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Macromol Biosci. Author manuscript; available in PMC 2016 July 01.

Published in final edited form as:

Macromol Biosci. 2015 July ; 15(7): 979–989. doi:10.1002/mabi.201400472.

Bioactive Microsphere-Based Scaffolds Containing Decellularized Cartilage

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Abstract

The aim of this study was to fabricate mechanically functional microsphere-based scaffolds containing decellularized cartilage (DCC), with the hypothesis that this approach would induce chondrogenesis of rat bone marrow-derived mesenchymal stem cells (rBMSCs) *in vitro*. The DCC was derived from porcine articular cartilage and decellularized using a combination of physical and chemical methods. Four types of scaffolds were fabricated: Poly(D,L-lactic-co-glycolic acid) (PLGA) only (negative control), TGF-β encapsulated (positive control), PLGA surface coated with DCC, and DCC-encapsulated. These scaffolds were seeded with rBMSCs and cultured up to 6 weeks. The compressive modulus of the DCC-coated scaffolds prior to cell seeding was significantly lower than all other scaffold types. Gene expression was comparable between DCC-encapsulated and TGF-β encapsulated groups. Notably, DCC-encapsulated scaffolds contained 70% higher glycosaminoglyan (GAG) content and 85% more hydroxyproline compared to the TGF-β group at week 3 (with baseline levels subtracted out from acellular DCC scaffolds). Certainly bioactivity was demonstrated in eliciting a biosynthetic response from the cells with DCC, although true demonstration of chondrogenesis remained elusive under the prescribed conditions. Encapsulation of DCC appeared to lead to improved cell performance relative to coating with DCC, although this finding may be a dose-dependent observation. Overall, DCC introduced via microsphere-based scaffolds appears to be promising as a bioactive approach to cartilage regeneration, although additional studies will be required to conclusively demonstrate chondroinductivity.

Introduction

Articular cartilage has limited capability for self repair after traumatic injury or osteoarthritis. Self-repair is limited in part because of the dense extracellular matrix (ECM), sparse chondrocyte population, and reduced access to systemic circulation. Current clinical treatments include osteochondral transplantation (mosaicplasty), autologous chondrocyte implantation (ACI), and microfracture.[1, 15] These current treatment options may produce inferior repair cartilage with respect to mechanical performance, tissue reintegration, and

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composition.[6, 22] They also have additional associated risks such as donor site morbidity and the need for multiple surgical procedures. Recently, acellular biomaterials have gained much popularity in the tissue engineering field due to the potential to create an off-the-shelf product with characteristics that aid in repairing cartilage tissue by enhancing stem cell recruitment, infiltration, and differentiation.[2, 5] One such material in particular, decellularized cartilage (DCC),[33] may be beneficial as it contains similar biochemical content as native cartilage and current problems associated with allograft implants (i.e., long term storage and immunogenicity) are mitigated.[4, 21, 23, 29] Previous studies have reported adipose derived stem cell (ASC) and bone marrow-derived mesenchymal stem cell (BMSC) differentiation in the presence of DCC.[6, 7, 34]

One challenge with DCC-based scaffolds is that the mechanical function of the scaffolds may be compromised during the decellularization process.[22, 28, 29] To help fabricate a material with a compressive strength suitable for articular cartilage repair, combining DCC with a polymeric scaffold has previously been shown to achieve greater mechanical performance than DCC scaffolds alone.[27] Using cartilage matrix to coat polymeric based scaffolds has also been investigated previously with electrospun scaffolds, but instead of using native cartilage, cell-derived cartilage matrix (CDM) secreted *in vitro* was used.[24]

We recently defined categories of cartilage matrix for tissue engineering in *Advanced Healthcare Materials*.[33] The difference between native DCC and CDM in particular must be emphasized here, as these matrices may vary in both composition and mechanical performance. Decellularization efficiency may be greater in CDM because the matrix is less dense, but the material may not contain the same composition as native cartilage ECM that comprises DCC. DCC was chosen for use in this study for the ease of acquiring the material and the ability to efficiently decellularize the tissue while maintaining biochemical content similar to native cartilage ECM.

Microsphere-based scaffolds for osteochondral tissue engineering are an attractive delivery vehicle for DCC due to the ability to control the morphology of the microspheres and, in turn, the properties of the bulk scaffold.[11, 26] The polymeric material can also be selected for desired degradation and release rates of a wide variety of encapsulated materials.[8, 17, 18, 32] Previously, chondroitin sulfate and bioactive glass as “raw materials” have been encapsulated in poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres that aided in BMSC differentiation and proliferation.[26] DCC, however, has never previously been incorporated into microsphere-based scaffolds.

In the present study, we investigated the encapsulation of DCC in PLGA microsphere-based scaffolds and the coating of the surface of PLGA microsphere scaffolds. Our hypothesis was that the DCC material would aid in chondrogenic differentiation of BMSCs *in vitro*.

Materials and Methods

Materials

All reagents for decellularization were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted. PLGA (50:50 D, L-PLGA with acid end group, intrinsic viscosity

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0.40-0.50 dL/g) was purchased from Lakeshore Biomaterials (Birmingham, AL). Human recombinant TGF-β_3 was purchased from PeproTech (Rocky Hill, NJ). 10 porcine knee and hip joints were obtained from crossbreed hogs (Cheshire White, Yorkshire, Berkshire, Duroc, Landrace, and Hampshire) with an approximate average mass of 120 kg. The joints were purchased from a local abattoir following sacrifice (Bichelmeyer Meats, Kansas City, KS).

Tissue Harvest and Decellularization

Articular cartilage was collected from joint surfaces using scalpels and immediately rinsed in phosphate buffered saline (PBS). No arthritic lesions were noted in joints from which the cartilage was collected. PBS was drained from the material and cartilage was stored at −20°C until further use. Decellularization of the cartilage was performed using a protocol adapted from Converse *et al*.[9] The cartilage was first coarsely ground using a cryogenic tissue grinder to reduce diffusion distances during the decellularization process (BioSpec Products, Bartlesville, OK). Following additional freezing at −20°C, the cartilage particles were packaged into dialysis tubing (3500 MWCO) and stored in hypertonic salt solution (HSS) overnight at 21°C with gentle shaking (70 rpm). All subsequent steps were performed at 21°C under agitation (200 rpm) unless otherwise noted. The tissue was then subject to two reciprocating washes of triton X-100 (0.01% v/v) followed with HSS to permeabilize intact cellular membranes. Overnight, the tissue was treated with benzonase (0.0625 KU ml^{-1}) to fragment nucleic acids at 37°C. Next, the tissue was treated with sodium-lauroylsarcosine (NLS, 1% v/v) overnight to further lyse cells and denature cellular proteins. Following NLS, the tissue was washed with ethanol (40% v/v). The tissue was then subjected to organic exchange resins to extract the organic solvents. Lastly, the tissue was washed in saline-mannitol solution (SMS). Following decellularization, the cartilage tissue was rinsed with deionized water and stored at −20°C. After freezing, the tissue was lyophilized and cryo-ground in a freezer-mill (SPEX Sample Prep, Metuchen, NJ).

Microsphere and Scaffold Fabrication

Three types of microspheres were produced (1) PLGA only (PLGA) (negative control), (2) TGF-β_3 encapsulated (TGF) (positive control), and (3) solubilized DCC encapsulated (DCC-encapsulated). All microspheres were fabricated using the patented precision particle formation method.[3, 12, 26, 32] Microspheres were approximately 350-400 μm in diameter.

