Tuning Cell Adhesion on Gradient Poly(2-hydroxyethyl methacrylate)-Grafted Surfaces

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A simple yet versatile method was developed to prepare a low-density polymerization initiator gradient, which was combined with surface-initiated atom transfer radical polymerization (ATRP) to produce a well-defined poly(2-hydroxyethyl methacrylate) (HEMA) gradient substrate. A smooth variation in film thickness was measured across the gradient, ranging from 20 Å to over 80 Å, but we observed a nonmonotonic variation in water contact angle. Fits of X-ray reflectivity profiles suggested that at the low graft density end, the polymer chain structure was in a "mushroom" regime, while the polymer chains at high graft density were in a "brush" regime. It was found that the "mushroom" region of the gradient could be made adhesive to cells by adsorbing adhesion proteins, and cell adhesion could be tuned by controlling the density of the polymer grafts. Fibroblasts were seeded on gradients precoated with fibronectin to test cellular responses to this novel substrate, but it was found that cell adhesion did not follow the expected trend; instead, saturated cell adhesion and spreading was found at the low grafting density region.

Introduction

Materials for controlling cell-material interactions have received significant attention recently because they offer the capability to guide cell differentiation and modulate host−biomaterial interactions. The main strategy currently used is to control cell adhesion by incorporating ligands for specific cell receptors in the material. Using such a targeted approach, it has been shown that cell migration,[1] proliferation, and phenotype can be tuned.[2,3] Ultimately this also leads to a greater understanding of tissue development, allowing realization of the potential to engineer living tissue. One of the challenges in controlling receptor−ligand interactions between an adherent cell and substrate is the design of substrates that are defined on the molecular level.[4] Alkane thiol or chlorosilane self-assembled monolayers (SAMs) satisfy many of the design criteria for creating such substrates. Using facile chemical techniques, it is possible to create close-packed substrates designed to promote or resist protein adhesion.[5−7] Although this approach has been widely used to provide the model surface for biomaterials to control cell behavior, the use of polymeric surfaces has several distinct advantages. The ability to prepare a polymer surface that consists of the same monomers as found in technologically relevant biomaterials provides a broad range of surface properties. It is also possible to synthesize surface-grafted copolymers that are organized on nanometer-length scales, thus creating a method for organizing cell-signaling functional groups. Although polymer brushes consisting of poly(ethylene glycol) (PEG) have been used by several research groups to modulate protein adsorption, only a few recent reports used the well-defined polymer surface prepared from various "controlled" polymerization techniques to study protein/cell material interactions.[8−10]

Recently, our group and others have demonstrated that gradient techniques provide a fast and convenient tool for high-throughput screening of polymer surfaces over a spectrum of material parameters.[11−14] In this paper, we present the use of atom transfer radical polymerization (ATRP) to produce surfaces of well-defined surface chemistry that offers means for controlling protein adsorption. Here, we combined these techniques to produce the well-defined gradient polymer surface. We chose 2-hydroxyethyl methacrylate (HEMA) as the monomer in this study because poly(HEMA) is an important polymer that has been used for ophthalmic uses, including contact lenses, as well as in many drug delivery and tissue engineering applications.[15] We have developed a simple yet powerful technique to prepare gradients of polymerization initiator to combine with surface-initiated HEMA polymerizations. By this method, well-defined gradient poly(HEMA) sur-

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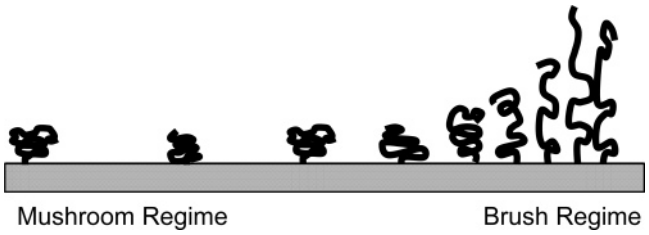
Scheme 1. Schematic Illustration of the Gradual Transition from Mushroom Regime to Brush Regime with Increased Grafting Density



faces were prepared to control cell adhesion and cell morphology.

