[24a] Devitalized cartilage has been

shown to have the ability to induce neocartilage formation when implanted subcutaneously.[29]

3. 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.[32] 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.[33] Perfusion bioreactors have been used to encourage cell deposition of ECM *in vitro* and may be used for increased ECM production.[34]

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.[35]

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.[36] 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.[25, 34a]

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.[34b, 37] The composition of CDM may also vary from that found in typical native tissue, and reproducibility may also become a concern.

3a. 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, 25] 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.[13] 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.[13] 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.[13]

3b. 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.*[25] 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.

4. 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.[38] 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™ NT Natural Tissue Graft, which is particulated juvenile human cartilage allograft for osteochondral defect repair.[39] 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.[40] The resulting neocartilage from juvenile human chondrocytes did not elicit a T-cell-mediated immune response in goats.[40] 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.[41] In a study with baboons, BioCartilage® was shown to promote chondral lesion regeneration without any adverse immunological reactions.[41]

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).[38-39, 42] 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.

5. 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.[43]

5a. 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*.[44] 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*.[44] 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*.[44] 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*.[45] 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.

5b. 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.[46] The alpha-Gal epitope is commonly found in xenograft materials originating from nonprimate animals and is a carbohydrate found within the ECM.[47] 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.[48] 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.[7a] Methods to reduce the antigenicity of xenogeneic materials have been studied including the solubilization of proteins to enhance antigen removal from decellularized bovine pericardium.[49] 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.[43c, 47] 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.[23] 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.[14a] 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.[14a] 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.[12]

5c. 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.[43b] 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.[50] 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.[50b] Hunt *et al*.[50b] 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.

6. 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.[8] For an in depth review of the rationale for using microscopic donor units, the reader is directed to Ghanavi *et al*.[8] 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.[10b, 24a] 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 μm) as used by Gong *et al*.[10a] The constructs with 10 μm 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.[34b, 51] CDM constructs fabricated using open-cell foams also result in scaffolds with high porosity to allow for cell migration and infiltration.[36] Decellularization of intact cartilage (no mechanical processing) may benefit from a significant decrease in GAG content to increase the porosity of the matrix.[12] 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.[50a] Studies exploring the use of DCC for cartilage regeneration have seen successful host integration when exogenous cells were added prior to implantation (Table 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.

7. 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.[19, 24a] 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*.[17] 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.[32a]

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.[52] 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.[37] 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*.[30] 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).[32a, 52-53] The decellularization or devitalization protocol used may affect either the biochemical content or the residual growth factors within the matrix. Wong *et al*.[49] 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.[26] 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.

8. 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.[54]

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.

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Biography

Michael Detamore is a Professor of Chemical & Petroleum Engineering and Director of the Biomaterials and Tissue Engineering Laboratory at the University of Kansas. His research interests revolve around translational regenerative medicine and biomaterials. Tissue engineering efforts focus primarily on bone and cartilage regeneration, including the temporomandibular joint (TMJ), knee, cranium, and trachea. Central research themes include umbilical cord stem cells, gradients in tissue engineering, and bioactive biomaterials.

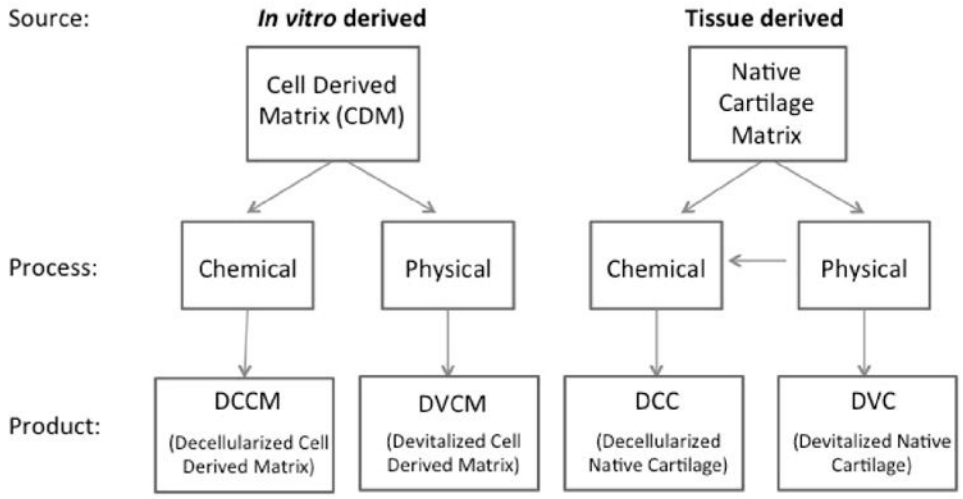

Figure 1.

A schematic depicting the distinctions between cartilage matrix final products dependent on the source and processing.

Table 1

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 sulfate (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

Table 2

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.

Matrix Type	Matrix Species	Implant Species	Defect Type	Exogenous Cells	Time	Results	Reference
DCC	Canine	Canine	Osteochondral	Canine BMSC	6 months	Unclear defect borders, stiffness was 70% of normal cartilage	Q. Yang *et al.*, 2011[10b]
DCC	Human	Rabbit	Chondral	Rabbit ASC	6 months	No defect boundary, basal integration, 83% stiffness of normal cartilage	Kang *et al.*, 2012[18]
DCC	Bovine	Rabbit	Osteochondral (patellar groove)	Rabbit MSC	3 months	Greater histological scoring and macrographic examination in cell seeded group than group without exogenous cells	Z. Yang *et al.*, 2010[10c]
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	Qiang *et al.*, 2014[20]

Abbreviations: DCC = Decellularized native cartilage (Figure 1), BMSC = bone-marrow derived mesenchymal stem cell, ASC = adipose derived stem cell, MSC = mesenchymal stem cell

Table 3

Summary of current patent applications and awarded patents describing formulations of cartilage matrices as of May 25, 2014.

Cartilage Matrix	Applicant	Year Filed	Country	Status	Patent Number
DVC	Theodore I. Malinin	2013	US	Application	US20130330391[42d]
DVC	Katherine G. Truncale, Moon Hae Sunwoo, Arthur A. Gertzman, William W. Tomford	2010	US	Application	US20110070271[42i]
DCC	Musculoskeletal Transplant Foundation	2009	US	Application	US20090291112[42h]
DCC	People's Liberation Army General Hospital (China)	2008	China	Application	CN101574540[55]
DCC	William Marsh Rice University	2009	World	Application	WO2010022074[42a]
DCC	Wake Forest University Health Sciences	2010	US	Awarded	US20110046732[42j]
DCCM	Byung Hune Choi, Filmiagen Co Ltd, Byoung-Hyun Min, So Ra Park	2007	US	Application	US20100137203[42f]
CDM	Regents of University of California	2012	US	Application	US20140023723[42c]

Abbreviations: DVC = devitalized native cartilage, DCC = decellularized native cartilage, DCCM = decellularized cell derived matrix, DVCM = devitalized cell derived matrix, CDM = cell derived matrix (Figure 1).