PLGA microspheres were fabricated at 20% w/v. DCC microspheres were fabricated by first solubilizing the DCC in an acid-pepsin solution for 24 hours. The acid pepsin solution contained 0.1 M HCl, 20 mg DCC / 1 mL HCl, and 1 mg pepsin / 1 mL HCl (pH = 5) (Sigma Aldrich, St. Louis, MO). After the solubilization period, the pH of the solution was raised by adding one-tenth the solubilized solution volume of 1 M NaOH and one-tenth the final solution volume of 10x PBS (pH = 8).[30] The solubilized solution was then frozen and lyophilized. The solubilized DCC was added to a PLGA solution at 10% w/w. TGF-β was reconstituted in 10 mM citric acid TGF microspheres were created with a concentration of 30 μg TGF-β_3/ g PLGA.[12, 26]

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Scaffolds were fabricated as previously described.[13, 26, 32] Briefly, microspheres were first loaded in a glass cylindrical mold. A 10 μm filter was used at the bottom of the mold and DI H_2O was pulled through the mold by a vacuum pump. The microspheres were sintered together in a 95% v/v ethanol-acetone solution for 45 minutes. Resulting scaffold dimensions were an average height of 4.43 ± .99 mm and an average diameter of 3.75 ± .08 mm. DCC-coated PLGA scaffolds were created using PLGA scaffolds (from PLGA-only microspheres; creating a DCC-coated group). The PLGA scaffolds were placed in the DCC acid-pepsin solution for 3 minutes and the pH was adjusted to neutral by adding 1 M NaOH. The scaffolds were then lyophilized. Following sintering, the scaffolds were lyophilized. All scaffolds were sterilized with ethylene oxide prior to cell seeding. Additionally, additional acid-treated scaffolds were created specifically for mechanical testing purposes. PLGA scaffolds were exposed to an acidic HCl solution (pH = 5) for 3 minutes and then lyophilized to mimic the effects of the acid-pepsin solution to which the scaffolds were exposed during the DCC-coating process.

Cell Isolation and Culture

Following a University of Kansas approved IACUC protocol (AUS 175-08), BMSCs were isolated from the femurs of 4 male Sprague-Dawley rats (200-250 g). Cells were isolated from the femurs by flushing the bones with cell culture media and immediately transferring the isolated bone marrow to tissue culture flasks. During expansion, the BMSCs were cultured in minimum essential media (MEM) alpha with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic. The BMSCs were expanded to passage 4 and suspended in cell culture media at a concentration of 12×10^5 cells/mL and 40 μL of cell suspension was pipetted onto constructs at 1×10^7 cells/cm^3 (500,000 cells/scaffold). After cells were seeded in the scaffolds, the scaffolds were placed in the incubator for 1 hour to allow the cells to attach before adding culture media. The cell seeded scaffolds were cultured in medium containing high glucose DMEM, 1% insulin-transferrin-selenium 100X (ITS), 50 μg/mL ascorbic acid, 40 μg/mL L-proline, 1% penicillin-streptomycin, 0.1 μM dexamethasone, 25 mM HEPES buffer, 1% non-essential amino acids (NEAA), and 100 μM sodium pyruvate.

SEM Imaging and Energy Dispersion Spectroscopy

Microspheres were imaged with a LEO 1550 scanning electron microscope (SEM) to observe the morphology of the microsphere surfaces. All microspheres were sputter-coated with 15 nm gold. To visualize the distribution of DCC, the presence of atomic nitrogen on the surface of the DCC-encapsulated microspheres was detected by SEM using energy dispersive spectroscopy (EDS) at 10 kV.

Biochemical Content Analysis

Biochemical content analysis was performed on solubilized DCC, acellular scaffolds, and cell seeded scaffolds (n = 5). For all analyses day 0 samples were collected at 24 hours. The biochemical content of the cell-seeded scaffolds was measured at 0, 3, and 6 weeks. Acellular scaffolds matched each time point and were used to subtract base values for all biochemical content. All samples were digested in 1 mL of papain solution containing 125

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μg/mL papain, 5mM N-acetyl cysteine, 5mM ethylenediaminetetraacetic acid (EDTA), and 100 mM PBS.[13, 16, 32]

Biochemical content was measured as previously described.[13, 16, 32] Briefly, double stranded DNA content was measured with the PicoGreen assay (Molecular Probes, Eugene, OR). The assay was performed according to the manufacture's instructions. Sulfated glycosaminoglycan (GAG) content was measured with the dimethylmethylene blue (DMMB) assay (Blyscan, Westbury, NY) according to the manufacturer protocol. Total hydroxyproline content was measured with the Sigma-Aldrich commercially available hydroxyproline assay kit (St. Louis, MO).

DCC Release Analysis

Acellular DCC-encapsulated and DCC-coated were cultured in the same conditions as cell seeded scaffolds. The scaffolds were used to determine the remaining amount of GAG and hydroxyproline as described above. The amount of GAG and hydroxyproline content in acellular scaffolds was used to approximate the release of the DCC from the scaffolds.

Mechanical Testing

Uniaxial unconfined compression testing was performed on acellular scaffolds (n=4-5) with a custom-built compression-bath assembly in an Instron 5848 microtester (Norwood, MA).[31] Five different scaffold groups were tested: PLGA, TGF, DCC-coated, DCC-encapsulated, and acid treated scaffolds. Following a tare load of 0.01 N, samples were compressed at a strain rate of 10%/min in PBS at 37 °C. The compressive modulus was calculated from the linear region of the stress-strain curve.

Gene Expression Analysis

Gene expression analysis was performed at 0, 1.5, 3, and 6 weeks (n=5). Day 0 was defined as 24 hours following cell seeding. RNA was isolated and purified with the Qiagen (Valencia, CA) RNeasy kit following manufacturer recommendations. RNA was reverse-transcribed using a high capacity reverse transcriptase kit (Invitrogen, Carlsbad, CA). RT-PCR was performed with a RealPlex thermocycler (Eppendorf, Hauppauge, NY) and TaqMan gene expression assays (Invitrogen, Carlsbad, CA). All primers were commercially available and purchased from Invitrogen. Gene expression analysis was performed for both chondrogenic and osteogenic gene markers including Coll1A1, Coll2A1, Aggrecan, Sox9, and Runx2. GAPDH was used as an endogenous control. All results are reported as relative expression to GAPDH using the $2^{-\Delta\Delta Ct}$ method.[13, 25]

Immunohistochemical Staining

Immunohistochemistry was performed on week 3 DCC-encapsulated scaffolds. Week 6 constructs did not withstand the initial processing and were not suitable for IHC use. The scaffolds were fixed in 10% formalin and embedded in optimal cutting temperature (OCT) medium (Tissue-Tek, Torrance, CA). 10 μm thick sections were cut using a cryostat (Micron Hm-500 OMP, Vista, CA). Primary antibodies for collagen I, collagen II, and aggrecan were obtained from Abcam (Cambridge, MA). Following the primary antibodies, biotinylated secondary antibodies were used followed with the ABC complex (Vector Labratories,

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Burlingame, CA). The antibodies were visualized with the diaminobenzidine (DAB) substrate per the manufacturer's protocol. Acellular scaffolds were also stained to provide background staining levels for the DCC-containing scaffolds.