Experimental Section

Certain commercial materials, instruments, and equipment are identified in this manuscript to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials, instruments, or equipment identified are necessarily the best available for the purpose.

Materials. All chemicals were purchased from Aldrich and used as received unless otherwise specified. High-purity 2-hydroxyethyl methacrylate (HEMA) was obtained from PolySciences, Inc, and octyltrichlorosilane (OTS) was purchased from Gelest, Inc. Copper(I) chloride (CuCl) was purified by stirring in acetic acid overnight, washing with ethanol, and filtering to collect the solids.

Formation of OTS Monolayer. The silicon wafers were cut into (3 × 1) cm^2 strips and treated with UV/ozone for 30 min. A solution of octyltrichlorosilane in mineral oil (mass fraction of 50%) was placed underneath the silicon wafers. As the OTS evaporated, it diffused into the vapor phase and generated a 10-Å-thick self-assembled monolayer (SAM) on the silica substrate. The formation of the SAM on the silicon substrate was confirmed by the contact angle (≈107°).

Preparation of a Gradient of Polymerization Iniator. The ATRP initiator was synthesized according to the literature from Matyjaszewski and co-workers.[16] The initiator included 2-bromoisobutyrate fragments to initiate ATRP of vinyl monomers, and chlorosilane segments to covalently bond to the substrates by reacting with silanol group on the silicon wafer surface.

The initiator concentration gradient was prepared by slowly pumping the initiator solution into the test tube, which contained the OTS SAM-covered silicon wafer. The width and position of the initiator gradient could be tuned by the pumping rate, which could be conveniently adjusted.

Fabrication of Poly(HEMA) Gradient. Homopolymer of HEMA from the surface was prepared according to the methodology proposed by Baker and co-workers.[17] Specifically, 18 mL of an aqueous solution of monomer (HEMA/H_2O, 1:1 v:v) was deoxygenated by purging argon through the solution for at least 50 min. Then the solution was transferred into a flask containing 110 mg (1.10 mmol) of CuCl, 72 mg (0.32 mmol) of $CuBr_2$, and 488 mg (3.12 mmol) of bipyridine (bpy), which was stirred until a homogeneous dark brown solution formed. The solution was then transferred into a rubber-septum-sealed vial containing the gradient silicon wafer that was degassed with three vacuum/argon fill cycles, and the vial was kept at room temperature for 30 min of polymerization.

Characterization of Polymer Gradients. Polymer film thickness was measured by a variable angle spectroscopic ellipsometer (J. A. Woollam, Inc.) using a refractive index of 1.5119. Contact angle measurements were performed at room temperature with a Krûss G2 contact angle measuring system using water as the probe fluid. X-ray reflectivity measurements were performed with a θ/θ diffractometer with Ni-filtered Cu K_α radiation (λ = 1.54Å) and soller slits on both the incident collimation and reflected beam. The real space density profile was determined by modeling the reflectivity using a multilayer, least-squares fitting algorithm.[18]

Protein Adsorption. Poly(HEMA) gradient samples were incubated in bovine fibronectin (25 μg/mL in Dulbecco's phosphate buffered saline (DPBS)) for at least 5 h at 4 °C, then rinsed with DPBS and water. Subsequently, the samples were dried under nitrogen. The thickness of the FN layer on a poly(HEMA) gradient sample was determined in two steps via spectroscopic ellipsometry. First, the optical constants of the substrate layer (i.e., poly(HEMA), SiO_2, Si) were determined by fitting ellipsometric data collected on the sample prior to protein exposure. Next, ellipsometric data were taken after protein exposure. These latter data were then modeled using two layers, one for the substrate and one for the adsorbed protein on top of it. The optical constants used for the substrate layer were those obtained from the initial ellipsometric measurements described previously. The optical constants of the protein layer were fixed (n = 1.45, k = 0).[19] The thickness of the protein layer in the model was then altered to fit the measured ellipsometric data.

