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Multifunctional hybrid three-dimensionally woven scaffolds for cartilage tissue engineering

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Abstract

The successful replacement of large-scale cartilage defects or osteoarthritic lesions using tissue-engineering approaches will likely require composite biomaterial scaffolds that have biomimetic mechanical properties and can provide cell-instructive cues to control the growth and differentiation of embedded stem or progenitor cells. This study describes a novel method of constructing multifunctional scaffolds for cartilage tissue engineering that can provide both mechanical support and biological stimulation to seeded progenitor cells. Three-dimensionally (3-D) woven poly (ϵ -caprolactone) (PCL) scaffolds were infiltrated with a slurry of homogenized cartilage-derived matrix (CDM) of porcine origin, seeded with human adipose-derived stem cells (ASCs), and cultured for up to 42 days in standard growth conditions. These constructs were compared to scaffolds derived solely from CDM as well as 3-D woven PCL fabric without CDM. While all scaffolds promoted a chondrogenic phenotype of the ASCs, CDM scaffolds showed low compressive and shear moduli and contracted significantly during culture. Fiber-reinforced CDM scaffolds and 3-D woven PCL scaffolds maintained their mechanical properties throughout the culture period, while supporting the accumulation of a cartilaginous extracellular matrix. These findings show that fiber-reinforced hybrid scaffolds can be produced with biomimetic mechanical properties as well as the ability to promote ASC differentiation and chondrogenesis *in vitro*.

Keywords

articular cartilage; tissue engineering; textile; stem cell; mesenchymal stem cell; fiber

Introduction

Tissue engineering seeks to repair or regenerate damaged or diseased tissues using combinations of scaffolds, cells, and environmental cues such as bioactive molecules and physical factors. A number of challenges remain in the development of tissue-engineered approaches for biomechanically functional tissues ^[1] such as articular cartilage ^[2]. In particular, the successful replacement of large-scale cartilage defects or osteoarthritic lesions will likely require composite biomaterial scaffolds that have biomimetic mechanical properties and can sustain physiologic joint loading soon after implantation ^[3]. Furthermore, tissue engineering approaches based on undifferentiated progenitor cells or stem cells may require “cell-instructive” cues to induce and maintain cellular differentiation over the life the construct *in vivo* ^[4].

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In this regard, multifunctional scaffolds for tissue engineering, which are capable of providing physical support while delivering bio-functional signals through cell-substrate interactions or biologic delivery, have been produced using numerous materials and manufacturing techniques [5]. Protein-modified polymers, surface immobilized proteins, drug-eluting polymers, and devitalized native tissues [6–10] are only a few examples. In earlier work, we have developed composite scaffolds consisting of a 3-D woven fabric infiltrated with a fibrin matrix that display anisotropic, nonlinear, and viscoelastic properties similar to those of articular cartilage, *a priori* [3, 11–13]. In particular, 3-D woven poly(ϵ -caprolactone) scaffolds seeded with human adipose-derived stem cells (ASCs) and cultivated *in vitro* for 28 days maintained the engineered initial properties of the scaffolds over time as new tissue was synthesized. While this neotissue was shown to enhance the mechanical behavior of the constructs, it appeared fibrocartilaginous in composition and lacked the proteoglycan-rich network that contributes to the compressive and frictional properties of native articular cartilage [14, 15]. Thus, we sought to modify the existing scaffold design to incorporate the use of a bioactive material capable of delivering the appropriate biochemical stimuli for cellular differentiation and cartilage-specific macromolecule production.

