Directional Migration of Vascular Smooth Muscle Cells Guided by a Molecule Weight Gradient of Poly(2-hydroxyethyl methacrylate) Brushes

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ABSTRACT: Directional migration of cells mediated by gradient cues in vitro can mimic the corresponding biological events in vivo and thereby provides a way to disclose the cascade responses in tissue regeneration processes and to develop novel criteria for design of tissue-inductive biomaterials. In this work, a molecular weight gradient of poly(2-hydroxyethyl methacrylate) (PHEMA) brushes with a thickness ranging from 3 to 30 nm and slopes of 0.8−3.2 nm/mm were fabricated by using surface-initiated atom transfer radical polymerization (ATRP) and a dynamically controlled reaction process. The PHEMA gradients were characterized by X-ray photoelectron spectrometry (XPS) and ellipsometry. The adhesion number, spreading area, adhesion force, and expression of focal adhesion and actin fibers of vascular smooth muscle cells (VSMCs) decreased along with the increase of the PHEMA brushes length. The VSMCs exhibited preferential orientation and enhanced directional migration toward the direction of reduced PHEMA thickness, whose extent was dependent on the gradient slope and polymer thickness. Most of the cells were oriented, and 87% of the cells moved directionally at the optimal conditions.

1. INTRODUCTION

Directional cell migration plays an essential role in many physiological processes, such as embryonic development, wound healing, and metastasis. Various biological, physical, and chemical cues such as cell growth factors, cytokines, and matrix stiffness are involved in the directional cell migration. Immobilization of these cues onto biomaterials can mimic the natural matrix in vivo and thereby can modulate the cell migration behaviors.

In particular, the biomaterials with gradient cues can better guide the cell orientation and migration. Previous strategies are mainly based on the application of biological cues due to the wide acknowledgment of the strong chemotaxis effect in vivo. For example, the extracellular matrix proteins (ECM), peptides, and growth factors are applied in microchannels, scaffolds, hydrogels, or immobilized on surfaces. Most of them can induce cell orientation, but only a few studies demonstrate the successful guidance of cell migration. For example, on a poly(ethylene glycol) (PEG) hydrogel with an Arg-Gly-Asp (RGD) peptide density gradient, fibroblasts align themselves along and prefer to move up to the gradient. Endothelial cells can sense the gradient of fibronectin and vascular endothelial cell growth factor (VEGF) on glass surfaces and move toward the direction with a higher protein density in the best case.

Despite the success to some extent, the complexity of natural macromolecules and their interactions with cells still challenge the design of biomaterials for tunable cell migration because a variety of factors will influence the cell fates. Also, these proteins and growth factors are expensive and easily denature with reduced activity, limiting their applications. On the other hand, physical and chemical gradients have their effectiveness, too, as cells can also respond to these kinds of cues in vivo, which are known as durotaxis and haptotaxis. For example, both vascular smooth muscle cells (VSMCs) and fibroblasts can undergo direct migration from the soft to stiff regions on surfaces with a stiffness gradient. The topographical gradient, with varying configuration of a surface, can largely affect cell migration behaviors as well. Kim et al. prepared a series of anisotropic substrates with variable local pattern density and found that cells preferentially migrate from the sparser topographical patterns toward the areas with denser ones. Moreover, the PEG grafting amount and the swelling gradient of polyelectrolyte multilayers can significantly influence the cell migration behavior, too.

It is expectable that the hydrophilic polymer brushes with a gradient increase of the molecular weight can have a precise control over the density of hydrophilic units along the gradient, thus generating a water amount gradient. They may provide a gradient cell adhesion force, which in turn tunes the directional cell migration. In this work, poly(2-hydroxyethyl methacrylate)
(PHEMA) brushes with a molecular weight gradient of different steepness shall be prepared by using the surface-initiated atom transfer radical polymerization (SI-ATRP). PHEMA is a hydrophilic polymer which has the so-called antifouling effect.\textsuperscript{22,23} Properly designed PHEMA surfaces can well modulate the protein adsorption and cell adhesion/spreading as well.\textsuperscript{24}–\textsuperscript{26} On the other hand, the ATRP results in narrow molecular weight distribution, allowing facile and precise control over the chain length via reaction time.\textsuperscript{27–30}

For the first time the directional migration of VSMCs is achieved under the guidance of a molecular weight gradient. The optimal directional migration percentage of >80\% is rarely achieved previously and is comparable to that achieved by using biomolecule cues.\textsuperscript{31}

