Controlling collagen fiber microstructure in three-dimensional hydrogels using ultrasound

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(Received 12 September 2012; revised 17 January 2013; accepted 22 January 2013)

Type I collagen is the primary fibrillar component of the extracellular matrix, and functional properties of collagen arise from variations in fiber structure. This study investigated the ability of ultrasound to control collagen microstructure during hydrogel fabrication. Under appropriate conditions, ultrasound exposure of type I collagen during polymerization altered fiber microstructure. Scanning electron microscopy and second-harmonic generation microscopy revealed decreased collagen fiber diameters in response to ultrasound compared to sham-exposed samples. Results of mechanistic investigations were consistent with a thermal mechanism for the effects of ultrasound on collagen fiber structure. To control collagen microstructure site-specifically, a high frequency, 8.3-MHz, ultrasound beam was directed within the center of a large collagen sample producing dense networks of short, thin collagen fibrils within the central core of the gel and longer, thicker fibers outside the beam area. Fibroblasts seeded onto these gels migrated rapidly into small, circularly arranged aggregates only within the beam area, and clustered fibroblasts remodeled the central, ultrasound-exposed collagen fibrils into dense sheets. These investigations demonstrate the capability of ultrasound to spatially pattern various collagen microstructures within an engineered tissue noninvasively, thus enhancing the level of complexity of extracellular matrix microenvironments and cellular functions achievable within three-dimensional engineered tissues.

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PACS number(s): 43.80.Gx, 43.35.Wa, 43.80.Sh [CCC]

Pages: 1491–1502

I. INTRODUCTION

Since the early 1960s, tissue transplantation has been a highly successful therapy for end-stage organ failure (Nasseri *et al.*, 2001; Atala, 2009). However, present demand for donor organs far exceeds the available supply. Currently, more than 115 000 patients in the United States are awaiting organ transplantation (U.S. Department of Health and Human Services). By developing methods for regenerating diseased or injured tissues and organs, tissue engineering seeks to provide an alternative supply of tissues and organs to balance supply and demand. Engineered tissues could also provide alternatives to traditional animal models used currently for product and procedure testing, toxicology screening, drug discovery, and biological and chemical warfare detection. One of the most common approaches for producing engineered tissue involves implanting cells within biologically or synthetically derived, three-dimensional scaffolds (Butler *et al.*, 2000; Stock and Vacanti, 2001). The current lack of available tissue analogs

reflects an inability to create three-dimensional scaffolds that have both biological activity and adequate mechanical strength.

The formation of new tissue depends upon dynamic interactions between cells and their surrounding extracellular matrix (Frantz *et al.*, 2010). The extracellular matrix is a complex network of interconnected proteins and polysaccharides that imparts structure and mechanical stability to tissues and provides cell adhesion sites, migration pathways, and proliferation signals to cells (Frantz *et al.*, 2010). In turn, the precise composition and organization of the extracellular matrix contribute to the mechanical and permeability properties of tissues and organs (Frantz *et al.*, 2010). Collagen is the primary fibrous component of the extracellular matrix, where it plays a central role in embryonic development and tissue repair (Rozario and DeSimone, 2009). Twenty-eight different types of collagen exist that are categorized by structure and organization into several different families (Kadler *et al.*, 2007). Approximately 90% of all collagens belong to the family of fibril-forming collagens (Kadler *et al.*, 2007). Of the fibrillar collagens, type I collagen is the most abundant. It is the principal component of bone, tendons, skin, and ligaments as well as cornea and is

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found in most interstitial connective tissues, where it provides tensile strength to tissues, regulates cell adhesion, and facilitates cell migration (Kadler *et al.*, 2007). Clinically, type I collagen is used widely in wound dressings, for skin substitutes, and as a natural component of biomaterials in tissue engineering and regenerative medicine applications (Lee *et al.*, 2001; Cen *et al.*, 2008). Collagen has numerous advantages as a biomaterial, including low toxicity and antigenicity, biodegradability, and high abundance (Lee *et al.*, 2001).

The incredible diversity of the functional properties of type I collagen arises from variations in the micro- and macromolecular structure of polymerized collagen fibers. Type I collagen molecules assemble as right-handed triple helices that bundle together in a staggered fashion to form long thin fibrils with diameters of $\sim 25\text{--}500\text{ nm}$ (Gelse *et al.*, 2003; Shoulders and Raines, 2009). *In vitro* type I collagen fibers can form spontaneously through a self-assembly process. The physical properties of self-assembled collagen fibers, including fibril density, thickness, and alignment, are influenced by several factors, namely collagen concentration, temperature, pH, ionic strength, and applied mechanical forces (Roeder *et al.*, 2002; Sander and Barocas, 2008). Differences in the physical parameters of fibrillar collagens, such as stiffness, fiber orientation, and ligand presentation, affect cell and tissue function (Gelse *et al.*, 2003). As such, controlling the structure of type I collagen provides a means to regulate the mechanical properties of biomaterials and control cellular responses in engineered tissues.

Type I collagen gels can be produced *in vitro* by increasing the temperature and neutralizing the pH of acid-solubilized collagen (Wood, 1960). Several groups have exploited critical collagen self-assembly parameters, including temperature and applied mechanical forces, to tune the density and diameter of collagen fibers (Isenberg and Tranquillo, 2003; McDaniel *et al.*, 2007; Yang *et al.*, 2010; Gillette *et al.*, 2011; Carey *et al.*, 2012). Ultrasound can produce localized heating and mechanical forces within the medium of propagation. Others have shown that exposure of fibrin gels to ultrasound decreases fibrin fibril diameter (Braaten *et al.*, 1997). Here we investigated whether exposure of type I collagen to ultrasound during the self-assembly process affects collagen fibril microstructure to in turn enhance cell function.

