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K. Mougin
University of Virginia - Main Campus

M. B. Lawrence
University of Virginia - Main Campus

E. J. Fernandez
University of Virginia - Main Campus

See next page for additional authors

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Abstract
Considerable effort has been expended in efforts to create surfaces that resist the adsorption of proteins and cells for biomedical applications. The majority of such work has focused on surfaces constructed from bulk polymers or thin polymer films. However, the fabrication of surfaces via self-assembled monolayers (SAMs) has attracted considerable interest because of the robustness, versatility, and wide-ranging applicability of these materials. SAMs are particularly appealing for biological systems where well-defined surface chemistries can be created to facilitate coupling, biorecognition, or cell adhesion along with a host of other applications in biochemistry and biotechnology.

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Authors
K. Mougin, M. B. Lawrence, E. J. Fernandez, and Andrew C. Hillier

Construction of Cell-Resistant Surfaces by Immobilization of Poly(ethylene glycol) on Gold

K. Mougin,[†,||] M. B. Lawrence,[‡] E. J. Fernandez,*[,†] and A. C. Hillier*[,§]

Department of Chemical Engineering, University of Virginia, Charlottesville, Virginia 22904, Department of Biomedical Engineering, University of Virginia, Charlottesville, Virginia 22908, and Department of Chemical Engineering and Department of Chemistry, Iowa State University, Ames, Iowa 50011

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Introduction

Considerable effort has been expended in efforts to create surfaces that resist the adsorption of proteins and cells for biomedical applications. The majority of such work has focused on surfaces constructed from bulk polymers or thin polymer films.[1,2] However, the fabrication of surfaces via self-assembled monolayers (SAMs) has attracted considerable interest because of the robustness, versatility, and wide-ranging applicability of these materials. SAMs are particularly appealing for biological systems where well-defined surface chemistries can be created to facilitate coupling,[3,4] biorecognition,[5,6] or cell adhesion[7−9] along with a host of other applications in biochemistry and biotechnology.[10,11]

The discovery that grafted poly(ethylene glycol) (PEG) produced surfaces with significant inertness to cell and protein adhesion has been extensively investigated.[12−14] Indeed, PEG is an important biomedical material because of its biocompatibility, low toxicity, nonimmunogenicity, and high water solubility.[14,15] Several studies have demonstrated that PEG can be tethered to surfaces via organosilane and organosulfur SAMs and other coupling agents to produce materials that reduce protein and cell adsorption.[12,13,16−19] Although several theories have been proposed to explain the inertness of these PEG-coated surfaces,[1,20] the correlations between protein adsorption and cell adhesion are complex[17] and the underlying mechanisms remain unclear.[17,21]

Ultimately, organosulfur SAMs represent a relatively simple and practical route for construction of model systems that are easy to design and have broad potential applications.[5,22] Their inherent flexibility allows surfaces to be modified in a controllable manner[23,24] and provides a straightforward way to molecularly engineer surfaces with specific and tailored chemical functionality.

In this work, we describe a simple method for construction of PEG-functionalized gold surfaces for studies of cell adhesion. Two different, high-molecular-weight PEG-tethered surfaces are created via simple coupling chemistry to an amine-terminated alkanethiol adsorbed on gold. Several surface-sensitive analytical measurements are used to characterize the surface structure and chemistry of the adsorbed films. In addition, cell adhesion tests using cultured endothelial cells under static conditions and leukocytes under flow conditions clearly illustrate that PEG-tethered surfaces significantly reduce adhesion as compared to the precursor surfaces. Notably, this is the first report quantifying the ability of alkanethiol-tethered PEG to resist attachment of highly adherent leukocytes as quantified in a parallel plate flow chamber assay.

Experimental Section

Materials. *Reagents.* Substrates were prepared from gold (99.999%, Ernest Fullam, Latham, NY), high-grade mica (Ted Pella, Redding, CA), and glass slides (VWR, Morrisville, NC). Cystamine (Aldrich, Milwaukee, WI) was used as received. Two commercially available PEG derivatives (Nektar Therapeutics, San Carlos, CA) were used as received. The PEG polymer with an *N*-hydroxysuccinimide ester (NHS) end group is referred as m-PEG-NHS or m-PEG (MW = 5261 Da) while a similar PEG possessing a fluorescein marker is referred as FITC-PEG-NHS or FITC-PEG (MW = 2025 Da). Unless noted otherwise, experiments were performed using reagent-grade solvents (Aldrich, Milwaukee, WI) and 18 MΩ deionized water (E-Pure, Barnstead, Dubuque, IA).