2. METHODS

2.1. Materials. 3-(Aminopropyl)triethoxysilane (APS) was purchased from J&K Co., α-Bromoisoobutyryl bromide (BIBB), N,N,N′,N″-pentamethyldiethylenetriamine (PMDETA), and 2-hydroxyethyl methacrylate (HEMA) were purchased from Sigma-Aldrich. HEMA, dichloromethane, toluene, and triethylamine (Et3N) were vacuum distilled prior to use. All other chemicals were of analytical grade and used as received. The water was purified via a Milli-Q Gradient System equipped with a quantum cartridge and had a resistivity of 18.2 mΩ/cm.

2.2. Immobilization of ATRP Initiators. The procedures of immobilization of ATRP initiators were performed according to the literature.\textsuperscript{27} Briefly, glass or silicon slides (25 × 10 mm) were consecutively washed in toluene, acetone, and ethanol, each for 15 min under ultrasonication. They were further treated with a “piranha” solution (a mixture of 30% hydrogen peroxide and 70% sulfuric acid (v/v)), thoroughly washed with water, and dried under a nitrogen flow. The slides were then immersed in the APS toluene solution (1%, v/v) for 30 min at room temperature. After being washed in toluene, acetone, and ethanol under ultrasonication, the slides were dried under a nitrogen flow and then baked at 120 °C for 4 h to stabilize the ultrathin silane layer. The NH\textsubscript{2}-functionalized slides were immersed into 30 mL of anhydrous CH\textsubscript{2}Cl\textsubscript{2} containing 0.6 mL of triethylamine, into which 0.6 mL of BIBB was dropwise added under gentle shaking in an ice–water bath. Then the reaction mixture was maintained at 0 °C for 2 h and at room temperature for 12 h. The ATRP initiator-functionalized slides were subsequently washed in CH\textsubscript{2}Cl\textsubscript{2}, acetone, and alcohol under ultrasonication, before they were dried under a nitrogen flow.

2.3. Preparation of PHEMA Gradient Brushes. As shown schematically in Figure 1, the initiator-immobilized slide (25 mm in length) was vertically placed in a sealed Pyrex test tube (diameter = 16 mm). 1.21 mL (1 mmol) of HEMA was added into 60 mL of water under a nitrogen atmosphere, into which the initiators were added with a molar ratio of HEMA:CuCl\textsubscript{2}:ascorbic acid:PMDETA of 100:1:2:1. The aqueous ATRP reaction solution was injected continuously to the Pyrex test tube by a microinfusion pump (WZS-50F2, Zhejiang University Medical Instrument Co., Ltd., China) at a desired rate. After reaction at room temperature for a predetermined time period, the slides were thoroughly washed in plenty of water and ethanol under ultrasonication three times.

The chemical compositions of the surfaces were characterized by X-ray photoelectron spectroscopy (XPS) using an Axis Ultra spectrometer (Kratos Analytical, UK) with a monochromatized Al K\textalpha{} source at the pass energy of 160 eV for survey scans and 80 eV for core level spectra. Data were analyzed by Kratos Vision Processing and XPS Peak software. The binding energy was corrected by setting the lowest binding energy of C 1s peak at 284.6 eV.

2.4. Characterization of the Films. 2.4.1. Film Thickness. The thickness of the PHEMA brushes was measured by a variable angle spectroscopic ellipsometer (model VASE; J.A. Woollam Inc., Lincoln, NE) at a 70° angle of incidence and a wavelength range of 300–1700 nm. A Cauchy model was used to fit the data.

2.4.2. Contact Angles and Surface Energy. The static contact angles of water and diiodomethane on the slides were measured by a sessile-drop method on a DSA 100 contact angle measuring system (Krüss, Germany). The volume of each droplet was 2 μL. The results were averaged from five independent measurements. The surface energy was calculated based on these data using the Owens–Wendt–Rabel–Kaelble method.

