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Cell Durotaxis on Polyelectrolyte Multilayers with Photogenerated Gradients of Modulus

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Abstract

Behaviors of rat aortic smooth muscle (A7r5) and human osteosarcoma (U2OS) cells on photocrosslinked polyelectrolyte multilayers (PEMUs) with uniform, or gradients of, moduli were investigated. The PEMUs were built layer-by-layer with the polycation poly(allylamine hydrochloride) (PAH) and a polyanion poly(acrylic acid) (PAA) that was modified with photoreactive 4-(2-hydroxyethoxy) benzophenone (PAABp). PEMUs with different uniform and gradients of modulus were generated by varying the time of uniform ultraviolet light exposure and by exposure through optical density gradient filters. Analysis of adhesion, morphology, cytoskeletal organization, and motility of the cells on the PEMUs revealed that A7r5 cells established a polarized orientation toward increasing modulus on shallow modulus gradients (approximately 4.7 MPa mm⁻¹) and durotaxed toward stiffer regions on steeper gradients (approximately 55 MPa mm⁻¹). In contrast, U2OS cells exhibited little orientation or durotaxis on modulus gradients. These results demonstrate the utility of photocrosslinked PEMUs to direct vascular and osteoblast cell behavior, a potential application for PEMU coatings on biomedical implants.

Keywords

PEMU; Durotaxis; Photosensitive Polyelectrolyte; Poly(acrylic acid) (PAA); 4-(2-Hydroxyethoxy) benzophenone; Poly(allylamine hydrochloride) (PAH); Ultraviolet Crosslinking

Introduction

Mechanical properties of microenvironments affect cell morphology, adhesion, motility, gene expression, and differentiation.¹ Biocompatible films with tunable mechanical properties have been useful for investigating cell activities in vitro and are being developed for biomedical applications, including as implantable device coatings and tissue scaffolds.^{2–6} Some coatings are designed to repel cell and protein adhesion, whereas other coatings support extracellular matrix deposition, cell recruitment, and induce or maintain specific cell phenotypes.^{7–11}

One particularly versatile strategy for generating biocompatible coatings is to build polyelectrolyte multilayers (PEMUs) layer-by-layer (LbL) with pairs of different polyelectrolytes (PE).^{12–13} PEMUs are stabilized by numerous ion-pairing interactions

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Supporting Information Available: Additional data and descriptions of analysis protocols are included in Supplemental Figures S1–S6. This material is available free of charge via the Internet at <http://pubs.acs.org>.

between PE layers and are relatively easy to build on irregularly shaped objects. Surface and mechanical properties of PEMUs can be tuned in various ways, such as pairing PEs with specific chemical characteristics, varying the number of layers (increasing number of layers decreases apparent modulus)¹⁴, setting specific ionic/pH conditions in the LbL building process, and introducing covalent bonds between the PE layers by thermal crosslinking,^{15–17} chemical crosslinking,^{18–21} or photocrosslinking.^{2,21–23} Photocrosslinking PEMUs containing either an exogenous photosensitive crosslinker or a PE modified partially with a photoreactive molecule^{2,16–17,22–23} is particularly advantageous in generating PEMU modulus gradients, because the degree of crosslinking can be stringently controlled using optical masks and can be patterned. Photocrosslinking minimizes changes in surface chemistry, wettability, and topography often associated with thermal and chemical crosslinking.²⁴

Many cell types respond to the mechanical properties of various surfaces including PEMUs. In general, cells adhere better and spread more on stiffer biocompatible substrates,^{13,25–26} but some cell types exhibit specific responses to a specific modulus or limited range of modulus, and some cell types can migrate up a modulus gradient through a process known as “durotaxis”.^{22,27–28} Mesenchymal stem cells, for example, differentiate into different cell types depending on the match between the culture surface modulus and the natural mechanical properties of the environmental niche of the differentiated cell *in vivo*.^{29–30}

In this investigation, the polycation poly(allylamine hydrochloride) (PAH) and a polyanion poly(acrylic acid) (PAA) that was partially modified with photoreactive 4-(2-hydroxyethoxy) benzophenone (PAABp) were used to build PEMUs that then were photocrosslinked to yield surfaces with uniform moduli or with modulus gradients. We previously have used PAH and PAA PEs in PEMUs and in solution to investigate cell and protein interaction and cytotoxicity.^{2,5,31–34} The behavior of A7r5 rat aorta smooth muscle cells and U2OS human osteoblast-like osteosarcoma cells on these PEMUs were investigated. These two cell types represent the vascular smooth muscle cells and osteoblast cells that could interact *in vivo* with PEMU-coated implantable devices, such as intravascular stents and bone implants, respectively.

Materials and Methods

Polyelectrolytes and Reagents

Poly(acrylic acid) (PAA, molar mass 100,000 g mol⁻¹) and poly(allylamine hydrochloride) (PAH, molar mass 56,000 g mol⁻¹) from Sigma-Aldrich, and poly(ethyleneimine) (PEI, molar mass 70,000 g mol⁻¹) from Polysciences, Inc. (Warrington, PA) were used as received. PAA grafted with photosensitive benzophenone (5% grafting degree in this investigation) was synthesized as described previously.¹⁴ Unless noted otherwise, all other reagents were used as received from Sigma-Aldrich.

