Poly-L-Arginine Based Materials as Instructive Substrates for Fibroblast Synthesis of Collagen

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Abstract

The interactions of cells and surrounding tissues with biomaterials used in tissue engineering, wound healing, and artificial organs ultimately determine their fate *in vivo*. We have demonstrated the ability to tune fibroblast responses with the use of varied material chemistries. In particular, we examined cell morphology, cytokine production, and collagen fiber deposition angles in response to a library of arginine-based polymeric materials. The data presented here shows a large range of vascular endothelial growth factor (VEGF) secretion ($0.637 \text{ ng}/10^6 \text{ cells/day}$ to $3.25 \text{ ng}/10^6 \text{ cells/day}$), cell migration ($\sim 15 \text{ minutes} < \text{persistence time} < 120 \text{ minutes}$, $0.11 \text{ }\mu\text{m}/\text{minute} < \text{speed} < 0.23 \text{ }\mu\text{m}/\text{minute}$), and cell morphology ($0.039 < \text{form factor (FF)} < 0.107$). Collagen orientation, quantified by shape descriptor (D) values that ranges from 0 to 1, representing completely random ($D = 0$) to aligned ($D = 1$) fibers, exhibited large variation both *in vitro* and *in vivo* ($0.167 < D < 0.36$ and $0.17 < D < 0.52$, respectively). These findings demonstrate the ability to exert a certain level of control over cellular responses with biomaterials and the potential to attain a desired cellular response such as, increased VEGF production or isotropic collagen deposition upon exposure to these materials in wound healing and tissue engineering applications.

Introduction

The extracellular microenvironment affects cells and is instrumental in controlling cellular behavior and responses [1]. Implanted biomaterials, tissue engineering constructs, and artificial organs all interact to some extent with surrounding tissues and cells. Ultimately, their *in vivo* fate depends on the outcome of this interaction. The impact of biomaterials on cells has been widely studied [2–5]; however, the ability to tune the response of fibroblasts for applications in wound healing and tissue engineering, in particular the substrate's ability to alter the distribution of collagen fiber orientation through changing chemical functional group alone, has not been explored.

Wound healing is a complex process involving the interaction of various cell lineages and tissue types [6,7]. While healing is often rapid and efficient, the resultant tissue is not always aesthetically appealing or functionally suitable [6]. Scar tissue is visually unpleasant and is mechanically weakened, sometimes dysfunctional. At maximum, remodeled tissue only reaches 80% of the strength of unwounded skin [7]. Reorganization of collagen by fibroblasts is directly responsible for this decrease in strength [8]. In healthy tissue, collagen is found to have isotropic fiber orientation, while scar tissue contains smaller bundles of collagen aligned parallel to one another [8]. This variation is especially problematic for tissue-engineered scaffolds and artificial organs because fibrotic tissue impedes nutrient transport [4].

Achieving random collagen deposition that more closely resembles young, healthy skin would be a vast improvement upon the imperfections of the natural wound healing process and the integration of tissue engineered scaffolds. One approach would be to develop a wound-healing polymer that can influence the way in which fibroblasts

synthesize and deposit collagen. Numerous wound dressings, other than standard bandages and gauzes, have aimed to provide an optimum environment for cells involved in wound healing [9]. These conditions include a warm, moist environment with oxygen circulation and low bacterial access [9]. Many newly developed dressings decrease pain levels and healing time; they can also serve an antibacterial, absorbent, adherent, or occlusive function. Preeminent dressings also elicit the appropriate cell-material interactions crucial for the immunomodulation and improved wound healing processes [9]. Unfortunately, none of the commercially available wound dressings target the remodeling phase of wound healing, in particular how fibroblasts respond to the biomaterials, to reduce scarring [9].

Here, we present a potential platform for engineering fibroblast responses to improve upon the wound healing process. This work directly targets and affects fibroblasts in the remodeling phase. The library of materials used here is based on the amino acid arginine. Arginine-rich peptides have been extensively explored as intracellular delivery vehicles as their guanidine functionality allows them to interact with the phosphate groups in cell membranes [10,11]. We have developed a library based on arginine on the premise that interactions between the arginine moieties and the cell membrane may result in altered cell morphology, cytokine production, and collagen fiber deposition distribution by fibroblasts.

Materials and Methods

PLR Modification. Fifteen different molecules (Figure 1a) (Sigma, St. Louis, MO), herein called amidine derivatives, were used to modify poly-L-arginine (PLR) with an 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Oakwood Chemical, West

Columbia, SC) coupling reaction. A generalized reaction scheme is given in Figure 1b. Briefly, 4 mL of a 2.5 mg/mL solution of PLR in phosphate buffered saline (PBS) were placed in glass scintillation vials with 100 molar equivalents of amidine derivative. 200 mg of EDC was added to the vial and the reaction was allowed to stir for 4 hours at room temperature. The polymers were then dialyzed in DI H₂O for 24 hours to remove EDC and unreacted amidine derivatives, and subsequently lyophilized (4.5 L, Labconco, Kansas City, MO). The modified PLR was re-suspended in PBS at 0.1% and stored at -20 °C.