2.5. Cell Culture Experiment. The human vascular smooth muscle cells (VSMCs) were obtained from the Cell Bank of Typical Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells were maintained with a regular growth medium consisting of high-glucose Dulbecco’s modified eagle’s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Sijiqing Inc., Hangzhou, China), 100 U/mL penicillin, and 100 μg/mL streptomycin and cultured at 37 °C in a 5% CO\textsubscript{2} humidified environment.
2.5.1. Cell Migration. The glass slides with PHEMA brushes were sterilized in 75% ethanol for 1 h, followed with six washes in phosphate buffered saline (PBS, pH 7.4). The cell migration behaviors were studied on the uniform and gradient surfaces with four different PHEMA thicknesses (3, 8, 15, and 30 nm). The VSMCs were seeded at a density of $5 \times 10^3$ cells/cm$^2$ in order to minimize the influence of cell–cell interactions. Approximately 8 h after the cell plating in 10% FBS DMEM, the cell migration behaviors were in situ monitored using a time-lapse phase-contrast microscope (IX81, Olympus) equipped with an incubation chamber (37°C and 5% CO$_2$ humidified atmosphere) over a period of 6 h. The VSMC trajectories were reconstructed from the center positions of individual cells over the whole observation time. The migration rate is calculated via dividing the total cell migration distance ($S$) by the migration time ($t$). 

\[
S = \sum_{i=1}^{t-1} \sqrt{(x_i - x_{i-1})^2 + (y_i - y_{i-1})^2}
\]

At least 30 cells were analyzed for each data point. All mitotic and spherical dead cells were excluded from the analyses.

2.5.2. Cell Adhesion. The glass slides were placed in the wells of a six-well culture plate. VSMCs were trypsinized and seeded into the wells at a density of $5 \times 10^3$ cells/cm$^2$ and incubated at 37°C under a 5% CO$_2$ humidified atmosphere. In order to study the adhesion behavior of VSMCs 24 h post cell seeding on the PHEMA surfaces, the images of cells were recorded under a phase-contrast microscope and the cell number and cell spreading area were analyzed. At least 15 photographs were taken at each appointed site of the gradient surface for statistical analysis.

The relative cell adhesion force of VSMCs on the substrates was measured according to the method suggested in Reyes’s work. After cell seeding for 24 h, the substrates were gently washed with PBS to remove the floating cells. The cell number was counted under a microscope. Then the glass slides were placed vertically at the bottom of centrifuge tubes which were filled with PBS. The number of cells remained on the glass slides was counted, and the fraction of adhesion cells was calculated after being centrifuged at 800 and 1500 rpm for 5 min, respectively. The forces exerted on cells were calculated according to the theory described previously.

2.5.3. Cell Orientation. The images of cells were recorded under a phase contrast microscope 24 h post cell seeding on the uniform and gradient PHEMA surfaces. The cell orientation of at least 100 cells was analyzed using the Image Pro Plus software to calculate the orientation angle of each cell.

2.5.4. Cell Focal Adhesion Complexes and Actin Organization. Fluorescent staining of actin, vinculin, and cell nucleus was carried out to reveal the cell morphology and skeleton organization. Briefly, after the VSMCs were cultured in the medium containing 10% FBS for 24 h, they were carefully washed with PBS three times and then were fixed with 4% paraformaldehyde at 37°C for 30 min, followed by three washes in PBS. The cells were further treated in 0.5% (v/v) Triton X-100/PBS at 4°C for 10 min to increase the permeability of the cell membrane. After being rinsed three times with PBS, they were incubated in 1% BSA/PBS at 37°C for 30 min to block the nonspecific interactions. Then the cells were incubated with a mouse monoclonal antibody against human vinculin (Sigma) for 1 h. After being washed twice in 1% BSA/PBS, they were further stained with FITC-labeled goat antimouse IgG (Beyotime, China), rhodamine phalloidin (Invitrogen), and 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) at room temperature for 1 h, followed by three washes in PBS. The cells were observed under confocal laser scanning microscopy (CLSM, SPS, Leica).

Figure 2. (a) C 1s core-level spectra of the PHEMA brushes with a thickness of 15 nm. (b) Thickness of the PHEMA brushes as a function of gradient position at various injection rates of the reaction solution. (c) Static water contact angle and surface energy as a function of the thickness of PHEMA gradient obtained at an injection rate of 5 mL/h (gradient slope 1.6 nm/mm).
Figure 3. Migration traces of the VSMCs on (a–d) uniform and (e–h) gradient PHEMA (slope 1.6 nm/mm) surfaces with different thicknesses: (a, e) 3 nm, (b, f) 8 nm, (c, g) 15 nm, and (d, h) 30 nm, respectively. At least 30 cells were tracked for each data point. The number on the bottom left side of each image indicates the percentage of cells moving to the −X direction. The mobility of VSMCs was continuously tracked every 30 min for 6 h.