Recent work by Cheng et al has shown that porous scaffolds produced from devitalized, full-thickness porcine cartilage can promote the chondrogenic differentiation of seeded ASCs, without the need for exogenously added growth factors [16, 17]. These studies found that scaffolds created entirely from a cartilage-derived matrix (CDM) upregulated positive markers of chondrogenesis (chondroitin 4-sulfate and collagen II) in ASCs at both the genetic and protein levels. However, constructs showed relatively low compressive moduli (~ 50 kPa at day 0), and cell-seeded scaffolds contracted significantly over time. Based on these findings, we hypothesized that CDM could be combined with a 3-D woven PCL reinforcement to form a functional, bioactive scaffold system capable of inducing a cartilaginous phenotype in ASCs. The principal advantages of this composite, hybrid scaffold system are twofold: first, the 3-D woven PCL fabric is designed to provide prescribed anisotropic and nonlinear mechanical properties, shape control, and long-term stability to the construct, and second, the CDM will act as a biologically active matrix providing chondrogenic cues to seeded ASCs, in the absence of exogenous growth factors that are typically necessary for chondrogenesis [18, 19].

In this study, we evaluated the cartilage forming potential of composite fiber-reinforced CDM (FR-CDM) scaffolds when seeded with human ASCs and cultured *in vitro* up to 42 days. For comparison, scaffolds were also formed from each of the constituent materials (PCL and CDM) alone and cultured in identical conditions. The functional properties of all constructs were assessed at 14, 28, and 42 day time points by gross morphology, histology, immunohistochemistry, biochemical composition, and biomechanical testing. The overall goal of this work is to create functional cartilage constructs for potential use in tissue engineered repair of the articular surface.

Materials and Methods

Scaffold Production

Disk-shaped CDM scaffolds used in this study were prepared by first harvesting full-thickness porcine cartilage from healthy femoral condyles and mincing it into fine pieces. The minced tissue was then suspended in distilled water at a concentration of 0.1 g/ml and homogenized using a tissue homogenizer. The cartilage slurry was then pipetted into the well of a 6-well plate (4 ml/well), frozen overnight at -80°C , and lyophilized for 24 h. A 6 mm diameter biopsy punch was then used to cut samples from the 2 mm thick casting. Porous, 3-D woven textile scaffolds were produced from 156 μm diameter multifilament

PCL yarns (EMS-Griltech, Domat, Switzerland) using a custom-built miniature weaving loom^[11]. The multilayer architecture consisted of 11 layers of axially oriented yarns, stacked in alternating x- and y-directions, and bound together by an interwoven set of vertically oriented yarns (z-direction) that repeatedly passed through the thickness of the fabric. Yarn spacing within each layer was controlled such that the resulting structure contained interconnected pores measuring approximately $850\ \mu\text{m} \times 1100\ \mu\text{m} \times 100\ \mu\text{m}$ with a total void fraction of approximately 70% and an overall thickness of approximately 900 μm . Prior to use, the PCL fabric was soaked in a 4M NaOH bath overnight to clean the fibers and increase their surface hydrophilicity^[20, 21]. FR-CDM composite scaffolds (Figure 1) were formed by spreading 1 ml of cartilage slurry onto a $3\ \text{cm} \times 3\ \text{cm}$ piece of 3-D woven scaffold and repeatedly working the mixture into both faces of the fabric and down into its pores using a spatula. 4 ml of cartilage slurry was then pipetted into the well of a 6-well plate, and the “wet-out” scaffold was suspended within the slurry, frozen overnight at -80°C , and lyophilized for 24 h. A 6 mm diameter biopsy punch was used to cut disk-shaped samples from the casting. Scaffolds were sterilized with ethylene oxide and given a minimum of 1 week to outgas prior to use.

