Published in final edited form as:

Biomaterials. 2016 June ; 91: 57–72. doi:10.1016/j.biomaterials.2016.03.012.

Fabrication of anatomically-shaped cartilage constructs using decellularized cartilage-derived matrix scaffolds

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Abstract

The native extracellular matrix of cartilage contains entrapped growth factors as well as tissue-specific epitopes for cell-matrix interactions, which make it a potentially attractive biomaterial for cartilage tissue engineering. A limitation to this approach is that the native cartilage extracellular matrix possesses a pore size of only a few nanometers, which inhibits cellular infiltration. Efforts to increase the pore size of cartilage-derived matrix (CDM) scaffolds dramatically attenuate their mechanical properties, which makes them susceptible to cell-mediated contraction. In previous studies, we have demonstrated that collagen crosslinking techniques are capable of preventing cell-mediated contraction in CDM disks. In the current study, we investigated the effects of CDM concentration and pore architecture on the ability of CDM scaffolds to resist cell-mediated contraction. Increasing CDM concentration significantly increased scaffold mechanical properties, which played an important role in preventing contraction, and only the highest CDM concentration (11% w/w) was able to retain the original scaffold dimensions. However, the increase in CDM concentration led to a concomitant decrease in porosity and pore size. Generating a temperature gradient during the freezing process resulted in unidirectional freezing, which aligned the formation of ice crystals during the freezing process and in turn produced aligned pores in CDM scaffolds. These aligned pores increased the pore size of CDM scaffolds at all CDM concentrations, and greatly facilitated infiltration by mesenchymal stem cells (MSCs). These methods were used to fabricate of anatomically-relevant CDM hemispheres. CDM hemispheres with aligned pores supported uniform MSC infiltration and matrix deposition. Furthermore, these CDM hemispheres retained their original architecture and did not contract, warp, curl, or splay throughout the entire 28-day culture period. These findings demonstrate that given the appropriate fabrication parameters, CDM scaffolds are capable of maintaining complex structures that support MSC chondrogenesis.

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Keywords

articular cartilage; decellularization; tissue engineering; mesenchymal stem cell; cell-mediated contraction; ice-templating

Introduction

In the field of cartilage tissue engineering, there is a growing interest in using the native cartilage extracellular matrix as a scaffold biomaterial due to its ability to retain active growth factors [1] as well as to provide cartilage-specific epitopes for cell-matrix interactions. Since initial reports that chondrocytes were capable of bonding devitalized articular cartilage slices [2], several studies have revealed that cartilage extracellular matrix extract can promote cartilage-specific differentiation of embryonic stem cells [3] and prevented dedifferentiation of chondrocytes [4, 5]. The chondroinductive properties of cartilage-derived matrix (CDM) scaffolds have been demonstrated in a variety of cell types including: adipose-derived [6–11], synovium-derived [12], infrapatellar fat pad-derived [13, 14], and bone marrow-derived stem cells (MSCs) [1, 7, 15–23], as well as chondrocytes [2, 21, 24–28]. Of particular note is the finding that, depending on the cell type, CDM can promote chondrogenic differentiation in the absence of exogenous growth factors [1, 6, 9, 12, 20, 24, 25], or exhibit a synergistic effect with growth factor supplementation [1, 7, 11–13, 18, 27, 28]. CDM has also been shown to enhance the *in vivo* repair of cartilage defects [8, 10, 16, 19, 26]. Furthermore, studies comparing collagen-hyaluronic acid scaffolds to CDM constructs illustrated that CDM constructs retained newly synthesized glycosaminoglycans better than collagen-hyaluronic acid scaffolds [13].

In order to mitigate potential immunogenic responses towards foreign cellular material, most native tissue biomaterials are decellularized [12, 15, 19, 22, 29–33]. To facilitate the removal of cellular debris, CDM is often pulverized into a fine power [12, 32], which can then be fabricated into a porous scaffold [14, 19, 21, 26]. While this processing improves decellularization and enhances repopulation with seeded cells, these treatments dramatically reduce the mechanical properties of CDM scaffolds, making them susceptible to cell-mediated contraction [6, 7, 9, 12–14, 18, 24, 34]. This contraction unpredictably alters the shape of CDM constructs and limits the space available for cellular proliferation and matrix deposition.

Previous studies have implemented chemical crosslinking treatments [8, 9, 13–18, 23, 26] or reinforced CDM with synthetic polymers [34] to mitigate cell-mediated contraction. Physical crosslinking techniques such as dehydrothermal treatment [18, 35, 36] or ultraviolet irradiation [18, 19, 21, 35, 36] have also been applied to prevent cell-mediated contraction of CDM scaffolds. Furthermore, these physical crosslinking treatments have been shown to preserve epitopes that participate in cell-matrix interactions, and supported greater chondrogenic differentiation than chemically crosslinked scaffolds [18]. Therefore in the current study, we sought to minimize the manipulation of the native cartilage extracellular matrix by only using dehydrothermal treatment to minimally crosslink CDM constructs.

In this study, we developed a method for fabrication of anatomically relevant, hemispherical CDM constructs seeded with human MSCs. To enhance the mechanical properties of these constructs, we altered the CDM concentration [14] and pore architecture [23] of the CDM scaffolds. Previous studies demonstrated that increasing CDM concentration dramatically increased the compressive modulus of CDM constructs [14]. However, increasing CDM concentration also corresponded with a concomitant decrease in pore size, which restricted cells to the surface of CDM constructs at high CDM concentrations [14]. Aligning the pores of CDM scaffolds via unidirectional freezing has also been shown to enhance the compressive modulus of CDM constructs and facilitate cellular infiltration resulting in uniform cellular distribution [23]. While these studies elucidated the prominent roles of CDM concentration and pore architecture in governing the mechanical properties of CDM scaffolds, each group examined these variables separately and both studies required chemical crosslinking treatments in order to prevent cell-mediated contraction. The current study investigated the synergistic effect of CDM concentration and pore architecture on the mechanical properties of CDM scaffolds, their ability to prevent cell-mediated contraction, and their influence on cellular infiltration. Using this defined scaffold fabrication method, we created anatomically-shaped CDM hemispheres that were seeded with MSCs and underwent chondrogenic differentiation in vitro.