2.6. Statistical Tests. Statistical analysis was performed by One-Way ANOVA with a Tukey posthoc method. A significant level of \( p < 0.05 \) was chosen for all the tests, unless otherwise stated.

3. RESULTS AND DISCUSSION

3.1. Preparation and Characterization of PHEMA Thickness Gradients. To obtain the PHEMA gradients for mediating the directional cell migration, bromoisobutyl bromide molecules (BIBB, which can initiate the ATRP) were uniformly immobilized onto the silicon surface (Figure 1a). For this purpose, the silicon wafer was aminosilanized with 3-(aminopropyl)triethoxysilane (APS). The thickness of the APS layer was measured to be \( \sim 7 \) nm by ellipsometry, and the water contact angle increased to \( \sim 57^\circ \) (Table S1). After immobilization of BIBB, the water contact angle increased further to \( \sim 74^\circ \). XPS characterization (Figure S1) found the appearance of N and Br elements after APS and BIBB immobilization, respectively. According to the change of the amino density before and after BIBB immobilization, the BIBB density was calculated to be \( 1.5 \) nm\(^2\) (Table S1).

Classical catalysts (the Cu(I) complexes) of ATRP reaction are very sensitive to oxygen, and thus the reaction rate is not easy to control during the injection process. Therefore, here the water-soluble ascorbic acid (VC) molecules were adopted as a reducing agent that reacts with oxidative Cu(II) precursors to generate active Cu(I) complexes.\(^{33,34}\) The excessive VC in the system can react with oxygen to prevent the oxidation of the Cu(I) complexes during the reaction, providing smoother control over the reaction rate and thereby the linear increase of the PHEMA chain length along with the time prolongation (Figure S2).

After the surface-initiated ATRP under a continuous injection of the reaction solution, the PHEMA brushes with gradually varied thickness are supposed to graft from the surfaces (Figure 1b). As a typical example, the C 1s XPS spectrum of a PHEMA surface with a thickness of about 15 nm is shown in Figure 2a. The peaks at 288.5, 286.2, and 284.6 eV are assigned to the ether bonds (O–C=O), carbon–oxygen bonds (C–O), and saturated carbons (C–C) of PHEMA, respectively. The peak area ratio of O–C=O, C–O, and C–C is 3:2.3:1, which is close to the theoretical value of 3:2:1 of PHEMA molecules. The XPS spectra at different positions along with the gradient are almost identical, suggesting that the chemical composition of the surface is homogeneous.

Figure 2b shows that the brushes grew almost linearly along with the position on the slide. Since the length of the gradient is predetermined to be 25 mm, a total of 5 mL reaction solution was needed. At three different injection rates of 5, 10, and 20 mL/h the total periods of time (i.e., the reaction time of the bottom substrate, which is defined as the 25 mm position (Figure 1)) were 60, 30, and 15 min, respectively. These three injection rates resulted in the gradients with a slope of 3.2, 1.6, and 0.8 nm/mm, respectively. The sharper gradient at a slower injection rate is caused by the larger difference of reaction time between the two ends of the substrate. The gradients with even sharper slopes could be generated by using a slower injection rate. However, in that case, the linearity of the gradient is not well controlled due to the reduced polymerization activity at longer reaction time. All the results confirm that the gradient PHEMA brushes with a linear gradient in thickness are successfully achieved. Since the initiators are uniformly immobilized on the surface and the nature of ATRP endows the same chance to be initiated for all the initiators, the thickness gradient should be only attributed to the molecular weight difference. As a hydrophilic polymer, the PHEMA films can swell in PBS and reach 2 times of the pristine thickness in a dry state along the entire gradient (data not shown).

It is known that the PHEMA is a typical hydrophilic polymer which can impede protein adsorption and cell adhesion. Figure 2c shows that the water contact angle on the PHEMA gradient surface decreased along with the increase of PHEMA thickness and reached a plateau (\( \sim 50^\circ \)) at 20 nm, which is consistent
with those of PHEMA surfaces obtained by coating (59°) and photografting (53°). As a hydrophilic polymer, the PHEMA film can swell 2 times in water compared to its initial thickness in air (data not shown).

Figure S3 shows the typical surface morphology of the PHEMA brushes. The PHEMA surfaces with different thickness are quite smooth, whose representative root-mean-squared (rms) roughness is less than 10 nm, suggesting that the possible influence of surface morphology on cell behaviors could be safely ruled out.

