Endothelial Cell Response to Chemical, Biological, and Physical Cues in Bioactive Hydrogels

Mary Beth Browning, PhD,¹ Viviana Guiza, PhD,² Brooke Russell, PhD,³ Jose Rivera, PhD,³ Stacy Cereceres, BS,¹ Magnus Höök, PhD,³ Mariah S. Hahn, PhD,² and Elizabeth M. Cosgriff-Hernandez, PhD¹

The highly tunable biological, chemical, and physical properties of bioactive hydrogels enable their use in an array of tissue engineering and drug delivery applications. Systematic modulation of these properties can be used to elucidate key cell–material interactions to improve therapeutic effects. For example, the rate and extent of endothelialization are critical to the long-term success of many blood-contacting devices. To this end, we have developed a bioactive hydrogel that could be used as coating on cardiovascular devices to enhance endothelial cell (EC) adhesion and migration. The current work investigates the relative impact of hydrogel variables on key endothelialization processes. The bioactive hydrogel is based on poly(ethylene glycol) (PEG) and a streptococcal collagen-like (Scl2-2) protein that has been modified with integrin $\alpha_1\beta_1$ and $\alpha_2\beta_1$ binding sites. The use of PEG hydrogels allows for incorporation of specific bioactive cues and independent manipulation of scaffold properties. The selective integrin binding of Scl2-2 was compared to more traditional collagen-modified PEG hydrogels to determine the effect of integrin binding on cell behavior. Protein functionalization density, protein concentration, and substrate modulus were independently tuned with both Scl2-2 and collagen to determine the effect of each variable on EC adhesion, spreading, and migration. The findings here demonstrate that increasing substrate modulus, decreasing functionalization density, and increasing protein concentration can be utilized to increase EC adhesion and migration. Additionally, PEG-Scl2-2 hydrogels had higher migration speeds and proliferation over 1 week compared with PEG-collagen gels, demonstrating that selective integrin binding can be used to enhance cell–material interactions. Overall, these studies contribute to the understanding of the effects of matrix cues on EC interactions and demonstrate the strong potential of PEG-Scl2-2 hydrogels to promote endothelialization of blood-contacting devices.

Introduction

BIOACTIVE HYDROGELS FABRICATED from natural and/or synthetic polymers are widely employed in regenerative medicine and drug delivery applications due to their tunable soft tissue-like properties and established biocompatibility. The ability to determine the effects of scaffold variables on cell–material interactions in a controlled manner is valuable in the development of improved bioactive scaffolds. The exceptional control that poly(ethylene glycol) (PEG)-based hydrogels provide over chemical, mechanical, and biological properties has facilitated their widespread use as biomaterials.^{1–4} Specifically, PEG hydrogel swelling ratio and modulus can be tuned over a wide range with facile alterations in molecular weight, concentration, and/or functionality.⁵ Furthermore, PEG hydrogels are intrinsically resistant to protein adsorption and cell adhesion.⁶ This property

provides scaffolds with an attenuated host response and controlled bioactivity through conjugation of specific bioactive agents, including proteins and peptides.^{7–10}

Streptococcal collagen-like (Scl2-1) proteins are a biomaterial platform with many advantages over traditional collagen- and extracellular matrix (ECM) peptide-based systems. These proteins, derived from group A streptococcus, contain the Gly-Xaa-Yaa motifs of native collagen, (where Xaa typically represents Pro, and Yaa typically represents Hyp [mammalian collagens] or any charged amino acid [bacterial collagens]), but lack hydroxyproline. This results in a stable triple helix without the need for posttranslational modification.^{11,12} Thus, recombinant expression in *Escherichia coli* can be utilized with Scl2-1 proteins, which reduces the batch variability seen with animal-derived collagen and decreases the cost associated with solid-phase synthesis of extracellular matrix peptides.¹³ One

¹Department of Biomedical Engineering, Texas A&M University, College Station, Texas.

²Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, New York.

³Institute of Biosciences and Technology, Texas A&M Health Science Center, Houston, Texas.

of the most valuable properties of Scl2-1 is that it serves as a biological blank slate resistant to cell adhesion and platelet aggregation.^{7,14} Scl2-1 can therefore serve as a template for insertion of desired receptor-binding motifs through site-directed mutagenesis to induce specific biological interactions. In the current work, a modified protein, Scl2-2, was utilized, in which the GFPGER sequence recognized by integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ was inserted into Scl2-1. This provides a protein with a complex tertiary structure that allows for endothelial cell (EC)-specific adhesion through a single integrin binding site while maintaining thromboresistance.^{15,16} These properties are particularly desirable in vascular applications, wherein generation of a quiescent EC layer and prevention of platelet adhesion and activation can increase success rates.^{17–19} We have previously developed the methodology to covalently incorporate Scl2 proteins into PEG hydrogel matrices.⁷ The high tunability and control over PEG-Scl2-2 hydrogel properties allow for independent exploration of the effects of both protein and matrix variables on cell–material interactions.

Recently, we have shown that reducing the density of functionalization linkers on proteins covalently incorporated into PEG hydrogels results in increased EC interactions due to reduced steric hindrance around integrin binding sites.²⁰ Furthermore, it is extensively acknowledged in current literature that increasing the bioactive factor concentration provides a means to increase cell adhesion and spreading.^{21–24} Whereas increased adhesion and spreading is generally desirable, cell migration is an additional component that is critical in the successful development of scaffolds for regenerative medicine applications. Previously, it has been shown that cell migration speed increases with adhesion and spreading to a maximum, and then begins to decrease as the adhesion strength overcomes the ability to migrate.^{25,26} PEG-Scl2-2 hydrogels provide a model matrix that can be utilized to independently determine the effects of functionalization density and protein concentration on EC adhesion, spreading, and migration. Additionally, collagen can be incorporated into PEG hydrogels using the same techniques as Scl2-2. This allows for investigation of the effects of protein type on EC binding affinity and the associated effects on adhesion, spreading, and migration. The information gained from these studies can then be translated into other bioactive hydrogel systems.

One of the most widely utilized properties of PEG hydrogels is the ability to alter their swelling and mechanical properties over wide ranges with simple changes in molecular weight or concentration.^{27–29} Multiple reports have demonstrated that mechanical stimuli and substrate modulus affect cell–material interactions with various cell types.^{26,30–38} Previous work with ECs, in particular, has demonstrated that shear stresses on their apical side caused by hemodynamic forces affect cell function and structure and induce changes throughout the cell interior.^{39,40} Additional studies have focused on evaluating the interactions between the extracellular matrix and the basal EC side for promotion of angiogenesis. In general, stiffer collagen and fibrin gels reduce vascular network formation.^{41–43} However, ECs have been found to have increased spreading on stiffer gels due to the ability of the cells to generate traction forces on their matrix.⁴⁴ Previous studies were primarily performed using natural polymer hydrogels, wherein the substrate modulus is

typically altered by changing the overall protein concentration. More recent work has utilized poly(acrylamide)-collagen hydrogels to independently determine the effects of matrix variables on EC spreading and microtubule formation.^{40,45} These studies utilized relatively soft (<10 kPa modulus) gels with a focus on promoting angiogenesis. Native vessels have reported modulus values of at least 170 kPa.⁴⁶ Little information is available on the effects of synthetic hydrogel matrix variables on EC interactions in this stiffness range for use in artificial vessels and other cardiovascular devices. PEG-Scl2-2 hydrogels provide a thromboresistant scaffold that has suitable stiffness for these types of applications.¹⁴ Thus, the substrate modulus can be varied independently of bioactive factor concentrations in these scaffolds to evaluate its effects on EC adhesion and spreading in higher stiffness ranges.

