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Electrospun Cartilage-Derived Matrix Scaffolds for Cartilage Tissue Engineering

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Abstract

Macroscale scaffolds created from cartilage-derived matrix (CDM) demonstrate chondroinductive properties, but many fabrication methods do not allow for control of nanoscale architecture. In this regard, electrospun scaffolds have shown significant promise for cartilage tissue engineering. However, nanofibrous materials generally exhibit a relatively small pore size and require techniques such as multi-layering or the inclusion of sacrificial fibers to enhance cellular infiltration. The objectives of this study were (1) to compare multi-layer to single-layer electrospun poly(ϵ -caprolactone) (PCL) scaffolds for cartilage tissue-engineering, and (2) to determine if incorporation of CDM into the PCL fibers would enhance chondrogenesis by human adipose-derived stem cells (hASCs). PCL and PCL-CDM scaffolds were prepared by sequential collection of 60 electrospun layers from the surface of a grounded saline bath into a single scaffold, or by continuous electrospinning onto the surface of a grounded saline bath and harvest as a single-layer scaffold. Scaffolds were seeded with hASCs and evaluated over 28 days in culture. The predominant effects on hASCs of incorporation of CDM into scaffolds were to stimulate s-GAG synthesis and *COL10A1* gene expression. Compared with single-layer scaffolds, multi-layer scaffolds enhanced cell infiltration and *ACAN* gene expression. However, compared to single-layer constructs, multi-layer PCL constructs had a much lower elastic modulus, and PCL-CDM constructs had an elastic modulus approximately 1% that of PCL constructs. These data suggest that multi-layer electrospun constructs enhance homogeneous cell seeding, and that the inclusion of CDM stimulates chondrogenesis-related bioactivity.

Keywords

Cartilage; Osteoarthritis; Nanofiber; Electrospun; Electrospinning; Extracellular Matrix; Mesenchymal Stem Cell; Chondrogenesis

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Conflict of Interest

Dr. Guilak owns stock and is an employee of Cytex Therapeutics Inc. Dr. Little is a paid consultant for Cytex Therapeutics Inc.

Introduction

Articular cartilage serves as the low-friction bearing surface of the diarthrodial joints, sustaining millions of cycles of joint loading with minimal wear or damage [1]. However, cartilage exhibits little or no capacity for self-repair, and thus a number of tissue-engineering approaches are being investigated as a means of treating cartilage injuries using combinations of biomaterial scaffolds, cells, and environmental signals [2]. Electrospun fibers have shown significant promise as a basis for forming cartilage tissue engineering scaffolds, providing a versatile technique to form various fiber architectures with fiber diameters on the micro- or nanoscale [3–10]. Such electrospun nanofibrous scaffolds appear to have beneficial effects on chondrocyte morphology and extracellular matrix production in comparison to microfibrous scaffolds [11]. However, as with most electrospinning techniques, achieving complete cellular infiltration through the full thickness of the scaffold represents an ongoing challenge. To this end, a number of techniques have been used to increase the effective pore size and improve cell infiltration, including combinations of nanofibers and microfibers [12], the use of sacrificial fibers [13, 14], salt leaching techniques [15], controlled fiber packing density [16] and laser ablation [17]. Multilayered scaffolds fabricated using wet electrospinning techniques have also been investigated for their ability to improve cell infiltration. In this technique, electrospun fibers accumulate on the surface of a liquid collecting media, instead of a solid ground plate [18–25]. Incorporation of specific proteins into electrospun scaffolds has also been used to improve cell infiltration and chondrogenic effects of electrospun scaffolds for cartilage tissue engineering [26, 27].

In other areas of tissue engineering, complex extracellular matrices have been incorporated into electrospun scaffolds to more closely replicate the extracellular environment [23, 28–30]. Furthermore, cartilage-derived matrix (CDM) has been shown to promote chondrogenesis by chondrocytes and adult stem cells [31–35], suggesting that incorporation of native cartilage proteins into electrospun scaffolds may enhance chondrogenesis. The objectives of this study were to compare multi-layer to single-layer electrospun poly(ϵ -caprolactone) (PCL) scaffolds for cartilage tissue-engineering, and to determine if incorporation of CDM into the PCL fibers would have a beneficial effect on chondrogenesis by human adipose-derived stem cells (hASCs). Chondrogenesis was assessed by gene and protein levels of major cartilage molecules, as well as by mechanical testing using atomic force microscopy.

