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*Emerging Trends in Biomaterials Research*

# Chondroinductive Hydrogel Pastes Composed of Naturally Derived Devitalized Cartilage

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**Abstract**—Hydrogel precursors are liquid solutions that are prone to leaking from the defect site once implanted *in vivo*. Therefore, the objective of the current study was to create a hydrogel precursor that exhibited a yield stress. Additionally, devitalized cartilage extracellular matrix (DVC) was mixed with DVC that had been solubilized and methacrylated (MeSDVC) to create hydrogels that were chondroinductive. Precursors composed of 10% MeSDVC or 10% MeSDVC with 10% DVC were first evaluated rheologically, where non-Newtonian behavior was observed in all hydrogel precursors. Rat bone marrow stem cells (rBMSCs) were mixed in the precursor solutions, and the solutions were then crosslinked and cultured *in vitro* for 6 weeks with and without exposure to human transforming growth factor $\beta$3 (TGF-$\beta$3). The compressive modulus, gene expression, biochemical content, swelling, and histology of the gels were analyzed. The DVC-containing gels consistently outperformed the MeSDVC-only group in chondrogenic gene expression, especially at 6 weeks, where the relative collagen II expression of the DVC-containing groups with and without TGF-$\beta$3 exposure was 40- and 78-fold higher, respectively, than that of MeSDVC alone. Future work will test for chondrogenesis *in vivo* and overall, these two cartilage-derived components are promising materials for cartilage tissue engineering applications.

**Keywords**—Devitalized cartilage, Hydrogel, Yield stress, Chondroinduction.

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## INTRODUCTION

Hydrogels have several advantages for cartilage tissue engineering, including the ease of formation, the ability to encapsulate cells, and the ability to fine tune mechanical properties.[5,13,16] Although hydrogels are promising materials for cartilage regeneration, they cannot be molded into a defect site by a surgeon because hydrogel precursors are liquid solutions that are prone to leaking after placement.[33,41] To address this drawback, we recently published a method to achieve paste-like hydrogel precursor solutions by combining hyaluronic acid nanoparticles with traditional crosslinked hyaluronic acid hydrogels, known as methacrylated hyaluronic acid (MeHA).[3] This paste-like behavior was induced by incorporating hyaluronic acid nanoparticles,[3] where the MeHA mixed with hyaluronic acid nanoparticles were together referred to as hydrogel pastes prior to crosslinking. The hydrogel pastes were then crosslinked to form a rigid traditional hydrogel structure. In this current study, in an effort to introduce bioactivity to the hydrogel itself, the two components of the hydrogel paste, both the MeHA and nanoparticles were substituted with components made from naturally derived cartilage extracellular matrix (ECM).

Materials derived from ECM are attractive for regenerative medicine because they may promote stem cell recruitment, infiltration, and differentiation without the need to supplement with additional biological factors.[4,6,31] We and other groups have recently established that cartilage ECM has chondroinductive

potential,[7,9,18,25,34,38] where we observed that rat bone marrow stem cells (rBMSCs) exposed to cartilage ECM outperformed those cells exposed to TGF-$\beta_3$ in chondroinductivity.[38] ECM materials in general can be obtained from cell-derived matrices, which are ECM materials secreted during *in vitro* culture, or they can be obtained from native tissue.[4,7,10,12,35,49] Additionally, ECM materials are generally either decellularized to remove cellular components and nucleic acids or they are devitalized to kill but not necessarily remove cells within the matrix.[39]

Decellularization processes are known to inevitably cause some disruption to the matrix architecture, orientation, and surface landscape.[22] Therefore, in this current work, only unaltered DVC was studied. The objective was to create a hydrogel paste that was entirely derived from DVC that was capable of inducing chondrogenesis. Prior studies have made traditional hydrogels entirely out of ECM by first solubilizing the ECM, and the solubilized matrix could then form a gel at body temperature.[17–19,37] One group even used solubilized cartilage matrix as a depot for delivering drugs, where the gel maintained enough structural integrity under physiological conditions to sufficiently deliver bioactive molecules.[24] When we tried using this thermoresponsive method to create solubilized cartilage hydrogels, the gels that formed were too compliant and left opportunity for improvement for load-bearing applications, so methods to further crosslink the cartilage were desired. The crosslinking of unsolubilized cartilage has been reported, including crosslinking cartilage ECM with genipin, dehydrothermal treatment, ultraviolet irradiation, or carbodiimide chemistry.[10,32] Using these methods, cartilage scaffolds were crosslinked and maintained some mechanical integrity throughout culture. Furthermore, cell-mediated contraction was able to be controlled depending on the method of crosslinking. However, the authors noted that the constructs would need added reinforcements to achieve functional biomechanical properties. In the current study, these added reinforcements were attained by first solubilizing and then further crosslinking the cartilage tissue. Solubilizing the cartilage tissue allows for more fine-tuning of mechanical properties through allowing the control of the solid content of the hydrogel. Furthermore, solubilizing cartilage can remove particles that may cause premature gel fracture and it may open up more reactive sites on the cartilage ECM for crosslinking, which may help reinforce the ECM-based gels once they are crosslinked.

Therefore, with prior experience with functionalizing GAGs, such as hyaluronic acid and chondroitin sulfate with glycidyl methacrylate,[3,23] which allows photocrosslinking of the hydrogel material, in the current study, the solubilized cartilage ECM was methacrylated. One pioneering study recently reported methacrylating solubilized cartilage matrix to make photocrosslinkable hydrogels, and they demonstrated for the first time that native tissues can be crosslinked forming hydrogels.[43] However, in that study, the solubilized cartilage matrix was reinforced with methacrylated gelatin (GelMA). In another study, solubilized cartilage ECM was cleverly reinforced by combining it with poly($\varepsilon$-caprolactone) and then electrospinning it into a scaffold,[18] although the biomechanics of the scaffolds in that study still fell short of that of native cartilage tissue. The purpose of the current study was to create, for the first time, a hydrogel entirely composed of cartilage ECM without the use of GelMA and to additionally mix the methacrylated cartilage ECM with particles to give the hydrogel precursor a yield stress before crosslinking. We hypothesized that the hydrogel precursors containing DVC particles would exhibit a yield stress, and would be more chondroinductive than that of the methacrylated cartilage alone.

## METHODS AND MATERIALS

### *DVC Processing and Characterization*

Twenty porcine knees were purchased from a local abattoir (Bichelmeyer Meats, Kansas City, KS). The knees came from Berkshire hogs, which were castrated males that were approximately 7–8 months old and 120 kg in weight. Articular cartilage from both the knee and hip joints was carefully removed and collected using scalpels and was then rinsed twice in deionized (DI) water and stored at −20 °C. After freezing overnight, the cartilage was thawed, mixed with dry ice and coarsely ground using a cryogenic tissue grinder (BioSpec Products, Bartlesville, OK). The dry ice was allowed to evaporate overnight in the freezer, where the cartilage was then referred to as devitalized cartilage (DVC), and then the DVC was lyophilized. The DVC was then cryoground into a fine powder using a freezer-mill (SPEX SamplePrep, Metuchen, NJ) and was lyophilized again overnight. The DVC powder was then filtered using a 106 $\mu$m mesh (ThermoFisher Scientific, Waltham, MA) to remove large particles and then frozen until use. The DVC powder was sputter coated with gold and imaged with a Versa 3D Dual Beam (FEI, Hillsboro, OR) to observe particle surface morphology and size. The size distribution of the DVC powder was determined by an electrical impedance technique with a Beckman Multisizer 4 Coulter Counter (Beckman Coulter Inc., Fullerton, CA) using a 200 $\mu$m aperture, and by dynamic light scattering with a ZetaPALS Omni particle

sizer (Brookhaven Instruments, Holtsville, NY) using DVC powder that had undergone further filtration using a 10 $\mu$m nylon mesh (Spectrum Laboratories, Inc., Rancho Dominguez, CA).

### *Synthesis and Characterization of MeSDVC*

DVC powder was first solubilized using an adapted protocol from our previously reported method.[36] First, DVC powder was mixed in 0.1 M HCl at a concentration of 10 mg DVC per 1 mL HCl. Pepsin was then added to the solution at a concentration of 1 mg/mL. The mixture was then stirred at 200 rpm at room temperature. After 2 days of stirring, the solution was then brought back to physiological pH by adding 1 M NaOH. This solubilized DVC powder (SDVC) was then centrifuged at 10,000$\times g$ for 3 min to pellet any unsolubilized particulates and the supernatant was frozen and lyophilized and later used to make methacrylated SDVC (MeSDVC).

MeSDVC was created by reacting SDVC with 20-fold molar excess glycidyl methacrylate (Sigma-Aldrich, St. Louis, MO) in the presence of trimethylamine and tetrabutyl ammonium bromide (Sigma-Aldrich) in a 1:3 acetone:water mixture at a concentration of 1 g SDVC for every 150 mL solution. This solution was then stirred at 200 rpm for 6 days. 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. After 6 days, the MeSDVC was then precipitated in excess acetone, was dialyzed for 2 days in DI water, and then was lyophilized. Successful methacrylation was confirmed using $^1$H NMR (Avance AV-III 500, Bruker).

### *Rat Bone Marrow Stem Cell Harvest and Culture*

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

### *Description of Experimental Groups*

Both acellular and cellular crosslinked formulations of 10% MeSDVC and 10% MeSDVC 10% DVC (w/v) were tested for 6 weeks *in vitro* along with one cellular group composed of 10% MeSDVC 10% DVC that was exposed to 10 ng/mL human transforming growth factor-$\beta_3$ (TGF-$\beta_3$, PeproTech Inc., Rocky Hill, NJ). In addition, one acellular group composed of 20% MeSDVC was tested at day 1 after crosslinking. The acellular formulations were analyzed along with the cellular groups to quantify the acellular biochemical content and to analyze the effect of cells encapsulated in the networks. Ten percent MeSDVC was chosen as it was a concentration previously reported in literature for methacrylated gelatin (GelMA) gels.[43] A concentration of 20% MeSDVC was chosen as that is the approximate concentration of dry mass in native cartilage matrix.[29] Prior to crosslinking, the aforementioned cellular and acellular groups (except the growth factor group) and additional groups of 5% DVC and 10% DVC were tested rheologically. However, DVC alone cannot be crosslinked into a hydrogel network, which is why these two DVC groups were only tested rheologically.