In these studies, EC adhesion, proliferation, spreading, and migration on bioactive PEG hydrogels were assessed in response to protein type (collagen vs. Scl2-2), protein concentration (1, 4, or 8 mg/mL), protein functionalization density (1×, 0.5×, or 0.1×), and substrate modulus (110 kPa with 10% PEG [10 kDa] hydrogels vs. 270 kPa with 10% PEG [3.4 kDa] hydrogels) (Table 1). Based on previous research, we hypothesized that EC interactions would be increased with increased concentration, decreased functionalization density, and increased substrate modulus. Additionally, it was hypothesized that PEG-collagen hydrogels would support enhanced cell adhesion and spreading and reduced cell migration based upon its increased number of integrin binding sites for ECs and our previous research.^{14,20} These studies include the first assessment of EC proliferation on PEG-Scl2-2 hydrogels and the first measurement of the effects of higher substrate

TABLE 1. EXPERIMENTAL MATRIX OF BIOACTIVE HYDROGEL COMPOSITIONS

	Matrix	Protein concentration	Functionalization density
Effect of ligand presentation on adhesion	PEG (3.4 kDa) DAA	1 mg/mL	1×
			0.5×
			0.1×
		4 mg/mL	1×
			0.5×
			0.1×
		8 mg/mL	1×
			0.5×
			0.1×
Effect of ligand presentation on migration and spreading	PEG (3.4 kDa) DAA	1 mg/mL	1×
		8 mg/mL	0.1×
			1×
			0.1×
Effect of substrate modulus on adhesion	PEG (3.4 kDa) DAA	1 mg/mL	1×
		8 mg/mL	0.1×
			1×
			0.1×
Effect of substrate modulus on migration and spreading	PEG (10 kDa) DAA	1 mg/mL	1×
		8 mg/mL	0.1×
			1×
			0.1×

PEG, poly(ethylene glycol).

moduli on EC interactions. As PEG-Sc12-2 hydrogels offer many benefits over PEG-collagen hydrogels for cardiovascular applications, this provides information about their relative potential to promote rapid endothelialization in cases where a thromboresistant protein is required. Overall, this systematic investigation offers valuable insight into the effects of scaffold variables on EC-material interactions that can be used to improve the rational design of bioactive hydrogels for a range of applications.

Materials and Methods

Materials

All chemicals and materials were purchased from Sigma Aldrich and used as received unless otherwise noted. PEG diamine (3.4 kDa and 10 kDa) was purchased from Jenkem Technology USA.

Synthesis of PEG diacrylamide

PEG diacrylamide (PEGDAA) was prepared, as previously described.⁴⁷ Briefly, acryloyl chloride (4 molar equivalents) was added dropwise to a solution of PEG (3.4 kDa) or (10 kDa) diamine (1 molar equivalent) and TEA (2 molar equivalents) in anhydrous DCM under nitrogen. The reaction was allowed to proceed for 24 h before being washed with a 2 M potassium bicarbonate solution. A sufficient volume of potassium bicarbonate solution was used to achieve a ratio of 8 molar equivalents of potassium bicarbonate per mole of PEG diamine in the reaction. Following drying with anhydrous sodium sulfate, the product was precipitated in cold diethyl ether, filtered, and dried under vacuum.

Successful synthesis of PEGDAA was confirmed with Fourier transform infrared (FTIR) and proton nuclear magnetic resonance (¹H-NMR) spectroscopy. Control and functionalized polymers were solution cast onto KBr pellets, and FTIR spectra were obtained using a Bruker TENSOR 27 spectrometer. The introduction of amide peaks at 1640 and 1675 cm⁻¹ indicated successful acrylamidation of PEG diamine. A mercury 300 MHz spectrometer with a TMS/solvent signal as an internal reference was utilized to obtain ¹H-NMR spectra of control and functionalized polymers. Greater than 90% conversions of amine to acrylamide endgroups were observed for all candidate macromers. ¹H-NMR (CDCl₃): 3.6 ppm (m, -OCH₂CH₂-); 6.5 ppm (s, -CH₂-NH-); 6.4 ppm (m, -CH=CH₂); 5.6 and 6.1 ppm (m, -CH=CH₂).

Synthesis of acrylamide-PEG-isocyanate linker

A hydrolytically stable photocrosslinkable linker was synthesized, as previously described.²⁰ One molar equivalent of PEG (3.4 kDa) diamine dissolved in anhydrous DCM was added dropwise to 2 molar equivalents of hexane diisocyanate under nitrogen to synthesize PEG diisocyanate (PEGDI). After 2 h of stirring, a solution with 1 molar equivalent of aminoethyl acrylamide (Enamine) in dimethyl sulfoxide was added dropwise to the reaction solution while stirring under nitrogen. The reaction was allowed to proceed for 2 h, and then the final product was precipitated in cold diethyl ether, filtered, and dried under vacuum. Successful synthesis of PEGDI and Aam-PEG-I was confirmed with FTIR spectroscopy in a method such as that described above. The introduction of an isocyanate peak at 2250 cm⁻¹ and urea peaks at

1560 and 1650 cm⁻¹ confirmed isocyanate endcapping of PEG diamine, and relative decreases in the isocyanate peak and increases in the urea peaks confirmed successful formation of the Aam-PEG-I linker. The Aam-PEG-I structure was confirmed with ¹H-NMR spectroscopy as described above. ¹H-NMR (CDCl₃): 3.6 ppm (m, -OCH₂CH₂-), 3.3 ppm (m, -NHCH₂CH₂NH-), 1.4 and 3.1 ppm (m, -NH[CH₂]₆N-), 5.1–5.4 and 7.3 ppm (s, -NH-), 6.1 ppm (dd, -CH=CH₂), 5.8 and 6.2 ppm (dd, -CH=CH₂).

Protein functionalization

Sc12-2 was synthesized and purified as previously described.¹² Sc12-2 and a rat tail collagen type I control were functionalized with Aam-PEG-I. Briefly, Aam-PEG-I was dissolved in anhydrous dimethylformamide and added dropwise to protein solutions in phosphate-buffered saline (PBS) at room temperature. The molar ratio of Aam-PEG-I:NH₂ was 0.1:1, 0.5:1, or 1:1 to provide proteins with low (0.1×), medium (0.5×), and high (1×) functionalization densities, respectively. After 2 h of stirring, functionalized proteins were purified using dialysis against reverse osmosis (RO) water for 24 h (MWCO = 20,000 Da). FTIR spectroscopy confirmed functionalization of proteins with varied Aam-PEG-I densities. The presence of ether peaks from the PEG linker at 1110 cm⁻¹ and amide peaks from the proteins at 1560 cm⁻¹ confirmed functionalization, and relative decreases in absorbance peak ratios of the ether of the Aam-PEG-I to the amide of the proteins confirmed variation in functionalization density.

Preparation and characterization of bioactive PEG hydrogels

PEG-Sc12-2 and PEG-collagen hydrogels were prepared by dissolving PEGDAA (10 wt%) and functionalized Sc12-2 or collagen in 20 mM acetic acid. A photoinitiator solution (1 mg Irgacure 2959 per 0.01 mL 70% ethanol) was added at 1 vol% of precursor solution. Solutions were pipetted between 0.5 or 1.5 mm spaced plates or into microcentrifuge tubes and crosslinked by 6-min exposure to long-wave UV light (Intelli Ray Shuttered UV Flood Light; Integrated Dispensing Solutions, Inc., 365 nm, 4 mW/cm²). Full details of the compositional variables used to generate hydrogel specimens are included in Table 1.

