HHS Public Access

Author manuscript

Acta Biomater. Author manuscript; available in PMC 2017 July 01.

Published in final edited form as:

Acta Biomater. 2016 July 1; 38: 94–105. doi:10.1016/j.actbio.2016.04.019.

Approaching the Compressive Modulus of Articular Cartilage With a Decellularized Cartilage-Based Hydrogel

Emily C. Beck, Ph.D[1], **Marilyn Barragan**[2], **Madeleine H. Tadros**[3], **Stevin H. Gehrke, Ph.D**[4,5], and **Michael S. Detamore, Ph.D**[4,5]

[1]Department of Surgery, University of Kansas Medical Center, Kansas City, KS 66160

[2]Department of Molecular Biosciences, University of Kansas, Lawrence, KS 66045

[3]Department of Chemical and Biomolecular Engineering, Rice University, Houston, TX 77005

[4]Bioengineering Program, University of Kansas, Lawrence, KS 66045

[5]Department of Chemical and Petroleum Engineering, University of Kansas, Lawrence, KS 66045

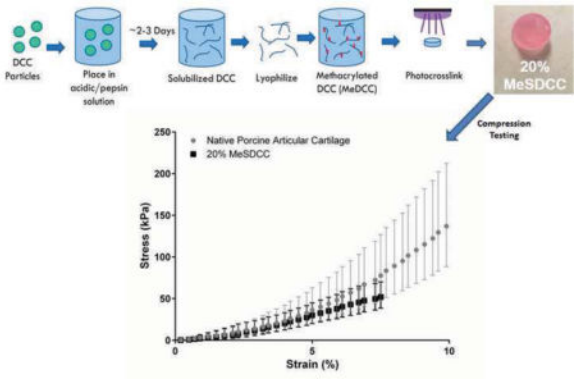
Abstract

ECM-based materials are appealing for tissue engineering strategies because they may promote stem cell recruitment, cell infiltration, and cell differentiation without the need to supplement with additional biological factors. Cartilage ECM has recently shown potential to be chondroinductive, particularly in a hydrogel-based system, which may be revolutionary in orthopedic medicine. However, hydrogels composed of natural materials are often mechanically inferior to synthetic materials, which is a major limitation for load-bearing tissue applications. The objective was therefore to create an unprecedented hydrogel derived entirely from native cartilage ECM that was both mechanically more similar to native cartilage tissue and capable of inducing chondrogenesis. Porcine cartilage was decellularized, solubilized, and then methacrylated and UV photocrosslinked to create methacrylated solubilized decellularized cartilage (MeSDCC) gels. Methacrylated gelatin (GelMA) was employed as a control for both biomechanics and bioactivity. Rat bone marrow-derived mesenchymal stem cells were encapsulated in these networks, which were cultured *in vitro* for 6 weeks, where chondrogenic gene expression, the compressive modulus, swelling, and histology were analyzed. One day after crosslinking, the elastic compressive modulus of the 20% MeSDCC gels was 1070 ± 150 kPa. Most notably, the stress strain profile of the 20% MeSDCC gels fell within the 95% confidence interval range of native porcine cartilage. Additionally, MeSDCC gels significantly upregulated chondrogenic genes compared to GelMA as early as day 1 and supported extensive matrix synthesis as observed histologically. Given that these gels approached the mechanics of native cartilage tissue, supported matrix synthesis, and induced chondrogenic gene expression, MeSDCC hydrogels may be promising materials for cartilage

Corresponding Author: Michael S. Detamore, Department of Chemical and Petroleum Engineering, University of Kansas, 4163 Learned Hall, 1530 W. 15th Street, Lawrence, KS 66045, Phone: (785) 864-4943, detamore@ku.edu.

tissue engineering applications. Future efforts will focus on improving fracture mechanics as well to benefit overall biomechanical performance.

Graphical abstract



Keywords

decellularized cartilage; hydrogel; compressive modulus

Introduction

Arthritis is one of the leading causes of disability among US adults [1]. Some of the current clinical treatments include autologous chondrocyte implantation, mosaicplasty, and microfracture [2, 3]. However, not only do these treatments involve high risk of donor site morbidity and/or the need for multiple surgeries, these treatments still lack the ability to regenerate fully functional cartilage tissue [4–6]. Tissue engineering approaches are therefore striving to fully regenerate cartilage tissue by utilizing a bioactive and bioresorbable construct that provides the necessary cues to facilitate cell growth, differentiation, and tissue integration, while providing the mechanical integrity and support to allow the tissue to sustain its load bearing function [3].

Hydrogels have several advantages in cartilage tissue engineering, which include ease of formation, the ability to fine tune mechanical properties, the ability to encapsulate cells, and vast array of conjugation options for degradability, bioactivity, etc. [7–9]. Hydrogels can be made from both synthetic (e.g., polyethylene glycol) and natural materials (e.g., collagen, gelatin), where both have their own inherent advantages and disadvantages. Synthetic materials have the advantage of the ability to more readily control the composition and mechanical properties of the hydrogel compared to hydrogels composed of natural materials, but natural materials have the additional advantage of providing biochemical cues and signals to facilitate cell attachment, growth, and differentiation [10].

One such natural material that is gaining attention in tissue engineering approaches is naturally derived extracellular matrix [11]. ECM materials can either be obtained from cell-derived matrices that are secreted during *in vitro* culture or they can be derived directly from native tissue [4, 12–16], and often they have been decellularized to remove cellular

components and nucleic acids that may have the potential to cause an adverse immunological response [11]. We and other groups have already established that decellularized cartilage has chondroinductive potential [11, 13, 17–20], and we recently reported the chondroinductive potential of decellularized cartilage (DCC) in pellet culture [11], where we observed increased chondroinductivity of rat bone marrow stem cells (rBMSCs) exposed to DCC as compared to those cells only exposed to TGF-β_3 [11].

Therefore, in this study we endeavored to create a material that was entirely derived from DCC to potentially make the material inherently chondroinductive, and we furthermore endeavored to design a material would have the mechanical properties necessary to be load-bearing. Several studies have made gels entirely out of ECM by first solubilizing the ECM, where the solubilized matrix would form a gel at body temperature [18, 21–23]. One group even utilized solubilized cartilage matrix gels for drug delivery, where they noted that the gel maintained enough structural integrity under physiological conditions to be a stable drug depot [24]. We tried using solubilized cartilage hydrogels, but the gels that formed were too compliant and left opportunity for improvement for load-bearing applications. Methods of crosslinking unsolubilized cartilage have been reported, including crosslinking cartilage ECM with genipin, dehydrothermal treatment, ultraviolet irradiation, and carbodiimide chemistry [4, 25]. Using these methods, cartilage scaffolds were able to be crosslinked and maintained some mechanical integrity throughout culture where cell mediated contraction was able to be controlled depending on the method of crosslinking. However, the authors of these previous studies noted that the constructs would require additional reinforcements to attain functional biomechanical properties and additionally, a sole ECM content of 10% was used to make the gels. In the current study, we sought to overcome this limitation through solubilizing and further crosslinking cartilage tissue. The rationale for solubilizing the cartilage tissue was to provide more control over mechanical properties through the ability to more finely tune the solid content of the hydrogel. Furthermore, solubilizing the cartilage may free up more reactive sites for crosslinking on the cartilage ECM, which may help reinforce the biomechanical properties of the solubilized cartilage once it is crosslinked. Therefore, based on our experience of functionalizing GAGs such as hyaluronic acid and chondroitin sulfate with glycidyl methacrylate [26, 27], which allows the hydrogel to be formed through photocrosslinking, we decided to methacrylate solubilized, decellularized cartilage ECM. Earlier in 2015, one pioneering study reported methacrylating solubilized cartilage matrix to make photocrosslinkable hydrogels, demonstrating for the first time that native tissues can be crosslinked to form hydrogels [28]. However, in that study, the solubilized cartilage matrix was mixed with methacrylated gelatin (GelMA) and the biomechanics of the hydrogels, evaluated via the compressive modulus, still fell short of native cartilage tissue. Garrigues *et al.* [18] cleverly reinforced solubilized cartilage ECM through combining it with poly(ε-caprolactone) and then electrospinning it into a scaffold. However, the Young's moduli of the cartilage-containing electrospun scaffolds were approximately 10 kPa, which again fall short of the biomechanics of native cartilage tissue. In this current study, the goal was to create the first hydrogel entirely derived from cartilage ECM without additional reinforcements and study its potential for cartilage tissue engineering over a period of 6 weeks, a length of time that should be sufficient to show chondrogenesis and matrix synthesis. We hypothesized that this MeSDCC hydrogel would

have a compressive modulus comparable to native cartilage and would be chondroinductive. Therefore, solubilized cartilage hydrogels were photocrosslinked and their mechanics as well as chondroinductive potential were analyzed.