Cell Culture and Cell Image Analysis. All cell culture reagents were from Sigma (Sigma, St. Louis, MO) unless indicated otherwise. Substrates were incubated in fibronectin (25 mg/mL in Dulbecco's phosphate buffered saline (DPBS)) for at least 5 h at 4 °C and rinsed with DPBS before use. NIH3T3 fibroblasts (ATCC, Manassas, VA), were maintained in Dulbecco's modified Eagles medium (DMEM; Mediatech, Herndon, VA) supplemented with nonessential amino acids, glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% (by volume fraction) fetal bovine serum (FBS; Gibco Invitrogen, Carlsbad, CA) and maintained in a humidified 5% (by volume fraction) CO_2 balanced-air atmosphere at 37 °C. Subconfluent cultures were switched to supplemented DMEM containing 5% (by volume fraction) FBS 24 h prior to an experiment. Cells were removed from tissue culture polystyrene flasks by trypsinization, washed with DMEM/5% FBS, centrifuged for 5 min at 105 rad/s and plated in DMEM/5% FBS onto the substrates at a density of 2000 cells/cm^2. Substrates were placed in four-well polystyrene plates, and NIH-3T3 cells were seeded on the substrates. Substrates were removed from the incubator after 8 h, rinsed with Hanks balanced salt solution (HBSS; ICN Biomedicals, Costa Mesa, CA) supplemented with 10 mM HEPES, and fixed for 24 h at room temperature in 100 mM PIPES, 1 mM EGTA, 4% PEG 8000, pH 6.9, containing 100 ug/mL 3-malemido-benzoic acid-NHS ester (MBS, Sigma) as the cross-linker. Cells were permeabilized in 0.05% Triton X-100 in DPBS, rinsed in DPBS, and incubated with DPBS containing Texas Red/C2-maleimide (10 ng/mL) as a general stain and 0.05% 4′,6-diamidino-2-phenylindole (DAPI) as a nuclear counterstain. After 2 h at RT, 1% bovine serum albumin (BSA) was added to quench the conjugation reaction, and the substrates were rinsed with DPBS. The substrates were mounted onto thin glass slides with 9:1 glycerol/Tris, pH 8.0, and the cells were imaged with an automated fluorescence microscope as previously described.[20] Images were collected with 1-mm step sizes over the entire area of the gradient samples, and individual cell morphology and cell density (number of cells/frame) were determined with image analysis software. Morphology data for individual cells were imported into spreadsheet software and manipulated so that cell data for each position along the gradient were grouped accordingly.[20]

Results and Discussion

Preparations and Characterizations of Poly(HEMA) Gradient. A vapor diffusion method similar to that of Wu et al. was used to prepare a gradient of polymerization initiator and resulted in a polymer graft-

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density gradient.[21] However, rather thick gradient poly(HEMA) films with thickness ranging from 162 to 337 Å were obtained, and no cell adhesion was observed, presumably due to complete blocking of serum adhesion proteins. Moreover, when using this method, it can be difficult to gain control over the linear gradient profile of grafting density due to the reliance on vapor diffusion. Therefore, we developed a new method based on the defects in SAMs in which an octyltrichlorosilane monolayer was prepared on the silicon wafer.[22,23] The gradient of polymerization initiator was established by gradually pumping the initiator solution to backfill the defects inside the OTS monolayer. The lower end has a longer reaction time between the silicon wafer and the initiator solution, and this resulted in higher initiator density, whereas the upper end has a shorter reaction time between the initiator solution and the silicon wafer and resulted in a lower initiator density. Various gradient profiles could be obtained by adjusting the pumping rate. In this study, the linear gradient profile was targeted by a fixed pumping rate. The grafting density profile could be evaluated by the polymer film thickness because of the following: all the polymers grafted on the substrate have the same degree of polymerization, which could be assumed because of the "controlled" polymerization nature of ATRP.[24] For example, Huck and co-workers reported that the polymerization rate is independent of initiator density.[25] The variation of the polymer film thickness can be attributed to the difference in the initiator grafting density, σ. The grafting density can be calculated from $\sigma = h\rho N_A/M_n$, where h is the polymer film thickness, ρ is the density of polymer, N_A is Avogadro's number, and M_n is the number average molecular weight of the polymer.[21] At the dense end of the gradient, we assume the polymer chains are packed tightly enough so as to form a "brush" structure, with chains extending normal to the surface. At the sparse end of the gradient, chains are spaced far enough from each other so that they are expected to spread parallel to the surface; the structure in this region is generally referred to as a "mushroom". The polymer chain conformation at different grafting density in the library will be discussed in more detail later. A schematic representation of the gradient is shown in Scheme 1. Since it is well-known that fibronectin (FN), a cell adhesive protein, adsorbed strongly on hydrophobic surfaces such as an OTS surface,[26,27] and poly(HEMA) can effectively resist protein adsorption,[15] we hypothesize that the "mushroom" region of the gradient library would allow adhesion-protein adsorption in the regions between polymer grafts and thus make it possible to tune the density of adhesion ligands on the substrate. The experiments described later support this hypothesis.