Materials and Methods

Cartilage-Derived Matrix Production

Articular cartilage was harvested from the femoral condyles of skeletally mature female pigs obtained from a local abattoir (n=20). The cartilage was frozen at -80°C overnight, lyophilized (Freezone 2.5L, Labconco, Kansas City, MO), and crushed to approximately 5 mm pieces, then pulverized to a powder using a 6750 Spex SamplePrep Freezer Mill (Spex CertiPrep, Metuchen, NJ). CDM powder was stored at room temperature until use, and a single batch of powder was used for all experiments.

Electrospinning

CDM powder was dissolved at 0.08 g/mL in hexafluoroisopropanol (Sigma Aldrich, St. Louis, MO) for 24 hours before being filtered twice through 84 mesh stainless steel (0.18 mm pores). To increase the viscosity of the CDM solution for electrospinning, PCL (Mn = 80,000) (Sigma Aldrich) was added at 0.08 g/mL and dissolved for 24 hours to prepare PCL-CDM solution for electrospinning. PCL-CDM scaffolds were electrospun at 1.2 mL/hr through a 21G needle fitted with a round focusing cage (3 cm diameter, needle tip protruding 4 mm) with applied voltage of 25kV, and fibers were collected on the surface of a grounded saline solution (NaCl 1.25 g/L in distilled water) at a distance of 20 cm. For multi-layer scaffolds, 60 layers were collected from the surface of the grounded solution on a 5cm × 7.5cm glass slide at 1-minute intervals. Single-layer scaffolds were collected after 60-minutes of continuous electrospinning. For PCL scaffolds, PCL was dissolved overnight at 0.1 g/mL in 70% dichloromethane and 30% ethanol. This solution was pumped at 1.2 mL/hr through a 25G needle with a round focusing cage at 17kV into a saline bath 20 cm away. Multi-layer PCL scaffolds consisted of 60 layers collected at 1 minute intervals. Single-layer PCL scaffolds were collected for 180 minutes to ensure scaffold thickness was similar to the other three scaffold groups. Immediately after collection, scaffolds were frozen and lyophilized, then stored sealed at room temperature in the dark until use.

Analysis of Fiber Diameter

Scaffolds were visualized using a scanning electron microscope (SEM) (FEI XL30 ESEM, Hillsboro, OR) after sputter coating with gold (DeskIV, Denton Vacuum, Moorestown, NJ). These images were used to measure the diameter of 183–190 individual fibers from each scaffold type using ImageJ (<http://imagej.nih.gov/ij/>).

Cell seeding and culture

Scaffolds from each group were cut into 8 mm diameter discs and sterilized in 70% ethanol then rinsed with phosphate buffered saline. Scaffold surfaces were sterilized with ultraviolet light for 10 minutes on each side and each sample was incubated in phosphate buffered saline for 18 hours at 37 °C before cell seeding. hASCs from 3 donors (female Hispanic and Caucasian, age 36–59, body mass index 24.6–33.1, posterior waist and thigh lipo-aspirations) were isolated and expanded as described previously [34, 36–39]. Equal numbers of cells from each donor were pooled at passage 4 and seeded on each side of the scaffolds at a final seeding density of 1 million cells/cm². Constructs were maintained in low-attachment tissue culture plates with chondrogenic medium changed every two days consisting of DMEM-high glucose (Life Technologies, Grand Island, NY), 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO), 1% penicillin/streptomycin (Life Technologies), 1% ITS+ Premix (Becton Dickinson, Bedford, MA), 100nM dexamethasone (Sigma Aldrich), 37.5 µg/mL ascorbate (Sigma Aldrich), 40 µg/mL proline (Sigma Aldrich), 10 ng/mL bone morphogenic protein-6 (BMP-6) (R&D Systems, Minneapolis, MN), and 10 ng/mL transforming growth factor β1 (TGF- β1) (R&D Systems) [38, 40–42]. Acellular scaffolds were maintained in similar medium without BMP-6 or TGF- β1.