### *Preparation of Hydrogel Pastes, Cell Encapsulation, and Hydrogel Culture Conditions*

Hydrogel pastes were created by first measuring the desired weight percents of MeSDVC and DVC in a mini-centrifuge tube. All materials used for future cell encapsulation were then sterilized with ethylene oxide prior to use and were handled under sterile conditions thereafter. The pastes were mixed in two stages (e.g., in photoinitiator solution overnight and then more photoinitiator solution or cell suspension the day of testing) due to the longer time it took for MeSDVC to dissolve (i.e., overnight) to ensure mixture homogeneity. This length of time was deemed too long for adequate cell survival for the groups incorporating cells. Therefore, cell mixtures were added the day after the MeSDVC was mixed and given a chance to dissolve in half of the final solution. For rheological testing of acellular groups, two stages of mixing was performed to maintain the same mixing process for both cellular and acellular groups. Sterile 0.01 M PBS containing 0.05% (w/v) Irgacure (I-2959) photoinitiator was added to the acellular groups until the concentration of MeSDVC and DVC was twice the desired concentration. The samples were then mixed, centrifuged at 3000 rpm, and stored at 4 °C overnight to allow the MeSDVC to adequately dissolve. Prior to rheological

testing, more photoinitiator solution was added until the desired final concentration of materials was reached. The samples were then again mixed and centrifuged to remove air bubbles. For example, to make a 10% MeSDVC solution, 40 mg MeSDVC and 200 $\mu$L photoinitiator solution were mixed and allowed to fully dissolve overnight and then another 200 $\mu$L photoinitiator solution was added to make the final concentration at 10% MeSDVC the following day. For cellular testing, the samples were first mixed with 0.1% (w/v) Irgacure photoinitiator in PBS until the concentration of MeSDVC and DVC was twice the desired final concentration, and then the solutions were centrifuged and stored at 4 °C overnight just like the acellular groups. The following day however, passage 4 rBMSCs were then suspended at 20 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 $\mu$g/mL ascorbic acid, and 0.25 mg/mL penicillin/streptomycin. This cell suspension was then added to the hydrogel paste solutions until the desired concentration of MeSDVC and DVC was reached and the final cell concentration and photoinitiator concentration were 10 million cells/mL and 0.05%, respectively. These solutions were then either tested rheologically or they were crosslinked with UV light and further characterized as solids. For pastes undergoing crosslinking, the paste solutions were 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 for a total of 5 min. Using a 4 mm biopsy punch, each gel was cut and placed in one well of a 24 well, non-tissue culture-treated plate (Corning Incorporated, Corning, NY). Each gel was then exposed to 1 mL of incomplete chondrogenic medium or 1 mL of complete chondrogenic medium, which consisted of incomplete chondrogenic medium and 0.1 mg/mL dexamethasone and 10 ng/mL TGF-$\beta_3$. The medium was replaced every other day throughout the 6 week study.

### *Rheological Testing of Hydrogel Precursors*

Prior to crosslinking the hydrogel precursor pastes, the precursor solutions were shaped into spheres to demonstrate their shaping capabilities, and they were then loaded into a 3 mL syringe and extruded onto a glass slide to macroscopically observe shape retention. The gels were extruded in a wavy line appearance to observe whether the formulations could maintain shaping after crosslinking.

The oscillatory shear stress of the precursor solutions ($n = 5$) was measured over an oscillatory shear stress sweep of 1–2500 Pa at 37 °C using an AR-2000 rheometer (TA instruments, New Castle, DE) and a gap of 500 $\mu$m. The rheometer was equipped with a 20 mm diameter roughened plate and a roughened Peltier plate cover. Frozen rBMSCs that were thawed and cultured to passage 4 were used to make the cellular samples for rheological testing. The pastes for rheological testing were created as previously mentioned for the *in vitro* culture. The yield stress was interpolated from the point at which the storage ($G'$) and loss ($G''$) modulus crossed.[44] Additionally, an oscillatory shear stress sweep of 0.1–10 Pa was performed to assess the linear viscoelastic region of the hydrogel precursors to determine the storage modulus of each solution.

### *Mechanical Testing of Crosslinked Hydrogels*

After swelling to equilibrium for 24 h in either complete or incomplete chondrogenic medium, mechanical testing was performed. In addition, the groups in the 6 week study were tested at 6 weeks as well. First, the geometric mean diameter of the gels was determined using forceps and a stereomicroscope (×20 magnification) and then the height of each gel was measured directly using a RSA-III dynamic mechanical analyzer (DMA, TA instruments, New Castle, DE). The gels ($n = 5$) were then compressed at a rate of 0.01 mm/s until mechanical failure. The compressive modulus was calculated as the slope of the linear portion of the stress–strain curve (i.e., 5–15% strain).

### *Swelling Degree and Volume*

Gels that were swollen to equilibrium were weighed 1 day after crosslinking and were then frozen and lyophilized ($n = 5$). The dry weight was then recorded and the swelling degree was calculated as the ratio of total wet mass to dry mass. From the diameter and height readings recorded during mechanical testing, the volume of each gel ($n = 5$) was calculated at 1 day and after 6 weeks of culture. Additionally from the diameter and height readings recorded during mechanical testing, the average conversion efficiency of the acellular 10% MeSDVC gels was determined by the ratio of the actual mass to the theoretical mass of the gels.

### *Biochemical Content Analysis*

The biochemical content of the starting materials, which include MeSDVC, SDVC, and DVC, and the biochemical content of the gels at 1 day, 3 weeks, and 6 weeks were quantified ($n = 5$). The gels were each

digested overnight 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 °C. The digested solutions were then frozen and stored at −20 °C. Prior to biochemical analyses, all digested gel solutions were thawed to room temperature and then vortexed and centrifuged at 10,000 rpm for 10 min to pellet fragments of polymers. The supernatant was then used to quantify DNA, GAG, and hydroxyproline contents. Using a Cytation 5 Cell-Imaging Multi-Mode reader (Bio-Tek, Winooski, VT) and according to manufacturer instructions, the DNA content was quantified with the PicoGreen assay (Molecular Probes, Eugene, OR), the glycosaminoglycan (GAG) content was determined with the dimethylmethylene blue (DMMB) assay (Biocolor, Newtownabby, Northern Ireland), and the hydroxyproline content was quantified using a hydroxyproline detection kit (Sigma-Aldrich). Neither the GAG or hydroxyproline contents were normalized to DNA and instead are shown in total because of the gels' inherent initial DNA contents (i.e., true normalization to DNA content of seeded cells is not possible).

### *Gene Expression Analysis*

RNA was isolated and purified using Qiagen QIAshredders followed by an RNeasy Kit (Valencia, CA) according to manufacturer guidelines ($n = 6$). 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 *Taq*Man gene expression assays from Applied Biosystems for Sox-9 (Rn01751070_m1), aggrecan (Rn00573424_m1), collagens type I (Rn01463848_m1) and II (Rn01637087_m1), and GAPDH (Rn01775763_g1). Relative gene expression levels for each gene were calculated using the $2^{-\Delta\Delta C_t}$ method where the 10% MeSDVC gels at day 1 were designated as the calibrator group and GAPDH expression was used as the endogenous control.[27] Last, RNA from DVC only (i.e., no rBMSCs) was isolated, converted to DNA, and then PCR was performed with the same previously mentioned *Taq*Man assays, where it was confirmed that all gene expression observed in the study was that of the rBMSCs.

### *Histological Analysis*

Cellular gels from day 1 and 6 weeks were fixed in 10% formalin for 15 min and then embedded in Optimal Temperature Cutting (OCT) medium (TissueTek, Torrance, CA) overnight at 37 °C. Then the gels were frozen at −20 °C and were sectioned at a thickness of 10 $\mu$m 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 the nuclei purple. In addition, sections were stained with the standard Safranin-O/Fast Green (Saf-O) stain, which stains negatively charged GAGs orange. Last, the sections were stained immunologically 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). The slides were first fixed in chilled acetone (−20 °C), treated with proteinase K (Abcam), and exposed to 0.3% hydrogen peroxide to suppress endogenous peroxidase activity. Then the sections were blocked with serum according to the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) following the manufacturer's instructions and were then incubated with primary antibody. Following primary antibody incubation, the sections were exposed to biotinylated secondary antibodies (horse anti-rabbit and mouse) and ABC reagent according to the manufacturer protocol. Antibodies were visualized using the ImmPact DAB peroxidase substrate (Vector). The sections were then rinsed in DI water, counter stained with VECTOR hematoxylin QS stain, and then were dehydrated and mounted. Negative controls consisted of omitting the primary antibody (for aggrecan) or substituting with 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.

### *Statistical Analysis*

Using GraphPad Prism 6 statistical software (GraphPad Software, Inc., La Jolla, CA), experimental groups were compared using a one-factor ANOVA (for analyses with one time point) or a two-factor ANOVA (for analyses with two or more time points) 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. Standard box plots were constructed to eliminate outliers. All quantitative results are reported as mean ± standard deviation within the text or as mean + standard deviation within the figures. Furthermore, unless otherwise stated, all groups discussed in the Results section are cell-encapsulated.

## RESULTS

### *Characterization of MeSDVC, SDVC, and DVC particles*

SEM imaging revealed that the DVC particles were approximately 110 $\mu$m in diameter or smaller and were noted to be heterogeneous in size and morphology (Fig. 1a). Success of the methacrylation procedure on MeSDVC was confirmed *via* $^1$H NMR by the emergence of methacrylate peaks between 5 and 6.5 ppm (Fig. 1b). The DNA content of the DVC particles was 1170 ± 68 ng DNA per mg dry DVC, where the SDVC and MeSDVC had DNA contents that were 92 and 97% less than DVC, respectively ($p < 0.05$) (Fig. 1c). The GAG content of the DVC particles was 380 ± 57 $\mu$g GAG per mg dry DVC, and the SDVC and MeSDVC had GAG contents that were 44 and 41% less than that of DVC, respectively ($p < 0.05$) (Fig. 1d). Last, the hydroxyproline content of DVC was 48.60 ± 0.58 $\mu$g hydroxyproline per mg dry DVC, where the hydroxyproline content of SDVC was 26% lower than that of DVC ($p < 0.05$) (Fig. 1e). The hydroxyproline content of MeSDVC was not significantly different from that of DVC, but was 41% higher than that of SDVC ($p < 0.05$).

Particle sizing further demonstrated the heterogeneity of the DVC particles beyond aforementioned SEM imaging, showing a large size distribution in both the micro- and nanoranges, with a significant number of particles with diameters below 200 nm (Fig. 2a), and the vast majority below 20 $\mu$m in diameter (Fig. 2b).

### *Macroscopic Observation and Rheological Testing of Hydrogel Precursors*

Macroscopic observation of hydrogel precursors revealed non-Newtonian and paste-like behavior in all precursors (Fig. 3). Furthermore, all solutions except the 5% DVC and 10% DVC groups were able to be shaped and molded into a sphere, where it was noted that the pastes incorporating particles were easier to shape and manipulate because the solutions containing only MeSDVC were sticky. Shape retention after extrusion through a 3 mL syringe was indicated by the fluids that retained the diameter of the syringe orifice. All pastes exhibited shape retention except the 5% DVC group, which spread out over 2 times the diameter of the syringe orifice. Furthermore, all formulations containing MeSDVC were able to be crosslinked to maintain extrusion shape (Fig. 3).

Additionally, all solutions exhibited a yield stress (Fig. 4a). The yield stress of 10% MeSDVC acellular group was 725 ± 55 Pa, where the difference in yield stress compared to its respective cellular group was not significant. The 5% DVC and 10% DVC groups had yield stresses that were 96 and 92% lower, respectively, than that of 10% MeSDVC ($p < 0.05$), while the MeSDVC + DVC acellular group had a yield stress that was 94% higher than that of 10% MeSDVC ($p < 0.05$) (Fig. 4a). Furthermore, when cells were mixed into the MeSDVC + DVC group, the yield stress was not significantly different from the acellular group, but it was 62% higher than that of the 20% MeSDVC acellular group ($p < 0.05$).

All solutions exhibited viscoelastic behavior, which was indicated by a measurable storage modulus, although the storage modulus of the 5% DVC was the lowest at 1.33 ± 0.80 Pa (Fig. 4b). The storage modulus of the 10% MeSDVC acellular group was 773 ± 84 Pa. The only groups that were significantly different from the 10% MeSDVC acellular group were the acellular and cellular MeSDVC + DVC groups, where their storage moduli were 5.7 and 7.2 times higher than that of the 10% MeSDVC acellular group, respectively ($p < 0.05$). Last, the storage modulus of the MeSDVC + DVC cellular group was 2 times higher than that of the 20% MeSDVC acellular group ($p < 0.05$).

