

Self-Assembled Monolayers That Resist the Adsorption of Proteins and the Adhesion of Bacterial and Mammalian Cells

Emanuele Ostuni,[†] Robert G. Chapman,[†] Michael N. Liang,[†] Gloria Meluleni,[‡] Gerald Pier,[‡] Donald E. Ingber,[§] and George M. Whitesides^{*,†}

Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138, Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, 181 Longwood Avenue, Boston, Massachusetts 02115, and Departments of Surgery and Pathology, Children's Hospital and Harvard Medical School, Enders 1007, 300 Longwood Avenue, Boston, Massachusetts 02115

Received April 16, 2001. In Final Form: June 28, 2001

This paper examines the hypothesis that surfaces resistant to protein adsorption should also be resistant to the adhesion of bacteria (*Staphylococcus aureus*, *Staphylococcus epidermidis*) and the attachment and spreading of mammalian cells (bovine capillary endothelial (BCE) cells). The surfaces tested were those of self-assembled monolayers (SAMs) terminated with derivatives of tri(sarcosine) (Sarc), *N*-acetylpiperazine, permethylated sorbitol, hexamethylphosphoramide, phosphoryl choline, and an intramolecular zwitterion ($-\text{CH}_2\text{N}^+(\text{CH}_3)_2\text{CH}_2\text{CH}_2\text{CH}_2\text{SO}_3^-$) (ZW); all are known to resist the adsorption of proteins. There seems to be little or no correlation between the adsorption of protein (fibrinogen and lysozyme) and the adhesion of cells. Surfaces terminated with derivatives of Sarc and *N*-acetylpiperazine resisted the adhesion of *S. aureus* and *S. epidermidis* as well as did surfaces terminated with tri(ethylene glycol). A surface that presented Sarc groups was the only one that resisted the adhesion of BCE cells as well as did a surface terminated with tri(ethylene glycol). The attachment of BCE cells to surfaces could be patterned using SAMs terminated with derivatives of Sarc, *N*-acetylpiperazine, phosphoramidate, and the ZW as the attachment-resistant component and methyl-terminated SAMs as the adhesive component.

Introduction

This paper examines six single-component self-assembled monolayers (SAMs) for their ability to resist the adsorption of proteins from solution and the adhesion of *Staphylococcus epidermidis*, *Staphylococcus aureus*, and bovine capillary endothelial (BCE) cells from suspension. We have recently described these SAMs as alternatives to SAMs terminating in oligomers of ethylene glycol $(\text{EG})_n\text{OR}$, $\text{R} = \text{H}, \text{CH}_3$ for the formation of surfaces that resist the adsorption of proteins.^{1,2} The ability to resist the adsorption of proteins is generally believed to be a prerequisite for the ability of a surface to resist the attachment of bacteria and mammalian cells.^{3,4} For brevity, we refer to surfaces that resist the adsorption of protein as "inert".

We tested the hypothesis that resistance to adsorption of proteins would correlate with resistance to attachment of bacterial and mammalian cells using six homogeneous SAMs with terminal groups that each belong to different structural classes. These SAMs allow us to search for relationships between the structures of surfaces and their

ability to resist the adsorption of proteins or the adhesion of bacterial and mammalian cells. Studies of this sort have not been possible in the past because only one inert surface (that based on SAMs terminating in $(\text{EG})_n\text{OH}$ groups) was available.

Adsorption of Proteins. The mechanism by which proteins interact with surfaces, whether biospecifically or based on hydrophobicity or other nonspecific interaction, has challenged many researchers to define biophysical parameters that characterize this process. Despite substantial effort, our understanding of the mechanisms of adsorption of proteins on surfaces remains incomplete.⁵ Nonspecific interactions between surfaces and proteins usually involve hydrophobic interactions, although electrostatic interactions may be important in some circumstances.⁶ More detailed questions about the mechanism—the nature of the initial adsorbed species, the mobility of this species on the surface, the rate at which it changes conformation on the surface, its interactions with other adsorbed species—are all still the subject of speculation. A clear understanding of adsorption has immediate applications in fields such as bioanalysis,³ micrototal analysis,⁷ tissue engineering,⁸ proteomics,⁹ and separations.⁷

Inert Surfaces. The early and serendipitous discovery of the inertness of surfaces having exposed $(\text{EG})_n\text{OH}$ chains led to the acceptance of these surfaces as the standard for

* Corresponding author tel: (617)495-9430; fax: (617)495-9857; e-mail: gwhitesides@gmwhgroup.harvard.edu.

[†] Department of Chemistry and Chemical Biology.

[‡] Channing Laboratory, Department of Medicine.

[§] Departments of Surgery and Pathology.

(1) Ostuni, E.; Chapman, R. G.; Holmlin, R. E.; Takayama, S.; Whitesides, G. M. *Langmuir*, in press.

(2) Chapman, R. G.; Ostuni, E.; Takayama, S.; Holmlin, R. E.; Yan, L.; Whitesides, G. M. *J. Am. Chem. Soc.* **2000**, *122*, 8303–8304.

(3) Ratner, B. D.; Hoffmann, F. J.; Schoen, J. E.; Lemons, F. *Biomaterials Science: An Introduction to Materials in Medicine*; Academic Press: New York, 1996.

(4) Chapman, R. G.; Ostuni, E.; Liang, M. N.; Meluleni, G.; Kim, E.; Yan, L.; Pier, G.; Warren, H. S.; Whitesides, G. M. *Langmuir* **2001**, *17*, 1225–1233.

