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Integration of layered chondrocyte-seeded alginate hydrogel scaffolds

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Abstract

Motivated by the necessity to engineer appropriately stratified cartilage, the shear mechanics of layered, bovine chondrocyte-seeded 20 mg/mL alginate scaffolds were investigated and related to the structure and biochemical composition. Chondrocyte-seeded alginate scaffolds were exposed to a calcium-chelating solution, layered, crosslinked in $CaCl_2$, and cultured for 10 weeks. The shear mechanical properties of the layered gels were statistically similar to those of the non-layered controls. Shear modulus of layered gels increased by approximately six-fold while toughness and shear strength increased by more than two-fold during the culture period. Hydroxyproline content in both layered gels and controls had statistically significant increases after 6 weeks. Glycosaminoglycan (GAG) content of controls increased throughout culture while GAG content in layered gels leveled off after 4 weeks. Hematoxylin and eosin histological staining showed tissue growth at the interface over the first 4 weeks. Shear mechanical properties in the engineered tissues showed significant correlations to hydroxyproline content. Dependence of interfacial mechanical properties on hydroxyproline content was most evident for layered gels when compared to controls, especially for toughness and shear strength. Additionally, interfacial properties showed almost no dependence on GAG content. These findings demonstrate the feasibility of creating stratified engineered tissues through layering and that collagen deposition is necessary for interfacial integrity.

Keywords: Cartilage tissue engineering; Interface; Alginate; Micropatterning; Mechanical properties

1. Introduction

Cartilage tissue engineering has been widely proposed as a method to repair injured or diseased cartilage. Most approaches to cartilage tissue engineering involve delivery of cells via biodegradable scaffolds to regenerate tissue. Hydrogels have been used as scaffolds since their diffusion properties allow for sufficient delivery of nutrients and oxygen to and removal of waste products and carbon dioxide from cells [1] while providing uniform cell seeding [2]. Alginate has been used for cartilage tissue engineering because it supports chondrocytes [3], can be molded into specific shapes [4,5], supports chondrogenesis in large animal models [6], and is biocompatible in delivering cells in human trials [7].

However, engineering cartilage with an appropriately stratified extracellular matrix (ECM) composition that matches native tissue in joints is a persistent challenge. Articular cartilage has a highly organized structure, leading to complex spatial variations in its mechanical properties and composition. In particular, ECM is highly organized throughout the cross-section of the tissue and is responsible for the mechanical properties of cartilage [8,9]. To date, no method exists for producing engineered cartilage with an appropriately stratified structure. Depositing chondrocytes in multiple layers [10] or layering multiple

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chondrocyte-seeded hydrogel scaffolds [11,12] are promising methods for producing stratified structures, but there has been little investigation into ECM deposition between these layers and how this affects the integrity of the interface. Layering chondrocyte-seeded hydrogel scaffolds provides an appropriate spatially distribution of cells, but this may not lead to ECM arrangement that is similar to that of solid homogeneous constructs or native tissue.

A recently developed microfluidic scaffold that can control delivery of different soluble factors on the micrometer scale provides another method to engineer appropriately stratified cartilage [13]. A main step in the soft lithographic fabrication of this biomaterial involves layering and sealing different hydrogel scaffolds. Understanding tissue growth and its relation to mechanical properties in this layered construct will be essential in evaluating the feasibility of this biomaterial. Because the mechanical properties of cartilage define its function, understanding the interfacial mechanics of stratified engineered tissues is critical to assess their functionality. Therefore, the objectives of this study were to quantify the mechanics of layered, chondrocyte-seeded alginate hydrogel scaffolds and to relate the interfacial properties to the structure and biochemical composition.

2. Methods

2.1. Chondrocyte isolation and seeding

Bovine cartilage was harvested from the femoropatellar groove of 1–3-day-old calves. The tissue was digested for 18 h in Dulbecco's Modified Eagle Medium (DMEM) [Sigma-Aldrich, St. Louis, MO] containing 3 mg/mL collagenase at 37 °C and 5% CO_2. The digest was filtered with a 100 μm cell strainer [BD Bioscience, Bedford, MA] and then centrifuged at 412*g* for 7 min. The resulting cell pellet was washed twice with Dulbecco's phospate-buffered saline (PBS) [Mediatech, Herndan, VA]. The cell number was determined using a hemocytometer [Hausser Scientific, Horsham, PA] and cell viability was determined using trypan blue dye [Sigma-Aldrich]. Chondrocytes were then suspended into 20 mg/mL of Protanal LF 10/60, a low viscosity alginate with a mean G/M ratio of 70% and mean molecular weight of 180 kDa [FMC Biopolymer, Drammen, Norway], in PBS at a seeding density of 5×10^7 cells/mL.