Methods and Materials

Tissue Retrieval, Devitalization, and Decellularization

Ten porcine knees obtained from Berkshire hogs (castrated males that were approximately 7–8 months old and 120 kg) were purchased from a local abattoir (Bichelmeyer Meats, Kansas City, KS). Articular cartilage from the knee and hip joints was carefully removed and collected using scalpels. The cartilage was then rinsed twice in DI water and stored at −20 ºC. After freezing overnight, the cartilage was thawed and then coarsely ground with dry ice using a cryogenic tissue grinder (BioSpec Products, Bartlesville, OK). Coarse grinding was performed to reduce diffusion distances during the decellularization process. The dry ice was then allowed to evaporate overnight in the freezer, at which point the cartilage was referred to as devitalized cartilage (DVC) [11], and then the DVC was packed into dialysis tubing (3500 MWCO) and decellularized using an adapted version of our previously established method using osmotic shock, detergent, and enzymatic washes [29]. The packets were placed under gentle agitation (70 rpm) in a hypertonic salt solution (HSS) overnight at room temperature. The packets were then subjected to 220 rpm agitation with two reciprocating washes of triton X-100 (0.01% v/v) followed with HSS to permeabilize intact cellular membranes. The tissue was then treated overnight with benzonase (0.0625 KU ml^{-1}) at 37 ºC and then with sodium-lauroylsarcosine (NLS, 1% v/v) overnight to further lyse cells and denature cellular proteins. After NLS exposure, the tissue was washed with ethanol (40% v/v) at 50 rpm and then was subjected to organic exchange resins at 65 rpm to extract the organic solvents. The tissue was then washed in saline-mannitol solution at 50 rpm followed by two hours of rinsing with DI water at 220 rpm. The tissue was then removed from the packets and was then frozen and lyophilized. The cartilage was then cryoground into a fine powder with a freezer-mill (SPEX SamplePrep, Metuchen, NJ) and was lyophilized overnight. The decellularized cartilage powder was then filtered using a 45 μm mesh (ThermoFisher Scientific, Waltham, MA) to remove large particles and then frozen until use.

Synthesis and Characterization of MeSDCC and GelMA

DCC powder was first solubilized using an adapted protocol from a previously reported method [30]. DCC powder was first mixed in 0.1M HCl at a concentration of 10mg DCC per 1 mL HCl. Pepsin was then added at a concentration of 1mg/mL and the solution was stirred at 200 rpm for 2 days at room temperature. The solution was then brought back to physiological pH, verified with litmus paper, by adding 1M NaOH. The solubilized DCC powder (SDCC) was then centrifuged at 10,000 × *g* for 3 min and the supernatant was frozen and lyophilized and used to make methacrylated SDCC (MeSDCC).

MeSDCC was prepared by reacting SDCC with 20 fold molar excess glycidyl methacrylate (Sigma-Aldrich, St. Louis, MO) in the presence of trimethylamine and tetrabutyl ammonium bromide (Sigma-Aldrich). The reaction solution was a 1:3 acetone:water mixture, which was

stirred at 200 rpm at a concentration of 1 g SDCC for every 150 mL solution. The molar excess was approximated based on reacting one glycidyl methacrylate group to every monomer present in the solution and with the assumption that all monomers were hyaluronic acid. The reaction continued stirring for 6 days, the MeSDCC was then precipitated in excess acetone, dialyzed for 2 days in DI water, and then lyophilized. Methacrylated gelatin (GelMA) was made with the same protocol used to make MeSDCC, except Type A gelatin from porcine skin (Sigma-Aldrich) was used in the reaction instead of SDCC. Methacrylation was confirmed using ^{1}H NMR (Avance AV-III 500, Bruker).

Rat Bone Marrow Stem Cell Harvest and Culture

Rat bone marrow stems cells (rBMSCs) were harvested from both femurs of five male Sprague-Dawley rats (200–250 g) following an approved University of Kansas IACUC protocol (AUS #175-08). The rBMSCs were first harvested in minimum essential medium-α (MEM-α, ThermoFisher) supplemented with 10% fetal bovine serum (FBS, MSC qualified, ThermoFisher) and 1% antibiotic-antimycotic (anti-anti, ThermoFisher) and were then cultured in this medium for one week to ensure no mycotic contamination from harvesting. After 1 week of culture, the anti-anti was substituted for 1% penicillin/streptomycin (ThermoFisher) and the cells were cultured in this medium until they reached passage 4 for cell encapsulation into the hydrogels.

Description of Experimental Groups

Formulations tested in the 6 week culture were both cellular and acellular formulations of 10% GelMA, 10% MeSDCC, and 20% MeSDCC (w/v). In addition, acellular GelMA was tested at a concentration of 20% under mechanical compression and swelling at day 1. Acellular formulations were prepared and analyzed with the cellular groups to quantify the acellular biochemical content and to analyze the effect of cells encapsulated in the networks. A concentration of 10% for GelMA and MeSDCC was chosen as it was a concentration previously reported in literature, and it was verified in our preliminary studies by evaluation of a wide range of concentrations [28]. A concentration of 20% was chosen as that is the approximate concentration of dry mass in native cartilage matrix [31].

Preparation of Hydrogels, Cell Encapsulation, and Hydrogel Culture Conditions

Hydrogels were made by first measuring out the desired weight percents of either GelMA or MeSDCC into a 50 mL centrifuge tube. The tubes with the weighed materials were then sterilized with ethylene oxide prior to use and from then on were handled under sterile conditions. All gels were mixed in two stages (e.g., in photoinitiator solution overnight and then more photoinitiator or cell suspension the day of testing). This two-stage mixing process was used because some of the samples required mixing with cells and the time it took for MeSDCC to dissolve to ensure mixture homogeneity (i.e., overnight) was deemed too long for adequate cell survival. Therefore, cell suspensions were added the next day after the MeSDCC was given a chance to dissolve in half of the final solution. For acellular testing, the first stage of mixing involved adding sterile 0.01 M PBS containing 0.05% (w/v) Irgacure (I-2959) photoinitiator until the concentration of MeSDCC or GelMA was twice the desired concentration. The acellular samples were then mixed, centrifuged at 3000 rpm, and stored at 4 ºC overnight to allow time for the MeSDCC to dissolve. Prior to testing, more

photoinitiator solution was added to the acellular samples until the desired concentration was reached. The samples were then mixed again and centrifuged to remove air bubbles. For example, to make a 10% MeSDCC solution, 40 mg MeSDCC and 200 μL photoinitiator solution were mixed and allowed to dissolve overnight, and then another 200 μL photoinitiator solution was added to make the final concentration at 10% MeSDCC. For cellular testing, the first stage of mixing involved adding 0.1% (w/v) Irgacure photoinitiator in PBS until the concentration of MeSDCC or GelMA was twice the desired final concentration, and then the solutions were centrifuged and stored at 4 ºC overnight. Passage 4 rBMSCs were then suspended at 10 million cells/mL in incomplete chondrogenic medium consisting of high glucose DMEM (ThermoFisher) with 4.5 g/L D-glucose supplemented with 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate, 50 μg/mL ascorbic acid, and 0.25 mg/mL penicillin/streptomycin. We refer to incomplete chondrogenic medium as medium that did not contain growth factors. The cell suspension in incomplete chondrogenic medium was then added to the cellular samples until the desired concentration of MeSDCC or GelMA was reached and the final cell concentration and photoinitiator concentration was 5 million cells/mL and 0.05%, respectively. Both cellular and acellular solutions were then loaded into 2 mm thick molds between glass slides and exposed to 312 nm UV light at 3.0 mW/cm^2 in a UV crosslinker (Spectrolinker XL-100, Spectronics Corporation, Westbury, NY) for 2.5 min on each side. Each gel was then cut using a 4mm biopsy punch and placed in one well of a 24 well, non-tissue culture-treated plate (Corning Incorporated, Corning, NY). Each gel was exposed to 1 mL of incomplete chondrogenic medium, which was replaced every other day throughout the 6 weeks of culture.

Mechanical Testing of Crosslinked Hydrogels and Native Cartilage

The gels were allowed to swell to equilibrium for 24 hours in incomplete chondrogenic medium and mechanical testing was performed at 1 day and 6 weeks. The geometric mean diameter of the gels was first determined using forceps and a stereomicroscope (20× magnification) and the height of each gel was measured directly with a RSA-III dynamic mechanical analyzer (DMA, TA instruments, New Castle, DE). The gels (n=5) were compressed until mechanical failure at a rate of 0.01 mm/s (i.e., 0.6% strain/s) until and the compressive modulus was calculated as the slope of the linear portion of the stress-strain curve (i.e., 4–10% strain).

To compare the compressive modulus to that of native porcine cartilage, cylindrical samples of native articular cartilage obtained from the load-bearing region of the femoral head of the same porcine tissue harvested to make MeSDCC, were cut to the same height as the gel samples using scalpels and were then cut to the appropriate diameter using a 4 mm biopsy punch. The compressive modulus of the native cartilage was determined from the same linear range as that of the 20% MeSDCC gels, as the 20% gels fractured at ~7–8% strain. Methacrylated hyaluronic acid (MeHA) gels were tested on the DMA as a control. MeHA was prepared by reacting hyaluronic acid (MW 1 MDa, Lifecore Biomedical, Chaska, MN) with 20 fold molar excess glycidyl methacrylate (Sigma-Aldrich) in the presence of triethylamine and tetrabutyl ammonium bromide (Sigma-Aldrich) in a 50:50 water:acetone mixture stirring at 200rpm for 12 days. MeHA was then dialyzed against deionized (DI) water for two days and was frozen and lyophilized. The degree of methacrylation was

determined to be 1.2% using ^{1}H NMR (Avance AV-III 500, Bruker) by calculating the ratio of the relative peak area of methacrylate protons to methyl protons [27]. MeHA was mixed to a 3% concentration using the same two step procedure as described prior and samples were cut using a 4 mm biopsy punch and were allowed to swell to equilibrium for 24 hours before testing on the DMA.

Swelling Degree and Volume

Gels were swollen to equilibrium for 24 hours and the swollen weight was recorded. The gels were then frozen and lyophilized. The dry weight was then recorded and the swelling degree was calculated as the ratio of total wet mass to dry mass. Gel volume was calculated at 1 day and 6 weeks from the diameter and height of the gels that were recorded during mechanical testing.