### *Mechanical Testing of Crosslinked Hydrogel Pastes*

One day after crosslinking, the compressive modulus of the 10% MeSDVC acellular group was 135 ± 37 kPa (Fig. 5). None of the groups were significantly different from the 10% MeSDVC acellular group except the 20% MeSDVC acellular group, which had a modulus of 675 ± 130 kPa ($p < 0.05$).

Six weeks after crosslinking, the compressive modulus of the 10% MeSDVC acellular group was 32 ± 12 kPa, although there were no significant differences compared to other groups (Fig. 5). However, over the 6 weeks of culture, while most of the groups did not deviate significantly from their original compressive modulus, the compressive modulus of the 10% MeSDVC acellular and cellular groups reduced by 77% and 86%, respectively ($p < 0.05$).

### *Swelling and Volume Analysis of Crosslinked Hydrogel Pastes*

The only group that had a significantly lower swelling degree than that of the 10% MeSDVC group, which had a swelling degree of 10.5 ± 3.5 after swelling to equilibrium, was the 20% MeSDVC acellular group, where its swelling degree was 36% lower than that of the 10% MeSDVC group ($p < 0.05$) (Fig. 6a).

At 1 day after crosslinking and swelling to equilibrium, the gel volume of the 10% MeSDVC group was

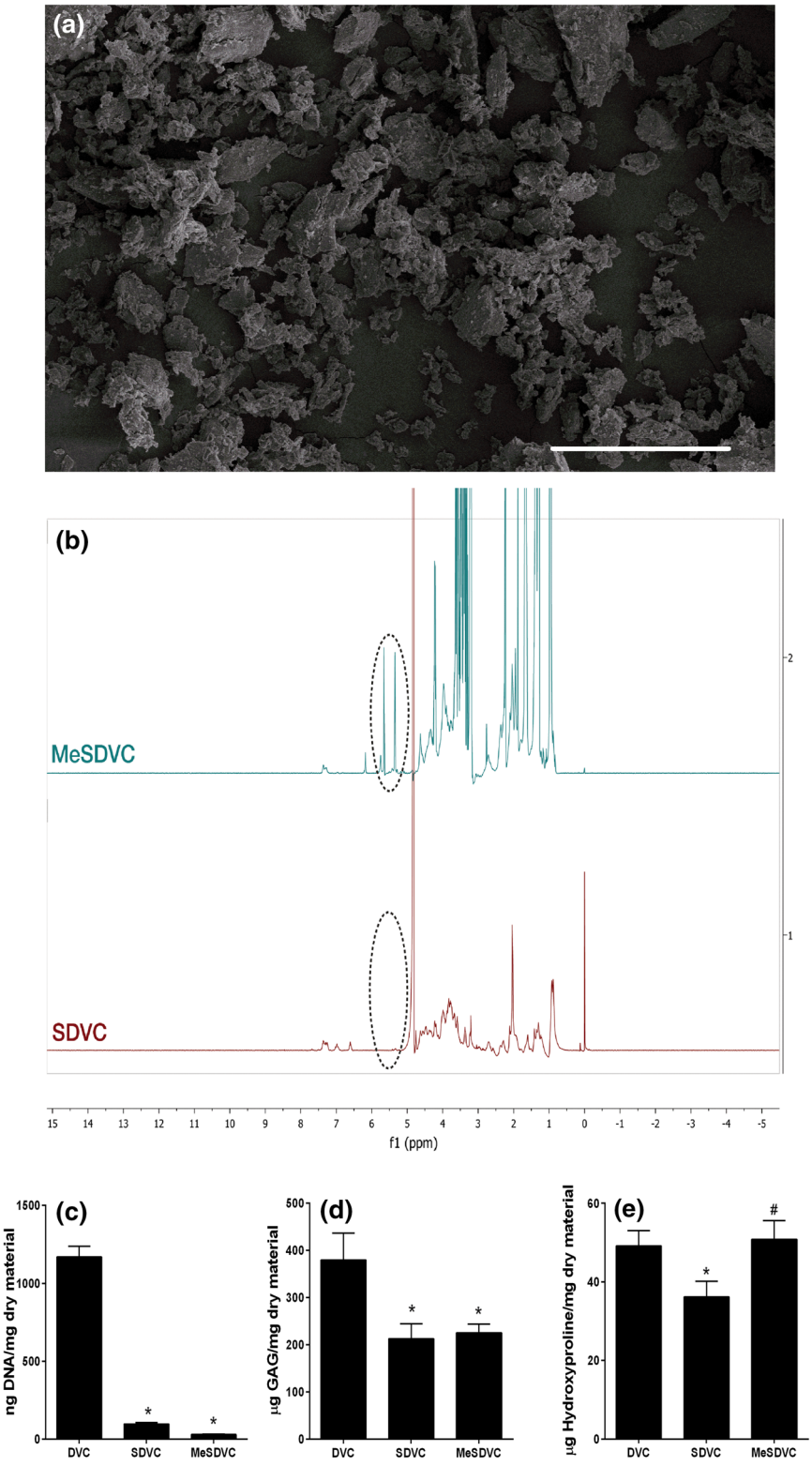


**FIGURE 1. Characterization of Hydrogel Components. (a) SEM image of DVC particles at ×500 magnification. DVC particles were heterogeneous in morphology and size. Scale bar is 100 $\mu$m. (b) Methacrylation was confirmed by the emergence of methacrylate peaks circled between 5 and 6.5 ppm. (c) PicoGreen content, (d) GAG content, and (e) Hydroxyproline content of each material. The SDVC and MeSDVC had DNA contents that were 92 and 97% less than DVC, respectively, and had GAG contents that were 44 and 41% less than that of DVC, respectively ($p < 0.05$). The hydroxyproline content of SDVC was 26% lower than that of DVC ($p < 0.05$). While the hydroxyproline content of MeSDVC was not significant from DVC, it was 41% higher than that of SDVC ($p < 0.05$). Data reported as mean + standard deviation ($n = 5$); statistically significant from DVC ($^{*}p < 0.05$), statistically significant from SDVC ($^{\#}p < 0.05$).**

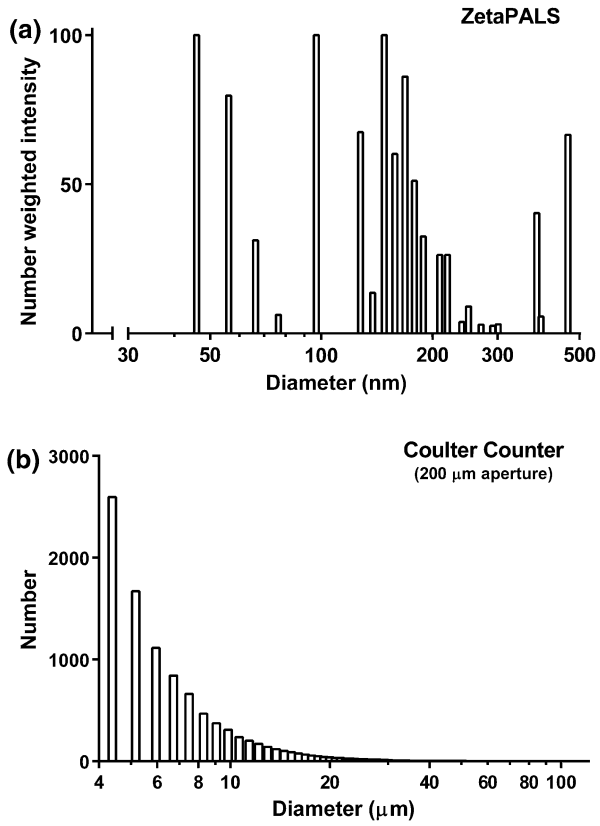


**FIGURE 2. DVC particle size distribution. (a) ZetaPALS DVC particle size distribution by number weighted intensity. Though particles are seen throughout the nanometer range, there is a greater presence of particles less than 200 nm. It should be noted that the DVC were filtered through a 10 $\mu$m nylon mesh before being analyzed. (b) Coulter counter DVC particle size distribution by number of particles. Using a 200 $\mu$m aperture (with a normal range of 4–120 $\mu$m) the majority of particles had diameters less than 20 $\mu$m.**

19.26 ± 0.54 $\mu$L, where the volumes of the MeSDVC + DVC cellular, acellular, and TGF-$\beta_3$ exposed groups were 17, 20, and 17% higher, respectively ($p < 0.05$) (Fig. 6b). Furthermore, the volume of the MeSDVC + DVC group was 22.6 ± 1.7 $\mu$L and it was not significant from its respective acellular and growth factor exposed group.

At 6 weeks after crosslinking, again all three of the MeSDVC + DVC groups had significantly higher volumes than that of the 10% MeSDVC group ($p < 0.05$) (Fig. 6b). The volume of the 10% MeSDVC group was 15.8 ± 2.1 $\mu$L, while the volume of the cellular MeSDVC + DVC group was 36% larger ($p < 0.05$).

Over the course of 6 weeks, the only groups that had a significant change in volume were the 10% MeSDVC acellular and cellular groups, where they each decreased in volume by 27% and 18%, respectively ($p < 0.05$) (Fig. 6b). The volumes of all three MeSDVC + DVC groups remained constant throughout the 6 week study.

Finally, the conversion efficiencies of the 10% MeSDVC acellular and the MeSDVC ± DVC acellular groups were determined to be approximately 89 and 53%, respectively.

### *Biochemical Content of Crosslinked Hydrogel Pastes*

All cellular groups had significantly higher DNA contents than their respective acellular groups at all time points ($p < 0.05$) (Fig. 7a). At 1 day after crosslinking, the 10% MeSDVC group contained 680 ± 170 ng DNA per gel, and both the MeSDVC + DVC cellular and growth factor exposed groups contained 26 and 28% more DNA, respectively, ($p < 0.05$). At 3 weeks after crosslinking, the 10% MeSDVC group had a DNA content of 386 ± 37 ng DNA per gel, which was not significantly different from any of the other cellular groups (Fig. 7a). After 6 weeks of culture, the 10% MeSDVC group contained 241 ± 18 ng DNA per gel, which was 42% lower than the DNA content of the MeSDVC + DVC + TGF-$\beta_3$ group ($p < 0.05$) (Fig. 7a). Over the course of the 6 week culture period, all cellular groups had a significant reduction in DNA content ($p < 0.05$), where after 3 weeks the DNA content in the 10% MeSDVC, MeSDVC + DVC, and the MeSDVC + DVC + TGF-$\beta_3$ groups decreased by 43, 43, and 49%, respectively ($p < 0.05$). By 6 weeks, the DNA contents of the 10% MeSDVC and MeSDVC + DVC groups were significantly lower than their 3 week values, where their total reductions in DNA over the entire culture period was 65 and 72%, respectively ($p < 0.05$). There was not a significant reduction in DNA content for the growth factor exposed group after 3 weeks. Finally, the acellular groups did not have any significant reduction in DNA content over the culture period (Fig. 7a).

