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Two surface gradients of polyethylene glycol for a reduction in protein adsorption

Nathan W. Gooch and

Department of Bioengineering, University of Utah, Salt Lake City, UT 84112, USA

Vladimir Hlady*

Department of Bioengineering, University of Utah, Salt Lake City, UT 84112, USA

Abstract

Polyethylene glycol (PEG) coatings have been commonly used in reducing protein adsorption with the intent of improving a biomaterial's biocompatibility. To elucidate the role of PEG surface density in reducing protein adsorption, two types of grafted PEG surface density gradients were evaluated for the adsorption and desorption of albumin and fibrinogen, two blood proteins. PEG density gradients were characterized using contact angle measurements and X-ray photoelectron spectroscopy. Total internal reflection fluorescence was used to measure protein adsorption kinetics and adsorption profiles on the two types of PEG gradients. The PEG gradient generated by the flow method decreased adsorption of both proteins in proportion to the PEG surface density; however, their desorption by buffer solution from the grafted PEG layer was not complete. In contrast, desorption of two proteins from the grafted PEG layer generated by a UV oxidation method resulted in near-zero adsorbed amount. The difference between the two types of gradients might have originated from counter-diffusion of PEG and water molecules occurring during the flow method procedure.

Keywords

adsorption; protein; surface

1. Introduction

The interactions between a host and biomaterials have long been studied.^{1–3} Although much progress has been made in the development of biomaterials, successful applications have been limited in scope due to a lack of consistency and understanding of biocompatibility.^{4–6} Medical device biocompatibility is a complex issue, but when these devices are blood contacting, the situation becomes significantly more complex.⁷ Typically a biomaterial, after being exposed to a protein solution such as blood, will become coated with a protein layer within seconds.^{8–11} The adsorption of certain blood proteins prompts the adherence and activation of platelets and blood cells.^{7,12–14} Platelet-initiated fibrin formation and cross-linking can cause fouling or disruption of a biomedical device and its function.^{5,7} These

*Corresponding author: vladimir.hlady@utah.edu.

interactions also initiate two of the major biomaterial challenges: the foreign body reaction and inflammation.^{5,15,16}

A large research effort has focused on the protein-inert properties of polyethylene glycol (PEG)^{17–22}; however, despite the investigation of underlying mechanisms, the protein-inert qualities of PEG are still not fully understood. The large extended volume which the hydrophilic polymer occupies in aqueous solutions and the polymer mobility form an enthalpic and entropic favored state.^{17,19,23–27} In order to adsorb to a PEG-coated surface, a protein would need to overcome the entropic and enthalpic favored state of PEG chains. This and the absence of strong attractive interactions between most proteins and PEG-containing coatings, appear to be the most important adsorption-reducing properties. The protein-inert properties of PEG-containing coatings depend on a range of parameters including PEG molecular weight, interfacial chain density, polymer chain structure, as well as protein size.^{17,18,25,28–30} The objective of the present study was to elucidate the effect of PEG grafting density on the inhibition of protein adsorption. Here, two novel methods of preparing PEG gradients were compared in protein adsorption experiments where albumin and fibrinogen were used as model blood proteins.

2. Materials and methods

2.1 Sulfhydryl surface formation

The initial substrates for the gradient surfaces were 3" × 1" × 1 mm fused silica microscope slides (Ted Pella, Inc.). After cleaning with Millipore® water and Alconox® solution, the slides were soaked in piranha solution (70% H₂SO₄, 30% H₂O₂) for 1 h. The slides were then rinsed again with Millipore® water, dried, and oxygen plasma (Plasmod, Tegal Inc., 50 W @200 mTorr) treated. Oxygen plasma treatment resulted in uniform surface coverage with reactive silanol groups. These surfaces were then immersed in a 1% solution of 3-mercaptopropyltrimethoxysilane (MTS) (M8500, UCT) in toluene for 4 h as previously described.¹⁴ The result was the formation of surface sulfhydryl chemistry (Figure 1).