(5) Ramsden, J. J. *Q. Rev. Biophys.* **1993**, *27*, 41–105.

(6) Claesson, P. M. In *Biopolymers At Interfaces*; Malmsten, M., Ed.; Marcel Dekker: New York, 1998; Vol. 75, pp 281–320.

(7) Manz, A.; Becker, H. *Microsystem Technology in Chemistry and Life Sciences*; Springer-Verlag: Berlin, Germany, 1998.

(8) Niklason, L. E.; Gao, J.; Abbott, W. M.; Hirschi, K. K.; Houser, S.; Marini, R.; Langer, R. *Science* **1999**, *284*, 489–493.

(9) Macbeath, G.; Schreiber, S. L. *Science* **2000**, *289*, 1760–1763.

many applications in drug delivery and biomaterials.¹⁰ Surfaces terminated with (EG)_nOH groups provided the first and, so far, only example that could be used in developing a mechanistic explanation of inertness. Andrade and de Gennes rationalized the resistance to adsorption of proteins at surfaces functionalized with poly(ethylene glycol) (PEG) using concepts developed to explain colloid stabilization; the compression of the long, hydrated, polymer chains, upon approach of the protein, causes unfavorable steric interactions that prevent adsorption.^{11–13} We established that SAMs terminated with as few as three repeat units of oligo(ethylene glycol) are inert to the adsorption of proteins.^{14,15}

The inertness of (EG)_nOR-terminated surfaces was unmatched until the recent development of mannitol-terminated SAMs by Mrksich and co-workers.¹⁶ The resistance of this type of surface to the adsorption of proteins appears to be comparable to an (EG)_nOH-terminated SAM, and Mrksich has reported that it resists the adhesion of certain mammalian cells for longer periods of time than a surface terminated with tri(ethylene glycol).¹⁶ We continue to use (EG)_n-terminated SAMs as the standard for inert surfaces, however, for two reasons: first, the (EG)_n-terminated SAMs are the most extensively characterized inert surfaces; second, the mannitol-terminated SAMs may be different in the mechanisms that make them inert from most other inert surfaces.^{14,15,17–20} We use surfaces that present tri(ethylene glycol) terminated with OH and OCH₃ groups interchangeably: their behavior in adsorption of proteins is similar.²⁰

Although (EG)_nOH is a very useful functional group, its applications are limited in some circumstances by some of its characteristics: (EG)_nOH is a polyether that can autoxidize in the presence of O₂ and transition metal ions (most biological fluids contain these substances).^{21–23} In vivo, the hydroxyl groups of (EG)_nOH can be oxidized to aldehydes and acids by alcohol dehydrogenase and aldehyde dehydrogenase, respectively.^{24,25} In applications in cell culture, patterns formed using (EG)_nOH-terminated SAMs eventually lose their definition and are overgrown.¹⁶ The shortcomings of PEG warrant the search for new types of inert surfaces.

We have recently described a protocol that combines SAMs and surface plasmon resonance (SPR) to screen

organic functional groups and to identify those that make surfaces inert.^{1,2,20,26} By screening ca. 60 surfaces, we found that the functional groups that are best at rendering surfaces inert, when they are installed on SAMs, share the following characteristics: (i) they contain hydrogen bond acceptor groups but not hydrogen bond donor groups, (ii) their overall charge is neutral,²⁷ and (iii) they are polar.^{1,2} (The mannitol-terminated surface of Mrksich is an obvious exception to this characterization.^{16,28} Other surfaces coated with derivatives of carbohydrates, however, also reduce or resist the adsorption of proteins and may share mechanistic and/or structural similarities with the mannitol-terminated surface.^{14,29})

We, Grunze, Mrksich, and others^{1,2,16,30} have accumulated evidence that points to the importance of the interaction of water with surfaces in determining their inertness. We found that inert surfaces were hydrophilic and contained hydrogen bond acceptor groups.^{1,2} Inertness, however, did not correlate with interfacial free energy; details of the molecular structure of the surfaces seemed to influence inertness strongly.¹ Grunze found that the conformations of (EG)_nOR that promote inertness are the same ones that interact most strongly with water;^{17,18} this result was confirmed by sum-frequency generation spectroscopy and simulations.^{31,32} Parsegian and Rau found that the interaction of carbohydrates with water was independent of the acetylation of the hydroxyl groups.³³

Adhesion of Bacterial Cells. The adhesion of bacteria to surfaces and host cells can occur by a number of mechanisms, both biospecific (protein–protein, carbohydrate–protein) and nonbiospecific (hydrophobic or electrostatic).³⁴ Examples of biospecific interactions are the attachment of type I pili of *Escherichia coli* to mannose groups³⁵ and the adhesion of microbial surface components recognizing adhesive matrix molecules of *S. aureus* to host plasma proteins adsorbed to bone matrix or bone implant materials.^{34,36–39}

It appears that the adhesion of bacteria to surfaces is facilitated by a layer of adsorbed protein. It is thus a plausible hypothesis that surfaces that resist the adsorption of proteins are possible candidates for surfaces that prevent bacterial adhesion; we have used this strategy successfully with thin polymeric films grafted to surfaces.⁴ Currently, no material exists that completely resists the

(10) Harris, J. M.; Zalipsky, S. *Poly(ethylene glycol): Chemistry and Biological Applications*; American Chemical Society: Washington, DC, 1997.

(11) Jeon, S. I.; Lee, L. H.; Andrade, J. D.; de Gennes, P. G. *J. Colloid Interface Sci.* **1991**, *142*, 149–158.