Methods

Preparation of Scaffolds

Articular cartilage was harvested from the femoral condyles of freshly slaughtered, skeletally-mature (over 18 months of age), female pigs (n = 200). Cartilage was shaved off of the bone in large pieces, frozen overnight at -80°C , and lyophilized (Freezone 2.5L, Labconco, Kansas City, MO) for 24 h. Lyophilized cartilage was pulverized into a fine powder using a 6770 freezer/mill (SPEX SamplePrep, Metuchen, NJ). Cartilage was precooled for 3 min prior to pulverization at 5 Hz for 10 cycles of 1 min run, 1 min cool. Cartilage powder was treated with 10 mM Tris-HCl (pH 7.5) containing 2.5 mM MgCl_2 , 0.5 mM CaCl_2 , and 50 U/mL DNase I (Sigma, St. Louis, MO) at a ratio of 20 mL decellularization solution per 1 gram cartilage powder for 24 h at 37°C , adapted from [15]. In order to preserve the GAG content of the cartilage powder, decellularized CDM was immediately frozen and lyophilized after DNase treatment. The lyophilized CDM was pulverized again using the settings described above to form a fine powder, which was then sieved through a mesh with a 97 μm mesh size to ensure that all particles were at most 97 μm in one dimension. Decellularized CDM powder from each pig joint was combined to form a single superlot of powder (n = 200) that was used for all experiments. In order to produce CDM concentrations of 11%, 10%, 9%, 8%, and 7% weight/weight, cartilage powder was weighed into aliquots of 1.1, 1.0, 0.9, 0.8, or 0.7 g, respectively, then distilled water was added to each until a final weight of 10 g was reached. Cartilage powder was suspended in distilled water using a homogenizer (PRO260, PRO Scientific Inc., Oxford, CT). Cartilage was homogenized for five cycles of 2 minutes homogenization at 30,000 rpm and cooling 2 minutes on ice to prevent overheating. Homogenized cartilage was pipetted into one of the following two-part delrin-silicone molds: 1) discs 6 mm in diameter, 2 mm deep with a flat silicone lid 2) hemispheres having an outer radius of 4.76mm with a silicone

lid containing hemisphere protrusions to generate an inner radius of 3.175mm (Fig. 1). In order to produce aligned pores, the two-part molds were placed directly into a -80°C freezer and frozen overnight. Since the silicone lids were much thinner than the delrin molds, they froze first producing a temperature gradient during the freezing process, which aligned the ice crystals and thus the pores in the scaffold [23, 37]. In order to produce uniform pores, the two-part molds were placed in a Styrofoam container filled with isopropyl alcohol and then placed in a -80°C freezer, which cooled both halves of the mold at the same rate, thus removing the temperature gradient. After freezing overnight, the silicone lids were removed and scaffolds were lyophilized (Freezone 2.5L) for 24 h. After lyophilization, scaffolds were crosslinked and sterilized via dehydrothermal treatment by heating scaffolds in a dry climate at 120°C for 24 h.

Porosity and Pore Size Measurement

CDM discs were scanned using micro-computed tomography (micro-CT) (SkyScan 1176, Bruker, Billerica, MA) at 40 kV, 600 μA , 16.67 μm isotropic spatial resolution. Micro-CT datasets were reconstructed with NRecon software (Bruker) using a dynamic range of 0.0171, ring artifact correction of 11, and beam hardening correction of 20%. Reconstructed images were binarized using thresholds that were calibrated to the Archimedes-based volume fractions as described previously [38]. Porosity and pore size were calculated via image processing executed with CT-Analyzer software (Bruker).

Analysis of Pore Architecture

CDM scaffolds were sputter-coated (Desk IV, Denton Vacuum, Moorestown, NJ) with gold at 18 mA for 600 seconds, which resulted in a gold sputter deposition of 20 nm in thickness. Coated samples were scanned (FEI XL30 ESEM, Hillsboro, OR) at an accelerating voltage of 30 kV. For scaffolds seeded with cells prior to sputter coating, constructs were fixed in 2.5% glutaraldehyde (Electron Microscope Sciences, Hatfield, PA) for 15 minutes, dehydrated in a graded ethanol series to 100% ethanol, and critical point dried using hexamethyldisilazane (Electron Microscope Sciences).

Cell Culture

Bone marrow was obtained in concordance with an approved Institutional Review Board exemption as discarded and de-identified waste tissue from adult bone marrow transplant donors at Duke University Medical Center. MSCs were expanded in DMEM-low glucose (Gibco, Grand Island, NY) supplemented with L-glutamine, sodium pyruvate, 1 ng/mL bFGF (Roche, Florence, SC), 1% penicillin-streptomycin (Gibco), and 10% lot-selected FBS (HyClone, Logan, UT). After passage two, MSCs from three donors (out of 11 donors collected) were combined in equal numbers to form a superlot and expanded together through passage four. Passage four MSCs were trypsinized, counted, and suspended in expansion medium at a density of $8.33 \times 10^6/\text{mL}$ for CDM discs and a density of $6.67 \times 10^6/\text{mL}$ for CDM hemispheres. The disc and hemisphere molds (Fig. 1) were sterilized in 70% ethanol for 15 min and used to hold the CDM scaffolds in order to prevent flipping during the seeding process. CDM discs were calculated to have a saturation volume of 60 μL , and were seeded by pipetting 30 μL of cell suspension (8.33×10^6 cells/mL) directly onto each side of the disc for a total volume of 60 μL (500,000 cells). CDM hemispheres were calculated to have

a saturation volume of 150 μ L, and were seeded by pipetting 75 μ L of cell suspension (6.67×10^6 cells/mL) directly onto each side of the hemisphere for a total volume of 150 μ L (1,000,000 cells). After seeding, constructs were placed in a vacuum chamber and pulled under vacuum for 60 seconds in order to facilitate cellular infiltration as described in previous studies [39]. Seeded constructs were placed in 24-well low attachment plates (Corning Life Sciences, Corning, NY), and cells were allowed 1 hr to attach before adding 1 mL of expansion medium for discs and 2 mL of expansion medium for hemispheres. Cells were expanded on the CDM scaffolds by culturing constructs in expansion medium for 6 days, and media was changed every 48 h. After 6 days of expansion, constructs were switched to chondrogenic medium consisting of DMEM-HG (Gibco), 1% pen/strep (Gibco), 50 μ g/mL L-ascorbic acid 2-phosphate (Sigma), 40 μ g/mL L-proline (Sigma), 1% ITS +Premix (Collaborative Biomedical-Becton Dickinson, Bedford, MA), 100 nM dexamethasone (Sigma), and 10 ng/mL human TGF- β 3 (R&D Systems, Minneapolis, MN). Constructs were harvested for mechanical testing, area determination, biochemical analysis, histology, and immunohistochemistry after 6 days of expansion (Day 0) and after 28 days of chondrogenic differentiation.

