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An Instrumented Bioreactor for Mechanical Stimulation and Real-Time, Nondestructive Evaluation of Engineered Cartilage Tissue

Mechanical stimulation is essential for chondrocyte metabolism and cartilage matrix deposition. Traditional methods for evaluating developing tissue in vitro are destructive, time consuming, and expensive. Nondestructive evaluation of engineered tissue is promising for the development of replacement tissues. Here we present a novel instrumented bioreactor for dynamic mechanical stimulation and nondestructive evaluation of tissue mechanical properties and extracellular matrix (ECM) content. The bioreactor is instrumented with a video microscope and load cells in each well to measure tissue stiffness and an ultrasonic transducer for evaluating ECM content. Chondrocyte-laden hydrogel constructs were placed in the bioreactor and subjected to dynamic intermittent compression at 1 Hz and 10% strain for 1 h, twice per day for 7 days. Compressive modulus of the constructs, measured online in the bioreactor and offline on a mechanical testing machine, did not significantly change over time. Deposition of sulfated glycosaminoglycan (sGAG) increased significantly after 7 days, independent of loading. Furthermore, the relative reflection amplitude of the loaded constructs decreased significantly after 7 days, consistent with an increase in sGAG content. This preliminary work with our novel bioreactor demonstrates its capabilities for dynamic culture and nondestructive evaluation. [DOI: 10.1115/1.4006546]

Keywords: ultrasound, glycosaminoglycan, compression, tissue engineering

1 Introduction

Functional tissue engineering involves the application of physical loads to promote the development of tissue constructs that can withstand the mechanical demands encountered in vivo [1]. Specifically, the goal of functional tissue engineering of articular cartilage is to develop an engineered cartilage construct that exhibits structure and function sufficient to replace or repair damaged articular cartilage. To accomplish this goal, bioreactors have been developed to apply mechanical stimulation to cell-laden constructs. Design strategies may impart various types of load including hydrostatic pressure, compression, or shear [2–5]. However, few bioreactors include instrumentation that allow for continuous monitoring of tissue development.

The successful in vitro development of functional tissue-engineered constructs could benefit from a method of assessment that allows for continuous evaluation of tissue while not compromising construct integrity, preserving the construct for continuous development and eventual implantation. Current methods for evaluating extracellular matrix (ECM) development and mechanical properties are time consuming and destructive to the construct, and require numerous replicates to obtain a comprehensive overview of construct quality. Nondestructive, continuous evaluation of a tissue construct during development can be useful not only for final clinical use, but also for determining appropriate bioreactor parameters to achieve sufficient structure and function.

Nondestructive measurement systems have been developed to assess construct mechanical properties as well as bulk-tissue development [6,7]. Preiss-Bloom et al. developed a bioreactor to mechanically stimulate tissue-engineered cartilage and measure real-time force response [6]. The bioreactor was outfitted with load sensors to continuously log construct resistance to deformation by the bioreactor. Such measurements give insight into the change in construct stiffness during stimulation and development in the bioreactor. Hagenmuller et al. developed a bioreactor that combines mechanical loading and online microcomputed tomography (μCT) for monitoring the development of engineered bone tissue [7]. Cartridge-like culture chambers were designed to allow for sterile mechanical stimulation and μCT monitoring of mineral deposition without removing the constructs.

Another potential method for nondestructive assessment of tissue formation is ultrasound. Ultrasonic techniques are sensitive to mechanical and biochemical properties of cartilage [8–10] and have the potential to nondestructively assess the quality of tissue-engineered cartilage during development. Ultrasonic waves are utilized to acquire acoustic images and make localized quantitative measurements of tissue properties. Propagation and scattering of ultrasonic waves depend on tissue composition and structure [11]. Specifically, the reflection coefficient, the fraction of ultrasound reflected from an interface with different acoustic impedances, is one parameter commonly used to evaluate tissue characteristics [12–16].

A number of studies have been conducted to examine the feasibility of ultrasound as a tool for diagnosis of osteoarthritis by measuring changes in ultrasonic parameters after spontaneous and selective enzymatic degradation of cartilage tissue [17–20]. Ultrasound has also been used as a tool for monitoring in vivo cartilage tissue development and repair [21–23]. However, ultrasound has

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Manuscript received November 18, 2011; final manuscript received February 29, 2012; published online April 26, 2012. Review conducted by: Gary L Bowlin.

only recently been used as a measurement tool for the evaluation of tissue-engineered cartilage [8,24] and has yet to be implemented for real-time evaluation of tissue development.