II. MATERIALS AND METHODS

A. Ultrasound exposure apparatus

The experimental set-up used for ultrasound exposures [Fig. 1(A)] has been described previously (Garvin *et al.*, 2010; Garvin *et al.*, 2011). Briefly, the acoustic source, either a 1-MHz (2.5 cm diameter) or 8.3-MHz (0.64 cm diameter) unfocused piezoceramic transducer, was mounted to the bottom of a plastic exposure tank filled with degassed, deionized water. The ultrasound signal driving the transducer was generated using a waveform generator (Hewlett Packard, Model 33120A, Palo Alto, CA, or Agilent, Model 33250A, Santa Clara, CA), an attenuator (Kay Elemetrics, model 837, Lincoln Park, NJ), and a radio-frequency (RF) power

FIG. 1. (A) Schematic of the experimental set-up used for ultrasound standing wave field exposures. The acoustic source, either a 1-MHz, 2.5-cm diameter or an 8.3-MHz, 0.64-cm diameter unfocused piezoceramic transducer, was situated at the bottom of a plastic exposure tank filled with degassed, deionized water. Well bottoms were placed near the air-water interface a distance, d (12.2 cm for the 1 MHz source and 6.7 cm for the 8.3 MHz source) from the transducer. (B) Schematic of the experimental set-up used for ultrasound traveling wave field exposures. The acoustic source was a 1-MHz, 2.5-cm diameter unfocused piezoceramic transducer. The three-axis positioner was used to place the front face of the sample holder a distance, d , of 12.2 cm from the transducer. A rubber absorber was placed behind the cuvette to reduce reflections.

amplifier (ENI, Model 2100L, Rochester, NY). Samples were contained in the wells of silicone elastomer-bottomed cell culture plates (FlexCell International Corporation, FlexI[®] or BioFlex[®] plates, Hillsborough, NC). In some experiments, well diameters of FlexI[®] culture plates were reduced to 1 cm using elastomer molds (Sylgard[®] 184 silicone elastomer; Dow Corning, Midland, MI). Sample holders were mounted to a three-axis positioner (Velmex, Series B4000 Unislide, East Bloomfield, NY) to allow precise control over sample location within the sound field. In this set-up, the presence of an air interface above the sample produced an ultrasound standing wave field within the sample volume (Garvin *et al.*, 2010; Garvin *et al.*, 2011).

Acoustic field calibrations were conducted prior to each experiment using a needle hydrophone (Onda, HNC-0400, Sunnyvale, CA) under traveling wave conditions. Acoustic pressures were measured at axial distances of 12.2 cm from the transducer for experiments at 1 MHz and 6.7 cm from the transducer for experiments at 8.3 MHz. The -6 dB transaxial beamwidths of the 1- and 8.3-MHz sound fields at these axial locations were 1.2 and 0.6 cm, respectively. Sample holder placement within the acoustic field has been described previously (Garvin *et al.*, 2010). Briefly, coordinates from a fixed point of reference to the field exposure location were obtained from the hydrophone calibration, and the three-axis positioner was used to locate well bottoms of the cell culture plates at the air-water interface, either 12.2 cm (1 MHz experiments) or 6.7 cm (8.3 MHz experiments) from the transducer. The ultrasound-exposed well of the sample

holder was positioned so that the maximum acoustic pressure in the transaxial direction was centered within the well at the desired axial locations listed in the preceding text.

In some experiments, the apparatus for ultrasound exposures was modified to produce a traveling wave field within the sample volume [Fig. 1(B)]. The acoustic source (1 MHz, 2.5 cm diameter piezoceramic transducer) was mounted to the side of the plastic exposure tank. Samples were contained within plastic cuvettes (1 cm × 1 cm × 4.5 cm; VWR, Radnor, PA) mounted to the three-axis positioner. Cuvettes were modified by replacing the front and back walls with acoustically transparent silicone elastomer membranes (FlexCell). A rubber absorber was placed behind the sample holder to reduce reflections. The front face of the cuvette was situated at an axial distance of 12.2 cm from the transducer, and the maximum acoustic pressure in the transaxial direction at this location was centered within the lower 1 cm of the cuvette.

B. Collagen sample preparation

Neutralized type I collagen solutions were prepared on ice by combining type I collagen (BD Biosciences, Bedford, MA; from rat tail) with 2x concentrated Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) and 1x DMEM such that the final mixture contained 1x DMEM and 0.8 mg/ml collagen (Garvin *et al.*, 2010; Garvin *et al.*, 2011).

C. Ultrasound exposures

For ultrasound exposures using the apparatus shown in Fig. 1(A), aliquots of the collagen solution were pipetted into two wells of the cell culture plate yielding samples 0.5 cm in height. One sample was exposed to ultrasound at either 1 or 8.3 MHz for 15 min in a room temperature (RT) water tank. The other sample was treated exactly as the ultrasound-exposed sample but was not exposed to the sound field and served as the sham exposure condition. The 15-min exposure was sufficient for collagen polymerization. This set-up [Fig. 1(A)] produced an ultrasound standing wave field within the sample volume (Garvin *et al.*, 2010; Garvin *et al.*, 2011). Acoustic pressures reported here are the maximum peak positive pressure at an antinode and reported intensities are the spatial peak temporal average intensity (I_{spta}) calculated at an antinode.