Biochemical Assays

After 0, 14 and 28 days of culture, constructs from each group (n=6) were harvested, lyophilized to obtain dry weight and digested with papain (125 µg/mL) at 60°C for 15 hours. dsDNA content was quantified using the Picogreen Assay (Life Technologies). Sulfated glycosaminoglycan (s-GAG) content was quantified spectrophotometrically using 1,9-dimethylmethylene blue (DMMB) dye (pH 3.0) with bovine chondroitin-4-sulfate (Sigma Aldrich) as a standard [43]. The hydroxyproline assay was used to determine total collagen content using a conversion factor of 1:7.46 to convert hydroxyproline to collagen [44]. All results were normalized to dry weight and reported as mean±SD.

Real-Time RT-qPCR

Total RNA was extracted from constructs in each of the four groups after 1, 3, 7 and 14 days of culture (n=3) and aliquots (n=3) of hASCs from day 0, which had not been seeded onto scaffolds. Constructs were pulverized in a freezer mill and resuspended in lysis buffer (Qiagen, Valencia, CA). RNA extraction was performed using the QiaShredder column (Qiagen) followed by the RNeasy Mini kit (Qiagen) with on-column DNAase treatment. Equal amounts of RNA were reverse transcribed using the Superscript VILO cDNA Synthesis Kit (Life Technologies). Equal aliquots of each cDNA sample were pooled and used to generate serial dilutions for standard curves from which efficiency was calculated for each gene of interest. Real Time PCR was performed on an iCycler (Biorad, Hercules, CA) using Express qPCR SuperMix (Life Technologies). Commercially available primer-probes (Applied Biosystems, Foster City, CA) were used to compare transcript levels for 5 different genes between each construct and time point compared to the unseeded hASC pellet. Genes examined were: 18S ribosomal RNA (endogenous control, assay ID Hs99999901_s1); aggrecan (*ACAN*, assay ID Hs00153936_m1); type II collagen (*COL2A1*, custom assay: forward primer, 5'-GAGACAGCATGACGCCGAG-3'; reverse primer, 5'-GCGGATGCTCTCAATCTGGT-3'; probe 5'-FAM TGGATGCCACACTCAAGTCCCTCAAC-TAMRA-3')[34, 45]; type I collagen (*COL1A1*, assay ID Hs00164004_m1); and type X collagen (*COLX1A1*, assay ID Hs00166657_m1). Data were corrected for efficiency, normalized to 18S and to the aliquots of hASCs from day 0 that were not seeded onto scaffolds and the relative expression ratio was used for analysis [46].

Histology and immunohistochemistry

hASC-seeded constructs harvested at day 0 and 28 from each of the four treatment groups (n=3) were embedded in optimal cutting temperature gel (Sakura, Torrance, CA), and frozen at -80°C. Samples were cut into 8 µm sections, mounted on slides and evaluated by light microscopy after staining with Safranin-O, Fast Green, and Hematoxylin. Additional sections were analyzed for collagen II content using immunohistochemistry with a mouse monoclonal antibody (III16B3; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), and antimouse IgG secondary antibody (Sigma-Aldrich) linked to horseradish peroxidase (Life Technologies).

Atomic Force Microscopy

All scaffolds were harvested, soaked in phosphate buffered saline and frozen at -20°C until the day before testing. Constructs were thawed, immobilized on glass slides and equilibrated with PBS overnight. The elastic moduli of hASC-seeded and acellular constructs from each group after 0 and 28 days of culture ($n=3$) were determined using an atomic force microscope (MFP-3D, Asylum Research, Santa Barbara, CA). A silicon nitride cantilever ($k = 1.75 \text{ N/m}$) with a $25 \mu\text{m}$ diameter polystyrene bead attached to its end (Novascan Technologies, Ames, IA) was used to test each construct. To address the differences in fiber diameter and distribution between testing sites on each construct, each testing site was imaged in contact mode prior to indentation to identify local height maxima ($25 \times 25 \mu\text{m}$ area, 0.6 Hz , 61.51 nN trigger force). Five sites were imaged on each construct, and two maxima were indented per site (10 indents/construct) at $20 \mu\text{m/s}$ indentation velocity and 150 nN trigger force. The elastic moduli of the constructs were determined by fitting a modified Hertz model to the force-indentation curves as described previously [47]. Consistent with prior work [47], the Poisson's ratio was assumed to be 0.04 for all modulus calculations [48].