Biochemical Content Analysis

The biochemical content of the initial DVC, DCC, SDCC, MeSDCC, and GelMA materials as well as the biochemical content of the gels at 1 day, 3 weeks, and 6 weeks of culture were quantified (n=5). The materials and gels were digested in a 1.5 mL papain mixture consisting of 125 mg/mL papain from papaya latex), 5 mM N-acetyl cysteine, 5 mM EDTA, and 100 mM potassium phosphate buffered saline at 65 ºC overnight. The digestion solutions were stored at −20 ºC until further testing. Prior to biochemical analysis, all digestion solutions were allowed to thaw to room temperature and then vortexed and centrifuged at 10,000 rpm for 10 min to pellet polymer fragments and the supernatant was then used to quantify biochemical contents. Using a Cytation 5 Cell-Imaging Multi-Mode reader (Bio-Tek, Winooski, VT), the DNA content was quantified with the PicoGreen assay (Molecular Probes, Eugene, OR), the glycosaminoglycan (GAG) content was analyzed with the dimethylmethylene (DMMB) assay (Biocolor, Newtownabby, Northern Ireland), and hydroxyproline content was determined with a hydroxyproline detection kit (Sigma-Aldrich), all according to the manufacturer's instructions. GAG and hydroxyproline contents were not normalized to DNA and are rather shown in total because of the gels' inherent initial GAG and hydroxyproline contents.

Gene Expression Analysis

Using Qiagen QIAshredders and an RNeasy Kit (Valencia, CA) according to the manufacturer's guidelines, RNA was isolated and purified (n=6). The isolated RNA was converted into cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time quantitative polymerase chain reaction (qPCR) was performed using a RealPlex MasterCycler (Eppendorf, Hauppauge, NY) and TaqMan gene expression assays from Applied Biosystems, which included Sox-9 (Rn01751070_m1), aggrecan (Rn00573424_m1), collagens type I (Rn01463848_m1) and II (Rn01637087_m1), and GAPDH (Rn01775763_g1). The $2^{-\Delta\Delta Ct}$ method was used to quantify relative expression levels for each gene where the 10% GelMA gels at day 1 were designated as the calibrator group and GAPDH expression was used as the endogenous control [32]. Finally, RNA from DVC and DCC only (i.e., no rBMSCs) was isolated, converted to DNA, and then PCR was performed with the same previously mentioned TaqMan assays, where it was confirmed that all gene expression observed in the study was that of the rBMSCs.

Histological Analysis

Both cellular and acellular gels at day 1 and cellular gels from 6 weeks were analyzed histologically. These gels were first fixed in 10% formalin for 15 min, were embedded in Optimal Temperature Cutting (OCT) medium (TissueTek, Torrance, CA) overnight at 37 °C, and were then frozen at −20 °C. Sections were cut at a thickness of 10 μm using a cryostat (Micron HM-550 OMP, Vista, CA). The sections were then stained with the standard Hematoxylin and Eosin (H&E) stain, which stains the cytoplasm, connective tissues, and other extracellular substances red or pink and stains the nuclei purple. The sections were stained for GAGs with the standard Safranin-O/Fast Green (Saf-O) stain, where the GAGs stain orange in color. Lastly, the sections were stained immunohistochemically using primary antibodies that target both rat and porcine tissues for collagen I (ThermoFisher, NB600408, 1:200 dilution), collagen II (Abcam, ab34712, 1:200 dilution), and aggrecan (ThermoFisher, MA3-16888, 1:100 dilution). Prior to primary antibody incubation, the slides were first fixed in chilled acetone (−20 °C) and then treated with proteinase K (Abcam). The slides were then exposed to 0.3% hydrogen peroxide (Abcam) to suppress endogenous peroxidase activity. The sections were then blocked with serum according to the manufacturer's instructions in the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and were then incubated with primary antibody. Then the sections were exposed to biotinylated secondary antibodies (horse anti-rabbit and mouse) and ABC reagent according to manufacturer protocol. The antibodies were visualized using the ImmPact DAB peroxidase substrate (Vector), and then the sections were rinsed in DI water, counter stained with VECTOR hematoxylin QS stain, and then dehydrated and mounted. Exposure to a rabbit IgG isotype control (for collagen I and II, Abcam, ab27478) at an antibody concentration calculated to be the same used for the corresponding antibodies or omitting the primary antibody (for aggrecan) was used as the negative control.

Statistical Analysis

Statistics were performed on GraphPad Prism 6 statistical software (GraphPad Software, Inc., La Jolla, CA). A one-factor ANOVA was used for analyses with one time point and a two-factor ANOVA was used for analyses with two or more time points. Both ANOVAs were followed by either a Sidak's *post hoc* test (for two-way ANOVAs with two time points only) or a *Tukey's post hoc* test (for all other ANOVAs), where $p \leq 0.05$ was considered significant. In addition, outliers were eliminated by constructing standard box plots. All quantitative results are reported as mean ± standard deviation within the text or as mean + standard deviation within the figures. Select significant differences between groups are highlighted in the Results section, with complete statistically significant differences reported in the figures.

Results

Characterization of Initial DVC, DCC, MeSDCC, and GelMA DNA and Matrix Content

Success of the methacrylation procedure for both MeSDCC and GelMA was confirmed via ^{1}H NMR by the emergence of methacrylate peaks between 5 and 6.5 ppm (Fig. 1A–B). The success of the methacrylation procedure was further confirmed with the formation of crosslinked GelMA and MeSDCC gels (Fig. 1C). The DNA, GAG, and hydroxyproline

contents of DVC were determined to be 1151 ± 51 ng DNA/mg dry DVC, 252 ± 17 μg GAG/mg dry DVC, and 56.1 ± 3.9 μg hydroxyproline/mg dry DVC, respectively (Fig. 2). Following decellularization and cryogrinding, there was a 44% reduction in DNA, a 23% reduction in GAG, and a 23% reduction in hydroxyproline ($p<0.05$) (Fig. 2). After solubilizing and after methacrylating, the DNA content further reduced to 4% and 1.7% of that of the original DVC DNA content, respectively ($p<0.05$), although there were no significant reductions in GAG content through the solubilization and methacrylation procedure. Following solubilization, the hydroxyproline content was reduced by 25% compared to DCC, and then increased by 59% after the methacrylation procedure compared to SDCC ($p<0.05$). The DNA, GAG, and hydroxyproline contents of GelMA were 10.10 ± 0.81 ng DNA/mg dry GelMA, 8 ± 15 μg GAG/mg dry GelMA, and 71.9 ± 1.0 μg hydroxyproline/mg dry GelMA, respectively (Fig. 2).

Mechanical Testing of Crosslinked Hydrogels

One day after crosslinking, the compressive modulus of the 10% GelMA was 55 ± 10 kPa, whereas that of the 10% MeSDCC and 20% MeSDCC groups were 5.3 and 20 times larger, respectively ($p<0.05$) (Fig. 3A). Furthermore, the compressive modulus of the 20% MeSDCC group was 3.7 times larger than that of the 10% MeSDCC group ($p<0.05$). In addition, the modulus of the 20% MeSDCC acellular group was 2.3 and 3.4 times larger than that of the 10% MeSDCC and 20% GelMA acellular groups, respectively ($p<0.05$). As a comparison, the native cartilage compressive modulus was determined to be 1.8 ± 1.1 MPa.

Six weeks after crosslinking, the compressive modulus of the 20% MeSDCC group was 560 ± 310 kPa, which was 7.4 and 3.0 times larger than that of the 10% GelMA and 10% MeSDCC groups, respectively ($p<0.05$) (Fig. 3A).

Over the 6 weeks of culture, the only groups that significantly deviated from their original compressive modulus were the 20% MeSDCC groups, where the modulus of the 20% MeSDCC acellular and cellular groups reduced by 30% and 48%, respectively ($p<0.05$) (Fig. 3A). Additionally, the modulus of the 10% GelMA group increased by 37% over the 6 week culture period, although the increase was not significant.

The stress-strain profiles of native porcine cartilage samples were compared with that of the 20% MeSDCC, 20% GelMA acellular, and 3% MeHA groups, where the 95% confidence intervals were compared at each level of strain tested. The only stress-strain profile that fell within the 95% confidence interval of the native porcine cartilage was that of the 20% MeSDCC group until it began to fracture at 7.5% strain (Fig. 3B).

Swelling and Volume Analysis

The only group that had a significantly different swelling degree than 10% GelMA, which had a swelling degree of 8.6 ± 1.2 after swelling to equilibrium, was the 10% MeSDCC acellular group, which had a swelling degree 56% higher than that of 10% GelMA ($p<0.05$) (Fig. 4A). In addition, all 20% GelMA and MeSDCC groups had between 15% and 38% lower swelling degrees than that of the 10% MeSDCC cellular and acellular groups, where

the swelling degrees of 20% GelMA, 20% MeSDCC acellular and 20% MeSDCC groups were 34%, 19%, and 15% lower than 10% MeSDCC ($p<0.05$) (Fig. 4A).

At one day after crosslinking and swelling to equilibrium, the gel volumes of the 20% GelMA and all MeSDCC gels were significantly higher than that of 10% GelMA ($p<0.05$) (Fig. 4B). The 10% MeSDCC group had a volume of 20.24 ± 0.47 μL, while the 20% MeSDCC group had a volume 9.6% greater. These 10% and 20% MeSDCC groups in turn had volumes that were 17% and 29% higher than that of 10% GelMA, respectively ($p<0.05$).