At 1 day after crosslinking, the GAG content of the 10% MeSDVC group was 86 ± 15 $\mu$g GAG per gel, where that of the MeSDVC + DVC group was 3.9-fold higher ($p < 0.05$) (Fig. 7b). Additionally, the MeSDVC + DVC and the TGF-$\beta_3$ exposed groups contained 45 and 27% more GAG than their acellular control ($p < 0.05$). At 3 weeks, the GAG content of the 10% MeSDVC was 40.7 ± 2.5 $\mu$g GAG per gel, whereas that of the MeSDVC + DVC group was 4.7-fold higher ($p < 0.05$) (Fig. 7b). At 6 weeks, the GAG content of the 10% MeSDVC group was 25.2 ± 3.0 $\mu$g GAG per gel, whereas that of the MeSDVC + DVC group was 3.7-fold higher ($p < 0.05$). In addition, the GAG content of the MeSDVC + DVC + TGF-$\beta_3$ group was 75% larger than that of the MeSDVC + DVC group ($p < 0.05$). From day 1 to 3 weeks, the GAG contents of the 10% MeSDVC, the

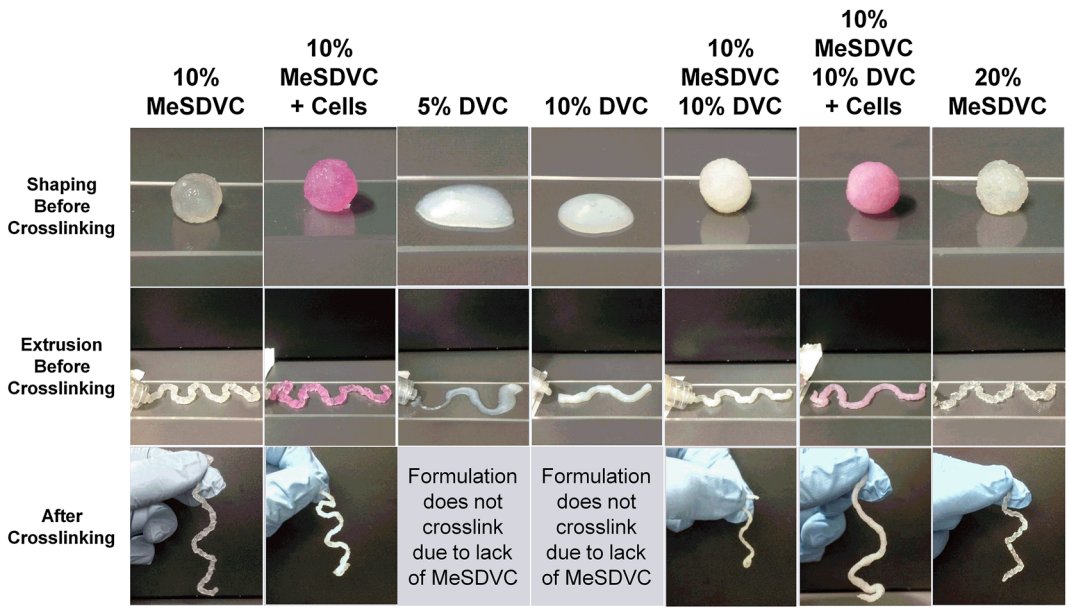


**FIGURE 3. Macroscopic rheological evaluation of hydrogel precursors before and after crosslinking. All formulations were acellular unless noted and cellular formulations were pink in color due to presence of cell culture medium. Non-Newtonian behavior was observed in all solutions. However, the 5% DVC and 10% DVC formulations were the only solutions that could not be molded and shaped into a sphere. Shape retention (indicated by the solution retaining extrusion orifice diameter) was noted in all solutions except the 5% DVC solution. Finally, all formulations containing MeSDVC retained shaping after crosslinking.**

MeSDVC + DVC, and the MeSDVC + DVC + TGF-$\beta_3$ group decreased by 53, 43, and 26%, respectively ($p < 0.05$). By 6 weeks, the GAG contents of the MeSDVC + DVC and the MeSDVC + DVC + TGF-$\beta_3$ groups decreased by 72 and 44%, respectively, compared to their original GAG contents ($p < 0.05$).

Finally, at day 1, the initial hydroxyproline content of the 10% MeSDVC group was 66.4 ± 2.8 $\mu$g hydroxyproline per gel, whereas that of the MeSDVC + DVC group was 2.7-fold higher ($p < 0.05$) (Fig. 7c). At 3 weeks, the MeSDVC + DVC group contained 144 ± 21 $\mu$g hydroxyproline per gel, which was 2 times higher than that of the 10% MeSDVC group and 22% lower than that of the MeSDVC + DVC + TGF-$\beta_3$ group ($p < 0.05$). At 6 weeks, the hydroxyproline content of the MeSDVC + DVC group was 114 ± 11 $\mu$g hydroxyproline per gel, which was 89% higher than that of the 10% MeSDVC group and 28% lower than that of the MeSDVC + DVC + TGF-$\beta_3$ group ($p < 0.05$). The only group that experienced a loss in hydroxyproline content from day 1 to 3 weeks was the MeSDVC + DVC group, where at 3 weeks the hydroxyproline content was 81% of its original content at day 1 ($p < 0.05$). By 6 weeks, all three of the DVC-incorporating groups experienced a significant loss in hydroxyproline, where the hydroxyproline contents for the MeSDVC + DVC and the MeSDVC + DVC + TGF-$\beta_3$ groups were 64 and 95% of their original contents at day 1 ($p < 0.05$).

### *Gene Expression Analysis*

At 1 day after crosslinking, the relative Sox-9 expression of 10% MeSDVC was 9.4 times larger than that of the MeSDVC + DVC group ($p < 0.05$) (Fig. 8a). Furthermore, growth factor exposure had no significant effect on Sox-9 expression compared to the MeSDVC + DVC group. At 1 week, the relative Sox-9 expression of the 10% MeSDVC group was 73% larger than that of the TGF-$\beta_3$ exposed group ($p < 0.05$). At 2 weeks, the relative Sox-9 expression of the MeSDVC + DVC group was 89% larger than that of the 10% MeSDVC group and 37% smaller than that of the TGF-$\beta_3$ exposed group ($p < 0.05$). At 3 weeks, the relative Sox-9 expression of the MeSDVC + DVC group was 5.2-fold higher than that of the 10% MeSDVC group ($p < 0.05$). At 6 weeks, the relative Sox-9 expression of the MeSDVC + DVC group was 15 and 5.6 times larger than that of the 10% MeSDVC and the TGF-$\beta_3$ exposed groups, respectively ($p < 0.05$) (Fig. 8a). From 1 day to 3 weeks, the relative Sox-9 expression of the 10% MeSDVC group decreased by 82% ($p < 0.05$), but did not change significantly thereafter. From 1 day to 1 week, the relative Sox-9 expression of the MeSDVC + DVC group increased by a factor of 6.6 ($p < 0.05$), and did not change significantly thereafter. Finally, the relative Sox-9 expression of the MeSDVC + DVC + TGF-$\beta_3$ group increased by a factor of 6.6 from 1 day to 1 week ($p < 0.05$),

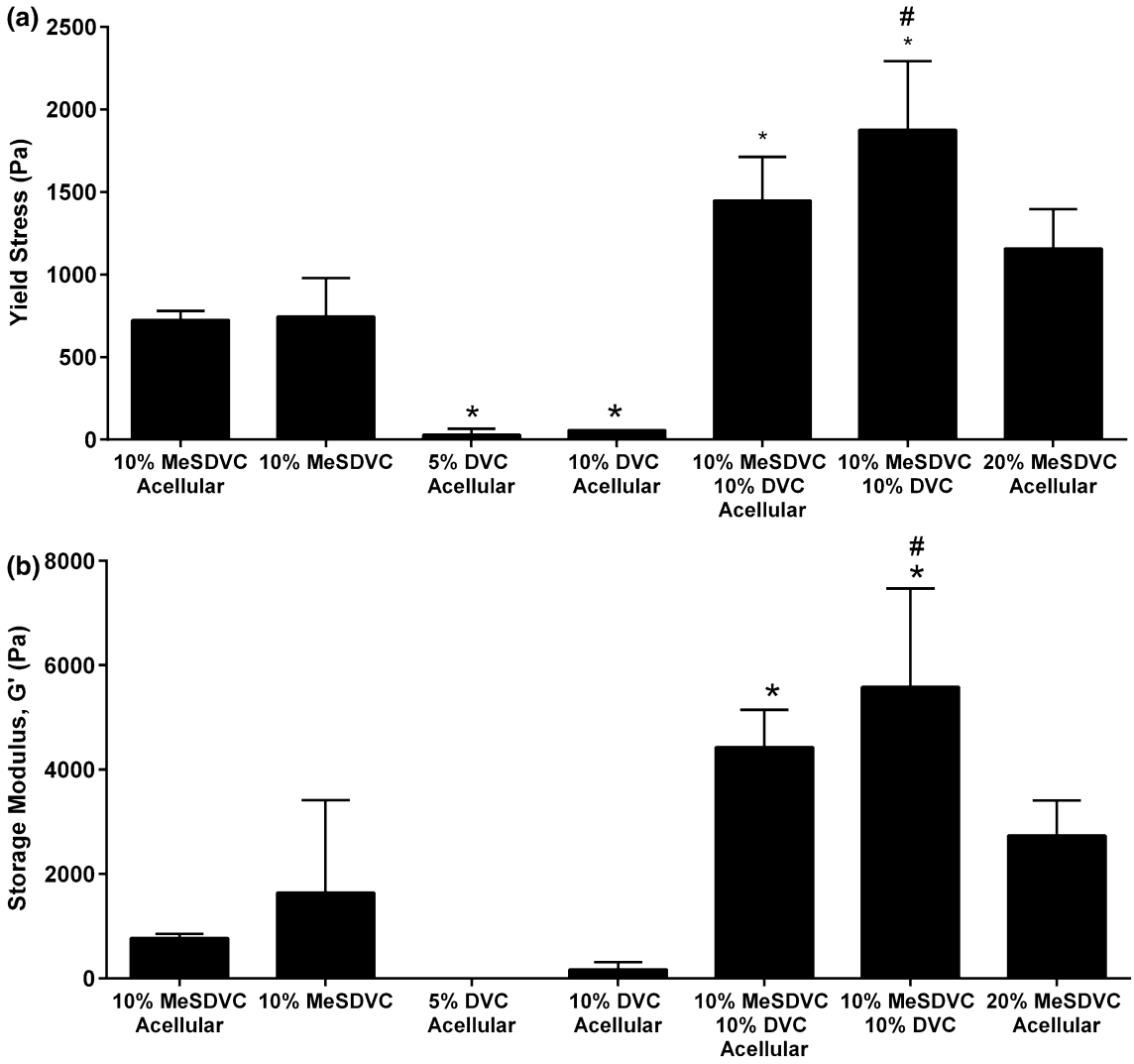


**FIGURE 4. Yield stress (a) and storage modulus (b) of hydrogel precursor solutions. All solutions had a measurable yield stress and storage modulus, while the groups containing both MeSDVC and DVC had the highest reported values. No significant differences were observed with the incorporation of cells. Data reported as mean + standard deviation ($n = 5$); *significantly different from 10% MeSDVC acellular, #significantly different from 20% MeSDVC acellular.**

increased by a factor of 2.9 from 1 to 2 weeks ($p < 0.05$), decreased by 54% from 2 to 3 weeks ($p < 0.05$), and then decreased again by 87% from 3 to 6 weeks ($p < 0.05$) (Fig. 8a).