2.2. Hydrogel scaffold fabrication

The protocol for casting alginate hydrogels was based on that previously described for injection molding [4]. Briefly, two alginate sheets were formed by mixing 2.5 mL of the seeded alginate suspension with 20 mg/mL $CaSO_4$ [Mallinckrodt Baker, Phillipsburg, NJ] at a 2:1 volume ratio. The mixing occured in two 10 mL syringes [Becton-Dickinson, Franklin Lakes, NJ, USA] connected by a three-way stopcock [Baxter, Deerfield, IL, USA] with two passages back and forth between the syringes in a span of two seconds. Once mixed, two 1 mm thick hydrogel sheets were cast, one between two glass plates and another between a glass plate and a poly(dimethylsiloxane) (PDMS) [Dow Corning, Midland, MI] sheet to assist in the layering process (Fig. 1).

Layering methods were similar to those used to create a microfluidic biomaterial [13] and were previously developed in creating acellular layered constructs with the strongest interfacial properties. After 5 min, the alginate sheet cast between glass plates was cut into 8.5 mm squares. The second sheet of alginate was partially de-molded by removing the top glass plate. The resulting exposed sheet was then treated with a calcium-chelating solution of 82 mM sodium citrate [Sigma-Aldrich] in 4 mg/mL of Protanal LF 10/60 using a sterile cotton pad saturated with the solution. The cotton pad was then removed after an exposure time of 2 min. Using the flexible PDMS sheet, the treated alginate was lowered onto the cut alginate squares, producing a structure of two separate alginate gels. This system was then placed in a mold consisting of two glass plates and 2 mm spacers. The layered hydrogels were kept in contact for 8 min; we hypothesize that this delay allows for molecular rearrangement and entanglement at the interface. Samples were then transferred into a bath of 80 mM $CaCl_2$ [EMD Chemicals, Darmstadt, Germany], 49 mM NaCl [Mallinckrodt Baker], and 25 mM HEPES buffer [Gibco]. Samples were maintained in the bath for 15 min to crosslink alginate at the interface.

Upon removal, layered alginate squares were cut out and incubated in DMEM with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Controls of 2 mm thick alginate hydrogels were created using the same method without layering. All chondrocyte-seeded hydrogel scaffolds were then incubated for 24 h. Layered gels were randomly removed from culture while others were cultured for 2, 4, 6, 8, and 10 weeks. Controls were also incubated for the initial 24 h, then for 2, 4, 6, 8, and 12 weeks. After incubation, samples were either fixed in formalin for histological staining or frozen at −20 °C for either mechanical testing or biochemical assays.

2.3. Mechanical lap-shear testing

Layered alginate hydrogels and controls were thawed in PBS at 37 °C for 10 min. Samples were then immediately tested mechanically using a lap-shear geometry to measure the interfacial and shear material properties [14]. Excess fluid was carefully removed from hydrogel samples with a kimwipe [Kimberly-Clark, Roswell, GA] as they were attached to the ends of 8.5 mm wide aluminum grips with a 1 mm offset bend using cyanoacrylate glue. Samples were then temporarily clamped to ensure adhesion between the hydrogel and grips (Fig. 2A). Once adhered to the grips, samples were loaded onto an EnduraTEC ELF3200 mechanical test frame and pulled to failure at a displacement rate of 0.025 mm/s, with load measured to within 1 g at a sampling frequency of 10 Hz (Fig. 2B). Stresses and strains were calculated from sample geometry, displacements, and