Gold Substrates. Ultrasmooth template-stripped gold (TSG) substrates were created using a published procedure.[25] Freshly cleaved 1 × 1 in. mica sheets were first washed with ethanol, dried under a nitrogen flow, and then placed, cleaved-side up, in the vacuum chamber of a metal coating system (Benchtop Turbo, Denton Vacuum, Moorestown, NJ). A gold film of 150-nm thickness was vapor-deposited at room temperature and a pressure of $\sim 7 \times 10^{-5}$ Torr at a rate of 1 Å s^{-1}. The gold film was subsequently glued to a glass slide using a UV-curable adhesive (Norland 61, Newport). The glue was cured by exposure to a

* To whom correspondence should be addressed. E-mail: erik@virginia.edu (E.J.F.); hillier@iastate.edu (A.C.H.).

† Department of Chemical Engineering, University of Virginia.

‡ Department of Biomedical Engineering, University of Virginia.

§ Iowa State University.

|| Current address: Institut de Chimie des Surfaces et Interface, Université de Haute Alsace, Mulhouse, 68093 France.

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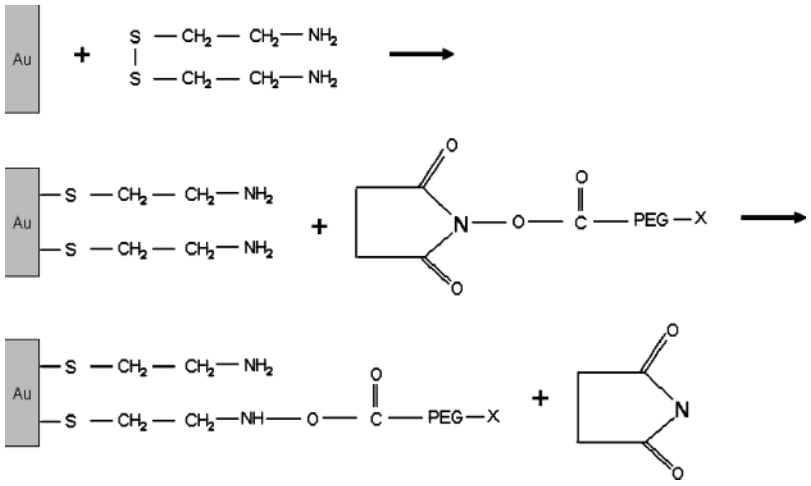
Scheme 1. Formation of the PEG-Terminated Gold Surface



100-W mercury lamp for 10 min. Optimum adhesion was achieved by aging the glue for a period of about 1 week at room temperature or 12 h at 50 °C. Once cured, the assembly was soaked in tetrahydrofuran for several hours to delaminate the mica and expose the underside of the gold. This procedure resulted in the formation of ultrasmooth gold possessing a (111) texture and an average roughness of ~3 nm/μm^2.

PEG Functionalization. Cystamine was adsorbed to the gold substrate by immersing it in a 3 mM solution of cystamine in deionized water at room temperature for 6 h. Samples were then rinsed with deionized water and dried under nitrogen flow. PEG was subsequently immobilized using an ester−amine reaction between the NHS end group of the PEG and the aminated solid surface presented by the cystamine-coated gold (Scheme 1).[26] The procedure involved dissolving m-PEG-NHS or FITC-PEG-NHS at a concentration of 4 mg/mL in phosphate-buffered saline (PBS), exposing the gold surfaces to this solution, and adding 0.1 μL of triethylamine/mg of PEG-NHS to drive the reaction to completion. The substrates were incubated under these conditions for about 6 h and then rinsed with PBS.

Methods. *Polarization−Modulation Infrared Reflection−Adsorption Spectroscopy (PM-IRRAS).* PM-IRRAS measurements were performed with a spectrometer (IF66S, Bruker Optics, Inc.) using a mercury−cadmium−tellurium detector. Typical data were derived from 2000 scans at a resolution of 1 cm^{-1}, 85° beam angle of incidence, and 74-kHz modulation frequency.

Ellipsometry. Film thicknesses were determined by null ellipsometry (Multiskop, Optrel, Berlin, Germany).[27] All measurements were done under ambient conditions at a 70° angle of incidence using a He−Ne laser (λ = 632.8 nm). Film calculations were performed using the Elli software (Optrel). The refractive index, n, and absorption coefficient, k, of the substrate were determined from bare gold surfaces using a two-phase model (air/substrate). The thickness of the grafted molecular film was then calculated using a three-phase model (air/film/substrate) assuming that film optical constants were $n = 1.46$ and $k = 0$.