Cellular Infiltration During Seeding

MSCs in monolayer were cultured in with polybrene (4 μ g/mL, Sigma) and lentiviral vectors containing a green fluorescent protein (GFP) plasmid. Centrifugation at 1,200G for 30 minutes was used to facilitate transduction. Successful transduction was verified via confocal microscopy using 488 nm excitation (LSM 510, Zeiss, Thornwood, NY, USA). The vacuum seeding technique described above resulted in successful seeding of GFP-expressing MSCs in CDM discs to achieve a final density of 500,000 cells/scaffold. Cells were allowed 1 hour to attach. Two PBS washes were used to remove non-adherent cells. Using a surgical blade, constructs were transversely bisected and their cross-sections were imaged at 488 nm excitation (LSM 510).

Area Determination

Immediately after culture, scaffolds were removed from wells and photographed. ImageJ was used to analyze and quantify the projected area of the scaffolds from the pictures.

Biochemical Analysis

One mL of papain buffer [125 μ g/mL papain (Sigma), 100 mM phosphate buffer, 10 mM cysteine, and 10mM EDTA, pH 6.3] was used to digest day 0 and day 28 biochemical samples (n=6 per group) for 16 hr at 65°C. Using the PicoGreen fluorescent double-stranded DNA assay (Invitrogen/Molecular Probes, Carlsbad, California), DNA content was measured fluorometrically (excitation wavelength, 485nm; emission wavelength, 535 nm). The dimethylmethylene blue assay [40] was used to quantify GAG content against a bovine chondroitin sulfate standard. Total collagen content was analyzed by quantifying the hydroxyproline content of the scaffolds after alkaline hydrolysis and reaction with chloramine-T and p-dimethylaminobenzaldehyde, using a ratio of 7.46 mg collagen to 1 mg of hydroxyproline [41].

Mechanical Testing

An ELF 3200 Series materials testing system (Bose, Minnetonka, MN) was used to perform stress-relaxation experiments in an unconfined-compression configuration. Unseeded and day-28 mechanical samples (n=6 per group) were cylindrical (6mm diameter, 2mm height) in shape. Test specimens were placed in a PBS bath and compressive loads were applied using a solid piston 9mm in diameter. Following equilibration of a 0.5 gf tare load, strains of $\epsilon = 0.04, 0.08, 0.12,$ and 0.16 were applied to the specimens. Strain steps were held constant for 1200s allowing the scaffolds to relax to an equilibrium level. Young's modulus (E) was determined by performing linear regression on the resulting equilibrium stress-strain plot.

Actin Staining, Histology, and Immunohistochemistry

For actin staining, histology, and immunohistochemistry, constructs (n=2 per group) were fixed overnight at 4°C in a pH 7.4 solution containing 4% paraformaldehyde and 100 mM sodium cacodylate. After fixing, constructs were cut in half in order to expose their cross-sections. Samples were treated with 0.2% Triton X-100 (Sigma) to permeabilize cell membranes. Alexa Fluor 488® Phalloidin (Life Technologies, Grand Island, NY) was used to stain specifically for filamentous actin (F-actin). Stained samples were visualized using 488 nm excitation with confocal microscopy (LSM 510). After confocal imaging, constructs were taken through a series of increasing ethanol solutions and xylene steps to clear the constructs prior to paraffin embedding. Embedded samples were cut into 5 μm sections. Sections were treated with pepsin (Digest-All; Zymed, San Francisco, CA) to expose epitopes for collagen staining. After epitope retrieval, sections were blocked with 10% goat serum (Histostain® Plus Broad Spectrum kit; Invitrogen, Carlsbad, California) to prevent non-specific staining. Samples were then treated with one of the following monoclonal antibodies: type I collagen (ab90395; Abcam, Cambridge, MA) (1:800 dilution), type II collagen (II-II6B3; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) (1:1 dilution), or type X collagen (C7974; Sigma-Aldrich) (1:200 dilution) overnight at 4°C. The anti-mouse IgG biotinylated secondary antibody (ab97021; Abcam, Cambridge, MA) (1:500 dilution) was then linked to horseradish peroxidase and reacted with aminoethyl carbazole (Histostain® Plus Broad Spectrum kit). Histological staining using 0.1% aqueous Safranin-O, 0.02% fast-green, and hematoxylin was also performed on xylene-cleared sections. Sections were also stained using Picro-Sirius Red Staining Kit (ScyTek Laboratories, Logan, UT) and viewed under polarized light to visualize aligned collagen [42]. Human osteochondral plugs were obtained in concordance with an approved Institutional Review Board exemption as discarded and de-identified waste tissue from adult donors undergoing total knee replacement surgery at Duke University Medical Center. The human osteochondral plugs were prepared in the same manner as samples and were used as positive controls for each antibody. Negative controls without primary antibody were also prepared for each slide.

Statistical Analysis

Two-factor analysis of variance (ANOVA) and Fisher's protected least significant difference (PLSD) *post hoc* test ($\alpha = 0.05$) were used to determine significance for each treatment

condition. Only differences that were statistically significant at this level are presented in the results section.

Results

Quantitative, micro-CT analysis revealed scaffold porosity decreased with increasing CDM concentration (Fig. 2B). However, porosity was not affected by pore architecture: uniform versus aligned pores (Fig. 2B). Pore size also decreased with increasing CDM concentration (Fig. 2C). Furthermore at each CDM concentration, scaffolds with uniform pores possessed smaller pores than respective scaffolds with aligned pores (Fig. 2C). The 3-D micro-CT images illustrated the large grooves present in the scaffolds with aligned pores (Supplemental Fig. 1).

SEM imaging showed a dramatic difference in pore architecture between CDM scaffolds with uniform versus aligned pores (Fig. 3). Uniform pores were small, round, and evenly distributed; whereas, aligned pores presented as large grooves that penetrated into the center of CDM constructs (Fig. 3). The transverse sections of CDM scaffolds seeded with GFP-expressing MSCs visualized cellular infiltration 1 hr after seeding (Fig. 3). In constructs with uniform pores, MSCs were restricted to the surfaces of constructs; however, constructs with aligned pores exhibited cellular infiltration into the center of constructs at all CDM concentrations (Fig. 3). Furthermore in the 10%, aligned, CDM constructs, the cellular infiltration mimicked the pattern produced by the aligned grooves (Fig. 3). At 24 hrs after cell seeding, scaffolds with higher CDM concentrations resulted in cells spreading to form a sheet over the surface of the constructs; however, at lower CDM concentrations cells remained rounded (Supplemental Fig. 2).