Statistical analysis

All data were assessed for normality, analyzed by factorial ANOVA for the effects of hASC-seeding, scaffold, number of layers and time, followed by Tukey's post-hoc test in cases where the main effect had $p \leq 0.05$.

Results

Scaffold appearance and fiber diameter

All four types of scaffolds had similar thickness, with single-layer PCL $0.87 \pm 0.09 \text{ mm}$, multilayer PCL $0.92 \pm 0.23 \text{ mm}$, single-layer PCL-CDM $0.97 \pm 0.26 \text{ mm}$ and multi-layer PCL-CDM $0.90 \pm 0.15 \text{ mm}$ (mean $\pm$ SD, $p > 0.29$). Grossly, the PCL scaffolds had a smooth surface texture. Single-layer PCL-CDM scaffolds had a grainy surface texture, observed during electrospinning and post-processing, probably due to incorporation of fragments of insoluble cartilage during the electrospinning process. However, in the multi-layer PCL-CDM scaffolds, these fragments did not have a noticeable effect on the surface appearance of the scaffold. Multi-layer scaffolds had noticeable voids between the layers, in contrast to single-layered scaffolds. By SEM imaging performed immediately after electrospinning, PCL-CDM fibers were more variable in appearance as compared to PCL fibers (Figure 1). In contrast to PCL fibers (Figure 1A,C), PCL-CDM fibers had intermittent branching and pieces of CDM were evident that were not completely incorporated within the fiber itself (Figure 1B). The surface of the largest PCL-CDM fibers also had a 'pitted' appearance (Figure 1D). Electrospun PCL-CDM fibers also demonstrated a sub-population of fibers with much smaller fiber diameter (approximately 50 nm) that was regional in distribution and therefore not possible to accurately quantify in fiber diameter analysis (Figure 1D). Fiber diameters (Figure 2) of the major population of fibers in PCL-CDM scaffolds were not different between single-layer ($0.58 \pm 0.02 \mu\text{m}$ diameter) and multi-layer ($0.56 \pm 0.01 \mu\text{m}$) scaffolds ($p = 0.60$), but were smaller than PCL fibers ($p < 0.0001$). Single-layer PCL had

smaller diameter fibers ($1.40 \pm 0.03 \mu\text{m}$) than multi-layer PCL ($2.21 \pm 0.04 \mu\text{m}$) ($p < 0.0001$).

Histology and Immunohistochemistry

Processing difficulties precluded evaluation of single- and multi-layer PCL constructs on day 0, but there was no apparent difference in histological appearance between single- and multi-layer PCL-CDM constructs on day 0 (Figure 3). PCL-CDM constructs demonstrated small focal regions of glycosaminoglycan and type II collagen that were not evident in PCL constructs, consistent with the fragments of cartilage that had been observed by SEM. After 28 days in culture, multi-layer PCL constructs demonstrated robust cell infiltration and non-proteoglycan protein synthesis through the full thickness of the construct, in contrast to single-layer PCL constructs which had reduced cell infiltration, and non-proteoglycan protein synthesis restricted largely to the outer third of the construct. However, in both single- and multi-layer PCL constructs, type II collagen immunolabeling was restricted to one surface of the construct. After 28 days in culture, single-layer PCL-CDM constructs showed comparable cellular infiltration to the single-layer PCL constructs, but multi-layer PCL-CDM constructs had reduced cell infiltration compared to the multi-layer PCL constructs. Neither single- nor multi-layer PCL-CDM constructs demonstrated non-proteoglycan protein synthesis through the full thickness of the construct in contrast to the multi-layer PCL constructs. Type II collagen labeling in the PCL-CDM constructs after 28 days in culture was restricted to small foci through the full thickness, attributed to the original porcine CDM. Additionally, there was a more diffuse type II collagen labeling pattern on the exterior of the constructs similar to that seen with the PCL constructs and consistent with new type II collagen synthesis. Negligible new type II collagen was observed in the interior of any of the four construct groups.