To measure the compressive modulus, six 8-mm-diameter discs were punched from hydrogel sheets (1.5 mm thick) and swollen in RO water overnight. Samples were subjected to mechanical testing under unconstrained compression at room temperature using a dynamic mechanical analyzer (RSAIII; TA Instruments) equipped with a parallel plate compression clamp. The linear viscoelastic range for each hydrogel formulation was determined using dynamic strain sweeps. A strain within the upper region of the linear viscoelastic range was used in a constant strain frequency sweep from 0.79 and 79 Hz. The compressive storage modulus was taken at 1.25 Hz.

For swelling assessments, six 8-mm-diameter discs were punched from hydrogel sheets (1.5 mm thick) directly after polymerization. The hydrogel discs were swollen in RO water overnight and weighed to determine the equilibrium swelling mass (*W_s*). Then, samples were dried under vacuum overnight and weighed to assess dry (polymer) mass

(W_d). The equilibrium volumetric swelling ratio, Q , was calculated from the equilibrium mass swelling ratio:

$$Q = \frac{W_s}{W_d} \quad [1]$$

Initial protein incorporation

PEGDAA-collagen (1, 4, or 8 mg protein/mL; $1 \times$, $0.5 \times$, or $0.1 \times$ functionalization density; 10% PEG [3.4 or 10 kDa] DAA) and PEGDAA-Sc12-2 gels (4 mg protein/mL; $1 \times$, $0.5 \times$, or $0.1 \times$ functionalization density; 10% PEG [3.4 kDa] DAA) were crosslinked directly into microcentrifuge tubes and swelled in PBS at 37°C for 24 h. The swelling solutions were then removed from the tubes, frozen at -80°C , and lyophilized to obtain the gel leachables. The lyophilized powders were dissolved in a known volume of PBS, and protein concentration of the resulting solutions were determined with a 3-(4-carboxy-benzoyl)-2-quinoline-carboxaldehyde (CBQCA) protein quantification kit (Molecular Probes, Life Technologies), as specified by the manufacturer. Briefly, 10 μL aliquots of each sample were diluted in 125 μL of 0.1 M sodium borate buffer. Then, 5 μL of 5 mM potassium cyanide (KCN) was added to each sample, followed by an addition of 10 μL of 5 mM CBQCA (dissolved to 40 mM in DMSO, then diluted to 5 mM in 0.1 M sodium borate buffer). Each sample was tested in triplicate and run against a standard of the same protein type and functionalization density. After 1 to 3 h of incubation in a black 96-well plate at room temperature with shaking, fluorescence was read using an Infinite M200 Pro plate reader (Tecan Group, Inc.; emission = 550 nm and excitation = 465 nm). The concentration of lost protein (C_L , mg/mL) in the hydrolyzed gels was determined by inserting the measured fluorescence into a best-fit linear equation for corresponding standard curves. Protein retention at 24 h (PR, mg/mL) was then calculated as follows:

$$PR = \frac{C_0 \times V_0 - C_L \times V_{\text{PBS}}}{V_0} \quad [2]$$

where C_0 and V_0 are the original protein concentration (mg/mL) and gel volume (mL), respectively, and V_{PBS} is the volume of PBS used to dissolve the lyophilized leachables (mL). Initial protein incorporation (I) was measured from PR as follows:

$$I = \frac{PR}{C_0} \times 100\% \quad [3]$$

EC adhesion and spreading

Bovine aortic endothelial cells (BAOECs; Cell Applications, Inc.) were used for all cell studies. *In vitro* culture was carried out at 37°C/5% CO_2 with Dulbecco's modified Eagle's medium (DMEM, high glucose GlutaMAX™; Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin solution (Gibco). Cells were used between passages 2 and 5 after 7–10 days of culture for all studies.

Bioactive hydrogels were crosslinked between 0.5 mm spaced plates, as described above, and swelled for 3 h in 70% ethanol to sterilize. Subsequently, gels were taken through an ethanol swelling ramp (15 min each in 50%, 25%, and 0% ethanol in water) to remove residual ethanol. Then, 6 mm diameter punches were taken from each hydrogel formulation and swelled in PBS for 1–3 h to ensure full hydration. BAOECs were seeded onto the surface of specimens at 10,000 cells cm^{-2} and cultured in 2D at 37°C/5% CO_2 for 3 h, 72 h, or 1 week. The medium was changed at 3 h and then every 48 h. Following the desired culture time, cells were fixed with 3.7% glutaraldehyde and stained with rhodamine phalloidin (F-actin/cytoplasm; Invitrogen) and SYBRGreen (DNA/nucleus; Invitrogen). Representative images were obtained with a Nikon Eclipse TE2000-S with three field views per specimen and four specimens per hydrogel formulation. Fluorescent images (three images per specimen, four specimens per sample) of SYBRGreen- and rhodamine phalloidin-stained cells were utilized to quantify cell adhesion, proliferation, and spreading. Cell adhesion was measured by manually counting the number of SYBRGreen-stained cell nuclei in each image. Proliferation was assessed by comparing the adhesion at 7 days to the initial (3 h) adhesion. Average cell spreading or cell area was quantified by applying the Photoshop magic wand tool to the image background and adjusting the tool tolerance so that all extracellular regions were selected. The histogram function was then utilized to evaluate the extracellular pixels (P_{Ex}). The average pixels per cell (A_{cell}) for that image were then quantified as follows:

$$A_{\text{cell}} = \frac{P_T - P_{\text{Ex}}}{N} \quad [4]$$

where P_T represents total image pixels and N is total number of cell nuclei. Pixels were then converted to microns using known objective scaling.

EC migration

To more fully analyze the effects of scaffold variables (protein concentration, protein functionalization density, and substrate modulus) on cell interactions, migration was measured on the surface of selected formulations. PEG-Sc12-2 and PEG-collagen hydrogels were prepared between glass plates separated by 0.5 mm spacers. The resulting hydrogels were sterilized by immersion in 70% ethanol and an ethanol ramp, as described above. After hydrating in PBS, a set of 2.54 cm punches were collected from the swollen gels.

BAOECs were harvested and seeded onto the surface of the hydrogels at 10,000 cells cm^{-2} . Gels were cultured for 24 h at 37°C/5% CO_2 to allow for cell adhesion and equilibrium cell spreading. EC migration on the surface was monitored for 1 h at 5 min intervals in at least six randomly selected locations of each hydrogel formulation using a Zeiss Axiovert microscope fitted with an incubation chamber at 37°C/5% CO_2 . For determination of single-cell migration parameters, only cells that remained in isolation ($> 100 \mu\text{m}$ from other cells) were tracked. The cell centroid position at each 5 min time increment was tracked using ImageJ software. These centroids were then used to calculate

the mean square displacement (MSD, $\langle D^2 \rangle$) for a range of time intervals. In brief,

$$\langle D^2 \rangle = \frac{1}{N} \sum_{i=1}^N d_i^2 \quad [5]$$

where N is the total number of 5 min time increments in the time interval under consideration, and d_i is the square of cell displacement during time increment i .⁴⁸ The speed, S , and direction persistence time (P) were determined by fitting the MSD ($\langle D^2 \rangle$) and the time interval, t , to the persistent random walk equation using the nonlinear least squares regression analysis⁴⁹:

$$\langle D^2 \rangle = 2S^2P \left[t - P \left(1 - e^{-\frac{t}{P}} \right) \right] \quad [6]$$