Material Characterization. In order to ensure that the reaction proceeded, NMR was used to characterize the reaction products (see supporting information Supplementary Figure 1). The modified PLR samples were dissolved in D₂O and freeze-dried three times to replace all OH groups with OD groups before 2mg of polymer were dissolved a final time in 600μL of D₂O. The ¹H spectra were recorded on a Bruker Avance III Spectrometer at 70 °C, a sweep width of 6602.1Hz, a 90° pulse, and an acquisition time of 2.48 s. 128 repetitive scans with 64k points were acquired and the data were processed in MNova with 128k points, zero filling, and exponential line broadening of 1.0Hz [12]. 16 repetitive scans were acquired at room temperature for the ¹H spectra of the amidine derivatives with all other parameters the same as above. In comparing spectra, additional peaks were found near 1.8, 3.5, and 3.8 ppm for each modified PLR. Primary amines typically appear in the region of 1 – 3 ppm, depending on H-bonding. The 1.8 ppm shift is assigned to the primary amine formed during the amidine reacting with the carboxylic acid. Because peaks were not present at these chemical shifts in the spectra of the amidine derivative reacted with PLR, it was determined that they were a result of the

formation of amide bonds in EDC coupling reactions. Protons associated with imidazole show shifts in the range of 7 – 8.4 ppm, which were not observed in the ^1H NMR spectra.

Water contact angle. The hydrophobicity of each modified PLR sample was measured by determining the water contact angle of each sample. The captive air bubble method was used as all the samples were very hydrophilic. The coated coverslips were attached to microscope slides and placed upside down on top of a container of water. An air bubble (100 μL) was then distributed under the coverslip and imaged with a digital camera (Canon EOS Rebel T3i, Canon, Melville, NY). This process was repeated to obtain five replicates before the angle between the glass and the bubble were measured using ImageJ (NIH, Bethesda, MD) software and subtracted from 180° to determine the final water contact angle. Water contact angles and corresponding standard deviations are shown for each modified PLR sample in Supplementary Table 1. The angle of 24° for a control glass coverslip closely matched other literature values for silicon dioxide [13]. With the highest angle measuring at 69° , this data indicates that all samples are reasonably hydrophilic. Although none of the samples are as hydrophilic as the control, modifications 4, 5, 7, 9 and PLL were the most hydrophilic with water contact angles between 34° and 38° which is in the conditionally non-adhesive region, as defined by Vogler and Saltzman [14]. Modifications 3, 11, and 12 were the most hydrophobic with angles greater than 55° , falling in the adhesive region. The remaining modifications were moderately hydrophilic with angles ranging from 41° - 53° .

Cell Culture. The mouse embryo fibroblast cell line NIH/3T3 (American Type Cell Collection, ATCC, Manassas, VA) was cultured in complete media (CM, Dulbecco's modified Eagle's medium (DMEM, Mediatech Inc., Manassas, VA) supplemented with

10% bovine calf serum (BCS, Mediatech Inc.), 100 U/mL penicillin, and 100 µg/mL streptomycin) at 37°C in 5% CO₂. Approximately every three days, the cells were passaged to ensure proper cell growth through 0.025% trypsin-EDTA detachment and subculturing at 5.4×10^3 cells/cm².

Cell Viability. Cytotoxicity of all materials synthesized was determined using MTT assays. This assay operates by converting a yellow tetrazole (MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Research Products International Corp., Mt. Prospect, IL) to a purple formazan in the mitochondria of living cells. During passaging, NIH/3T3 cells were seeded at 250,000 cells/cm² in 24-well plates (KSE Scientific, Durham, NC). Prior to this, the wells were coated with 0.05% solutions of the modified PLR samples by incubating 200µL/well at 37°C for one hour and washing twice with sterile PBS. After 24 hours, the supernatant was removed from each well to be assayed. 500 µL of media were then added to each well along with 50 µL of an MTT solution (5mg/mL in DI water). After incubation for 2 h at 37°C, 425 µL of media were removed from each well and 500µL of dimethyl sulfoxide (DMSO, Fisher, Waltham, MA) were added to dissolve the purple formazan crystals. The absorbance was read at 540 nm with a reference at 690 nm with a BioTek Synergy HT Multidetector Microplate Reader (BioTek, Winooski, VT). Positive controls of cells in the absence of any PLR were used to normalize all samples. Negative controls containing media and PLR were also done. All experiments were done in quadruplicate. Results were expressed as the mean value ± standard error of the mean normalized to the positive control.

Measurement of VEGF Production. Secretion levels of VEGF in cell culture supernatant were measured using ELISA assays. 96 well ELISA plates (eBioscience Inc.,

San Diego, CA) were coated with 100 μ l/well of polyclonal goat anti-VEGF₁₆₄ antibody (R&D Systems; AF 493 NA, Minneapolis, MN) at 0.6 μ g/mL diluted in PBS and incubated for 12 -16 hours at room temperature (rt). Subsequently, the wells were washed with PBS and blocked for 1 h at rt with 200 μ l of assay diluent (eBioscience Inc.). After washing, serial dilutions of VEGF₁₆₄ standards (R&D Systems; 493-MV) and supernatant samples were prepared in assay diluent and incubated at rt for 12-16 hours. Plates were washed and incubated for 12-16 hours at 4 °C with biotinylated polyclonal goat anti-VEGF₁₆₄ antibody at 0.4 μ g/mL (R&D Systems; BAF 493). After washing the plates, streptavidin/horseradish peroxidase (ThermoScientific, Prod# N504, 1:1000, Waltham, MA) was added to the wells and incubated for 30 minutes at rt. A substrate solution (tetramethylbenzidine (TMB), ThermoScientific, Prod#34029) was added to each well and developed for 5 minutes. The reaction was stopped with 2M H₂SO₄. Absorbance was measured at 450 nm, with a reference at 650 nm. All experiments were performed in quadruplicate. Results were expressed as the mean value $\pm$ standard error of the mean.