For experiments at 1 MHz, samples were subjected to either continuous wave (CW) or pulsed exposures (20- μ s pulse duration, duty cycles of 0.12, 0.24, or 0.5) at various peak positive acoustic pressures (0–1 MPa) and spatial peak temporal average intensities ($I_{\text{spta}} = 0$ –2.4 W/cm²). For experiments at 8.3 MHz, samples were exposed to CW ultrasound at I_{spta} values of 0 or 30 W/cm².

Following ultrasound exposure, collagen gels were incubated at 37 °C, 5% CO₂ for 20 min. Samples were then fixed for 1 h using 4% paraformaldehyde and then washed 3x with phosphate-buffered saline. In some experiments, collagen gels were fixed 20 h after ultrasound exposure. In other experiments, the collagen solution was allowed to polymerize within sample holders at 37 °C, 5% CO₂ for 1 h prior to ultrasound exposure.

For traveling wave field exposures [Fig. 1(B)], an aliquot of the collagen solution was pipetted into the cuvette sample holder and exposed to, or not exposed to (sham condition) ultrasound (1 MHz) for 15 min in a RT water tank. Samples were exposed at 3 W/cm² I_{spta} using either CW or pulsed (100 μ s pulse duration, duty cycle of 0.5) ultrasound. Collagen gels were then fixed after a 20-min incubation at 37 °C, 5% CO₂ as described in the preceding text.

D. Microscopy

To visualize type I collagen fibers, collagen gels were examined using either second-harmonic generation microscopy (Garvin *et al.*, 2010) or scanning electron microscopy. Second-harmonic generation microscopy was performed using an Olympus Fluoview 1000 AOM-MPM microscope equipped with a 25x, 1.05 NA water immersion lens (Olympus, Center Valley, PA). Samples were illuminated with 780 nm light generated by a Mai Tai HP Deep See Ti:Sa laser (Spectra-Physics, Mountain View, CA), and the emitted light was detected with a photomultiplier tube using a bandpass filter with a 390 nm center wavelength (Semrock, Filter FF01-390/40-25, Rochester, NY). Collagen fibers were photographed using a CMOS digital camera (Motic, Moticam 1000, Xiamen, China). For ultrasound standing wave field experiments at 1 MHz, images were collected in 5- μ m steps in the z direction through a 100- μ m depth at both the first pressure antinode from the center of the gel surface (375 μ m) and the first pressure node from the center of the gel surface (750 μ m). Images were then projected onto the z plane using IMAGEJ software (NIH, Bethesda, MD) to create three-dimensional projections of collagen fiber morphology. For ultrasound standing wave field experiments at 8.3 MHz, additional sets of images were collected near the periphery of the collagen gel. For traveling wave field experiments, images were collected at a depth of 900–1000 μ m from the center of the front face of the gel surface.

Scanning electron microscopy was performed using a Zeiss Auriga focused ion beam-scanning electron microscope equipped with high vacuum secondary electron imaging and TIFF file recording (Carl Zeiss Microscopy, Peabody, MA). Collagen samples were prepared for electron microscopy by fixation in 2% glutaraldehyde followed by dehydration in a graded series of ethanol washes. Samples were washed in a graded series of hexamethyldisilazane to sequentially exchange the ethanol and were allowed to air dry. Dried collagen samples were mounted on scanning electron microscope sample stages and sputter coated with gold to an approximate thickness of 3 nm (Desk II Sputter Coater, Denton Vacuum, LLC, Moorestown, NJ). Prepared collagen samples were imaged at 10 kV and magnifications of 10 000x and 50 000x.

E. Quantification of collagen fiber density and diameter

Images collected using second-harmonic generation microscopy were analyzed using IMAGEJ software (NIH) to quantify density of type I collagen fiber networks. For each z stack of images, images were thresholded and converted

from grayscale to binary using an Otsu black and white filter. The number of white pixels in each image series, representing collagen fibers, was divided by the total number of black and white pixels to calculate collagen fiber density. A total of three z stacks of images were analyzed at both imaging locations (pressure antinode and node) within three separate collagen gels per condition that were fabricated on three different experimental days.

Scanning electron microscopy images were analyzed using IMAGEJ software to quantify collagen fiber diameter. A 64×64 pixel grid was overlaid onto images collected at 50 000x magnification. At each grid intersection point, collagen fiber diameter was measured. A total of 18 images per condition were used to quantify collagen fiber diameters. Three images were collected from each of six samples per condition that were fabricated on three different experimental days. For each collagen sample, a histogram of fiber diameters was compiled to display the frequency of occurrence of fiber diameters as a percentage of the total number of fibers measured.

F. Thermocouple measurements

Temperature changes within collagen samples were monitored during ultrasound exposure using a 50- μ m copper-constantan thermocouple situated at the location of maximum acoustic pressure within the samples. Temperature was monitored using a digital laboratory thermometer (Physitemp Instruments, Model BAT-12, Clifton, NJ), sensitive to changes of 0.1°C. For exposures at 8.3 MHz, temperature changes at both the gel center (maximum acoustic pressure) and gel periphery were measured simultaneously using two thermocouples and thermometers.