3.2. Cell Migration on the PHEMA Surfaces. The surface-grafted PHEMA brushes can behave the so-called “antifouling effect”, leading to strong repellence of protein and blood cell adsorption. However, their effect on adhesion and, in particular, migration of anchorage-dependent cells such as VSMCs has rarely been addressed so far. The cell adhesion and migration is a basic process for the development and regeneration of tissues such as blood vessel, which is significantly influenced by the biomaterials’ properties and thereby should be paid preferential attention.

The cell migration trajectories on various PHEMA surfaces were recorded by a time lapse microscope and reconstructed into an X−Y plane for intuitive comparison (Figure 3). For the ease of discussion, the direction of the gradient with increasing PHEMA thickness (from top to bottom) was set as the X coordinate (Figure 4a). The same X coordinate is assigned to the uniform PHEMA surface as the gradient substrate during cell culture. Figure 3a−d shows that the cells moved randomly without a preferential direction on the uniform PHEMA surfaces regardless of the PHEMA thickness, although those on the surface with a moderate PHEMA thickness traveled a longer distance. By contrast, most of the cells moved toward the −X direction on the gradient PHEMA brushes with a moderate thickness (Figure 3e−g and Figure S4), confirming the haptotactic effect of the PHEMA gradients. As shown in Figure 4b, the degree of the haptotactic effect is influenced by both the thickness and gradient steepness of the PHEMA brushes. At the place with 3 nm thick PHEMA brushes, only 58% of the cells move to the −X direction when the steepness of the gradient is the smallest (0.8 nm/mm), suggesting that the haptotactic effect is not obvious. The haptotactic effect was significantly enhanced at the sharper gradient steepness such as 1.6 and 3.2 nm/mm, on which the directional migrating cells increased to nearly 70%. At the place with 8 nm thick PHEMA brushes, the cell percentage moving to the −X direction is improved from 53% on the uniform surface to 71%, 87%, and 83% on the gradient brushes with the steepness of 0.8, 1.6, and 3.2 nm/mm, respectively (see also Video S1). The haptotactic effect of the gradient PHEMA brushes was still maintained to some extent on the thicker places (30 nm) compared to that of the uniform one but was basically lowered down compared with that on the 8 nm thick gradient brushes. Generally, the haptotactic effect of PHEMA gradient brushes is more obvious at the larger steepness (1.6

Figure 4. (a) Schematic illustration of cell migration parameters. d represents the total migration trajectory recorded by the time-lapse microscope. x represents the final distance between the end of the observation time and original position. y and z stand for the component of d along X-axis and Y-axis, respectively. The positive orientation of the X-axis is the increase of PHEMA thickness. The numbers indicate the percentages of cells moving to the −X direction. (b) The percentage of cells moving to the −X direction and (c) the migration rate of VSMCs on the uniform and gradient surfaces with variable gradient slope (0.8, 1.6, and 3.2 nm/mm) and PHEMA thickness. Asterisk (*) indicates a significance difference at p < 0.05.
and 3.2 nm/mm) with a moderate PHEMA thickness (8 and 15 nm). The maximal directional cell migration reached up to 87% within ±90° of the −X direction and 70% within ±60° of the −X direction with only the PHEMA thickness gradient cue, respectively (Figure 4b and Figure S5a). The real-time X-axis moving ratio (XMR, the ratio of the cells moving to the −X direction averaged 12 times within 6 h) confirms further the directional VSMCs migration (Figure S5b).

Different from the migration direction, the cell migration rate was only dominated by the thickness of the PHEMA brushes regardless of their distribution manner on the surface (Figure 4c and Figure S6). It decreased monotonously from ∼25 μm/h (p < 0.05) on the 3 nm PHEMA brushes to 8 μm/h (p < 0.05) on the 30 nm surfaces. A detailed characterization on the uniform PHEMA brushes (Figure S6) revealed that the largest migration rate (∼26 μm/h) appeared on the 5 nm PHEMA brushes, which is significantly larger than that on the BIBB-immobilized surface (15 μm/h) as well (p < 0.05).

The migration behaviors of mesenchymal stem cells (MSCs) on the PHEMA brush gradients were also studied. As shown in Figure S7ef, the MSCs showed preferable migration toward the −X direction of the gradient as well. In contrast, they moved randomly on the uniform PHEMA brushes (Figure S7a−d). These results suggest that the PHEMA brush gradients can guide the directional migration of different types of cells. Again, the migration rate of MSCs was influenced by the thickness of the PHEMA brushes, too (Figure S8).