At 6 weeks after crosslinking, the volume of the 20% MeSDCC group was 21.8 ± 1.2 μL, which was 14% and 9.3% higher than that of 10% MeSDCC and 10% GelMA, respectively ($p<0.05$) (Fig. 4B). In addition, the volume of the 20% MeSDCC group was 92% of its acellular control ($p<0.05$).

Over the course of the 6 weeks, the only group that had a significant change in volume was the 10% MeSDCC acellular group, which experienced an 11% reduction in volume ($p<0.05$).

Biochemical Content Analysis

As expected, all cellular groups had significantly higher DNA contents than their respective acellular groups at all time points ($p<0.05$) (Fig. 6A). At 1 day after hydrogel formation, the 10% GelMA group contained 650 ± 160 ng DNA per gel, and the only gel with a significantly different DNA content was the 20% MeSDCC group, which had 21% more DNA per gel ($p<0.05$) (Fig. 5A). At 3 weeks after crosslinking, the 20% MeSDCC group had a DNA content of 833 ± 88 ng DNA per gel, which was 3.2 and 1.7 times higher than that of the 10% GelMA and 10% MeSDCC groups, respectively ($p<0.05$) (Fig. 5A). After 6 weeks of culture, the 20% MeSDCC group contained 660 ± 80 ng DNA per gel, which was 2.1 and 1.3 times higher than that of the 10% GelMA and 10% MeSDCC groups, respectively ($p<0.05$) (Fig. 5A). Over the course of the 6 week culture period, all cellular groups had a significant reduction in DNA content ($p<0.05$), where the DNA content in the 10% GelMA, 10% MeSDCC, and 20% MeSDCC groups reduced by 51%, 30%, and 16%, respectively ($p<0.05$). The acellular groups did not have any significant reduction in DNA content over the culture period (Fig. 5A).

Throughout the culture period, there was no detectable level of GAG in the 10% GelMA group (Fig. 5B). At 1 day after crosslinking, the GAG content of the 10% MeSDCC group was 74 ± 23 μg GAG per gel, and the GAG content of the 20% MeSDCC group was 92% higher ($p<0.05$) (Fig. 5B). At 3 weeks, the GAG content of the 10% MeSDCC group was 22.3 ± 7.6 μg GAG per gel, which was not significantly different from the 20% MeSDCC group (Fig. 5B). In addition, the GAG content of the 20% MeSDCC group was 55% less than its respective acellular control ($p<0.05$). At 6 weeks, the GAG content of the 10% MeSDCC group was 23.7 ± 9.2 μg GAG per gel, and the GAG content of the 20% MeSDCC group was 4.1 times larger ($p<0.05$). In addition, the GAG content of the 10% MeSDCC group was 68% less than that of its respective acellular control ($p<0.05$). Over the 6 week culture period, both the 10% MeSDCC group and the 20% MeSDCC groups experienced 68% and 32% reductions in GAG content, respectively ($p<0.05$) (Fig. 5B).

Finally, at 1 day, the initial hydroxyproline content of 10% GelMA was 108 ± 11 μg hydroxyproline per gel, where that of the 20% MeSDCC group was 66% higher ($p<0.05$) (Fig. 5C). At 3 weeks, the 10% GelMA group contained 111 ± 19 μg hydroxyproline per gel, which was 53% higher than that of the 10% MeSDCC group and 22% lower than that of the 20% MeSDCC group ($p<0.05$). Furthermore, the 20% MeSDCC group contained 95% more hydroxyproline than that of the 10% MeSDCC group ($p<0.05$). At 6 weeks, the hydroxyproline content of the 10% GelMA group was 118 ± 17 μg per gel, which was 44% higher than that of the 10% MeSDCC group ($p<0.05$). Furthermore, the hydroxyproline content of the 20% MeSDCC group was 80% higher than that of the 10% MeSDCC group ($p<0.05$). Over the 6 week culture period, the only group that experienced a significant loss in hydroxyproline was the 20% MeSDCC group, where the hydroxyproline loss was 18% ($p<0.05$) (Fig. 5C).

Gene Expression Analysis

At 1 day after crosslinking, the relative Sox-9 expression of 10% MeSDCC and 20% MeSDCC were 8.5 and 3.4 times larger than that of 10% GelMA ($p<0.05$) (Fig. 6A). The relative Sox-9 expression of the 10% MeSDCC group was 2.5 times larger than that of the 20% MeSDCC group ($p<0.05$). At 1 week, the relative Sox-9 expression of the 10% GelMA group was 2.6 times larger than that of the 20% MeSDCC group ($p<0.05$). For the rest of the study, there were no significant differences in Sox-9 expression among groups within each time point. From 1 day to 1 week, the relative Sox-9 expression of the 10% GelMA group increased by a factor of 2.5 ($p<0.05$), but then decreased by 54% from 1 week to 2 weeks ($p<0.05$), and did not change significantly thereafter. From 1 day to 1 week, the relative Sox-9 expression of the 10% MeSDCC group decreased by 69% ($p<0.05$), and then further decreased by 80% from 1 week to 2 weeks ($p<0.05$). The relative Sox-9 expression of the 10% MeSDCC group did not change significantly after 2 weeks. Last, the relative Sox-9 expression of the 20% MeSDCC group decreased by 72% from 1 day to 1 week ($p<0.05$) and did not change significantly thereafter (Fig. 6A).

The relative aggrecan expression of 10% MeSDCC and 20% MeSDCC were 6.5 and 2.8 times higher than that of 10% GelMA, respectively at 1 day ($p<0.05$) (Fig. 6B). There were no significant differences among groups at each time point thereafter. By 1 week, the relative aggrecan expressions of 10% GelMA, 10% MeSDCC, and 20% MeSDCC were reduced by 85%, 96%, and 89%, respectively, compared to their expression levels at 1 day ($p<0.05$), and there were no significantly different changes in expression thereafter.

The 10% MeSDCC and 20% MeSDCC groups had 8.1 and 2.9 fold higher relative collagen II expressions at 1 day ($p<0.05$), and by 1 week, the relative aggrecan expression of the 10% MeSDCC group was 2.7 times higher than that of 10% GelMA ($p<0.05$). Furthermore, at one week, the relative collagen II expression of the 10% MeSDCC group was 2.7 times higher than that of the 20% MeSDCC group. There were no significant differences among groups at each time point thereafter and collagen II expression was not detected at all at 6 weeks (Fig. 6C). Over the culture period, there were no significant changes in collagen II expression for the 10% GelMA group, but the relative collagen II expression of the 10% MeSDCC group decreased by 73% from 1 day to 1 week ($p<0.05$), and then it did not

change significantly thereafter. The relative collagen II expression of the 20% MeSDCC group decreased by 81% from 1 day to 1 week ($p<0.05$), was not detectable at 2 weeks, but was detectable at 3 weeks, although the expression level at 3 weeks was not significantly different from the expression level detected at 1 week (Fig. 6C).

The relative collagen I expressions of the 10% MeSDCC and 20% MeSDCC groups were 23% and 67% lower than that of the 10% GelMA group at day 1, respectively ($p<0.05$) (Fig. 6D). There were no significant differences among groups observed thereafter within each time point in the culture period. From 1 day to 1 week, the relative collagen expression levels of the 10% GelMA, 10% MeSDCC, and 20% MeSDCC groups decreased by 94%, 93%, and 72%, respectively ($p<0.05$), and did not change significantly thereafter (Fig. 6D).

Histological Analysis

H&E staining revealed regions of tissue growth within the 10% MeSDCC and 20% MeSDCC groups at 6 weeks. Saf-O did not stain for GAGs in any of the 10% GelMA stains; however, an increase in Saf-O staining intensity was notably observed over the 6 week culture period in the 10% and 20% MeSDCC groups, particularly in the regions surrounding rBMSCs (Fig. 7). All MeSDCC groups stained for collagen II, although no increase in collagen II staining intensity was observed for those groups. However, the 10% GelMA group had an increase in collagen II staining intensity over the culture period (Fig. 7). Collagen I staining was noted in the 10% GelMA group, although there were no apparent changes in staining over the culture period and minimal collagen I staining was observed in the other groups (Fig. 7). Finally, the 10% GelMA and the 20% MeSDCC groups had an apparent slight increase in aggrecan staining over the culture period (Fig. 7).

Discussion

In the current study, we were the first to create hydrogels derived entirely from solubilized cartilage ECM and test their chondroinductivity. ECM-based materials are attractive for tissue engineering strategies because they can potentially aid in stem cell recruitment, cell infiltration, and cell differentiation without supplementing with additional biological factors [12, 33, 34]. However, one of the major limitations of using natural polymers in hydrogels is their reduced mechanical integrity [35]. While native human articular cartilage has an elastic compressive modulus ranging from 240–1000 kPa [31, 36, 37], the compressive modulus of hydrogels composed of natural materials are typically an order of magnitude less than native cartilage tissue [35]. However, it must be noted that biomechanical properties of cartilage can vary depending on parameters such as the method of testing, the strain rate of testing, and cartilage zone depth [38]. Although certainly other mechanical properties have been explored and analyzed in cartilage tissue engineering, including the aggregate modulus, hydraulic permeability, and fracture stress [35, 39], in the current study the compressive modulus and the overall stress-strain profile of the gel constructs were the primary emphases. Gels composed entirely of crosslinked solubilized cartilage matrix were created that had a compressive modulus in the same range of values reported for native cartilage. The 20% MeSDCC gels had a compressive modulus of 1070 ± 150 kPa after one day of culture, which was more than 3 fold higher than that of the 20% GelMA acellular group.