Statistical Analysis

All results are reported as mean ± standard deviation. Box plots were created to remove all statistical outliers. Statistical analyses were performed using one way analysis of variance (ANOVA) and Tukey's *post-hoc* tests. Significance was reported for $p<0.05$. SPSS statistical software was used for all analyses (Armonk, NY).

Results

Microsphere Morphology

PLGA microspheres had a smooth, even surface without any pores (Fig. 1). TGF microspheres showed small pores on the surfaces. The DCC microspheres also had numerous small pores on the surface. To view the distribution of DCC, EDS showed that atomic nitrogen was present on the surface of the DCC-encapsulated microspheres (Fig. 2), and that the nitrogen was fairly evenly distributed, although not perfectly uniform.

Tissue Decellularization

Following decellularization and cryo-grinding the DNA content was reduced by 86%. The GAG content was reduced by 55% and the hydroxyproline content was not significantly changed (results not shown).

Biochemical Analysis

Dry SDCC contained 42.6 ± 2.6 μg GAG/mg prior to incorporation into scaffolds. Acellular scaffolds were used to determine the remaining GAG and hydroxyproline content in the DCC and coated scaffolds. At t = 0, the acellular DCC-encapsulated scaffolds contained nearly 4 times as much GAG as the acellular DCC-coated scaffolds ($p<0.001$) (Fig. 3). The acellular DCC-encapsulated group showed an approximately 50% decrease in GAG content from day 0 to week 3 ($p<0.001$) (Fig. 3). After week 3, the GAG content remained roughly equal through week 6 in DCC-encapsulated scaffolds (no significant difference). The hydroxyproline content in the DCC-encapsulated scaffolds was approximately 10 times greater than in the DCC-coated group at day 0 ($p<0.001$). The DCC-encapsulated scaffold hydroxyproline content remained similar between weeks 3 and 6. Relative GAG loss in both DCC-encapsulated and DCC-coated scaffolds was similar by week 6 (Fig. 4).

Moving on to the cell-seeded scaffolds, at day 0 (24 hours after seeding), the cell-seeded DCC-coated scaffold group had approximately 30% more DNA than all of the other groups at all times ($p<0.001$) (Fig. 5). By week 3, all of the groups had similar amounts of DNA and remained constant through week 6 at approximately 3.5 μg DNA/scaffold. Since there were no statistically significant differences in DNA content among groups, other than the day 0 DCC-coated group, the hydroxyproline and GAG totals are reported here on a basis of total content per scaffold.

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The DCC-encapsulated scaffolds contained more hydroxyproline than both the PLGA (blank) and TGF scaffolds at day 0 and week 3, even with baseline values subtracted out from acellular scaffolds ($p<0.001$) (Fig. 6). Specifically, the DCC-encapsulated group had nearly 7 times as much hydroxyproline as the blank group ($p<0.001$) and almost 10 times as much hydroxyproline as the TGF group ($p<0.001$) at day 0. At week 3, the DCC-encapsulated scaffold group contained approximately 8 times the amount of hydroxyproline as the TGF scaffolds and 40 times the amount of hydroxyproline as the blank scaffolds ($p<0.001$). At week 6, DCC-encapsulated scaffolds exhibited a significant 70% reduction in the amount of hydroxyproline per scaffold ($p<0.005$), although here was no significant change in hydroxyproline content in the DCC-coated scaffolds with time. At week 6, the DCC-encapsulated group had over 10 times as much hydroxyproline as the blank group ($p<0.05$) and over 4 times more hydroxyproline than the TGF group ($p < 0.05$).

In blank, DCC-encapsulated, and DCC-coated cell seeded scaffolds the GAG contents significantly decreased from week 3 to week 6 ($p<0.001$) (Fig. 6). Similar trends were seen between GAG and hydroxyproline content, e.g., decreased amount of GAG content at week 6 compared to week 3. DCC-encapsulated scaffolds had 3 times greater GAG content and 8 times greater hydroxyproline content at 3 weeks compared to the TGF group at the same time ($p<0.001$). At week 6, the GAG content of the DCC-encapsulated scaffolds decreased by 65% from the week 3 value ($p<0.005$).

Mechanical Testing

The blank, TGF, and DCC-encapsulated scaffolds groups at week 0 had compressive elastic moduli around 80 kPa that were not significantly different from one another. However, the DCC-coated group compressive elastic modulus was approximately 75% less than all of the other groups ($p<0.05$) (Fig. 7). As a basis of comparison for the DCC-coated group, the acid treated scaffolds were not statistically significant from the blank scaffolds or the DCC-coated scaffolds. The acid treated scaffold compressive elastic modulus was over 3 times greater than that of the DCC-Coated scaffolds, however, there was no statistical significance between the groups.

Gene Expression

At day 0, the TGF group had a mean value of collagen II gene expression that was over 3 times higher than the mean value for the DCC-encapsulated group, however, the difference was not statistically significant (Fig. 8). The TGF group had nearly 16 times the expression of collagen II compared to the blank group at day 0 ($p<0.001$). There was no statistically significant difference between collagen II expression in the DCC-encapsulated group and TGF group. The DCC-coated group had significantly less expression than the TGF group at day 0 ($p<0.001$). After day 0, no group showed a detectable expression of collagen II.

At day 0, day 10, and week 3, the DCC-encapsulated group had a similar expression of Sox9 compared to the blank group. Additionally, the DCC-encapsulated group had similar expression of Sox9 as the TGF group at day 10 and week 3. At day 0, the TGF group had nearly 3 times the expression of Sox9 compared to the DCC-coated group ($p<0.001$).

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There were no statistically significant differences in aggrecan expression among groups at any time point. However, it was worth noting that the aggrecan expression in the blank group decreased 87% at week 3 compared to days 0 and 10 ($p<0.01$).

Both TGF and DCC-encapsulated groups had nearly 80% less expression of Collagen I at day 0 compared to the blank and DCC-coated groups ($p<0.001$). At day 0, the blank group had 2.5 times greater expression of Runx2 compared to the TGF group ($p<0.001$). There were no significant differences among the TGF, DCC-coated, and DCC-encapsulated groups with respect to Runx2 expression.

Immunohistochemical Staining

Immunohistochemical staining of week 3 DCC-encapsulated scaffolds was positive for collagen II, collagen I, and aggrecan (Fig. 9). Collagen I staining was more intense in the cell-seeded DCC-encapsulated scaffolds compared to the acellular DCC-encapsulated scaffolds. Collagen II and aggrecan staining were comparable between cell-seeded and acellular scaffolds at week 3.

Discussion

In the current study, DCC was incorporated into microsphere-based scaffolds either by coating PLGA microsphere scaffold surfaces or by encapsulating the DCC within the PLGA microspheres. Although cellular response characterization to the DCC in the microsphere-based scaffolds did not overwhelmingly indicate chondrogenesis in BMSCs, the DCC material did induce some level of bioactivity with the cells. The difference between the loading amounts achieved by different DCC incorporation methods (coating vs. encapsulating) may have contributed to the difference in cellular responses to the respective scaffold types, as the acellular DCC-encapsulated scaffolds contained significantly greater amounts of GAG and hydroxyproline than the acellular DCC-coated scaffolds, which may be viewed as an inherent limitation in the coating method. Although the processing of the DCC material in both DCC-coated and DCC-encapsulated groups was the same, the presentation of the material to the BMSCs was different and may have also contributed to differences in cell response. The DCC-encapsulated group relied more greatly on diffusion of the DCC out of PLGA microspheres and degradation of these microspheres, even though there was visible distribution of DCC on the surface of the DCC-encapsulated microspheres with SEM-EDS. In contrast, DCC on the DCC-coated group was available for cells on the surface of the scaffolds and did not rely on diffusion or degradation.