G. Cell culture and cell seeding onto ultrasound-exposed collagen gels

Fibronectin-null mouse embryonic myofibroblasts (MEFs) were cultured in a 1:1 mixture of AimV (Invitrogen) and CellGro (Mediatech, Herndon, VA) on tissue culture dishes pre-coated with type I collagen as described previously (Garvin *et al.*, 2010). Fibronectin-null MEFs were seeded at a density of 5.2×10^4 cells/cm² onto ultrasound- and sham-exposed collagen gels and cultured at 37°C and 8% CO₂. Cells were observed at various times using an Olympus BX60 microscope and images were collected using a digital camera (QImaging, ExiBlue, Surrey, BC, Canada).

Collagen fiber structure of cell-seeded collagen samples was examined using second-harmonic generation microscopy. Fibronectin-null MEFs were simultaneously visualized using a second bandpass filter with a 519 nm center wavelength (Olympus, Filter BA 495-540HQ from MPFC1) by exploiting the intrinsic auto-fluorescence of cells. Images were collected in the z direction in 1- μ m steps from the gel surface to a depth of 150 μ m into the collagen sample.

H. Statistics

Unless otherwise noted, all experiments were performed on three separate occasions. Data are presented as

means $\pm$ SE. Statistical comparisons between experimental conditions were performed using one-way analysis of variance in GRAPHPAD PRISM software (LaJolla, CA). Differences were considered significant for p values <0.05 .

III. RESULTS

A. Ultrasound exposure affects collagen fiber structure

To investigate effects of ultrasound on collagen fiber microstructure, unpolymerized solutions of type I collagen were exposed to 1 MHz, CW ultrasound of various peak positive pressures and intensities using the apparatus diagrammed in Fig. 1(A). This exposure geometry produces an acoustic standing wave field throughout the collagen sample volume (Garvin *et al.*, 2010). Therefore collagen fiber morphology of polymerized gels was assessed at imaging depths corresponding to either pressure antinodes or nodes using second-harmonic generation microscopy imaging. Sham-exposed collagen gels were characterized by long, thick, loosely packed collagen fibers [Fig. 2(A)]. In contrast, as the ultrasound exposure pressure increased, collagen fibers became visibly thinner, shorter, and more numerous at both pressure antinodes [Fig. 2(A), upper panel] and nodes [Fig. 2(A), lower panel]. The onset for visible differences in collagen fiber structure occurred at 0.2 MPa, as measured at a pressure antinode [Fig. 2(A)], which corresponds to a spatial peak, temporal average intensity (I_{spta}) of 1.2 W/cm².

Changes in collagen fiber morphology were quantified through image processing [Fig. 2(B)]. Results indicated a ~ 1.5 - and ~ 2.4 -fold increase in fiber density over sham for samples exposed at 0.2 and 0.3 MPa, respectively. Similar changes in collagen fiber structure were observed at depths corresponding to both antinodes and nodes [Fig. 2(B)]. Ultrasound-induced changes in collagen fiber microstructure persisted for at least 20 h after exposure (Fig. 3), indicating that the observed changes were not readily reversible. Collagen gels polymerized prior to ultrasound exposure did not show changes in collagen microstructure compared to sham samples (Fig. 3).

B. Ultrasound-mediated changes in collagen fiber structure depend on I_{spta}

Other studies have shown that collagen fiber microstructure is affected by polymerization temperature (Roeder *et al.*, 2002; Sander and Barocas, 2008). Thus experiments were conducted to investigate the role of ultrasound-induced heating in the observed effects on collagen fiber microstructure. Two investigations were performed to independently assess the roles of acoustic pressure and I_{spta} . To examine the role of I_{spta} , solutions of type I collagen were exposed to 1-MHz ultrasound standing wave fields of various I_{spta} but constant pressure amplitude. A constant peak positive pressure amplitude of 0.3 MPa was utilized as this value was above the threshold for ultrasound-mediated changes in collagen fiber structure (Fig. 2). The ultrasound was pulsed using a 20- μ s pulse duration and duty cycles of 0 (sham), 0.12, 0.24, 0.5, or 1 (CW), corresponding to I_{spta} values of 0,

FIG. 3. Ultrasound effects on collagen are not transient. Unpolymerized solutions of type I collagen were exposed during the polymerization process to 1-MHz CW ultrasound using an I_{spta} of 2.4 W/cm² (0.3 MPa). Resultant collagen gels were incubated at 37 °C for either 20 min [(A) and (B)] or 20 h [(C) and (D)], then fixed and imaged using second-harmonic generation microscopy. (E) and (F) Polymerized type I collagen gels were exposed to ultrasound and processed for imaging as above. Representative images of collagen fibers collected at the antinode imaging depth are shown ($n = 3$). Scale bar, 50 μ m.

FIG. 2. Ultrasound standing wave field exposure alters collagen fiber microstructure. Solutions of type I collagen were exposed during the polymerization process to 1-MHz CW ultrasound using various peak positive pressure amplitudes and I_{spta} values. Resultant collagen gels were analyzed for collagen fiber structure using second-harmonic generation microscopy. (A) Representative images of collagen fibers collected at the antinode (top panel) and node (bottom panel) imaging depths are shown. Scale bar, 50 μ m. (B) Images were analyzed to quantify collagen fiber density. Data for antinode (left panel) and node (right panel) imaging depths are presented as average fold difference in fiber density $\pm$ SE normalized to sham average fiber density values ($n = 3$; * $p < 0.05$).