Construct Culture

Human ASCs from seven different donors (female, non-smoking, non-diabetic, ages 27–51, and BMI 22.5–28.2) were obtained from liposuction waste (Zen-Bio, Durham, NC) and pooled for use in this study. The cells were plated on $225\ \text{cm}^2$ culture flasks (Corning, Corning, NY) at an initial density of 8,000 cells/ cm^2 and cultured at 37°C at 5% CO_2 in expansion medium consisting of DMEM/F12 (Cambrex Bio Science, Walkersville, MD), 10% fetal bovine serum (FBS) (Atlas Biologicals, Ft. Collins, CO), 1% penicillin-streptomycin-fungizone (Gibco), 0.25 ng/ml TGF- β 1 (R&D Systems, Minneapolis, MN), 5 ng/ml EGF (Roche Diagnostics, Indianapolis, IN), 1 ng/ml bFGF (Roche Diagnostics). Expansion medium was replaced every 2–3 days as needed until cells became 90% confluent, at which time they were trypsinized (0.05% Trypsin/EDTA, Gibco), resuspended in DMEM/F12 plus 10% FBS, counted with a hemocytometer, and replated. Cell viability was assessed using a trypan blue exclusion assay. ASCs were passaged four times after which they were resuspended in culture medium at a concentration of 500,000 cells per 30 μl and seeded by directly pipetting 30 μl of suspension onto the disk-shaped scaffolds. The cell seeded constructs were placed into 24-well low-attachment plates (Corning) in 1 ml of culture medium per well consisting of DMEM-high glucose (Gibco), 10% FBS (Atlas Biologicals), 37.5 $\mu\text{g/ml}$ ascorbic-2-phosphate (Sigma), 1% ITS+ premix (Collaborative Biomedical, Becton-Dickinson, Bedford, MA), 1% penicillin-streptomycin (Gibco), and 100 nM dexamethasone (Sigma). Media was completely changed every 2–3 days. Cultures were terminated at 14, 28, and 42 day time points for analysis. To assess baseline mechanical and biochemical properties, constructs were harvested after allowing cells 24 hours to attach to the scaffolds.

Histology and Immunohistochemistry

Disk-shaped constructs ($n = 2$ per time point) were fixed overnight at 4°C in a solution containing 4% paraformaldehyde in a 100 mM sodium cacodylate buffer (pH 7.4), dehydrated in graded ethanol steps, embedded in paraffin wax, cut into 10 μm thick cross-sections using a Reichart-Jung microtome, and mounted on SuperFrost microscope slides (Microm International AG, Volketswil, Switzerland). Samples were stained for sulfated glycosaminoglycans (GAGs) and the production of a collagenous matrix using a 0.1% aqueous safranin-O solution and 0.02% fast green solution, respectively, while also using hematoxylin as a nucleus counter-stain. Human osteochondral tissue was used as a positive control. For immunohistochemistry, pepsin digestion of the sections to be labeled for types I and II collagen was performed using Digest-All (Zymed, South San Francisco, CA), while

those sections to be stained for chondroitin 4-sulfate were digested with trypsin, followed by a soybean trypsin inhibitor, and finally with chondroitinase (all from Sigma). Monoclonal antibodies were used to identify type I collagen (ab6308; Abcam, Cambridge, MA), type II collagen (II-II6B3; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), and chondroitin 4-sulfate (2B6; gift from Dr. Virginia Kraus, Duke University Medical Center). Reagent A from the Histostain-Plus ES kit (Zymed) was used on all sections for serum blocking prior to primary and secondary antibody labeling (anti-mouse IgG antibody; Sigma Catalog No. B7151), while subsequent linkage to horseradish peroxidase was accomplished using Reagent C. The enzyme substrate/chromagen used for staining was aminoethyl carbazole (Zymed). Human osteochondral samples were used as positive controls for each antibody, and the cartilage region of each sample was inspected to ensure antibody specificity. Negative controls for each slide were also prepared to rule out nonspecific labeling. These sections were prepared by omitting the primary antibody incubation step.

Mechanical Testing

Six tissue-engineered constructs per group (CDM, PCL, and FR-CDM) were harvested at days 1, 14, 28, and 42 for mechanical and biochemical evaluation. 3 mm diameter samples were cored from the central region of each construct and subjected to confined compression, unconfined compression, shear, and frictional mechanical testing.

