Self-Assembled Monolayers of Alkanethiolates Presenting Mannitol Groups Are Inert to Protein Adsorption and Cell Attachment

Yan-Yeung Luk, Mihoko Kato, and Milan Mrksich*

Department of Chemistry The University of Chicago, Chicago, Illinois 60637

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This paper reports on a model surface that is inert in biological fluids and that is important for studies in biointerfacial science. A self-assembled monolayer (SAM) terminated in the mannitol group was found to prevent the adsorption of proteins and the attachment of cells. Surface plasmon resonance spectroscopy showed that the mannitol-terminated SAM prevented the adsorption of several proteins and was indistinguishable from a SAM presenting tri(ethylene glycol)groups. In a second set of experiments, monolayers were patterned into circular regions of hexadecanethiolate with the surrounding area terminated in the mannitol group in order to evaluate the inert surface for patterning cell attachment. 3T3 fibroblasts, attached to the circular regions, proliferated to occupy these regions completely and remained patterned on these regions for 25 days. The use of oligo(ethylene glycol)-terminated SAMs, by contrast, showed a loss in fidelity of the pattern after one week in culture. The mannitol-terminated monolayers significantly extend the time course for maintaining patterned cells and will have immediate utility in fundamental and applied biology.

Introduction

Self-assembled monolayers (SAMs) of alkanethiolates on gold are an important class of model substrate for mechanistic studies of the interactions of proteins and cells with surfaces.[1] A primary reason for the importance of these model substrates is the availability of SAMs that are inert, that is, SAMs that *prevent* the adsorption of protein and the attachment of cells.[2] Monolayers terminated in short oligomers of the ethylene glycol group ($[OCH_2CH_2]_nOH$, $n = 3-6$) prevent the adsorption of virtually all proteins under a wide range of conditions and are currently the most effective inert surface chemistry.[3] This class of SAMs has been critical for developing methods to pattern the adhesion of cells,[4,5] to prepare surfaces that present ligands for selective interactions with proteins,[6] and to design electroactive dynamic substrates that can modulate the selective recognition of proteins.[7]

Given the importance of inert surfaces, it is surprising that there are so few functional groups that have been identified that render materials inert—and none that are as effective at preventing protein adsorption as are oligo(ethylene glycol) groups when presented on SAMs. We reported that a monolayer presenting a tri(propylene sulfoxide) group was inert to protein adsorption and cell attachment.[8] The selection of the propylene sulfoxide group was largely based on preserving three properties believed to be important with the glycol group: (i) the repeating unit is conformationally flexible; (ii) the sulfoxide group is a hydrogen bond acceptor but not a donor; and (iii) the sulfoxide group is water-soluble and self-repulsive. Whereas monolayers terminated in this propylene sulfoxide group prevented protein adsorption, they were generally less effective and less stable over time than were SAMs presenting oligo(ethylene glycol) groups. The synthetic route to the propylene sulfoxide oligomers is laborious, which further limits the utility of these monolayers. Another early example was reported by Prime and Whitesides, who showed that a monolayer terminated in the carbohydrate maltose prevented protein adsorption.[2] In that work, the lack of adsorption was demonstrated with ellipsometric measurements, which required the substrate to be removed from solution, rinsed, and then dried prior to the measurement. Because this and related ex situ techniques cannot detect weak and reversible adsorption, it is not clear whether these monolayers will ultimately prove inert in settings where the substrate is in contact with a protein-containing solution for long periods of time.

The development or identification of alternative functional groups that can render SAMs inert to protein adsorption and cell attachment is important for at least

* To whom correspondence should be addressed.