(12) Jeon, S. I.; Andrade, J. D. *J. Colloid Interface Sci.* **1991**, *142*, 159–165.

(13) Andrade, J. D.; Hlady, V. *Adv. Polym. Sci.* **1986**, *79*, 1–63.

(14) Prime, K. L.; Whitesides, G. M. *Science* **1991**, *252*, 1164–1167.

(15) Prime, K. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1993**, *115*, 10714–10721.

(16) Luk, Y.-Y.; Kato, M.; Mrksich, M. *Langmuir* **2000**, *16*, 9604–9608.

(17) Harder, P.; Grunze, M.; Dahint, R.; Whitesides, G. M.; Laibinis, P. E. *J. Phys. Chem. B* **1998**, *102*, 426–436.

(18) Feldman, K.; Hahner, G.; Spencer, N. D.; Harder, P.; Grunze, M. *J. Am. Chem. Soc.* **1999**, *121*, 10134–10141.

(19) Pertsin, A. J.; Grunze, M.; Kreuzer, H. J.; Wang, R. L. C. *Phys. Chem. Chem. Phys.* **2000**, *2*, 1729–1733.

(20) Chapman, R. G.; Ostuni, E.; Yan, L.; Whitesides, G. M. *Langmuir* **2000**, *16*, 6927–6936.

(21) Hamburger, R.; Azaz, E.; Donbrow, M. *Pharm. Acta Helv.* **1975**, *50*, 10–17.

(22) Gerhardt, W.; Martens, C. *Z. Chem.* **1985**, *25*, 143.

(23) Crouzet, C.; Decker, C.; Marchal, J. *Makromol. Chem.* **1976**, *177*, 145–157.

(24) Herold, D. A.; Keil, K.; Bruns, D. E. *Biochem. Pharmacol.* **1989**, *38*, 73–76.

(25) Talarico, T.; Swank, A.; Privalle, C. *Biochem. Biophys. Res. Commun.* **1998**, *250*, 354–358.

(26) Yan, L.; Marzolin, C.; Terfort, A.; Whitesides, G. M. *Langmuir* **1997**, *13*, 6704–6712.

(27) Holmlin, R. E.; Chen, X.; Chapman, R. G.; Takayama, S. T.; Whitesides, G. M. *Langmuir* **2000**, *17*, 2841–2850.

(28) Recently, Mrksich and co-workers found that the inertness of SAMs terminated with mannitol derivatives increased as their melting point and packing density decreased. Mrksich, M. Personal communication.

(29) Holland, N. B.; Qui, S. X.; Ruegsegger, M.; Marchant, R. E. *Nature* **1998**, *392*, 799–801.

(30) Schwendel, D.; Hayashi, T.; Steitz, R.; Dahint, R.; Pipper, J.; Pertsin, A.; Grunze, M. Submitted for publication, 2001.

(31) Pertsin, A. J.; Grunze, M.; Garbuzova, I. A. *J. Phys. Chem. B* **1998**, *102*, 4918–4926.

(32) Zolk, M.; Eisert, F.; Pipper, J.; Herrwerth, S.; Eck, W.; Buck, M.; Grunze, M. *Langmuir* **2000**, *16*, 5849–5852.

(33) Rau, D. C.; Parsegian, V. A. *Science* **1990**, *249*, 1278–1281.

(34) Habash, M.; Reid, G. *J. Clin. Pharmacol.* **1999**, *39*, 887–898.

(35) Liang, M. N.; Smith, S. P.; Metallo, S. J.; Choi, I. S.; Prentiss, M.; Whitesides, G. M. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13092–13096.

(36) McKenney, D.; Hubner, J.; Muller, E.; Wang, Y.; Goldmann, D. A.; Pier, G. B. *Infect. Immun.* **1998**, *66*, 4711–4720.

(37) McKenney, D.; Pouliot, K. L.; Wang, Y.; Murthy, V.; Ulrich, M.; Doring, G.; Lee, J. C.; Goldmann, D. A.; Pier, G. B. *Science* **1999**, *284*, 1523–1527.

(38) Hudson, M. C.; Ramp, W. K.; Frankenburg, K. P. *FEMS Microbiol. Lett.* **1999**, *173*, 279–284.

(39) Joh, D.; Wann, E. R.; Kreikemeyer, B.; Speziale, P.; Hook, M. *Matrix Biol.* **1999**, *18*, 211–223.

printing (μ CP).^{40,46,48,67–70} To pattern cells using μ CP, two types of alkanethiols are patterned: one that generates a SAM that adsorbs the protein and one that generates a SAM that resists the adsorption of protein. In the most common use of μ CP for patterned cell culture, islands of hexadecanethiolate (HDT) are surrounded by an inert SAM terminated with (EG)₃OH groups. We tested the six SAMs described in this paper in studies of unpatterned and patterned cell adhesion.

Our objective in this work was to test the correlation between the adsorption of protein and the attachment of cells with surfaces that are structurally more diverse and molecularly more well-defined than those that have been studied to date. Historically, the adsorption of protein and the adhesion of cells have been tested on polymeric surfaces that are heterogeneous and are difficult subjects to use in constructing structure–property relationships.³

Results and Discussion

SPR Protocol. SPR is an optical technique that is sensitive to changes in the index of refraction at an interface defined by a metal and a solution.⁷¹ The Biacore instrument that we use measures changes in the index of refraction as a function of the amount of light reflected by the sample (RU = reflection units, 1 RU = 10⁻⁴). Hence, the amount of protein that adsorbs to SAMs is proportional to the difference in the signal measured after and before the surfaces were exposed to solutions of protein (Δ RU, Figure 1).