Cell Migration. NIH/3T3 cells were seeded on coated dishes at 8,400 cells/cm² and incubated overnight at 37 °C in 5% CO₂ to permit cell adhesion. Fresh medium with 12 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma) was added at the beginning of each experiment to buffer the solution in the absence of a CO₂ environment. In a control experiment, cells were incubated on the surface of unmodified PLR, PLL, and uncoated petri dishes.

Cell tracking and migration analysis: Cell migration experiments were performed using time-lapse microscopy. Briefly, petri dishes were placed on the heating stage at 37 °C and imaged every 5 minutes for 6 hours with a 10x objective using an inverted microscope

(Zeiss Axio Vert.A1, Jena, Germany) and digital camera (Canon EOS Rebel T3i). The motion of individual cells was tracked by marking cell centroids using ImageJ software. A minimum of 50 cells were tracked for each surface modification or control. Cell locomotion can be characterized by cell speed and persistence time. These parameters are calculated by fitting the mean-squared displacement to the persistent random walk model:

$$\langle D^2 \rangle = 2S^2P^2 \left(\frac{t}{P} - 1 + \exp\left(-\frac{t}{P}\right) \right) \quad (1)$$

where t is time, S is the root mean square speed of the cell population, and P is the persistence of cell movement.[15] Persistence describes the time period over which cell movement continues in the same direction. Values of mean squared displacement $\langle D^2 \rangle$ were calculated using the overlapping interval method. Pooled averages of displacements for cell paths in all replicate experiments were used for fitting with equation 1.[16,17] Matlab (MathWorks, Natick, MA) was used to calculate the pooled average of displacements from loaded coordinates. The displacements were fit with a Levenberg-Marquardt algorithm using Matlab [18].

Cell Staining for Fluorescent Imaging. NIH/3T3 cells were cultured on glass coverslips that had been cleaned for 2 h in 200 mL ethanol, 50 g NaOH, and 300 mL DI water before being rinsed vigorously for 10 min with DI water. The glass coverslips were then placed in treated $35 \times 10 \text{ mm}^2$ Petri dishes (Corning, Tewksbury, MA). Using 600 μL of 0.05% modified PLR in PBS, the coverslip was covered and incubated at 37°C for 1 h. Unmodified PLR, PLL, and uncoated coverslips were used as controls. The coverslips were rinsed twice with 1 mL of PBS and seeded with 25,000 cells/ cm^2 in 2 mL of CM and incubated at 37°C in 5% CO_2 for 48 h. After 48 hours, the coverslips were rinsed

twice with PBS and fixed using methanol at -20°C for 7 minutes. After rinsing twice with ice cold PBS, the samples were blocked with 1% BSA and 0.3% Tween-20 in PBS and incubated at rt for 1 h. The cells were stained with 1:100 α -tubulin rabbit mAb Alexa Fluor® 488 Conjugate (5063, Cell Signaling Technology, Danvers, MA), and incubated at 4°C overnight in the dark. After washing 3 times with PBS for 5 minutes, the coverslips were incubated in 0.1 µg/mL DAPI for 2 minutes and rinsed with PBS. Then, the samples were mounted on microscope slides with 50 µL of glycerin jelly, sealed with clear nail polish, and imaged with an EVOS® FLoid® Imaging Station (Life Technologies, Grand Island, NY) using the blue channel (excitation/emission 390/446 nm) and the green channel (482/532 nm).

Morphology Analysis. CellProfiler (Broad Institute, www.cellprofiler.org, Cambridge, MA) was used to analyze the morphology of the adherent cells. A more detailed description on the software can be found at www.cellprofiler.org. This software was used to analyze the shape and morphology of the NIH/3T3 cells on surfaces coated with each modified PLR and control. The shape was analyzed using a form factor, FF:

$$FF = 4 * \pi * area/perimeter^2 \quad (2)$$

The pipeline for this analysis included four modules: IdentifyPrimaryObjects, IdentifySecondaryObjects, IdentifyTertiaryObjects and MeasureObjectSizeShape. Adjustments were made to the threshold correction factor in the first two modules for each image set.

Ethics Statement. The research protocol was approved by the local animal ethics committees at Iowa State University (Institutional Animal Care and Use Committee) prior to initiation of the study.

Animals. 6-week-old female SKH1-E mice were obtained from Charles River Laboratories (Wilmington, MA). The mice were maintained at the animal facilities in the College of Veterinary Medicine at Iowa State University, accredited by the American Association of Laboratory Animal care, and were housed under standard conditions with a 12-hour light/dark cycle. Both water and food were provided *ad libitum*.