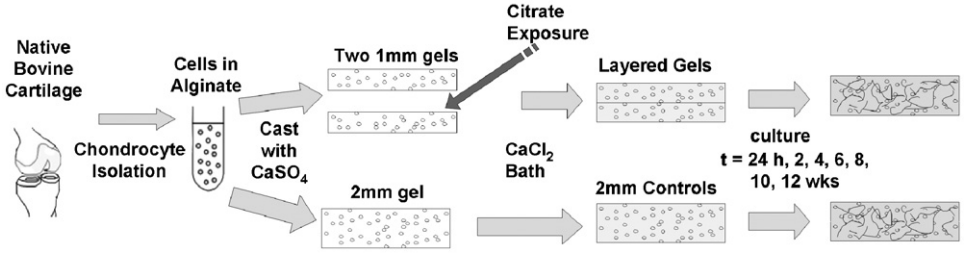

Fig. 1. Schematic of layering process. Bovine articular cartilage was digested in collagenase. Chondrocytes were then suspended in an alginate solution and mixed with $CaSO_4$ to initiate crosslinking. Two 1 mm gels and one 2 mm gel were then cast and demolded. Citrate solution was then applied to one of the 1 mm gel and the two gels were layered. The layered gel and 2 mm gel were then placed in a $CaCl_2$ bath to finalize crosslinking. The samples were then placed in culture for 24 h, 2, 4, 6, 8, 10, and 12 weeks.

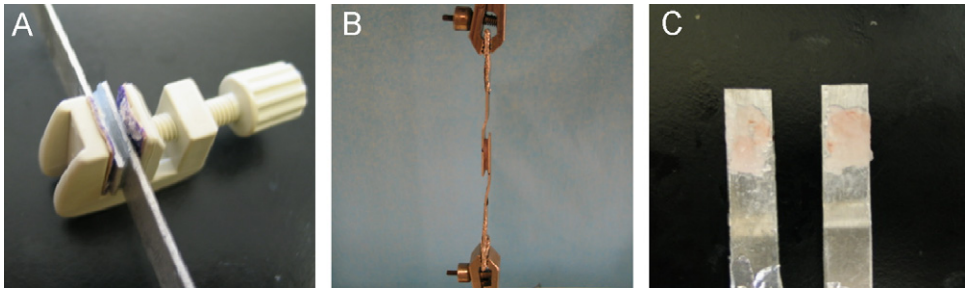

Fig. 2. Mechanical testing procedure. (A) Samples were attached to aluminum grips and temporarily clamped. (B) Hydrogels were then loaded onto mechanical test frame and then pulled to failure. (C) Failure locations of samples were noted.

loads. Shear strength, shear modulus, and toughness were then calculated from the resulting stress-strain curves. Shear strength was calculated as the maximum value of shear stress the sample endured prior to failure. Shear modulus was determined as the slope of the linear region of the stress-strain curve. Toughness was the area under the stress-strain curve and was calculated by using a rectangular Riemann sum technique bounded by zero strain and the failure strain [15,16]. The failure locations of the layered hydrogels and controls were documented throughout mechanical testing of samples (Fig. 2C).

2.4. Biochemical analysis

Frozen samples were weighed on a microbalance and digested in 1 mL of 0.1 M sodium phosphate, 10 mM disodium ethylenediaminetetraacetic acid (Na_2EDTA) [Sigma-Aldrich], 10 mM cysteine hydrochloride [Sigma-Aldrich], and 3.8 U/mL papain [Sigma-Aldrich] at 60 °C for 18 h. Resulting biochemical contents were then normalized to the wet weight of the sample.

The hydroxyproline content of digests were measured using previously described methods [17]. In summary, 100 μL of each sample's digest was hydrolyzed in 100 μL of 2 N NaOH at 110 °C for 18 h. Afterwards, 20 μL 5 N HCl, 100 μL 0.01 M $CuSO_4$, 100 μL 2.5 N NaOH, and 100 μL 6% H_2O_2 were added to the digested hydrolyzed sample in microfuge tubes. The tubes were allowed to sit at room temperature for 5 min, vortexed, and placed in a heat block at 80 °C for 5 min. The tubes were then placed in an ice bath to cool to room temperature and 400 μL of 3 N H_2SO_4 and 200 μL of 5 mg/mL *p*-dimethylaminobenzaldehyde in *n*-propanol (DMAB) were added to each tube. All assays were then carried out in 96-well plates [Nalge Nunc, Rochester, NY]. Each well of a 96-well plate was filled with 200 μL of a treated sample and absorbance was measured at 540 nm.

The digest was also analyzed for glycosaminoglycans (GAG) as a marker for proteoglycans using well-established methods [18]. The assay was carried out in 96-well plates. In each well, 50 μL of digest was mixed with 250 μL of dye containing 16 mg/L 1,9-dimethylmethylene blue (DMMB) and 3.04 g/L glycine (pH 1.5). The absorbance was read at 595 nm using a microplate reader. Chondroitin-6-sulfate from shark cartilage [Sigma] was used to construct the standard curve.