The objective of this work was to develop an instrumented bioreactor that could be utilized to stimulate and nondestructively evaluate tissue-engineered cartilage. Our dynamic compression bioreactor is instrumented with an ultrasonic transducer, load cells, and a video microscope for assessing ECM development and mechanical properties of tissue-engineered cartilage. Chondrocyte-laden hydrogel constructs were placed in the bioreactor and subjected to a three-part loading regime including: (1) a ramp, (2) sinusoidal compression, and (3) no load. This regime was repeated twice per day for 7 days. Constructs were nondestructively evaluated with ultrasound on days 0 and 7. Constructs were also evaluated on days 0 and 7 for cell viability, cell number, sulfated glycosaminoglycan (sGAG), and collagen content. Histological sections were stained for sGAG and collagen with safranin O and Masson's trichrome, respectively.

2 Materials and Methods[2]

2.1 Bioreactor Instrumentation. The bioreactor (Fig. 1) is designed to fit on the shelf of a standard incubator. Five cubic samples (5 mm on a side) are placed in wells in the sample tray. The wells are filled with appropriate cell culture medium. The sample wells can be removed from the sample tray for sterilization of the wells before use. Nonporous stainless-steel platens contact the samples from above and apply compressive, mechanical stimulation. Individual platens can be manually adjusted to the desired height. The platens have a significantly larger surface area than the hydrogel samples, such that the sample does not slip out and over the platen during compression. Lids with holes just wide enough for the push rods keep the samples from being contaminated. Software allows for displacement-controlled stimulation (strain) or for force-controlled stimulation (stress) of the linear actuator, such that all five samples are displaced or loaded equally. Software allows any waveform and duty cycle to be applied. The force can be recorded continuously during the stimulation, or the peak forces for each waveform can be collected. When needed, the bioreactor can be removed from the incubator and fitted in the stand of a horizontal microscope, where the mechanical property and ultrasonic attenuation measurements are made.

The force applied through the platens is measured with load cells of 9.8 N capacity and an rms error of less than 0.1%. The linear actuator can be controlled to 1 μm and can apply compressive strains with a displacement range of ± 2.5 mm up to a force capacity of 4 N per sample. Two small, angular-contact bearings inserted in the attachment mechanism between the actuator and the linear displacement guide ensure that the actuator is not damaged by rotation of the sample tray. The linear displacement guide is a "double D" slider that allows precise alignment of the load cell and push rods with the sample wells. An adjustable spring in the hollow core of the linear displacement guide is used to compensate for the weight of the guide, load cells, and push rods [Fig. 1(*b*)].

A rotation stage is used to position the sample well over the ultrasonic transducer (UT) that is attached to a linear actuator [Fig. 1(*c*)]. The UT moves radially in a slot machined in the base of the bioreactor. Coupled with the rotation stage, the entire specimen can then be imaged (scanning in the radial [r] and circumferential [θ] directions) with through-thickness ultrasonic propagation. Optical flats are machined on the sides of the wells to allow the samples to be imaged with a video microscope [Fig. 1(*c*)]. To measure the mechanical properties of the sample, the platen compresses the sample at a predetermined strain rate and amplitude.

[2]The full description of the procedures used in this paper requires the identification of certain commercial products and their suppliers. The inclusion of such information should in no way be construed as indicating that such products or suppliers are endorsed by NIST or are recommended by NIST or that they are necessarily the best materials, instruments, software or suppliers for the purposes described.