Gross images of unseeded scaffolds showed that all constructs possessed uniform dimensions prior to cell seeding (Fig. 4). In the unseeded constructs, the large grooves of the scaffolds with aligned pores were visible without magnification (Fig. 4). Minimal matrix synthesis was observed after the 6-day, cell-expansion period (Fig. 4). By day 28 of culture, all cell-seeded constructs showed a white, opaque appearance, and a smooth, shiny texture (Fig. 4). Cell-free (CF) scaffolds did not demonstrate any matrix accumulation (Fig. 4). Only the 11% CDM concentration scaffolds retained their original dimensions throughout the 28-day culture period, while scaffolds with lower CDM concentrations contracted over the course of chondrogenic culture (Fig. 4 & 5A).

After 6 days of cell-expansion on the CDM discs, which corresponded to the “day 0” of chondrogenic induction, there was no difference in DNA content (1.7 $\mu\text{g}/\text{construct}$) across any of the treatment groups (Supplemental Fig. 3A). After 28 days of chondrogenic differentiation, all constructs exhibited cellular proliferation relative to day 0 (Fig. 5B). However, the 7% CDM concentration group possessed the lowest degree of cellular proliferation (Fig. 5B). Since the CDM scaffolds were fabricated from native cartilage, which is rich in glycosaminoglycans (GAG) and collagens, the GAG (Fig. 5C) and collagen (Fig. 5D) data largely followed the dry weight of the CDM scaffolds. After 28 days of chondrogenic culture, all constructs possessed higher GAG (Fig. 5C) and collagen (Fig. 5D) contents compared to their respective day 0 controls (Supplemental Fig. 3B & 3C). After 28

days, unseeded cell-free constructs possessed less GAG (Supplemental Fig. 3D) but maintained their collagen content (Supplemental Fig. 3E) compared to their respective day 0 controls (Supplemental Fig. 3B & 3C). DNase treatment dramatically reduced foreign DNA from the CDM, while preserving the native GAG content (Supplemental Fig. 4). All scaffolds used in this study were fabricated from decellularized CDM.

For unseeded constructs, CDM concentration and pore architecture had profound effects on scaffold mechanical properties (Fig. 6). Compressive moduli dramatically increased with increasing CDM concentration (Fig. 6). Furthermore at each CDM concentration, uniform pores yielded substantially higher compressive moduli compared to respective constructs with aligned pores (Fig. 6). After 28 days of chondrogenic culture, 7% constructs possessed higher compressive moduli than 11% and 9% constructs, and there was no difference between scaffolds with aligned or uniform pores (Fig. 6).

F-actin staining revealed even cell-distribution throughout the entire thickness of all constructs (Fig. 7). Seeded MSCs produced mature cartilaginous matrix that stained positive for GAG and type II collagen, and completely filled the pores of the CDM scaffolds (Fig. 7). Unseeded, cell-free constructs did stain for GAG; however, the porcine cartilage that composed the CDM scaffolds did not stain for type I, II, or X collagen (Supplemental Fig. 5). Since the type II collagen antibody preferentially stained human type II collagen, the remnants of the CDM discs, which were fabricated from porcine cartilage, appeared as white holes amongst the intensely stained, newly synthesized matrix from human MSCs (Fig. 7). Type I collagen staining was most intense in 7% scaffolds (Fig. 7). Type X collagen was not detected in any sample (Fig. 7). Collagen of the CDM scaffold, derived from native cartilage, possessed alignment capable of being visualized under polarized light (Fig. 7). While the newly synthesized matrix stained positive for type II collagen, it possessed a limited capacity to be visualized under polarized light, and dark holes in the scaffolds colocalized with regions of new matrix deposition (Fig. 7).

SEM images of 11% CDM concentration hemispheres with aligned pores not only illustrated the convex and concave architecture of the hemispheres, but also visualized the large, aligned grooves on both the convex and concave surfaces of the hemispheres (Fig. 8 & Supplemental Fig. 6). Gross pictures demonstrated that the 11% CDM hemispheres were able to maintain the hemispherical shape throughout chondrogenic culture, and did not contract, curl, warp, or splay (Fig. 8). By day-28, hemispheres adopted smooth, glistening surfaces, which indicated new cartilaginous matrix deposition (Fig. 8).

Quantitative analysis of the circular area of the CDM hemispheres verified that the hemispheres did not contract and retained their original dimensions (Fig 9A). MSCs proliferated over the course of the 28-day chondrogenic culture (Fig. 9B). CDM hemispheres maintained their GAG and collagen content (Fig. 9C & 9D). At day 28, F-actin staining revealed that MSCs evenly distributed throughout the full-thickness of the hemispheres (Fig. 10). MSCs synthesized mature cartilaginous matrix that completely filled the pores of the hemispheres and stained positive for GAG and type II collagen (Fig. 10). Again since the type II collagen antibody did not stain the porcine cartilage in cell-free CDM constructs (Supplemental Fig. 5), the remnants of the porcine CDM hemispheres

appeared as white holes amongst the intensely stained, newly deposited matrix (Fig. 10). While the newly synthesized cartilaginous tissue stained positive for type I collagen, there were no signs of type X collagen staining (Fig. 10). While the aligned collagen of the CDM hemispheres was visible under polarized light, the newly synthesized matrix was generally undetectable, and dark holes in the scaffolds colocalized with regions of new matrix deposition (Fig. 10).

Discussion

Our findings show that a multistep processing method can be used to fabricate anatomically-shaped scaffolds with aligned pore architecture from decellularized and dehydrothermally crosslinked CDM. A controlled directional freezing method yielded scaffolds with uniform dimensions, as well as aligned grooves in CDM discs and hemispheres. These aligned grooves increased pore size and enhanced cellular infiltration. Furthermore, CDM concentration was shown to govern porosity, pore size, mechanical properties, and most importantly cell-mediated contraction. Cell-mediated contraction not only unpredictably altered the size and shape of CDM discs, but also decreased cellular proliferation and encouraged a fibrocartilaginous phenotype. The results of the CDM disc studies indicated that the 11% CDM concentration with aligned pores prevented cell-mediated contraction while facilitating cellular infiltration. CDM hemispheres fabricated with these conditions maintained their predefined geometry throughout chondrogenic culture. Additionally, MSCs distributed evenly throughout the hemispheres, which resulted in uniform production of mature cartilaginous matrix. These findings demonstrated that given the appropriate fabrication parameters scaffolds synthesized purely from CDM were capable of maintaining more complex, anatomically relevant shapes without excessive chemical crosslinking or support from synthetic materials.

