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Construction of a Tethered Poly(ethylene glycol) Surface Gradient For Studies of Cell Adhesion Kinetics

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Surface gradients can be used to perform a wide range of functions and represent a novel experimental platform for combinatorial discovery and analysis. In this work, a gradient in the coverage of a surface-immobilized poly(ethylene glycol) (PEG) layer is constructed to interrogate cell adhesion on a solid surface. Variation of surface coverage is achieved by controlled transport of a reactive PEG precursor from a point source through a hydrated gel. Immobilization of PEG is achieved by covalent attachment of the PEG molecule via direct coupling chemistry to a cystamine self-assembled monolayer on gold. This represents a simple method for creating spatial gradients in surface chemistry that does not require special instrumentation or microfabrication procedures. The structure and spatial distribution of the PEG gradient are evaluated via ellipsometry and atomic force microscopy. A cell adhesion assay using bovine arteriole endothelium cells is used to study the influence of PEG thickness and chain density on biocompatibility. The kinetics of cell adhesion are quantified as a function of the thickness of the PEG layer. Results depict a surface in which the variation in layer thickness along the PEG gradient strongly modifies the biological response.

Introduction

Controlling the properties of biological interfaces is essential for constructing the next generation of biomedical materials and devices. A variety of strategies have been used to create sophisticated and functional interfaces with biomimetic characteristics.[1,2] Further developments will require an improved understanding of the physicochemical parameters that control the structure of functionalized surfaces as well as an improved understanding their interactions with proteins and cells.

A critical factor in many biomedical technologies involves the ability to control and minimize biomolecular adhesion through surface engineering strategies. Poly(ethylene glycol) (PEG)-coated surfaces represent an important class of biomaterials that possess the ability to reduce nonspecific adsorption of proteins and cells.[3−6] Previous studies have demonstrated that the adhesion resistance of PEG-coated surfaces increases with chain packing density and increasing length of the polymer chains.[7] Nevertheless, the mechanisms by which proteins and cells interact with these surfaces remain unclear. Better control of the physical chemistry of the cell−substrate interface is a necessary step in performing well-defined studies of specific and nonspecific binding to elucidate these mechanisms.

Spatial gradients have received increasing attention as a novel means of interrogating the complexity of processes occurring at surfaces. For biological processes, surfaces that possess a gradient in chemistry or structure allow one to interrogate behaviors such as surface binding or adhesion over a well-defined variation in surface properties. In addition, such surfaces allow investigation of the effects of surface chemical gradients on biological phenomena important for applications related to drug delivery, tissue engineering, and biosensor development.[8−14]

A variety of methods have been used to generate confined and surface-immobilized gradients. Examples include gas-phase diffusion of silane-coupling agents to silicon,[15] gel-based diffusion of reactive alkanethiols to gold surfaces,[16,17] diffusion in microfluidic channels,[13,14] spatially localized corona discharge treatment of polymer surfaces,[18,19] plasma discharge methods,[20] and in-plane electric field gradients to spatially desorb alkanethiols[11,21] or electrodeposit metals.[22] These and other gradient

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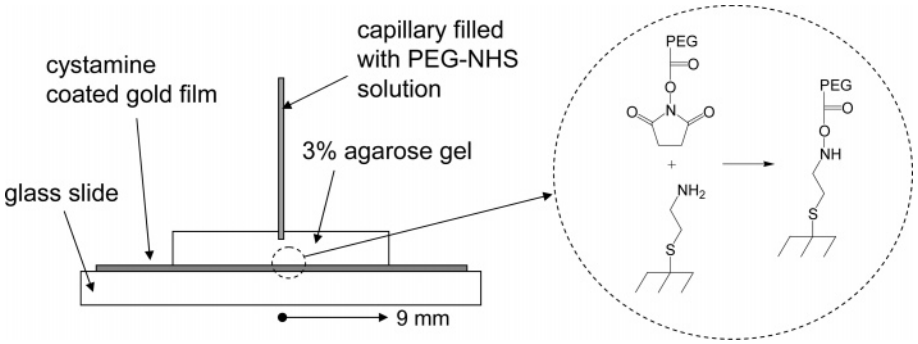
Scheme 1. Schematic of the Gel Diffusion Method for Construction of the Tethered PEG Surface Gradient[a]