The relative aggrecan expression of 10% MeSDVC was 42 times higher than that of the MeSDVC + DVC group at day 1 ($p < 0.05$) (Fig. 8b). There were no significant differences among groups at 1 week. At 2 weeks, the relative aggrecan expression of the MeSDVC + DVC group was 2.8 times higher than that of 10% MeSDVC and 41% lower than that of the TGF-$\beta_3$ exposed group ($p < 0.05$). At 3 weeks, the relative aggrecan expression of the MeSDVC + DVC group was 8.6 times higher than that of the 10% MeSDVC group ($p < 0.05$). At 6 weeks, there was no detectable aggrecan expression in the TGF-$\beta_3$ exposed group. However, the relative aggrecan expression of the MeSDVC + DVC group was 53 times higher than that of the 10% MeSDVC group ($p < 0.05$). From day 1 to 1 week, the relative aggrecan expression of the 10% MeSDVC group decreased by 94% ($p < 0.05$), and did not change significantly thereafter. The relative aggrecan expression of the MeSDVC + DVC group increased by a factor of 3.3 from 1 day to 1 week ($p < 0.05$), and did not change significantly thereafter. Last, from 1 day to 2 weeks, the relative aggrecan expression of the MeSDVC + DVC + TGF-$\beta_3$ group increased by a factor of 5.9 ($p < 0.05$), and then decreased by 45% from 2 to 3 weeks ($p < 0.05$) (Fig. 8b).

There were no significant differences between groups for collagen II expression from day 1 through

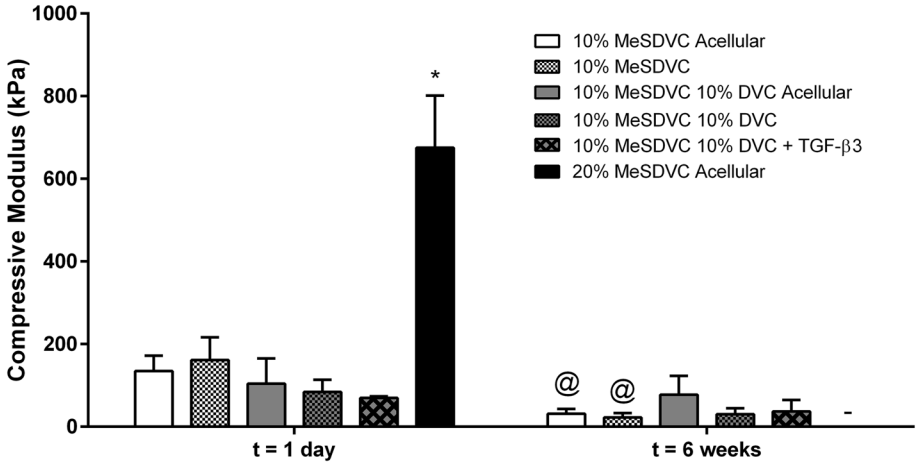


**FIGURE 5. Compressive modulus of crosslinked hydrogels after 1 day and 6 weeks of culture. None of the groups were significantly different from the 10% MeSDVC acellular group except the 20% MeSDVC acellular group, which had a modulus of 675 ± 130 kPa ($p < 0.05$). Additionally, the only groups that significantly deviated from their original compressive modulus over the 6 week period were the 10% MeSDVC acellular and cellular groups. Data reported as mean + standard deviation ($n = 5$); *significantly different from all other groups at same time point ($p < 0.05$), [@]significantly different from same group at first time point ($p < 0.05$), - not tested.**

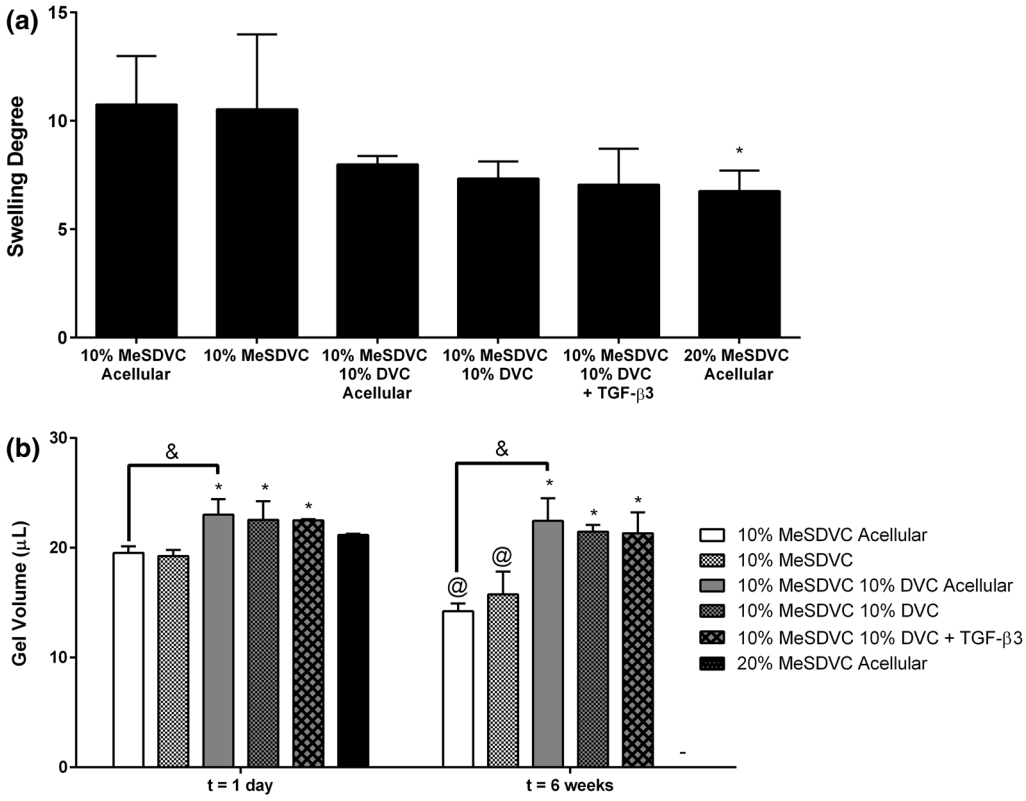


**FIGURE 6. Swelling degree (a) and volume (b) of crosslinked hydrogel pastes. (a) The only group that had a significantly lower swelling degree than that of the 10% MeSDVC group was the 20% MeSDVC acellular group. (b) Over the course of the 6 weeks, the only groups that had a significant reduction in volume were the 10% MeSDVC acellular and cellular groups. Data reported as mean + standard deviation ($n = 5$); statistically significant from 10% MeSDVC at same time point (*$p < 0.05$), statistically significant from acellular group at same time point ([!]$p < 0.05$), [&]$p < 0.05$ for specified comparison, statistically significant from same group at first time point ([@]$p < 0.05$), - not tested.**

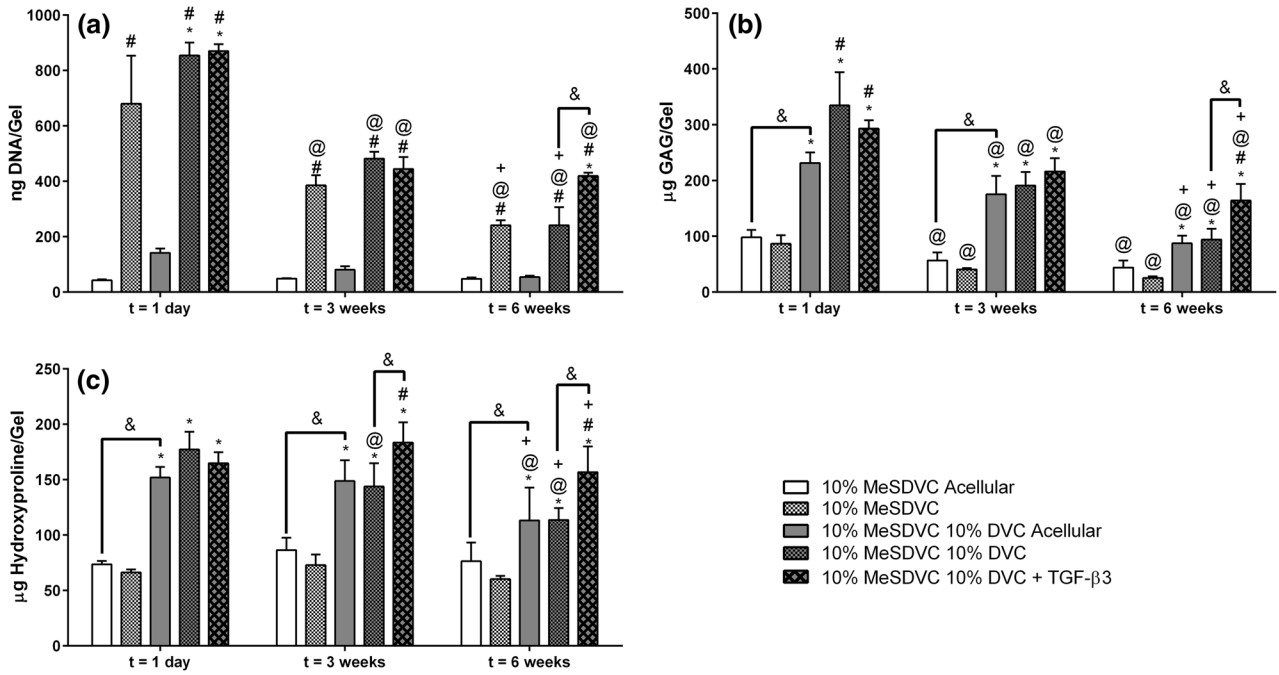


**FIGURE 7. Biochemical content of gels over the 6 week culture period. (a) DNA content, (b) GAG content, and (c) hydroxyproline content. All cellular groups had significantly higher DNA contents than their respective acellular groups at all time points. Over the course of the 6 week culture period, all groups had significant reductions in biochemical content ($p < 0.05$), except for the TGF-$\beta_3$ exposed group, which did not have a significant reduction in hydroxyproline. Data reported as mean + standard deviation ($n = 5$); significantly different from 10% MeSDVC 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 ($^{+}p < 0.05$).**

6 weeks (Fig. 8c). Additionally, there was no detectable collagen II expression in the DVC-incorporated groups at 2 and 3 weeks, and there was no detectable collagen II expression in the 10% MeSDVC group at week 3. However, at 6 weeks, the relative collagen II expressions of the MeSDVC + DVC and the TGF-$\beta_3$ exposed groups were 78- and 40-fold higher than that of the 10% MeSDVC group, respectively ($p < 0.05$). Due to many groups not having detectable collagen II expression throughout the culture period, for observing differences within groups throughout the culture period, only differences from day 1 to 6 weeks were reported here. From 1 day to 6 weeks, the relative collagen II expression for 10% MeSDVC did not change significantly. However, the relative collagen II expression for the MeSDVC + DVC and the MeSDVC + DVC + TGF-$\beta_3$ groups increased by a factor of 131.1 and 92.9, respectively ($p < 0.05$) (Fig. 8c).