A benefit to the microsphere-based scaffolds and encapsulation technique used in the study was that the DCC-encapsulated scaffolds had comparable mechanical properties to the PLGA scaffolds. The DCC-coated scaffolds, however, had a significantly reduced compressive modulus compared to all other scaffold types. The decrease in mechanical stiffness of the DCC-coated scaffolds may have been partially due to the coating procedure and exposure to acid as the acidic conditions could increase the rate of degradation of the PLGA. However, the acid-treated scaffolds evaluated for purpose of comparison did not exhibit the same decrease in modulus as the DCC-coated scaffolds, meaning some aspect of the DCC coating itself may have adversely affected mechanical performance. Previous

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studies have also reported decreased mechanical properties in cartilage matrix-polymer constructs compared to polymer-only constructs.[14, 27]

The porous morphology of the microspheres allowed for diffusion of DCC out of the respective scaffold types. Based on quantification of remaining hydroxyproline and GAG content in acellular scaffolds, by week 6, the remaining GAG content reduced by 50% and the remaining hydroxyproline content reduced by 75% in the DCC-encapsulated scaffolds. At week 6, the total hydroxyproline and GAG content in cell-seeded DCC-encapsulated scaffolds also significantly decreased compared to previous time points. Additionally, at week 3, chondrogenic markers Sox9, aggrecan, and collagen II all decreased in the DCC-encapsulated group. The decrease in chondrogenic gene markers at week 3 in the DCC-encapsulated scaffold group was consistent with the more intense staining of collagen I at week 3 in the DCC-encapsulated scaffolds. However, although chondrogenic markers decreased at week 3, collagen I and the osteogenic marker Runx2 remained low and not significantly different from that of the TGF group. At all gene expression time points, although the DCC-encapsulated scaffolds did not differ significantly from the TGF group, the hydroxyproline content was greater in DCC-encapsulated constructs at all time points compared to the TGF scaffolds. The GAG content was also greater in DCC-encapsulated scaffolds than in TGF scaffolds at day 0 and week 3. These higher biochemical contents in the DCC-encapsulated group may suggest that the encapsulated DCC was more effective than TGF-β at inducing biosynthesis in BMSCs, although there was not sufficient evidence of outperforming TGF-β in inducing chondrogenesis. Once less than half of the originally encapsulated DCC remained in the scaffolds, a decrease in GAG and hydroxyproline production rate was observed as well as a decrease in chondrogenic gene markers. The significant reduction in remaining DCC encapsulated within the scaffolds suggests that the bioactivity that was observed through week 3 was due to the encapsulated DCC. *In vivo*, the DCC in an osteochondral defect would be better confined to the defect and may thus possibly be more potent in its bioactivity, although future studies will be required to evaluate this possibility.

The greater cell number as evaluated by DNA content at the initial time point for DCC-coated than for DCC-encapsulated constructs may be due to the immediate exposure to DCC, which may contain cellular adhesion sites to aid in cell attachment and migration. Such an advantage with immediate cell response may be of major importance *in vivo* as initial BMSCs infiltrate the scaffold from an osteochondral defect.

The mechanism by which DCC would induce chondrogenesis is still unclear at this time. In the current study, we showed that encapsulated DCC and TGF-β had similar effects on BMSC gene expression *in vitro*, but encapsulated DCC had a greater effect on BMSC production of GAG and collagen than TGF-β. The encapsulation of DCC compromised the macro-structure of the matrix during solubilization and the DCC effect remained positive on the cells, this may indicate that the structure of the matrix is not vital to bioactivity induced by DCC. Additionally, the difference between the amounts of DCC material each scaffold type contained (coated vs. encapsulated) may have had an effect on the differences seen in cellular response. The response to DCC may be dose dependent. Additional work to identify a preferred loading dose may be beneficial for future work.

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Microsphere-based scaffolds are a promising alternative to current cartilage repair techniques due to the ability to control both their mechanical properties and to encapsulate a wide variety of materials.[8, 12, 26, 32] Encapsulated materials can be selected to aid in stem cell differentiation and cartilage tissue repair.[26] DCC was chosen for encapsulation and coating because of previous studies citing cartilage matrix as a potentially chondroinductive material.[10, 14, 24, 27, 34] The use of decellularized cartilage instead of native or devitalized cartilage is advantageous from clinical and commercial standpoints because of decreased immunogenicity and long term storage of the material. Successful decellularization of tissues, i.e., complete removal of residual DNA and immunogenic antigens, may also eventually lead to safer xenogeneic tissue implants for all tissue types.[19, 20, 29]

In summary, DCC microsphere-based scaffolds led to gene expression and mechanical performance comparable to that of TGF-β, while outperforming both the TGF-β and control groups in biosynthesis, suggesting that DCC in a microsphere-based scaffold may indeed be bioactive, but additional work remains in terms of method of incorporation (i.e., coated vs. encapsulated) and dose to determine whether indeed a chondroinductive approach is achievable. Encapsulation of DCC carries the advantage of delivering greater amounts of DCC, whereas coating with DCC has the advantages of immediate exposure without relying on diffusion or degradation. In terms of the method of incorporation, the DCC-encapsulated group generally outperformed the DCC-coated group with the techniques presented. However, perhaps a combination of coating and encapsulation to leverage the advantages of each would provide the greatest overall effect. Overall, using microsphere-based scaffolds as a means to incorporate and deliver DCC to regenerating cartilage may be a powerful tool in the future for treatment of cartilage defects.

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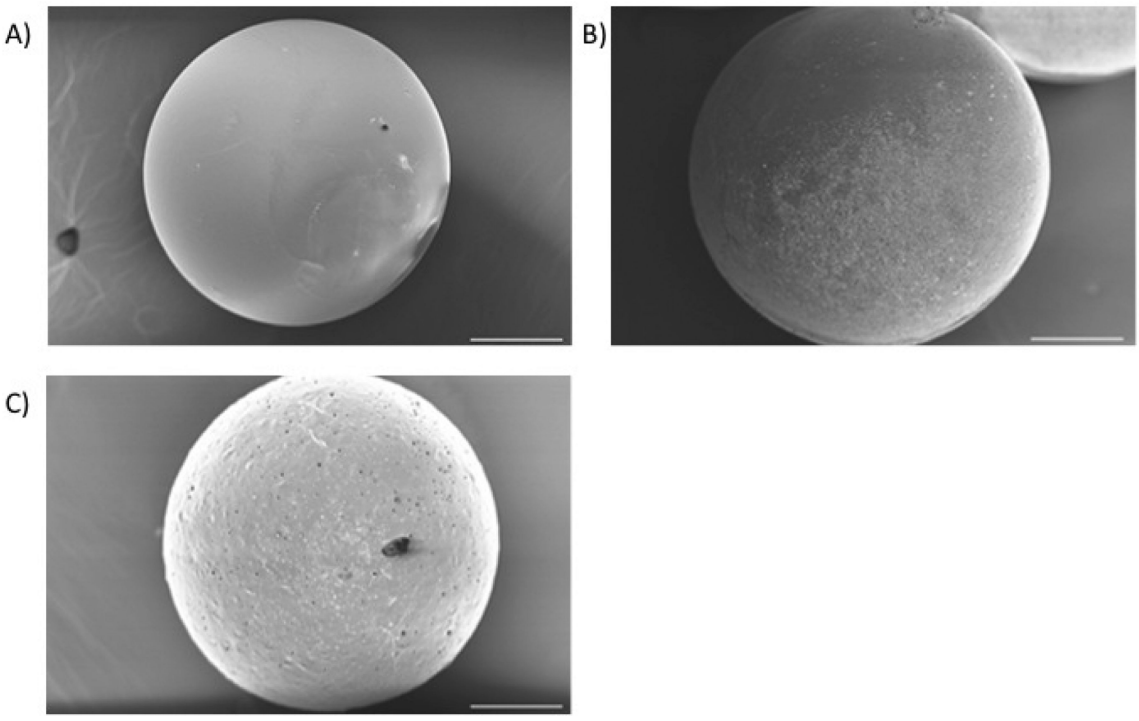