Biochemical composition

There was no significant difference between total dsDNA content (Figure 4A) in acellular PCL and PCL-CDM scaffolds throughout 28 days of culture. In hASC-seeded constructs, DNA content increased over time in culture, with no difference identified between single- and multilayer constructs. dsDNA content of all hASC-seeded PCL constructs was greater than hASC-seeded PCL-CDM constructs. Sulfated glycosaminoglycan content (Figure 4B) was significantly greater in acellular PCL-CDM scaffolds compared acellular PCL scaffolds, but the s-GAG content of these acellular PCL-CDM scaffolds decreased over 28 days, and was consistently lower in multi- compared to acellular single-layer PCL-CDM scaffolds. In hASC-seeded PCL scaffolds, s-GAG content increased over time in culture, but remained significantly lower at all time points than the s-GAG content of PCL-CDM constructs. In hASC-seeded PCL-CDM constructs, s-GAG content after 28 days in culture was similar to both acellular and hASC-seeded constructs at day 0, suggesting that hASCs either mitigated s-GAG loss from the PCL-CDM constructs, synthesized s-GAG at a rate similar to the rate of loss, or both. There was no effect of layer in PCL constructs, but in cell-seeded PCL-CDM constructs, s-GAG content was increased in multi-layer compared to single-layer constructs. There was a small synergistic effect of incorporation of CDM on s-GAG synthesis compared to PCL alone, as hASC-seeded PCL-CDM constructs showed a larger increase in s-GAG content at day 28 (compared to similar acellular constructs at Day 28)

than that expected from evaluation of day 28 hASC-seeded PCL constructs. Collagen content (Figure 4C) increased in hASC-seeded PCL constructs compared to acellular and was increased by day 14 compared to day 0, after which there was no further increase. Collagen content in PCL-CDM constructs was significantly greater than all PCL constructs, and in hASC-seeded constructs compared to acellular PCL-CDM scaffolds. In acellular PCL-CDM scaffolds, collagen content was decreased at day 28 compared to day 0 and 14, whereas in hASC-seeded PCL-CDM constructs, collagen content increased at day 14 and 28 compared to day 0. In contrast to s-GAG results, there was no synergistic effect of CDM on collagen synthesis in PCL-CDM constructs compared to PCL constructs.

Gene Expression

In both PCL and PCL-CDM constructs, compared to day 0 unseeded cells, *ACAN* did not increase until day 7 and continued to increase until day 14. There was no effect of scaffold material, but in both PCL and PCL-CDM constructs, there was a significant increase in *ACAN* expression at day 14 in multi- compared to single-layer constructs, consistent with s-GAG results in PCL-CDM constructs. *COL2A1* expression was increased compared to day 0 unseeded cells at day 1 after seeding, returned to baseline levels by day 3 and 7, and by day 14 was not different from Day 1. There was no effect of scaffold material or layer on *COL2A1* expression. *COL1A1* expression was not different between day 0 unseeded cells and day 1, but by day 3, multi-layer PCL demonstrated increased expression compared to baseline, and PCL-CDM (single- and multi-layer) had increased expression compared to single-layer PCL-CDM at day 1. At day 7, for PCL, single-layer constructs had a significant increase in *COL1A1* expression compared to baseline (day 0 unseeded cells and day 1), whereas at day 14, multi-layer constructs were increased relative to baseline. For PCL-CDM at days 7 and 14, *COL1A1* expression for both single- and multi-layer constructs was increased compared to baseline. There was no difference in *COL1A1* expression at any time point between PCL and PCL-CDM constructs. *COL10A1* expression was not different from baseline until day 7 for both PCL and PCL-CDM, and continued to increase for both construct groups, but this increase was greatest in PCL-CDM and was significantly different from PCL at day 14. There was an opposite effect of layer for each of the two construct groups. For PCL, across all time-points, multi-layer constructs reduced *COL10A1* expression compared to single-layer, whereas for PCL-CDM, multi-layer constructs stimulated *COL10A1* expression compared to single-layer.

Atomic Force Microscopy

Elastic modulus (Figure 6) of PCL-CDM constructs was approximately 100-fold lower than that of PCL constructs. There was no effect of time in culture, layer or hASC-seeding on elastic modulus of PCL-CDM constructs. In PCL constructs, single-layer constructs had a significantly greater modulus than multi-layer constructs, and elastic modulus for all PCL constructs was greater at day 0 than day 28. Acellular scaffolds had a greater modulus than hASC-seeded constructs for 1-layer constructs, but hASC-seeded 60-layer constructs had a greater modulus than acellular 60-layer scaffolds.