[a] See text for details.

approaches have been exploited to examine a variety of biological processes.[10] Wettability gradients have been generated on glass and silicon surfaces to study protein adsorption.[23,24] A series of publications involving corona discharge to modify polymer surfaces examined the adsorption of various proteins and the adhesion of cells on gradients in hydrophobicity and chemical functionality.[19,25−27] Surface-bound protein gradients formed by laminar flow in microchannels were used to examine the oriented growth of rat hippocampal neurons.[13] Gradients in surface-bound extracellular matrix proteins have been created using electrochemical desorption methods to study cell adhesion.[11] This electrochemical method has also been employed to create gradients of surface-immobilized peptides and proteins.[12]

This report describes a method for construction of a PEG-coated surface possessing a gradient in surface coverage, which represents a model surface for studies of cell adhesion. Our method represents a simple and robust technique for gradient construction that complements earlier work using corona discharge/graft polymerization of PEG on polyethylene surfaces,[27] silanization of a silica surface followed by PEG grafting,[8] or diffusion of oligo(ethylene glycol)-terminated thiols through a gel matrix[16] to create PEG surface gradients. We employ a simple fabrication method that relies on transport of a reactive PEG molecule from a point source through a hydrated gel followed by covalent attachment to a functionalized gold surface. This approach eliminates the need for elaborate instrumentation or complex microfabrication techniques. The reagents are also readily available, and no custom synthesis is required. The structure and properties of the resulting surface gradient are illustrated. In particular, ellipsometry and atomic force microscopy (AFM) are used to measure the spatial variation in thickness and morphology of this PEG layer. Cell culture results using bovine endothelial cells depict an adhesion rate that is a strong function of the PEG coverage.

Experimental Section

Materials. Gold (99.999%) was purchased from Ernest Fullam (Latham, NY), high-grade mica was purchased from Ted Pella (Redding, CA), and the glass slides were acquired from VWR (Morrisville, NC). Cystamine and (3-mercaptopropyl)-trimethoxysilane (Aldrich, Milwaukee, WI) were used as received. A PEG derivative (molecular weight = 5261) possessing a *N*-hydroxysuccinimide ester end group, referred to as m-PEG-NHS or m-PEG, was purchased from Nektar (San Carlos, CA).

Sample Preparation. Gold-coated glass slides were used as substrates in these studies. Glass slides were cleaned in 3:7 (v/v) 30% H_2O_2/H_2SO_4 solutions carefully thermostated at 50 °C for 30 min (caution: these solutions react violently with organic materials and must be handled with extreme caution!!!). The slides were thoroughly rinsed, sonicated several times in deionized water, and dried under a nitrogen flow. This treatment produced a glass surface with a high coverage of silanol (SiOH), to which functional silanes could adsorb upon hydrolysis. The glass slides were then soaked overnight in a 5 mM toluene solution of (3-mercaptopropyl)-trimethoxysilane.[28] They were rinsed and sonicated with the same solvent to remove weakly attached molecules and then dried under a nitrogen flow. A 50-nm gold film was then thermally deposited onto the glass surface using a high vacuum coating system (Benchtop Turbo, Denton Vacuum, Moorestown, NJ), as described previously.[5] The samples were then immediately subjected to the further processing steps described below.

Cystamine Adsorption. A cystamine monolayer was created by immersing the gold substrates in a 3 mM solution of cystamine in deionized water at room temperature for 6 h. The sample was then rinsed with deionized water to remove weakly adsorbed molecules and dried under nitrogen flow. The monolayers produced by this method have been previously characterized using ellipsometry, contact angle, and reflectance infrared spectroscopy.[5]

Deposition of PEG-NHS. Grafting of PEG to the cystamine layer was achieved using a well-established ester−amine chemistry.[29] The PEG molecule used here possessed a *N*-hydroxysuccinimide ester end group (NHS) that reacts with aminated solid surfaces (Scheme 1). This reaction is convenient to use in aqueous media and can be driven to completion by addition of triethylamine (TEA), which is a deprotonating agent that reacts with the surface amine group to facilitate attachment.