Figure 1.

SEM images of microsphere morphology. Both TGF-β3- and decellularized cartilage (DCC)-encapsulated microspheres had slightly porous surfaces, whereas PLGA microspheres (i.e., blank, nothing encapsulated) had smooth surfaces. Scale bars are 100 μm. A) PLGA microsphere, B) DCC-encapsulated microsphere, and C) TGF-β3-encapsulated microsphere.

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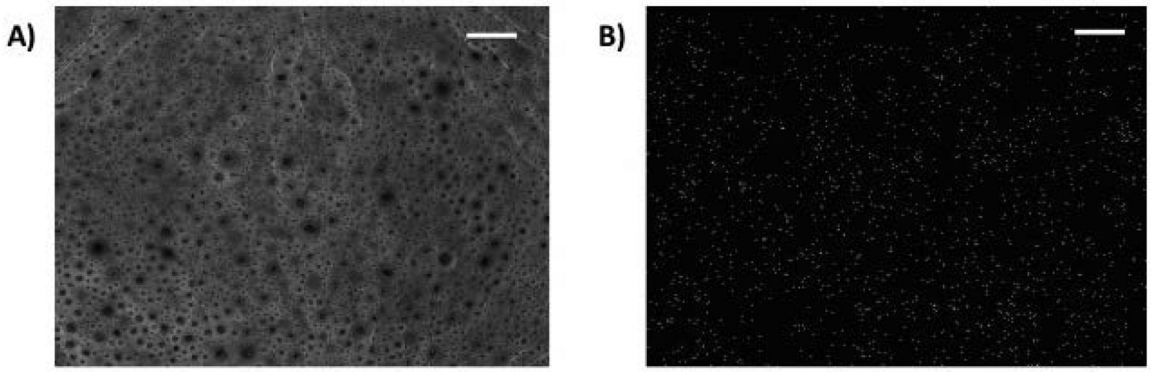

Figure 2.

A) Scanning electron microscopy (SEM) image of a representative decellularized cartilage (DCC)-encapsulated microsphere, and B) energy dispersive spectroscopy (EDS) pixel map depicting the location of atomic nitrogen on the surface of the DCC-encapsulated microsphere. Given that the PLGA polymer does not contain atomic nigrogen, it can be seen that DCC was distributed across the surface of the microsphere. Scale bars are 10 μm.

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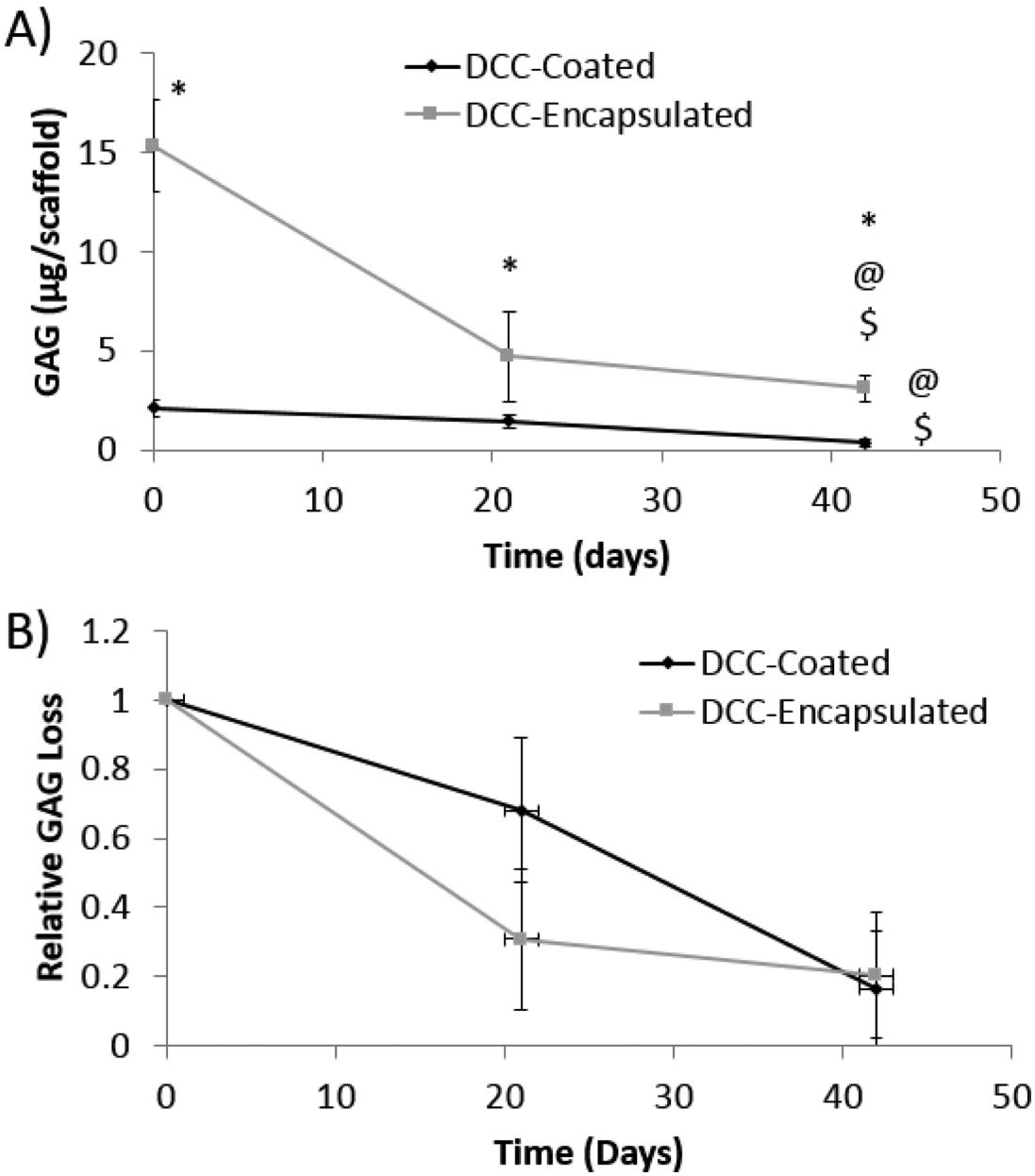

Figure 3.