DNA content was measured as a marker of cell quantity using a Hoechst dye [19]. In each well, 190 μL of 0.1 μg/mL Hoechst 33258 dye in *tris*(hydroxymethyl)aminomethane EDTA saline (TES) buffer was added to 10 μL of the digested samples. Calf thymus DNA was used as a standard and DNA contents were determined by reaction with the Hoechst dye. Fluorescence was measured with an excitation wavelength at 348 nm and emission wavelength at 456 nm.

2.5. Histology

Cross-sections of the sample containing the gel-gel interface were fixed in 10% phosphate-buffered formalin containing 10 mg/mL $CaCl_2$ for 48 h, then embedded in paraffin. Using standard histochemical techniques, five-micrometer-thick serial sections were obtained via a rotary microtome and stained with hematoxylin and eosin (H&E) stain to highlight cells and ECM. Images of sections were obtained with a Spot Jr Digital camera attached to a Nikon TS 200 microscope [MicroVideo Instruments, Avon, MA].

2.6. Statistics

Mechanical properties and composition between the layered gels and controls at 0, 2, 4, 6, and 8 weeks were compared with a two-way analysis of variance ($p<0.05$) with a post-hoc Tukey test for pairwise comparison using Sigma Stat 3.0.1 [SPSS, Chicago, IL]. The relationship between mechanical properties and ECM was investigated by linear correlation analysis. This value for each parameter comparison was compared with a critical value for the number of time points in the study to determine statistical significance (1-tail analysis $p<0.05$) [20].

3. Results

3.1. Mechanical

The mechanical shear properties of both the layered gels and controls gradually increased over the entire period of culture (Fig. 3). The toughness and shear strength of layered gels more than doubled over the 10-week culture (Fig. 3B, C) while shear modulus increased by approximately six-fold (Fig. 3A). Enhancement of mechanical properties in the controls was not as pronounced, with toughness and shear strength nearly doubling and shear modulus increasing by 75% after 12 weeks in culture. Additionally, layered gels had higher shear modulus, toughness, and shear strength compared to controls at the end of the culture period. Layered gels showed statistically significant increases in shear modulus at 2 ($p<0.043$), 6 ($p<0.009$), 8 ($p<0.016$), and 10 ($p<0.001$) weeks compared to the initial time point. Additionally, toughness of layered gels showed significant enhancements at 6 ($p<0.039$) and 10 ($p<0.009$) weeks. Although the shear strength of layered gels did not show any statistically increases over the 10-week culture period, layered gels and controls together showed a statistical increase over the entire culture period ($p<0.012$). Mechanical properties of layered gels and controls were statistically similar except for toughness values at 6 weeks ($p<0.006$). At all time points, the most common site of failure in the layered gels