3.3. Cell Adhesion and Orientation on Gradient Surface. The cell mobility is intrinsically tied with the adhesion and orientation behaviors of cells on the substrates. According to our observation, there is no significant difference of cell orientation between 12 and 24 h post cell seeding. Therefore, the cell orientation was analyzed at 24 h due to the ease of experimental operation. Figure 5a shows that along with the increase of PHEMA thickness the cell adhesion density decreased almost linearly regardless of the distribution manner of the PHEMA molecules, i.e., uniform or gradient. When the PHEMA brushes were the thickest (30 nm), the cell adhesion density reached the smallest value of 90 cells/mm² on both the uniform and gradient surfaces. The cell spreading area (Figure Sb) and cell adhesion force (Figure Sc; the values are calculated from the remained cell numbers after centrifugation as shown in Figure S919,32) show a similar alteration pattern along with the increase of PHEMA thickness and reached the smallest values at the end position (30 nm) regardless of the molecular distribution, too. However, no significant difference was found between the 0 and 3 nm positions in terms of the cell adhesion density and spreading area on both the gradient and uniform surfaces (p > 0.05). It is known that the anchorage-dependent cells must adhere onto a substrate and form focal adhesions before the subsequent cellular events such as spreading and migration. Along with the increase of the thickness of PHEMA with the “antifouling” ability, the substrate provides gradually weakening interaction with cells, leading to the progressively decreased cell adhesion force and thereby the smaller cell density and cell spreading area. The strong adhesion of VSMCs on the BIBB-immobilized surface will impair the release of their rear ends, leading to a slower migration rate. By contrast, the poorly adhered cells cannot grasp the surface with enough dragging force by the leading...
Figure 6. CLSM images showing the VSMCs on (a–d) 16 nm/mm gradient (slope 1.6 nm/mm) and (a′–d′) uniform PHEMA surfaces with a thickness of (a, a′) 3 nm, (b, b′) 8 nm, (c, c′) 15 nm, and (d, d′) 30 nm. F-actin was stained by phalloidine (row 3, red), vinculin was stained by its monoclonal antibody (row 4, green), and nucleus was stained by DAPI (row 5, blue). Row 1 (low magnification) and row 2 (high magnification) are merged images. The cells were cultured on the surfaces for 24 h. The scale bars are 50 μm for row 1 and 25 μm for others. The cartoons and numbers on the top of images represent the types of surfaces and the thicknesses of the PHEMA brushes.

Figure 7. Distribution of the VSMCs angles to the gradient direction on the 1.6 nm/mm PHEMA thickness gradient with a thickness of (a) 3, (b) 8, (c) 15, and (d) 30 nm. At least 100 cells were analyzed for each data point.
edge (the lamellipodia), resulting in a slower migration, too. However, these results cannot sufficiently explain the strongest haptotactic effect on the gradient PHEMA surfaces with thicknesses of 8 and 15 nm (Figure 4b).

The focal adhesion is universally required for most of the cellular events such as adhesion, spreading, survival, proliferation, migration, and differentiation by tuning cytoskeleton reorganization and intracellular signaling, allowing force transfer from the contractile actomyosin cytoskeleton inside the cell to the outside surface. Actin is also the major structure in microfilaments whose polymerization and depolymerization are key steps in the cell adhesion and movement. Therefore, the morphology of VSMCs 24 h post cell seeding on the gradient PHEMA brushes was observed by staining F-actin, vinculin, and nucleus (Figure 6). The cell orientation was further quantified by measuring the angular separation between the major axis of the cell and the gradient direction 24 h post cell seeding (Figure 7).