Discussion

The findings of this study demonstrate that CDM incorporated into electrospun PCL fibers influences s-GAG synthesis and *COL10A1* gene expression by hASCs, while maintaining overall strong s-GAG and type II collagen expression of native cartilage as assessed by histology. Compared with a continuously-collected single-layer construct, collection of 60 separate layers to form a single multi-layer construct enhanced cell infiltration and *ACAN* gene expression and, when combined with CDM, promoted *COL10A1* gene expression. However, multi-layer PCL constructs had a much lower elastic modulus compared to single-layer constructs, and PCL-CDM constructs had an elastic modulus approximately 1% that of PCL constructs. Together these data suggest that multi-layered electrospun constructs achieve homogeneous cell seeding, and that the inclusion of CDM in these constructs can enhance chondrogenesis-related bioactivity. Further investigation is needed to incorporate these features into a mechanically robust system for *in vivo* applications.

This method of CDM preparation and electrospinning resulted in incorporation of approximately 15% cartilage proteins by dry weight within the PCL-CDM scaffold. The ratio of collagen to proteoglycan inclusion was approximately 5:1, consistent with articular cartilage, in which the ratio is ~4:1 [49]. The similarity of this ratio to native tissue suggests that neither of the two major cartilage components were preferentially lost in the lyophilizing, pulverizing, filtering, and electrospinning process. In this study, the CDM was not uniformly distributed within the scaffold (as seen in the scanning electron microscopy and histology data). While more uniform distribution of the CDM into the composite fiber may be desirable in some respects and is achievable through more processing of the CDM, electrospinning of solutions containing microparticles of up to 10µm diameter have recently been evaluated for phage or viral gene therapy delivery, protein or pharmaceutical delivery, and to increase the surface area of the scaffold [50, 51]. It is known that native collagen structure may be permanently disrupted during electrospinning [52, 53]; therefore in this initial study we elected not to evenly distribute all of the CDM within composite fibers in an attempt to protect some native proteins and matrikines from the electrospinning process. The CDM that was incorporated into these electrospun scaffolds showed chondrogenic effects despite this processing, indicating that this electrospinning process retains some important bioactive components of CDM.

Fiber diameters of the PCL-CDM scaffold were significantly smaller than the fibers in the PCL scaffolds, and those of single-layer PCL scaffolds were smaller than those of multi-layer scaffolds. The difference between PCL-CDM and PCL fiber diameter is most likely attributed to different PCL concentrations and solvent combinations used in preparation of the two scaffolds as well as the inclusion of CDM within the composite fiber. The presence of the CDM microparticles is not likely to have influenced fiber diameter by itself [50]. In PCL scaffolds, the difference in fiber diameter between single- and multi-layer PCL scaffolds may be due to protracted accumulation of insulating PCL fibers on the surface of the liquid ground in the single-layer group. This may have resulted in 'masking' of the ground solution to incoming positively charged fibers, greater instability and increased 'drawing out' of the whipping fibers before deposition and ultimately a reduction in fiber diameter.

PCL constructs had increased dsDNA content compared to PCL-CDM constructs, largely as a result of improved cell seeding. This is in contrast to previous studies examining the effects of CDM on PCL scaffolds, where dsDNA content of woven PCL constructs seeded with hASCs was not different from woven PCL constructs containing CDM at day 0, but CDM containing constructs had enhanced dsDNA content compared to PCL-only constructs at day 28 [54]. In another study, cell seeding on multi-layered electrospun constructs was not enhanced by tendon-derived matrix (TDM) compared to PCL, and dsDNA content was not different between TDM coating and PCL alone after 28 days [23]. The reason for this is unknown, but may be due to chemical alteration of sites critical for cell-matrix interactions in the CDM during the electrospinning process [35]. In the current study, PCL-CDM constructs had increased s-GAG content after cell seeding over time in culture compared to PCL constructs, and this effect was greatest in multi-layer PCL-CDM constructs compared to single-layer PCL-CDM constructs. Collagen and s-GAG content of PCL-CDM constructs was also increased relative to PCL constructs, although this effect appeared to be an additive effect of underlying CDM collagen content and collagen produced by hASCs on the constructs. Together, these findings suggest that multi-layer PCL-CDM constructs promote new cartilage-related ECM synthesis, and supports our previous findings of a chondroinductive effect of CDM on hASCs, in the presence or absence of exogenous growth factors [32, 34]. Limited studies of cell infiltration into multilayered constructs collected using an aqueous bath have shown rapid cell infiltration through the full thickness of the construct [19, 23, 24]. However, larger fiber diameter (~8µm) constructs produced by this technique demonstrate improved cell infiltration compared to those with smaller fiber diameters (~0.8µm) [19]. Enhanced cellular infiltration compared to single-layer constructs was observed in the current study for multi-layer PCL constructs (mean diameter 2.21 µm), but was less robust for multi-layer PCL-CDM constructs (mean diameter 0.56 µm), suggesting micro-, rather than nanoscale fibers may be critical for early and complete cell infiltration, even in multi-layered constructs.