Polyelectrolyte Multilayer Preparation

The 31 layer PAH-terminated PEI(PAABp/PAH)₁₅ PEMUs used in this investigation were built layer by layer with the aid of a robot (StratoSequence V, nanoStrata Inc., Tallahassee, FL) using 10 mM (with respect to the repeat unit) PE solutions made in 0.15 M NaCl, 25 mM Tris-HCl, pH 7.4, as described previously.¹⁴ The PEMUs were built on one-inch diameter single-sided polished silicon wafers for AFM force spectroscopy, on Fisherbrand Cover Glass No.1 (Fisher Scientific) 22×22×0.17 mm coverslips for uniform modulus and edge modulus gradient assays, and on 50×22×0.17 mm coverslips for continuous modulus gradient assays. Prior to PEMU coating, the silicon wafers were cleaned with piranha (70% H₂SO₄ and 30% H₂O₂) for 20 min (*caution*: piranha is a strong oxidizer and should not be

stored in closed containers), rinsed vigorously with H₂O, and dried with a stream of N₂. The glass coverslips were cleaned by soaking in 70% ethanol overnight, rinsing extensively with H₂O, dried with a stream of N₂, and exposed to air plasma for 1 min.

To build the PEI(PAABp/PAH)₁₅ PEMUs, these surfaces were first primed with a layer of PEI by dipping into PEI solution for 30 min and rinsing with water. This initial PEI layer promoted a more uniform coating by subsequent PE layers. The PEI-coated surfaces were mounted in the robot on a shaft that rotated at 300 rpm, and the robot built the PEMUs for 15 cycles of dipping in alternating baths of PAABp and PAH for 5 min each, followed by three 1 min water-rinsing steps between each PE bath. Before use in cell culture, the PEMU-coated coverslips were incubated overnight in 0.15 M NaCl, 25 mM Tris-HCl, pH 7.4. All PE solutions used for building the cell culture PEMUs contained 0.02 w/v% sodium azide to prevent microbial contamination. PEMUs were handled and stored using sterile laboratory practices. Microbial contamination of polyelectrolyte solutions lacking sodium azide was rare, and its inclusion was only precautionary and unnecessary if PEMUs were handled following sterile practices. No significant differences were found by AFM spectroscopy or cell culture analysis in PEMUs built with or without sodium azide (data not shown).

Photocrosslinking

For photocrosslinking, PEI(PAABp/PAH)₁₅ PEMUs were dried after the final water rinse and irradiated in a 12.70×30.5×25.4 cm CL-1000 Model 254 nm UV box (Ultra-Violet Products Ltd., Upland, CA,) equipped with five 8 W 200–280 nm wavelength UV lamps and a calibration photodiode. The PEMUs were placed at the center of the box where the UV intensity at the surface level was 4.9 mW cm⁻². Irradiating a PEMU transforms the benzophenone to a biradical (ketyl radical), which either can form a crosslinking covalent bond with PAH or return to ground state by phosphorescence.¹⁴

To determine the resulting crosslinking density (percentage of crosslinked benzophenone) by UV irradiation, a PEI(PAABp/PAH)₁₅ PEMU built on a quartz substrate was crosslinked with UV irradiation for cumulative times of 0, 2, 10, 35, and 100 minutes, with modulus determination between each exposure. The absorption of the benzophenone carbonyl peak, measured by UV-Vis spectroscopy at 280 nm, decreased with increasing crosslinking irradiation time as the carbonyl became crosslinked. The percentage of crosslinked benzophenone for each crosslinking time was calculated from Equation 1,¹⁴

$$\%Bp_{crosslinked} = \frac{(A_0 - A_t)}{A_0} \times 100 \quad (1)$$

in which %*Bp_{crosslinked}* is the percentage of crosslinked benzophenone within the multilayer; *A*₀ is the initial absorption of benzophenone before crosslinking irradiation; and *A*_{*t*} is the absorption of benzophenone after crosslinking irradiation for a given time.

Shallow modulus gradients in the PEI(PAABp/PAH)₁₅ PEMUs on one inch diameter silicon wafers and on 50×22×0.17 mm glass coverslips were formed by irradiating for 200 min through a rectangular continuously variable neutral density gradient filter composed of a UV-fused silica glass substrate with a metallic Inconel gradient surface coating (NDL-10C-2, Thorlabs, Newton, NJ). The coated part of the filter is 45 mm long, over which the optical density along the coating varies from 0.04 to 2. To cover the entire crosslinking range on the PEMU-coated silicon wafers, two wafers were placed side by side under the filter for irradiation. Steeper modulus gradients were generated by masking half the multilayer with a non-transparent black shield and exposing the other half to UV irradiation for 100 min. The position of the mask edge was marked for future reference.

Force Spectroscopy

The apparent modulus of PEMUs was determined by force indentation measurements (force curves) using an MFP-3D atomic force microscope (AFM) equipped with a Multi 75AI silicon tip (Budget Sensors, spring constant of $\sim 2 \text{ N m}^{-1}$ and a tip radius of 10 nm), an ARC2 controller (Asylum Research Inc., Santa Barbara, CA), and Igor Pro software at room temperature. The optical lever sensitivity (OLP) of the tip was calibrated on glass in air, and its spring constant was determined by the thermal fluctuation technique. The OLP of the tip was recalibrated in 0.15 M NaCl. For standard measurement of PEMU apparent modulus (modulus recorded at a specific tip velocity), force maps of a 4×5 matrix of points in a scan range of $10 \times 10 \text{ }\mu\text{m}$ were performed with a tip velocity of $1 \text{ }\mu\text{m s}^{-1}$ and a starting distance from the surface of 500 nm. For characterization of PEMU viscoelasticity, force curves were recorded at different tip indentation velocities (equivalent to different strain rates) ranging from 50 nm s^{-1} to $3.17 \text{ }\mu\text{m s}^{-1}$.