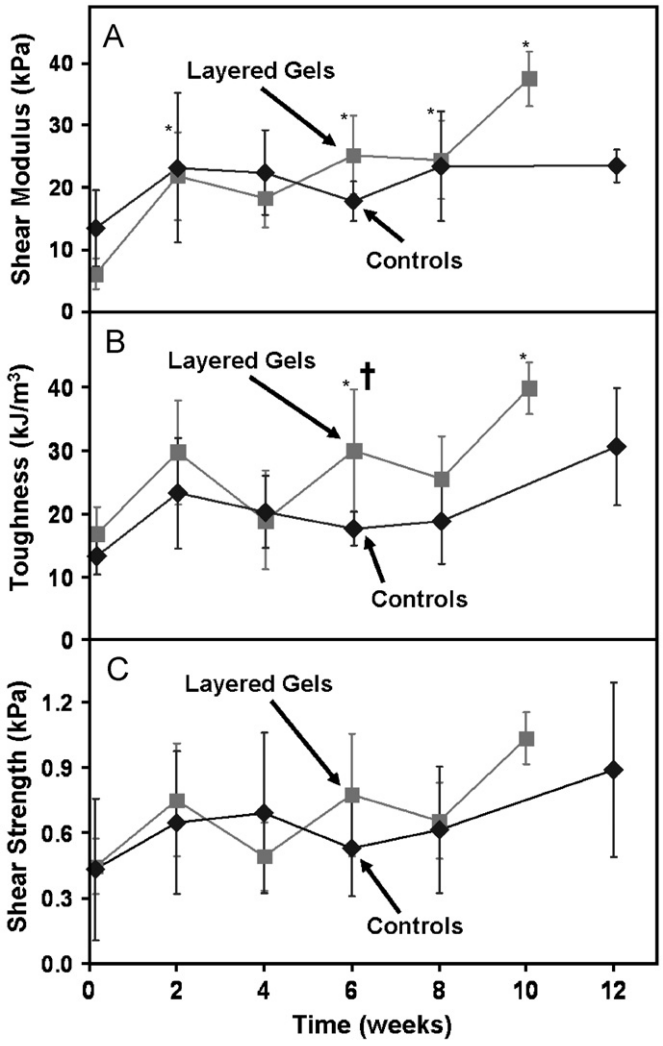

Fig. 3. (A) Shear modulus, (B) shear toughness, and (C) shear strength of layered gels and controls at 24 h, 2, 4, 6, 8, 10, and 12 weeks. Each data point represents $n = 3–7 \pm$ standard deviation. (*) $p < 0.05$ for layered gel compared to initial time point, (†) $p < 0.05$ layered gel compared to control.

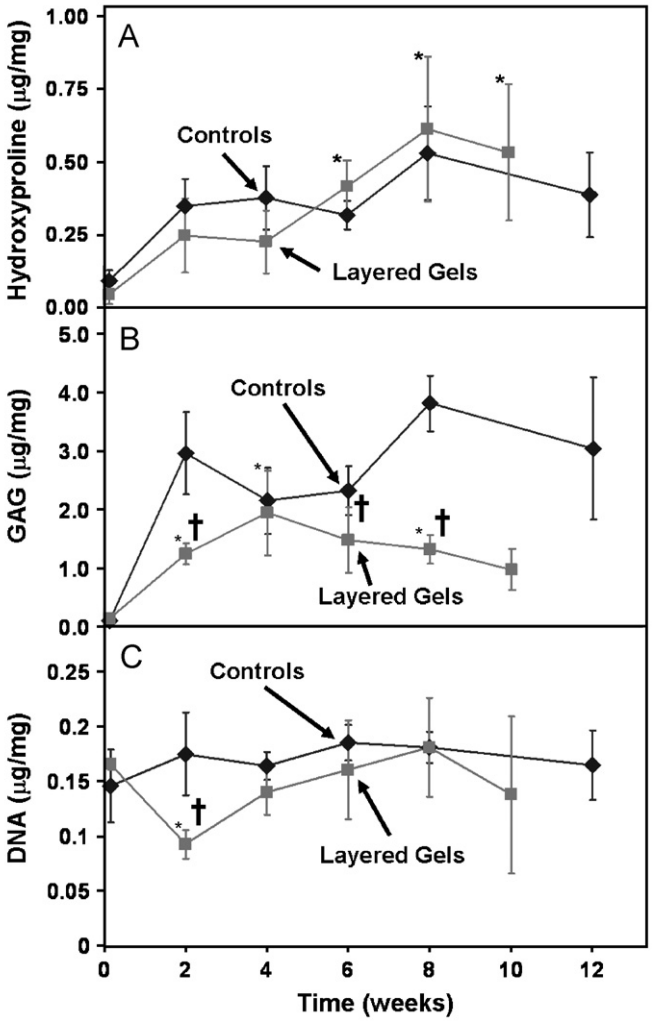

Fig. 4. (A) Hydroxyproline content, (B) GAG content, and (C) DNA content of layered gels and controls at 24 h, 2, 4, 6, 8, 10, and 12 weeks normalized to wet weight. Each data point represents $n = 3–7 \pm$ standard deviation. (*) $p < 0.05$ for layered gel compared to initial time point, (†) $p < 0.05$ layered gel compared to control.

was at the interface whereas the controls failed randomly throughout the cross-section of the gel.