0.28, 0.56, 1.2, and 2.4 W/cm², respectively. Collagen gels exposed to ultrasound at or above 1.2 W/cm² consisted of thinner, shorter, and more densely packed fibers compared to sham-exposed gels [Fig. 4(A)]. Similar results were observed at imaging depths corresponding to pressure antinodes [shown in Fig. 4(A)] and nodes (not shown).

To next investigate the role of acoustic pressure, solutions of type I collagen were exposed to an ultrasound standing wave field at 1-MHz with various pressure amplitudes but at a constant I_{spta} (0.28 W/cm²), which was below the threshold for ultrasound-induced effects [Fig. 4(A)]. The exposures were pulsed using a 20- μ s pulse duration and duty cycles of 0 (sham), 0.12, 0.24, 0.5, or 1 (CW), corresponding to acoustic pressure amplitudes of 0, 0.3, 0.2, 0.15, and 0.1 MPa, respectively. No differences in collagen fiber structure were observed for any of the ultrasound-exposed gels compared to sham-exposed collagen samples [Fig. 4(B)], including those exposed at a pressure amplitude of 0.3 MPa. Taken together, these data indicate that I_{spta} provides a predictor of ultrasound-induced changes in collagen fiber structure, suggesting that the effects of ultrasound on collagen microstructure are mediated through a thermal mechanism.

C. Temperature measurements and thermal effects of ultrasound

Temperatures of collagen gels were measured during ultrasound exposure. Sham-exposed gels reached a steady-state temperature of 18.6 ± 0.3 °C [Fig. 5(A); “Sham, RT water bath”]. In contrast, the steady-state temperature within collagen gels exposed at 2.4 W/cm² was 26.3 ± 1.7 °C [Fig. 5(A)]. This temperature rise was simulated in sham-exposed samples by

FIG. 4. Temporal average intensity of the sound field correlates with changes in collagen fiber structure. Unpolymerized solutions of type I collagen were exposed to a 1-MHz pulsed or CW ultrasound field [20 μ s pulse duration with duty cycles from left to right of (A) 0, 0.12, 0.24, 0.5, and 1, and (B) 0, 1, 0.5, 0.24, and 0.12] using (A) a constant ultrasound standing wave field peak positive pressure amplitude of 0.3 MPa and various I_{spta} or (B) a constant I_{spta} of 0.28 W/cm² and various ultrasound standing wave field peak positive pressure amplitudes. Resultant collagen gels were fixed and analyzed for collagen fiber structure using second-harmonic generation microscopy. Representative images of collagen fibers collected at the antinode imaging depth are shown ($n = 3$). Scale bar, 50 μ m.

bulk heating using a water bath heated to 28.5 °C [Fig. 5(A)]. The morphology of collagen fibrils of gels polymerized in the 28.5 °C water bath was similar to that of collagen gels exposed to ultrasound at 2.4 W/cm² [Fig. 5(B)]. In these gels, fibrils appeared thinner, shorter, and more densely packed than those of sham-exposed collagen gels polymerized at RT [Fig. 5(B)].

Quantitative analysis of images showed a 2.5-fold increase in fiber density for samples polymerized in the 28.5 °C water tank and ultrasound-exposed samples at 2.4 W/cm² as compared to sham collagen gels polymerized at RT (not shown).

As a further test of the role of ultrasound-induced heating in collagen fiber morphology, solutions of type I collagen

FIG. 5. Ultrasound-induced heating of collagen solutions. Solutions of type I collagen were exposed during the polymerization process to 1-MHz, CW ultrasound using an I_{spta} of 2.4 W/cm². (A) Temperatures of ultrasound and sham-exposed collagen gels are plotted as means $\pm$ SE ($n = 3$). (B) Representative images of collagen fibers collected using second-harmonic generation microscopy ($n = 3$). Scale bar, 50 μ m.

FIG. 6. Ultrasound-induced heating of collagen affects fiber microstructure. Solutions of type I collagen were exposed to a 1-MHz pulsed or CW ultrasound field ($20 \mu\text{s}$ pulse duration with duty cycles of 0, 1, 0.5, 0.24, and 0.12 from left to right) using a constant I_{spta} of 2.4 W/cm^2 and various ultrasound standing wave field peak positive pressure amplitudes. Representative images of collagen fibers collected using second-harmonic generation microscopy at a pressure antinode imaging depth ($n = 3$). Scale bar, $50 \mu\text{m}$.

were exposed during polymerization to 1 MHz ultrasound standing wave fields of varying pressure but constant I_{spta} (2.4 W/cm^2) using pulsed exposures with a $20\text{-}\mu\text{s}$ pulse duration and duty cycles of 0 (sham), 0.12, 0.24, 0.5, or 1 (CW). Duty cycles of 0, 0.12, 0.24, 0.5, and 1 corresponded to acoustic pressures of 0, 1.0, 0.7, 0.5, and 0.3 MPa, respectively. Each of the ultrasound exposures produced nearly identical temperature profiles with final temperatures of $\sim 28^\circ\text{C}$ (not shown); this was expected given that the I_{spta} was the same for each condition. Similar dense, short collagen fibrils were observed in all ultrasound-exposed samples compared to the thicker fibers produced in sham gels polymerized at 19°C in a RT water bath (Fig. 6). These studies indicate that ultrasound-induced heating during collagen polymerization can control collagen fiber microstructure within hydrogels.