Furthermore, when the 95% confidence intervals of the stress strain profiles of the 20% MeSDCC gels were compared to native porcine cartilage, it was found that the stress strain profile of the 20% MeSDCC gels actually fell within the confidence interval of native cartilage, and they were the only gels to do so. Although the 20% gels fractured early at 7.5% strain, the fact that they fell within the stress strain profile of native cartilage tissue was promising. Certainly the early fracture stress needs to be addressed, however [39]. Furthermore, the early fracture stress precluded time-dependent property analysis, which will be important to consider for future work with these materials to better understand their viscoelastic behavior. Modifications to the hydrogel may be made such as increasing the solid content or methacrylation efficiency, to improve the fracture stress. At this stage, due to not knowing the exact biochemical content of MeSDCC, the degree of methacrylation could not be calculated through the NMR spectra, so this is one limitation of using MeSDCC as a hydrogel material. However, the ability to modulate the mechanical properties through the solid content is a tremendous advantage compared to crosslinking cartilage particles, where the solid content would be confined due to particles only crosslinking in the vicinity of other particles.

The mechanical properties of MeSDCC hydrogels may be able to be improved through mechanical stimuli *in vivo*, as mechanical stimulation alone is known to induce chondrogenesis [40]. Therefore, once the material is implanted *in vivo,* there could be less of a decrease in the compressive modulus long-term like what was observed after 6 weeks of *in vitro* culture in the current study. However, an apparent increase in matrix synthesis was observed in the MeSDCC gels over the GelMA gels even *in vitro* via hematoxylin and eosin staining and Saf-O staining, even though the biochemical content analysis did not show an increase in the amount of matrix produced. This lack of increase in the biochemical content could be due to the cells assisting in the biodegradation and remodeling of the ECM, whereby even though some matrix is being lost, new matrix is simultaneously being formed. Additionally, it is also possible that new matrix may have been impossible to detect due to the large amount of matrix initially present in the hydrogel. Low overall matrix production is consistent with findings from Visser *et al.* [28]; however, they observed an increase in collagen I expression when exposed to MeSDCC as opposed to the current study, where a decrease in collagen I expression was noted while exposed to MeSDCC in reference to GelMA gels.

Compared to GelMA gels, an apparent increase in collagen II, Sox-9, and aggrecan expression was observed in the MeSDCC gels. Although a significant reduction in all chondrogenic gene expression was noted after 1 day, this reduction does not necessarily mean aggrecan and collagen II synthesis stopped. GelMA is widely used in the field of tissue engineering for its low cost, its abundant cell adhesion sites, and for its ability to support chondrocyte differentiation [28, 41]. Therefore, it is possible that all gels had sufficient cartilage ECM production and the low chondrogenic gene expression levels were low only in reference to GelMA at day 1, the calibrator group. Through collagen II IHC analysis for example, the GelMA gels were noted to have an apparent increase in collagen II staining at 6 weeks compared to day 1, even though the collagen II expression after 1 day was significantly reduced. Because the MeSDCC gels contained so much collagen II initially, it was difficult to discern any new collagen II production, but at least the relative

level of collagen II staining remained the same throughout culture. It must be noted that an apparent increase in matrix synthesis and an increase in chondrogenic gene expression at day 1 was observed without any growth factor supplementation. ECM-based materials, like these MeSDCC hydrogels, are attractive for regenerative medicine because of their ability to potentially aid in stem cell recruitment, infiltration, and differentiation without supplementing with additional biological factors [11, 12, 33, 34]. The ability to cause some differentiation shows great promise in using these materials for cartilage tissue engineering and may even make these gels more economical than using other natural materials such as hyaluronic acid or gelatin [6]. However, future work is certainly required to further understand the relationship between the quantitative data (e.g., biochemical content and gene expression) and the qualitative data (e.g., IHC) to determine to what extent matrix remodeling is occurring.

Of note was the limited removal of DNA in a mild decellularization process, which may need to be addressed in future work if it is deemed that a higher degree of decellularization is required for successful cartilage regeneration *in vivo*. However, non-decellularized products, such as Zimmer's DeNovo® product, rely on the immunoprivileged environment and so far there have been no reports to the best of our knowledge of allograft rejection or disease transmission even though the product is composed of living allogeneic cells. Additionally, although the DeNovo® product is composed of human juvenile cartilage, it has been observed to create hyaline-like cartilage in goats, where no T-cell-mediated response was noted [42]. Furthermore, even though the decellularization process in the current study only removed 44% of the DNA, the DNA content of the SDCC and MeSDCC was reduced to 4% and 1.7% of that of the original DVC DNA content, respectively ($p<0.05$). At this stage, it is unknown whether the solubilization and methacrylation process were removing DNA, or if the DNA was modified to a degree where the PicoGreen assay could no longer detect the DNA. Due to the low pH and pepsin exposure during the solubilization process, the DNA would likely be denatured to a single-stranded state and would in addition, be hydrolyzed and further degraded [43]. Furthermore, the dialysis step after methacrylation would likely remove these degraded DNA segments and low molecular weight nucleotides and amino acids, leaving behind higher molecular weight methacrylated GAGs and collagen. Future work will certainly need to address how solubilization and methacrylation affect cartilage DNA and other biochemical contents and how they affect the retained growth factors. However, depending on the application of MeSDCC gels, decellularization may not even be necessary. Furthermore, knowing more about the biochemical contents and which specific GAGs are present within the MeSDCC may allow for better approximations for the amount of reactants to add during the methacrylation process to achieve better mechanical properties after crosslinking.

In addition to mechanics and gene expression, the swelling and volume of the materials were analyzed throughout culture. One major concern of using hydrogels for tissue engineering is cell-mediated contraction of the gel construct throughout culture [4, 28]. Contraction of gels can cause disintegration with host tissue, which could potentially hinder successful cartilage regeneration and may even dislodge the hydrogel from the defect site. In the current study, however, the only gels that had a significant reduction in volume were the 20% MeSDCC gels, where the gels only reduced in volume by 2%, which is unlikely a concern for cartilage

tissue engineering applications. In addition to volume, the swelling of the materials is important as well due to a drastic increase in swelling after surgical placement is undesirable. The swelling degree was significantly lowered by increasing the amount of material in the hydrogels from 10% to 20%, which is to be expected since additional material would increase the solid content.

Conclusion

We created crosslinkable hydrogels derived entirely from native cartilage ECM. The cartilage was first solubilized and then methacrylated to create photocrosslinkable gels. Compared to the traditional GelMA hydrogels, these MeSDCC gels supported rBMSC growth, ECM production, caused significant upregulation of chondrogenic genes at 1 day after crosslinking, and remarkably, the mechanics of the MeSDCC gels were characteristically similar to that of native porcine cartilage until their failure. The concentration of MeSDCC was found to affect chondroinduction and mechanical properties, where the 20% MeSDCC gels were superior in mechanical performance and promoting ECM synthesis, while the 10% MeSDCC gels were superior in chondroinduction. Clinically, these results could potentially translate to a surgeon being able to inject the MeSDCC paste into a cartilage defect, where the procedure could be performed in conjunction with microfracture as a means of a source for stem cells. The materials could then be crosslinked into a gel with sufficient strength that would allow the patient to walk after the procedure, where the MeSDCC materials as well as the biomechanical stimulation received by the cells through walking would result in chondrogenesis. Therefore, future work will address improving the fracture mechanics, and chondrogenesis and immune compatibility *in vivo*. Additionally, the ability of these materials to support zonal organization through *in vivo* biomechanical stimulation as well as their ability to promote lubrication of the superficial zone will be important to consider in future work. Overall in the current study, we have shown that MeSDCC may prove to be a promising biomaterial for cartilage tissue engineering applications.

Acknowledgments

We gratefully acknowledge funding support from the NIH (R01 DE022472 to C.J.B, S10 RR024664), the NSF for a Graduate Research Fellowship (E.B.) and a Major Research Instrumentation Grant (0320648), and the Kansas Bioscience Authority Rising Star Award (M.D.). In addition, we are thankful for the help of Emi Kiyotake and Francisca Acosta with IHC and for the members of the KU Biomaterials and Tissue Engineering Lab who helped with porcine cartilage harvesting.