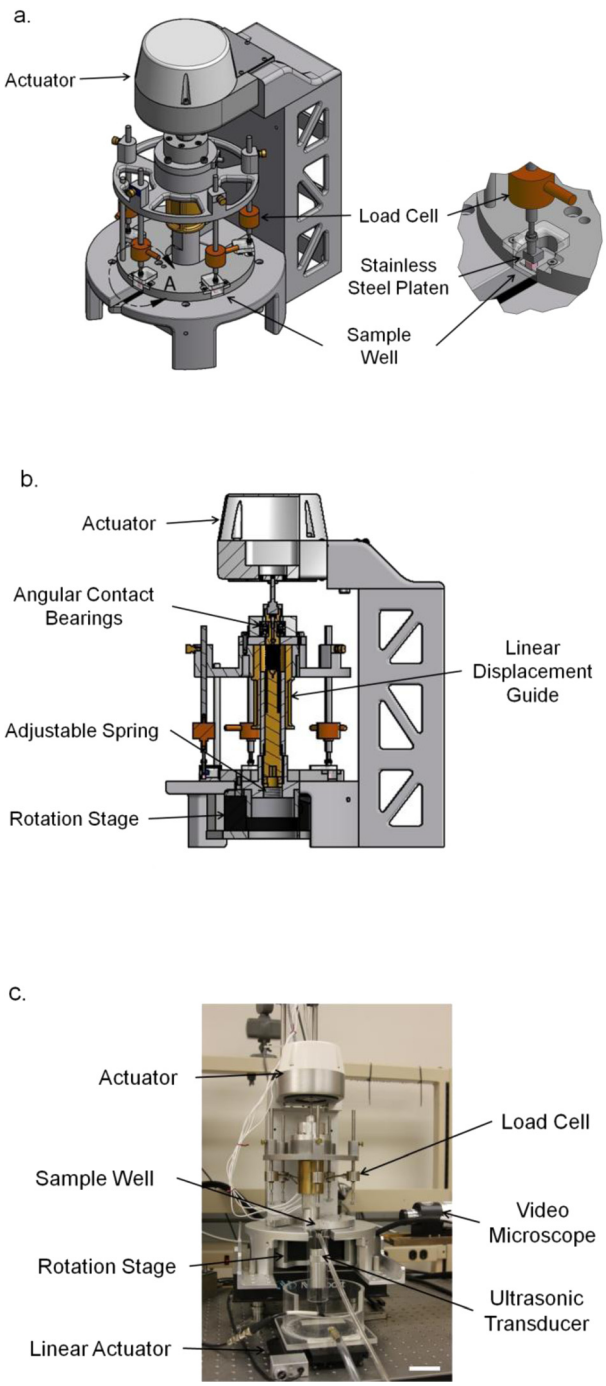

Fig. 1 Instrumented bioreactor. (*a*) The loading mechanism consists of an actuator that applies force to the constructs through impermeable stainless steel platens attached to load cells to measure applied force. The portion labeled (A) corresponds to the cutout of the sample well. (*b*) A cross-sectional view depicts the linear displacement guide and mechanism. (*c*) Photograph of the bioreactor and additional instruments. A rotation stage is used to position the sample well over the ultrasonic transducer that is attached to a linear actuator or the video microscope located adjacent to the bioreactor. Scale bar: 5 cm.

The stress is calculated from the force measured by the load cell, and strain is calculated from displacement measured by the actuator. One sample can be tested at a time.

2.2 Mechanical Stimulation. Chondrocyte-laden hydrogel constructs were subjected to displacement-controlled intermittent cyclic compression in a three-part loading regime: (1) ramp

[0.0017 s^{-1} strain rate to a maximum 10% strain], (2) 1 h of sinusoidal compression [1 Hz, 0%–10% strain], and (3) 11 h with no load. This regime was repeated twice per day for 7 days. Load and displacement data were collected during the ramp and compression stages. Load and displacement data collected during each ramp stage were used to calculate compressive modulus. Video microscope images of the sample were analyzed with image correlation software to confirm that bulk deformation of the gel was consistent with actuator measurements of displacement. Free-swelling gels (free-floating in well plates with chondrocyte medium and not subjected to mechanical stimulation) served as the control.

2.3 Nondestructive Ultrasound Analysis. High-frequency ultrasound in pulse-echo mode was used to interrogate the loaded constructs on days 0 and 7. The ultrasonic pulse from the transducer traveled through the sample well, passed through the construct, was reflected from the surface of the stainless steel platen, passed through the construct a second time, and traveled back to the transducer, where it was digitized as a time-dependent voltage waveform. A commercial pulser/receiver (UTEX 340, Mississauga, Ontario, Canada) was used to create the pulse, and a high speed digitization card (STR1G Sonix, Springfield, VA) digitizing at one gigasample per second with 8-bit resolution was used to capture the waveform. A 30 MHz acoustic transducer (V3346-SU/RM, Olympus, Waltham, MA) with a focal length of 12.7 mm and depth of field of 1.2 mm was employed. Scanning step sizes of 0.5 mm radially and 0.5° circumferentially were chosen to cover an area of 6.5 mm by 4.5°. From the acquired data, a plot was obtained of the peak amplitude of the largest pulse echo. All control, scanning, and saving of acquired data were performed with Labview software (National Instruments, Austin, TX). For scanning, the construct was immersed in chondrocyte medium.