Remaining GAG and hydroxyproline in acellular DCC-encapsulated and DCC-coated scaffolds at day 0, week 3 and week 6. All scaffolds exhibited a decrease in biochemical content by week 3 ($p<0.05$). n = 5. Data is reported as mean ± standard deviation. * denotes statistical significance from DCC-coated group at the same time point, @ denotes statistical significance from the day 0 time point, $ denotes statistical significance from the previous time point.

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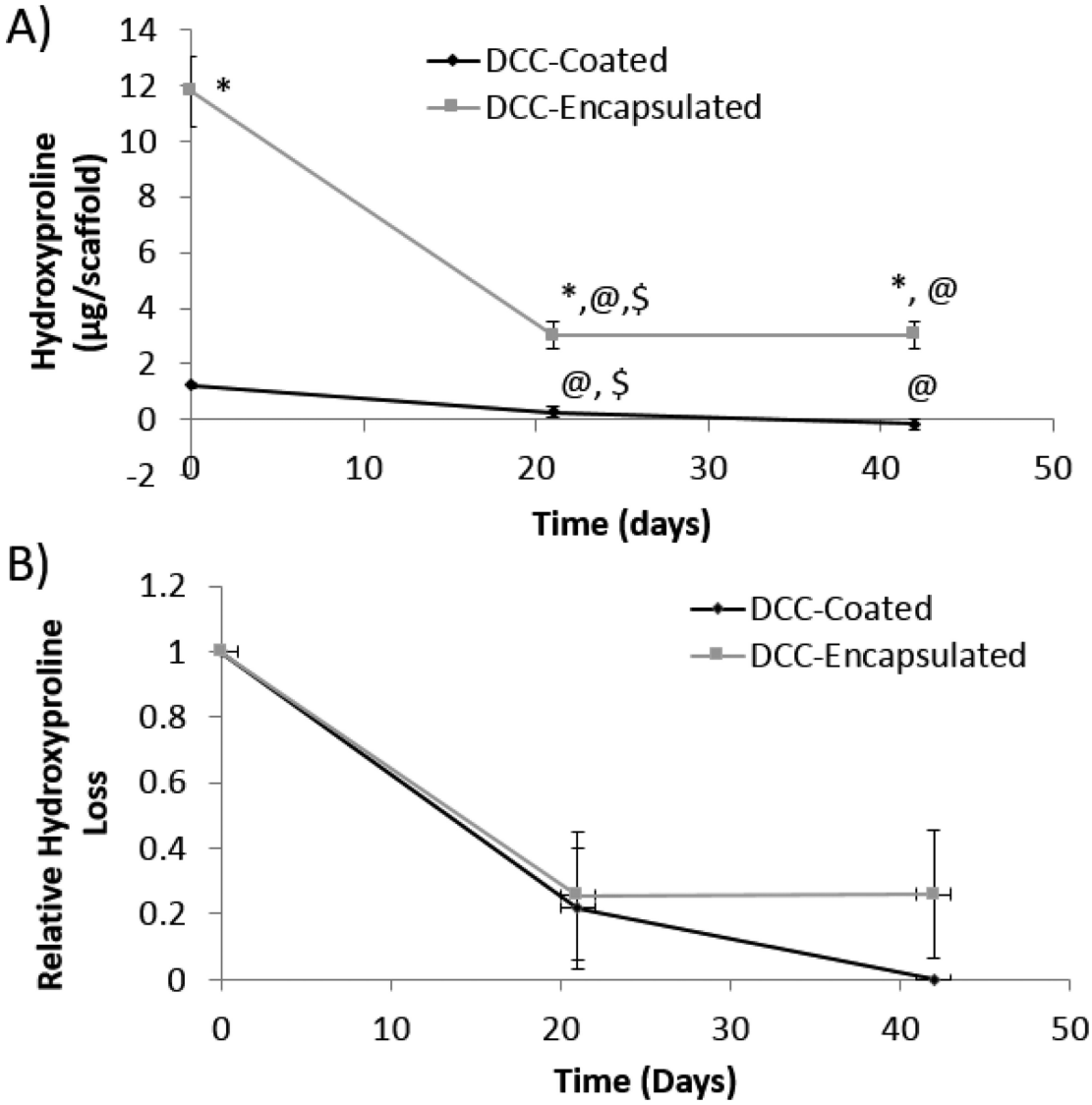

Figure 4.

Relative loss of A) hydroxyproline and B) GAG from acellular DCC-Coated and DCC-Encapsulated scaffolds. By week 6, a greater proportion of hydroxyproline was lost from the DCC-Coated scaffolds and an equal proportion of GAG was lost from each type of scaffold. n = 5. Data is reported at mean ± standard deviation. * denotes statistical significance from DCC-coated group at the same time point, @ denotes statistical significance from the day 0 time point, $ denotes statistical significance from the previous time point.

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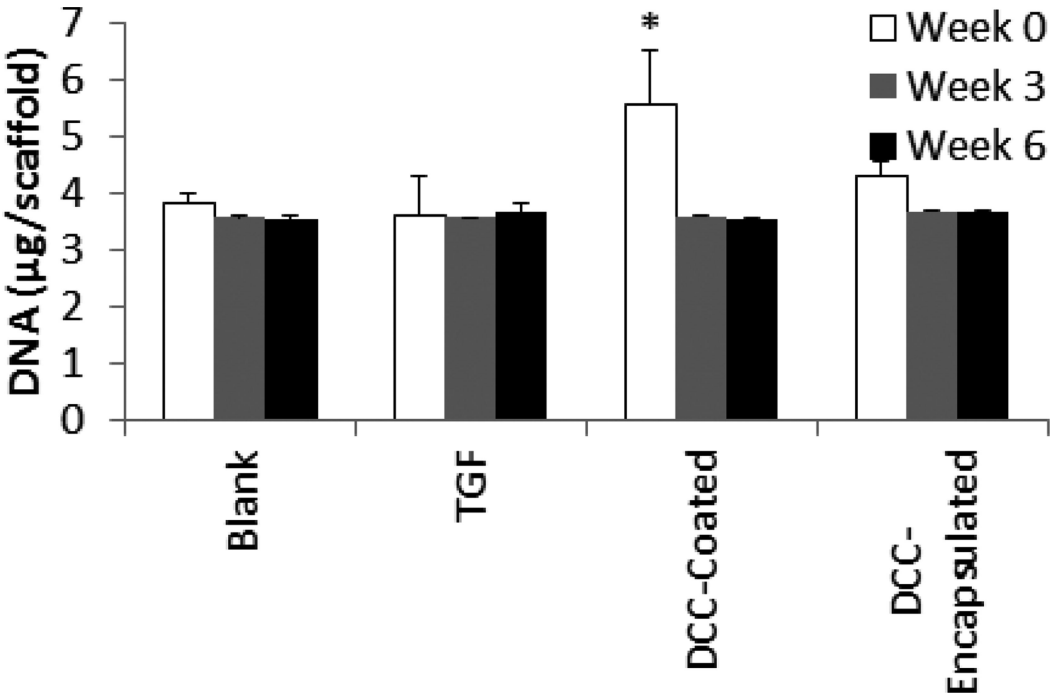

Figure 5.