The magnitude of the heating observed in the measurements in the preceding text was not a result of direct absorption of ultrasound in the collagen. The -6 dB beam width (1.2 cm) of the 1-MHz ultrasound field at the exposure site was comparable to the diameter (1 cm) of the Sylgard[®] mold used to contain the collagen gel. Sylgard[®] is a polymer with an absorption coefficient of $1.4 \pm 0.03 \text{ dB/cm}$ at 1 MHz (Garvin *et al.*, 2010). Thus it was hypothesized that ultrasound-induced heating of the Sylgard[®] mold was the predominant source of thermal increases within the collagen samples in the preceding experiments. To test this, the Sylgard[®] mold was removed from the sample holder wells, and collagen gel samples were exposed to ultrasound in wells that were 2.5 cm in diameter. In these samples, the collagen solution filled the 2.5 cm diameter well, and thus, only ultrasound-induced heating of the collagen, if any, was measured. Temperature profiles of collagen gels exposed to ultrasound at 2.4 W/cm^2 in either 1- or 2.5-cm wells are shown in Fig. 7(A). Sham and ultrasound-exposed 2.5-cm diameter gels showed nearly identical increases in temperature to $\sim 19^\circ\text{C}$ [Fig. 7(A)], indicating limited ultrasound heating of collagen gels under these exposure conditions. No differences in collagen fiber structure were observed in gels exposed to ultrasound in the 2.5 cm wells compared to sham conditions [Fig. 7(B)]. This lack of an effect on collagen fiber microstructure is consistent with the lack of ultrasound-induced temperature rise in the collagen samples contained within the larger wells under the stated exposure condition.

D. Ultrasound traveling wave field exposures

In the studies described thus far, similar changes in collagen fiber morphology were observed at both the pressure antinodes and nodes of the sample, suggesting that the ultrasound-induced effects are not dependent upon the standing wave field. To clearly demonstrate that standing wave fields are not necessary for effects of ultrasound on collagen fiber structure, the exposure geometry was reconfigured such that the collagen samples were exposed to an ultrasound traveling wave field [Fig. 1(B)]. Temperature profiles of collagen gel samples exposed to ultrasound traveling wave fields or standing wave fields and the corresponding sham conditions are shown in Fig. 8(A). Temperature profiles were similar for collagen solutions exposed to ultrasound using the following three conditions: (1) 1 MHz, CW ultrasound standing wave field with I_{spta} of 2.4 W/cm^2 at a pressure antinode, (2) 1 MHz, CW ultrasound traveling wave field with I_{spta} of 3 W/cm^2 , and (3) 1 MHz, pulsed ($100\text{-}\mu\text{s}$ pulse duration, duty cycle of 0.5) ultrasound traveling wave field with I_{spta} of 3 W/cm^2 [Fig. 8(A)]. For all ultrasound exposure conditions, the final steady-state temperature of the gels was approximately 25°C [Fig. 8(A)]. Collagen fibers of samples exposed to both ultrasound traveling wave fields conditions (pulsed and CW) were short, thin, and dense as compared to sham conditions [Fig. 8(B)] and comparable to collagen samples exposed to ultrasound standing wave fields at 2.4 W/cm^2 [Fig. 5(B)]. These results demonstrate that effects of ultrasound on collagen fiber structure are not unique to either standing wave fields or traveling wave fields. Rather it is the extent of the resultant ultrasound-induced heating during polymerization that non-invasively controls collagen fiber structure.

E. Spatial patterning of collagen microstructure using ultrasound

The absorption coefficient of ultrasound in soft-tissues is approximately proportional to acoustic frequency. Thus, to heat collagen directly with ultrasound, a higher frequency ultrasound source was employed. For these studies, an 8.3-MHz source with a -6 dB beam width of 0.6 cm at the exposure location was used. Figure 9(A) illustrates the dimensions of the ultrasound beam width that was located centrally in a 4-cm diameter well containing the collagen

FIG. 7. Tests with and without elastomer mold. Solutions of type I collagen were exposed to 1-MHz CW ultrasound using an I_{spta} of 2.4 W/cm². Samples were either contained within 1-cm (elastomer mold present) or 2.5-cm diameter (elastomer mold absent) wells. (A) Sample temperatures are plotted as means $\pm$ SE ($n=3$). (B) Representative images of collagen fibers collected using second-harmonic generation microscopy at the antinodal imaging depth ($n=3$). Scale bar, 50 μm .

FIG. 8. Ultrasound traveling wave fields produce changes in collagen microstructure. Solutions of type I collagen were exposed to 1-MHz ultrasound using either an ultrasound standing wave field or traveling wave field experimental set-up. (A) Temperature measurements were obtained under various exposure conditions and are plotted as means $\pm$ SE ($n=3$). (B) Representative images of collagen fibers collected using second-harmonic generation microscopy ($n=3$). Scale bar, 50 μm .