Resistance to Protein Adsorption. Table 1 summarizes the amount of protein that adsorbed to the SAMs as a percentage of the amount of protein that adsorbed to a SAM of HS(CH₂)₁₅CH₃. Most proteins adsorb to hydrophobic surfaces.⁷² We normalized the values of Δ RU that we measured on the SAMs with those obtained using a hydrophobic surface using eq 1:

$$\%ML_{\text{protein}} = \% \text{monolayer} = \frac{\Delta RU_{\text{SAM}(\text{HS}(\text{CH}_2)_{10,15}\text{-R})}}{\Delta RU_{\text{SAM}(\text{HS}(\text{CH}_2)_{15}\text{CH}_3)}} \times 100 \quad (1)$$

In this equation, $\%ML_{\text{protein}}$ is the percentage of a monolayer that adsorbed to the SAM terminated in the R group relative to that adsorbed on a SAM of HDT. We base our definition of $\%ML_{\text{protein}}$ on the assumption that a complete monolayer of protein adsorbed to the hydrophobic surface of a SAM of HDT; on the basis of previous work, we believe that this assumption is reasonable, but we note that the surface that adsorbs the largest quantity of protein is not necessarily the most hydrophobic one.^{1,2,72} The quantities of protein that adsorbed on single-component SAMs of HDT are, however, only 10–20% lower than the maximum values that were measured during the screening protocol.^{1,73}

(67) Dike, L. E.; Chen, C. S.; Mrksich, M.; Tien, J.; Whitesides, G. M.; Ingber, D. E. *In Vitro Cell. Dev. Biol.: Anim.* **2000**, *35*, 441–448.
(68) Huang, S.; Chen, C. S.; Ingber, D. E. *Mol. Biol. Cell* **1998**, *9*, 3179–3193.

(69) Mrksich, M.; Chen, C. S.; Xia, Y.; Dike, L. E.; Ingber, D. E.; Whitesides, G. M. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 10775–10778.

(70) Mrksich, M.; Dike, L. E.; Tien, J.; Ingber, D. E.; Whitesides, G. M. *Exp. Cell Res.* **1997**, *235*, 305–313.

(71) Raether, H. In *Physics of Thin Films*; Hass, G., Francombe, M., Hoffman, R., Eds.; Academic Press: New York, 1977; Vol. 9, pp 145–261.

(72) Ostuni, E.; Grzybowski, B. A.; Roberts, C. S.; Mrksich, M.; Whitesides, G. M. Submitted for publication, 2001.

(73) Hydrophobic surfaces generally cause adsorbed proteins to spread onto surfaces by undergoing conformational changes.

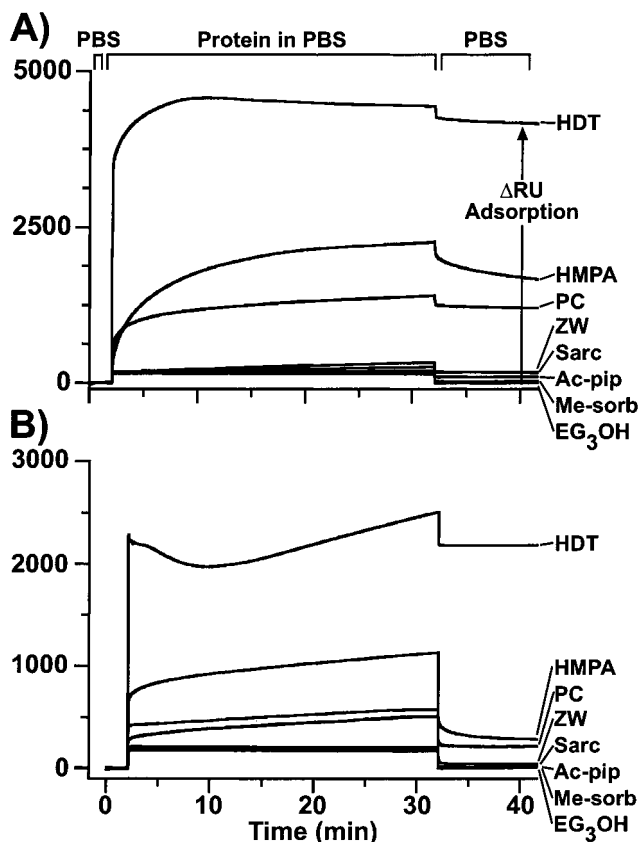


Figure 1. SPR sensorgrams that illustrate the adsorption of fibrinogen (A) and lysozyme (B) to different SAMs. We compare the surfaces that resist the adsorption of protein with the hydrophobic surface of a SAM of HDT. The functional groups that correspond to each curve are labeled on the figure (Table 1). We refer to the amount of protein adsorption (Δ RU) as the difference in signal between the beginning of the experiment and the end of the flow of protein solution (see labels on figure). The nonmonotonic shapes of adsorption of proteins on HDT probably reflect rearrangement and spreading of proteins once adsorbed.⁷⁰

The values of $\%ML_{\text{protein}}$ measured with the single-component SAMs are sufficiently low to be useful for applications in cell patterning and biosensors (Table 1). The inertness of SAMs that presented derivatives of permethylated sorbitol (Me-sorb) and acetylpiperazine (Ac-pip) was comparable to that of surfaces terminated with (EG)₃OH groups (Figure 1; Table 1). Single-component SAMs that presented a derivative of hexamethylphosphoramide (HMPA) adsorbed more protein than the mixed SAMs that presented the same group installed at the surface by the reaction of an amine derivative with a SAM terminated with carboxylic anhydride groups ($\%ML_{\text{Fib}} = 4$).¹ The bulky phosphoramidate derivative may form a disordered single-component SAM that does not resist the adsorption of proteins as well as the mixed SAM.