Uniform Coverage Surface. A uniformly coated PEG surface was created by dissolving m-PEG-NHS in a phosphate buffered solution (PBS) at a concentration of 4 mg/mL. Immobilization of PEG to the cystamine-coated gold surface was then achieved after addition of 0.1 μL/mg of TEA per mg of PEG-NHS to this solution and incubation for a period of 12 h. A surface possessing a nominal coverage of 0.32 PEG nm^{-2} resulted. Details of the structure and chemistry of the resulting film were the subject of a previous study.[5]

Gradient Formation. Fabrication of a PEG surface chemical gradient was achieved using a gel-diffusion method that was recently reported for the construction of catalyst gradients.[30] The cystamine-modified gold surface was coated with an ~1 mm thick layer of a 3% agarose gel. A small capillary was attached to a syringe and used to pierce the gel at a central location of the

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sample. The capillary served as a point source for m-PEG-NHS, which traveled from the syringe tip into the gel. A syringe pump was used to provide a nominal delivery rate of the PEG solution into the gel at a value of 10 μL/h for a period of 8 h (Scheme 1). This process allowed PEG-NHS to travel to the sample surface, where it reacted with the cystamine monolayer and was covalently linked. An estimate of the diffusion rate of PEG suggests that delivery of this molecule to the sample surface occurs by diffusion, as well as having a small contribution from transport due to flow. The gel was peeled from the surface, and the sample was rinsed with PBS to remove excess PEG and gel. The sample was then dried under nitrogen flow. Ellipsometry, polarization modulation infrared reflection−absorption spectroscopy (PM-IRRAS), and AFM were used to confirm the successful grafting of PEG to the surface and the complete removal of agarose gel (Supporting Information).

Methods. *Ellipsometry.* Ellipsometry measurements were performed by null ellipsometry with a SENTECH SE 800 variable angle ellipsometer (Carrollton, TX). All measurements were performed in ambient air, using an incidence angle of 70° versus the sample surface normal at room temperature (20 °C). The film thickness and refractive indices were calculated from the measured ellipsometric angles. Calculations were performed using software supplied by the ellipsometer manufacturer, where parameters were optimized to give a best fit of the experimental data.

The optical constants of the gold substrate (refractive index n and absorption coefficient k) were first determined using a two-phase model (air/substrate). The thickness of the grafted molecular film was then measured using a three-phase model (air/polymer/substrate). Because the film could be partially hydrated, the optical constants could not be determined precisely. However, values of $n = 1.46$ and $k = 0$ produced excellent fits of the ellipsometer data analysis models.

AFM. Surface images were acquired with an atomic force microscope (Picostat, Molecular Imaging, Inc., Phoenix, AZ, and Nanoscope E, Digital Instruments, Santa Barbara, CA) in contact mode in air using Si_3N_4 AFM tips (Nanoprobe, Park Scientific) with a spring constant of $k \sim 0.1$ N m^{-1}.

Static Cell Culture Adhesion Assay. Bovine arteriole endothelium cells (BAVEC-1) from passage 13 were grown in RPMI medium and harvested after reaching a concentration of approximately 500 000 cells mL^{-1}. The gold substrates were immersed into the culture media and placed in a Queue Stabil-Therm incubator at 37.2 °C with 5.0% CO_2. Cell growth was recorded using an optical microscope (Nikon Diaphot 300) with 40× and 10× objective lenses, a camera, and a video recorder. The videos were then analyzed, and the adherent cells were counted for the different samples.