Statistical analysis

All modulus, swelling, and protein retention data were expressed as the mean $\pm$ standard deviation of the mean. Statistical analysis was performed by an unpaired two-tailed Student's t -test. All cell adhesion, spreading, and migration data were expressed as the mean $\pm$ standard error of the mean. ANOVA was used to determine statistical significance in cell studies. Tukey's *post hoc* test was applied for independent variables with greater than two levels. All of the calculated p -values for the effects of combined variables on cell adhesion can be found in Supplementary Tables S1 and S2 (Supplementary Data are available online at www.liebertpub.com/tea). Statistical significance was accepted at $p < 0.05$ for EC adhesion and spreading and at $p < 0.1$ for EC migration.⁴⁸

Results

Effects of PEG molecular weight, protein functionalization density, and protein concentration on initial protein incorporation

Loss of protein from hydrogels after swelling for 24 h was used to estimate the initial protein incorporation as a function of PEGDAA molecular weight, protein functionalization density, and protein concentration. We have previously reported that decreased protein functionalization can result in a 10–30% reduction in protein incorporation.²⁰ In this study, protein retention varied from 53% for $0.1 \times$ collagen gels to 59% for $1 \times$ collagen gels (Table 2). Similar results were observed in Scl2-2 gels with protein incorporation ranging from 41% to 60% as functionalization density was varied. Percent protein incorporation also decreased with increased protein concentration (80% for 1 mg/mL to 49% for 8 mg/mL) and increased PEGDAA molecular weight (49% for 3.4 kDa to 22% for 10 kDa).

Effects of protein type, functionalization density, and concentration on EC adhesion and proliferation

BAOEC adhesion and spreading on PEG-Scl2-2 and PEG-collagen gels at 3 h, 72 h, and 1 week were analyzed as a function of protein concentration (1, 4, and 8 mg protein mL⁻¹ hydrogel solution) with three different functionalization densities for each protein ($1 \times$, $0.5 \times$, and $0.1 \times$) (Table 1). In general, increases in protein concentration resulted in increases in EC adhesion at 3 h for all functionalization densities of both protein types, and lower func-

TABLE 2. INITIAL PROTEIN INCORPORATION OF AAM-PEG-I-FUNCTIONALIZED COLLAGEN IN PEGDAA HYDROGELS

	Sample	Initial incorporation (%)	Concentration
Effect of concentration	1 mg/mL	80 $\pm$ 1	0.80 $\pm$ 0.02
	4 mg/mL	66 $\pm$ 1	2.65 $\pm$ 0.09
	8 mg/mL	49 $\pm$ 1	3.92 $\pm$ 0.04
Collagen $0.1 \times$ PEG (3.4 kDa) DAA			
Effect of functionalization	$1 \times$	59 $\pm$ 1	2.36 $\pm$ 0.04
	$0.5 \times$	56 $\pm$ 4	2.24 $\pm$ 0.16
	$0.1 \times$	53 $\pm$ 2	2.12 $\pm$ 0.08
4 mg/mL collagen PEG (3.4 kDa) DAA			
Effect of PEGDAA MW	3.4 kDa	49 $\pm$ 1	3.92 $\pm$ 0.04
	10 kDa	22 $\pm$ 1	1.78 $\pm$ 0.18
Collagen $0.1 \times$ 8 mg/mL			

PEGDAA, PEG diacrylamide.

tionalization densities ($0.1 \times$) supported greater BAOEC adhesion than higher functionalization densities ($1 \times$) (Fig. 1). All protein variables (type, concentration, and functionalization density) had a significant effect on adhesion ($p < 0.001$). The observed concentration effects were expected based on the multitude of reports in literature demonstrating that increasing bioactive factor concentration can be used to increase cell interactions.^{21–23} Additionally, the effects of functionalization density correlate with our previous results and were attributed to reduced steric hindrance around the integrin ligand.²⁰

PEG-Scl2-2 hydrogels exhibited BAOEC proliferation throughout the study to provide significant increases in EC adhesion between 3 h and 1 week for all samples except for the 1 and 4 mg/mL samples at $0.1 \times$ functionalization density. It was found that time had a significant effect on adhesion to PEG-Scl2-2 hydrogels for all three time points ($p < 0.001$). Although PEG-collagen hydrogels had greater initial adhesion than corollary Scl2-2 samples, there were no significant increases in EC adhesion on PEG-collagen gels between 3 h and 1 week, and *post hoc* statistical analysis revealed a significant decrease in adhesion over this time frame ($p = 0.002$). As a result, greater BAOEC adhesion was observed on PEG-Scl2-2 gels at 1 week than on PEG-collagen gels, especially at higher protein concentrations.

Effects of protein type, functionalization density, and concentration on EC migration and spreading

BAOEC migration on PEG-Scl2-2 and PEG-collagen hydrogels with high and low functionalization densities ($1 \times$ and $0.1 \times$, 8 mg/mL) and protein concentrations (1 and 8 mg/mL, $0.1 \times$) was tracked over 1 h to compare migration on collagen and Scl2-2 gels and to determine the ability to tune migration speeds by altering the number and availability of integrin binding sites. It was found that BAOEC migration rates on the hydrogels were affected by protein type, concentration, and functionalization density (Fig. 2A). Specifically, increasing the concentration of Scl2-2 from 1 to 8 mg/mL, while maintaining a constant functionalization density

FIG. 1. Effects of protein concentration, functionalization density, and type on BAOEC adhesion over 1 week. ANOVA analysis revealed significant effects of all protein variables (concentration, functionalization density, and type) and time on adhesion levels ($p < 0.001$). Tukey's *post hoc* test was applied for independent variables with greater than two levels with significance noted by the following: * $p < 0.001$ relative to all other time points for given protein type; ** $p < 0.003$ relative to the 3-h time point for given protein type; † $p < 0.001$ relative to all other concentrations of given protein type; • $p < 0.001$ relative to all other functionalization densities of given protein type. $n = 4$ samples, 3 images per sample for a total of 12 images; measurements are expressed as mean $\pm$ standard error. BAOECs, bovine aortic endothelial cells. Color images available online at www.liebertpub.com/tea

(0.1 $\times$), resulted in an 80% increase in migration speed. Similarly, a reduction in Scl2-2 functionalization density with maintained protein concentration at 8 mg/mL provided a 60% increase in migration speed. Corresponding alterations in collagen concentration and functionalization density did not significantly affect migration rates. In comparing the two proteins, a 70% increase in migration rate on the PEG-Scl2-2 gel was observed relative to the PEG-collagen gel at 0.1 $\times$ functionalization density and 8 mg/mL.

BAOEC spreading at 3 h on PEG-collagen and PEG-Scl2-2 hydrogels was assessed in response to functionalization density and concentration (Fig. 2B). Both variables had a significant effect on spreading ($p < 0.004$). Furthermore, the combined effect of changing both protein type and either functionalization density or concentration had a significant effect on BAOEC spreading ($p < 0.04$). Decreasing Scl2-2 functionalization density from 1 $\times$ to 0.1 $\times$, while maintaining concentration at 8 mg/mL, resulted in a 44% increase in EC spreading. Correspondingly, increasing the concen-

tration of 0.1 $\times$ -functionalized Scl2-2 from 1 to 8 mg/mL provided a 61% increase in spreading. For the PEG-collagen formulations, functionalization density and concentration did not have any significant effects on spreading. The two collagen functionalization densities tested provided spreading measurements with only 1% difference, and for the collagen concentration increase, a corresponding 22% increase in spreading was measured.

