COMBINATORIAL STUDIES OF THE EFFECT OF POLYMER GRAFTING DENSITY ON PROTEIN ABSORPTION AND CELL ADHESION

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Introduction

Surface modifications by grafting a non-fouling polymer such as poly(ethylene glycol) (PEG) on a surface were extensively used for various biomedical and biotechnological applications, and the effect of the grafted PEG layer on protein absorption was studied by various research groups.[1] The grafting density of PEG is well-known to significantly influence protein absorption, however, the experimental results are not always clear, and this is partially due to the fact that grafted PEG layers in many cases were not well defined and resulted in poorly controlled grafting density.[1]

Our group and others have demonstrated that gradient techniques provide a fast and convenient tool for high throughput screening of polymeric surfaces over a spectrum of material parameters.[2] Similar to PEG, poly(2-hydroxyethyl methacrylate) (HEMA) is a well-established biomaterial, which can resist non-specific protein absorption and cell adhesion.[3] In this study, we combined surface initiated polymerization with gradient formation technology to develop a poly(HEMA) grafting density gradient, which covers a broad range from the "mushroom" regime to the "brush" regime to explore different protein absorption and cell adhesion. Here, we focused on the physical characterization of the low grafting density gradients and initial cell adhesion studies.

Experimental•

The details of the preparation and the characterization of poly(HEMA) gradients were available elsewhere.[4] In brief, a self-assembled monolayer of octyltrichlorosilane (OTS) was established on a silica substrate by vapor evaporation. The polymerization initiator solution was slowly pumped into a test tube, which contained the OTS self-assembled monolayer (SAM) covered silicon wafer, to backfill the defects inside the OTS SAM and generate an initiator density gradient. Surface-initiated atom transfer radical polymerization (ATRP) was employed to prepare poly(HEMA) grafting density gradient surfaces.[5] The thickness of poly(HEMA) film and protein layer were measured by a variable angle spectroscopic ellipsometer (J. A. Woollam, Inc.).

Cell Culture and Cell Imagine Analysis: Substrates were incubated in fibronectin (25 mg/mL) for at least 5 h at 4 ºC and rinsed with Dulbecco's Phosphate Buffered Saline (DPBS) before use. NIH3T3 fibroblasts were maintained in Dulbecco's Minimum Essential Media (DMEM) supplemented with nonessential amino acids, glutamine, penicillin, streptomycin and fetal bovine serum, and maintained in a humidified 5 % CO_2 balanced-air atmosphere at 37 °C. Substrates were placed in four-well tissue culture polystyrene plates and NIH-3T3 cells were seeded on the substrates at (2000 cells/cm^2). Substrates were removed from the incubator after 8 h, and fixed for 24 h at room temperature. Cells were stained by Texas Red-C2-Maleimide and 4',6-diamidino-2-phenylindole (DAPI). Images were collected with 1 mm step sizes over the entire area of the gradient samples, and individual cell morphology and cell density were determined with image analysis software.

Results and Discussion

It was reported that protein resistance increases dramatically when PEG grafting density changing from the "mushroom" regime, where chains are spaced far enough from each other so that they are expected to spread parallel to the surface, to the "brush" regime, where polymer chains are packed tightly enough to extend normal to the surface. (Figure 1)[6]

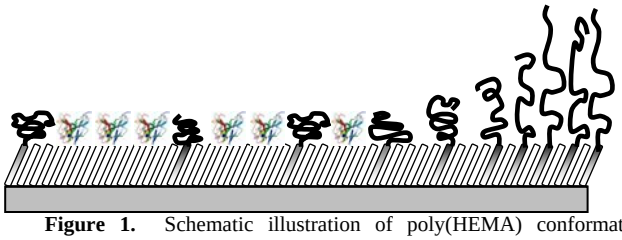
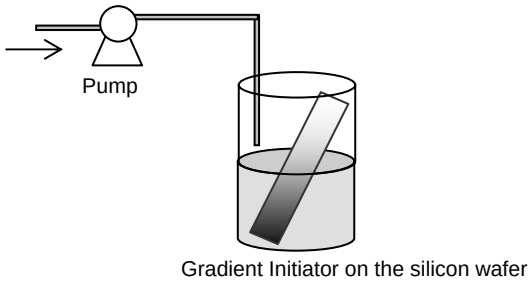


Figure 1. Schematic illustration of poly(HEMA) conformational change from "mushroom" regime to "brush" regime, and fibronectin (FN) density gradient established by backfilling the open space between poly(HEMA) chains.

At first, 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-density gradient.[7] However, rather thick gradient poly(HEMA) films with thickness ranging from 162 Å to 337 Å were obtained, and no cell adhesion was observed because poly(HEMA) brushes completely covered the surface and thus blocked cell adhesive protein (FN) adsorption. Moreover, 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 where an OTS monolayer was prepared on the silicon wafer. The gradient of polymerization initiator was established by gradually pumping the initiator solution to backfill the defects inside the OTS monolayer. (Scheme 1)



Scheme 1. Schematic illustration of the preparation of the gradient of polymerization initiator.