An ELF 3200 Series precision controlled materials testing system (Bose, Minnetonka, MN) was used to perform creep experiments in a confined-compression configuration. The cylindrical test specimens were placed in a confining chamber and compressive loads were applied using a solid piston against a rigid porous platen (porosity = 50%, pore size = 50–100 μm). Following equilibration of a 0.5–10gf tare load, a step compressive load of 2–30gf was applied to the sample and allowed to equilibrate for 3600s. Different load levels were selected for each group to ensure that the final equilibrium strain was less than or equal to 10%. The aggregate modulus (H_A) and hydraulic permeability (k) were determined numerically by matching the solution for axial strain (ϵ_z) to the experimental data for all creep tests using a three-parameter, nonlinear least-squares regression. For unconfined compression, strains of $\epsilon = 0.04, 0.08, 0.12$, and 0.16 were applied to the specimens after equilibration of a 4gf tare load. Strain steps were held constant for 900s 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.

For determining shear properties, dynamic frequency sweeps were performed using an ARES Rheometrics System (Rheometric Scientific, Piscataway, NJ). Samples were placed between two rigid porous platens in a PBS bath at room temperature and a compressive offset strain of 10% was applied. After equilibration, a sinusoidal shear strain profile, $\gamma = \gamma_o \sin(\omega t)$ at an amplitude γ_o of 0.05 and an increasing angular frequency, ω , from 1 to 50 rad/s was applied. The magnitude of the complex shear modulus was then calculated from $|G^*| = \tau_o/\gamma_o$, based on the assumption of linear, viscoelastic behavior, where the complex modulus, G^* , is obtained from the storage (G') and loss moduli (G'') as $G^* = G' + i G''$.

The equilibrium friction coefficient, μ_{eq} , was determined using a shear friction testing method described by Wang and Ateshian^[22]. Prior to the start of the test, samples were fixed to an impermeable bottom platen in a PBS bath using cyanoacrylate glue and subjected to a 10% compressive tare strain using a stainless steel top platen. Once the imparted stress reached an equilibrium level (~1800s), a series of sequential angular velocities, $\omega = 0.01, 0.1, 1$, and 10 rad/s, were subsequently applied through the bottom platen for a duration of 120s each. Normal force, N , and frictional torque, T , were recorded during each step and used for calculation of the equilibrium friction coefficient given by $\mu_{eq} = F/N$. By assuming

that the distribution of unknown frictional shear is zero at the center and varies linearly along the radial direction of the cylindrical test specimen, the average frictional force, F , is given by $F = 4T/3r_0$, where r_0 is the radius of the specimen.

Biochemical Analysis

After completing all mechanical tests, samples were digested in 1 ml of a papain solution (papain [125 µg/mL; Sigma], 100 mM sodium phosphate buffer, 5 mM cysteine hydrochloride, and 5 mM EDTA, pH 6.5) at 60°C for 15 h. Total DNA content was determined fluorometrically using the PicoGreen double-stranded DNA (dsDNA) assay (Molecular Probes, Eugene, OR). Sulfated glycosaminoglycan (GAG) content was measured using the dimethylmethylene blue assay (DMB) as previously described [23]. Hydroxyproline (OHP) was used to determine total collagen content of the cultured constructs. Briefly, sample digest was acid hydrolyzed and reacted with *p*-dimethylaminobenzaldehyde and chloramine-T to measure OHP content per construct. Total collagen was then determined using 0.134 as the ratio of OHP to collagen [24].

Statistical Analysis

Analysis of variance (ANOVA) with Fisher's PLSD post-hoc test was performed to compare the results of mechanical and biochemical tests for each construct between time points ($\alpha = 0.05$).

Results

Gross morphology, histology, and immunohistochemistry

PCL and FR-CDM constructs maintained their initial size and disk shape throughout the entire 42 day culture, while CDM constructs became rounded and showed a marked reduction in size by day 14 with further reduction by day 28 (Figure 2). CDM and FR-CDM constructs had developed smooth, glistening surfaces by day 14; however, the surfaces of the PCL constructs showed the raised topography of the woven scaffold beneath a thin layer of newly synthesized extracellular matrix (ECM). As observed grossly and in cross-sectional images, this surface texture appeared to fill in and become less pronounced as culture time increased.