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two reasons. First, it is probable that no one surface chemistry will be best suited to give an inert interface under *all* conditions. The availability of several inert surface chemistries would permit optimization of the substrate properties for each unique application. Second, additional examples of inert surfaces would aid in developing a mechanistic understanding of the key factors that underlie protein resistance. This improved understanding will certainly lead to the development of surface modifications that are more practical to apply to common materials—for example, those based on polymer grafting—but retain the effectiveness of the model substrates. In this paper, we show that SAMs presenting the mannitol group are highly effective at preventing the adsorption of proteins and attachment of cells and that these monolayers are superior to SAMs of EG_nOH for patterning the long-term adhesion of cells.

Experiments and Results

Experimental Design. We used two experimental procedures to evaluate the monolayers as inert surfaces.[9] Surface plasmon resonance (SPR) spectroscopy measures the adsorption—or in the case of inert SAMs, the lack of adsorption—of protein to the monolayer.[10] SPR is well suited for characterization of protein adsorption because it measures adsorption in situ and can detect weak, readily reversible protein adsorption. For all monolayers, we investigated a panel of five proteins that spanned a range in molecular weight and p*I*. In a second set of experiments, we evaluated the ability of the monolayers to pattern the long-term adhesion of Swiss 3T3 Fibroblast cells. These experiments used monolayers that are patterned into hydrophobic regions of hexadecanethiolate (HDT) surrounded by the inert monolayer. Cells initially attach only to the hydrophobic regions and proliferate to completely fill those regions after 2−3 days in culture. We compared the periods of time that the cells remain confined to the pattern—because the surrounding monolayer prevents attachment—to assess the effectiveness of the inert monolayer. The cell patterning experiments impose a more demanding environment than do the protein adsorption experiments, and hence, provide a more stringent test of inertness.

We compared protein adsorption and cell attachment on four different monolayers (Figure 1). As a control monolayer that is not inert, we used a SAM of hexadecanethiolate that presents hydrophobic methyl groups at the surface. Monolayers terminated in the tri(ethylene glycol) group were used as the current standard for inert surfaces. Each of the two additional monolayers presents the mannitol group and differs only in the length of alkyl chain to which the mannitol group is appended (Figure 1). Alkanethiol **1a** has an alkyl chain of eleven methylene units, which is the standard length in SAMs that are used in bio-interfacial science. Alkanethiol **1b** has three fewer methylene units in the alkyl chain and was investigated with the goal of finding inert monolayers that are thinner overall.

Measuring Adsorption of Protein. We used SPR to measure the adsorption of five proteins—fibrinogen, pepsin, lysozyme, insulin, and trypsin—to the four monolayers.[11] SPR measures changes in the refractive index of a solution near the interface with a gold film by measuring changes in the angle ($\Delta\Theta_m$) at which p-polarized light reflected from the glass/gold interface has a minimum in intensity.[10] SAMs were mounted in a flow cell and experiments were performed by first flowing a phosphate-buffered saline (PBS) through the cell for 5 min, then a solution of protein in the same buffer (0.5 mg/mL) for 5 min, and then PBS for 5 min. The rise in $\Delta\Theta_m$ upon introduction of the protein solution is the sum of two contributions. The first is due to the increase in refractive index of the solution caused by the dissolved protein—the "bulk effect"—and does not represent protein adsorption. The second contribution is due to the adsorption of protein to the SAM. The amount of protein that adsorbs irreversibly to the SAM is determined by comparing the shift in Θ_m before and after the SAM is exposed to protein. An increase in Θ_m of 0.1° corresponds to an increase in density of adsorbed protein of 1 ng/mm^2.[9,10]