Data were analyzed with Matlab software (Mathworks, Natick, MA). The dominant feature of each waveform was the echo off the stainless steel platen. The peak amplitude of the pulse echo reflected from the stainless steel platen was calculated for each scan point by finding the maximum amplitude of the analytic representation in the appropriate interval using the Hilbert transform. The amplitude of this echo was affected by the fraction of acoustic energy reflected at the media/construct interfaces, scattering in the construct, and damping in the construct; in addition to the transducer sensitivity and acoustic impedance. No attempt was made in this study to quantitatively separate these effects. The analysis was focused, instead, on the net amplitude of the echo from the stainless steel platen, after passing in both directions through the media and the construct. This echo amplitude with the construct present was referenced to measurements of echo amplitude with the construct absent. The relative reflection amplitude (RRA) was defined to be the ratio of these two measurements (with the reference measurement in the denominator). Loaded samples were not compared to free-swelling controls because ultrasonic evaluation is performed within the bioreactor wells. It was not possible to test free-swelling controls in the bioreactor and maintain sterility. Therefore, relative reflection amplitude was evaluated only with respect to time in culture.

2.4 Macromer. Poly(ethylene glycol) dimethacrylate (PEGDM) macromer was synthesized by reacting methacrylic anhydride to PEG (3000 Da, Fluka, St. Louis, MO) in the presence of hydroquinone via microwave methacrylation [25]. Macromer was dissolved in methylene chloride and purified by repeated precipitations in ethyl ether. The degree of methacrylate functionalization was determined by ^{1}H-NMR to be greater than 80%.

2.5 Chondrocyte Isolation and Encapsulation. Full-depth articular cartilage was harvested from the patellar-femoral groove and femoral condyles of a one-to-three-week old calf (Research 87, Boylston, MA) within 24 h of slaughter [26]. The tissue was digested in 500 units/mL type II collagenase (Worthington, Lakewood, NJ) in Dulbecco's Minimal Essential Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS, Invitrogen) for 16 h at 37 °C on an orbital shaker. The digest was passed through a 100 μm cell strainer, centrifuged, and rinsed with PBS containing 1% penicillin/streptomycin, 0.5 μg/mL fungizone, and 20 μg/mL gentamicin. Isolated chondrocytes were rinsed with phosphate buffered saline (PBS) and resuspended in chondrocyte medium (DMEM supplemented with 20% FBS, 0.04 mmol/L L-proline, 50 mg/L L-ascorbic acid, 10 mmol/L HEPES buffer, 0.1 mol/L MEM-nonessential amino acids, 1% penicillin-streptomycin, 0.5 μg/mL fungizone, and 20 μg/mL gentamicin (Invitrogen)) and counted via the trypan blue exclusion assay.

Chondrocyte-laden hydrogel constructs (5 mm × 5 mm × 5 mm) were formed via photopolymerization by dissolving PEGDM in chondrocyte medium to a final concentration of 10% by weight, with 0.05% photoinitiator (Irgacure I2959, Ciba Specialty Chemical) and chondrocytes (5×10^7 cells/mL). The macromer/photoinitiator/cell solution was added to a custom Teflon mold and exposed to 365 nm light (~6 mW/cm^2) for 10 min [27]. Cell-hydrogel constructs were allowed to equilibrate under free-swelling culture conditions (free-floating in well plates with chondrocyte medium) for 24 h at 37 °C and 5% CO_2. After 24 h, constructs were removed from free-swelling culture and placed in the bioreactor, denoted as day 0. Free-swelling controls were placed in well plates and were maintained in static culture in the incubator for seven days.

2.6 Offline Mechanical Testing. Mechanical testing with a materials testing system (MTS 858 Mini Bionix II, Eden Prairie, MN) provided a comparison for real-time measurements in the bioreactor. Compressive modulus was determined by applying strain at a constant rate of 0.0017 s^{-1} to a maximum of 10% strain to the hydrated constructs in unconfined compression. The compressive modulus was determined by analyzing the linear region of the stress versus strain curve.