The applied force and the resulting indentation are given by Equations 2 and 3,

$$F_{\text{applied}} = Kd = K(z - \delta) \quad (2)$$

$$\delta = (z - d) \quad (3)$$

in which δ is the indentation distance of the tip into the PEMU, up to approximately 50 nm in these investigations, z is the distance of the tip relative to the PEMU in the z -direction, d is the deflection of the tip, and K is the spring constant of the cantilever. All force curves were analyzed using Hertzian contact mechanics for a punch model with Equation 4^{35–36}

$$F_{\text{punch}} = 2E_c R(z - d) \quad (4)$$

in which E_c is the convoluted modulus of the material and R is the radius of the indenter tip (10 nm for a Multi75AI tip, according to the manufacturer). Equation 4 predicts that force is proportional to indentation, which was observed in this investigation.

By fitting the force vs. indentation curves, E_c was obtained and related to the apparent modulus E_1 of the PEMU and E_2 of the indenter using Equation 5,

$$E_c = \left(\frac{1 - \nu_1^2}{E_1} + \frac{1 - \nu_2^2}{E_2} \right)^{-1} \quad (5)$$

in which ν_1 and ν_2 are the Poisson ratios of the PEMU and the silicon indenter, respectively. In Equation 5, ν_1 was assumed to be 0.5, as the PEMUs were considered to be isotropic elastic materials in the range of loads applied, ν_2 was set at 0.27, and E_2 was set at 150 GPa for the silicon tip.

Cell Culture

Rat aortic smooth muscle A7r5 cells (originally ATCC CRL-1444) and U2OS osteosarcoma cells (originally ATCC HTB-96) were purchased from American Type Culture Collection (ATCC, Manassas, VA) and propagated as cell lines in the lab for several years. The A7r5 cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) (D5648, Sigma-Aldrich) prepared from powder with sterile H_2O and supplemented with 1.5 g L^{-1} NaHCO_3 , 10% fetal bovine serum (HyClone Standard Bovine Serum, Thermo Scientific), $10 \text{ }\mu\text{g mL}^{-1}$ gentamicin (Gibco Gentamicin Reagent Solution, Invitrogen), and an antibiotic-antimycotic supplement providing final concentrations of 100 units mL^{-1} penicillin G, 100 Pg mL^{-1} streptomycin, $0.25 \text{ }\mu\text{g mL}^{-1}$ amphotericin (Gibco Antibiotic-Antimycotic,

Invitrogen). The U2OS cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM F-12) (D8900, Sigma-Aldrich) prepared from powder with sterile H₂O and supplemented with 1.5g L⁻¹ NaHCO₃, 10% Cosmic Calf Serum (HyClone Cosmic Calf Serum, Thermo Scientific), and the penicillin, streptomycin, amphotericin, and gentamicin as described above. During live cell analysis (see Imaging) cells were maintained under the microscope at 37°C, 5% CO₂, and 40% relative humidity in a LiveCell™ Chamber (Pathology Devices, Westminster, MD).

To facilitate DIC imaging of live cells on PEMUs, a 22×22×0.17 mm coverslip (uncoated or coated with uniform or edge gradient PEMUs) was attached using Gorilla Glue (Gorilla Glue Inc.) over the hole in the bottom of 35 mm tissue culture dish that was drilled with a variable speed bench drill press fitted with a smooth-finish wood bit (3/4" bit size, 3 1/2", 5/16" shank diameter, McMaster-Carr, Cleveland, OH). The drilled culture dishes were sterilized with 70% ethanol and washed extensively with sterile phosphate-buffered saline (PBS, pH 7.4) before gluing on the PEMU coated and uncoated coverslip windows. The glue was allowed to cure for at least 24 h, after which the culture dishes windowed with uncoated or PEMU-coated coverslips were washed extensively with PBS to remove any remaining particulate material.

Fluorescent Localization of Vinculin and Actin

For fluorescent localization of vinculin and actin, A7r5 cells were seeded at 5×10^4 cells per well on uncoated or PEMU-coated coverslips in six-well plates (BD Falcon multiwell flat-bottom plates). The cells were cultured for 48 h, then fixed, and stained for vinculin and actin localization by washing free of culture medium with three rapid washes with PBS at 37°C and fixing for 15 min with 3.7% formaldehyde (molecular-biology grade formaldehyde, Fisher Scientific) prepared in PBS. The formaldehyde solution was removed, and the cells were washed with PBS at room temperature for three 5 min washes, extracted for 10 min with 0.2% Triton X-100-PBS, rinsed three times for 5 min each with 0.05% Triton X-100-PBS, and blocked for 1 h at 37°C in 1% bovine serum albumin prepared in 0.05% Triton X-100-PBS.

After blocking, the staining protocol described in Moussallem et al.² was followed using the hVIN-1 anti-vinculin mouse monoclonal primary antibody (Sigma-Aldrich, at a 1:200 dilution) and Alexa 488 goat anti-mouse IgM secondary antibody (Invitrogen, at a 1:200 dilution) and Texas Red phalloidin (Invitrogen) all diluted in 1% bovine serum albumin (BSA) prepared in 0.05% Triton X-100-PBS buffer. The cover slips were mounted with Pro-Long Gold Antifade Reagent with DAPI (Invitrogen) onto microscope slides and allowed to cure for 24 hours. The slides were stored at 4 °C in the dark.