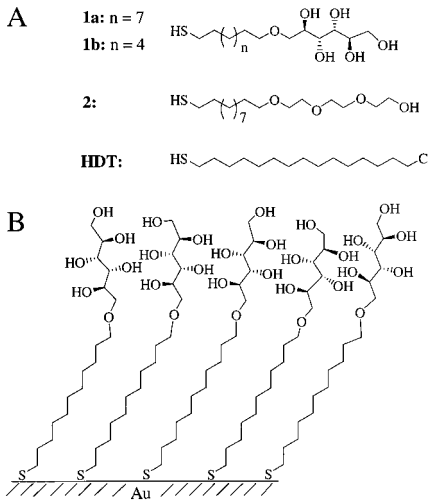

Figure 1. (A) Structures of the alkanethiols used to prepare monolayers in this work. Alkanethiols **1a** and **1b** are both substituted with the mannitol group but differ in the length of the poly(methylene) chain. Alkanethiol **2** is substituted with the tri(ethylene glycol) group. Hexadecanethiol (HDT) was used to prepare control monolayers that are not inert. (B) Schematic representation of the mannitol-terminated SAM. The conformation of the mannitol groups appended on the SAM has not been established.

Figure 2 shows data for the adsorption of fibrinogen to the four SAMs. A complete monolayer of protein adsorbs to the SAM of hexadecanethiolate. The change in Θ_m of 0.35° corresponds to a density of protein of 3500 pg/mm^2. The two monolayers presenting mannitol groups, by contrast, show essentially no adsorption of fibrinogen. This lack of adsorption is indistinguishable from that on monolayers presenting tri(ethylene glycol) groups. Experiments with the four other proteins gave similar results and demonstrated that the mannitol group is broadly effective at preventing protein adsorption (Table 1). For all five proteins, the amount of irreversible adsorption was less than 2% of the total amount that adsorbed on the methyl-terminated SAMs (HDT).[10]

Patterning the Adhesion of Cells. We evaluated the degree to which these monolayers could prevent the adhesion and growth of cells that were confined to a patterned substrate. We patterned gold substrates by microcontact printing[4] a set of circular regions of hexadecanethiolate that were 200 μm in diameter and then immersed the substrates in a solution of **1a**, **1b**, or **2** to assemble an inert monolayer on the nonprinted areas. When a suspension of cells was added to culture wells containing the substrates,[12] cells attached only to the methyl-terminated regions. For each

(9) The alkanethiols **1a** and **1b** were each synthesized in 3 steps from commercially available reagents. All intermediates gave satisfactory ^{1}H NMR and mass spectral data. Gold substrates were prepared by evaporation of titanium (1.5 nm) and then gold (40 nm) onto glass cover slips (0.20 mm, No. 2, Corning). The SAMs were prepared by immersing gold substrates of approximately 1 cm^2 in ethanolic solutions of alkanethiol (2 mM) for 9 h. The SAMs were rinsed with ethanol and dried with nitrogen before use.

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(11) All SPR experiments were performed on a BIAcore 1000 instrument. Monolayer substrates were glued into plastic BIACore cassettes with a two-part epoxy (Devcon). Phosphate-buffered saline (PBS; 10 mM phosphate, 150 mM sodium chloride, pH 7.6) was degassed under vacuum, and all protein solutions were filtered through 0.45-μm filters before use.

(12) Albino 3T3-Swiss fibroblasts (American Type Culture Collector) were harvested from culture dishes with trypsin-EDTA, washed, and resuspended in DMEM supplemented with 10% fetal bovine serum. The suspended cells were added to wells (10 000/ well) containing patterned monolayer substrates immersed in DMEM supplemented with serum. Serum-supplemented medium was exchanged with fresh medium after 12 h and then every 5 days. Cell cultures were maintained at 37 °C and photographed daily with a Hamamatsu CCD camera through a Zeiss axiovert phase-contrast microscope (5× magnification).