2.7 Biochemical and Histological Analysis. On days 0 and 7, constructs were removed from culture and cut in half. One half of each gel was designated for biochemical analysis and one half for histology. For biochemical analysis, the mass of each of the hydrated gels was obtained. Gels were then freeze-dried, and the mass of each dry construct was obtained. Gels were homogenized and enzymatically digested with papain solution (Worthington Biochemical, Lakewood, NJ, 125 μg/mL papain, 10 mmol/L L-cysteine, 100 mmol/L Na_2HPO_4, 10 mmol/L EDTA, *p*H 6.5) for 16 h at 60 °C. DNA content was determined by Hoechst 33258 (Polysciences, Warrington, PA) fluorescence assay [28]. sGAG content was determined by 1,9-dimethylmethylene blue dye method [29]. Collagen content was determined by hydroxyproline detection with p-dimethylaminobenzaldehyde [30].

For histological analysis, the remaining half of each hydrated gel was fixed in 4% paraformaldehyde, dehydrated, paraffin-embedded, and sectioned. The sections were stained with safranin-O/fast green, which stains sGAG red-orange or Masson's trichrome, which stains collagen blue. Cell nuclei were counterstained with hemotoxylin. Sections were mounted on glass slides, and images were acquired.

2.8 Statistical Analysis. Statistical analysis was performed using unpaired, single-factor analysis of variance with a confidence interval of 0.05. All values are reported as the average plus or minus one standard deviation.

3 Results

Chondrocyte-laden hydrogel constructs were subjected to displacement-controlled intermittent cyclic load for 7 days, resulting in maximum loads of (0.186 ± 0.035) N (Fig. 2). Compressive modulus values were calculated from data collected during the ramp stage for each construct in the bioreactor every 12 h. Modulus did not significantly change over the course of 7 days in the

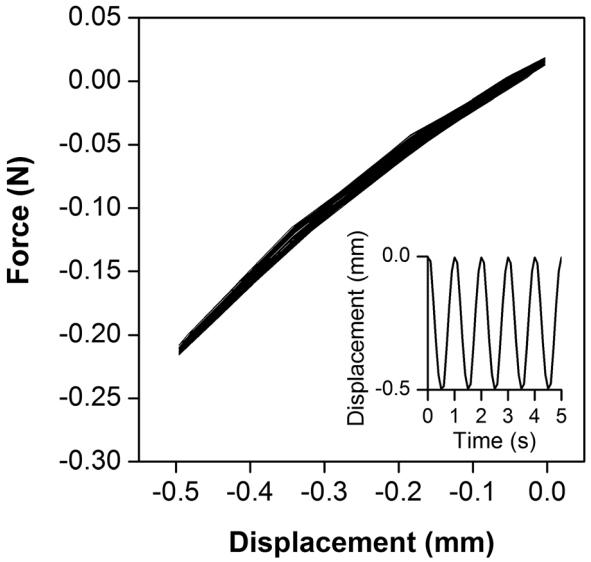

Fig. 2 Representative force-displacement curve for a loaded sample. Samples (5 mm tall) are subjected to 1 h of sinusoidal compression at 1 Hz from 0% to 10% strain. The figure depicts force-displacement data for 200 s of the 1 h sinusoidal loading condition. Force measured by the load cell increases with increasing displacement of the platen. Inset depicts displacement versus time for a portion of the 1 h sinusoidal loading condition.

bioreactor [Fig. 3(*a*)]. Further mechanical testing was performed on a materials testing system (MTS) outside the bioreactor on days 0 and 7 to validate real-time measurements [Fig. 3(*b*)]. Compressive modulus measured on the MTS was not significantly different from real-time measurements of compressive modulus in the bioreactor. Other exploratory data revealed that the bioreactor was able to detect a significant decrease in compressive modulus for degradable PEG hydrogels over the course of a 10 day culture period (data not shown).

Histological staining [Figs. 4(*a*)–4(*c*)] confirmed an increase in pericellular sGAG between days 0 and 7. However, there was no visible difference between day 7 controls and day 7 bioreactor constructs. sGAG content, as quantified with dimethylmethylene blue, increased significantly after 7 days, independent of compression [Fig. 4(*d*)]. Collagen was not detected with histological staining or the hydroxyproline assay.

Loaded constructs were evaluated with ultrasound on days 0 and 7. Figure 5 demonstrates the decrease in signal amplitude between day 0 [Fig. 5(*a*)] and day 7 [Fig. 5(*b*)] constructs. In each figure, the pixel brightness corresponds to the amplitude of the ultrasound signal reflected back to the detector. The day 7 construct [Fig. 5(*b*)] had overall lower pixel brightness in the area corresponding to the construct. The average ultrasonic signal through the construct was then normalized by the average ultrasonic signal through chondrocyte medium only (no construct in place) to calculate RRA (Fig. 6). There was a significant decrease, approximately 42% ($p = 0.006$), in average RRA between day 0 (0.637 ± 0.213) and day 7 (0.369 ± 0.125). Furthermore, there was a significant decrease in RRA between days 0 and 7 for each construct ($p < 0.031$).