Injections. Injections were performed in accordance with ISO 10993-6:2001. Prior to injection, 105-150 μm glass beads (Polysciences, Warrington, PA) were autoclaved for sterilization. They were then coated with each of the fifteen modified PLRs, PLL, and unmodified PLR via electrostatic interactions between the negatively charged glass beads and the positively charged PLRs [19]. Unmodified glass beads were also tested. The mice were anesthetized via isoflurane inhalation at a concentration of 1–4% isoflurane/balance O_2 to minimize movement. Their backs were scrubbed with 70% isopropyl alcohol and the animals were injected with five different modified 10%-v/v glass beads in an array format on the mouse's back and a sixth sample of unmodified PLR coated glass beads as a control. All injection volumes were 100 μL and experiments were conducted in quintuplicate.

Histology. Mice were euthanized via CO_2 asphyxiation and the injected biomaterial and surrounding tissue were excised. One or two tissue sections were fixed in 10% formalin, embedded in paraffin, cut into 5mm sections, and either stained using Mason's Trichrome for histological analysis or used for SHG.

SHG. Sample preparation: Samples for SHG were initially prepared in the same way as those for fluorescence imaging at a seeding density of 60,000 cells/ cm^2 . After fixing the cells with ice-cold methanol for 7 min and washing twice with cold PBS, the coverslips

were mounted on a glass microscope slide with glycerin jelly (10 g gelatin, 60 mL DI water, 70 mL glycerin).

Collagen Gel Preparation. Collagen type I (BD Biosciences, Franklin Lakes, NJ) at concentration 9.87 mg/mL was mixed on ice with 10x PBS, 1 M NaOH, and DI H₂O to obtain final concentration of 4 mg/mL. Microscope glass slides were covered with 600 μ L of 0.05% PLR in PBS. Collagen gel solution was pipetted on microscope glass slides and were gelled at 37°C for 30 min. Microscope slides were mounted with glass coverslips.

Microscopy equipment and SHG experiments: The laser system is a mode-locked Ti:Sapphire laser (100 fs pulse width, 1 kHz repetition rate, Libra, Coherent, Santa Clara, CA) that produces an 800 nm fundamental. The average power at the samples was controlled using a combination of a half-wave plate and a Glan-Thompson polarizer (Thorlabs, Newton, NJ) and kept between 1-10 mW, as experimentally determined to be below the damage threshold of the cells. SHG signal was collected in the transmission mode. For this setup, an inverted microscope stand (AmScope, Irvine, CA) and 20X Nikon Plan Fluorite objective (0.50 NA, 2.1 mm) were used to focus the beam and the SHG light was collected with a 40x Nikon water immersion objective (0.8 NA, 3.5 mm). The transmitted SHG signal was reflected by a dichroic mirror (Thorlabs) and separated from the fundamental beam with a short pass filter < 450nm (Thorlabs) and a 808 nm notch filter (NF-808.0-E-25.0M, Melles Griot), before detection by an intensified CCD camera (iCCD, iStar 334T, Andor, Belfast, UK).

Polarization analysis of SHG signal from samples was accomplished using a Glan-Thompson polarizer and a half-wave plate mounted on a motor driven rotational

stage (Thorlabs). These optics were inserted before the focusing objective. The Glan-Thompson polarizer was used to achieve linear polarization, which was rotated with a half-wave plate [20]. An additional polarizer was inserted into the optical path in front of the iCCD to ensure the polarization state of the transmitted SHG signal. Images were acquired every 10° from 0° to 350° .

Theoretical background and image processing: A model describing SHG in collagen based on cylindrical symmetry, which reduces independent second-order susceptibility tensor elements to four tensors, was used to fit the acquired images [21–25]. In our setup, the laser beam is normally incident on a sample x-z plane. Considering these assumptions and our configuration, the SHG intensity can be written as a function of polarization angle of the incident laser beam:

$$I_{SHG} = c \cdot \left\{ \left[\sin^2(\theta_e - \theta_o) + \left(\frac{\chi_{zzz}}{\chi_{zzx}} \right) \cos^2(\theta_e - \theta_o) \right]^2 + \left(\frac{\chi_{xzx}}{\chi_{zzx}} \right)^2 \sin^2(2(\theta_e - \theta_o)) \right\} \quad (3)$$

where $\frac{\chi_{zzz}}{\chi_{zzx}}$ and $\frac{\chi_{xzx}}{\chi_{zzx}}$ are second-order susceptibility tensor element ratios, θ_e and θ_o are incident polarization angle and collagen fiber angle, respectively.