Table 1. Adsorption of Protein on Monolayers

SAM	thickness[a]	fibrinogen[b] 40 kD,[d] 5.5[e]	pepsin 35 kD, <1	lysozyme 14 kD, 11.4	insulin 5.4 kD, 5.4	trypsin 24 kD, 10.5
1a	18.0 Å	27[c]	<10	19	15	<10
1b	15.7 Å	45	12	10	25	16
2	19.5 Å	29	<10	<10	<10	<10
HDT	20.0 Å	3432	1337	1023	688	661

[a] Thickness of the SAM measured by ellipsometry (±0.5 Å). [b] All protein solutions were 0.5 mg/mL in PBS (10 mM phosphate, 150 mM sodium chloride, pH = 7.6). [c] The amount of protein that remains adsorbed to the SAM was determined by SPR and is reported in units of pg/mm^2. Each value for absorbed protein was taken from a single experiment and has a variance of approximately 5% across independent experiments. [d] Molecular weight of the protein. [e] p*I* of the protein.

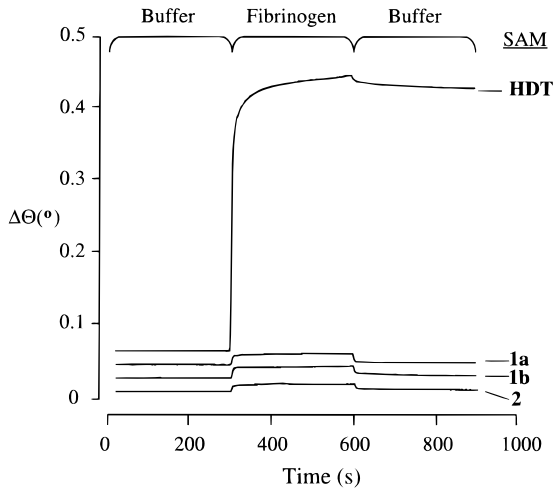

Figure 2. Data from surface plasmon resonance spectroscopy for the adsorption of fibrinogen (0.5 mg/mL protein in 10 mM phosphate, 150 mM sodium chloride, pH = 7.6) to each of the four SAMs. The monolayer is indicated to the right of each plot. The *relative* change in angle of minimum reflectivity ($\Delta\theta_m$) is plotted as solutions are flowed over the SAM. PBS buffer was first flowed, replaced with a solution of protein in PBS, and then finally replaced with PBS. The periods of time during which protein and buffers were flowed over the SAM are indicated above the plot. The curves are offset vertically for clarity.

of the substrates patterned with the inert monolayers (**1a**, **1b**, and **2**), cells spread and proliferated to completely occupy these regions but did not invade the surrounding inert regions. The period of time that cells remain confined to the patterned monolayers provides a relative measure of the effectiveness of inert surface chemistries and a direct measure for identifying inert surfaces that are most effective for applications requiring long-term cell patterning.

Albino 3T3-Swiss fibroblasts patterned on the monolayers were kept at 37 °C in serum-containing media for four weeks. The media was exchanged every 5 days, and cells were photographed daily (Figure 3). In all cases, cells remained patterned to the circular regions of hexadecanethiolate for at least 6 days in culture. The tri(ethylene glycol)-terminated monolayers began to fail after 7 days, with cells spreading onto the inert regions. After 12 days, cells had migrated from the circular regions and divided to give a confluent monolayer of cells, with a complete loss of pattern. Monolayers terminated in the mannitol group (**1a**) were substantially more effective at confining cells for longer periods. Even after 21 days, cells remained completely confined to the patterns. After 25 days in culture, the viability of the adherent cells decreased and therefore prevented an assessment of the monolayer over longer periods of time. Monolayers that presented the mannitol group on the shorter polymethylene chain (prepared from alkanethiol **1b**) were also more effective than monolayers presenting the tri(ethylene glycol) group but were not as effective as monolayers of **1a**. Monolayers of **1b** generally failed at two weeks in culture. We repeated these long-term patterning experiments on three separate occasions with consistent results: monolayers of **2** fail at approximately one week, monolayers of **1b** fail at approximately two weeks, and monolayers of **1a** are effective for at least three weeks.