Ellipsometry was used to measure the film thickness of gradient poly(HEMA) as a function of position, the results of which are shown in Figure 1. A linear increase in film thickness ranging from (18 ± 6) to (80 ± 12) Å was measured across the gradient. The water contact angle data are also shown in Figure 1. Although a monotonic

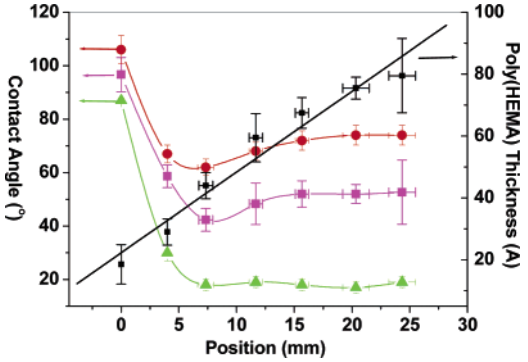

Figure 1. Water contact angle. Circles, rectangles, and triangles represent the advancing, static, and receding contact angles, respectively.

decrease in water contact angle was expected with increasing polymer thickness, a minimum was found in static and advancing contact angle measurements at a polymer film thickness around 40 Å before they reached the plateaus, and no minimum was found in receding contact angle measurements. It is important to point out that the large difference in advancing and receding contact angle was consistent with the literature report. For example, Holly and Refojo investigated wettability of poly(HEMA) hydrogels, and they attributed the large hysteresis to the functional group reorientation. They proposed that in the dehydrated state, polar side groups are buried in the bulk, and hydrophobic backbone methyl groups are pointed outward from the surface, whereas in the hydrated state, the hydrophilic hydroxyethyl groups were reoriented outward and hydrophobic methyl groups were buried inside.[28] This dynamic behavior of a poly(HEMA) surface has been observed by different research groups with various surface characterization techniques.[29–30] Since OTS is more hydrophobic than air, it is energetically favorable for methyl groups oriented toward a poly(HEMA)/OTS interface. We propose that the "forced" exposure of pendant hydroxyethyl groups to the air in the "mushroom" regime is responsible for this unusual dependence of contact angle on graft density. In the brush regime, there could be intermolecular hydrogen bonding between the hydroxyethyl groups, preventing them from orienting toward air; however, to the best of our knowledge, the low graft density region synthesized in this study has not been investigated previously.

Information regarding the polymer chain conformation can be inferred from the density profiles obtained from X-ray reflectivity. Due to the large footprint of the X-ray beam, uniform poly(HEMA) films were required for these measurements. The thickness of these uniform films was controlled by the exposure time of the OTS-covered silicon wafer to the initiator solution. Figure 2a shows the reflectivity profiles as a function of the momentum transfer vector, q, where $q = 4\pi \sin(\theta)/\lambda$, θ is the incident angle, and λ is the radiation wavelength, in terms of Rq^4, where R is the fraction of the incident beam specularly reflected, to compensate for the q^{-4} decay due to Fresnel's law. The best-fit electron density profile ($Q_c^2 = 16\pi Nb$, where N is the number of electrons and b is the average atomic scattering length) for each of the reflectivity curves is shown as a function of distance from the silicon substrate