Effects of substrate modulus on EC adhesion

To assess the effects of substrate modulus on EC interactions with bioactive PEG hydrogels, collagen and Scl2-2 were incorporated into gels fabricated with varied PEG molecular weights. Two formulations at the high and low ends of the adhesion data were chosen, and protein concentration (1 or 8 mg/mL) and functionalization density (1 $\times$ or 0.1 $\times$, respectively) were kept constant, while the molecular weight of the PEGDAA matrix was varied (3.4 or 10 kDa) to generate

FIG. 2. Effects of protein concentration and functionalization density on BAOEC (A) migration and (B) 3-h spreading on PEG-Scl2-2 and PEG-collagen gels. (C) Representative images of BAOECs adhered to samples at 3 h. Scale bar applied to all images. (A) $n=3$ samples, 9 images per sample for a total of 27 images; (B) $n=3$ samples, 3 images per sample for a total of 9 images; measurements are expressed as mean $\pm$ standard error; * $p<0.1$ relative to all other samples; † $p<0.05$ relative to Scl2-2 1X, 8 mg/mL sample; ‡ $p<0.05$ relative to collagen and Scl2-2 0.1X, 1 mg/mL samples. PEG, poly(ethylene glycol). Scl2-2, Streptococcal collagen like. Color images available online at www.liebertpub.com/tea

different modulus hydrogels (Table 1). Increasing the PEG molecular weight from 3.4 to 10 kDa resulted in a modulus reduction and swelling ratio increase of $\sim 60\%$ (Fig. 3). BAOEC adhesion was measured at 3 h to determine the initial effect of substrate modulus (Fig. 4). It was found that altering the PEG molecular weight had a significant effect on adhesion ($p=0.000$). Additionally, the combined effects of changing PEG molecular weight, with either protein type (collagen to Scl2-2) or protein formulation (1X, 1 mg/mL to 0.1X, 8 mg/mL), significantly altered adhesion levels ($p<0.036$). Significant increases in BAOEC adhesion were observed with increased substrate modulus for all formula-

tions tested except for the collagen 1X, 1 mg/mL sample. The higher modulus (270 kPa vs. 110 kPa) Scl2-2 gels displayed larger percent increases with fourfold increases in cell numbers for both formulations compared with the twofold increase in cell numbers observed on the higher modulus collagen gels (0.1X, 8 mg/mL).

Effects of substrate modulus on EC migration and spreading

BAOEC migration on PEG-collagen and PEG-Scl2-2 hydrogels with varied PEG molecular weight was analyzed

FIG. 3. Effects of PEG molecular weight on compressive modulus and volumetric swelling ratio. $n=6$; measurements are expressed as mean $\pm$ standard deviation; * $p<0.05$ relative to 10% 10 kDa sample. Color images available online at www.liebertpub.com/tea

to determine the effects of substrate modulus (Fig. 5A). There was a 213% increase in migration on PEG-Scl2-2 gels with a higher modulus, while a much smaller, insignificant change in migration was measured on PEG-collagen gels (20% increase). Thus, the increased modulus enhanced migration speeds on PEG-Scl2-2 gels with minimal effects on PEG-collagen gels. Spreading as a function of substrate modulus was assessed at 3 h (Fig. 5B). No significant increases in spreading were measured with increased modulus, but both collagen and Scl2-2 had generally increased spreading on the higher modulus 3.4 kDa gels, with larger increases seen on the PEG-Scl2-2 gels (45% increase vs. 10% increase on PEG-collagen). A note should be made on the variations in absolute magnitudes of cell adhesion, spreading, and migration between the different sets of experiments. These are due to the interexperimental variations commonly seen with cell data. The relationships between the data on samples presented here are representative of those that we have seen in previous studies.

Discussion

Effects of protein type, functionalization density, and concentration on EC adhesion and proliferation

The measurement of cell adhesion provides an initial assessment of cell interactions with a given substrate. In general, it is desired to maximize EC adhesion to vascular devices, as this would potentially translate to recruitment of both adjacent and circulating ECs to the device surface. It can be seen in Figure 1 that for the most part, concentration effects on BAOEC adhesion to PEG-Scl2-2 hydrogels were

enhanced over 1 week, while the impact of functionalization density effects was less pronounced. It was hypothesized that this could be due to reduced initial protein incorporation that resulted from reducing functionalization density, as the effects of increased access to integrin binding sites and decreased concentration that occur with altering this variable are conflicting. Although we have previously shown that reduced protein incorporation that results from reducing functionalization density does not affect the initial adhesion levels, it is possible that the concentration differences between high and low functionalization densities have a greater effect over time. Normalizing initial concentrations for each functionalization density could provide enhanced functionalization density effects in the long term that are more similar to the observed short-term effects.

FIG. 4. Effects of substrate modulus on BAOEC adhesion at 3 h. $n=3$ samples, three images per sample for a total of nine images; measurements are expressed as mean $\pm$ standard error; * $p<0.05$ relative to corollary 10% 10 kDa sample. Color images available online at www.liebertpub.com/tea

FIG. 5. Effects of substrate modulus on BAOEC (A) migration and (B) 3 h spreading. (A) $n=3$ samples, 9 images per sample for a total of 27 images; (B) $n=3$ samples, 3 images per sample for a total of 9 images; measurements are expressed as mean $\pm$ standard error; * $p<0.05$ relative to all samples; † $p<0.05$ relative to Scl2-2 10 kDa sample. Color images available online at www.liebertpub.com/tea

Similar to adhesion measurements, the ability to maximize EC proliferation on a surface indicates the potential for that surface to support rapid endothelialization, as adhered cells will proliferate more quickly to cover the substrate. The results of the 1-week adhesion studies presented here demonstrate for the first time the ability of Scl2-2 to support significant BAOEC proliferation. In addition to higher cell numbers on the Scl2-2 substrates, there were qualitatively larger areas of confluent ECs in contact with PEG-Scl2-2 hydrogels at 1 week than on PEG-collagen hydrogels. We hypothesize that the enhanced proliferation induced by Scl2-2 relative to collagen is due to the directed signaling provided by the single GFPGER integrin binding site. Integrin $\alpha_1\beta_1$ contributes to angiogenesis by regulating EC migration (p38 MAPK activation), tubulogenesis (p38 MAPK and PI3K activation), and proliferation (ERK activation) on collagen substrates.⁵⁰ Additionally, it has been shown that $\alpha_1\beta_1$ is the primary collagen receptor that mediates the Shc-mediated growth pathway to promote cell proliferation.⁵¹ Collagen type I contains at least three different $\alpha_1\beta_1$ binding sites each with their own affinity that can, in turn, regulate pathway activations, and it contains a host of alternate integrin binding sites.^{52,53} Thus, it is possible that the controlled presence of only the GFPGER-based integrin $\alpha_1\beta_1$ binding site in Scl2-2 provides more direct and robust cell signaling to promote EC proliferation, and the multitude of integrin binding sites in collagen results in mixed signaling that produces marginal proliferation effects.