Discussion

The most important result from this work is that SAMs presenting mannitol groups are highly effective as inert surfaces. SPR showed that monolayers of **1a** and **1b** prevented the adsorption of several different proteins, including the "sticky" protein fibrinogen. In this respect, the monolayers were indistinguishable from monolayers presenting tri(ethylene glycol) groups, which are the current standard for inert model surfaces. When evaluated for the ability to maintain the patterned adhesion of cells, we found that SAMs of **1a** were superior to SAMs presenting tri(ethylene glycol) groups. In this and other work, we found that SAMs presenting tri(ethylene glycol) groups fail at approximately 7 days in culture. The mannitol-terminated SAMs, by contrast, maintained the pattern of adherent cells over as many as 25 days. We do not know whether the pattern fidelity persists for longer periods since these experiments were limited by the viability of the cells. We also do not understand the reasons for which SAMs terminated in oligo(ethylene glycol) groups fail. It is generally believed that oxidation of the glycol chains is involved,[13] but this supposition remains to be proven.

The mechanisms by which inert monolayers prevent protein adsorption are not yet established. Theoretical considerations suggest that the absence of protein adsorption to poly(ethylene glycol) of high molecular weight is due to steric repulsion in the polymer, which is disfavored for two reasons.[14] First, adsorption of protein to the polymer results in a compression of the glycol chains and is entropically unfavorable since the conformational dynamics of the polymer chains are restricted. Second, this compression also results in desolvation of the polymer—as water molecules are transferred to the bulk—with a corresponding enthalpic penalty. These same factors, however, are probably not of central importance with *monolayers* presenting short oligomers of the ethylene glycol groups, since these short chains are confined at high density and probably cannot accommodate a high degree of solvation and conformational dynamics.

Recent work by Grunze and co-workers suggests that the conformations of the ethylene glycol chains are crucial for inertness.[15] This work compared the structures of tri(ethylene glycol)-terminated monolayers on gold and on silver substrates; the latter were not inert to protein adsorption. Fourier transform infrared reflection—ad-

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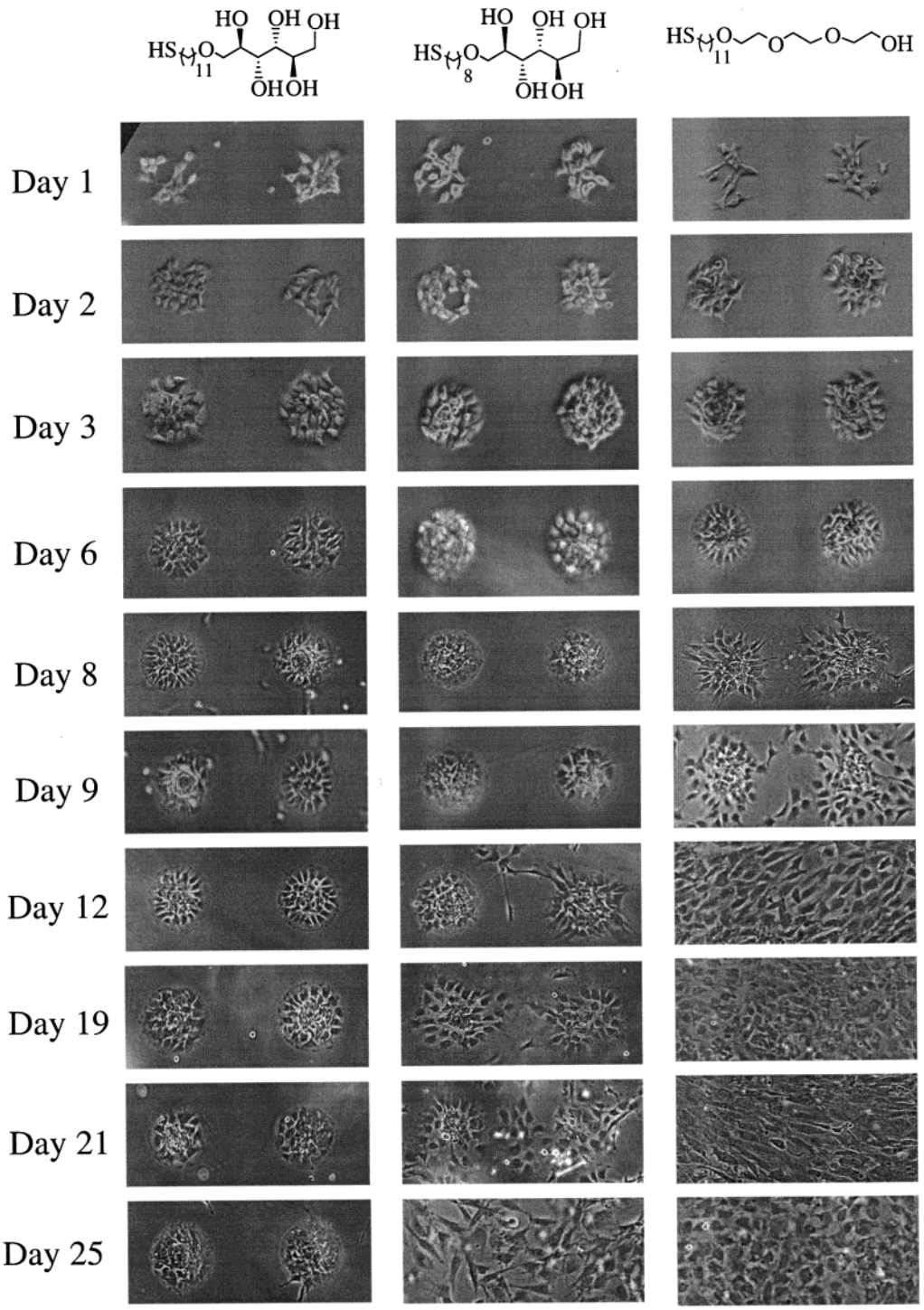