All construct groups demonstrated an increase in *ACAN* expression over time, but 60-layer constructs demonstrated the greatest increase. *COL2A1* expression increased immediately after seeding compared to a baseline unseeded cell pellet, but then decreased over time in culture, even though type II collagen deposition was observed by immunohistochemistry. PCL-CDM constructs stimulated *COL10A1* expression to a greater extent than PCL constructs over time in culture, and *COL1A1* expression was increased over time, but with no effect of layer or construct type. Together, these gene expression data suggest that multi-layered constructs seeded with hASCs may promote a more hypertrophic chondrocyte phenotype [55], which was slightly increased by inclusion of the CDM. The increase in *COL1A1* expression may have resulted from the flattened, elongated fibroblastic morphology which we and others have observed from chondrocytes, MSCs and ASCs in monolayer culture and on electrospun constructs [9, 55, 56]. In support of these data, MSCs differentiated towards a chondrogenic lineage on electrospun constructs can maintain chondrogenic behavior upon implantation in osteochondral defects or progress to ossification upon subcutaneous implantation [56, 57]. These data suggest that the ultimate site of *in vivo* implantation may be important for maintenance of a chondrogenic rather than a fibrocartilage phenotype and caution against the over-reliance on interpretation of *in vitro*

differentiation data. Chondrogenic differentiation as assessed by gene expression may also not be immediately apparent *in vitro*. Rabbit MSCs seeded on PVA/PCL electrospun constructs did not express *COL2A1* until day 21 of chondrogenic differentiation, whereas *COL2A1* expression in a concurrent chondrogenic pellet culture was identified much earlier. In contrast, *ACAN* was expressed continuously in both pellet and constructs, but proteoglycan deposition was only identified in the presence of exogenous growth factors [56]. Further, in our previous studies, CDM did not induce upregulation in *COL2A1* expression by hASCs until day 14 of culture [32]. Similarly, MSCs seeded on electrospun PCL did not show significant upregulation of chondrogenic genes, despite increased cartilaginous matrix accumulation [5]. Thus, although some chondrogenic genes were shown to be upregulated in the current study, evaluation of these genes over longer periods of *in vitro* culture may be required to fully evaluate the capacity of these constructs to support chondrogenesis.