The two-part, delrin-silicone molds (Fig. 1) produced freezing conditions that generated aligned ice crystals, which in turn yielded aligned pores in CDM constructs after lyophilization. Unidirectional freezing is a well-established technique for generating aligned pores in scaffolds [37, 43–45]. This process requires a temperature gradient to be formed across the freezing suspension so that ice crystal growth aligns parallel to the temperature gradient [37, 43–45]. In order to generate a temperature gradient, the silicone lid (9 mm thick) was made thinner than the delrin mold (25 mm thick), which ensured that ice nucleation would initiate only on the inner surface of the silicone lid thus resulting in unidirectional freezing. Furthermore, the inner surface of the silicone lid was patterned with a lamellar pattern of 50 μm ridges spaced 90 μm apart that served as a template for the aligned pores (Fig. 1E). Templating the nucleation surface with a unidirectional pattern has been shown to prevent the formation of a denser, reduced-pore region typically observed with unidirectional freezing, and instead produce a lamellar architecture throughout the entire construct [46]. In contrast to previous studies that did not observe aligned pores until 20 mm away from the nucleation surface [47], the current study observed aligned pores immediately on the nucleation surface (Fig. 3). Submerging the mold in isopropyl alcohol cooled both halves of the mold at the same rate, which eliminated the temperature gradient and led to the formation of uniform pores (Fig. 3). These results demonstrated that the aligned grooves were the direct result of unidirectional freezing produced by the differential

freezing rates of the two halves of the two-part mold. The molds not only resulted in uniform scaffold dimensions, which minimized sample variability, but also enabled the fabrication of more complex anatomically relevant hemispheres (Fig. 8 & Supplemental Fig. 6). Our findings elucidated the ability of delrin-silicone molds to generate unidirectional freezing conditions that aligned pores in CDM discs (Fig. 3) and hemispheres (Fig. 8 & Supplemental Fig. 6). Furthermore, the templated surface of the silicone lid (Fig. 1E) oriented pores throughout the entire thickness of CDM constructs and prevented the formation of a denser region along the nucleation surface.

CDM concentration, but not freezing technique, influenced scaffold porosity; however, both CDM concentration and pore architecture (freezing technique) independently regulated pore size (Fig. 2). These trends in porosity and pore size agreed with previous results reported for freeze-cast CDM constructs [13, 14], collagen matrices [48], hydroxyapatite scaffolds [49], and alumina structures [50]; and followed the general properties observed across a variety of freeze-cast biomaterials [51]. Increasing CDM concentration led to a concomitant decrease in porosity (Fig. 2B) and pore size (Fig. 2C), which has been observed previously [13, 14]. While previous studies have shown no change in porosity [24] or increasing pore size [35] with increasing CDM concentration, these discrepancies may be attributable to differences in the fabrication process of the CDM scaffolds. Studies that reported changes in porosity [24] and pore size [35] that conflicted with the findings of the current study fabricated CDM scaffolds via homogenization alone, which has been shown to result in larger CDM particle sizes of 322 μm [14]. In contrast, the current study synthesized CDM constructs from pulverized CDM power, which leads to fine particle sizes of 97 μm and has been shown to produce more consistent pore size and morphology compared to CDM scaffolds fabricated via homogenization alone [14]. Previous studies comparing CDM scaffolds fabricated via homogenization or pulverization did not find differences in the chondroinductive capacity between CDM constructs produced using either of the two fabrication techniques [14]. Interestingly, the current study demonstrated that aligned pores produced a larger pore size than the uniform pores (Fig. 2C), which contrasted with previous findings in CDM constructs that reported aligned pores were smaller than uniform pores [23]. These discrepancies could be the result of differences in the molds and their corresponding freeze rates used to fabricate the aligned pores. While the current study used two insulating materials to generate a temperature gradient, the previous study used a metal plate equilibrated to -196°C to produce unidirectional freezing [23]. The metal plate likely resulted in much faster freeze rates than the delrin-silicone mold, and slower freeze rates have been shown to generate larger pores [52–58].

Intriguingly, cellular infiltration was independent of pore size. Specifically, 11% and 10% CDM scaffolds with aligned pores, which possessed significantly smaller pore sizes than 8% and 7% scaffolds with uniform pores (Fig. 2), exhibited greater cellular infiltration into the center of constructs compared to any of the CDM scaffolds with uniform pores (Fig. 3). These results contrasted with previous studies that found smaller pore sizes inhibited cellular infiltration and restricted cells to the surface of CDM constructs [14]; however, it should be noted that the pore sizes produced in the current study (128 – 198 μm) were substantially larger than the pores fabricated in previous studies (32 – 65 μm) [14]. In addition to the larger pore sizes, these discrepancies could be attributed to the aligned pore architecture

implemented in the current study. The previous study only produced CDM scaffolds with uniform pores [14], and the results of the current study demonstrated that scaffolds with uniform pores restricted cells to the periphery of CDM constructs (Fig. 3). The finding that the aligned pore architecture corresponded with enhanced cell infiltration (Fig. 3) has been demonstrated previously [59]. Furthermore, studies with lyophilized collagen sponges demonstrated that cells were restricted to the surface of constructs unless templated to form channels into the center of the scaffolds [60]. The results of the current study revealed that the aligned grooves provided a mechanism for enhancing cellular infiltration as evidenced by the cellular distribution pattern in the 10% aligned scaffolds matching the striated pattern of the aligned grooves (Fig. 3). Through cellular proliferation and migration, all constructs exhibited uniform cell distribution after 28 days of culture (Fig. 7); however, this finding does not undermine the importance of the rapid initial cellular infiltration facilitated by the aligned pores. Upon *in vivo* implantation into an osteochondral defect, the aligned pores could encourage rapid engraftment of chondro-progenitor cells from the subchondral bone, which could help anchor the implanted CDM construct within the harsh mechanical environment of the joint.