2.2 PEG gradient formation

Two methods were compared for the formation of PEG density gradients. The first 'flow' method of surface gradient formation was based on a controlled exposure of uniform sulfhydryl surfaces to maleimide-terminated poly (ethylene glycol) (5 kDa, 63187, Sigma-Aldrich) (Figure 2). A silica slide with sulfhydryl groups was placed in a vertical flow cell and a 0.1 mg/ml solution of a maleimide-terminated PEG was progressively added to the cell. This PEG solution, which also contained in 11% K₂SO₄ w/w to collapse PEG chains, was flowed from the bottom of the flow cell, displacing water upwards along the length of the silica slide. The bottom third of the slide was first covered by PEG solution for 5 min to generate a uniformly coated PEG region. After that, the PEG solution was slowly added from the bottom of the cell over 2 min to transiently cover the middle third of the slide to maleimide-PEG and thus create a surface PEG gradient whose steepness depended on the time during which the sulfhydryl surface was in contact with the maleimide-PEG molecules. After 2 min elapsed, water was added from the top to completely wash the PEG solution out. In this way, the PEG solution never contacted the upper third of the silica slide, the middle

third was exposed to PEG between 0 (top) and 2 min (bottom) and the lower third was in contact with PEG solution for 7 min.

The second 'oxidation' method generated the gradient through a gradual oxidation of reactive surface sulfhydryl species into sulfonate moieties as previously described^{14,31} (Figure 3). A uniform sulfhydryl surface was placed into a slide holder and the central 1 cm region of the fused silica slide was gradually exposed to UV light (ELC-4000, Electrolite Corp) by the linear translation of a mask at a constant rate of 0.25 cm/min for 4 min. Afterwards, the slides with the sulfhydryl-sulfonate gradients were reacted with maleimide-terminated PEG + 11% K₂SO₄ w/w solution for 5 min.

2.3 Surface characterization

X-ray photoelectron spectroscopy (XPS) analyses were performed at the University of Utah Nanofabrication laboratory. High-resolution scans were collected using 0.1 eV steps, 300 ms dwell time at a pass energy of 40 eV. Advancing and receding water contact angles were measured by the sessile drop method using a contact angle goniometer (CAM 100, KSV Instruments). Five microlitre Milipore filtered water droplets were dispensed along the length of the samples and imaged using the instrument camera. The KSV software was used to calculate the contact angle of the water droplet using the image of each drop.

2.4 Protein adsorption kinetics

Albumin (HSA) (fraction V, fatty acid free) from human serum and fibrinogen (FGN) were from Sigma-Aldrich and used as received. Each protein solution in Dulbecco's phosphate-buffered saline (DPBS) was fluorescently labeled using Alexa Fluor 488 carboxylic acid, succinimidyl ester (A2000, Invitrogen). After conjugation, the protein solution was passed through a column (PD-10 desalting column, GE Healthcare) to separate the free dye from the protein fraction. After determining the degree of labeling, DPBS was added to generate protein solutions at concentrations that were equivalent to their respective 1 and 10% concentrations in blood plasma.

Total internal reflection fluorescence (TIRF) spectroscopy was used to measure protein adsorption kinetics along the gradients.^{32,33} The excitation light source was a 0.5 mW Ar⁺-ion laser beam. The laser beam was expanded and collimated into a Gaussian profile beam of light (~2 cm diameter). The beam was then passed through a 1 cm × 1 mm rectangle mask to selectively illuminate the whole gradient region with the evanescent wave generated by total internal reflection. The flow cell dimensions were determined by a silicon rubber gasket that separated the gradient silica slide from a flow cell support. Protein solutions were flowed through the TIRF flow cell at 0.7 ml/min using a syringe pump for 10 min followed by a flow of DPBS for another 10 min.

The fluorescence emitted along the gradient was focused on the slit of a monochromator (1681C, SPEX Industries, Inc., #4, 2 mm slit, 300 grooves mm⁻¹ grating) and imaged by a thermo-cooled charge-coupled device (CCD, C200, Photometrics) camera cooled to -46 °C to minimize dark current. The fluorescence signal was binned across a wavelength range to improve the S/N ratio and allow for a faster readout of the CCD camera. A shutter was used to control the exposure to the laser excitation light. A blank image taken with a closed

shutter was used to subtract background noise from the fluorescence at each acquisition time point. The resulting profiles of fluorescence versus gradient length versus time were then combined in a single data file. The data were flat-fielded to correct for the Gaussian profile of the laser excitation light. Twenty lines of binned CCD data from such files were integrated to form the protein adsorption and desorption kinetics and average profiles of adsorbed protein along the PEG gradient.