The relative collagen I expression of the 10% MeSDVC group at 1 day was 17.9-fold higher than that of the MeSDVC + DVC group ($p < 0.05$) (Fig. 8d). The relative collagen I expression of the 10% MeSDVC group was 84 and 92% less than that of the MeSDVC + DVC group at 1 and 2 weeks, respectively ($p < 0.05$). At 3 weeks and at 6 weeks, there were no significant differences among groups. From 1 day to 1 week, the relative collagen I expression of the 10% MeSDVC group decreased by 98% ($p < 0.05$) and did not change significantly thereafter. Finally, the relative collagen I expression level for the DVC-incorporating groups did not change significantly throughout the culture period (Fig. 8d).

### *Histological and Immunohistochemical Evaluation*

At 6 weeks, there were no discernable changes in any of the constructs other than the DVC-containing groups appeared to have a decreased cell density compared to their respective cell densities at day 1. However, throughout culture, the cells remained evenly distributed. Saf-O stained MeSDVC and DVC particles a dark red/orange color and the staining intensity of the DVC particles appeared to fade over the 6 weeks in culture (Fig. 9). All groups stained for collagen II, where the collagen II staining for the DVC-incorporated groups was slightly darker at 6 weeks compared to 1 week (Fig. 9). The 10% MeSDVC group had a slight increase in collagen I staining over the culture period, whereas the DVC-incorporating groups had a slight decrease in collagen I

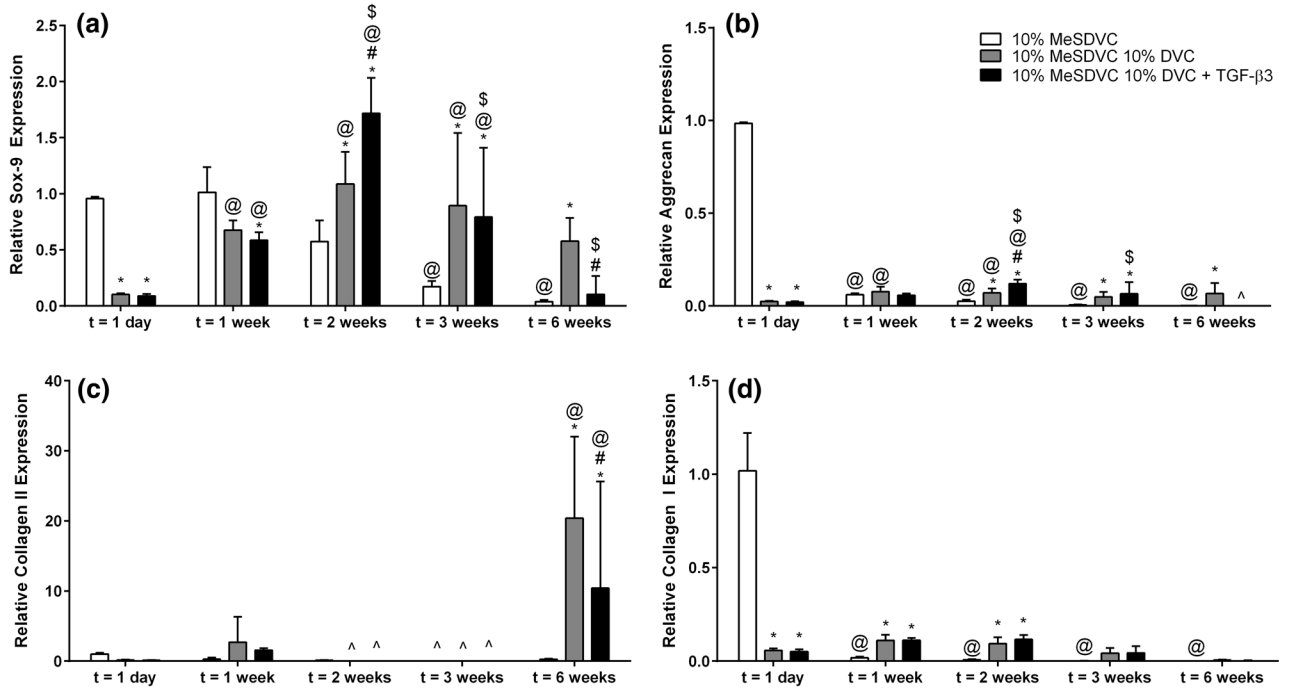


**FIGURE 8. Relative gene expression of (a) sox-9, (b) aggrecan, (c) collagen II, and (d) collagen I. From 2 weeks onward, the DVC-incorporating groups repeatedly outperformed the MeSDVC group in chondrogenic gene expression, especially at 6 weeks with collagen II. Data reported as mean + standard deviation ($n$ = 5); statistically significant from 10% MeSDVC at same time point ($^{*}p < 0.05$), statistically significant from 10% MeSDVC 10% DVC at same time point ($^{\#}p < 0.05$), statistically significant from same group at first time point ($^{@}p < 0.05$), statistically significant from same group at previous time point ($^{\$}p < 0.05$), ^below detectable limit.**

staining (Fig. 9). The MeSDVC + DVC group had the least amount of collagen I staining at 6 weeks. Aggrecan staining revealed a slight increase in aggrecan deposition in the 10% MeSDVC gels over the 6 week culture period (Fig. 9). Additionally, the MeSDVC + DVC group had an increase in aggrecan staining over the course of the 6 weeks (Fig. 9). Finally, no discernable changes in aggrecan staining was observed at 6 weeks for the MeSDVC + DVC + TGF-$\beta_3$ group compared to its aggrecan staining at day 1 (Fig. 9). Higher magnification images of H&E stains are shown in Supp. Fig. 1.

## DISCUSSION

To the best of our knowledge, we were the first group to create a covalently crosslinked hydrogel composed entirely of cartilage ECM and we are the first to additionally add cartilage matrix particles to give the hydrogel precursor a yield stress before crosslinking. Although the major focuses of hydrogel technologies are on hydrogels in their crosslinked form, our group additionally focuses on the fluid behavior of the hydrogel precursor solutions by fabricating colloidal gels instead, which are dynamically paste-like materials prior to crosslinking that can be molded into place and will 'set' after placement.[47] Colloidal gels are cohesive through disruptable particle interactions, we have previously shown that these gels are capable of successfully filling tissue defects, delivering bioactive signals, and promoting new tissue formation in non-load bearing cranial defect applications.[14,45,46,48] Preliminary work demonstrated that these colloidal gels did not retain their integrity over time in culture and recently, we published a method to combine colloidal gel systems with traditional crosslinked hyaluronic acid hydrogels to form a hydrogel suitable for load-bearing applications that exhibits a yield stress prior to crosslinking.[3] This yield stress, the threshold level where a solution transitions from an elastic solid to a pseudoplastic liquid, is crucial as it will enable a surgeon to mold and shape the material into the defect site without the concern that the material will flow or leak from the defect, which is a major concern for traditional hydrogel precursor solutions.[33,41]

In prior work from our group, only the colloidal solutions employing particulates were observed to exhibit a yield stress,[3] and therefore in the current study, it was hypothesized that particulates would be necessary to achieve a paste-like precursor solution. All samples tested in the current study, including the non-particulate samples composed of MeSDVC, were no-

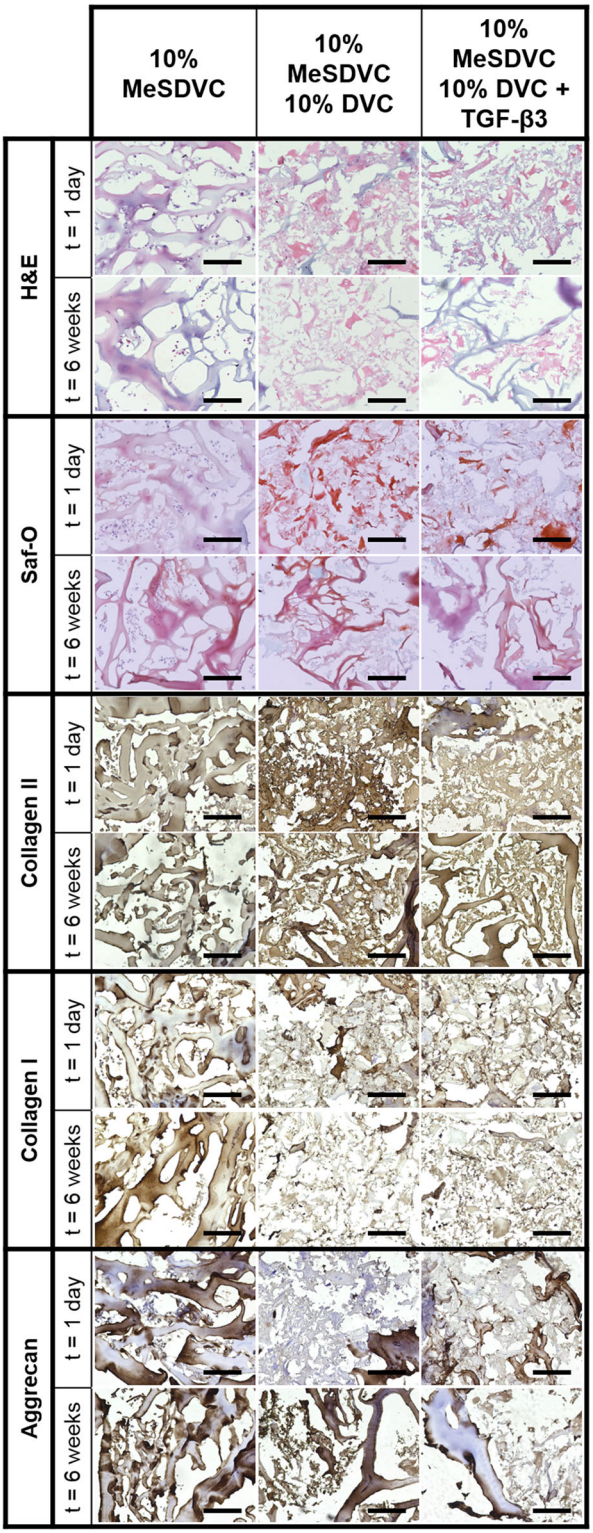


**FIGURE 9. Histological evaluation of gels. H&E staining revealed the cells remained evenly distributed throughout culture. MeSDVC and DVC particles were stained a dark red/orange color with Saf-O staining and the color of the DVC particles appeared to fade over the 6 weeks. A slight increase in collagen II staining was noted in the DVC-incorporating groups at 6 weeks. A slight increase in collagen I staining was observed for the 10% MeSDVC group at 6 weeks, whereas the DVC-incorporated groups had a slight decrease in staining at 6 weeks. A slight increase in aggrecan staining at 6 weeks was observed next to the cells of the 10% MeSDVC group. Additionally, the 10% MeSDVC 10% DVC group had a slight increase in aggrecan staining near the location of the rBMSCs at 6 weeks. Last, the 10% MeSDVC + 10% DVC + TGF-$\beta_3$ group had no discernable changes in aggrecan staining over the 6 weeks. Scale bars are 200 $\mu$m.**

ted to have a yield stress. Ten percent MeSDVC alone had a yield stress of over 700 Pa, and when it was combined with 10% DVC particles, which had a yield stress of only 58 Pa, the combined materials had a yield stress of over 1800 Pa. This synergistic effect was similarly noted in prior work,[3] which suggests that there may be some physical or chemical interactions between DVC and MeSDVC, which will be important to evaluate further in future work. For context, toothpaste, a common paste-like material, has a yield stress of approximately 200 Pa. Additionally, when cells were mixed in with the materials, the cells did not significantly affect the yield stress value, which is advantageous because these materials can be mixed with cells if necessary in a surgical context and would still allow for appropriate shaping and contouring. Although the MeSDVC + DVC precursor solutions are easily molded, shaped, and extruded through a syringe, there may be applications where the yield stress may need to be reduced. In this case, the concentrations of MeSDVC and DVC can be altered. Furthermore, future quantification of syringeability, the ability of a product to be successfully administered by a syringe, would be of value.