FIG. 9. Spatial patterning of collagen fiber microstructure. (A) Schematic of the method utilized to locally control collagen fiber microstructure. A high frequency, 8.3-MHz acoustic source, with a narrow beam width ($-6 \text{ dB} = 0.6 \text{ cm}$) was directed at the center of a collagen sample. (B) Solutions of type I collagen were exposed to 8.3-MHz CW ultrasound using an ultrasound standing wave field with an I_{spta} of 30 W/cm^2 . Samples were contained within 4-cm diameter wells of BioFlex[®] culture plates. Temperatures were measured at the gel center and periphery (outside the -6 dB beam width) using two thermocouples. Temperatures are plotted as means $\pm$ SE ($n = 3$). (C) Representative second-harmonic generation microscopy images of collagen fibers collected at the center and periphery of sham-exposed samples and samples exposed to 30 W/cm^2 I_{spta} ($n = 3$). Scale bar, $50 \mu\text{m}$. (D) Representative scanning electron microscopy images of collagen fibers from sham and ultrasound-exposed (30 W/cm^2) samples ($n = 3$). Scale bar, $1 \mu\text{m}$. (E) Scanning electron microscopy images were used to quantify collagen fiber diameters. Data are presented as histograms to display the frequency of occurrence of fiber diameters as a percentage of the total number of fibers measured in both sham- and ultrasound-exposed collagen gel centers ($n = 3$).

solution. We hypothesized that collagen microstructure could be controlled site-specifically such that ultrasound-induced heating within the central region of the gel would produce short, dense collagen fibers while longer, thicker fibrils would be produced outside the beam width at the gel periphery. Temperature measurements were obtained at the center and periphery of collagen samples contained within 4-cm wells and exposed at 8.3 MHz, CW, and I_{spta} of 30 W/cm^2 [Fig. 9(B)]. As expected, temperatures in the center of samples exposed to ultrasound were significantly higher than temperatures at the periphery [Fig. 9(B)]. Furthermore, temperatures in the center of these gels were comparable to temperatures measured within gels exposed at

1 MHz and 2.4 W/cm^2 and contained within 1-cm diameter Sylgard[®] molded wells [Fig. 9(B)].

Second-harmonic generation images were obtained at the center and periphery of polymerized collagen samples exposed to 8.3-MHz ultrasound within the larger wells. As expected from temperature profiles, dense networks of short, thin collagen fibers were observed within the central core of the collagen gel [Fig. 9(C)]. In contrast, longer, thicker fibers were found outside the central ultrasound beam area [Fig. 9(C)]. These experiments demonstrate the ability to design ultrasound exposure conditions to control collagen fiber structure noninvasively and site-specifically within a three-dimensional hydrogel.

Scanning electron microscopy images revealed decreased collagen fibril diameter in response to ultrasound exposure compared to sham [Fig. 9(D)], confirming changes observed by second-harmonic generation microscopy. Quantification of collagen fiber diameter from scanning electron microscopy images revealed that sham-exposed collagen samples contained a uniform distribution of fiber diameters ranging from ~ 25 to 600 nm [Fig. 9(E), left panel]. In contrast, ultrasound exposure shifted the collagen fiber diameter distribution to a higher occurrence of thinner fibers with sizes ranging from ~ 25 to 200 nm [Fig. 9(E), right panel]. Importantly, the absence of thick (200 – 600 nm) fibrils in ultrasound-exposed collagen samples provides additional evidence that ultrasound exposure produces thinner collagen fibers within these collagen hydrogels.

F. Cellular response to ultrasound-exposed collagen hydrogels

Collagen fiber structure affects cell behaviors that are critical to the fabrication of functional engineered tissue *in vitro* (Gelse *et al.*, 2003). As such, studies were conducted to determine whether ultrasound-induced changes in collagen fiber microstructure would produce spatially defined differences in cell behavior. For these experiments, fibronectin-null embryonic myofibroblasts (MEFs) were seeded onto 4-cm diameter collagen gels that had been exposed to ultrasound at 8.3 MHz and 30 W/cm². As shown in Fig. 9, this exposure produces a central cylinder (~ 1 cm diameter) of dense, thin, short collagen fibrils within the

FIG. 10. Cell response to ultrasound-exposed collagen gels. Solutions of type I collagen were exposed to 8.3 -MHz CW ultrasound using an ultrasound standing wave field I_{spta} of 30 W/cm². Fibronectin-null mouse embryonic myofibroblasts were seeded onto resultant collagen gels, and cell location and morphology were monitored over time using phase-contrast microscopy. Shown are representative images collected at the gel center and gel periphery of ultrasound-exposed and sham-exposed samples 1 h and 1 day post-cell seeding. Arrow denotes area of the collagen substrate devoid of cells ($n = 3$). Scale bar, 200 μm .

4 -cm diameter gel. Fibronectin-null MEFs were utilized as they do not produce fibronectin and are cultured under serum-free conditions. Thus initial cell adhesion in these experiments is mediated solely by the type I collagen substrate. Fibronectin-null MEFs adhered equally well to sham- and ultrasound-exposed collagen gels at both the gel center and periphery (Fig. 10, 1 h). Immediately after seeding, cells were homogeneously distributed over both the central and peripheral regions of ultrasound-exposed gels (Fig. 10, 1 h). However, within 1 day of seeding, cells within the central, ultrasound-exposed region of the collagen gel had migrated into small, circularly arranged aggregates (Fig. 10, 1 day, arrows). This spatial pattern remained evident up to 28 days after seeding (not shown). In contrast, cells seeded onto sham-exposed collagen, or cells adherent to the periphery of ultrasound-exposed samples, remained in a homogeneous distribution of single cells (Fig. 10, 1 day).