SHG images of the 15 samples and 3 controls (PLL, unmodified PLR, and uncoated coverslips) were obtained using the software package provided with the iCCD camera (Solis, Andor). Final images were acquired by averaging at least 15 images for each polarization angle. A portion of the image processing was done using ImageJ software. A small background, likely due to the ambient light noise, was subtracted from all images. After background subtraction, images were filtered using a median noise filter (3×3) to attenuate the salt-and-pepper noise in SHG images. Matlab was used to determine the deposited collagen orientation angle and tensor susceptibilities for every region of interest (ROI) by fitting equation 2 with the Levenberg-Marquardt algorithm

[18]. Images were binned to obtain 4×4 pixel areas (i.e. 4×4 ROIs) and Matlab was used to determine the mean signal intensity per pixel and per ROI. Photon counts below 5 counts per pixel were excluded from analysis, which was found to be below the limit of detection for this setup. The Matlab script also provides the susceptibility tensor element ratios as a heat map image to present the spatial distribution of these parameters and orientation angle maps.

Collagen fiber orientation was analyzed using SHG radar graphs in which SHG intensity was plotted as a function of incident polarization angle. SHG polarimetry profiles can be used for determining degree of organization of the collagen fibers secreted by fibroblast cells on different polymer substrates [26]. Fourier transformation was used for extracting shape characteristics of SHG radar graphs. Fourier space representation of can be expressed as follows:

$$D = \sqrt{\sum_{u=1}^M (A_u^i - A_u^j)^2} \quad (u = 1, 2, \dots, N-1) \quad (4)$$

Where A_u^i and A_u^j are normalized amplitude of the shape i and j . D is defined as normalization amplitude roughness (NAR). For $D = 1$ collagen orientation is uniaxial, while for collagen fibers with $D = 0$ orientation is completely random [27].

Statistical analysis: The statistical significance of the mean comparisons was determined by ANOVA using the JMP Pro statistical software package. Pair-wise comparisons were analyzed with Turkey HSD. Differences were considered statistically significant for $P < 0.05$. Partial least squares (PLS) regression was performed on the material descriptor data set to determine the influence of material properties on collagen orientation. The material

descriptors (Supplementary Table 6) were developed based on descriptors defined by Bicerano [28].

Results

Polymer synthesis and Characterization. A library of 15 modified poly-L-arginines (PLRs) were synthesized through carbodiimide chemistry and were characterized through NMR (Supplementary Figure 1). Figure 1a shows the structures of the amidine derivatives that were covalently attached to PLR. They were determined to be hydrophilic through water contact angle (WCA) measurements (Supplementary Table 1). Some correlations have been found between wettability and protein adsorption strength leading to differences in cell migration and morphology [14,29]. This is to be expected as cells migrate using lamellipodia or filopodia, which are developed by actin polymerization and stabilized by adherence of transmembrane receptors to the extracellular matrix (ECM) *in vivo* [30]. In the presence of biomaterials, cells may not effectively migrate if the substrate is not chemically or physically similar to the ECM and does not allow for binding of transmembrane receptors. Morphology of cells on substrates is also dependent on transmembrane receptors and the ability of cells to rearrange attachment sites. If cells cannot detach from a surface, they will remain spread out and unable to migrate, as is the case on materials with higher surface energy, increased wettability, lower amine content, and increased material stiffness [31,32].

Cell Viability. Materials must be cytocompatible to be useful in tissue engineering and wound healing applications. Cell viabilities were normalized to cells cultured on tissue culture polystyrene (TCPS). Cells were at least 70% viable on the different substrates

(Supplementary Figure 2), indicating that the materials synthesized here are not cytotoxic.

VEGF Secretion. VEGF is a key component in angiogenesis, epithelization, and collagen deposition, which are integral in the wound healing process [33]. VEGF has prominent applications in localized angiogenesis for therapeutic purposes; for example, promoting neovascularization in ischemic regions [34]. Ennet *et al.* reported VEGF concentrations up to 10 ng/mL at the implant site and significant upregulation of local angiogenesis *in vivo* [35]. The VEGF concentration required to initiate normal neovascularization ranges from approximately 5 to 70 ng/10⁶ cells/day [36]. These concentrations were produced by primary myofibroblasts.

VEGF production by fibroblasts in response to modified surfaces and controls were evaluated (Figure 2a). All three controls (PLR, PLL and TCPS) showed similar levels of VEGF expression (~ 0.55 ng/10⁶ cells/day). Surfaces with modifications 4, 6 and 15 significantly increased VEGF secretion compared to all controls (p<0.001). Modification 6 elevated VEGF release ~ 3 fold, compared to controls, while 4 and 15 showed ~ 2 fold increase. Modification 3 upregulated VEGF expression compared to PLR and PC. The remaining modifications did not significantly affect VEGF expression; however, differences were observed when comparing modifications to one another. For example, culturing cells on 1, 2, 5 and 7 led to an increase of VEGF compared to 10, 11, and 13 (p<0.05).

Migration. The ability of cells to attach and migrate through the ECM plays a crucial role in homeostasis, tissue development, and wound healing [29,37]. Cell adhesion receptors link to adhesion ligands, providing traction and signaling for locomotion [38].