PicoGreen results depicting greater DNA content on DCC-coated scaffolds at week 0 (corresponding to 24 hours after seeding). Blank = PLGA microspheres with nothing encapsulated, TGF = PLGA microspheres with TGF-β3 encapsulated, DCC-coated = PLGA microspheres with nothing encapsulated but coated in decellularized cartilage (DCC), and DCC-encapsulated = PLGA microspheres with DCC encapsulated. *denotes statistically significant difference from all other groups at same time and subsequent times of same group ($p<0.05$). Data are reported as mean ± standard deviation ($n = 5$).

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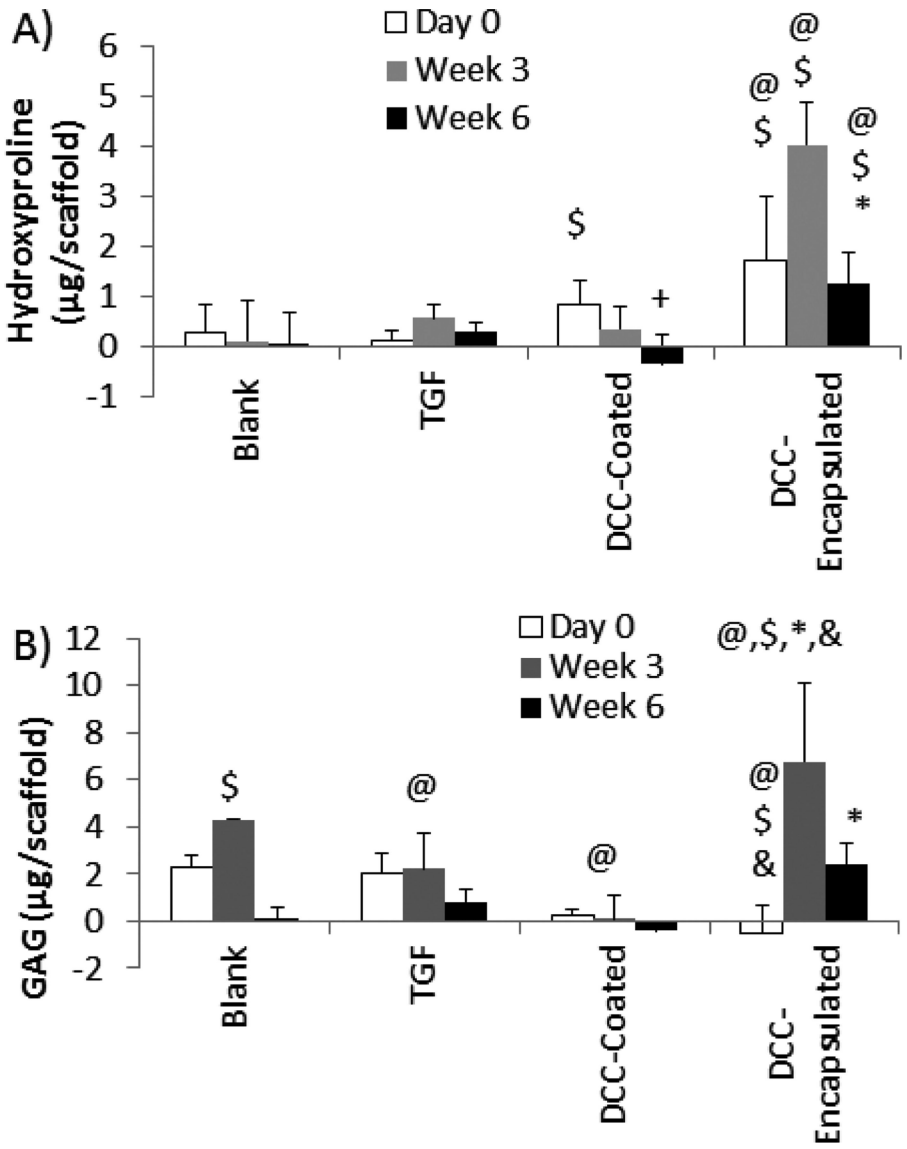

Figure 6.

Biochemical contents of engineered constructs (n=5). Note that hydroxyproline and GAG contents were measured for acellular scaffolds containing DCC and subtracted out as a baseline value. A) Hydroxyproline content on all scaffolds, a decrease in hydroxyproline is observed following week 3 on all scaffolds. B) GAG content on all scaffolds, also with a decrease in content following week 3. Blank = PLGA microspheres with nothing encapsulated, TGF = PLGA microspheres with TGF-β3 encapsulated, DCC-coated = PLGA microspheres with nothing encapsulated but coated in decellularized cartilage (DCC), and DCC-encapsulated = PLGA microspheres with DCC encapsulated. *denotes statistically significant difference from day 0 value, @denotes statistically significant difference from blank group at same time point, $denotes statistically significant difference from TGF group at same time point, & denotes statistically significant from DCC-Coated at the same time point, and + denotes statistically significant difference from DCC at same time point. All

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significance reported for $p<0.05$. Data are reported as mean ± standard deviation, and day 0 corresponds to 24 hours after seeding.

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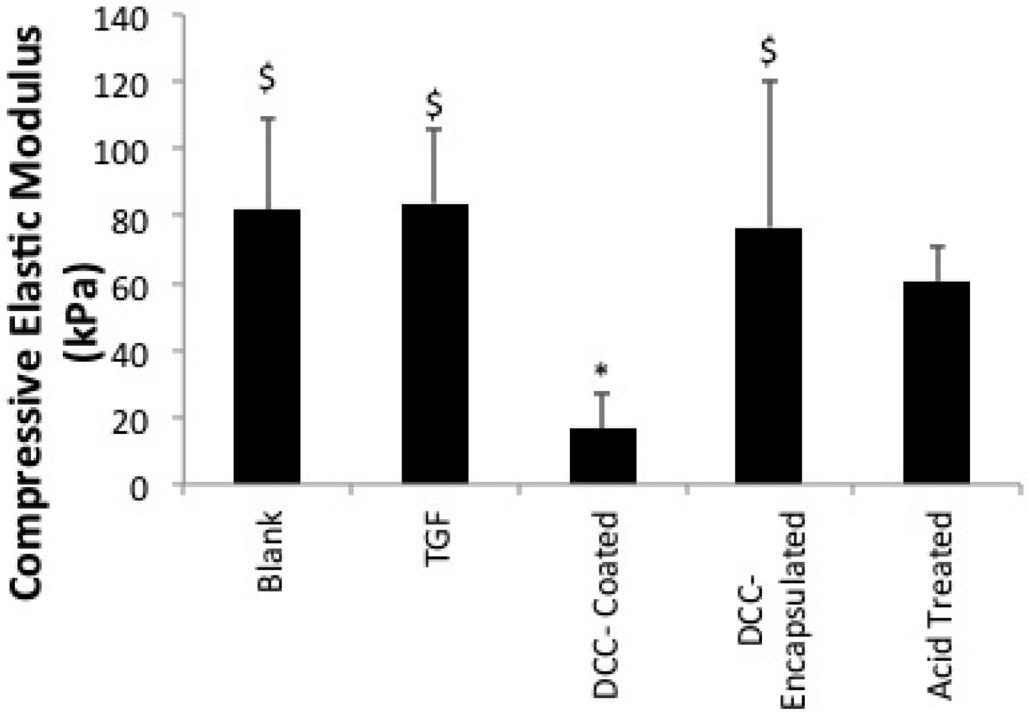

Figure 7.