Gross morphology (Fig. 4) and quantitative analysis of scaffold areas (Fig. 5A) demonstrated that cell-mediated contraction was influenced by CDM concentration, and only 11% CDM scaffolds were able to maintain the original scaffold shape and dimensions. All constructs received dehydrothermal crosslinking, which corroborated previous results demonstrating that physical crosslinking techniques were capable of preventing cell-mediated contraction in CDM scaffolds while preserving the chondrogenic properties of the scaffold [18]. However, the current findings emphasized the importance of scaffold density in empowering dehydrothermal treatment to confer resistance to cell-mediated contraction. Previous work has also reported that scaffolds produced with lower CDM concentrations underwent greater cell-mediated contraction and required chemical crosslinking treatments in order to prevent contraction [14]. While these studies concluded that dehydrothermal treatment was insufficient to resist contraction, they did report that scaffolds produced from higher CDM concentration underwent less contraction than lower CDM concentrations [14]. These findings suggested that given an appropriately dense CDM scaffold, dehydrothermal treatment alone can confer resistance to cell-mediated contraction, as demonstrated in the current study (Fig. 5A & 9A). Furthermore, discrepancies could also arise from differences in the concentration of seeded cells, as cell contractile forces have been shown to vary with cell density [61]. Previous studies have correlated cell-mediated contraction with scaffold mechanical properties, and reported higher degrees of contraction with lower mechanical properties [62, 63]. This notion was supported by the results of the current study that demonstrated the 11% CDM scaffolds, which possessed the highest compressive moduli (Fig. 6) were able to resist contraction (Fig. 5A). However, the 9% and 7% CDM constructs, which possessed lower compressive moduli (Fig. 6), underwent significant contraction (Fig. 5A). Interestingly, the 11% CDM scaffolds with uniform and aligned pores had very different compressive moduli (Fig. 6); however, both were able to prevent cell-mediated contraction (Fig. 5A). These findings suggested that there existed a threshold for mechanical properties above which cell-mediated contraction was prevented. While previous studies have found that cell-mediated contraction can enhance chondrogenic differentiation [64–66]

in the current study, contraction was associated with a fibrocartilaginous phenotype as 7% CDM constructs, which experienced the highest degree of cell-mediated contraction (Fig. 5A) also possessed the most intense type I collagen staining (Fig. 7). These findings were consistent with previous results that reported more intense type I collagen staining in CDM scaffolds that underwent higher degrees of contraction [9]. Furthermore, 7% CDM constructs supported the lowest amount of cellular proliferation (Fig. 5B), which agreed with previous studies that demonstrated cell-mediated contraction limited cellular proliferation [62, 67]. Taken together, these results confirmed that given the appropriate scaffold fabrication parameters, such as CDM concentration, dehydrothermal treatment was sufficient to confer resistance to cell-mediated contraction, which was critical in order to maintain the original scaffold dimensions, prevent a fibrocartilaginous phenotype, and enhance cellular proliferation.

Both CDM concentration and pore architecture (freezing technique) influenced the compressive moduli of CDM scaffolds; however, the initial mechanical properties did not persist after 28 days of chondrogenic culture (Fig. 6). These results agreed with previous findings in freeze-cast CDM [14], hydroxyapatite [49], silk [45], and gelatin [37] scaffolds that reported increasing slurry concentration decreased porosity, which in turn increased compressive strength. While previous studies only examined the effect of CDM concentration on the compressive modulus [14, 35], the current study investigated the effect of different pore architectures (Fig. 3) in addition to CDM concentration. Interestingly, the aligned pores possessed lower compressive moduli than the uniform pores (Fig. 6), which contrasted with previous findings in CDM [23] and PLGA [59, 68] scaffolds, which demonstrated that oriented pore architectures produced higher compressive moduli compared to constructs with uniform pores. These discrepancies could be attributed to differences in the pore shape and size. While the oriented pores in previous CDM studies adopted a cylindrical microtubule conformation and possessed an average diameter of 105 μm [23], the aligned pores in the current study adopted the conformation of large lamellar grooves that traversed across the width of the constructs (Fig. 3) and possessed a minimum pore size of 140 μm at the 11% CDM concentration (Fig. 2). Furthermore, decreasing the distance between lamellae in scaffolds with aligned pores has been shown to exponentially increase scaffold compressive strength [46]. These findings emphasized the importance of pore size in dictating the mechanical properties of scaffolds with lamellar pores [46] like the lamellar grooves found in our constructs. Unfortunately, the initial mechanical properties were not retained throughout chondrogenic culture, and after 28 days the compressive moduli of 11% and 9% CDM scaffolds substantially decreased compared to their respective unseeded scaffolds (Fig. 6). However, the compressive moduli of the 7% constructs increased after chondrogenic culture (Fig. 6). Previous studies have also reported that scaffolds fabricated from higher CDM concentrations decreased in mechanical properties over the course of chondrogenic culture, while CDM constructs from the lowest CDM concentration possessed the highest compressive modulus at the end of the culture period [14]. However, these findings contrasted with other studies that revealed CDM scaffolds with oriented pores possessed higher compressive moduli than constructs with uniform pores at both 2 weeks and 4 weeks of chondrogenic culture [23]. The observed decrease in mechanical properties experienced by the 11% and 9% scaffolds was likely due to the

enzymatic degradation of CDM constructs, which will be investigated in future studies. This effect was not observed in previous studies because the CDM was crosslinked with genipin [23], which has been shown to prevent enzymatic degradation [69]. The increase in the 7% CDM compressive moduli following chondrogenic culture was likely due to cell-mediated contraction that compacted the constructs. Cell-mediated contraction has been previously reported to increase the mechanical properties of both CDM [6, 34] and collagen-GAG [66] scaffolds, presumably due to consolidation of the porous scaffold. The compressive moduli values of the CDM constructs after chondrogenic culture (13 – 20 kPa) agreed with previously reported values of 17 kPa [34]. While these values were an order of magnitude lower than the compressive modulus of native cartilage 0.3–0.8 MPa [70], previous studies have insinuated that longer culture periods may be necessary to generate higher compressive moduli as mechanical properties continue to increase over time in chondrogenic culture [6, 34].

In conjunction with exogenous growth factors, decellularized CDM scaffolds were shown to promote chondrogenic differentiation and prevent a hypertrophic phenotype in MSCs as evidenced by gross morphology (Fig. 4), biochemical analysis (Fig. 5), as well as histological and immunohistochemical staining (Fig. 7). The addition of pulverization and DNase treatment to the CDM fabrication process varied from previous studies in our group [6, 7, 9, 18, 24, 34], and was necessary to remove foreign DNA from the CDM (Supplemental Fig. 4). Despite these additional processing steps, the decellularization treatment did not alter the native GAG content of the CDM (Supplemental Fig. 4), which was corroborated by previous studies [17, 19]. Most importantly, decellularization did not alter the chondroinductive properties of the CDM, which has been extensively documented in previous studies that demonstrated CDM retained its chondroinductive capacity after decellularization [1, 8, 10, 12, 15–17, 19, 21, 22, 25–27, 30, 32, 33, 71]. After chondrogenic culture, CDM constructs adopted a smooth shiny texture indicative of new matrix synthesis (Fig. 4). Furthermore, the deposited tissue was evenly distributed throughout the entire thickness of CDM scaffolds, completely filled in the pores of CDM constructs, and stained positive for GAGs as well as type II collagen indicative of a mature cartilaginous phenotype (Fig. 7). All of the type II collagen staining observed was the direct result of synthesis from the seeded human MSCs, as the CDM scaffold fabricated from porcine cartilage did not stain for type II collagen (Supplemental Fig. 5). While the GAG and collagen contents were dominated by the composition of the CDM scaffolds, the CDM constructs maintained GAG and collagen ratios to dry weight of $16.5\% \pm 0.7\%$ and $82\% \pm 3\%$, respectively, throughout chondrogenic culture; and these ratios were similar to those found in native cartilage [72]. Previous studies have also reported that increasing the CDM concentration led to a corresponding increase in GAG and collagen content [13, 14]. The absence of type X collagen staining (Fig. 7) demonstrated that the decellularized CDM scaffolds were able to prevent a hypertrophic phenotype in MSCs, which has been touted as a hallmark feature of using CDM as a scaffold [1, 7, 12, 18, 28]. While the newly synthesized matrix stained positive for cartilage specific proteins, visualization of picrosirius red staining under polarized light revealed that newly synthesized matrix possessed a much lower birefringence compared to the collagen of the CDM scaffold, which was derived from native cartilage (Fig. 7). Polarization of picrosirius red-stained collagens has been shown to be dependent