Figure 3. Attachment of 3T3 fibroblast cells to SAMs patterned into circular regions of hexadecanethiolate (HDT) with surrounding regions of either **1a**, **1b**, or **2**. Optical micrographs are shown for cells attached to each of three patterned monolayers over a period of 25 days. The structures of the alkanethiols that comprise the inert regions of the patterned monolayers are indicated above each column of photographs. The number of days that substrates were in culture is indicated to the left.

sorption spectroscopy showed that the glycol chains adopt helical conformations when supported on gold substrates but adopt an extended, all-trans conformation when supported on silver substrates (in part because of the higher adsorbate density on silver). Further studies that used scanning force microscopy to compare the monolayers on gold and on silver revealed a striking difference in the properties of water at the interface.[16] A scanning probe tip, modified to render it hydrophobic, experienced a repulsive interaction with the monolayer on gold—even at distances greater than 20 nm. For the monolayer on silver, however, the tip experienced a long-range attractive interaction starting at about 35 nm from the interface. Theoretical calculations are in agreement with these results[17] and suggest that the inert monolayers template an ordered solvent structure that prevents the approach

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of protein to the surface, and hence, prevents protein adsorption.[16] We are now pursuing similar mechanistic studies to determine whether mannitol-terminated monolayers prevent adsorption through a common mechanism.

The mannitol-terminated SAMs are significant because they extend the time course over which cells can be patterned in culture. This enhancement is important for several applications that use cells as functional components.[18] A primary example is the use of genetically engineered cells for screening libraries of drug candidates.[19] There is now substantial effort directed at transferring cell-based assays from 96-well titer plates to chip format. This and other applications require substrates that can maintain patterned cell populations with excellent fidelity and over long periods of time.

The mannitol-terminated SAMs described here are highly effective at preventing protein adsorption and extend the times over which cultured cells can be maintained in patterns. We believe these monolayers will find immediate utility for fundamental studies in cell biology and for patterning of cells onto chips for applications in diagnostics and high throughput screening.

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