References

1. Murphy L, Helmick CG. The impact of osteoarthritis in the United States: a population-health perspective. AJN The American Journal of Nursing. 2012; 112:S13–S9. [PubMed: 22373741]
2. Athanasiou, KAD.; Eric, M.; DuRaine, Grayson D.; Hu, Jerry C.; Hari Reddi, A. Articular Cartilage. Boca Raton, FL: CRC Press; 2013.
3. Hunziker E. Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. Osteoarthritis and cartilage. 2002; 10:432–63. [PubMed: 12056848]
4. Cheng N-C, Estes B, Young T-H, Guilak F. Genipin-crosslinked cartilage-derived matrix as a scaffold for human adipose-derived stem cell chondrogenesis. Tissue Eng Part A. 2013; 19:484–96. [PubMed: 23088537]

5. Kheir E, Stapleton T, Shaw D, Jin Z, Fisher J, Ingham E. Development and characterization of an acellular porcine cartilage bone matrix for use in tissue engineering. Journal of biomedical materials research Part A. 2011; 99:283–94. [PubMed: 21858917]
6. Sridharan B, Sharma B, Detamore MS. A Roadmap to Commercialization of Cartilage Therapy in the United States of America. Tissue engineering. 2015
7. Brigham M, Bick A, Lo E, Bendali A, Burdick J, Khademhosseini A. Mechanically robust and bioadhesive collagen and photocrosslinkable hyaluronic acid semi-interpenetrating networks. Tissue Eng Part A. 2009; 15:1645–53. [PubMed: 19105604]
8. DeKosky B, Dormer N, Ingavle G, Roatch C, Lomakin J, Detamore M, Gehrke S. Hierarchically designed agarose and poly(ethylene glycol) interpenetrating network hydrogels for cartilage tissue engineering. Tissue engineering Part C, Methods. 2010; 16:1533–42. [PubMed: 20626274]
9. Elisseeff J, Puleo C, Yang F, Sharma B. Advances in skeletal tissue engineering with hydrogels. Orthodontics & craniofacial research. 2005; 8:150–61. [PubMed: 16022717]
10. Weiss, P.; Fatimi, A.; Guicheux, J.; Vinatier, C. Biomedical Applications of Hydrogels Handbook. Springer; 2010. Hydrogels for Cartilage Tissue Engineering; p. 247-68.
11. Sutherland AJ, Converse GL, Hopkins RA, Detamore MS. The bioactivity of cartilage extracellular matrix in articular cartilage regeneration. Advanced healthcare materials. 2015; 4:29–39. [PubMed: 25044502]
12. Benders K, van Weeren P, Badylak S, Saris D, Dhert W, Malda J. Extracellular matrix scaffolds for cartilage and bone regeneration. Trends Biotechnol. 2013; 31:169–76. [PubMed: 23298610]
13. Cha M, Do S, Park G, Du P, Han K-C, Han D, Park K. Induction of re-differentiation of passaged rat chondrocytes using a naturally obtained extracellular matrix microenvironment. Tissue Eng Part A. 2013; 19:978–88. [PubMed: 23157423]
14. Decaris M, Binder B, Soicher M, Bhat A, Leach J. Cell-derived matrix coatings for polymeric scaffolds. Tissue Eng Part A. 2012; 18:2148–57. [PubMed: 22651377]
15. Schwarz S, Koerber L, Elsaesser AF, Goldberg-Bockhorn E, Seitz AM, Durselen L, Ignatius A, Walther P, Breiter R, Rotter N. Decellularized cartilage matrix as a novel biomatrix for cartilage tissue-engineering applications. Tissue Eng Part A. 2012; 18:2195–209. [PubMed: 22690787]
16. Yang Z, Shi Y, Wei X, He J, Yang S, Dickson G, Tang J, Xiang J, Song C, Li G. Fabrication and repair of cartilage defects with a novel acellular cartilage matrix scaffold. Tissue engineering Part C, Methods. 2010; 16:865–76. [PubMed: 19891538]
17. Cheng N-C, Estes BT, Young T-H, Guilak F. Engineered cartilage using primary chondrocytes cultured in a porous cartilage-derived matrix. Regenerative medicine. 2011; 6:81–93. [PubMed: 21175289]
18. Garrigues NW, Little D, Sanchez-Adams J, Ruch DS, Guilak F. Electrospun cartilage-derived matrix scaffolds for cartilage tissue engineering. Journal of Biomedical Materials Research Part A. 2014
19. Schwarz S, Elsaesser AF, Koerber L, Goldberg-Bockhorn E, Seitz AM, Bermueller C, Dürselen L, Ignatius A, Breiter R, Rotter N. Processed xenogenic cartilage as innovative biomatrix for cartilage tissue engineering: effects on chondrocyte differentiation and function. Journal of tissue engineering and regenerative medicine. 2012
20. Levorson E, Hu O, Mountziaris P, Kasper F, Mikos A. Cell-derived polymer/extracellular matrix composite scaffolds for cartilage regeneration, part 2: construct devitalization and determination of chondroinductive capacity. Tissue engineering Part C, Methods. 2014; 20:358–72. [PubMed: 24117143]
21. Seif-Naraghi SB, Salvatore MA, Schup-Magoffin PJ, Hu DP, Christman KL. Design and characterization of an injectable pericardial matrix gel: a potentially autologous scaffold for cardiac tissue engineering. Tissue Engineering Part A. 2010; 16:2017–27. [PubMed: 20100033]
22. Gershlak JR, Resnikoff JI, Sullivan KE, Williams C, Wang RM, Black LD. Mesenchymal stem cells ability to generate traction stress in response to substrate stiffness is modulated by the changing extracellular matrix composition of the heart during development. Biochemical and biophysical research communications. 2013; 439:161–6. [PubMed: 23994333]

23. Freytes DO, Martin J, Velankar SS, Lee AS, Badylak SF. Preparation and rheological characterization of a gel form of the porcine urinary bladder matrix. Biomaterials. 2008; 29:1630–7. [PubMed: 18201760]
24. Kwon JS, Yoon SM, Shim SW, Park JH, Min KJ, Oh HJ, Kim JH, Kim YJ, Yoon JJ, Choi BH. Injectable extracellular matrix hydrogel developed using porcine articular cartilage. International journal of pharmaceutics. 2013; 454:183–91. [PubMed: 23834831]
25. Rowland C, Lennon D, Caplan A, Guilak F. The effects of crosslinking of scaffolds engineered from cartilage ECM on the chondrogenic differentiation of MSCs. Biomaterials. 2013; 34:5802–12. [PubMed: 23642532]
26. Beck EC, Lohman BL, Tabakh DB, Kieweg SL, Gehrke SH, Berkland CJ, Detamore MS. Enabling Surgical Placement of Hydrogels Through Achieving Paste-Like Rheological Behavior in Hydrogel Precursor Solutions. Annals of biomedical engineering. 2015:1–8. [PubMed: 25527321]
27. Khanlari A, Detamore MS, Gehrke SH. Increasing Cross-Linking Efficiency of Methacrylated Chondroitin Sulfate Hydrogels by Copolymerization with Oligo (Ethylene Glycol) Diacrylates. Macromolecules. 2013; 46:9609–17.
28. Visser J, Levett PA, te Moller NC, Besems J, Boere KW, van Rijen MH, de Grauw JC, Dhert WJ, van Weeren PR, Malda J. Crosslinkable Hydrogels Derived from Cartilage, Meniscus, and Tendon Tissue. Tissue Engineering Part A. 2015; 21:1195–206. [PubMed: 25557049]
29. Converse G, Armstrong M, Quinn R, Buse E, Cromwell M, Moriarty S, Lofland G, Hilbert S, Hopkins R. Effects of cryopreservation, decellularization and novel extracellular matrix conditioning on the quasi-static and time-dependent properties of the pulmonary valve leaflet. Acta Biomaterialia. 2012; 8:2722–9. [PubMed: 22484150]
30. Seif-Naraghi SB, Horn D, Schup-Magoffin PJ, Christman KL. Injectable extracellular matrix derived hydrogel provides a platform for enhanced retention and delivery of a heparin-binding growth factor. Acta biomaterialia. 2012; 8:3695–703. [PubMed: 22750737]
31. Mansour JM. Biomechanics of cartilage. Kinesiology: the mechanics and pathomechanics of human movement. 2003:66–79.
32. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. methods. 2001; 25:402–8. [PubMed: 11846609]
33. Burdick JA, Mauck RL, Gorman JH 3rd, Gorman RC. Acellular biomaterials: an evolving alternative to cell-based therapies. Science translational medicine. 2013; 5:176ps4.
34. Renth AN, Detamore MS. Leveraging “raw materials” as building blocks and bioactive signals in regenerative medicine. Tissue engineering Part B, Reviews. 2012; 18:341–62. [PubMed: 22462759]
35. Tatman PD, Gerull W, Sweeney-Easter S, Davis JI, Kim D-H, Gee A. Multi-scale Biofabrication of Articular Cartilage: Bioinspired and Biomimetic Approaches. Tissue engineering. 2015
36. Armstrong C, Mow V. Variations in the intrinsic mechanical properties of human articular cartilage with age, degeneration, and water content. The Journal of Bone & Joint Surgery. 1982; 64:88–94. [PubMed: 7054208]
37. Little CJ, Bawolin NK, Chen X. Mechanical properties of natural cartilage and tissue-engineered constructs. Tissue Engineering Part B: Reviews. 2011; 17:213–27. [PubMed: 21406046]
38. Huang C-Y, Stankiewicz A, Ateshian GA, Mow VC. Anisotropy, inhomogeneity, and tension–compression nonlinearity of human glenohumeral cartilage in finite deformation. Journal of biomechanics. 2005; 38:799–809. [PubMed: 15713301]
39. Xiao Y, Friis EA, Gehrke SH, Detamore MS. Mechanical testing of hydrogels in cartilage tissue engineering: beyond the compressive modulus. Tissue engineering Part B, Reviews. 2013; 19:403–12. [PubMed: 23448091]
40. Villanueva I, Weigel CA, Bryant SJ. Cell–matrix interactions and dynamic mechanical loading influence chondrocyte gene expression and bioactivity in PEG-RGD hydrogels. Acta biomaterialia. 2009; 5:2832–46. [PubMed: 19508905]
41. Schuurman W, Levett PA, Pot MW, van Weeren PR, Dhert WJ, Hutmacher DW, Melchels FP, Klein TJ, Malda J. Gelatin-Methacrylamide Hydrogels as Potential Biomaterials for Fabrication of Tissue-Engineered Cartilage Constructs. Macromolecular bioscience. 2013; 13:551–61. [PubMed: 23420700]

42. Adkisson HD, Martin JA, Amendola RL, Milliman C, Mauch KA, Katwal AB, Seyedin M, Amendola A, Streeter PR, Buckwalter JA. The potential of human allogeneic juvenile chondrocytes for restoration of articular cartilage. The American journal of sports medicine. 2010; 38:1324–33. [PubMed: 20423988]

43. McLennan, A.; Bates, A.; Turner, P.; White, M. BIOS Instant Notes in Molecular Biology. Taylor & Francis; 2012.

Statement of Significance

Extracellular matrix (ECM)-based materials are appealing for tissue engineering strategies because they may promote stem cell recruitment, cell infiltration, and cell differentiation without the need to supplement with additional biological factors. One such ECM-based material, cartilage ECM, has recently shown potential to be chondroinductive; however, hydrogels composed of natural materials are often mechanically inferior to synthetic materials, which is a major limitation for load-bearing tissue applications. Therefore, this work is significant because we were the first to create hydrogels derived entirely from cartilage ECM that had mechanical properties similar to that of native cartilage until hydrogel failure. Furthermore, these hydrogels had a compressive modulus of 1070 ± 150 kPa, they were chondroinductive, and they supported extensive matrix synthesis. In the current study, we have shown that these new hydrogels may prove to be a promising biomaterial for cartilage tissue engineering applications.