This is not the first time yield stress has been reported in hydrogel precursors for tissue engineering purposes. One group used a dual component, "dock-and-lock", self-assembling gelation mechanism to create shear-thinning, self-healing, and injectable hydrogels.[28] Elder *et al.*[15] reported a method to modify the viscosity of hyaluronic acid hydrogel precursors by attaching peptides that self-assemble into $\beta$-sheets onto the hyaluronic acid. Although methods to induce yield stress in hydrogel precursor solutions have been reported, to the best of our knowledge, this current study is the first example of inducing yield stress in hydrogel precursors with ECM-based materials.

ECM-materials were used in the current study to not only impart a yield stress on the materials, but to further make the material inherently chondroinductive. A few other groups have recently reported the chondroinductivity of cartilage ECM.[7,9,18,25,34,38] For example, Cheng *et al.*[8] developed a porous cartilage matrix composed of homogenized and then lyophilized

cartilage matrix, which induced chondrogenic differentiation even without growth factor supplementation. However, these matrices contracted *in vitro*, so the cartilage matrix was then further crosslinked with genipin and found the crosslinking degree affected matrix synthesis and cell-mediated contraction. Although at a 0.05% genipin concentration, they found that the materials did not exhibit contraction and were chondroinductive.[10] Rowland *et al.*[32] further studied the crosslinking of these matrices, where it was reported that the crosslinking method affected the chondrogenesis and matrix synthesis of MSCs. Visser *et al.*[43] solubilized cartilage ECM and functionalized it with methacrylate groups, and demonstrated for the first time that hydrogels could be formed from ECM materials. However, these materials were not found to significantly affect the chondrogenic differentiation of MSCs. In the current study, compared to MeSDVC alone, an upregulation of Sox-9 and aggrecan was noted at 2 weeks when rBMSCs were exposed to DVC particles, with or without TGF-$\beta_3$ supplementation. Furthermore, at 6 weeks, the DVC particle groups with and without TGF-$\beta_3$ had a relative collagen II expression that was 40- and 78-fold higher, respectively, than that of MeSDVC alone ($p < 0.05$). Additionally, collagen II expression of the DVC particle groups significantly increased over the culture period, whereas the relative collagen II expression of the MeSDVC group alone did not change. Although a significant increase was not observed in matrix production of the cells either histologically or through biochemical analysis, which could be either a result of simultaneous bioabsorption and remodeling of the ECM or the inability to detect any increase in matrix due to the large amount of matrix initially present in the hydrogel, a slight increase in collagen II staining was observed in the DVC particle groups, in agreement with the gene expression data. A slight increase in aggrecan staining was noted in the 10% MeSDVC group, and this group was noted to have a significantly higher aggrecan gene expression than the other groups at day 1. Although the TGF-$\beta_3$ group had no discernable changes in aggrecan staining over the 6 weeks, it had the highest aggrecan gene expression at 2 weeks. Even though at weeks 1 and 2, the relative collagen I expression in the DVC particle groups was higher, at day 1, the relative collagen I expression in the DVC particle groups was significantly lower. Furthermore, collagen I staining actually increased slightly in the MeSDVC group over the culture period, while it decreased in the DVC particle groups. Overall, the gene expression and histological data pointed toward the DVC particles as an important component for upregulating chondrogenic genes, even though the particles are not necessary for inducing a yield stress in these MeSDVC and DVC systems.

Not only are the particles likely necessary for chondrogenesis, but they are successful in reducing hydrogel contraction. Hydrogel contraction is a major concern for tissue engineering because it can cause disintegration with host tissue, which could potentially hinder successful cartilage regeneration and may even dislodge the hydrogel from the defect site.[10,43] In prior work, Guilak and colleagues observed how the crosslinking degree and method of crosslinking affected gel contraction.[10,32] However, in the current study, we observed that the inclusion of particles affected gel contraction and swelling. In the current study, gels composed only of MeSDVC contracted by 18% over the culture period, but the gels containing DVC particles did not have a significant change in volume.

Another important feature for hydrogels in cartilage tissue engineering is their ability to withstand mechanical loading and one major disadvantage of using natural materials is their decreased mechanical integrity.[40] The compressive modulus of hydrogels composed of natural materials are typically an order of magnitude less than that of native cartilage tissue,[40] which has a compressive elastic modulus ranging from 240 to 1000 kPa.[2] However, the biomechanical properties of cartilage can vary depending on parameters such as the method of testing, the cartilage zone depth, and the strain rate of testing.[20] In the study performed by Visser *et al.*,[43] the solubilized cartilage matrix was reinforced with methacrylated gelatin (GelMA); however, the biomechanics of the hydrogels, evaluated *via* the compressive modulus, still fell short of native cartilage tissue. Another group used poly($\varepsilon$-caprolactone) to reinforce solubilized cartilage ECM by combining them and then electrospinning them into a scaffold.[18] However, the Young's moduli of these scaffolds were only approximately 10 kPa. In the current study, although we only observed compressive moduli in the range from 70 to 170 kPa for all of the 10% MeSDVC and MeSDVC + DVC groups, we did observe a compressive modulus of approximately 675 kPa in the acellular 20% MeSDVC group, which is on the same order of magnitude as native articular cartilage. Because there was such a difference in the modulus from 20% MeSDVC to MeSDVC + DVC even though both gels had a solids content of 20%, future work will be necessary to determine ratios of DVC particles and MeSDVC that still allow for sufficient chondrogenesis and reduced contraction, while maintaining the appropriate biomechanics. Both of the 10% MeSDVC groups had a significant reduction in their mechanical properties over the course of the 6 weeks and at this

stage, it is unknown at what point this change in properties occurred. We hypothesize the change occurred early on in culture as the portions of the hydrogel that were not converted into the network leached out, and future work will try to discern how and why these mechanical properties changed throughout culture. However, it is possible that once these materials are implanted *in vivo*, the biomechanical stimulation may help increase matrix synthesis and improve the mechanical properties.[42]

Even though DVC particles may hinder the biomechanical performance and are not necessarily needed to induce a yield stress if using MeSDVC as a hydrogel material, we have still demonstrated that the particles are likely contributing to enhanced chondrogenesis and the elimination of hydrogel contraction. Because the DVC particles contain mostly unaltered cartilage ECM, other than the DVC particles are cryoground, they may retain more of the bioactivity of the cartilage matrix than MeSDVC since MeSDVC is altered cartilage ECM, where it contained 97% less DNA and 41% fewer GAGs than DVC. Proteoglycans, specifically aggrecan in cartilage matrix, are found extensively in native cartilage matrix and are thought to be a reservoir of several growth factors.[11,21] It can therefore be hypothesized that some of the growth factors inducing chondrogenesis within cartilage ECM may have been altered or removed in the processing of MeSDVC are retained in DVC.

Of interest is that the processing of MeSDVC appears to have removed much of the DNA content. It is uncertain at this time whether or not the DNA was altered and unable to be detected *via* the PicoGreen assay, or if the MeSDVC process actually decellularizes the tissue as well and future work will certainly need to address this issue. The low pH exposure during the solubilization process would likely denature the DNA to a single-stranded state, and would in addition hydrolyze and further degrade the DNA.[30] 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. Furthermore, a recent study found that pepsin, although previously known to only digest protein, is in addition, capable of digesting nucleic acids.[26] These results are consistent with our findings since there was no mass loss during the solubilization/pepsin digestion process. Therefore, future work will certainly need to address what immunological effects may occur by digesting tissues in pepsin. Currently, it is unknown whether decellularizing of cartilage tissue is necessary or to what exact degree cells must be removed to enable the material to be implanted *in vivo* without an adverse immunological response. For example, Zimmer's DeNovo® product, which is composed of living, allogeneic human cells, has no reports of allograft rejection or disease transmission and it has been observed to create hyaline-like cartilage in goats, where no T cell-mediated response was noted.[1] Therefore, because cartilage tissue may be immunoprivileged for osteoarthritis applications, and because decellularization can result in changes in matrix architecture and surface ligand landscape,[22] decellularization may not even be necessary for some cartilage tissue engineering applications.

In addition, it must be noted that the inclusion of cells may or may not be necessary for future clinical application of these materials. It is possible that a cell source could come from the subchondral bone if microfracture is performed prior to implanting the acellular pastes. Therefore, future work will also consider the ability of cells to migrate into and remodel the pastes to determine whether or not cell encapsulation is necessary.

In summary, we created a potentially chondroinductive hydrogel that is entirely composed of cartilage-derived ECM and we have shown that by mixing in DVC particles, we can modulate the yield stress of the hydrogel precursors and prevent contraction after crosslinking. Furthermore, these two-component gels induced chondrogenic gene expression after 2 weeks compared to MeSDVC gels alone, and they had reduced cell-mediated contraction. Although the focus of this current work was not to identify which chemical groups were modified during the methacrylation process, we hypothesize these modified groups were the carboxylic acid groups on collagen and the GAGs within the cartilage ECM. Quantifying and determining the exact chemical structure of MeSDVC will be a focus of upcoming work. In addition, future work will address combinations of DVC and MeSDVC components that yield mechanics closer to that of native tissue and will further address tissue integration and regeneration *in vivo*. Although major differences in biochemical content were not observed among groups, this current *in vitro* study was an important first step in elucidating cell response (i.e., establishing chondroinductivity, shape retention, etc.), which provides the confidence necessary to proceed to *in vivo* testing where these factors will be of tremendous importance for successful regeneration. Ultimately, the combination of these two cartilage-derived components is promising for cartilage tissue engineering applications.

## ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (doi:10.1007/s10439-015-1547-5) contains supplementary material, which is available to authorized users.

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## REFERENCES

[1]Adkisson, H. D., J. A. Martin, R. L. Amendola, C. Milliman, K. A. Mauch, A. B. Katwal, M. Seyedin, A. Amendola, P. R. Streeter, and J. A. Buckwalter. The potential of human allogeneic juvenile chondrocytes for restoration of articular cartilage. *Am. J. Sports Med.* 38:1324–1333, 2010.

[2]Armstrong, C., and V. Mow. Variations in the intrinsic mechanical properties of human articular cartilage with age, degeneration, and water content. *J. Bone Joint Surg.* 64:88–94, 1982.

[3]Beck, E. C., B. L. Lohman, D. B. Tabakh, S. L. Kieweg, S. H. Gehrke, C. J. Berkland, and M. S. Detamore. Enabling surgical placement of hydrogels through achieving paste-like rheological behavior in hydrogel precursor solutions. *Ann. Biomed. Eng.* 7:1–8, 2015.

[4]Benders, K., P. van Weeren, S. Badylak, D. Saris, W. Dhert, and J. Malda. Extracellular matrix scaffolds for cartilage and bone regeneration. *Trends Biotechnol.* 31:169–176, 2013.

[5]Brigham, M., A. Bick, E. Lo, A. Bendali, J. Burdick, and A. Khademhosseini. Mechanically robust and bioadhesive collagen and photocrosslinkable hyaluronic acid semi-interpenetrating networks. *Tissue Eng. A* 15:1645–1653, 2009.