Adhesion of Bacterial Cells. We used an assay that measured a quantity proportional to the number of *S. aureus* and *S. epidermidis* cells that adhered to SAMs. The bacterial cells that attached to the homogeneous SAMs under trypticase soy broth (TSB) were removed from the surfaces of the substrates by sonication and grown on agar plates for a fixed amount of time. The resulting colonies were counted under a microscope. We assume that the number of colonies grown on the agar plates is proportional to the number of colony-forming units (cfu's) that attached to the SAMs. Sonication was not always efficient at removing the adhered bacterial cells; especially

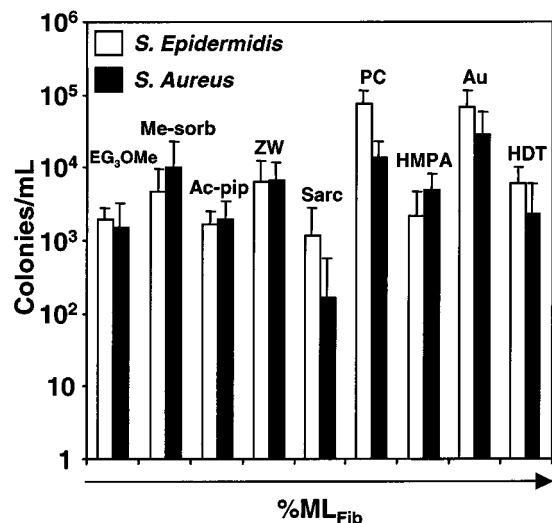


Figure 2. Adhesion of *S. aureus* and *S. epidermidis* to SAMs of the indicated alkanethiolates (see Table 1 for abbreviations). The horizontal axis is schematic only; the actual values of %ML_{Fib} are given in Table 1. The vertical axis summarizes a quantity that is approximately proportional to the average density of bacteria that bind to each substrate. The error bars are the standard deviation of experiments that were performed in triplicate on two different days.

in the case of the control surfaces—so-called bare gold—we noticed that many colonies remained attached to the surface. The number of colonies that were counted on bare gold was, therefore, artificially low; we believe that the correct number could be as much as 10-fold higher.

SAMs that presented derivatives of tri(sarcosine) (Sarc) and Ac-pip resisted the adhesion of both strains of bacteria to an extent comparable to SAMs terminated with EG₃-OCH₃ groups (Figure 2). Interestingly, a SAM terminated with Sarc was more resistant to the adhesion of *S. aureus* than a SAM that presented (EG)₃OCH₃ groups. The number of bacterial cells that adhered to SAMs terminated with derivatives of HMPA, Me-sorb, and the intramolecular zwitterion (−CH₂N⁺(CH₃)₂CH₂CH₂CH₂SO₃[−]) (ZW) was slightly larger than to EG₃OCH₃-terminated surfaces, but these surfaces were nonetheless sufficiently inert to be useful. Baumgartner et al. have reported the decreased adhesion of *S. aureus* to polyurethane surfaces functionalized with zwitterionic groups.⁷⁴ The relatively low adhesion of bacterial cells to hydrophobic SAMs was surprising; as in previous papers, however, we expect that the incubation of the bacteria with the surfaces for periods of time longer than we used here (<30 min) would cause greater numbers of bacteria to adhere to the hydrophobic substrates than to the inert substrates.⁷⁵ The large number of bacteria that attached to surfaces terminated with phosphoryl choline (PC, Figure 2) may be explained by the considerable amount of protein (%ML_{Fib} = 32) that adsorbed to these surfaces and the presence of PC derivatives on the outer surface of these bacteria.⁷⁶ The presentation of phospholipids at surfaces is known to reduce, but not completely inhibit, the adsorption of proteins and the adhesion of bacterial cells;^{77–79} this

(74) Baumgartner, J. N.; Cooper, S. L. *J. Biomed. Mater. Res.* **1998**, *40*, 660–670.

(75) Cunliffe, D.; Smart, C. A.; Alexander, C.; Vulfson, E. N. *Appl. Environ. Microbiol.* **1999**, *65*, 4995–5002.

(76) Foreman-Wykert, A. K.; Weinrauch, Y.; Elsbach, P.; Weiss, J. *J. Clin. Invest.* **1999**, *103*, 715–721.

(77) Hsiue, G.-H.; Lee, S.-D.; Chang, P. C.-T.; Kao, C.-Y. *J. Biomed. Mater. Res.* **1998**, *42*, 134–147.

(78) Millsap, K. W.; Reid, G.; van der Mei, H. C.; Busscher, H. J. *Biomaterials* **1996**, *18*, 87–91.