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 could be evaluated by the polymer film thickness from the following equation: $\sigma = h\rho N_A/M_n$, where h is the polymer film thickness, ρ is the density of polymer, N_A is Avogadro's number, and M_n is the relative number average molecular mass of the polymer.[8] Ellipsometry was used to measure the film thickness of the gradient poly(HEMA) as a function of position, and the grafting density was calculated from the equation above. The thicknesses along gradient samples measured by ellipsometry were summarized in Figure 2.

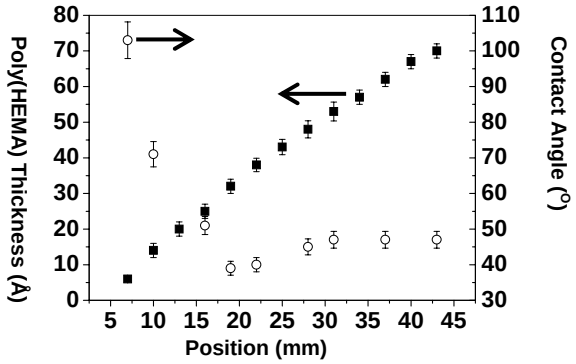


Figure 2. The plot of contact angle and polymer film thickness versus deposition position on the silicon wafer. The error bars denote the experimental uncertainties.

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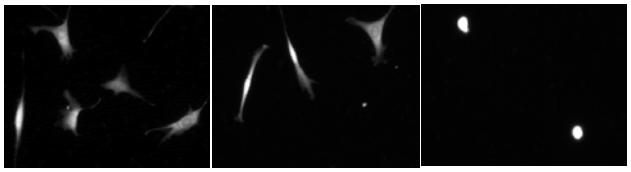
Certain commercial materials, instruments, and equipment are identified in this manuscript to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply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

A linear increase in film thickness ranging from 6 Å to 69 Å was found across the gradient library, which corresponded to the grafting density from 0 chain/nm^2 to 0.07 chain/nm^2.

The water contact angle data is also shown in Figure 2. Although a monotonic decrease in water contact angle was expected with increasing polymer thickness/grafting density, a minimum contact angle of 40º was found at a polymer film thickness around 33 Å, which correspond to the grafting density of 0.03 chain/nm^2, before the contact angle stabilized at (47 ± 2)º. Holly and Refojo investigated functional group reorientation of poly(HEMA), and 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.[9] While in the hydrated state, the hydrophilic hydroxyethyl groups were reoriented outward and hydrophobic methyl groups were buried inside. This dynamic behavior of poly(HEMA) surface has been confirmed by different research groups with various surface characterization techniques.[10] Since OTS is more hydrophobic than air, it is energetically favorable for methyl groups oriented toward the poly(HEMA)/OTS interface. We propose that the 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.

Information regarding the polymer chain conformation was inferred from the density profiles obtained from x-ray reflectivity (XRR), however the details could not be presented here due to limited space. The fitting of XRR profiles confirmed this grafting density library covered a broad range from the "mushroom" regime to the "brush" regime, and that the minimum contact angle corresponds to the transition from partial to complete surface coverage of poly(HEMA) on the OTS layer in dry state.

Cell adhesion and spreading experiments were performed with the fibroblast NIH-3T3 cell line to investigate the effect of grafting density on cellular response.[11] Figure 3 showed the representative cell adhesion and spreading on a FN pre-coated poly(HEMA) grafting density gradient after 8 h cell culture. Cell adhesion and cell spreading were found at the low graft density end and little cell adhesion and spreading was found at high graft density end. Further studies include detailed quantitative analysis of cell adhesion and spreading on FN pre-coated poly(HEMA) grafting density gradients using an automatic fluorescence microscopy technique developed at NIST.[12]



Low Grafting Density High Grafting Density

Figure 3. Membrane staining for fibroblast NIH-3T3 cells seeded on FN pre-coated poly(HEMA) grafting density gradient.

Conclusions:

A simple yet versatile method was developed to prepare a low-density polymerization initiator gradient, which was combined with surface-initiated ATRP to produce a well-defined poly(HEMA) gradient substrate. A smooth variation in film thickness was measured across the gradient, ranging from 6Å to over 69 Å, but we observed a non-monotonic variation in water contact angle. Fittings of x-ray reflectivity profiles suggested that at low graft density end, the polymer chain structure was in the "mushroom" regime, while the polymer chains at high graft density were in the "brush" regime. Fibroblasts were seeded on gradients pre-coated with FN to test cellular responses to this novel substrate. Cell adhesion and cell spreading were found at the low graft density end and little cell adhesion and spreading was found at high graft density end.

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