At day 14, histological analysis of CDM constructs revealed isolated areas of GAG (as stained by safranin-O) within a loosely assembled, collagen-rich matrix (as stained by fast green) (Figure 3). Positive GAG staining was less intense by day 42, but constructs appeared denser as new matrix was accumulated and completely filled the interstitial voids within the scaffold. Similar results were observed in the thick regions of CDM that surrounded the reinforcing fiber scaffold in FR-CDM constructs. PCL constructs showed no positive staining for GAG, but a collagenous ECM had accumulated over time within the pore structure of the 3-D woven scaffold. All constructs stained positively for type I collagen (Figure 4), type II collagen (Figure 5), and the chondroitin 4-sulfate epitope (Figure 6) at each time point.

Mechanical Testing

Each construct group displayed significantly different aggregate (Figure 7A, $p < 0.0001$) and Young's (Figure 7B, $p < 0.05$) moduli in confined and unconfined compression, respectively. On average, the aggregate modulus for FR-CDM constructs was six-fold higher than CDM constructs, while aggregate modulus for PCL constructs was over twofold higher than FR-CDM constructs. Similarly, the average Young's modulus of FR-CDM constructs was threefold higher than CDM constructs, with Young's modulus for PCL constructs nearly fivefold higher than FR-CDM constructs. With the exception of the PCL group, compressive

moduli showed no statistically significant changes over time within groups. When compared to baseline (day 1) values, cultured PCL constructs showed a significant increase in Young's modulus over time (Figure 7B, $p < 0.0001$). CDM constructs showed an increasing trend in stiffness with time as aggregate modulus increased 24% and Young's modulus increased 106% between days 14 and 42. Like modulus values, hydraulic permeability for the CDM and FR-CDM groups showed no significant changes over time; however, the permeability of the PCL constructs decreased by two orders of magnitude after day 1 (Figure 7C, $p < 0.0001$).

Within groups, cultured constructs displayed significant increases in complex shear modulus when compared to baseline values (Figure 8A, $p < 0.0001$). CDM and FR-CDM constructs displayed similar values of complex shear modulus at an angular velocity of 10 rad/sec, while values for PCL constructs were approximately two times higher (Figure 8A, $p < 0.0001$). After day 14, CDM and FR-CDM showed no significant changes in complex shear modulus over time; however, CDM constructs increased in stiffness by 43% between days 14 and 42. PCL constructs showed a 50% increase in complex shear modulus between day 14 and day 28 (Figure 8A, $p < 0.05$), and appeared to maintain this elevated level through day 42. The loss angle measured at 10 rad/sec was significantly different for each scaffold type (Figure 8B, $p < 0.0001$). Values ranged between 14° and 25°, indicating viscoelastic solid-like behavior.

As measured under steady frictional shear, the average equilibrium coefficient of friction for PCL constructs was significantly lower than CDM and FR-CDM constructs (Figure 8C, $p < 0.001$). PCL and FR-CDM constructs showed no statistically significant changes in μ_{eq} over time; however, values for CDM constructs decreased 28% from day 14 to day 28 and remained at this reduced level through day 42.

Biochemical Testing

The DNA content of PCL and FR-CDM constructs increased significantly from day 1 to day 42, reaching peak values of 11.7 $\mu\text{g}/\text{construct}$ and 12.2 $\mu\text{g}/\text{construct}$, respectively (Figure 9A, $p < 0.05$). CDM constructs, however, did not display a similar statistically significant increase from day 1 to day 42. The DNA content of CDM constructs peaked on day 28 at 5.5 $\mu\text{g}/\text{construct}$, but decreased to 4.5 $\mu\text{g}/\text{construct}$ on day 42. When normalized by the dry weight of the construct, GAG content was observed to be significantly different for each scaffold group (Figure 9B, $p < 0.05$). From day 1 to day 42, CDM and FR-CDM constructs showed a 48% and a 51% overall decrease in total GAG, respectively. Conversely, GAG content of the PCL constructs increased 112% from day 1 to day 42. Total collagen content was also observed to be significantly different for each scaffold group (Figure 9C, $p < 0.05$). When normalized to dry weight, collagen content for CDM and FR-CDM constructs increased significantly from day 1 to day 14. After 42 days in culture, the total collagen content of CDM constructs had increased 42-fold over baseline values. Similarly, FR-CDM constructs had increased by 18-fold, and PCL constructs had increased by 62-fold.