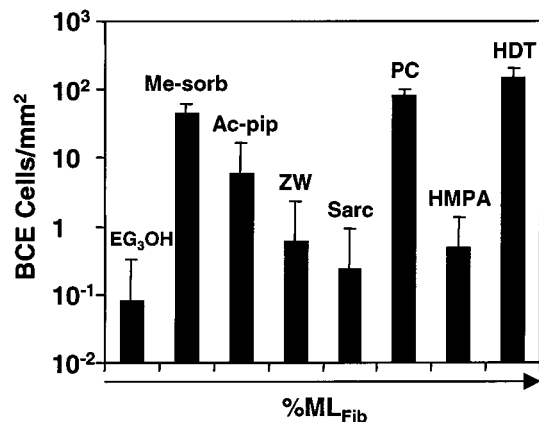


Figure 3. Number of BCE cells that adhered per millimeter squared of the surface of the SAMs formed with the indicated alkanethiolates (see Table 1 for abbreviations). The horizontal axis is schematic only; the actual values of %ML_{Fib} are given in Table 1. On the vertical axis, we plot the number of cells that adhered to at least 10 separate areas of a sample (1.26 mm² each) on two different days; those values were averaged. The error bars are the standard deviation of those measurements. The values of BCE cells per millimeter squared are plotted in order of increasing values of %ML_{Fib} for each homogeneous SAM (Table 1).

observation made with the zwitterionic PC SAMs is consistent with those made by us with other SAMs terminated with zwitterionic groups.²⁷ Recently, Tegoulia et al. showed that the adhesion of *S. aureus* to a number of SAMs that included PC did not follow any trends.⁸⁰

The values of colonies per milliliter did not decrease linearly with the decreasing amounts of adsorbed protein in a manner that would allow us to correlate resistance to protein adsorption with resistance to bacterial adhesion. Assuming that protein adsorption in our assays using fibrinogen correlated with protein adsorption from the media used to grow *S. aureus* and *S. epidermidis* or from the secretion of bacterial proteins, we infer that bacterial adhesion appears to be influenced by characteristics of a surface other than the amount of protein that can adsorb to those surfaces.

Adhesion of Mammalian Cells. We tested the attachment of BCE cells to the SAMs after incubating them with fibronectin—an extracellular matrix protein—for 1 h; the BCE cells were allowed to attach to and spread on the SAMs under modified Eagle's medium for 24 h before fixing and counting them.

The Sarc and (EG)₃OH surfaces resisted the adhesion of cells to a comparable extent; only ~1 cell/mm² adhered to these surfaces (Figure 3). The low number of cells that adhered to the HMPA surfaces is in contrast with the large amount of fibrinogen that adsorbed to this surface. We are unsure of the mechanism underlying this observation; it is known, however, that on some surfaces proteins adsorb in conformations that do not promote cell adhesion.^{81,82} The large number of BCE cells that adhered to SAMs of PC was expected based on the observation that large amounts of fibrinogen adsorbed to this surface (Table 1); the diminished adhesion of cells to PC as compared to control surfaces was recently shown by

(79) van der Heiden, A. P.; Willems, G. M.; Lindhout, T.; Pijpers, A. P.; Koole, L. H. *J. Biomed. Mater. Res.* **1998**, *40*, 195–203.

(80) Tegoulia, V. A.; Rao, W.; Kalambur, A. T.; Rabolt, J. F.; Cooper, S. L. *Langmuir* **2001**, *17*, 4396–4404.

(81) Culp, L. A.; Sukeinik, C. N. *J. Biomater. Sci. Polym. Ed.* **1998**, *9*, 1161–1176.

(82) McClary, K. B.; Ugarova, T.; Grainger, D. W. *J. Biomed. Mater. Res.* **2000**, *50*, 428–439.

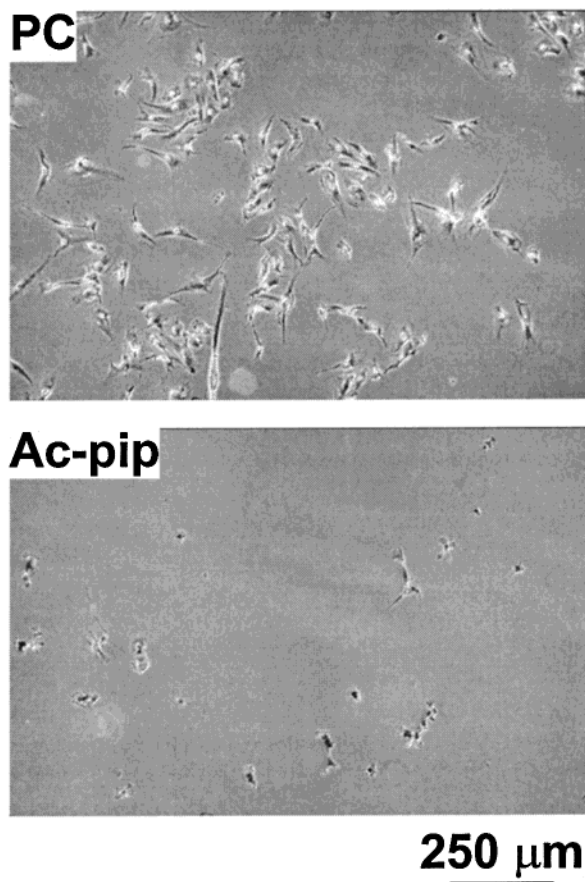


Figure 4. Extent of cell spreading on homogeneous SAMs is affected by the amount of fibronectin that adsorbed to those surfaces. (A) BCE cells that attached to the surface of a SAM terminated with PC groups. (B) BCE cells that attached to the surface of a SAM terminated with Ac-pip spread much less than on a substrate that adsorbed large amounts of protein (Table 1).

Tegoulia et al.⁸³ We do not understand the relatively large number of BCE cells that adhered to SAMs terminated with Me-sorb. Our results show no correlation between the number of cells that attach to a surface and the amount of protein that adsorbs to that surface (Figure 3).