Imaging

A Nikon Ti-E inverted microscope equipped with a Nikon Intensilight C-HGFI illuminator and a Photometrics Cool Snap HQ2 camera (Photometrics) was used to obtain differential interference contrast (DIC) images of fixed cells and to record live cell activity. Phase contrast images were captured using a Nikon TS100 microscope equipped with a Nikon Digital Sight DS-Ri1 digital camera. Fixed cell images and live cell recordings were analyzed and processed using NIS-Elements Advanced Research (Nikon), ImageJ (NIH), and Photoshop (Adobe) software.

Cellular Aspect Ratio and Orientation

The length:width aspect ratio (major:minor axis) and orientation of cells cultured on optical mask-generated modulus gradients were determined using the ImageJ EllipseFitter plugin to fit a model ellipse to each cell (Supporting Information Figure S1). Mean values of the

major:minor axes ratios of cells on the various surfaces were calculated and compared. Two independent trials analyzing 40 cells for each respective compliance condition were conducted. The EllipseFitter plugin also was used to assess cell orientation on gradients by determining the absolute angle of each cell major axis with respect to gradient axis. The angle of each cell on a surface was then binned into one of two groups with major axis angles of 0°–45° or 46°–90°. A cell with its major axis angle in the range 0–45° was considered orientated along the gradient.

Wind Rose Plots of A7r5 Cell Locomotion on PEMUs

Motility of cells was recorded at a rate of one image each 10 min (6 images per hour) for up to 48 h after seeding onto surfaces. The ImageJ Manual Tracking plugin was used to track the (x,y) coordinates of each cell nucleus over time, and Excel was used to generate Wind rose plots of the locomotion of each cell. For the Wind rose plots, the (x,y) starting position for each cell on a uniform modulus PEMUs was set to the origin (coordinates 0,0). For each cell on the edge modulus gradient, the starting x-coordinate plot position reflects the actual horizontal starting location of the cell along a 1 mm stretch centered at the position of the UV crosslinking exposure mask edge, and the starting y-coordinate position was arbitrarily set to enhance visualization of the migration pattern of each cell while representing changes in the y-coordinate position over time. The velocity of cell migration was determined by imaging the cells at 10 min intervals over 24 hours and mapping the distance each cell moved from image to image.

Results and Discussion

Several previously characterized properties of the PAH-terminated PEI(PAABp/PAH)₁₅ PEMU guided selection of this PEMU for investigation of cell interactions with surfaces of different uniform moduli and gradients of modulus.¹⁴ One important property is that PEI(PAABp/PAH)₁₅ PEMUs, with an uncrosslinked dry thickness of ~225 nm and a modest degree of swelling (about 22%) when wet³⁷, are sufficiently thick to isolate cells grown on the surface from the modulus of the underlying glass coverslip.¹⁴ During PEI(PAABp/PAH) PEMU buildup, the apparent modulus measured by AFM indentation decreases with addition of PAA/PAH bilayers up to 15 bilayers but decreases little more with additional bilayers.¹⁴ Another property is that, although the surface modulus changes significantly, the surface charge changes little with PEI(PAABp/PAH)₁₅ PEMU photocrosslinking, negating surface charge as a significant variable in comparing crosslinked and uncrosslinked PEMUs. The issue of surface charge was further mitigated by using only PAH-terminated PEI(PAABp/PAH)₁₅ PEMUs in these investigations, to focus on the effects of different moduli and gradients of moduli on cell interaction rather than on previously characterized differences in cell interactions with PAH- and PAA-terminated PEMUs.³¹

To determine the effect of UV crosslinking on modulus, five PEI(PAABp/PAH)₁₅ PEMU PEMUs built on quartz substrates were UV light irradiated for 0 (native), 2, 10, 35, and 100 minutes, which respectively caused covalent crosslinking of 0, 40, 58, 67, and 78% of the benzophenone, as measured by loss of benzophenone absorbance (as in ref 14, results not shown). The resulting apparent modulus of the uniformly crosslinked PEMUs determined from AFM force curve measurements with a tip velocity of 1 $\mu\text{m s}^{-1}$ varied from approximately 21 MPa for the non-irradiated native PEI(PAABp/PAH)₁₅ PEMUs up to more than 100 MPa for the 78% crosslinked PEMUs irradiated for 100 min (Figure 1).

Continuous PEI(PAABp/PAH)₁₅ PEMU modulus gradients were prepared by UV exposure through a neutral density optical gradient filter. AFM force curve measurements showed that the apparent modulus along the PEMU gradient varied from approximately 55 MPa at the end masked by the 2.0 density end of the filter to approximately 140 MPa at the end masked

by the 0.04 density end of the filter (Figure 2). The gradient was relatively linear over 18 mm with a modulus change of approximately 4.7 MPa mm^{-1} .

A much steeper modulus gradient was prepared by masking half the PEI(PAABp/PAH)₁₅ PEMU with a black shield and exposing the other half to UV irradiation for 100 minutes. AFM indicated that modulus along the masked edge varied continuously from ~38 MPa for the fully masked side to ~120 MPa for the fully irradiated side of the PEMU, with the steepest rate of change of about $\sim 55 \text{ MPa mm}^{-1}$ (Figure 3). Use of non-collimated light in the UV illumination box and possible light guiding effects within the PEMU may account for the difference in the slope of this gradient from a sharper step-like profile. Additionally, variations in experimental protocols of UV exposure and substrates used to generate PEMUs for Figure 1, 2, and 3 are likely to account for differences in modulus between the most compliant and stiffest uniformly crosslinked PEMU and the ends of the shallow and steep gradients.