Results and Discussion

PEG-modified surfaces were prepared using a standard coupling reaction between an amine-functionalized self-assembled monolayer (cystamine) and a terminal *N*-hydroxysuccinimide ester (NHS) group on PEG.[5,29] Gradient formation was achieved by transport of PEG from a point source through an ~1 mm thick agarose gel deposited onto a cystamine-modified gold substrate (Scheme 1). This produced a spatially nonuniform PEG concentration within the gel having a high concentration directly under the capillary and a decreasing concentration away from that point. Subsequent covalent attachment to the surface via NHS−amine coupling provided a high PEG coverage near the center of the gel and a decreasing coverage toward the edges. Following gradient formation, the gel was removed by peeling.

The PEG thickness profile for a surface gradient prepared from a 3% agarose gel using an 8-h diffusion−reaction period (Figure 1) exhibits an expected Gaussian shape with the highest values located near the capillary injection point. The PEG film thickness varied continuously from 0 at the edges of the sample to a maximum of 16 nm at the center of the gradient. This corresponds to a PEG chain density ranging from 0 up to 2.3 chains nm^{-2}.

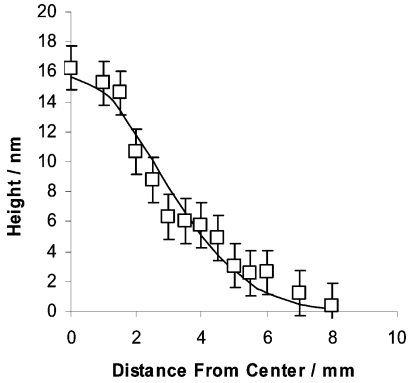

Figure 1. Height of PEG layer versus position measured by null-ellipsometry mapping.

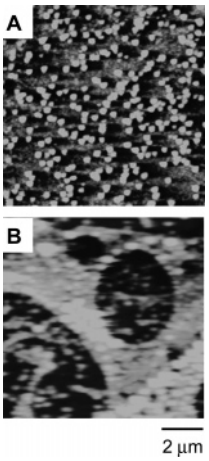

Figure 2. Atomic force microscope images of PEG layer measured at (A) 6 mm and (B) 0 mm from the center of the gradient.

The maximum measured chain density for this sample is significantly larger than that obtained by directly grafting PEG molecules from solution onto cystamine-coated surfaces.[5] In addition, this chain density exceeds that reported for other PEG-functionalized surfaces involving monolayers.[8] AFM images near the edge of the gradient (Figure 2A) depict isolated PEG clusters. However, a more complex, multilayer structure is observed toward the center of the gradient (Figure 2B). These observations suggest that the surface created by this gel-diffusion technique ranges from a sub-monolayer coverage of isolated PEG clusters at the edges to a complex, multilayer structure in the center. It is speculated that the multilayer structure consists of intertwined PEG molecules, possibly including strongly bound agarose. Notably, subsequent processing with thorough rinsing and sonication had little effect on the film's properties. Only aggressive processing sufficient to remove the gold substrate layer impacted the PEG layer.

A further test of the surface chemistry presented by the PEG gradient was assessed by a cell adhesion assay. The kinetics of cell adhesion were followed via growth of bovine arteriole endothelium cells (BAVEC-1) under static conditions. PEG gradient substrates were immersed into culture media and placed in an incubator at 37.2 °C with 5.0% CO_2.[5] In these static experiments, endothelial cells were exposed to the substrate and then allowed to adhere, grow, and spread across the surface for a period of 72 h.

A series of images along the PEG gradient at various times is depicted in Figure 3. These results clearly demonstrate that the regions with the highest PEG density near the center of the gradient provide the lowest cell

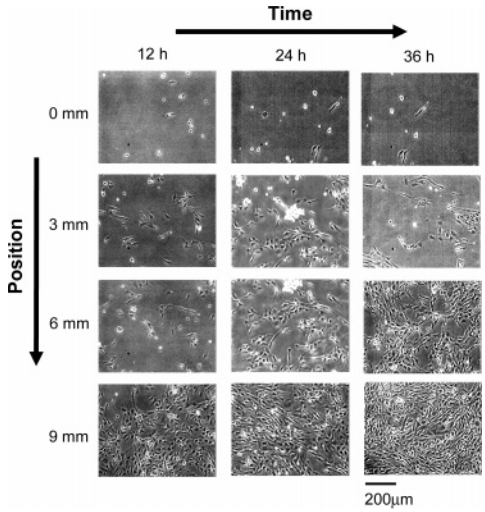

Figure 3. Optical micrographs of adhering bovine arteriole endothelium cells (BAVEC-1) at different positions along the PEG gradient after different exposure times (12, 24, and 36 h) in a culture medium.