2.5 Protein adsorption quantification

Experimental TIRF results (fluorescence versus time) need to be converted to the adsorbed protein amount versus time.^{34,35} In general, the rate of protein adsorption is a function of the protein's transport to the surface and the actual adsorption rate to the surface. Hence, the process of adsorption could be limited by either the transport rate or the adsorption rate. In the case of low protein concentrations and for relatively large proteins such as FGN, a transport-limited rate is initially expected. If the protein desorption rate is very slow, the flux of protein molecules to the surface (J_P) can be used to calibrate the fluorescence.^{31,35} When protein solution flows through a channel with a rectangular cross-section the flux of protein molecules to the surface is:

$$J_P = \left[\Gamma \left(\frac{4}{3} \right) \right]^{-1} \cdot 9^{-\frac{1}{3}} \left(\frac{6q}{b^2 w l D_P} \right)^{\frac{1}{3}} D_P c_P \quad 1$$

where D_P is the diffusion coefficient of the protein, c_P is the concentration of protein in solution, $\Gamma(4/3)$ is the gamma function of 4/3, l is the distance along the flow channel, b is the channel thickness, w is the channel width, and q is the volumetric flow rate of the protein solution.³⁵

In a transport-limited regime and in the absence of fast desorption, initial J_P from Equation 1 is approximately equal to the adsorption rate $d\Gamma/dt$, where Γ is the adsorbed amount (units: mass/area).^{34,35} Accordingly, the TIRF measured fluorescence increase in the initial part of the adsorption kinetics can be converted into adsorbed amount versus time by using a conversion factor:

$$X = J_P \cdot \left(\frac{dF}{dt} \right)^{-1} \quad 2$$

where dF/dt is an initial rate of fluorescence intensity increase and X is a conversion constant (units: $\text{kg m}^{-2} \text{ counts}^{-1}$). This method was used to find X for HSA and FGN adsorption from lower solution concentrations (equivalent to 1% in blood plasma) onto the sulhydryl end of the gradient. The conversion factors were then used to convert fluorescence intensity into adsorbed protein amount at all time points and gradient positions (i.e. it was assumed that the fluorescence quantum yields of the conjugated dye did not depend on gradient position). The same conversion factors were also applied to the adsorption from higher solution concentrations. Adsorbed amounts of FGN and HSA obtained by this

method agreed with literature data for sulfhydryl-sulfonate gradients as verified by autoradiography.³¹

3. Results and discussion

3.1 Surface characterization

Each procedure for generating PEG surface gradients began with a uniform sulfhydryl terminated silica surface. The first method of gradient formation utilized the control of the reaction time between reactive PEG and surface sulfhydryl groups using a timed flow protocol. In the second method, the availability of PEG reaction sites was controlled by UV oxidation of the surface sulfhydryls into sulfonate groups.^{14,36} Creating sulfhydryl-sulfonate gradients resulted in wettability changes that were easily verified by measuring water contact angle measurements (Table 1).

Only where sulfhydryl groups persisted on the surface could the maleimide-PEG be grafted to the surface. Prior to PEG grafting, the advancing water contact angles gradually decreased from $\approx 69^\circ$ on the sulfhydryl side to $\approx 32^\circ$ on the sulfonate side which was consistent with previous reports.^{14,28} Figure 4 shows advancing water contact angles after PEG grafting on the two types of gradients as a function of position along the silica slide. Although the flow methods were expected to generate 2-5 cm long gradients, the actual contact angles showed a much shorter (≈ 1 cm) gradient between the unmodified sulfhydryl and PEG-grafted ends of the gradient. The UV oxidation method generated ≈ 1 cm long gradients as expected.

Since contact angle measurements were only an indirect indicator of surface chemistry, high-resolution XPS spectra of C1s and S2p peaks were collected to verify formation of the grafted PEG layer, and the reaction of sulfhydryl oxidation into sulfonate moieties and subsequent PEG grafting (Figures 5 and 6). Uniform sulfhydryl coated silica surfaces were immersed in 0, 0.01, 0.1, or 1 mg/ml maleimide-terminated PEG solutions containing 11% K₂SO₄ w/w for 5 min to determine which concentration of PEG would be most effective for the grafting reaction. Figure 5 shows the XPS spectra of C1s and S2p peaks. The C1s peak changed from ≈ 284 eV (C-C bonds) to ≈ 286 eV (C-O bonds), thus confirming the successful PEG grafting reaction (Figure 5(a)). The spectra also indicated that the 10-fold increases in PEG concentration resulted in little or no change in the surface chemistry. For this reason, it was determined to use a PEG solution of 0.1 mg/ml for the generation of the gradients. The decrease of the ≈ 163 eV S2p sulfur (-SH) doublet peak was caused by formation of an adlayer of PEG polymer on the top of sulfhydryl surface (Figure 5(b)).