These edge gradient PEMUs were analyzed further to determine whether modulus change was accompanied by a change in PEMU viscoelastic properties. Viscoelasticity of PEMUs is poorly addressed in the literature, but viscoelasticity recently was shown to play an important role in the behavior of cells on PA hydrogels.³⁸ For analysis of the PEMU edge gradient viscoelasticity, AFM force curve measurements were obtained on the most and least compliant ends and in the middle of the gradient using different tip indentation velocities, which generated different strain rates (Figure 4). At all three positions on the gradient, the apparent Young's modulus decreased significantly with decreasing tip indentation velocity, demonstrating that there is a significant viscous component in the viscoelasticity of all regions along the PEMU gradient. This indicates that although the apparent moduli measured by AFM force curve analysis of these PEMUs are high compared to reported hydrogel moduli, cells may detect a much lower effective modulus range on the time scale of cell response owing to the PEMU viscoelastic properties.^{39–40} Cells assess the mechanical nature of their microenvironments with internal force production by stress fibers on focal adhesions that are dynamically forming, maturing, and turning over. Traction slip analysis⁴¹ has shown that focal adhesion traction rates during initial surface assessment are approximately 16 nm s^{-1} , which would deform the substrate at a rate significantly slower than the slowest indentation rate used in the PEMU viscoelasticity measurements (50 nm s^{-1} , Figure 4B). Additionally, the AFM analysis of PEMU modulus was performed at room temperature, but the cell interaction with the PEMUs is at 37°C , which also may decrease the effective modulus experienced by a cell even more. Shamoun et al.⁴² demonstrated polyelectrolyte complex viscoelasticity is temperature dependent.

To investigate cell interaction with these PEMUs, A7r5 cells were cultured on a series of uniform 0%, 40%, 58%, 67%, and 78% crosslinked PEI(PAABp/PAH)₁₅ PEMUs. The A7r5 cells spread and adhered through the formation of vinculin-containing focal adhesions to all the uniformly crosslinked PEI(PAABp/PAH)₁₅ PEMUs tested (Figure 5). Focal adhesions provide cell-substrate attachment points in which cell membrane integrin receptors bind to extracellular matrix proteins, such as fibronectin, and to intracellular proteins, including vinculin, that link the integrins to the ends of actin filaments in force-producing stress fibers.^{58–60} The A7r5 cells spread more and had more vinculin-rich focal adhesions on the stiffer PEMUs (Figure 5; Supporting Information Figure S2). The A7r5 cells on uniform modulus PEMUs at varying UV exposure (Supporting Information Figure S2) showed no significant difference in orientation or overall elongation compared to the cells on the uncrosslinked PEI(PAABp/PAH)₁₅ PEMU, except for the cells on glass coverslips, which were more rounded as well as more spread, and the cells on the 40% crosslinked PEMU, which were more elongated. The cells on the 40% crosslinked PEMU appear to be able to elongate more than the cells on the uncrosslinked PEMU, but not spread as much as the cells

on the 58% crosslinked PEMU, which would decrease their aspect ratios. Similar modulus-dependent morphological differences were found by Moussallem et al.², where A7r5 cell phenotype also varied from a motile "synthetic" phenotype to a sessile "contractile" phenotype when cultured on PAH/PAA PEMUs in which surface compliance was varied by thermally crosslinking the PAH amine groups and the PAA carboxyl groups to form amide linkages.³² Others have shown that PEMUs made with the polycation poly (L-lysine) (PLL) and the polyanion hyaluronan (HA) crosslinked either by forming amide bonds using water soluble EDC/NHS as the coupling agent or by incorporating photocrosslinking HA biopolymer grafted with a vinylbenzyl group (VB), affects the adhesion, spreading, and differentiation responses of other cell types, including chondrosarcoma cells (HCS2/8) and myoblast stem cells.^{3,4,6,29,43}

To investigate whether cells can sense and respond differentially along a PEMU modulus gradient, A7r5 cells were plated across a shallow continuous gradient similar to that shown in Figure 2. The elongation aspect ratio (ratio of the major:minor axes for an ellipse fitted to each cell) and orientation (angle of the major axis of each cell to the gradient axis) for cells located at specified positions along the PEMU were determined using the ImageJ EllipseFitter plugin (Supporting Information Figure S3). Virtually all the cells on the gradient and on the native uncrosslinked PEMU control had an elongation aspect ratio of at least 2 (Figure 6, A). The cells in the 19–21.4 mm range of the gradient were significantly more elongated than the cells in the other parts of the gradient, and the cells on all the PEMU surfaces were significantly more elongated than the cells on the uncoated glass coverslip control surface.

The orientation of each cell was binned into one of two groups based on whether its major axis was oriented at an angle to the gradient axis of 0–45° or 46–90° (Figure 6, B). Cells on the uniform native PEI(PAABp/PAH)₁₅ PEMU, on the most compliant (9.4 mm) and stiffest (26.2 mm) ends of the gradient, and on the glass control surfaces exhibited no significant orientation with respect to the gradient axis, with approximately 50% of the cells in each of the two angle ranges. The cells on the other regions of the gradient exhibited a propensity for orientation along the gradient axis, with cells in the 11.8 mm ($p < 0.05$) region near the more compliant end and in the 21.4 mm ($p < 0.1$) region near the stiffer end of the gradient exhibiting the greatest statistical significance for orientation along the gradient. It remains unclear whether the cells are more sensitive to gradients in these two specific modulus ranges or undetected variations in the steepness of the gradients account for these differences.