Discussion

The goal of this study was to develop a new scaffold system for the functional tissue engineering of articular cartilage that combined a bioactive, native tissue-derived matrix with load-bearing fiber reinforcement. Our rationale for choosing this hybrid design was to provide a relatively simple means of controlling the biological activity of the ASCs within a mechanically functional polymeric scaffold, thus avoiding the need to administer exogenous growth factors for chondrogenic differentiation of human ASCs while in 3-D culture. Although low levels of growth factors are used in the expansion phase for ASCs [25], previous studies have shown that relatively high concentrations of bone morphogenetic protein 6 (BMP-6) or transforming growth factor- β (TGF- β) are required to induce

chondrogenesis in this cell population [18, 26, 27], introducing concerns of expense, safety, and efficacy arise regarding their use *in vivo*. The results of this study demonstrate that a 3-D woven PCL reinforcing structure can be embedded within a cartilage-derived matrix to improve its functional properties, while conferring shape control and long-term dimensional stability to the tissue-engineered construct. Nonetheless, ASCs appeared to synthesize a similar, collagen-rich neotissue independent of scaffold type.

Decellularized organs and tissues have been used as scaffolding biomaterials in numerous regenerative medicine applications as they provide a “pre-assembled” three-dimensional ECM native to the tissue being recreated. In this regard, decellularized ECM scaffolds may provide seeded progenitor cells with the appropriate biophysical signals required for differentiation and metabolism. This approach has been utilized with tissues such as bone, ligament, tendon, small intestinal submucosa, blood vessels, bladder, heart, liver, trachea, and cartilage [28–38]. Because the dense ECM of articular cartilage contains an effective pore structure of only a few nanometers in size [39], cells are unable to infiltrate and repopulate a decellularized cartilage scaffold in its native form. Cheng and co-authors devised a physical processing technique utilizing homogenization and lyophilization to produce a highly porous, devitalized matrix that could be easily seeded and presumably retain its biochemical composition and activity. Yet one limitation of their manufacturing method is that the resulting sponge-like scaffold is incapable of resisting the cell-mediated contractile forces generated by developing tissue, and therefore the constructs displayed poor dimensional stability during cultivation [16]. Consistent with these results, the CDM constructs used in this study also showed substantial shape distortion by day 14, contracting in diameter and developing a rounded cross-section (Figure 2). This behavior continued through day 28, after which no further contraction was observed. The PCL and FR-CDM constructs, both of which contained fiber reinforcement, showed negligible contraction and maintained their original size and geometry throughout the 42 day culture.

Furthermore, one potential approach for treating osteoarthritis is to resurface the entire joint, rather than focusing on the repair of articular cartilage lesions. In order to achieve this goal, scaffolds must be precisely formed into complex, anatomically accurate geometries that maintain their shape until the time of surgical implantation. The unique orthogonal architecture of the 3-D weave lends itself well to this application because it allows the high-strength fabric scaffold to be formed to curved surfaces without folding or wrinkling. In the manufacturing technique we developed for producing FR-CDM scaffolds, the fabric was saturated with cartilage slurry prior to molding while still flat, allowing for maximum penetration of the homogenized tissue into the pores of the scaffold. After lyophilization, the slurry formed a porous matrix that bound the fibers and locked the scaffolds in shape. The formed FR-CDM scaffolds had excellent structural integrity and could be easily handled without compromising their geometry.