upon collagen fiber thickness as well as the packing density and alignment of collagen fibers [73]. These data suggested that while the newly deposited tissue mimicked cartilaginous composition, it lacked the structural organization found in native cartilage, which could further explain the poor mechanical properties observed after chondrogenic culture (Fig. 6). Dynamic loading during *in vitro* culture has been shown to promote collagen alignment that mimics the structural and functional properties of the native tissue [74, 75], and could ameliorate the lack of organization observed in the current study. Together, these findings demonstrated that the decellularized CDM retained its chondroinductive capacity and ability to prevent a hypertrophic phenotype in MSCs; however, further work is needed to achieve hierarchical organization of the newly synthesized tissue.

Hemispheres fabricated using the 11% CDM concentration along with aligned pores did not contract, warp, or splay throughout chondrogenic culture, and supported uniform cartilaginous matrix deposition. Since the 11% CDM discs demonstrated the ability to prevent cell-mediated contraction (Fig. 4 & 5A), we sought to investigate the ability of CDM constructs to maintain a more complex, anatomically relevant architecture, which possessed a greater propensity to be altered by cellular-contraction forces. Additionally since the aligned pores enhanced cellular infiltration in CDM discs (Fig. 3), we opted to generate aligned pores in the CDM hemispheres. Despite the decreased mechanical properties associated with the aligned pores (Fig. 6), CDM hemispheres were able to retain their original shape (Fig. 8) and dimensions (Fig. 9A) throughout chondrogenic culture, which enabled a doubling of seeded cells by day 28 (Fig. 9B). While scaffolds fabricated from pre-shaped acellular cartilage sheets have also been shown to retain their shape throughout chondrogenic culture [25], these scaffolds only maintained simple two-dimensional architectures, which were analogous to the CDM discs of the current study. Furthermore, decellularized meniscus [30], nasal cartilage [27, 30], and ear cartilage [22] have been used as scaffolds with the specific objective of recapitulating the complex architecture of the native tissue. While these decellularized tissues maintained their native architecture throughout *in vitro* culture in the presence of seeded cells, the dense extracellular matrix of the native tissues prevented cellular infiltration, and seeded cells remained restricted to the surface of constructs even after 21 days of culture [22, 30]. In stark contrast, the CDM hemispheres with aligned pores enabled robust cellular infiltration throughout the entire thickness of the constructs as visualized by actin staining (Fig. 10). The even cell distribution resulted in uniform cartilaginous matrix production that stained intensely for GAG and type II collagen (Fig. 10). These results demonstrated that given the appropriate fabrication parameters, scaffolds produced from CDM alone were capable of withstanding cellular contraction forces and maintaining complex anatomically relevant architectures. Maintenance of the anatomical architecture is critical for clinical application, as preservation of congruity with the surrounding host tissue has been cited as a key requirement for the success of implanted constructs [76]. The importance of maintaining congruity with the articulating surface has motivated others to fabricate various anatomically relevant constructs from agarose [77] or through the mechanical compression of cell pellets [78, 79].

Conclusions

Overall, these results highlighted the importance of CDM concentration and pore architecture in governing scaffold porosity, pore size, cellular infiltration, mechanical properties, and the prevention of cell-mediated contraction. The aligned pores increased pore size and substantially enhanced cellular infiltration; however, they led to a concomitant decrease in mechanical properties. While the aligned pore architecture decreased mechanical properties, the 11% CDM scaffolds with aligned pores were still able to withstand cellular contraction forces, which suggested that there existed a mechanical threshold for resisting cell-mediated contraction. Resistance of cell-mediated contraction not only maintained the original scaffold dimensions, but also enhanced cellular proliferation and discouraged a fibrocartilaginous phenotype. Furthermore, prevention of cell-mediated contraction enabled the fabrication of anatomically relevant hemispheres solely from CDM. The CDM hemispheres not only maintained a complex three-dimensional architecture, but also facilitated cellular infiltration, which led to uniform matrix deposition. Together these results demonstrated that the mechanical properties of CDM constructs could be tailored via CDM concentration, and cellular infiltration could be facilitated through the generation of aligned pores. While the initial mechanical properties did not persist throughout chondrogenic culture, future work is necessary to recapitulate the native collagen alignment, which could lead to better final mechanical properties.

Supplementary Material

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Acknowledgments

We thank Dr. Nelson Chao for providing bone marrow (NIH grant P01 CA47741), Dr. Brad Estes, Dr. Frank Moutos, and Steven Earp of the Pratt Student Shop for assistance in machining the hemisphere molds, and Katherine Glass and Jonathan Brunger for assistance with various aspects of the project. This work was supported in part by NIH grants AR50245, AR48852, AG15768, AR48182, AG46927, OD01070, the Collaborative Research Center, AO Foundation, Davos, Switzerland, the Arthritis Foundation, the Nancy Taylor Foundation for Chronic Diseases, and the Lord Foundation Grant through the Shared Materials Instrumentation Facility (SMIF).

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Figure 1.

Low (A&B) and high (C&D) magnification images of disc (A&C) and hemisphere (B&D) molds. The wells of the disc mold were 6mm in diameter and 2mm in depth. The hemispheres had an outer radius (delrin mold) of 4.76mm and an inner radius (silicone lid) of 3.175mm. (E) 1000X SEM image of the inner surface of silicone lid visualized the lamellar pattern of 50μm ridges spaced 90μm apart. Scale bar: 50μm. The two-part nature of the delrin mold with a silicone lid produced a temperature gradient during the freezing process that resulted in unidirectional freezing. The lamellar pattern on the inner surface of the silicone lid provided a template for the aligned pores. The machined molds ensured uniform dimensions across constructs.