These two analyses combined indicate that A7r5 cells responded most to the 21.4 mm region in the stiffer region of the gradient by both elongating more and orienting more along the gradient. Overall, the slight but statistically significant differences in cell orientations and aspect ratios all along the shallow gradient appear to indicate that the cells respond specifically not only to the slope of modulus change but also to the range of modulus change.

Differential cell spreading on surfaces with soft and stiff moduli is well known, but different relationships between the degree of cell spreading and elongation along the gradient, especially in the 19–21.4 mm range, where cell spreading is less but elongation is greater than in neighboring softer and stiffer areas of the gradient, reveals a more complicated cell response to specific regions of the gradient. It appears that cells on certain more compliant regions of the modulus gradient (e.g., Fig. 6, position 11.8) that are less conducive to cell spreading and elongation nevertheless can align with respect to modulus gradient (Figure 6, A and B). As in Figure 5 and Supporting Information Figure S2, the A7r5 cells on the more compliant regions of the gradient were smaller in adhesion area, spindle shaped, lack

orientation, and contained smaller focal adhesions compared to the well spread cells with robust focal adhesions on the higher modulus regions of the gradient.

Differences in cell morphology on different surface moduli also were reflected in differences in actin filament organization in the cells. The effect of PEMU uniform modulus and steep modulus gradients on A7r5 actin cytoskeleton organization was determined by fluorescently staining actin filaments in the cells (Figure 7). Cells on the more compliant uniform native PEI(PAH/PAABp)₁₅ PEMUs and on the more compliant end of the edge gradient contained fewer actin filament-containing stress fibers but had more actin filament-rich lamellipodia than were present in the cells on the stiffer uniform crosslinked PEMUs and on the stiffer end of the gradient. A7r5 cells on uncrosslinked PEMUs and on the more compliant ends of shallow and steep modulus gradients spread less than on the maximally crosslinked and stiffer ends of the gradients (Supporting Information Figure S4). Variation in the degree of cell spreading between A7r5 cells on the uniform (Supporting Information Figure S2B) and on the steep modulus gradients (Supporting Information Figure S4B) most likely reflects the ~20MPa higher modulus for the stiffest end of the steep gradient (Figure 2) compared to that of 78% crosslinked uniform PEMU modulus (Figure 1). Nevertheless, the A7r5 cells respond to the ~80MPa difference between the softest and stiffest moduli of the uniformly crosslinked PEMUs and the ends of the steep gradient by spreading ~2.5 fold more on the stiffest moduli. Not all the factors controlling cell spreading and elongation are known. One intriguing suggestion is that energy homeostasis is a driving factor in cell adhesive behavior.⁴⁴ The actin distribution results are consistent with the A7r5 cells being more motile on compliant PEMUs. The cell morphologies and orientation of cells along the steep compliance gradient (Supporting Information Figure S5) raised the possibility that the A7r5 cells could durotax up the gradient, perhaps to a point at which they assemble robust focal adhesions and stress fibers, and they become less directed and motile.

To investigate the possibility of durotaxis, the motility of cells on uniform modulus and steep modulus gradient PEMUs was recorded for 24 hours, and Wind rose plots representing cell migration tracks were generated (Figure 9). For each cell analyzed, the cell nucleus was designated as the centroid, and the two-dimensional position of each cell centroid over a 24 h period was plotted. The A7r5 cells on the uniform native and crosslinked PEMUs and outside the gradient region of the edge gradient PEMU exhibited little directional migration (Figure 9, A and B). In contrast, A7r5 cells on the more compliant end of the steep modulus gradient migrated progressively toward the stiffer side of the gradient (Figure 9, C), with exception of one cell (C5) which initiated migration toward the increasing modulus end but then returned back to its starting position. Velocities of A7r5 cell motility on uniform uncrosslinked, crosslinked, and steep modulus gradients were similar (Supporting Information Figure S6). In general, U2OS cells were highly immotile and showed no durotaxis along the modulus gradient (results not shown).

Durotaxis was first defined in 3T3 fibroblasts migrating unidirectionally over a modulus boundary from the softer side onto the stiffer side on a PA gel coated with collagen.⁴⁵ Since then, the behaviors of several cell types on modulus gradients have been investigated using elastomeric hydrogels composed of a variety of components including polyacrylamide (PA), polyethylene glycol (PEG), polydimethylsiloxane (PDMS), or polyethylene glycol diacrylate (PEGDA). In PA gels, varying the concentration of PA or varying the amount of bis-acrylamide crosslinking changes the uniform modulus of a gel. PA modulus gradients have been made by using a microfluidic device to generate a bis-acrylamide gradient in a constant concentration of PA⁴⁶ or by activating a photosensitive polymerization PA gel initiator through an optical gradient mask,⁴⁷ similar to the strategy employed in this investigation. Microfluidic-based lithography has been used to prepare a polyethylene glycol

diacrylate (PEGDA) modulus step gradient with a step size as small as 27 μ m, on which human foreskin fibroblasts durotaxed toward the stiffer end of the gradient.⁴⁸

On hydrogel gradients, fibroblasts, epithelial cells, and smooth muscle cells migrated faster, exhibited more lamellipodia activity on soft substrates, adhered better, and formed more stable focal adhesions on rigid substrates, and durotaxed across boundaries or along modulus gradients toward the stiffer substrate.^{47,48,62} Intriguingly, mesenchymal stem cells cultured on hydrogels differentiated into neuronal cells on the more compliant region, muscle cells on the intermediate stiffness region, and bone cells on a stiffer region.^{10,66,49–50}

Several characteristics of the PEMUs used in this investigation are different from those of chemically crosslinked elastomeric materials. One difference is that neutral hydrogels made from PA and PEG are highly cell repellent and typically must be coated with a layer of collagen or other types of biofunctionalization to promote cell interaction with the gel and its mechanical properties.⁵¹ In contrast, no such coatings or other functionalizations are required for the cell interactions and responses to the PEMU investigated here.