adhesion levels. At short time scales irreversibly adhered endothelial cells are clearly observed, with the greatest number appearing at the lowest PEG density regions. Increasing exposure time results in rapid cell attachment at low PEG regions and slow attachment at high PEG regions. After 36 h, the highest PEG density regions remain free of cells. However, after 72 h of exposure, the majority of the surface is completely covered with endothelial cells.

The rate of cell adhesion can be quantitatively assessed by monitoring the total number of adhered cells versus time. Figure 4A shows the number of adhered cells at various surface locations over the course of the cell culture assay. The increase in cell number is fastest at the edges of the gradient and decreases toward the center. These results can be used to extract a cell adhesion rate constant using a Langmuir adsorption model in which the rate of cell adhesion is proportional to the cell-free surface area. The resulting adhesion rate constant is plotted as a function of PEG layer thickness in Figure 4B. The adhesion rate drops rapidly with increasing thickness. The solid line in Figure 4B represents a model in which the adhesion rate decreases with increasing layer thickness.

A comparison of the cell adhesion kinetics for this gel-prepared gradient sample with previous work using a uniformly coated and gel-free PEG surface is shown (Figure 4B). Notably, the adhesion rate is somewhat lower for a PEG surface created in the absence of agarose gel. However, a much higher "effective" thickness (and lower adhesion rate) is achievable in this gradient system.

Conclusions

In conclusion, these static cell culture experiments demonstrate that a PEG coating will not indefinitely prevent cell adhesion. Rather, PEG reduces endothelial cell adhesion by decreasing the rate constant for this

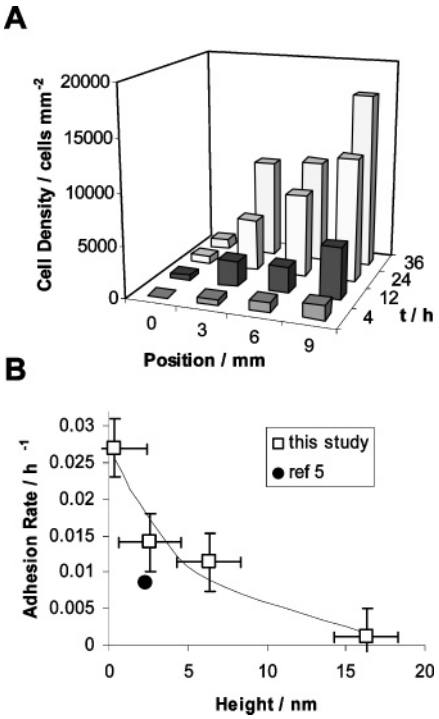

Figure 4. (A) Cell density of adherent BAVEC-1 cells along the PEG gradient versus exposure time. (B) Cell adhesion kinetics versus thickness of the PEG layer.

process. In addition, this work demonstrates a gradient fabrication method based upon a simple diffusion/reaction system. Ultimately, the nature of the gradient can be readily modified by tuning the assembly parameters, including the diffusion time, agarose concentration, or system geometry. Combined with detailed surface characterization, gradient surfaces provide a quantitative tool to optimize the concentration of desired ligands for the purposes of controlling cellular responses such as cell adhesion, migration, and proliferation. A suite of such tools is critical for developing novel, well-defined materials for drug delivery and tissue engineering.

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Supporting Information Available: Fluorescence microscopy and PM-IRRAS results for a similarly prepared PEG gradient utilizing a fluorescently labeled F-PEG molecule. Data for a dynamic cell culture assay on PEG gradient using a cell flow chamber under moderate shear conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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