Figure 2.

(A) μ CT reconstructions; (B) Porosity; (C) Pore Size of 11%, 10%, 9%, 8%, and 7% concentration (weight/weight) CDM scaffolds with uniform and aligned pores. Porosity was inversely proportional to CDM concentration, but was not affected by freeze rate. Pore size was also inversely proportional to CDM concentration. At each CDM concentration, pore size was smaller in scaffolds with uniform pores than aligned pores. There was no interaction effect between concentration and freeze rate. Bars represent means $\pm$ standard error of the means ($n=6$). Groups not sharing same letter have p -values < 0.05 . Scale bars: 1mm.

Figure 3. Scanning electron microscope (grayscale) and confocal (green) images of 11%, 10%, 9%, 8%, and 7% concentration (weight/weight) CDM scaffolds with uniform and aligned pores. Scanning electron microscope images are of the surface of the CDM scaffolds, while confocal images are transverse sections, showing the full-thickness of each construct. Green visualizes adherent GFP-expressing MSCs at 1 hour post-seeding. Pore size was visibly smaller in scaffolds with uniform pores, and cells (green) were restricted to the surface of scaffolds. Scaffolds with aligned pores possessed large grooves, which facilitated cellular infiltration into the center of the scaffolds. Scale bars: 500µm.

Figure 4.

Gross morphology of 11%, 9%, and 7% concentration (weight/weight) CDM scaffolds with uniform and aligned pores prior to seeding (unseeded), after 6 days of cell expansion (Day 0), and after 28 days of chondrogenic culture with cells and cell free (CF). The large grooves were visible in all aligned scaffolds, but were absent in the scaffolds with uniform pores. After 28 days of chondrogenic culture, constructs adopted a smooth, shiny surface indicative of cartilage-like matrix synthesis. The white dots on the Day 28 9% & 7% uniform constructs are reflected light. Scale bars: 1mm.

Figure 5.

(A) Scaffold Areas; (B) DNA content; (C) GAG content; (D) Collagen content of 11%, 9%, and 7% concentration (weight/weight) CDM scaffolds with uniform and aligned pores after 28 days of culture. All CDM scaffolds possessed the same original area. Only the 11% scaffolds were able to retain the original dimensions and resist cell-mediated contraction. The 7% scaffolds, which contracted the most, exhibited the lowest cellular proliferation. GAG and Collagen content followed the dry weight of the CDM scaffolds. Bars represent means $\pm$ standard error of the means (n=6). Groups not sharing same letter have p-values < 0.05.

Figure 6.

Compressive moduli of unseeded 11%, 10%, 9%, 8%, and 7% concentration (weight/weight) CDM scaffolds with uniform and aligned pores and 11%, 9%, 7% concentration (weight/weight) CDM scaffolds with uniform and aligned pores after 28 days of chondrogenic culture (Day 28). For unseeded scaffolds, the compressive modulus was directly proportional to CDM concentration. Furthermore at each concentration, scaffolds with uniform pores possessed higher compressive moduli than respective scaffolds with aligned pores. After 28 days of culture, the compressive moduli of the 11% scaffolds decreased, while the compressive moduli of the 7% scaffolds increased. Bars represent means $\pm$ standard error of the means ($n=6$). Groups not sharing same letter have p -values < 0.05 .

Figure 7.

Alexa Phalloidin staining (Actin); histology (Safranin-O and Fast Green staining); immunohistochemistry for type II collagen (COL2); type I collagen (COL1); type X collagen (COLX); Picrosirius red staining visualized under polarized light (Aligned Collagen) on human osteochondral (OC) sections and CDM scaffolds after 28 days of culture in the presence of TGF- β 3. All images are transverse sections, showing full-thickness of each construct. MSCs distributed evenly throughout all constructs. MSCs produced mature, cartilaginous matrix that stained positive for GAGs and type II collagen. Type I collagen staining was most intense in the 7% scaffolds, which underwent the highest degree of contraction. MSCs did not enter into a hypertrophic phenotype as shown by the absence of type X collagen staining. Collagen of the CDM scaffold was aligned and able to be visualized under polarized light; however, the collagen of the newly synthesized matrix was more difficult to detect. Aminoethyl carbazone (AEC) produces red color. Scale bars: 500 μ m.

Figure 8.

Scanning electron microscope (SEM) images and gross morphology of 11% concentration (weight/weight) CDM hemispheres with aligned pores prior to seeding (unseeded), after 6 days of cell expansion (Day 0), and after 28 days of chondrogenic culture. Hemispheres possessed both convex and concave surfaces. SEM images illustrated that the hemispheres with aligned pores contained large grooves similar to those seen in the discs. After 28 days of chondrogenic culture, hemispheres retained their original dimensions, thickness, and shape, and did not splay, warp, or contract. Scale bars: 2mm.

Figure 9.

(A) Scaffold Areas; (B) DNA content; (C) GAG content; (D) Collagen content of 11% concentration (weight/weight) CDM hemispheres with aligned pores at day 0 and after 28 days of culture. Hemispheres retained their original area, and did not succumb to cell-mediated contraction. MSCs proliferated throughout the 28-day culture period. GAG and collagen content were preserved throughout chondrogenic culture. Bars represent means $\pm$ standard error of the means (n=6). Groups not sharing same letter have p-values < 0.05.

Figure 10.

Alexa Phalloidin staining (Actin); histology (Safranin-O and Fast Green staining); immunohistochemistry for type II collagen (COL2); type I collagen (COL1); type X collagen (COLX); Picrosirius red staining visualized under polarized light (Aligned Collagen) on human osteochondral (OC) sections and 11% concentration (weight/weight) CDM hemispheres with aligned pores at day 0 and after 28 days of culture in the presence of TGF- β 3. All images are transverse sections, showing full-thickness of each construct. At day 28, MSCs had uniformly distributed throughout the entire thickness of the hemispheres. At day 0, the aligned pores were clearly visible in the histology sections. MSCs produced mature, cartilaginous matrix that stained positive for GAGs and type II collagen. MSCs did not enter into a hypertrophic phenotype as shown by the absence of type X collagen staining. Collagen of the CDM hemispheres was aligned and able to be visualized under polarized light; however, the collagen of the newly synthesized matrix was more difficult to detect. Aminoethyl carbazone (AEC) produces red color. Scale bars: 500 μ m.