A second difference is that in elastomeric substrates, which are capable of resuming their original shape and physical properties after force is applied, have a higher storage modulus than loss modulus over a broad frequency range, because the mechanical responses of the elastic components of the modulus are typically much stronger than the responses of the viscous components. Elastomeric materials, for example, would show much less variation in E1 at different indentation velocities than found in PEMUs (Figure 4). The mechanical nature of ECMs has a significant viscous component and therefore exhibits frequency responsivity.^{52–55} A frequency/speed-dependent modulus caused by viscous contributions (greater loss modulus) would provide more effective energy dissipation if cells rely on energy use as well as static stresses to gauge the mechanical properties of their environment. There also could be a positive feedback mechanism for durotaxis on viscous surfaces: the faster a cell travels the greater the modulus it experiences. Additionally, cell type-specific rate of focal adhesion turnover may contribute to the ability of a cell to respond to the specific modulus of a viscoelastic material. Cells with faster focal adhesion turnover and constantly forming focal adhesions, for example, may perceive a higher effective modulus through generation of higher levels of tension than do cells in which longer interaction of focal adhesions with the viscoelastic substrate dissipates the effect of tension production over time. The frequency response of the mechanical properties of PEMUs, therefore, may be more similar to those of the ECM than are the properties of the elastomeric films. Whether focal adhesion turnover is faster in A7r5 cells than in U2OS cells, which would support this possibility, will be assessed in future investigations.

Osteoblasts interact with a microenvironment that is stiffer than that of smooth muscle cells *in vivo*. To investigate whether these cell types behave differently on PEMUs of various compliances, osteoblast-like U2OS osteosarcoma cells and the A7r5 smooth muscle cells were compared at 24, 48, and 72 h after plating on uniform and steep compliance gradients (Figure 9). The behaviors of both cell types on the most compliant and stiffest ends of the edge modulus gradient were similar to their behaviors on the uniform native and maximally crosslinked PEMUs, respectively. Both cell types adhered and spread well on the uniform maximally crosslinked PEMUs and on the maximally crosslinked side of the modulus gradient PEMUs. Compared to the A7r5 cells, the U2OS cells adhered and spread far less well on the uniform native PEMUs and on the masked side of the modulus gradient PEMUs. On these more compliant surfaces, many of the U2OS cells failed to adhere or released from the surface and eventually underwent apoptosis, most likely anoikis, within 72 hours. Consequently, orientation of the U2OS cells along the gradient was far less apparent than in the A7r5 cells, in part because of the poor adherence of the U2OS cells to the more

compliant end of the modulus gradient. Previous investigations demonstrated U2OS cells response to uncrosslinked PEMUs to be a direct result of poor adhesion rather than a cytotoxicity effect.³¹

Conclusion

PEMUs built layer by layer with PAH and the photosensitive PE PAABp can be photocrosslinked to yield biocompatible surfaces with uniform, or continuous gradients of, modulus. Both A7r5 smooth muscle cells and the U2OS osteoblast-like cells spread and adhere well to stiffer PAABp/PAH PEMUs with no terminal layer biofunctionalization, but A7r5 cells spread and adhere better than do U2OS cells on the more compliant PEMUs, which reflects the compliance difference between the natural microenvironments for smooth muscle cells and osteoblasts in vivo. The A7r5 cells also exhibited the greater sensitivity to both shallow and steep modulus gradients by elongating and orienting along the shallow gradient and durotaxing up the steep gradient. The feasibility demonstrated here of generating stable, thin, conformable, and tunable modulus gradients that influence cell behavior adds to the potential utility of PEMUs as biocompatible coatings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Apparent uniform moduli of a PEI(PAABp/PAH)₁₅ PEMU built on quartz as a function of benzophenone crosslinking percentage. The PAABp used to build the PEMU contained 5 mol% benzophenone. The PEMU was UV-irradiated for cumulative times of 0, 2, 10, 35, and 100 min. The AFM force curve measurements for modulus determination of the PEMUs immersed in 0.15 M NaCl, Tris-HCl pH 7.4 were obtained with a tip indentation velocity of $1\mu\text{m s}^{-1}$. Error bars represent standard deviations from the mean modulus ($n=20$) on each PEMU.

Figure 2.

Gradient of apparent modulus along a PEI(PAABp/PAH)₁₅ PEMU on a silicon wafer that was UV-photocrosslinked through a neutral density gradient optical mask for 200 min. The AFM force curve measurements for apparent modulus determination of the PEMU immersed in 0.15 M NaCl, Tris-HCl pH 7.4 were obtained with an indentation velocity of $1\mu\text{m s}^{-1}$. The linear fit has a slope of $\sim 4.7\text{ MPa mm}^{-1}$ ($R^2 = 0.971$). Error bars represent standard deviations for $n=20$ measurements at each indicated position on the PEMU.

Figure 3.