The presence of large amounts of proteins at a surface facilitates the attachment of cells as well as their spreading.^{84,85} Ingber has shown that the spreading of BCE cells increased with an increasing concentration of fibronectin at a surface.⁸⁵ Hence, we expected attached cells to spread least on SAMs that adsorbed the least amount of protein. Figure 4 illustrates this point by showing that cells attached to SAMs that present PC (%ML_{Fib} = 32) spread over larger areas than those attached to SAMs terminated with Ac-pip (%ML_{Fib} = 2).⁸⁶

Relationship between Adhesion of Bacterial and Mammalian Cells. It is again evident that there is little or no correlation between the ability of surfaces to resist the attachment of bacterial cells and the attachment and spreading of mammalian cells (Figure 5).

Patterning BCE Cells. We tested the patterning of BCE cells by μ CP using SAMs made with the alkanethiols

(83) Tegoulia, V. A.; Cooper, S. L. *J. Biomed. Mater. Res.* **2000**, *50*, 291–301.

(84) Ingber, D. E.; Madri, J. A.; Folkman, J. *In Vitro Cell. Dev. Biol.* **1987**, *23*, 387–394.

(85) Ingber, D. E. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 3579–3583.

(86) We did not quantify the secretion of matrix proteins by the cells onto the SAMs.

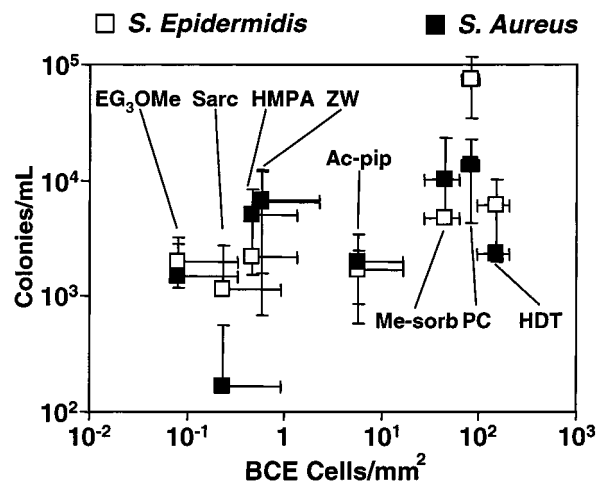


Figure 5. Plot of the number of colonies per milliliter vs the number of BCE cells per millimeter squared that adhered to each homogeneous SAM labeled according to the abbreviations in Table 1. The quantities are displayed on a log/log plot to facilitate the visualization of the data.

described in this paper; cell patterning is a unique application that requires inert surfaces. A hydrophobic alkanethiol was printed in 50 μ m wide lines (with edges separated by 50 μ m) onto gold-coated substrates to generate an adsorptive surface using procedures described elsewhere;^{46,70} the remaining surface of the substrates was then exposed to solutions of one of the alkanethiols listed in Table 1. BCE cells could be patterned well using alkanethiols that allowed minimal protein adsorption and cell attachment (Figure 3) as the inert component of the surface. BCE cells adhered preferentially to the hydrophobic surfaces in patterns created with each resistant SAM (Figure 6A,B), except for those terminated with PC and Me-sorb (Figure 6C). The poor patterning obtained with PC and Me-sorb is consistent with the results of protein adsorption and BCE cell adhesion measurements obtained on homogeneous surfaces (Table 1; Figure 2); large numbers of cells adhered to both surfaces.

Conclusions

This paper quantifies the inertness of six SAMs, each terminated with a different functional group (Table 1), in three biologically relevant assays: adsorption of fibrinogen and lysozyme, adhesion of *S. aureus* and *S. epidermidis*, and attachment and spreading of BCE cells. The SAMs we describe all reduce the adsorption of proteins and the adhesion of bacterial and mammalian cells relative to control, hydrophobic, or gold surfaces. Of these surfaces, those terminated with Sarc, Ac-pip, and ZW resisted the adhesion of bacterial cells in a manner comparable to surfaces terminated with (EG)₃OH—the most inert surface presently known. Only surfaces that presented Sarc resisted the adhesion of BCE cells as well as surfaces that presented (EG)₃OH groups. Surfaces terminated with (EG)_nOR groups are, therefore, not unique in their ability to resist the adhesion of cells; more such surfaces could, we believe, be discovered. The surfaces that we have described may prove useful in applications where (EG)_nOR-terminated surfaces are not ideal. They may also provide an alternative to (EG)_nOR-terminated surfaces for mechanistic studies.

Previous investigations of the relationship between the properties of a surface and its resistance to the adsorption of proteins and the adhesion of cells were not carried out using methods that allowed molecular-level control of the