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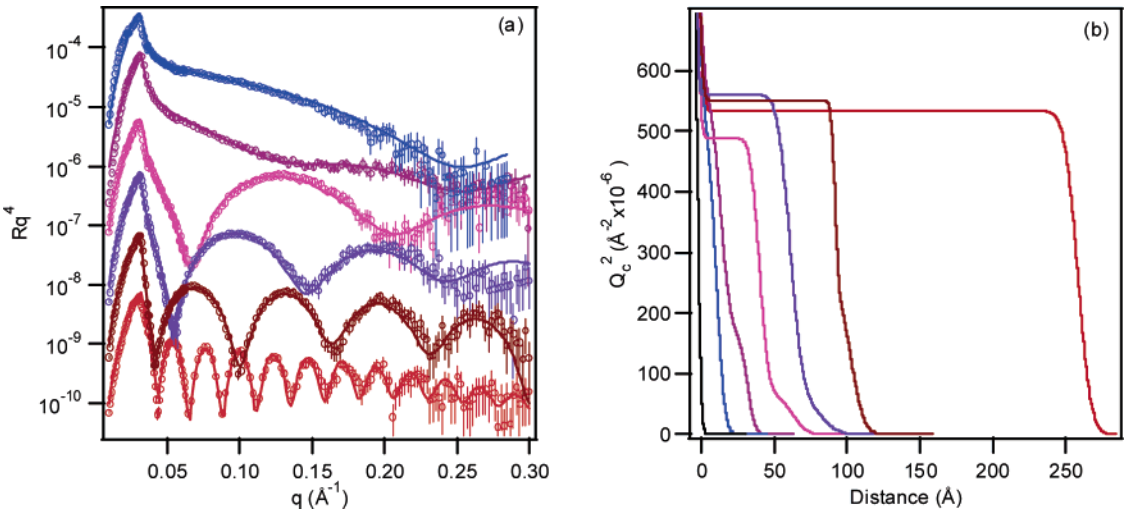

Figure 2. (a) Reflectivity profiles for different poly(HEMA) thicknesses. From top to bottom: the blue curve corresponds to the OTS-treated wafer without poly(HEMA), followed by poly(HEMA) film with thickness 30, 50, 70, 100, and 250 Å. Curves are offset for clarity. (b) From left to right: the electron density profile of wafer; OTS-treated wafer without poly(HEMA); and poly(HEMA) films with thickness 30, 50, 70, 100, and 250 Å. The lines in (a) are the best fits of the reflectivity corresponding to the electron density profiles in (b). The black line is typical for a silicon wafer. The distance is in terms of distance from the silicon substrate.

in Figure 2b. These density profiles correspond to the solid lines in Figure 2a. It is important to note that the thickness obtained from fitting the ellipsometry data with the bulk refractive index for poly(HEMA) is in agreement with those obtained from XRR, which considers heterogeneities resultant from incomplete surface coverage. Although it is not accurate to model the films using the refractive index for bulk poly(HEMA) at low grafting densities, the change in refractive index is small enough that the thickness measured by ellipsometry agrees well with XRR.

It can be clearly seen that the thinnest poly(HEMA) film, corresponding to the 30-Å-thick film from ellispometry, only partially covered the OTS layer. As the grafting density increases (thickness), a plateau in Q_c^2 is observed, corresponding to complete surface coverage. This strongly suggests that at the low graft density end, the polymer chain structure was in a "mushroom" regime, and the polymer chains at high graft density were in "brush" regime. The change in the electron density profile suggests that the transition from partial to complete surface coverage of the poly(HEMA) on the OTS layer in the dry state corresponds to the minimum contact angle.

Protein Adsorption and Cell Culture Studies. In this study, the amount of adsorbed fibronectin on a poly(HEMA) grafted surface was evaluated by ellipsometry because it was extensively used to measure protein adsorption on various surfaces.[31,32] The adsorbed fibronectin layer thickness along the poly(HEMA) gradient library is shown in Figure 3. It is clear that a fibronectin gradient was established by backfilling the open space between poly(HEMA) grafts. In the "mushroom" region of the gradient library, fibronectin can get adsorbed between poly(HEMA) grafts, but no detectable fibronectin was found at the high grafting density end of the gradient library.