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

Contact Angle Measurements. Contact angles were measured under ambient conditions (20 °C and 40% relative humidity) by depositing ~2-μL droplets of deionized water onto the different substrates. Advancing and receding contact angles were measured by recording images of the water droplet under positive (advancing) and negative (receding) pressures as manually controlled with a syringe. The results represent the average of at least four measurements.

Cell Culture. Bovine aortic endothelium cells (BAVEC-1) from passage 13 were grown in a solution of 5% fetal bovine serum (Gibco Invitrogen, Carlsbad, CA) in a Roswell Park Memorial Institute cell culture medium. The gold films were placed in the culture media and incubated at 37.2 °C with 5.0% CO_2 (Queue Stabltherm). Cell growth was recorded using an optical microscope (Nikon Diaphot 300) equipped with a camera and a video recorder using a 10× objective lens.

Cell Adhesion in a Flow Cell. Neutrophils were isolated from whole blood and resuspended in Hanks balanced salt solution (pH 7.4) containing 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Sigma-Aldrich). The final concentration of the white blood cells was estimated to be 500 000 cells mL^{-1}. Cell adhesion experiments were carried out at room temperature in a single channel flow cell (parallel plate flow chamber, Glycotech, Rockville, MD) at a flow rate of 0.645 mL min^{-1} (1 dyn cm^{-2}). An optical microscope (Nikon Diaphot 300) equipped with a charge-coupled device camera and a video recorder was used to monitor the number of adherent cells. Image analysis and cell counting were performed using NIH Image.

Results and Discussion

TSG substrates were used to provide a well-defined substrate for further investigation.[25] These surfaces are remarkably smooth over very large length scales, as shown in Figure 1a. This image depicts a topologically uniform surface over a 1 × 1 μm region. A vapor-deposited gold layer of similar thickness exhibits surface roughness approaching 30 nm μm^{-2} (Supporting Information), while TSG is on the order of 3 nm μm^{-2}. The ultrasmooth gold provides a surface that facilitates further surface characterization and structural imaging.

Construction of PEG-tethered surfaces proceeded through a standard coupling reaction (Scheme 1). An amine-terminated surface was created by adsorption of an amine-functionalized alkane dithiol (cystamine). Electrochemical blocking experiments (Supporting Information) indicated that a monolayer approaching 88% cystamine coverage could be created. This coverage equates to 10.6 cystamine molecules nm^{-2} on the basis of the surface density of Au(111) ($\Gamma = 1.2 \times 10^{15}$ atoms cm^{-2}), which provides a high density of exposed amino groups. Coupling of PEG was achieved by reaction of the NHS terminal group of a functionalized PEG molecule (m-PEG-NHS or FITC-PEG-NHS) to the surface amine groups. Two distinct PEG moieties were chosen to provide both an optical signal of surface coupling (FITC-PEG) and two different-molecular-weight polymers.

Functionalization with cystamine and PEG was confirmed through a combination of measurements. The fluorescently tagged PEG (FITC-PEG-NHS) was initially

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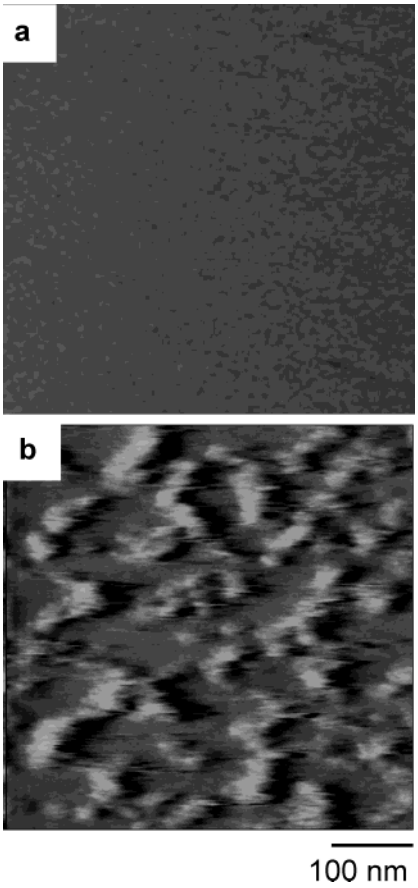

Figure 1. AFM contact mode images of (a) a bare TSG surface and (b) PEG-tethered gold. Height scales for the images are (a) z range = 2 nm and (b) z range = 40 nm.