On the gradient (Figure 6a) and uniform (Figure 6a') PHEMA surfaces with a thickness of 3 nm, the VSMCs possessed a polygon shape without preferential orientation. The stressed actin framework and vinculin plaques were clearly visible. By contrast, the VSMCs tended to align along the 8 nm gradient PHEMA (Figure 6b), and 67% of the elongated cells oriented in ±45° to the X-direction (Figure 7b). The cell orientation was further improved to 74% (Figures 6c and 7c) on the 15 nm gradient surface. Comparatively, the VSMCs on the 8 and 15 nm uniform PHEMA brushes still showed random alignment although they were similarly elongated with bundle-shaped actin filaments on the cell edges (Figures 6b'c'). Figures 6c and 7c also show that most of the cells aligned within a small angle, and even into a line (Figure 6c) on the 15 nm PHEMA gradient, suggesting the strongest inducing effect of the gradient surface on the cell alignment. This perfect alignment is caused by the synergic effect of gradient signal and polymer thickness. If the cell−substrate interactions are too strong such as on the thinner PHEMA brushes, the cells will spread in all directions without preferable orientation. When the cell−substrate interactions become rather poor, i.e., on the 15 nm PHEMA surface, the cells tend to minimize the contacting area especially on the thicker part of PHEMA brushes. Therefore, the cell adhesion force is different along the gradient. The focal adhesion will transfer this signal to cytoskeleton, which in turn influences its distribution and leads to the parallel alignment to the gradient and even form a line as also observed previously. This kind of cell alignment would enhance the cell−cell interaction and provide signaling cues to guide the directional cell migration.

Figures 6d,d' shows that the VSMCs became polygon again on the 30 nm PHEMA brushes regardless of the molecular distribution, accompanied by the significant reduction of the spreading area, actin filaments, and vinculin plaques compared to those on the 3 nm PHEMA brushes. Although Figure 7d shows a high percentage of cells aligned within a small angle of the gradient direction, the absolute number of the aligned cells is very small. Indeed, 64% of the cells were round without orientation (Figure S10) and were not calculated in the statistics. At such a case, some of the cells are able to sense the gradient and protrude their lamellipodia, but the observed alignment is likely the direction of lamellipodia instead of the nomenclon of cells (Figure 6d). It is conceivable that the very poor adhesion of cells cannot provide strong enough force to drive the reorganization of the cytoskeleton along the gradient, leading to the poorer directional migration.

So far many gradient materials with gradual changes in physical properties, chemical compositions, and density of biomolecules have been prepared. However, only very a few of them are used to guide the cell directional migration, and even fewer have achieved remarkable success. For example, Gunawan et al. fabricated a series of laminin density gradients, on which about 65% cells showed directional movement (within ±60° in the direction of gradient). DeLong et al. prepared gradient hydrogels with tethered basic fibroblast growth factor (bFGF), on which the percentages of directional cell migration was improved by 15% in maximum. Without introducing any expensive and easy-to-denature biomolecules, over 80% of the cells (70% of the cells within ±60° in the direction of gradient) move toward the direction with reduced PHEMA chain length at the optimal conditions in the present study. Moreover, the gradients can be further modified by introducing other functional segments to implement complex cellular functions, such as selective cell attachment and/or guided differentiation.

4. CONCLUSION

The thickness gradients of PHEMA brushes with controllable slopes (0.8−3.2 nm/mm) were fabricated by a surface-initiated ATRP reaction combined with an injection method. The cell adhesion number, spreading area, and adhesion force as well as expression level of focal adhesion and actin fibers decreased along with the increase of PHEMA molecule length. The VSMCs exhibited preferential orientation and enhanced directional migration behaviors on the gradient surface toward the reverse direction of PHEMA gradient with a proper slope and brush length. For example, most of the cells were oriented and 87% cells moved directionally toward the reverse direction of the PHEMA gradient on the 1.6 nm/mm gradient with 8 nm PHEMA brushes. The results demonstrate a versatile strategy to control the cell directional migration by the polymer brush length gradient, providing a new perspective on designing complex biomaterials with advanced functions for tissue regeneration.

ASSOCIATED CONTENT

Supporting Information
Physicochemical characterizations of the Si-NH2 and Si-BIBB substrates by XPS, AFM, water contact angle, and chemical analysis, and of uniform PHEMA brushes by ellipsometry and AFM; migration traces of the VSMCs on 0.8 and 3.2 nm/mm gradient PHEMA surfaces; the percentage of cells moving to the −X direction within ±60° of the gradient, and real-time X-axis moving ratio; the migration rate of VSMCs on uniform PHEMA surfaces; migration traces and rates of MSCs on uniform and gradient PHEMA surfaces with different thicknesses; remained percentages of cells after centrifugation with different rates; percentage of nondirectional cells on PHEMA gradient; and videos of cell migration. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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REFERENCES

(21) Han, L.; Mao, Z.; Wu, J.; Gao, Y.; Ren, T.; Gao, C. Directional cell migration through cell–cell interaction on polyelectrolyte multilayers with swelling gradients. Biomaterials 2013, 34, 975–984.