Cell adhesion experiments were performed with the fibroblast 3T3 cell line to investigate how these surfaces are capable of modulating cellular responses. An incubation period of 8 h was chosen in this study because it is adequate for good cell adhesion and cell spreading, and

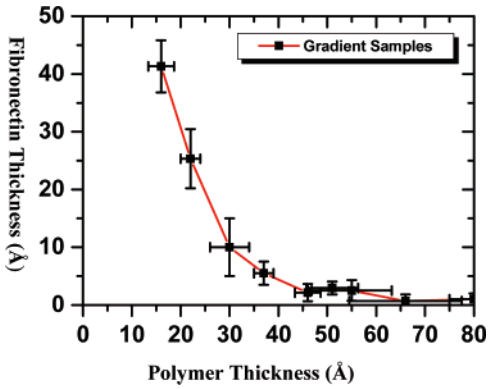

Figure 3. The effect of polymer film thickness on the fibronectin adsorption. The error bars in the figure denote the standard uncertainties from the triplicate runs. The lines are drawn to aid the readers' eyes.

the surface modification induced by cells appears to be minimal during this time period. To provide a defined adhesive surface, the gradient poly(HEMA) film was pretreated with the adhesion protein fibronectin for 5 h. We hypothesized that regions of the library that had incomplete coverage of poly(HEMA) would allow fibronectin adsorption and that cell adhesion would follow a pattern similar to that of fibronectin adsorption. Similar blocking experiments involving adsorbed albumin, an abundant blood protein that lacks cell adhesion domains, on copolymer films of hydroxyethyl methacrylate (HEMA) and ethyl methacrylate (EMA) have been performed.[33] Feuerstein et al. observed a maximum in platelet adhesion on poly(HEMA-*co*-EMA) films at compositions near equal molar concentrations of HEMA and EMA. At higher concentrations of HEMA, protein adsorption was inhibited due to the hydrophilic character of the substrate, whereas at higher concentrations of EMA, irreversible albumin adsorption blocked the adsorption of blood-borne adhesion proteins.

To compare our system with that of Feuerstein et al., control experiments were conducted in which cells were seeded onto OTS and poly(HEMA) samples pretreated

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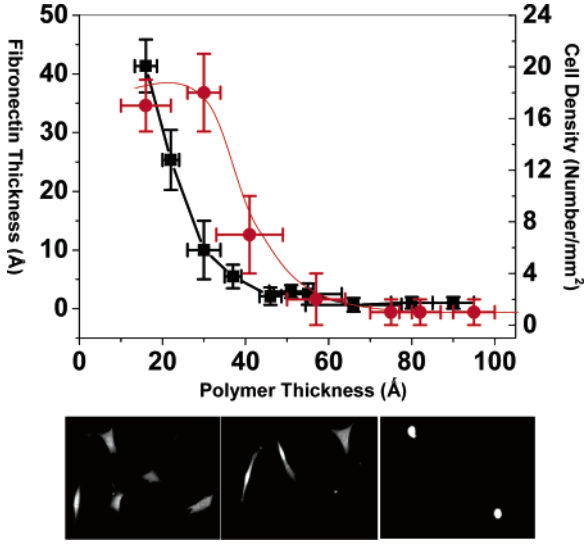

Figure 4. The effect of polymer film thickness on the thickness of adsorbed fibronectin, cell density, and cell morphology. The black line and the red line represent the fibronectin thickness and cell density, respectively. The error bars in the figure denote the standard uncertainties from the triplicate runs. The lines are drawn to aid the readers' eyes.

with fibronectin solution (25 μg/mL). It was found that no cells adhered to the poly(HEMA) samples and that cells adhered and spread on the OTS samples. Cell adhesion and spreading on the OTS samples may be due to the fibronectin adsorbed on the OTS samples in the fibronectin precoating process or cell adhesion protein adsorbed on the OTS from the serum-containing medium during cell seeding. To differentiate between these two processes, another control experiment was performed: cells were seeded onto the OTS and poly(HEMA) samples which were pretreated with serum-containing medium for 3 h. No cell adhesion was found on either the OTS or poly(HEMA) samples. These control experiments indicate only precoated FN mediate the initial cell adhesion, and these two control experiments on the compositions at the ends of the library are consistent with the results on the poly(HEMA-*co*-EMA) films; however, the results from the mushroom region of the gradient library do not follow the expected trend.