4 Discussion

Mechanical stimulation is important for chondrocyte metabolism and is a necessary element for successful functional tissue engineering of cartilage [1]. The goal of culturing tissue-engineered constructs under dynamic load is to produce a tissue replacement that is robust enough to withstand physiological loads within the joint after implantation, while also mimicking the mechanical and biochemical functionality of native tissue. Mechanical stimulation of chondrogenic cells has been shown to stimulate ECM production in vitro [31–33]. However, the use of traditional techniques for evaluating mechanical properties and ECM development of engineered tissue are destructive and time consuming. More recently, bioreactors have been designed and employed to measure mechanical properties during culture [6,34,35]. Here we demonstrate that our instrumented bioreactor can be utilized to stimulate and nondestructively evaluate tissue-engineered cartilage over the course of a one-week culture period.

Bioreactors for cultivating cartilage tissue are designed to mimic the mechanical loading experienced by cartilage in the body. Physiological loading of cartilage is a function of various forces within the joint, including sheer, hydrostatic pressure, and compression. A number of bioreactors have been designed specifically for controlled compression of cartilage explants or cell-seeded polymer constructs. These designs typically include a mechanism to impart a precise displacement or force to the material of interest. Mauck et al. designed a bioreactor with a cam-follower system to simultaneously impose dynamic loading on multiple chondrocyte-seeded agarose disks [3]. Another custom bioreactor was designed for simultaneous dynamic loading of multiple chondrocyte-laden hydrogel constructs by utilizing a stepper motor and lifting mechanism [2,26]. These designs offer precise control over loading of the constructs. However, it is rare that the design of compression bioreactors also includes load cells to measure the reaction force and evaluate material stiffness.

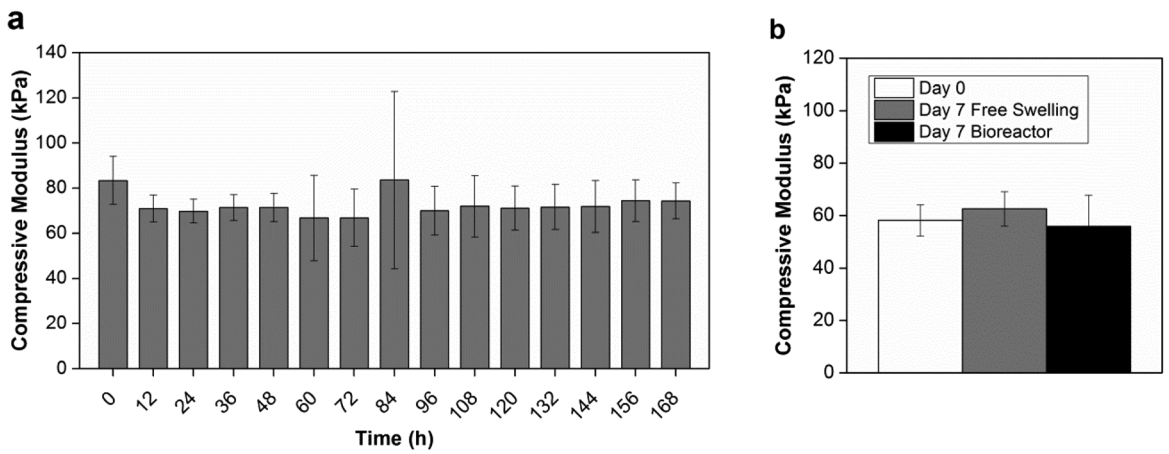

Fig. 3 The compressive modulus was measured (*a*) online in the bioreactor every 12 h and (*b*) offline in a standard mechanical testing machine on days 0 and 7. Data represent the mean plus or minus one standard deviation for $n = 4$ or $n = 5$ constructs.