Effects of protein type, functionalization density, and concentration on EC migration and spreading

Cell migration from surrounding tissue onto a bioactive scaffold is a critical factor in a number of regenerative medicine applications. Specifically, vascular graft endothelialization has shown promise as a method to improve long-term patency, and a vascular graft that promotes *in situ* EC migration from the anastomoses could provide improved performance over current clinically-available grafts. Overall, EC migration speeds on PEG-Scl2-2 hydrogels were found to be more susceptible to changes in matrix cues than those on PEG-collagen hydrogels. It has been previously demonstrated that cell adhesion strength increases to a saturation point as ligand density and affinity increases, whereas the cell migration rate responds in a biphasic manner as these variables are increased.²⁶ Specifically, there is a maximum ligand density/affinity, at which the migration speed is increased and beyond which the migration speed is reduced due to an increased adhesion strength.⁵⁴ For applications that are subjected to the shear stresses of blood flow, it is desired to find a balance of cell spreading indicative of good adhesion strength and high migration speeds to promote endothelialization without delamination of the cell layer. This can be preliminarily substantiated using measurements of cell spreading at 3 h on these formulations (Fig. 2B). Namely, there was no significant difference in spreading on any of the collagen gels at 3 h, which correlates with the similar migration rates between these samples. These small changes in spreading would likely indicate similar adhesion strengths and correlate to the lack of changes in migration on PEG-collagen gels.

Conversely, the increases in migration speeds observed on PEG-Scl2-2 gels with decreases in functionalization

density and increases in concentration correlated with significant increases in spreading. Increasing the Scl2-2 concentration increases ligand density, whereas decreasing the functionalization density increases ligand availability through a reduction in steric hindrance around the integrin binding sites. The increased cell affinity that occurs with these changes is shown in both the spreading and migration data. Additionally, it has been previously shown that integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ binding provide an important role in promoting angiogenesis and EC migration.⁵⁵ Thus, it is possible that the controlled presence of selective integrin binding sites partially accounts for the enhanced BAOEC migration on PEG-Scl2-2 gels relative to PEG-collagen gels. These studies demonstrated that Scl2-2 functionalization density and concentration can be used to control EC migration rates, and PEG-Scl2-2 hydrogels have improved EC migration relative to PEG-collagen gels. Elucidation of these interactions provides valuable information for designing a range of bioactive hydrogel systems for tissue engineering and regenerative medicine applications. Future work will assess EC migration in response to these variables under flow to better model the migration of ECs within native vasculature.

Effects of substrate modulus on EC adhesion

The ability to tune PEG hydrogel properties over a wide range with simple alterations in the molecular weight and concentration while maintaining a constant protein concentration enables facile investigation of the effects of scaffold properties on cell behavior. Numerous literature reports demonstrate that increasing substrate modulus increases cell adhesion and spreading with a range of cell types that include epithelial cells and fibroblasts.³³ However, substrate modulus can have the opposite effect on some cell types, including neurites, which have reduced extension with increased modulus, and hepatocytes, which demonstrate reduced adhesion and function as the modulus increases.^{37,38,56,57} While previous studies with very low modulus gels indicate that EC adhesion increases with increased modulus, this has not been confirmed within the modulus range tested here.⁴⁰ The results presented in the current studies provide a correlation for ECs with the more commonly demonstrated cellular trend of increased adhesion with increased stiffness.

It should be noted that although protein concentrations were held constant in these gel formulations, the 10% PEG (10 kDa) displayed a large reduction in protein incorporation that resulted in a decreased protein level as compared to the 3.4 kDa gel (Table 2). This is likely due to the reduced crosslinking efficiency of the lower crosslink density 10 kDa gels. Furthermore, the 10% PEG (10 kDa) gels have higher swelling ratios than the 10% PEG (3.4 kDa) gels. Based on the swelling ratios and known dry component mass, the apparent local protein concentrations in the volume expanded 10% 10 kDa gels at their equilibrium swelling states would be ~63% of those in the 10% 3.4 kDa gels. Thus, it is possible that the increases in swelling produce larger distances between integrin binding sites in the gel, which would result in the reduced adhesion shown in these studies. In our previous work, we utilized a four-arm PEG crosslinker to create local increases in the crosslink density to increase modulus with minimal effects on the mesh size.⁵ In

future investigations, compositions with similar swelling ratios and different modulus values could be utilized to fully decouple the effects of swelling from the effects of modulus on EC adhesion. Similarly, initial protein concentrations can be adjusted to yield comparable levels of protein incorporation based on the relationships elucidated here. This would provide definitive information on the effects of substrate modulus in our system; however, we have shown here that the PEG molecular weight can be utilized as an additional tool to alter bioactivity of PEG-Scl2-2 hydrogels.

Effects of substrate modulus on EC migration and spreading

The general trends of increased migration speed with increased substrate modulus that were demonstrated in the current studies agree with literature reports with similar observations.^{33,58} The previously measured increases in migration are commonly attributed to the enhanced ability of a cell to generate traction forces on stiffer surfaces. These results also correlate with the variations in BAOEC spreading that were seen with changes in modulus. Although increasing modulus did not produce significant effects on EC spreading, PEG-Scl2-2 hydrogels promoted larger increases in spreading with increased substrate modulus than PEG-collagen hydrogels. Based on our adhesion data and literature reports, we expected to see larger effects on spreading in response to modulus; however, the significant decreases in spreading that have been shown in previous research to occur with decreased scaffold modulus were obtained on scaffolds with much lower modulus values than the 10% PEG (10 kDa) gels in this study.^{30,36,40} Therefore, it is possible that even the low modulus gels have sufficient stiffness for cells to generate traction forces and spread and that further reductions in modulus would produce greater effects on spreading. As a whole, these studies consistently demonstrate a trend between cell spreading and migration speed over a range of manipulations in protein and matrix variables.

In assessing the ANOVA statistical analysis of the data presented here (Supplementary Tables S1 and S2), the value of the ability to simultaneously tune the matrix and protein properties of bioactive PEG hydrogels in a controlled manner is apparent. For example, in the adhesion data for Scl2-2 gels in Figure 1, functionalization density does not have a significant effect on its own ($p=0.883$); however, when the effects of functionalization density and concentration are combined, there is a significant effect on adhesion ($p=0.041$). Similarly, when considering adhesion as a function of substrate modulus (PEG molecular weight) and protein type (Fig. 4), altering the protein type does not produce a significant change in BAOEC adhesion ($p=0.206$), while the combined effects of changing both the protein type and PEG molecular weight significantly alter adhesion ($p=0.036$). Simultaneous manipulation of multiple scaffold properties enables finer control over cell-material interactions to both extend the basic understanding of how cells interact with their environment and to improve the implantable device design.

Although target *in vitro* values for successful promotion of *in vivo* device endothelialization are unknown, the value of these studies is the identification of the effect of compositional variables on each of the key cellular responses to

these bioactive hydrogels. This provides a means to selectively modify the bioactive hydrogel variables after initial *in vivo* study results to achieve specific device goals. For example, if a PEG-Scl2-2-based vascular graft was utilized in a porcine animal model and insufficient EC coverage was noted after 30 days of implantation, researchers could quickly provide grafts that include gels with higher modulus values to promote increased EC migration. Alternatively, if evidence of EC detachment due to shear forces was noted, an increase in Scl2-2 concentration could be used to increase adhesion strength. It is expected that different device designs will have different requirements in terms of cell interactions that successfully promote endothelialization (e.g., small-diameter vascular graft vs. hemodialysis catheter). These studies provide the necessary tools for the rational design of bioactive hydrogels to meet the design goals of a wide range of applications.