3.2. Biochemical

Hydroxyproline content exhibited similar trends in both the layered hydrogels and controls (Fig. 4A). In both samples, hydroxyproline content continually increased over time in culture. Hydroxyproline content in layered sheets at 6, 8, and 10 weeks were significantly higher than that at the initial time point ($p < 0.001$). At each time point throughout the culture period, hydroxyproline content in the layered sheets and controls were statistically similar. GAG content trends in the layered gels and controls over the entire course in culture were statistically different (Fig. 4B, $p < 0.001$). GAG accumulation in the layered gels leveled off after 4 weeks in culture, but GAG content in the controls continued to increase. GAG content in the layered samples showed significant increases after 2 ($p < 0.030$), 4 ($p < 0.001$), 6 ($p < 0.005$), and 8 weeks ($p < 0.003$) but not after 10 weeks. Additionally, GAG content in the layered gels and controls had significant differences at 2 ($p < 0.001$), 6 ($p < 0.013$), and 8 weeks ($p < 0.001$). DNA content was consistent throughout the entire period of culture for both the layered gels and controls (Fig. 4C). The only aberration occurred with a decrease in DNA content after 2 weeks in the layered gels ($p < 0.001$).

3.3. Histological

H&E staining demonstrated a well-defined interface between layers at the start of culture (Fig. 5A). The region between layers appeared to contain alginate with few cells. At 2 weeks, this region had begun to be filled with cells and

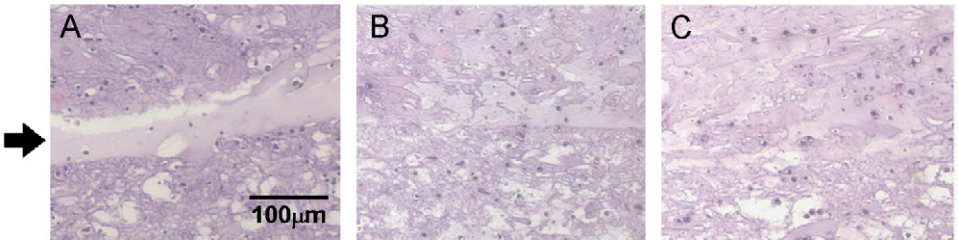

Fig. 5. Hematoxylin and eosin staining (original magnification ×100) of layered gels at interface after (A) 0 weeks, (B) 2 weeks, and (C) 4 weeks.

ECM (Fig. 5B). This pattern was even more pronounced after 4 weeks (Fig. 5C), and the location of a defined interface was difficult to determine.

3.4. Interfacial-biochemical relationships

Comparing the average shear mechanical properties and hydroxyproline contents for cultured samples at each time point showed a linear correlation between shear modulus and hydroxyproline in both the layered gels ($p<0.01$) and controls ($p<0.0025$) individually (Fig. 6A). Toughness and shear strength of layered gels also correlated with hydroxyproline content (Fig. 6B, C, $p<0.05$). Linear correlations were also seen between all average shear mechanical properties and hydroxyproline content when the sample set included both controls and layered gels (Fig. 6A–C). Comparing average mechanical properties and GAG contents at each time point showed little significant correlation between shear mechanical properties and GAG (Fig. 7). This lack of correlation was especially true for toughness and shear strength for sample sets that included either only layered gels or both layered gels and controls (Fig. 7B, C, $p>0.25$). Only the shear modulus of controls showed a correlation to GAG content (Fig. 7A, $p<0.0025$).

4. Discussion

This study demonstrates that integration occurs between two chondrocyte-seeded alginate layers through the deposition of ECM over time, thereby strengthening the interface. With time in culture, increases in shear modulus and shear strength in both the controls and layered gels coincided with the accumulation of proteoglycan and collagen (Fig. 4A, B).

The combined analysis of mechanical and biochemical data revealed a significant dependence of interfacial properties on the collagen content, but not on the proteoglycan content. These data are consistent with studies of interfacial repair between layers of intact cartilage in which collagen deposition correlated with interfacial shear strength [21,22] and the inhibition of collagen crosslink formation abolished the development of mechanical integration [23]. These studies, together with the present study, collectively point to controlling collagen deposition and organization as major mechanisms to enhance interfacial integration of cartilaginous tissues.