Uniform sulfhydryl-coated silica surfaces were also UV oxidized for 0, 1, or 2 min and subsequently immersed in a maleimide-terminated PEG solution for 5 min to graft PEO chains (Figure 6). The changes between the ≈ 284 eV (C-C bonds) and the ≈ 286 eV (C-O bonds) peaks showed a progressive decrease of C-O concentration and thus PEG surface concentration as the oxidation time increased (Figure 6(a)). The C-O contribution to total surface carbon content was 69% for samples that were not UV irradiated. This content of C-O bonds falls within the range of non-fouling PEG coatings.³⁷ The magnitudes of the ≈ 163 eV S2p sulfur (-SH) peak and the ≈ 168 eV oxidized sulfur (-SO₃⁻) peak (Figure 6(b)) also changed as a function of the UV oxidation time.

The ratio of S2p oxidized sulfur ($-\text{SO}_3^-$) peak (at ≈ 168 eV) area to sulfur ($-\text{SH}$) peak (at ≈ 163 eV) area, as determined by deconvoluting each S2p peak into 2 components, i.e., S2p_{1/2} and S2p_{3/2},³⁷ increased monotonically from 0.16 (no UV irradiation) to 2.85 (5 min UV irradiation). Such an increase of ($-\text{SO}_3^-$)/($-\text{SH}$) ratio was a signature of the progressive conversion of reactive ($-\text{SH}$) surface chemistry into non-reactive ($-\text{SO}_3^-$) moieties over the UV irradiation time.³⁶

3.2 Protein adsorption kinetics

Figure 7 shows the FGN and HSA adsorption/desorption kinetics from lower protein concentrations onto the two ends of the gradients prepared by the flow method. At these low concentrations, adsorption to the sulfhydryl surface was faster and exceeded the adsorption rate onto the grafted PEG side of the gradient. When buffer was used to replace the HSA protein solution, a partial desorption took place (Figure 7(a)) from both surface regions, while in the case of FGN, almost no change in the FGN adsorbed amount was observed (Figure 7(b)). The results showed that for both HSA and FGN, grafted surface PEG chains were effective at reducing protein adsorption but not eliminating it completely. The effect of PEG surface density in the region of the PEG-sulfhydryl gradient is shown in Figures 7(c) and 7(d). The adsorbed protein profiles were taken at the end of adsorption cycle (showing the adsorption average between 9th and 10th minute while protein solution is flowed) and at the end of desorption cycles (adsorption average between 19th and 20th minute while buffer solution is flowed).

In the HSA experiment, a difference between the two profiles was observed (Figure 7(c)) while the two FGN profiles were indistinguishable (Figure 7(d)). It is known that the evanescent wave used in TIRF method also excites a minor fluorescence contribution from the bulk protein in the proximity of the solid-liquid interface.³⁸ This could be the reason for the difference of the two HSA profiles as the dye-protein molecules were present in higher concentration than in the case of FGN.

Figure 8 shows the HSA and FGN adsorption and desorption kinetics from the protein solutions equivalent to their 10% blood plasma concentrations on two ends of the PEG gradients generated by the oxidation method, i.e. to grafted PEG and sulfonate regions. As in the case of adsorption from the lower solution concentration, protein surface adsorption was fast during the adsorption cycle of the experiment. For both HSA (Figure 8(a)) and FGN (Figure 8(b)), once the protein solution was replaced with a buffer solution, a larger fraction of protein was desorbed from the surface in a matter of a minute; much more than what was observed in the experiments using a lower concentration of proteins (Figure 7). This could be due in part to the removal of bulk protein solution fluorescence excited by the evanescent wave. Another, more important difference was a complete desorption of both HSA and FGN from the grafted PEG side of the gradient. During the flow of buffer solution after a few minutes, both proteins desorbed from the grafted PEG region to such a degree as to render them undetectable by TIRF, i.e. to near-zero surface adsorption. This behavior, a weak binding followed by (almost) complete wash-off, has been predicted by theoretical models of protein adsorption to grafted polymer chains.^{39,40} Figure 8(c) shows the profiles of adsorbed proteins from a higher solution concentration at the end of desorption cycles

(average amount between 19th and 20th minute while buffer solution is flowed). Both adsorbed amount profiles approached zero adsorption towards the PEG side of the gradient while the adsorbed amounts at the sulfonate region were at the levels comparable to previously reported data.³¹ Figure 8(d) shows two HSA adsorption profiles taken in different experiments illustrating how reproducible PEG gradients were when prepared by the oxidation method.

When comparing protein adsorption onto the two types of gradients, one notices two important differences.