Cell migration on solid substrates occurs through amoeboid movement involving a sequence of cell protrusions and retractions using actin filaments [39]. For implanted biomaterials rapid healing is vital for a successful outcome. Increasing cell migration speed can significantly promote the healing process following implantation [32], necessitating an understanding of the way cells interact with synthetic surfaces when designing materials for wound healing and tissue engineering applications [40]. We examined the effect of the PLRs on fibroblast migration behavior. The persistence of directionality and cell migration speed results are shown in Figure 2b. Modifications 3, 10, and 13 significantly increased directionality of cell migration compared to TCPS, and maximum migration rates were found for TCPS coated with modification 3. Fibroblasts also migrated faster on modifications 8 and 5. Modifications 4, 7, 9, 12, 14, 15, and PLL only slightly affected migration rate, while decreasing persistence. Modification 2 inhibited the migration of fibroblasts without any change in persistence compared to TCPS. The degree of migration of cells on other modifications 1, 6, 11, 13, and PLR was approximately the same, with 13 having higher persistence of directionality. Overall, the persistence varied from ~ 15 to 120 minutes and the speed ranged from 0.11 to 0.23 $\mu\text{m}/\text{minute}$. Cells cultured on TCPS were in the middle of these regions with a P of 60 minutes and an S of 0.18 $\mu\text{m}/\text{minute}$. This compares well with other literature values of ~ 0.2 $\mu\text{m}/\text{minute}$ for NIH/3T3 cells [41], ~ 0.17 $\mu\text{m}/\text{minute}$ for primary fibroblasts [42], and directional persistence of ~ 40 minutes for secondary fibroblasts [43].

Cell Morphology. Most cell types require adhesion to a surface for viability and growth [37]. Thus, adhesion typically precedes cell spreading, migration, and ultimately cell function [14]. Cell morphology is thought to impact biomaterial integration and wound

healing, as normal cell morphology is typically indicative of proper cell adhesion leading to cell function and biocompatibility [29]. For this reason, cell morphology was characterized in response to PLR substrates. A spectrum of cell morphologies that qualitatively fit into three categories can be seen in Figure 2c. Cells cultured on modifications 1, 6, 13, 14, 15, and PLL, were similar to the cells cultured on glass coverslips, mostly rounded with very little cytoplasm was visible in the images. Very few elongated cells were observed on these surfaces. Cells cultured on a second group of modifications, 8, 11, 5, 9, 3, and 4 exhibited an elongated and linear morphology. When cultured on modifications 2, 7, 10, 12, and PLR, the majority of cells were spread out in an unrestricted manner, adopting more triangular or rectangular shapes. Quantitative analysis of the cell morphology is shown in Supplementary Table S2, where a form factor (FF) value of 1 indicates a perfectly circular object and 0 corresponds to linear or star shaped morphologies. These values were used to arrange the images and histograms in Figure 2c and d in a spectrum based on their shape beginning with the most circular. Histograms (Figure 2d) illustrate the percent of cells portraying each FF value. Based on the mode of the form factor, modifications 2, 4, 7, 10, 12, and PLR showed a large decrease when compared to the glass coverslip indicating that the cells cultured on these samples were more spread out, not circular, with a larger perimeter. There was only a moderate shift in this value from the control for modifications 3, 5, 6, 8, 9, 11, and 15, which had form factors between 0.07 and 0.09. Lastly, 1, 13, 14, and PLL produced values very similar to that of the control.

Second Harmonic Generation (SHG). Self-assembly of collagen plays an essential role in tissue repair and growth [26], making the orientation angle of deposited collagen a key

parameter in determining the *in vivo* fate of tissue engineered constructs. It has been shown that the amount of collagen produced by fibroblasts depends on surface chemistry of the material *in vitro* [44]. Collagen deposited on biomaterial surfaces and its orientation can dictate the successful outcome of implanted devices. A thin and well-oriented collagen capsule around the implant has low vascular density and can hinder the function of encapsulated cells for tissue engineering applications or artificial organs [45]. SHG is a nonlinear optical technique in which isotropic or centrosymmetric molecules do not generate signal, meaning that cell culture media will not interfere with the images. This technique is perfectly suited to studying collagen, which has a triple helical structure, rendering it non-centrosymmetric. Furthermore, since SHG is a second-order phenomenon, orientation angles of molecules can be measured [22].

Representative SHG images of collagen deposited by fibroblasts *in vitro* on the modified PLR surfaces can be seen in Figure 3a. Polarimetry profiles of collagen orientation angles are shown in Figure 3b. These plots represent the integrated intensity of images taken every 10° from 0 to 360° . A shape descriptor, D , was used to quantify the shape of the polarimetry profile and is shown in Figure 3c. D ranges from 0 being perfectly circular, or an isotropic collagen distribution, to 1 being aligned collagen. Modifications 2, 3, 4, 7, 12, 14, and PLR exhibit oval polarimetry plots ($0.17 < D < 0.22$). Modifications 1, 10, and 11 show slightly higher order in collagen orientation, with the D values very closely resembling the cells cultured on glass coverslips (Figure 3c ($0.27 < D < 0.32$)). Modification 6 had extremely round polarimetry profiles with the lowest D values among all samples (0.17 ± 0.01). On the other hand, modifications 5, 8, and 13 showed the highest degree of collagen orientation with D values of 0.30 , 0.36 and

0.33, respectively. In examining the profiles in Figure 3b, modification 5 appears to have four-fold symmetry. Several other materials appear to be oriented at 135° (8 and 10).