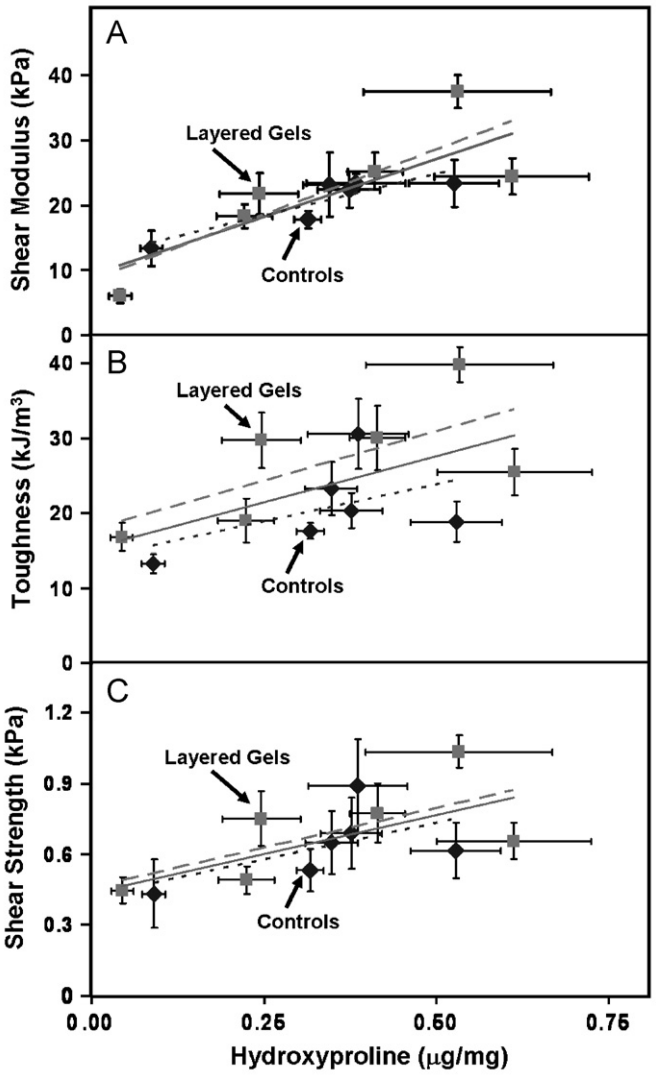

Fig. 6. Hydroxyproline content normalized to wet weight of layered gels and controls versus (A) shear modulus: (– – –) layered gel correlation $r^2=0.690/p<0.01$, (- - -) control correlation $r^2=0.781/p<0.0025$, (—) overall correlation $r^2=0.673/p<0.0005$; (B) shear toughness: (– – –) layered gel correlation $r^2=0.448/p<0.05$, (- - -) control correlation $r^2=0.237/p<0.25$, (—) overall correlation $r^2=0.313$, $p<0.025$; and (C) shear strength: (– – –) layered gel correlation $r^2=0.447/p<0.05$, (- - -) control correlation $r^2=0.328/p<0.10$, (—) overall correlation $r^2=0.397/p<0.01$, at each time point. Each data point represents $n=3$–$7\pm$standard error of the mean.

The dependence of interfacial mechanical properties on collagen content was most evident for the layered samples and less pronounced in control hydrogels. This difference