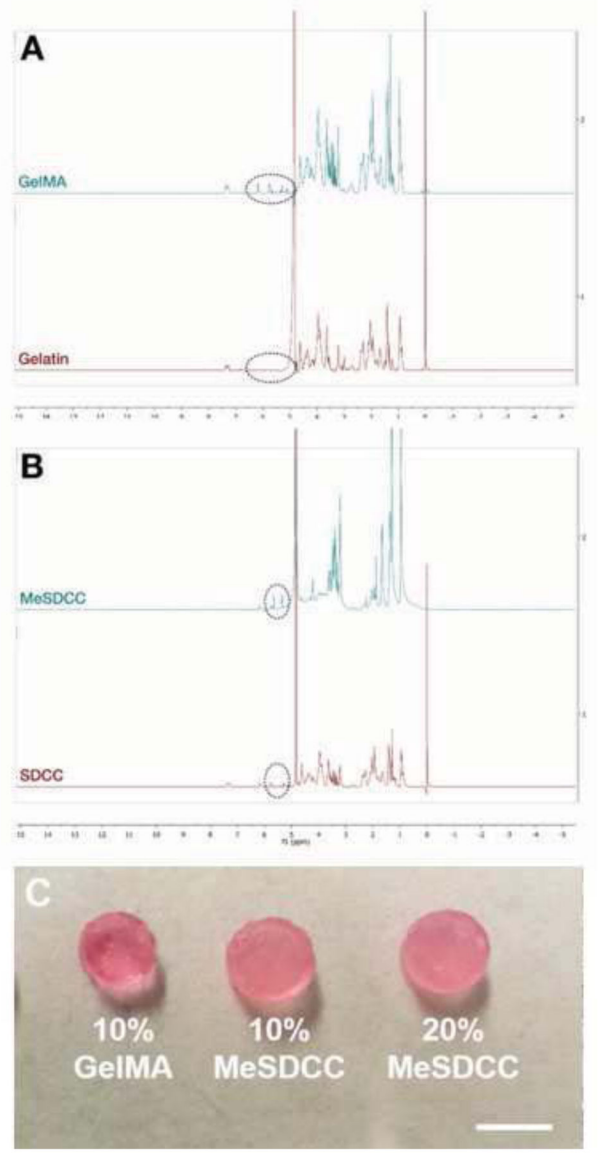


Figure 1.

NMR of GelMA (A) and MeSDCC (B) Before and After Methacrylation and (C) Gross Morphology of Crosslinked Hydrogels. Methacrylation was confirmed on both materials by the emergence of methacrylate peaks between 5 and 6.5 ppm. The GelMA and MeSDCC were successfully crosslinked into hydrogels. The photograph is of the GelMA and MeSDCC gels 6 weeks after crosslinking and they are pink from soaking in cell media. The scale bar is 5 mm.

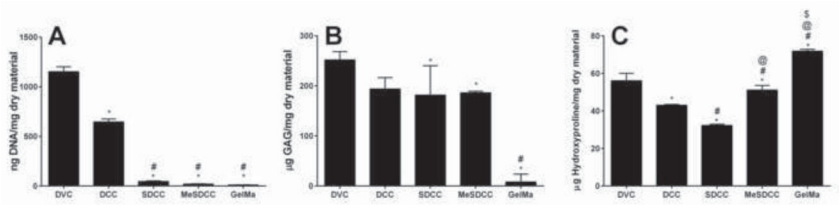


Figure 2.

Biochemical Contents of DVC, DCC, SDCC, MeSDCC, and GelMA. A) PicoGreen content, B) GAG content, and C) Hydroxyproline content of each material. Decellularization removed 44% of the DNA, 23% of the GAGs, and 23% of the hydroxyproline ($p<0.05$). After solubilizing and after methacrylating, the DNA content further reduced to 4% and 1.7% of that of the original DVC DNA content, respectively ($p<0.05$). Data reported as mean + standard deviation (n=5); *significantly different from DVC ($p<0.05$), #significantly different from DCC ($p<0.05$), @significantly different from SDCC ($p<0.05$), $significantly different from MeSDCC ($p<0.05$).

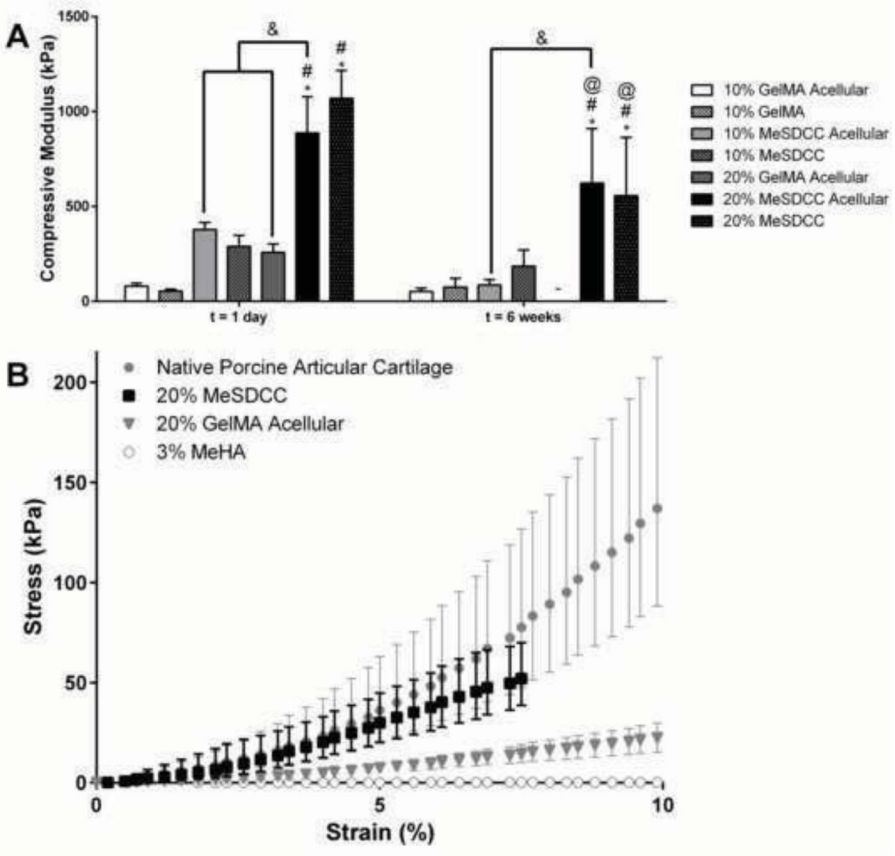


Figure 3.

Mechanical Testing of Crosslinked Hydrogels. A) Compressive modulus of gels after 1 day and 6 weeks of culture. At day 1, the compressive modulus of the 10% MeSDCC and 20% MeSDCC cellular groups were 5.3 and 20 times larger than the 10% GelMA gels, respectively. Data reported as mean + standard deviation (n=5); * significantly different from 10% GelMA at same time point ($p<0.05$), #significantly different from 10% MeSDCC at same time point ($p<0.05$), &$p<0.05$ for specified comparison, @significantly different from same group at first time point ($p<0.05$), -not tested. B) Stress-Strain Curves of Native Porcine Cartilage Compared to Select Hydrogels. Data are reported as mean ± 95% confidence interval. The stress strain profile of native porcine cartilage were compared to that of 20% MeSDCC, 20% GelMA acellular, and 3% MeHA gels, where 20% MeSDCC was the only hydrogel that fell within the 95% confidence interval of native porcine cartilage until they began to fracture at 7.5% strain on average.