ASC-seeded constructs synthesized a fibrous, collagen-rich tissue that appeared to accumulate over time within the pores of all scaffold types. As observed in the histologic sections, this neotissue contained the cartilage-specific macromolecules type II collagen, the predominant collagenous component of articular cartilage, type I collagen, and chondroitin 4-sulfate (Figures 4–6). However, s-GAGs were not detected in the neotissue of either of the three scaffold types by histological methods. Instead, the intact pieces of native cartilage in the CDM and FR-CDM scaffolds stained positively for s-GAG (by safranin-O) at day 14, but showed decreased staining intensity by day 42 (Figure 3). This reduction in staining correlated with the drop in total GAG content of CDM and FR-CDM constructs as measured by DMB (Figure 9B). These findings suggest that the ASCs synthesized a phenotypically similar tissue regardless of scaffold type. Still, scaffold type was shown to affect the biochemical composition of the tissue-engineered constructs; yet, this is likely due to the

native proteoglycans and collagens present in the CDM scaffold. For example, CDM constructs contained the highest amounts of total GAG and collagen when normalized to dry weight, followed by FR-CDM constructs, and finally PCL constructs (Figures 9B,C). It should be noted that the lower reported values of total GAG and collagen content for the FR-CDM and PCL constructs results from their increase in overall weight due to the embedded fiber reinforcement. It is interesting to note that the PCL constructs displayed a slight increasing trend in total GAG and collagen content over time, suggesting that ASCs may be deriving chondrogenic stimuli from interacting with the PCL scaffold alone (Figure 9B,C). Further, PCL and FR-CDM scaffolds appeared to promote ASC proliferation, most likely due to their retention of volume and surface area (Figure 9A).

Mechanical testing revealed that tissue constructs grown on PCL scaffolds displayed higher compressive and shear moduli than constructs grown on CDM or FR-CDM scaffolds. As expected, the compressive stiffness of CDM constructs was significantly increased by adding 3-D woven PCL reinforcement to the matrix. However, the composite (FR-CDM) constructs displayed values for aggregate and Young's moduli that were 61% and 78% less than values for PCL constructs, respectively. Cross-sectional images revealed that the manufacturing technique utilized to produce the disk-shaped FR-CDM scaffolds resulted in thick zones of CDM surrounding the PCL scaffold on both upper and lower surfaces. These un-reinforced layers, which comprised a significant portion of the overall scaffold thickness, acted to reduce the stiffness of the constructs by reducing the effect of the load-bearing PCL. Future work entails the modification of the manufacturing technique to optimize the amount of CDM deposition on the scaffold to minimize this effect. Aggregate moduli measured for PCL (0.453 ± 0.033 MPa) and FR-CDM (0.169 ± 0.012 MPa) compared well to those of native articular cartilage ($0.1\text{--}2.0$ MPa) [40], while CDM values were an order of magnitude less (0.028 ± 0.003 MPa). A similar trend was observed when comparing Young's modulus of native cartilage ($0.4\text{--}0.8$ MPa) [41, 42] to values of the constructs; PCL constructs compared well (0.224 ± 0.024 MPa), while FR-CDM (0.048 ± 0.003 MPa) and CDM (0.017 ± 0.002 MPa) were an order of magnitude less. There have been relatively few studies that have examined the mechanical properties of stem cell tissue-engineered cartilage constructs. Comparatively, however, the mechanical properties of the engineered tissues are generally reported as being grossly inferior to native cartilage properties. For example, the compressive moduli of MSCs or ASCs cultured in agarose, gelatin, hyaluronic acid, poly(l-lactic acid), puramatrix, and silk derived scaffolds are an order of magnitude lower than that of articular cartilage [43–48] and that which we measured in this study for PCL and FR-CDM constructs, alluding to the importance of the biomimetic mechanical properties of the orthogonally woven scaffold. The apparent hydraulic permeability of the constructs also compared well to that of native cartilage ($k = 0.0005\text{--}0.005$ mm⁴/ (N·s)) [49, 50]. Shear mechanical testing revealed that fiber-reinforcement did not have a significant effect on the complex shear modulus (i.e. CDM vs. FR-CDM). This unexpected observation is likely due to shear deformation occurring primarily in the un-reinforced CDM layers of the composite constructs, where G^* is approximately 50% lower than it is within the reinforced PCL zone.