Conclusions

PEG-Scl2-2 hydrogels are an ideal system for the independent investigation of the effects of a wide range of protein and network variables on bioactivity. Increases in protein concentration and decreases in functionalization density can be used to increase EC adhesion, spreading, and migration. Additionally, it was observed that PEG-Scl2-2 hydrogels promote significant EC proliferation over 1 week, resulting in adhesion levels that are greater than corollary PEG-collagen gels. Significant increases in migration speeds were also observed as compared to PEG-collagen gels. This is most likely due to the specificity in signaling that occurs with Scl2-2 relative to collagen, as it only contains one GFPGER-based binding site for integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$, whereas collagen contains numerous integrin binding sites. Furthermore, substrate modulus can be easily increased by reducing the PEG molecular weight, which results in increased EC adhesion, spreading, and migration and provides an additional tool for manipulation of cell interactions with bioactive hydrogels. A strong correlation existed between increased spreading and migration speed for all tested formulations. These studies demonstrate the potential utility of PEG-Scl2-2 hydrogels in regenerative medicine applications. Furthermore, they serve to elucidate key effects of matrix cues on cell interactions to improve the rational design of both synthetic and natural polymer-based bioactive hydrogel systems.

Acknowledgments

This work was supported by NIH R01 EB013297 and the National Science Foundation Graduate Research Fellowship Program.

Disclosure Statement

The authors disclose equity holdings in ECM Technologies, which seeks to commercialize Scl2-2.

References

- Hoffman, A.S. Hydrogels for biomedical applications. *Adv Drug Deliv Rev* **43**, 3, 2002.
- Lee, K.Y., and Mooney, D.J. Hydrogels for tissue engineering. *Chem Rev* **101**, 1869, 2001.

3. Nguyen, K.T., and West, J.L. Photopolymerizable hydrogels for tissue engineering applications. *Biomaterials* **23**, 4307, 2002.
4. Peppas, N.A., Keys, K.B., Torres-Lugo, M., and Lowman, A.M. Poly(ethylene glycol)-containing hydrogels in drug delivery. *J Control Release* **62**, 81, 1999.
5. Browning, M.B., Wilems, T., Hahn, M., and Cosgriff-Hernandez, E. Compositional control of poly(ethylene glycol) hydrogel modulus independent of mesh size. *J Biomed Mater Res Part A* **98**, 268, 2011.
6. Gombotz, W.R., Guanghai, W., Horbett, T.A., and Hoffman, A. Protein adsorption to poly(ethylene oxide) surfaces. *J Biomed Mater Res* **25**, 1547, 1991.
7. Cosgriff-Hernandez, E., Hahn, M., Wilems, T., Munoz-Pinto, D.J., Browning, M.B., Rivera, J., *et al.* Bioactive hydrogels based on designer collagens. *Acta Biomater* **6**, 3969, 2010.
8. Cruise, G.M., Hegre, O.D., Scharp, D.S., and Hubbell, J.A. A sensitivity study of the key parameters in the interfacial photopolymerization of poly(ethylene glycol) diacrylate upon porcine islets. *Biotechnol Bioeng* **57**, 655, 1998.
9. Deible, C.R., Petrosko, P., Johnson, P.C., Beckman, E.J., Russell, A.J., and Wagner, W.R. Molecular barriers to biomaterial thrombosis by modification of surface proteins with polyethylene glycol. *Biomaterials* **19**, 1885, 1998.
10. Mann, B., Schmedlen, R., and West, J. Tethered-TGF- β increases extracellular matrix production of vascular smooth muscle cells. *Biomaterials* **22**, 439, 2001.
11. Brown, F.R., Hopfinger, A.J., and Blout, E.R. The collagen-like triple helix to random-chain transition: experiment and theory. *J Mol Biol* **63**, 101, 1972.
12. Xu, Y., Keene, D.R., Bujnicki, J.M., Hook, M., and Lukomski, S. Streptococcal Sc11 and Sc12 proteins form collagen-like triple helices. *J Biol Chem* **277**, 27312, 2002.
13. Han, R., Zwiefka, A., Caswell, C., Xu, Y., Keene, D., Lukomska, E., *et al.* Assessment of collagen-like sequences derived from Sc11 and Sc12 proteins as a source of recombinant GXY polymers. *Appl Microbiol Biotechnol* **72**, 109, 2006.
14. Browning, M.B., Dempsey, D., Guiza, V., Becerra, S., Rivera, J., Russell, B., *et al.* Multilayer vascular grafts based on collagen-mimetic proteins. *Acta Biomater* **8**, 1010, 2012.
15. Knight, C.G., Morton, L.F., Peachey, A.R., Tuckwell, D.S., Farndale, R.W., and Barnes, M.J. The collagen-binding I-domains of integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ recognize the same specific amino acid sequence, GFOGER, in native (triple-helical) collagens. *J Biol Chem* **275**, 35, 2000.
16. Sweeney, S.M., DiLullo, G., Slater, S.J., Martinez, J., Iozzo, R.V., Lauer-Fields, J.L., *et al.* Angiogenesis in collagen I requires $\alpha 2\beta 1$ ligation of a GFP*GER sequence and possibly p38 MAPK activation and focal adhesion disassembly. *J Biol Chem* **278**, 30516, 2003.
17. Burkel, W.E. The challenge of small diameter vascular grafts. *Med Prog Technol* **14**, 165, 1989.
18. Kader, K., Akella, R., Ziats, N., Lakey, L., Harasaki, H., Ranieri, J., *et al.* eNOS-overexpressing endothelial cells inhibit platelet aggregation and smooth muscle cell proliferation *in vitro*. *Tissue Eng* **6**, 241, 2000.
19. Dole, V.S., Bergmeier, W., Mitchell, H.A., Eichenberger, S.C., and Wagner, D.D. Activated platelets induce Weibel-Palade-body secretion and leukocyte rolling *in vivo*: role of P-selectin. *Blood* **106**, 2334, 2005.
20. Browning, M.B., Russell, B., Rivera, J., Höök, M., and Cosgriff-Hernandez, E.M. Bioactive hydrogels with enhanced initial and sustained cell interactions. *Biomacromolecules* **14**, 2225, 2013.
21. Civerchia-Perez, L., Faris, B., LaPointe, G., Beldekas, J., Leibowitz, H., and Franzblau, C. Use of collagen-hydroxyethylmethacrylate hydrogels for cell growth. *Proc Natl Acad Sci U S A* **77**, 2064, 1980.
22. Hern, D.L., and Hubbell, J.A. Incorporation of adhesion peptides into nonadhesive hydrogels useful for tissue resurfacing. *J Biomed Mater Res* **39**, 266, 1998.
23. Mann, B., Gobin, A., Tsai, A., Schmedlen, R., and West, J. Smooth muscle cell growth in photopolymerized hydrogels with cell adhesive and proteolytically degradable domains: synthetic ECM analogs for tissue engineering. *Biomaterials* **22**, 3045, 2001.
24. Reinhart-King, C.A., Dembo, M., and Hammer, D.A. The dynamics and mechanics of endothelial cell spreading. *Biophys J* **89**, 676, 2005.
25. DiMilla, P., Stone, J., Quinn, J., Albelda, S., and Lauffenburger, D. Maximal migration of human smooth muscle cells on fibronectin and type IV collagen occurs at an intermediate attachment strength. *J Cell Biol* **122**, 729, 1993.
26. Peyton, S., and Putnam, A. Extracellular matrix rigidity governs smooth muscle cell motility in a biphasic fashion. *J Cell Physiol* **204**, 198, 2005.
27. Anseth, K.S., Bowman, C.N., and Brannon-Peppas, L. Mechanical properties of hydrogels and their experimental determination. *Biomaterials* **17**, 1647, 1996.
28. Bryant, S.J., Chowdhury, T.T., Lee, D.A., Bader, D.L., and Anseth, K.S. Crosslinking density influences chondrocyte metabolism in dynamically loaded photocrosslinked poly(ethylene glycol) hydrogels. *Ann Biomed Eng* **32**, 407, 2003.
29. Temenoff, J.S., Athanasiou, K.A., LeBaron, R.G., and Mikos, A.G. Effect of poly(ethylene glycol) molecular weight on tensile and swelling properties of oligo(poly(ethylene glycol) fumarate) hydrogels for cartilage tissue engineering. *J Biomed Mater Res* **59**, 429, 2001.
30. Discher, D.E., Janmey, P., and Wang, Y.-L. Tissue cells feel and respond to the stiffness of their substrate. *Science* **310**, 1139, 2005.
31. Georges, P.C., and Janmey, P.A. Cell type-specific response to growth on soft materials. *J Appl Physiol* **98**, 1547, 2005.
32. Gray, D.S., Tien, J., and Chen, C.S. Repositioning of cells by mechanotaxis on surfaces with micropatterned Young's modulus. *J Biomed Mater Res* **66A**, 605, 2003.
33. Pelham, R.J., Jr., and Wang, Y. Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proc Natl Acad Sci U S A* **94**, 13661, 1997.
34. Thompson, M.T., Berg, M.C., Tobias, I.S., Rubner, M.F., and Van Vliet, K.J. Tuning compliance of nanoscale polyelectrolyte multilayers to modulate cell adhesion. *Biomaterials* **26**, 6836, 2005.
35. Yeung, T., Georges, P.C., Flanagan, L.A., Marg, B., Ortiz, M., Funaki, M., *et al.* Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cell Motil Cytoskeleton* **60**, 2005.
36. Lawyer, T., McIntosh, K., Clavijo, C., Potekhina, L., and Mann, B. Formulation changes affect material properties and cell behavior in HA-based hydrogels. *Int J Cell Biol* **2012**, 1, 2012.
37. You, J., Park, S.-A., Shin, D.-S., Patel, D., Raghunathan, V.K., Kim, M., *et al.* Characterizing the effects of heparin gel stiffness on function of primary hepatocytes. *Tissue Eng* **19**, 2655, 2013.