AFM was used to determine the Young's modulus of the constructs due to their small size and irregular geometry. A microspherical tip was used to evaluate properties over an area of several square microns, as opposed to highly localized (e.g., single fiber properties) obtained through use of a sharp tip. Use of the microspherical tip avoided problems previously associated with nanoindentation testing of electrospun scaffolds, such as poor contact of the tip with fibers, slipping of the tip off the fibers, displacement of the fibers by the tip, or inhomogeneous influence of nearby fibers [58]. The Young's moduli reported here are more consistent with bulk material properties than those of individual nanofibers [59, 60] [4]. The differences in Young's modulus identified between PCL and PCL-CDM scaffolds in this study at day 0 may be attributed to several factors. First, there were differences in material properties of the two polymers and preparation methods. Second, differences in fiber diameter, since Young's modulus increases with fiber diameter [59]. hASC-seeded PCL constructs were less stiff than acellular scaffolds after 28 days in culture, presumably reflecting the microscale mechanical properties of the hASCs or newly formed tissue on the construct surface [61, 62]. A similar softening of chondrocyte-seeded microfibrillar electrospun PCL constructs compared to acellular scaffolds has recently been reported following culture, suggesting that this may be a macroscale, rather than a microscale characteristic [3, 4]. The Young's modulus of these constructs did not improve upon previously reported results for electrospun constructs at the macroscale [63], and after 28 days in culture were far inferior to the elastic modulus of human articular cartilage tested using a similar AFM-based method [64]. Thus, future studies may wish to focus on methods to enhance the compressive mechanical properties of these constructs. The effect of a single freeze-thaw cycle on the microscale mechanical properties of these PCL and PCL-CDM scaffolds is not known. However, estimation of the Young's modulus of the pericellular matrix of chondrons using a similar AFM technique is consistent across many measurement techniques, including frozen section and immunolabeling [65]. The specific effects of extracellular and intracellular ice formation on tissue microstructure and on fluid matrix interactions are not fully understood, but compared to unfrozen tissue, freezing increases ECM pore size [66, 67]. In this study, we imaged each testing sites in contact mode, prior to determining micromechanical properties, thus while we cannot eliminate any possible

influence of changes to polymer structure or fluid matrix interactions on our results, we consistently tested void-free regions.

In conclusion, this study demonstrated beneficial effects of using a multi-layered approach that includes CDM in electrospun constructs for cartilage tissue engineering. Future studies will investigate additional methods of CDM incorporation, fiber diameter variation and alignment patterns to promote chondrogenesis while improving and maintaining compressive mechanical properties.

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Figure 1.

Scanning electron micrographs of PCL (A,C) and PCL-CDM scaffolds (B,D). Arrowhead in (B) annotates piece of CDM within matrix. Arrow in (D) annotates subpopulation of very small fibers in PCL-CDM scaffolds, * Pitted appearance to some fibers in PCL-CDM scaffold. Scale bars in A, B are 20 μm , scale bars in C, D are 5 μm .

Figure 2.

Distribution of fiber diameter of PCL (A) and PCL-CDM (B) scaffolds. PCL-CDM scaffolds were composed of smaller fibers than PCL scaffolds ($p < 0.0001$), and for PCL scaffolds only, multilayered scaffolds had greater fiber diameter than single-layer scaffolds ($p < 0.0001$). $n = 183\text{--}190$ fibers, $N = 3$ scaffolds. Factorial ANOVA, Tukey's post-hoc test.

Figure 3.

Single-layer PCL constructs (C,I), multi-layer PCL constructs (D,J), single-layer PCL-CDM constructs (A,E,G,K) and multi-layer PCL-CDM constructs (B,F,H,L) at 0 (A,B, G,H) and 28 days (C–F, I–L) after seeding with human adipose stem cells, and stained with safranin-O/fast green (A–F, 4x objective) or immunohistochemistry for type II collagen (G–L, 10x objective). Inset in B is safranin-O/fast green section of porcine cartilage, and insets at G–L represent negative controls. Scale bar 250um.

Figure 4. Mean ± SD dsDNA (A), s-GAG (B) and collagen (C) content of single- and multilayer PCL and PCL-CDM constructs over 28 days in culture. Factorial ANOVA, Tukey's post-hoc test. n=6, p<0.05. Within each panel, groups that do not share the same letter are different. +, #, *: culture time significantly different within seeding and scaffold group. † effect of layer for PCL-CDM only.

Figure 5.

Gene expression aggrecan (ACAN)(A), type II collagen (COL2A1) (B), type I collagen (COL1A1)(C), and type X collagen (COL10A1)(D) of hASCs on single- and multilayer PCL and PCL-CDM constructs over 14 days in culture, normalized to hASC cell pellet at Day 0 (mean±SD). Within each panel, groups that do not share the same letter are different, $p < 0.05$, $n=3$. * indicates that there is an effect of layer.

Figure 6.

Young's Modulus of PCL and PCL-CDM constructs. PCL-CDM significantly different to PCL constructs (different letters), # single- and multi-layer constructs significantly different, * acellular and hASC seeded significantly different, + Day 0 significantly different to Day 28. Factorial ANOVA, Tukey's post-hoc test, $p < 0.05$, $n = 3$.