The compressive elastic moduli of engineered scaffolds prior to cell seeding (n=4-5). The DCC-coated scaffolds had significantly lower compressive moduli than the blank, TGF, and DCC-encapsulated scaffolds. Blank = PLGA microspheres with nothing encapsulated, TGF = PLGA microspheres with TGF-β3 encapsulated, DCC-coated = PLGA microspheres with nothing encapsulated but coated in decellularized cartilage (DCC), and DCC-encapsulated = PLGA microspheres with DCC encapsulated. *denotes significant difference from blank scaffolds ($p<0.05$) and $denotes significance from DCC-coated scaffolds. Data are reported as mean ± standard deviation.

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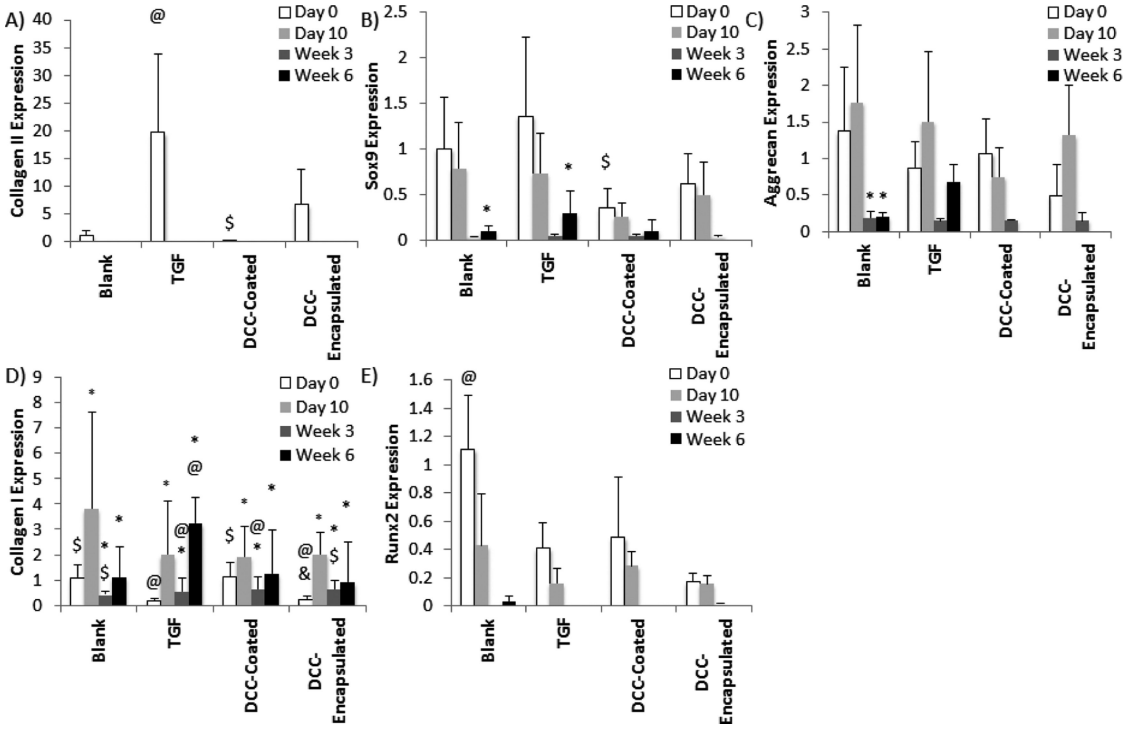

Figure 8.

RT-PCR results for all scaffolds and time points (n=5). A) Collagen II expression was significantly greater in TGF scaffolds compared to all others. B) Sox9 expression was significantly lower in DCC-coated scaffolds at day 0 compared to all other scaffolds. C) Aggrecan expression was nearly equal among all scaffold types during culture. D) Collagen I expression was lower in both TGF and DCC-encapsulated scaffolds at day 0. E) Runx2 expression was greatest in blank scaffolds at day 0. Blank = PLGA microspheres with nothing encapsulated, TGF = PLGA microspheres with TGF-β3 encapsulated, DCC-coated = PLGA microspheres with nothing encapsulated but coated in decellularized cartilage (DCC), and DCC-encapsulated = PLGA microspheres with DCC encapsulated. * denotes significant from time 0 value (same group), @ denotes significant from blank at same time point, $ denotes significant from TGF at same time point, & denotes significant from DCC-coated at same time point. For all significance noted ($p<0.05$). Data are reported as mean ± standard deviation, and Day 0 corresponds to 24 hours after seeding.

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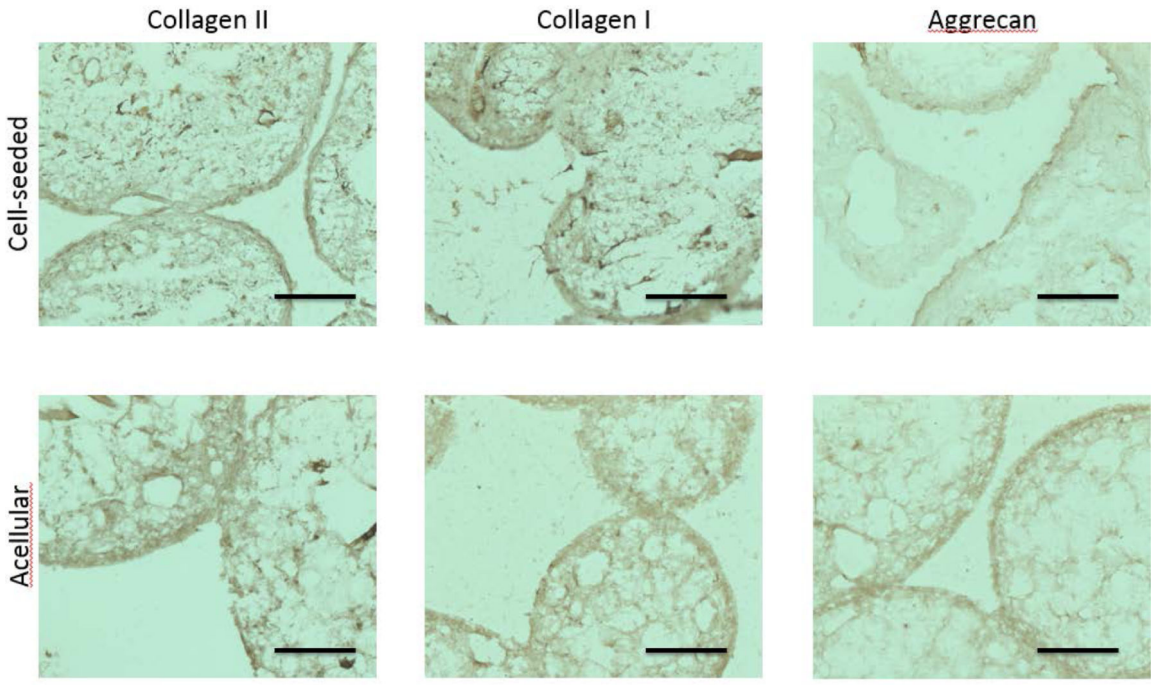

Figure 9.

Immunohistochemical staining of decellularized cartilage (DCC)-encapsulated scaffolds at week 3. Staining was positive for collagen II, collagen I, and aggrecan. Collagen I staining was more intense and present between microspheres in the cell-seeded group compared to the acellular group. Round objects are intact microspheres, material outside of microspheres can be seen in collagen I stained cell-seeded group. Scale bars represent 100 μm.