Apparent modulus gradient across the boundary between masked and UV irradiated sides of a PEI(PAABp/PAH)₁₅ PEMU that was photocrosslinked using an edge mask. Force curve measurements on PEMUs in 0.15 M NaCl, Tris-HCl pH 7.4 were obtained with a tip indentation velocity of $1\mu\text{m s}^{-1}$. Position zero corresponds to the position of the mask edge on the PEMU during UV irradiation. The linear fit has a slope of $\sim 55\text{ MPa mm}^{-1}$, ($R^2 = 0.976$).

Figure 4.

PEMU viscoelasticity. (A) Variation of apparent modulus with AFM tip indentation velocity at different positions along a PEI(PAABp/PAH)₁₅ PEMU that was photocrosslinked using an edge mask. (B) Expansion of the lower indentation velocity region of plot A. The AFM force curve measurements for modulus determination were obtained on the masked, most compliant side, ($\diamond$), in the middle of the gradient between the two sides ($\square$), and on the maximally irradiated, stiffest side (Δ), with the PEMU immersed in 0.15 M NaCl, Tris-Cl pH 7.4. Error bars indicate standard deviations for $n=20$ force curves determined at each gradient position.

Figure 5.

Morphology and focal adhesions of A7r5 cells on PEI(PAABp/PAH)₁₅ PEMUs with various uniform moduli. A7r5 cells were cultured for 24 h on PEI(PAABp/PAH)₁₅ PEMUs crosslinked with: (A) no UV exposure, 0% crosslinked; (B) 2 min of UV exposure, 40% crosslinked; (C) 10 min of UV exposure, 58% crosslinked; (D) 35 min of UV exposure, 67% crosslinked; (E) 100 min of UV exposure, 78% crosslinked; or (F) directly on a glass coverslip with no PEMU. The cells were immunofluorescently stained for vinculin (green) localization in focal adhesions (scale bar = 20μm).

Figure 6.

Orientation and elongation of A7r5 cells on a photocrosslinked PEI(PAABp/PAH)₁₅ PEMU modulus gradient. The morphology of each cell ($n = 80$, 40 cells in each of two independent trials analyzed) on a uniform native, uncrosslinked PEMU (PAABp/PAH), on an uncoated glass coverslip (Glass), and on each indicated region of the modulus gradient was fit with a model ellipse using the ImageJ EllipseFitter plugin. (A) Aspect ratio of A7r5 cells determined by the axial ratio of the major axis to minor axis length for the ellipse fitted for each cell. Asterisks indicate Student's T-test P values of <0.05 for significance of difference compared to the aspect ratio of cells on the native, uncrosslinked PEMU with a uniform modulus. (B) Percentage of A7r5 cells, at indicated positions along the gradient, with an absolute major axis angle orientation to the gradient axis of between $0-45^\circ$ or $46-90^\circ$, with corresponding Chi Squared P values for the significance of difference from a 50:50 (i.e. random) distribution. (C) DIC images of A7r5 cells on the indicated regions of the PEMU modulus gradient superimposed on fluorescent images of DAPI-stained cell nuclei (scale bar = $100\mu\text{m}$).

Figure 7.

Actin filament organization in A7r5 cells on PEI(PAH/PAABp)₁₅ PEMUs with uniform modulus or a steep modulus gradient. Actin filaments were immunofluorescently stained with Texas Red Phalloidin in cells cultured on (A) a uniform, native PEI(PAH/PAABp)₁₅ PEMU, (B) the most compliant region of edge modulus gradient, (C) at the position of the edge mask on a modulus gradient, (D) the stiffest region of edge modulus gradient, (E) a uniform, maximally-crosslinked PEI(PAH/PAABp)₁₅ PEMU, (F) an uncoated glass coverslip, and (G) a PEI(PAH/PAABp)₁₅ PEMU edge modulus gradient (a composite of sequential images taken along the gradient). The dotted line (Edge) indicates position of the optical mask edge gradient along the 1mm region from Figure 3 (A–F scale bars = 20μm and G scale bar = 100μm).

Figure 8.

Wind rose plots of A7r5 cell migration on native and crosslinked PEI(PAH/PAABp)₁₅ PEMUs with uniform modulus and a modulus gradient. The motility of ten cells each on (A) a native, uniform modulus PEMU, (B) a crosslinked, uniform modulus PEMU, and (C) a modulus gradient at the masked edge of crosslinking were mapped with Wind rose plots. Points on each plot represent the relative position of the cell nucleus at 10-minute intervals over 24 hours. The relative starting point for each cell on the two uniform compliance PEMUs was set to 0,0 (A and B). For each cell on the modulus gradient (C), the x-axis starting point (on the left most side for each cell) represents the initial position of the cell relative to the gradient, and the y-axis starting point is arbitrarily positioned to optimize

visualization of non-overlapping tracks. Respective modulus values for real distance (μm) along the Edge gradient are from Figure 3.

Figure 9.

A7r5 and U2OS cell adhesion and spreading on uniform modulus and steep modulus gradient PEI(PAH/PAABp)₁₅ PEMUs. A7r5 cells (top) and U2OS (bottom) were cultured on steep edge modulus gradients (Edge; dotted line indicates position of the optical mask edge gradient along the 1 mm region from Figure 3); uniform uncrosslinked (Native); and uniform maximally photocrosslinked (Crosslinked) PEMUs; and uncoated glass coverslips (Glass). Images of the cells were acquired at 24, 48, and 72 h after plating the cells on the PEMUs (scale bar = 100μm).

PAH **PAABp**

Scheme 1.
Structures of polyelectrolytes used in this study ($m=0.05$).