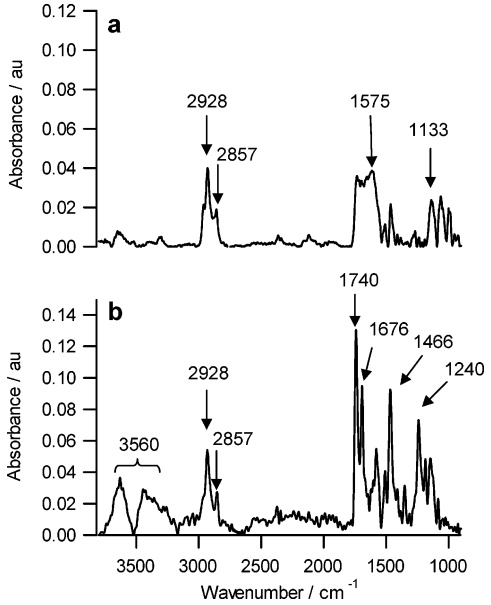

Figure 2. PM-IRRAS spectra of (a) cystamine-coated gold and (b) PEG-tethered gold versus the bare gold reference surface. For the cystamine-coated surface (Figure 2a), adsorption bands are attributed to deformation of the NH_2 group (1575 cm^{-1}), asymmetric CH_2 stretch (2928 cm^{-1}), symmetric CH_2 stretch (2857 cm^{-1}), and asymmetric C—C—N stretch (1133 cm^{-1}). The m-PEG-coated surface (Figure 2b) shows new absorption bands corresponding to CH_2—O—CH_2 and CH_2—OH stretches of PEG (1240 and 1460 cm^{-1}) and deformation of N—O—C in the covalent bond between PEG-NHS and cystamine (1676 cm^{-1}). Additional bands at 1740, 2857, and 2928 cm^{-1} can be attributed to a strong C═O deformation of the NHS end group of PEG. Spectra for the FITC-PEG surface (not shown) exhibited several additional absorbance peaks between 900 and 1100 cm^{-1}, cut to the fluorescein moiety.

used to provide a simple optical verification of the PEG-coupling step. AFM images also provided direct evidence of surface coupling. AFM images of the cystamine-coated gold surface indicated no discernible topological differences as compared to the bare gold (not shown). In contrast, an AFM image of a FITC-PEG layer depicts a globular morphology of tethered PEG molecules (Figure 1b). The morphology is consistent with a layer containing numerous PEG bundles. The fraction of the surface covered by PEG based upon the AFM image is ~35%.

Changes in surface chemistry were confirmed by contact angle and PM-IRRAS measurements. The equilibrium contact angle for water on gold (47°) decreased following the adsorption of cystamine (35°) and then PEG (28°), indicating an increase in the surface hydrophilicity. PM-IRRAS measurements on the cystamine- and m-PEG-coated surfaces (Figure 2) showed new absorbance peaks consistent with the relevant surface chemistry.

The average thickness of the cystamine and PEG-coated surfaces was measured in the dry state by ellipsometry. Using a three-layer model for the gold—cystamine—air interface, a thickness of 4.4 Å was determined for the cystamine film. Similar measurements for the m-PEG and FITC-PEG layers provided thicknesses of 26.1 and 19.1 Å, respectively. Notably, m-PEG has a larger molecular weight (5261 Da) as compared to FITC-PEG (2025 Da). Using these values, the average grafting density (σ) of the adsorbed PEG molecules can be estimated according to eq 1.

$$\sigma = \frac{\tilde{N}_A d \rho_{dry}}{MW} [=] \text{ PEG molecules nm}^{-2} \qquad (1)$$

where d is the layer thickness, ρ_{dry} is the density of the dry polymer, MW is the molecular weight, and $\tilde{N}_A$ is Avogadro's number. The grafting density for FITC-PEG is 0.61 PEG nm^{-2} and m-PEG is 0.32 PEG nm^{-2}, indicating that less than 10% of the available cystamine surface sites are tethered with PEG. In addition, the calculated separation distances between PEG molecules, based upon the dry density (12.7 and 17.2 Å for FITC-PEG and m-PEG), are smaller than the radius of gyration of these molecules (18.07 and 30.82 Å). This suggests that these layers are dense with significant overlap of PEG and may be best described as a "brush border" morphology.[28,29]

The kinetics of endothelial cell adhesion and proliferation on the various functionalized surfaces was assessed with static cell culture experiments. Figure 3 demonstrates that, after 12 h, the gold and cystamine are fully covered with adhered cells while the PEG-coated surfaces remain relatively cell-free. Quantitative analysis in Figure 4 over a 72-h period shows that all of the surfaces ultimately permitted the attachment and growth of cells. However, rates of cell growth on the PEG-tethered surfaces were dramatically reduced and the m-PEG yielded the greatest resistance to cell attachment and growth. Notably, the attachment of PEG did not arrest the growth of cells but rather delayed the onset of growth and also lowered the proliferation rate.