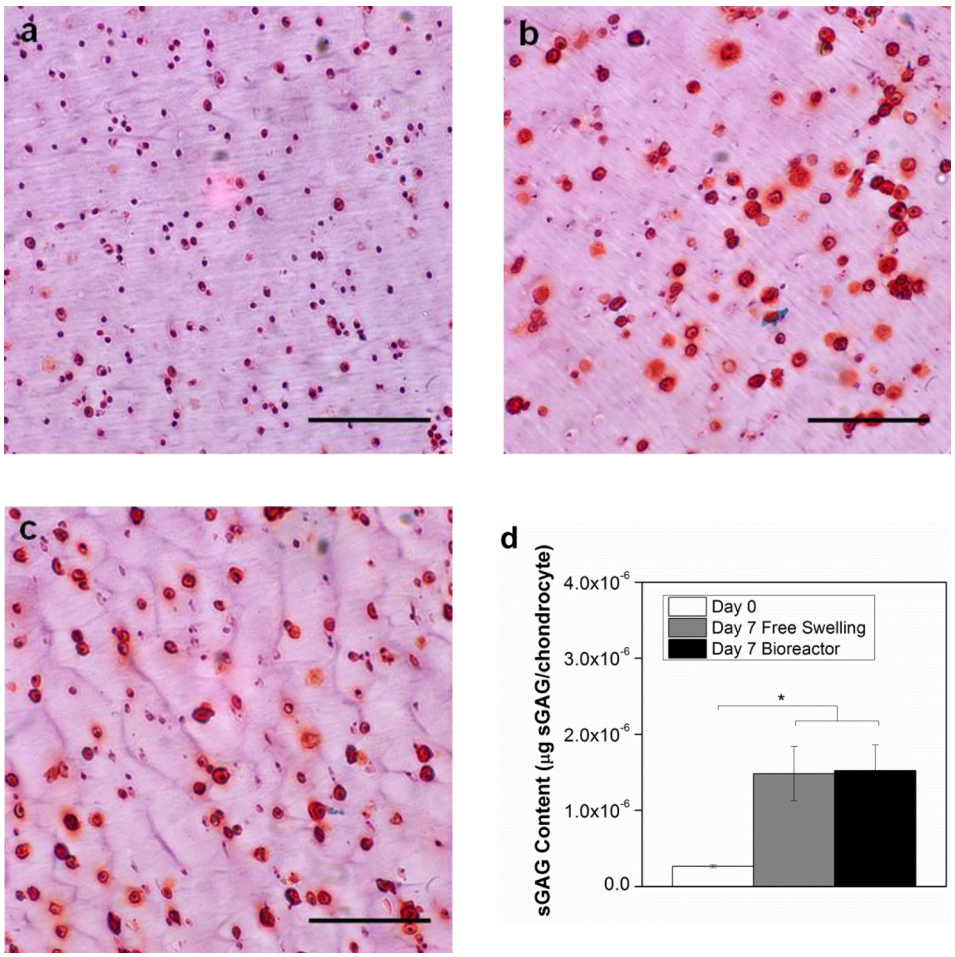

Fig. 4 Sulfated glycosaminoglycan. Histological sections stained with safranin O on (*a*) day 0 control, (*b*) day 7 control, and (*c*) day 7 bioreactor. Scale bars are 100 μm. sGAG content was quantified with dimethylmethylene blue at days 0 and 7 (*d*). Data represent the mean plus or minus one standard deviation for $n = 4$ constructs. Asterisk denotes significant difference from day 0, $p < 0.05$.

In the current work, an instrumented bioreactor was designed for dynamic compression of multiple chondrocyte-laden hydrogels by use of a computer-controlled actuator for control over frequency, waveform, and displacement. This design is also being utilized for validating biokinetic models of engineered tissue development [36]. Here the addition of load cells to the bioreactor allows for measurement of the real-time force response of the chondrocyte-laden hydrogel constructs under dynamic compression. In our evaluation of this instrumentation, the compressive modulus of loaded constructs did not change over the course of the 7 day study [Fig. 3(*a*)]. However, PEG hydrogel scaffolds are considered nondegradable on the time scale of this study, and a change in compressive modulus was not expected. Furthermore, the amount of ECM secreted by the cells was not expected to