[6]Burdick, J. A., R. L. Mauck, J. H. Gorman, 3rd, and R. C. Gorman. Acellular biomaterials: an evolving alternative to cell-based therapies. *Sci. Transl. Med.* 5:176ps4, 2013.

[7]Cha, M., S. Do, G. Park, P. Du, K.-C. Han, D. Han, and K. Park. Induction of re-differentiation of passaged rat chondrocytes using a naturally obtained extracellular matrix microenvironment. *Tissue Eng. A* 19:978–988, 2013.

[8]Cheng, N.-C., B. T. Estes, H. A. Awad, and F. Guilak. Chondrogenic differentiation of adipose-derived adult stem cells by a porous scaffold derived from native articular cartilage extracellular matrix. *Tissue Eng. A* 15:231–241, 2008.

[9]Cheng, N.-C., B. T. Estes, T.-H. Young, and F. Guilak. Engineered cartilage using primary chondrocytes cultured in a porous cartilage-derived matrix. *Regen. Med.* 6:81–93, 2011.

[10]Cheng, N.-C., B. Estes, T.-H. Young, and F. Guilak. Genipin-crosslinked cartilage-derived matrix as a scaffold for human adipose-derived stem cell chondrogenesis. *Tissue Eng. A* 19:484–496, 2013.

[11]Chun, S. Y., G. J. Lim, T. G. Kwon, E. K. Kwak, B. W. Kim, A. Atala, and J. J. Yoo. Identification and characterization of bioactive factors in bladder submucosa matrix. *Biomaterials* 28:4251–4256, 2007.

[12]Decaris, M., B. Binder, M. Soicher, A. Bhat, and J. Leach. Cell-derived matrix coatings for polymeric scaffolds. *Tissue Eng. A* 18:2148–2157, 2012.

[13]DeKosky, B., N. Dormer, G. Ingavle, C. Roatch, J. Lomakin, M. Detamore, and S. Gehrke. Hierarchically designed agarose and poly(ethylene glycol) interpenetrating network hydrogels for cartilage tissue engineering. *Tissue Eng. C* 16:1533–1542, 2010.

[14]Dennis, S., M. Detamore, S. Kieweg, and C. Berkland. Mapping glycosaminoglycan-hydroxyapatite colloidal gels as potential tissue defect fillers. *Langmuir* 30:3528–3537, 2014.

[15]Elder, A., N. Dangelo, S. Kim, and N. Washburn. Conjugation of $\beta$-sheet peptides to modify the rheological properties of hyaluronic acid. *Biomacromolecules* 12:2610–2616, 2011.

[16]Elisseeff, J., C. Puleo, F. Yang, and B. Sharma. Advances in skeletal tissue engineering with hydrogels. *Orthod. Craniofac. Res.* 8:150–161, 2005.

[17]Freytes, D. O., J. Martin, S. S. Velankar, A. S. Lee, and S. F. Badylak. Preparation and rheological characterization of a gel form of the porcine urinary bladder matrix. *Biomaterials* 29:1630–1637, 2008.

[18]Garrigues, N. W., D. Little, J. Sanchez-Adams, D. S. Ruch, and F. Guilak. Electrospun cartilage-derived matrix scaffolds for cartilage tissue engineering. *J. Biomed. Mater. Res. A* 102:3998–4008, 2014.

[19]Gershlak, J. R., J. I. Resnikoff, K. E. Sullivan, C. Williams, R. M. Wang, and L. D. Black. 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. *Biochem. Biophys. Res. Commun.* 439:161–166, 2013.

[20]Huang, C.-Y., A. Stankiewicz, G. A. Ateshian, and V. C. Mow. Anisotropy, inhomogeneity, and tension–compression nonlinearity of human glenohumeral cartilage in finite deformation. *J. Biomech.* 38:799–809, 2005.

[21]Kanematsu, A., S. Yamamoto, M. Ozeki, T. Noguchi, I. Kanatani, O. Ogawa, and Y. Tabata. Collagenous matrices as release carriers of exogenous growth factors. *Biomaterials* 25:4513–4520, 2004.

[22]Keane, T. J., I. T. Swinehart, and S. F. Badylak. Methods of tissue decellularization used for preparation of biologic scaffolds and in vivo relevance. *Methods* 84:25–34, 2015.

[23]Khanlari, A., M. S. Detamore, and S. H. Gehrke. Increasing cross-linking efficiency of methacrylated chon-

droitin sulfate hydrogels by copolymerization with oligo (ethylene glycol) diacrylates. *Macromolecules* 46:9609–9617, 2013.
[24]Kwon, J. S., S. M. Yoon, S. W. Shim, J. H. Park, K. J. Min, H. J. Oh, J. H. Kim, Y. J. Kim, J. J. Yoon, and B. H. Choi. Injectable extracellular matrix hydrogel developed using porcine articular cartilage. *Int. J. Pharm.* 454:183–191, 2013.
[25]Levorson, E., O. Hu, P. Mountziaris, F. Kasper, and A. Mikos. Cell-derived polymer/extracellular matrix composite scaffolds for cartilage regeneration, Part 2: Construct devitalization and determination of chondroinductive capacity. *Tissue Eng. C* 20:358–372, 2014.
[26]Liu, Y., Y. Zhang, P. Dong, R. An, C. Xue, Y. Ge, L. Wei, and X. Liang. Digestion of nucleic acids starts in the stomach. *Sci. Rep.* 5:11936, 2015.
[27]Livak, K. J., and T. D. Schmittgen. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25:402–408, 2001.
[28]Lu, H., M. Charati, I. Kim, and J. Burdick. Injectable shear-thinning hydrogels engineered with a self-assembling Dock-and-Lock mechanism. *Biomaterials* 33:2145–2153, 2012.
[29]Mansour, J. M. Biomechanics of cartilage. In: Kinesiology: The Mechanics and Pathomechanics of Human Movement, edited by C. A. Oatis. Baltimore: Lippincott Williams & Wilkins, 2003, pp. 66–79.
[30]McLennan, A., A. Bates, P. Turner, and M. White. BIOS Instant Notes in Molecular Biology. New York: Taylor & Francis, 2012.
[31]Renth, A. N., and M. S. Detamore. Leveraging "raw materials" as building blocks and bioactive signals in regenerative medicine. *Tissue Eng. B* 18:341–362, 2012.
[32]Rowland, C., D. Lennon, A. Caplan, and F. Guilak. The effects of crosslinking of scaffolds engineered from cartilage ECM on the chondrogenic differentiation of MSCs. *Biomaterials* 34:5802–5812, 2013.
[33]Rughani, R. V., M. C. Branco, D. J. Pochan, and J. P. Schneider. De novo design of a shear-thin recoverable peptide-based hydrogel capable of intrafibrillar photopolymerization. *Macromolecules* 43:7924–7930, 2010.
[34]Schwarz, S., A. F. Elsaesser, L. Koerber, E. Goldberg-Bockhorn, A. M. Seitz, C. Bermueller, L. Dürselen, A. Ignatius, R. Breiter, and N. Rotter. Processed xenogenic cartilage as innovative biomatrix for cartilage tissue engineering: effects on chondrocyte differentiation and function. *J. Tissue Eng. Regen. Med.* 2012. doi: 10.1002/term.1650.
[35]Schwarz, S., L. Koerber, A. F. Elsaesser, E. Goldberg-Bockhorn, A. M. Seitz, L. Durselen, A. Ignatius, P. Walther, R. Breiter, and N. Rotter. Decellularized cartilage matrix as a novel biomatrix for cartilage tissue-engineering applications. *Tissue Eng. A* 18:2195–2209, 2012.
[36]Seif-Naraghi, S. B., D. Horn, P. J. Schup-Magoffin, and K. L. Christman. Injectable extracellular matrix derived hydrogel provides a platform for enhanced retention and delivery of a heparin-binding growth factor. *Acta Biomater.* 8:3695–3703, 2012.
[37]Seif-Naraghi, S. B., M. A. Salvatore, P. J. Schup-Magoffin, D. P. Hu, and K. L. Christman. Design and characterization of an injectable pericardial matrix gel: a potentially autologous scaffold for cardiac tissue engineering. *Tissue Eng. A* 16:2017–2027, 2010.
[38]Sutherland, A. J., E. C. Beck, S. C. Dennis, G. L. Converse, R. A. Hopkins, C. J. Berkland, and M. S. Detamore. Decellularized cartilage may be a chondroinductive material for osteochondral tissue engineering. *PLoS One* 10:e0121966, 2015.
[39]Sutherland, A. J., G. L. Converse, R. A. Hopkins, and M. S. Detamore. The bioactivity of cartilage extracellular matrix in articular cartilage regeneration. *Adv. Healthc. Mater.* 4:29–39, 2015.
[40]Tatman, P. D., W. Gerull, S. Sweeney-Easter, J. I. Davis, D.-H. Kim, and A. Gee. Multi-scale biofabrication of articular cartilage: bioinspired and biomimetic approaches. *Tissue Eng. B* 21:543–559, 2015.
[41]Todd, R. H., and S. K. Daniel. Hydrogels in drug delivery: progress and challenges. *Polymer* 49:1993–2007, 2008.
[42]Villanueva, I., C. A. Weigel, and S. J. Bryant. Cell–matrix interactions and dynamic mechanical loading influence chondrocyte gene expression and bioactivity in PEG-RGD hydrogels. *Acta Biomater.* 5:2832–2846, 2009.
[43]Visser, J., P. A. Levett, N. C. te Moller, J. Besems, K. W. Boere, M. H. van Rijen, J. C. de Grauw, W. J. Dhert, P. R. van Weeren, and J. Malda. Crosslinkable hydrogels derived from cartilage, meniscus, and tendon tissue. *Tissue Eng. A* 21:1195–1206, 2015.
[44]Wan, Y. S., S. Wei-Heng, and A. A. Ilhan. Elastic and yield behavior of strongly flocculated colloids. *J. Am. Ceram. Soc.* 82:616–624, 2004.
[45]Wang, Q., Z. Gu, S. Jamal, M. S. Detamore, and C. Berkland. Hybrid hydroxyapatite nanoparticle colloidal gels are injectable fillers for bone tissue engineering. *Tissue Eng. A* 19:2586–2593, 2013.
[46]Wang, Q., S. Jamal, M. Detamore, and C. Berkland. PLGA-chitosan/PLGA-alginate nanoparticle blends as biodegradable colloidal gels for seeding human umbilical cord mesenchymal stem cells. *J. Biomed. Mater. Res. A* 96:520–527, 2011.
[47]Wang, Q., L. Wang, M. S. Detamore, and C. Berkland. Biodegradable colloidal gels as moldable tissue engineering scaffolds. *Adv. Mater.* 20:236–239, 2008.
[48]Wang, Q., J. Wang, Q. Lu, M. Detamore, and C. Berkland. Injectable PLGA based colloidal gels for zero-order dexamethasone release in cranial defects. *Biomaterials* 31:4980–4986, 2010.
[49]Yang, Z., Y. Shi, X. Wei, J. He, S. Yang, G. Dickson, J. Tang, J. Xiang, C. Song, and G. Li. Fabrication and repair of cartilage defects with a novel acellular cartilage matrix scaffold. *Tissue Eng. C* 16:865–876, 2010.