Surfaces were also tested for their ability to resist adhesion of leukocytes in the form of neutrophil cells under

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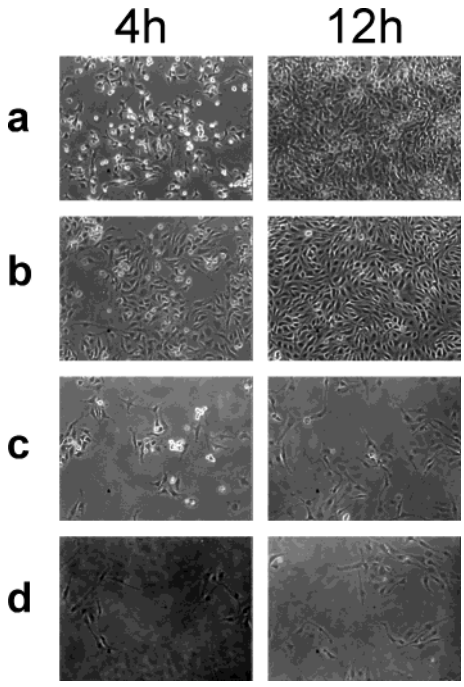

Figure 3. Optical micrographs of adhering bovine arteriole endothelium cells (BAVEC-1) in a culture medium after exposure times of 4 and 12 h for (a) bare gold, (b) cystamine-coated gold, (c) FITC-PEG, and (d) m-PEG surfaces.

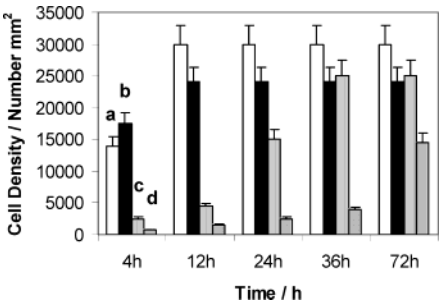

Figure 4. Histogram depicting the cumulative density of BAVEC-1 cells adhered to (a) bare gold, (b) cystamine, (c) FITC-PEG, and (d) m-PEG as a function of time.

flow conditions with defined shear (see Methods). Neutrophils adhere strongly to a wide variety of surfaces and, thus, represent a stringent test for the ability of a surface to inhibit adhesion.[2] The percent of adhered cells was determined by counting the number of nonmoving cells under steady-state flow conditions in a parallel plate flow chamber. Bare gold adhered 99% of the neutrophils while the cystamine-coated surface adhered 97% of the cells. FITC-PEG reduced cell adhesion markedly (33%) and m-PEG reduced it to an even greater extent (11% adherent). The two likely explanations for this difference are that the lower molecular weight of FITC-PEG (2025 Da) and addition of the hydrophobic fluorescent group made FITC-PEG less effective than m-PEG (5261 Da). Notably, contact angle measurements showed that the addition of the fluorescein group did not significantly alter the hydrophobicity of the surface. Therefore, the difference in these surfaces is likely the result of the difference in the molecular weights, which is consistent with previous reports on the influence of PEG molecular weight on adhesion.[17,30]

Conclusions

We have described a practical method for the construction of PEG-functionalized gold surfaces via a simple coupling chemistry with commercially available reagents. The use of TSG substrates facilitates characterization of the morphology of immobilized structures. Surface structural and chemical measurements illustrate the evolution of surface properties following each step of functionalization. The dense layer of PEG covering the surface reduced adhesion of endothelial and even highly adherent neutrophils under shearing conditions. Notably, these results show that PEG immobilization did not completely prevent but rather slowed the kinetics of cell adhesion. This surface construction method represents a simple, robust, and practical surface engineering technique based upon commercially available reagents that can be used to construct a variety of model surfaces for studies of cell and protein adhesion. Notably, this is also the first report quantifying the ability of alkanethiol-tethered PEG to resist leukocyte adhesion as quantified in a parallel plate flow chamber assay. Further studies of surfaces with well-defined surface chemistry, topology, and adhesive function such as those created and characterized here are required to determine the effect of surface chemical and structural features on cell adhesion to PEG-tethered surfaces.

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Supporting Information Available: Values of water contact angle measurements and film thicknesses, comparison of FITC-PEG and m-PEG film thicknesses, average distances between grafted polymers and polymer grafting density via nullellipsometry, percentage of Neutrophil cells attached to various surfaces, AFM images, and cyclic voltammetric response. This material is available free of charge via the Internet at http://pubs.acs.org.

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