All constructs displayed relatively stable mechanical properties during culture; however, CDM constructs showed an increasing trend in stiffness over time. This result is likely due to compaction of the tissue constructs by cell-mediated contraction and deposition of new extracellular matrix. PCL constructs, whose properties are dominated by the behavior of the 3-D weave, showed increases in compressive and shear stiffness when compared to baseline values. The mechanism responsible for this stiffening effect is likely the accumulation of new ECM within the construct. The ECM acts to bind and limit the relative movement of the fibers within the orthogonally woven scaffold, which has an inherently low resistance to shear deformation due to a lack of interweaving between its layers. It is important to note that construct mechanical properties never declined below day 1 levels, suggesting that

scaffolds achieved near maximal properties early in the culture, and maintained their structural integrity over time as ASCs synthesized new tissue.

In summary, this study describes a novel method of constructing multifunctional scaffolds for cartilage tissue engineering that may provide both mechanical support and biological stimulation to seeded progenitor cells.

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Figure 1. Fiber-reinforced cartilage-derived matrix (FR-CDM) scaffolds (SEM). (A,B) Surface views, (C) Cross-sectional view. Arrows indicate CDM within pores of the woven PCL scaffold.

Figure 2.

Gross morphology of cultured CDM, PCL, and FR-CDM constructs. Initial diameter of all constructs measured 6 mm. PCL and FR-CDM constructs maintained their original size over 42 days of cultivation. CDM constructs showed marked contraction by day 14.

Figure 3. Histology of CDM, PCL, and FR-CDM constructs at days 14, 28, and 42 (safranin-O and fast green). Top row: Blank CDM scaffold prior to seeding. Scale bar = 200 μ m.

Figure 4. Immunohistochemical staining of type I collagen in CDM, PCL, and FR-CDM constructs at days 14, 28, and 42. Top row: Blank CDM scaffold prior to seeding. Scale bar = 200 μ m.

Figure 5. Immunohistochemical staining of type II collagen in CDM, PCL, and FR-CDM constructs at days 14, 28, and 42. Top row: Blank CDM scaffold prior to seeding. Scale bar = 200 μ m.

Figure 6. Immunohistochemical staining of chondroitin 4-sulfate epitope in CDM, PCL, and FR-CDM constructs at days 14, 28, and 42. Top row: Blank CDM scaffold prior to seeding. Scale bar = 200 μ m.

Figure 7. Compressive biomechanical properties of cultured constructs at days 1, 14, 28, and 42. (A) Aggregate modulus (H_A) and (B) Young's modulus (E) as determined by confined and unconfined compression, respectively. (C) Hydraulic permeability (k) as determined by curve-fitting creep tests using a numerical least-squares regression procedure. Time points not sharing the same letter are statistically different from one another (ANOVA, $p < 0.05$). Data represented as mean $\pm$ SEM.

Figure 8. Shear biomechanical properties of cultured constructs at days 1, 14, 28, and 42. (A) Complex shear modulus (G^*) and (B) loss angle (δ) measured at $\omega = 10$ rad/sec and $\gamma_0 = 0.05$. (C) Equilibrium coefficient of friction (μ_{eq}) measured under steady frictional shear. Time points not sharing the same letter are statistically different from one another (ANOVA, $p < 0.05$). Data represented as mean $\pm$ SEM.

Figure 9.

Biochemical analysis of CDM, PCL and FR-CDM constructs over time. (A) Total dsDNA per construct, (B) total s-GAG and (C) total collagen normalized to dry weight. Time points not sharing the same letter are statistically different from one another (ANOVA, $p < 0.05$). Data represented as mean $\pm$ SEM.