In the range of film thickness from 20 to 40 Å, the amount of adsorbed fibronectin decreases rapidly, which is shown in Figure 4. The cell adhesion density does not follow the same trend; cell density is essentially constant up to a thickness of 30 Å. As shown in Figure 4, the increasing poly(HEMA) grafting density reduces the area available for fibronectin adsorption. Instead, we observed a constant cell adhesion as the film thickness increased in the sparse region of the gradient library. This could be attributed to a saturation effect. For example, Garcia and co-workers have reported that there is a minimum functional fibronectin density for maximum cell adhesion,[34] and Hubbell et al. has found a minimum RGD peptide density required for cell spreading and focal adhesion density.[35] The amount of adsorbed fibronectin at the sparse region of the gradient library may be simple above this saturation level, and cell adhesion would not decline until the amount of adsorbed fibronectin is below the saturation level. However, we cannot completely rule out the possibility of a conformational change induced in fibronectin, which could improve the exposure of the cell-adhesion site, when fibronectin was forced to adsorb between poly(HEMA) grafts. Further investigation is under way to explore the details of the interactions between polymer grafts and adsorbed proteins.

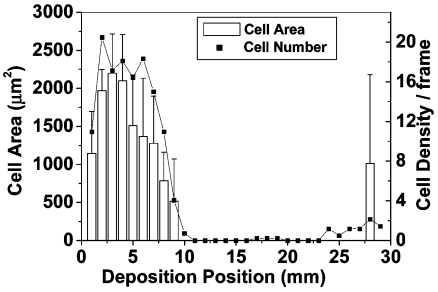

Figure 5. Cell adhesion and area as a function of position on the fibronectin-coated poly(HEMA) gradient.

The cell density and average cell area along a typical poly(HEMA) gradient are shown in Figure 5. It is not surprising that the cell density and average cell area follow a similar trend.[36–37] Therefore, we focused on the cell adhesion density in this study. We correlated the poly(HEMA) film thickness with the fibronectin adsorption, cell adhesion density, and cell morphology in Figure 4. From Figure 4, there are three different regions of cell density and cell morphology with the increase of the poly(HEMA) film thickness. In the first region (polymer thickness from ≅15 to ≅30 Å), the thickness of adsorbed fibronectin decreased from ≅40 to ≅10 Å. A saturated cell density is essentially constant at ~17/mm^2 and cells spread well in this region. In the second region, the polymer thickness ranged from ≅30 to ≅65 Å. In this region, the thickness of adsorbed fibronectin decreased from ≅10 Å to below detection limit of ellipsometry. Although cells were found to be adherent and spreading in this region, cell density was gradually decreased from ≅18/mm^2 to ≅1/mm^2, and cells were not spread well as compared to the cell spreading in the first region. In the last region (polymer thickness higher than 65 Å), the amount of adsorbed fibronectin was below the detection limit of ellipsometry, and little cell adhesion ($<$1/mm^2) was found.

Conclusions

The goal of this research was to develop a novel polymer substrate to regulate cell adhesion using technologically relevant materials. We developed a robust and versatile method to prepare a low grafting density initiator concentration gradient and combined it with the surface-initated atom transfer radical polymerization (ATRP) to produce a well-defined polymer surface. In this study, we pretreated the gradient poly(HEMA) film with a fibronectin solution. It was found that fibronectin adsorbed on the low graft density end and repelled at the high graft density end. Cell adhesion and cell spreading were found at the low graft density end and little cell adhesion and spreading was found at the high graft density end. In this way, we demonstrated that cells could adhere and spread at a

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poly(HEMA) surface with low grafting density. By fine-tuning the grafting density, the cell adhesion and cell morphology could be controlled. Further studies are necessary to better understand the interactions between polymer grafts and adhesion proteins, but this approach offers a potent method for tuning cell-material interactions at the molecular level.

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