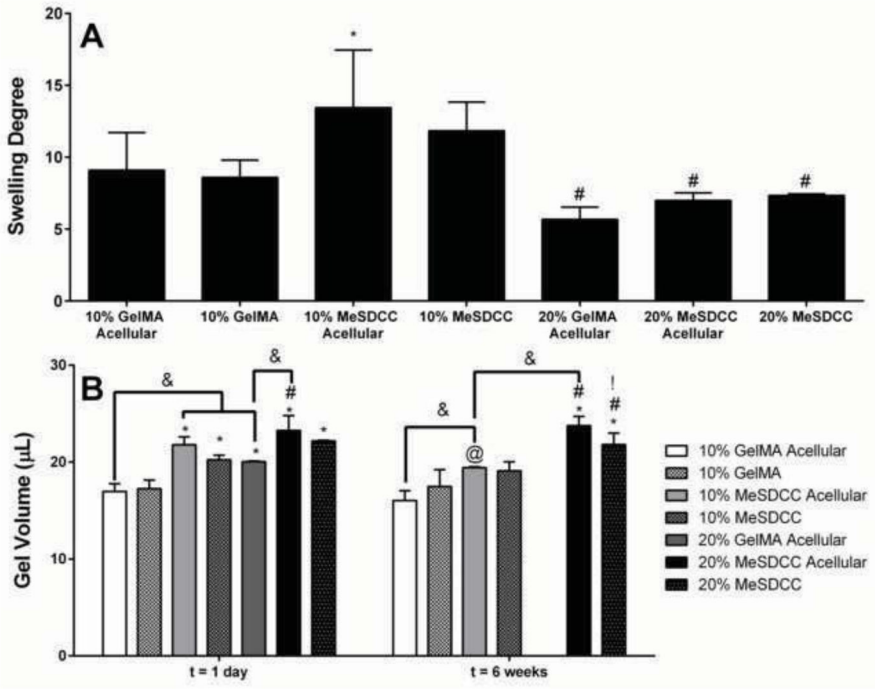


Figure 4.

Swelling Degree (A) and Volume (B) of Crosslinked Hydrogels. A) The 10% MeSDCC gel had a significantly higher swelling degree compared to 10% GelMA, while the 20% GelMA and 20% MeSDCC groups had significantly lower swelling degrees compared to 10% MeSDCC. B) The only group that had a significant change in volume was the 10% MeSDCC acellular group, which experienced an 11% volume reduction ($p<0.05$). Data reported as mean + standard deviation (n=5); *significantly different from 10% GelMA at same time point ($p<0.05$), #significantly different from 10% MeSDCC at same time point ($p<0.05$), !significantly different from acellular group at same time point ($p<0.05$), &$p<0.05$ for specified comparison, @significantly different from same group at first time point ($p<0.05$), -not tested.

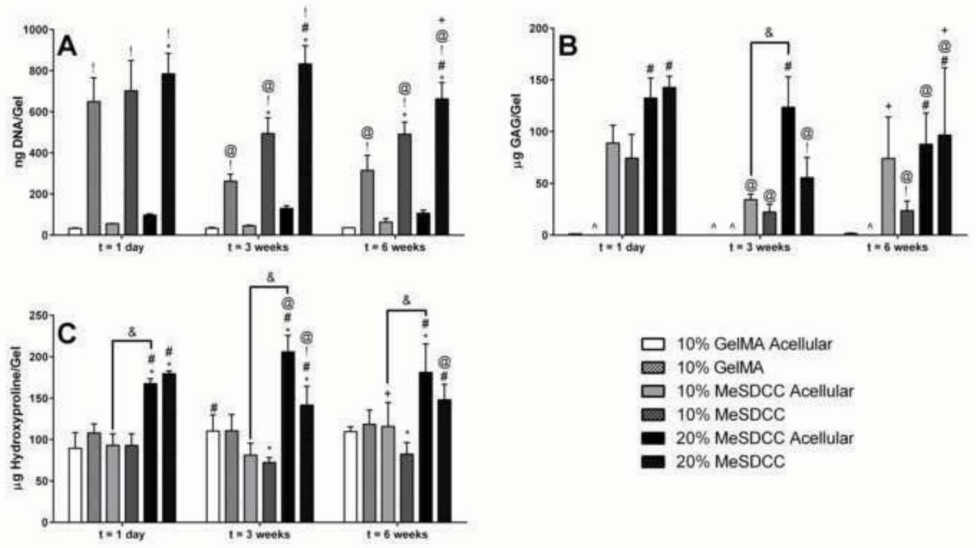


Figure 5.

Biochemical Content of Gels over the 6 Week Culture Period. A) DNA content, B) GAG content, and C) Hydroxyproline content. All cellular groups had significantly higher DNA contents than their respective acellular groups at all time points ($p<0.05$). Over the course of the 6 week culture period, all cellular groups had a significant reduction in DNA content, both the 10% MeSDCC group and the 20% MeSDCC groups experienced a significant reduction in GAG content, and the only group that experienced a significant loss in hydroxyproline was the 20% MeSDCC group. Data reported as mean + standard deviation (n=5); ^below detectable limit, *significantly different from 10% GelMA at same time point ($p<0.05$), #significantly different from 10% MeSDCC at same time point ($p<0.05$), ! significantly different from acellular group at same time point ($p<0.05$), &$p<0.05$ for specified comparison, @significantly different from same group at first time point ($p<0.05$), +significantly different from same group at previous time point ($p<0.05$).

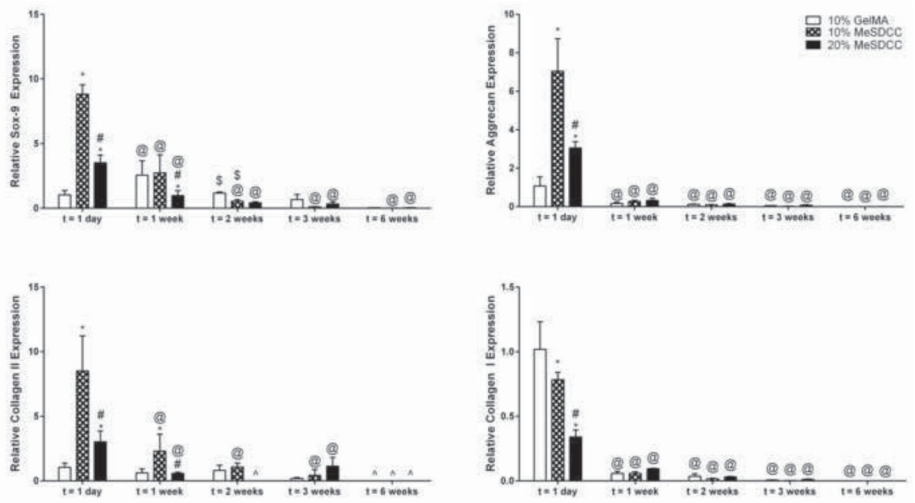


Figure 6.

Relative Gene Expression of A) Sox-9, B) Aggrecan, C) Collagen II, and D) Collagen I. MeSDCC gels significantly upregulated chondrogenic genes compared to GelMA as early as day 1. Data reported as mean + standard deviation (n=5); *significantly different from 10% GelMA at same time point ($p<0.05$), #significantly different from 10% MeSDCC at same time point ($p<0.05$), @significantly different from same group at first time point ($p<0.05$), $significantly different from same group at previous time point ($p<0.05$), ^below detectable limit.

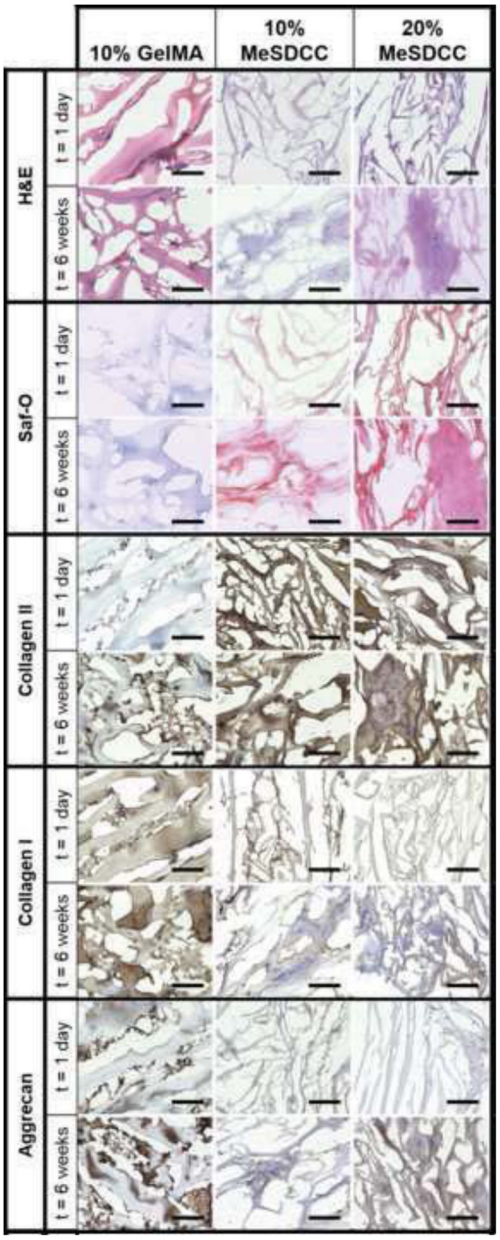


Figure 7.

Histological Evaluation of Gels. H&E stained the nuclei dark purple and MeSDCC light purple. GelMA and new tissue formation in the 20% MeSDCC group at 6 weeks was stained pink. Regions of new tissue formation can be observed within the 20% and 20% MeSDCC groups at 6 weeks. All MeSDCC gels stained red/orange for GAGs, while no GAG staining was observed in the 10% GelMA group. Regions of new tissue formation surrounding rBMSCs were observed to stain for GAGs. All MeSDCC groups stained for collagen II, although no increase in collagen II staining was observed for those groups throughout culture. However, the 10% GelMA group had an increase in collagen II staining at 6 weeks. Minimal collagen I staining was observed in the MeSDCC groups. Collagen I staining was noted in the 10% GelMA group, although there were no significant changes in staining over

the culture period. Last, a slight increase in aggrecan staining was observed in the 10% GelMA and 20% MeSDCC groups over the culture period. Scale bars are 200 μm.