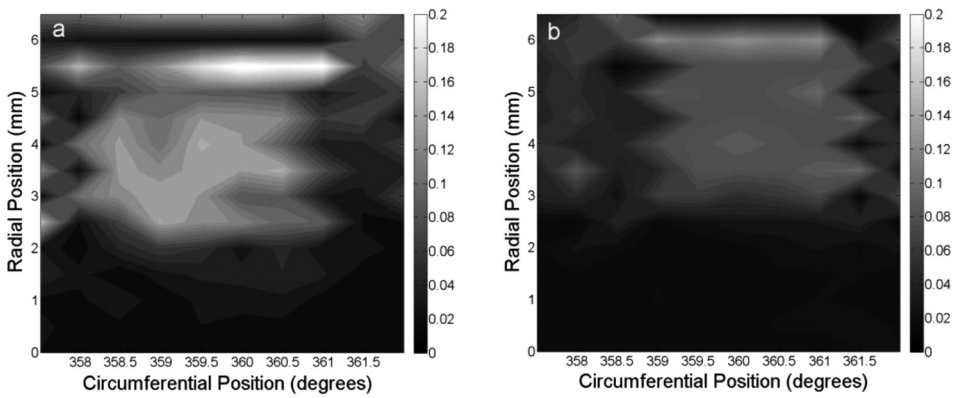

Fig. 5 Representative ultrasonic amplitude maps of hydrogel constructs at (*a*) day 0 and (*b*) day 7. Two-dimensional acoustic images were constructed with the brightness of each pixel corresponding to the amplitude of the acoustic signal reflected from the stainless steel platen.

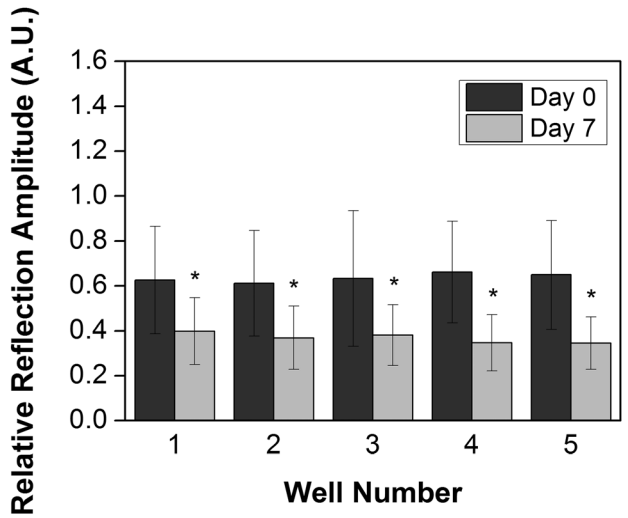

Fig. 6 Relative reflection amplitude for each construct on days 0 and 7. Data represent the mean plus or minus one standard deviation for $n = 4$ constructs. Asterisk denotes significant difference between day 0 and day 7 for the same construct, $p < 0.05$.

impact compressive modulus after only 7 days. Proteoglycan deposition has been detected within the first week of culture. However, the deposition and diffusion of collagen molecules into the matrix may take several weeks.

From the real-time force measurements we can calculate and monitor tissue stiffness over the course of an experiment, which has clear advantages over endpoint testing. Online monitoring in the bioreactor provides a measure of the transient development of the tissue. Furthermore, it may be possible to make adjustments to bioreactor conditions in response to changes in construct stiffness in order to tailor final construct properties. Such online monitoring is of considerable importance when utilizing degradable polymer scaffolds over longer culture periods.

The addition of a nondestructive measurement system for evaluating ECM development has the potential to provide great advantages for developing viable engineered replacement tissues. Hagenmuller et al. have developed a bioreactor to nondestructively monitor bone tissue development with μCT [7]. In the work presented here, ultrasound was used as a nondestructive technique for monitoring ECM development. As an ultrasonic wave travels through a medium, the signal is attenuated due to reflection, scattering, and absorption. Scattering of the propagating acoustic wave, and a subsequent decrease in the RRA, is indicative of the presence of scattering particles and material in the sample matrix [8]. The significant decrease in RRA for each construct after 7 days (Fig. 6) is consistent with the increase in scattering from deposited ECM molecules, specifically sulfated glycosaminoglycans.

For longer-term studies in our bioreactor, RRA should be measured at several discrete time points throughout the study to obtain a transient measure of ECM development. A logical extension of this study may be to determine direct quantitative correlations between RRA and the content of collagen and proteoglycan for our system. Such correlations will allow for measurement of ECM content solely by ultrasound, avoiding the need for traditional and destructive endpoint biochemical assays.

5 Conclusions

We developed an instrumented bioreactor for mechanical stimulation of tissue-engineered cartilage with the capability to measure real-time tissue stiffness and ECM content. Increases in pericellular sGAG were seen after 7 days, independent of compression. Furthermore, RRA decreased significantly after 7 days, likely due to increases pericellular sGAG. However, the short duration of this experiment led to no differences in modulus or collagen content. We expect our bioreactor to be particularly powerful when combined with degradable hydrogels in longer-term studies.

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