To examine the morphology of collagen fibers of cell-seeded collagen gels, second-harmonic generation microscopy images were collected at various times post-cell

FIG. 11. Cell-mediated reorganization of ultrasound-exposed collagen fibers. Solutions of type I collagen were exposed to 8.3 -MHz, CW ultrasound using an ultrasound standing wave field I_{spta} of 30 W/cm². Fibronectin-null mouse embryonic myofibroblasts were seeded onto resultant collagen gels and samples were fixed 1 or 28 days post-cell seeding. Samples were analyzed using two-photon and second-harmonic generation microscopy. Representative merged images show reorganization of ultrasound-exposed collagen fibers at gel centers, first into aligned fiber bundles between cellular aggregates at day 1 (arrows), and into densely packed sheets at 28 days (arrowheads) ($n = 3$). Cells, green; collagen fibers, red. Scale bar, 50 μm .

seeding. Cellular aggregates were present at the center of ultrasound-exposed samples at 1 day, and cells within this region reorganized their underlying collagen substrate into collagen bundles aligned between neighboring cell clusters (Fig. 11, 1 day, arrows). Over a 28-day period, cells extensively remodeled only the central, ultrasound-exposed collagen fibrils into dense sheet-like structures (Fig. 11, 28 days, arrowheads). This cell-mediated collagen reorganization was absent at the periphery of ultrasound-exposed gels (Fig. 11, 28 days). Similarly, collagen fibril structure of sham-exposed, cell-seeded gels was similar 1 and 28 days post-cell seeding (Fig. 11), indicating little cell-mediated reorganization of the collagen fibrils in sham samples. These data indicate that cells specifically remodel ultrasound-exposed collagen fibrils into dense collagen sheets. Furthermore, these results provide evidence that cells are capable of sensing ultrasound-induced changes in collagen microstructure and can respond to localized variations in fiber structure within the same three-dimensional hydrogel by exhibiting spatial differences in cell behavior.

IV. DISCUSSION

In this paper, we demonstrate that ultrasound can be used to noninvasively control the microstructure of collagen fibers within three-dimensional hydrogels. Under appropriate conditions, exposure of soluble collagen to ultrasound during the self-assembly process resulted in collagen gels with shorter and thinner fibers compared to collagen gels that were not exposed to ultrasound. These changes in collagen microstructure were produced using both ultrasound standing wave fields and traveling wave fields. The observed effects were localized to the ultrasound beam area and occurred throughout the three-dimensional gel volume. The effect of ultrasound on collagen microstructure occurred only when soluble collagen was exposed during the polymerization process; no effects of ultrasound on collagen fiber microstructure were observed in collagen gels that were polymerized prior to ultrasound exposure. The ultrasound-induced alterations in collagen microstructure were not transient or readily reversible. Braaten *et al.* (1997) observed changes in the microstructure of fibrin in fibrin clots exposed to ultrasound. In that work, effects of ultrasound on fibrin structure were transient.

Results of a series of mechanistic experiments were consistent with a thermal mechanism for the effects of ultrasound on collagen fiber microstructure. Ultrasound exposure conditions utilized in some experiments produced indirect heating of collagen samples due to absorption of sound in the elastomer mold surrounding the collagen. In other experiments, the mold was removed, and a higher frequency and intensity were employed to heat the collagen directly. In all cases, the ultrasound-induced heating was relatively mild, producing final temperatures within the collagen gel of $\sim 30^{\circ}\text{C}$ for the highest intensities investigated in this study.

These results are consistent with literature reports demonstrating that collagen polymerized at different temperatures results in different fiber structures (Wood, 1960). During the self-assembly process, collagen fiber thickness is affected by both pH and temperature, where lower pH and temperature

provides a longer nucleation phase to produce thicker fibrils (Wood, 1960; McPherson *et al.*, 1985). Interestingly, the mechanical stiffness of collagen hydrogels is dependent upon collagen fiber microstructure, where thinner fibers result in stiffer gels (Roeder *et al.*, 2002; Raub *et al.*, 2007; Yang *et al.*, 2009). Physical parameters of collagen gels, such as fiber density, and bulk gel stiffness affect cell proliferation, viability, differentiation, and migration (Hansen *et al.*, 2006; Sung *et al.*, 2009; Baker *et al.*, 2010; Miron-Mendoza *et al.*, 2010). Thus there has been strong interest in developing technologies that can tune the physical properties of collagen gels to control cell behavior (Cen *et al.*, 2008).

Ultrasound holds numerous technological advantages over bulk heating. The magnitude of heating produced by ultrasound can be controlled noninvasively through design of acoustic exposure parameters. Additionally, ultrasound heating can be produced site-specifically resulting in a single collagen hydrogel with spatial variations in fiber microstructure. Further, more complex spatial patterns of collagen microstructure within a hydrogel could be produced with the use of multiple focused ultrasound fields. In the present study, a high frequency ultrasound beam was directed within the center of a large collagen sample producing dense networks of short, thin collagen fibrils within the central core of the gel and longer, thicker fibers outside the beam area. Fibroblasts seeded onto these gels migrated rapidly into small, circularly arranged aggregates only within the beam area, and clustered fibroblasts remodeled the central, ultrasound-exposed collagen fibrils into dense sheets. The observed differences in cell motility and collagen fibril remodeling activity likely occurred in response to regional differences in collagen gel stiffness (Zaman *et al.*, 2006; Hadjipanayi *et al.*, 2009) and/or collagen fiber density (Grinnell and Petroll, 2009). Thus ultrasound technologies that can noninvasively and site-specifically control the microstructure of collagen fibrils have the potential to produce three-dimensional scaffolds with defined mechanical and biological properties.

ACKNOWLEDGMENTS

This work was supported in part by the National Institutes for Health (R01 EB008996 and R01 EB008368). The authors thank Sally Z. Child and John Nicosia for technical assistance and Brian McIntyre for assistance with scanning electron microscopy.

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