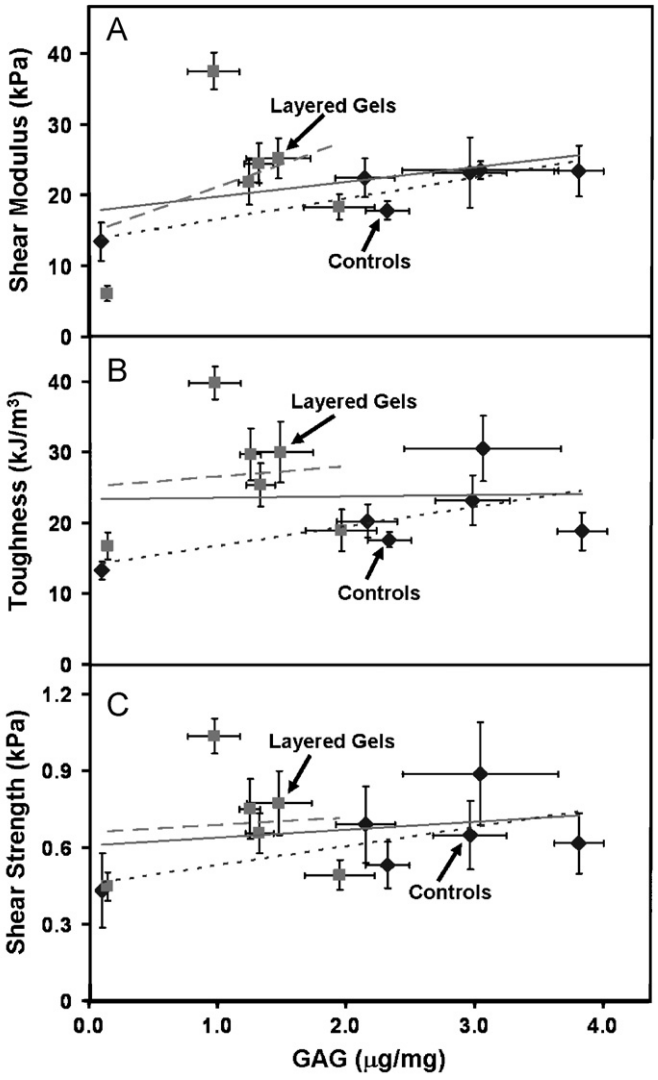

Fig. 7. GAG content content normalized to wet weight of layered gels and controls versus (A) shear modulus: (– – –) layered gel correlation $r^2 = 0.145/p < 0.25$, (- - -) control correlation $r^2 = 0.804/p < 0.0025$, (—) overall correlation $r^2 = 0.098/p < 0.25$; (B) shear toughness: (– – –) layered gel correlation $r^2 = 0.011/p > 0.25$, (- - -) control correlation $r^2 = 0.366/p < 0.10$, (—) overall correlation $r^2 = 0.001/p > 0.25$; and (C) shear strength: (– – –) layered gel correlation $r^2 = 0.007/p > 0.25$, (- - -) control correlation $r^2 = 0.376/p < 0.10$, (—) overall correlation $r^2 = 0.039/p > 0.25$, at each time point. Each data point represents $n = 3–7 \pm$ standard error of the mean.

in behavior between layered samples and controls was evident in the toughness and shear strength (Fig. 6B, C). This is likely due to the fact that the initial interface of the layered samples was less stiff than the controls (modulus of 6 vs. 13 kPa). As such, collagen deposited at this interface would be expected to have more of a stiffening effect on the interface of the layered gels.

The leveling off of GAG content in the layered gels after 4 weeks may be due to proteoglycans being lost to the media, which has been observed in previous studies with chondrocytes cultured in hydrogel scaffolds [24]. The integrity of the scaffold may have been compromised by the chelating and subsequent annealing processes, allowing GAG loss to be even more prominent. Additionally, the decrease in DNA content in the layered gels after the first 2 weeks in culture may be due to cell death during the chelating process, but the recovery in subsequent weeks indicates that any cytotoxic effects during processing are not long term. Future studies should investigate ways to improve the layering technique to maximally enhance engineered layered tissue development.

The shear strength of this interface was on the order of 1 kPa, which was significantly lower than that of intact cartilage [25], and significantly lower than that observed in healing of cartilage explants [26]. Strategies for enhancing interfacial properties should focus on increasing production of ECM components, particularly collagen. These strategies may include supplementing media with ascorbate [27] or growth factors during culture [28], placing samples under dynamic shear [29], or using viral vectors that have the potential to increase gene expression of different collagen components [30] specifically collagen IIA for articular cartilage.

The ability to integrate separate layers of chondrocyte-seeded alginate hydrogels has many applications for cartilage tissue engineering. This study has shown that layering different chondrocyte-seeded hydrogel scaffolds is a feasible way to engineer stratified cartilage since interfacial mechanical properties increase over culture time and correlate to collagen deposition. Besides creating heterogeneous cell populations by depositing layers, this method can also be used to create mechanically or chemically anisotropic structures that more appropriately match the different regions in articular cartilage. This layering technique may also be applied to engineer other types of tissues that require a stratified structure. Additionally, this study supports the feasibility of a microfluidic alginate hydrogel scaffold [13] for long-term culture periods since the main mode of failure will occur at the interface.

5. Conclusions

Chondrocyte-seeded alginate hydrogel scaffolds were layered with the aid of a calcium-chelating solution and the mechanical shear interfacial properties over a 10-week culture period were related to the biochemical composition and structure of the sample. Mechanical shear properties at the interface, particularly shear modulus, increased over time in culture. These mechanical properties were similar to those exhibited by solid gel controls. ECM deposition was also seen throughout the entire culture period. Upon further analysis, mechanical shear properties of the interface correlated to collagen deposition while showing little correlation to proteoglycan deposition. Mechanical properties also increased with visible tissue development between the layered hydrogel scaffolds as the location of a defined interface was difficult to determine after 4 weeks. By successfully demonstrating the integration of separately deposited engineered tissues, even more incentive has been

provided to consider using layered hydrogel scaffolds to create more functional engineered tissues.

Acknowledgments

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