In vivo characterization of the orientation of the collagen adjacent to the materials 28 days after implantation was also performed. SKH1-E, an immunocompetent wound healing mouse model was injected with 105-150 μm glass beads coated with PLR modified with the molecules in Figure 1a. Controls of unmodified PLR, PLL, and uncoated particles were also injected. Animals were sacrificed at 28 days after material implantation, and the tissue surrounding the implant was excised, fixed, and either stained with Masson's trichrome or imaged with SHG. Representative images and polarimetry profiles are shown in Figure 4a and b. Modifications 3, 6, 7, 10, 14, and PLL have collagen orientation similar to that of the unaffected skin (0.28 ± 0.07). Modifications 4, 5, 9, 11, and 12 elicit higher collagen orientation compared to the unaffected skin section but similar to the uncoated glass beads (0.37 ± 0.02). From the polarimetry profiles it can be noticed that modification 13 has the highest collagen orientation degree (0.52 ± 0.07) and was significantly higher compared to the controls ($p < 0.05$). All D values for *in vivo* data are given in Figure 4c.

Standard histological evaluation around the implants was used to evaluate the tissue response after implantation. Representative Masson's trichrome images are shown in Figures 4d and e, with the remaining images in Supplementary Figure 3. The implanted beads were dislodged during the processing, however, collagen fibrils can be seen surrounding voids that remain from the implanted materials. New blood vessels are observed in Figure 4e, which is modification 14.

Very little correlation was observed between the *in vitro* and *in vivo* results ($R^2=0.304$). However, modifications 2, 3, 6, and 7 exhibit a low degree of collagen orientation for both *in vitro* and *in vivo* studies. The same trend was noticed for modification 5, which has four-fold symmetry and high D values for both cases. It was also determined that the orientation of collagen gels deposited on modified PLR surfaces have no dependence on surface chemistry (Supplementary Figure 4). Taken together this illustrates the ability for polymers to control the orientation of deposited collagen, a result that has never previously been seen.

Discussion

The impact of different physiochemical properties on cell adhesion, migration, and proliferation is of great interest for biomedical applications. As mentioned above, many different parameters are important in engineering fibroblast function for wound healing and medical device implantation, such as the orientation of collagen deposition and VEGF production. Thin and well oriented collagen is associated with scar tissue and can hinder proper function of implanted devices due to low vascular density [8,26]. This is also why angiogenesis is instrumental in a successful wound healing or incorporation process [33]. Discussed below are some of the ways hydrophobicity and chemical structure impact each of these factors, as well as how these cellular functions correlate with one another.

As previously mentioned, the attachment of cells to a surface is necessary for proliferation and functionality [37]. In the body, this surface is the ECM. In cell culture, almost any surface could provide the mechanical stability necessary for cellular attachment, ergo differences in cell behavior in response to materials must be due to the

physiochemical properties [37]. A plethora of work has examined how chemical groups and physical properties of substrates affect cell behavior [1,31,46–48]. Some work has shown hydrophobicity to be important in cell proliferation rate, adhesion, and collagen synthesis, with peaks in all three around WCAs of 70° [14,49]. In this work, hydrophobicity appears to impact cell morphology similarly. FF, a measure of morphology also indicates adhesion [46], where lower values correspond to spread out morphologies that are better adhered to the surfaces. It was found that FF reached a minimum at WCAs between 34° and 48°, and again for more hydrophobic surfaces. There was low adhesion for some WCAs between 41° and 53° (Supplementary Figure 5). Taken together, this means that very different cell adhesion can result from materials with similar WCAs. Hydrophilicity is not the only factor influencing cell adhesion since polymers with similar WCAs exhibited similar cell morphologies. Other studies have found that positively charged functional groups contribute to better cell attachment [46].

During the final stage of the foreign body response to implanted biomaterials collagen is deposited to form a fibrous capsule with unidirectional collagen fibers [45,50]. Our results demonstrate that functional groups are able to affect collagen fiber orientation, as deposited by fibroblasts. Previously, collagen alignment was attributed to collagen molecule interaction with hydrophobic/hydrophilic surfaces [51], to traction forces generated by cell movement [52], or to the orientation of the cell layer [53]. Our results do not show a dependence of collagen orientation on the hydrophobicity of the surface, which might be due to the narrow range of hydrophilicity of our samples. Modifications with further spread out morphologies have a low degree of collagen orientation. For example, PLL and modification 6 exhibited compact cell morphologies with extremely

isotropic collagen orientation angles, while modifications 8 and 5 deposit collagen with the highest degree of order in our experimental set and show moderate cell spreading. This observation may result from initial cell adhesion dictating collagen fiber orientation [53], suggesting that optimal substrate material interactions with the cell can lead to more isotropic collagen orientation.

There does not seem to be a complete trend between the speed or persistence of cells and the form factor or anisotropy of collagen fibers for the various modified PLRs. There are, however, several regions in Figure 2b that mildly correlate with the WCAs. For example, 1, 2, 13, and PLR all show lower WCAs on the volcano plot (Supplemental Figure 5), and have the slowest speeds. In contrast, 3 and 8 are considerably more hydrophobic with greater speeds in comparison to other samples. This suggests that the hydrophobicity of a material does have a specific impact on the migration of cells, but not one that can be easily identifiable trend based on chemical moieties.