1. The flow gradient preparation method generates PEG regions that retain both adsorbed proteins even when they adsorbed from lower solution concentrations (Figure 7(c)), while the two adsorbed proteins completely desorbed from the PEG regions prepared by the oxidation method even if adsorption was carried out from more concentrated protein solutions (Figure 8(c)).
2. Adsorbed amount profiles on the PEG gradients prepared by the oxidation method appear smoother than profiles observed on the flow method gradients. Both of the HSA and FGN profiles on the flow method gradients show a small 'bump' (marked by dashed vertical error in Figure 7(d)) which may have originated at the boundary between the middle and the lower third of silica slide exposed to PEG solution for 2 and 5 + 2 min, respectively.

It is difficult to pinpoint the causes for these differences as both PEG grafting reaction conditions were apparently identical. It is possible that in the flow gradient preparation method the PEG solution flown to displace water in the flow chamber might have created a diffuse boundary layer across which water diffused into PEG (and PEG into water) during the 5 + 2 min of reaction. Such counter-diffusion would dilute maleimide-PEG concentration and could have possibly diminished the extent of the grafting reaction which then resulted in a lower PEG surface density. Such scenario seems to be contradicted by the identical water contact angles on the PEG side of the gradients (Figure 4). This may also indicate that water contact angles are not as sensitive to PEG surface density as adsorbing proteins are.

4. Summary

In order to further understand the mechanisms of PEG's protein inertness, two types of grafted PEG density gradient surfaces were generated and characterized using contact angle measurements and XPS. TIRF was used to measure adsorption kinetics and the adsorption profiles of fluorescently-labeled human serum albumin and fibrinogen on the two types of PEG gradients. Each protein solution was diluted to 1 or 10% of their respective blood concentrations. The grafted PEG layer generated by the flow method decreased adsorption of both proteins as compared with silylhydride terminated silica surface. However, the protein desorption from PEG-coated regions was not complete as seen by residual adsorbed amounts of both proteins after 10 min of desorption by buffer solution. This result was contrasted by the desorption kinetics and adsorbed protein profiles measured on the gradients created by UV oxidation of reactive surface silylhydride groups and a subsequent maleimide-PEG grafting reaction. On this type of gradient, the desorption in the buffer solution was complete

even when protein adsorption took place from more concentrated solutions (10%) resulting in a near-zero adsorbed amount towards the PEG side of the gradient. The difference between the two types of gradients might have originated from counter-diffusion of PEG and water molecules in the flow method procedure.

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Figure 1.
Diagram of the MTS reaction with surface silanols forming uniform sulfhydryl surfaces

Figure 2.
Diagram of the reaction for grafting PEG chains to silica surface with reactive sulfhydryl groups

Figure 3.

Diagram showing the progressive oxidation of surface sulfydryl groups for the formation of PEG gradients (adapted from ref. 14)

Figure 4.
Water contact angles observed on two types of PEG gradients

Figure 5.

XPS spectra of the C1s carbon peak (a) and S2p sulfur peak (b) showing the changes of C-C to C-O bonds and decrease of -SH signal after the PEG grafting reaction from different solution concentrations

Figure 6.

XPS spectra of the C1s carbon peak (a) and S2p sulfur peak (b) showing the changes of C-C to C-O bonds and -SH to -SO_3^- moieties after PEG grafting reactions to sulfhydryl surfaces as function of UV oxidation duration

Figure 7.

HSA (a) and FGN (b) adsorption kinetics from the solutions equivalent to 1% blood protein concentrations to sulfhydryl and PEG surface regions of the PEG gradient generated by the flow method. Data between 600 and 700 s during the switch of protein solution flow to buffer were removed. HSA (c) and FGN (d) adsorbed amounts profiles along PEG gradient at the end of the adsorption and desorption cycles of the experiments

Figure 8.

HSA (a) and FGN (b) adsorption kinetics from the solutions equivalent to 10% blood protein concentrations to sulfonate and PEG surface regions of the PEG gradient generated by the oxidation method. Data between 600 700 s during the switch of protein solution flow to buffer were removed. HSA and FGN (c) adsorbed amounts profiles along PEG gradient at the end desorption cycle of the experiments. (d) Two HSA desorption profiles measured from 1% solution in separate experiments on PEG gradients prepared by the oxidation method

Table 1

Contact angles measured on each side of sulfhydryl-sulfonate gradients

Contact angles	Sulfhydryl region	Sulfonate region
Advancing	69±4°	32±5°
Receding	53±3°	10±3°