38. Balgude, A.P., Yu, X., Szymanski, A., and Bellamkonda, R.V. Agarose gel stiffness determines rate of DRG neurite extension in 3D cultures. *Biomaterials* **22**, 1077, 2001.
39. Helmke, B.P., and Davies, P.F. The cytoskeleton under external fluid mechanical forces: Hemodynamic forces acting on the endothelium. *Ann Biomed Eng* **30**, 284, 2002.
40. Califano, J.P., and Reinhart-King, C.A. Exogenous and endogenous force regulation of endothelial cell behavior. *J Biomech* **43**, 79, 2010.
41. Vailhé, B., Ronot, X., Tracqui, P., Usson, Y., and Tranqui, L. *In vitro* angiogenesis is modulated by the mechanical properties of fibrin gels and is related to $\alpha v\beta 3$ integrin localization. *In Vitro Cell Dev Biol Anim* **33**, 763, 1997.
42. Vernon, R.B., Angello, J.C., Iruela-Arispe, M.L., Lane, T.F., and Sage, E.H. Reorganization of basement membrane matrices by cellular traction promotes the formation of cellular networks *in vitro*. *Lab Invest* **66**, 536, 1992.
43. Rao, R.R., Peterson, A.W., Ceccarelli, J., Putnam, A.J., and Stegmann, J.P. Matrix composition regulates three-dimensional network formation by endothelial cells and mesenchymal stem cells in collagen/fibrin materials. *Angiogenesis* **15**, 253, 2012.
44. Sieminski, A.L., Hebbel, R.P., and Gooch, K.J. The relative magnitudes of endothelial force generation and matrix stiffness modulate capillary morphogenesis *in vitro*. *Exp Cell Res* **297**, 574, 2004.
45. Califano, J.P., and Reinhart-King, C.A. A balance of substrate mechanics and matrix chemistry regulates endothelial cell network assembly. *Cell Mol Bioeng* **1**, 122, 2008.
46. Driessen, G.K., Scheidt-Bleichert, H., Sobota, A., Inhoffen, W., Heidtmann, H., Haest, C.W.M., *et al.* Capillary resistance to flow of hardened (diamide treated) red blood cells (RBC). *Pflugers Arch* **392**, 261, 1982.
47. Browning, M.B., and Cosgriff-Hernandez, E. Development of a biostable replacement for PEGDA hydrogels. *Biomacromolecules* **13**, 779, 2012.
48. Stokes, C.L., and Lauffenburger, S.K.W. Migration of individual microvessel endothelial cells: Stochastic model and parameter measurement. *J Cell Sci* **99**, 419, 1991.
49. Reinhart-King, C.A., Dembo, M., and Hammer, D.A. Cell-cell mechanical communication through compliant substrates. *Biophys J* **95**, 6044, 2008.
50. Abair, T.D., Bulus, N., Borza, C., Sundaramoorthy, M., Zent, R., and Pozzi, A. Functional analysis of the cytoplasmic domain of the integrin $\alpha 1$ subunit in endothelial cells. *Blood* **112**, 3242, 2008.
51. Pozzi, A., Wary, K.K., Giancotti, F.G., and Gardner, H.A. Integrin $\alpha 1\beta 1$ mediates a unique collagen-dependent proliferation pathway *in vivo*. *J Cell Biol* **142**, 587, 1998.
52. Shi, M., Pedchenko, V., Greer, B.H., Van Horn, W.D., Santoro, S.A., Sanders, C.R., *et al.* Enhancing integrin $\alpha 1$ inserted (I) domain affinity to ligand potentiates integrin $\alpha 1\beta 1$ -mediated down-regulation of collagen synthesis. *J Biol Chem* **287**, 35139, 2012.
53. Xu, Y., Gurusiddappa, S., Rich, R.L., Owens, R.T., Keene, D.R., Mayne, R., *et al.* Multiple binding sites in collagen type I for the integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$. *J Biol Chem* **275**, 38981, 2000.
54. Rasmussen, L., and Ledet, T. Aortic collagen alterations in human diabetes mellitus. Changes in basement membrane collagen content and in the susceptibility of total collagen to cyanogen bromide solubilisation. *Diabetologia* **36**, 445, 1993.
55. Senger, D.R., Claffey, K.P., Benes, J.E., Perruzzi, C.A., Sergiou, A.P., and Detmar, M. Angiogenesis promoted by vascular endothelial growth factor: regulation through $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins. *Proc Natl Acad Sci U S A* **94**, 13612, 1997.
56. Gunn, J.W., Turner, S.D., and Mann, B.K. Adhesive and mechanical properties of hydrogels influence neurite extension. *J Biomed Mater Res Part A* **72A**, 91, 2005.
57. Willits, R.K., and Skornia, S.L. Effect of collagen gel stiffness on neurite extension. *J Biomater Sci Polym Ed* **15**, 1521, 2004.
58. Lo, C.-M., Wang, H.-B., Dembo, M., and Wang, Y.-l. Cell movement is guided by the rigidity of the substrate. *Biophys J* **79**, 144, 2000.

Address correspondence to:
 Elizabeth M. Cosgriff-Hernandez, PhD
 Department of Biomedical Engineering
 Texas A&M University
 5045 Emerging Technologies Building
 3120 TAMU
 College Station, TX 77843-3120

E-mail: cosgriff.hernandez@tamu.edu

Received: September 24, 2013

Accepted: May 20, 2014

Online Publication Date: August 1, 2014