Another parameter that can be controlled by substrate-cell interactions is VEGF secretion. A range between 0.637 ng/10⁶ cells/day and 3.25 ng/10⁶ cells/day was found. Using materials to control VEGF secretion, rather than or in addition to controlled release, remains a largely unexplored avenue. This work clearly demonstrates the ability of material interactions with fibroblasts to induce such an effect. Interestingly, modifications 15 and 6 secrete 2 and 3 times the amount of VEGF compared to the control sample and induce very isotropic collagen fiber orientations.

Statistical analysis was performed on *in vivo* collagen D values in order determine the presence of any existing correlations with *in vitro* results or other material parameters. Correlation coefficients and material parameters measured are given in Supplementary

Figure 6 and Table 6, respectively. There are several correlations with the *in vivo* D values that are made apparent with the results of this analysis. The *in vivo* D values have relatively strong correlations with VEGF secretion ($R = -0.451$), FF values ($R = 0.420$), and *in vitro* D values ($R = 0.541$). As for material parameters, both number of carbons and N_{PS} have positive correlations ($R = 0.459$ and 0.436 , respectively) with *in vivo* D values. These correlations are not surprising as the number of carbon atoms, the number of sp^3 carbon atoms, and the number of carbonyl atoms are all important in determining the hydrophobicity of the material. As previously discussed, the hydrophobicity of materials plays an important role in cell adhesion leading to varied collagen deposition.

The data presented here shows important progress in the ability to tune cellular responses through biomaterials. This feature was exemplified through the large variation of VEGF secretion, collagen fiber orientation, cell migration, and cell morphology. These findings demonstrate extremely promising avenues for future work in tissue engineering and wound healing. The limited library of materials used here illustrates the need for additional studies in structure/function relationships to build predictive models for rational biomaterial design.

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Author Contributions

H.C.B., D.A., and K.M.B. designed the study. H.C.B., D.A., K.D.F. and A.M. performed experiments. H.C.B., D.A., and K.D.F. analyzed data. H.C.B., D.A., and K.M.B wrote the manuscript.

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Figure 1

Figure 2

Figure 3

Figure 4

Figure Legend

Figure 1. Synthesis of a new library of poly-L-arginine (PLR) derivatives. Chemical structures of molecules used to modify PLR. The amidine derivatives shown here are numbered for easier identification in experiments and discussion throughout the paper.

Figure 2. The affect of PLR substrates on persistence time and speed, VEGF secretion, and morphology of NIH/3T3 cells. (a) VEGF expressions are normalized to the number of viable cells. Error bars represent the 95% confidence interval based on the standard deviation. (*) indicates $p < 0.05$ compared with PC by pairwise comparison and (**) $p < 0.05$ compared with other samples by pairwise comparison. (b) Mean speeds and persistence times are reported for cells ($n \geq 50$) migrating on PLR modified substrates. Error bars represent the 95% confidence interval based on the standard error of the mean. (c) Representative images of fibroblasts morphology in response to different surfaces of modified PLRs. Images are arranged to show the spectrum of FF values and morphology found. Green is α -tubulin rabbit mAb Alexa Fluor[®] 488 Conjugate and blue is DAPI. The scale bar represents 100 μ m. (d) Quantitative measurements of fibroblast morphology cultured on PLR substrates. Histograms of FF values for each sample (red) plotted with that of cells cultured on a glass coverslip (black). The y-axis represents the percent of cells ($n > 750$) on each sample that were determined to have each FF value.

Figure 3. SHG emanates from collagen secreted by fibroblasts cultured on modified PLR substrates, which cause fibroblasts to secrete collagen fibers with different orientation angles. (a) Representative SHG images of collagen secreted by fibroblast

samples on different surfaces of modified PLRs. SHG intensity is expressed in blue pseudocolor. The scale bar represents 25 μ m. **(b)** Representative SHG polarimetry profiles for fibroblast cells on different modified PLR surfaces. Y scale corresponds to SHG intensity in arbitrary units and the azimuthal angles correspond to polarization angles. Arrows are a guide to the eye for the major axes of the collagen fiber orientation. **(c)** Shape descriptor values for collagen secreted by fibroblasts on modified PLR surfaces. Error bars represent standard deviation.

Figure 4. SHG emanates from collagen secreted *in vivo* by cells adjacent to the implant sites containing modified PLR substrates coated on glass beads. (a)

Representative SHG images of collagen secreted *in vivo* near the surfaces of glass beads with modified PLR coatings. SHG intensity is expressed in blue pseudocolor. The scale bar represents 25 μ m. **(b)** Representative SHG polarimetry profiles of collagen secretion at modified PLR glass bead implant sites. Y scale corresponds to SHG intensity in arbitrary units and the azimuthal angles correspond to polarization angles. Arrows are a guide to the eye for the major axes of the collagen fiber orientation. **(c)** Shape descriptor values for collagen secreted adjacent to implant sites for modified PLR coated glass beads. Error bars represent standard deviation. **(d)** and **(e)** representative Masson's trichrome staining images of tissue sections containing implanted glass beads coated with modified PLR 1 and 14, respectively. Arrowhead indicates the presence of blood vessels. The scale bar represents 150 μ m.