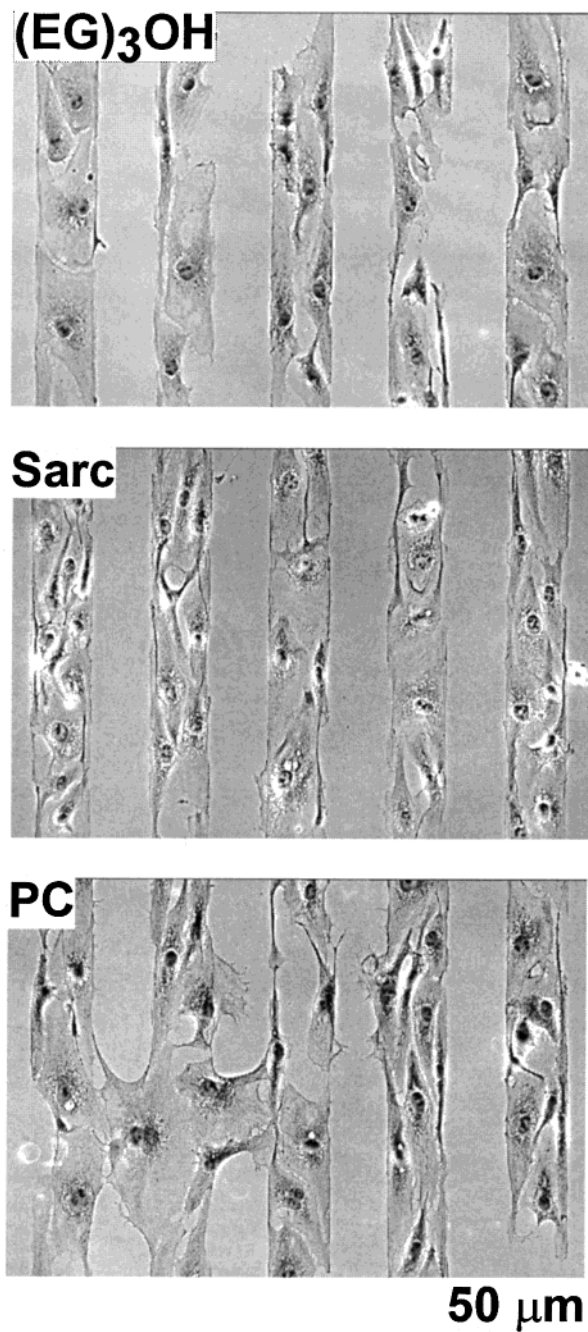


Figure 6. Patterned adhesion of BCE cells onto gold-coated substrates that were patterned by μ CP lines of hexadecanethiol. The remainder of the gold surfaces were exposed to an alkanethiol terminated with (EG)₃OH, Sarc, or PC groups. Patterning was least efficient with the PC alkanethiol; this alkanethiol also adsorbed the largest amount of fibrinogen of the three (Table 1).

structure of surfaces. The systems studied here differed substantially in composition of the media in contact with the surfaces. Further study will be required to test whether the conclusions from this study also apply in more closely parallel experiments. Our observation is that the resistance of surfaces to protein adsorption does not correlate, in the assays used here, with their resistance to cell adhesion (bacterial and mammalian) and that the resistance to the adhesion of bacterial cells did not correlate with the resistance to the adhesion of mammalian cells.

The principles that we have used relatively successfully to design surfaces that resist the adsorption of proteins are, thus, not sufficient to design surfaces inert to the

adhesion of bacterial and mammalian cells. Much remains to be understood about mechanisms of resistance of surfaces to cell adhesion before structure–property relationships can be established and a set of predictive rules can be devised to guide the rational design of these surfaces. Our results leave open the question of whether adsorption/attachment of proteins, bacteria, and mammalian cells occurs by different mechanisms or whether the details of the composition (especially, the protein composition) of the fluid media are important in ways we do not yet understand.

Experimental Section

Materials. Unless stated otherwise, all chemicals were reagent grade. Fibrinogen (from bovine plasma, F8630), lysozyme (egg white, EC 3.2.1.17, L6876), and sodium dodecyl sulfate were purchased from Sigma (St. Louis, MO). Absolute ethanol was purchased from Pharmco Products (Brookfield, CT). Phosphate-buffered saline (PBS: 10 mM phosphate, 138 mM NaCl, and 2.7 mM KCl) was prepared with distilled, deionized water and filtered through 0.22 μ m filters prior to use. The alkanethiols were available from previous studies.^{1,27}

Preparation of SAMs. Glass substrates were coated with 1.5 nm of titanium and 38 nm of gold by e-beam evaporation as described previously and used within 3 weeks of preparation.^{50,87,88} SAMs were prepared by immersion of the freshly evaporated gold substrates (24 \times 50 mm glass coverslips) in a 2 mM ethanolic solution of alkanethiol at room temperature overnight. These substrates were dried in a stream of nitrogen after being removed from the solution and rinsed with ethanol. The preparation of the substrates for use in SPR studies and for the measurement of the adhesion of *S. aureus*, *S. epidermidis*, and BCE cells was identical.

SPR Spectroscopy. All SPR measurements were performed on a Biacore 1000 SPR instrument. The gold-coated substrates, derivatized with SAMs, were mounted on a modified SPR cartridge as described previously.^{50,87} The adsorption of proteins to SAMs was measured by allowing a solution of sodium dodecyl sulfate (40 mM in PBS) to flow over the surface of the SAM for 3 min, followed by rinsing with a solution of PBS buffer for 10 min. After this washing procedure, PBS buffer was allowed to flow over the surface for 2 min, followed by a solution of protein (1 mg/mL in PBS) for 30 min, before allowing PBS buffer to flow over the surface for an additional 10 min (Figure 1). All experiments were carried out at a flow rate of 10 μ L/min.

In Vitro Adhesion Model for *S. epidermidis* and *S. aureus*.⁴ Gold-coated glass coverslips functionalized with a SAM (18 mm²) were rinsed with 100% ethanol immediately before use and dried in sterile 100 \times 15 mm polystyrene dishes (Fisher). An inoculum of either *S. epidermidis* M187 or *S. aureus* MN8M (100 μ L of a 2.5×10^8 bacteria/mL suspension) was spread over the entire surface of the gold substrates with a sterile pipet tip and incubated at 37 $^\circ$ C for 30 min. The gold-coated substrates were washed 5 \times in sterile PBS after being removed from the medium, and they were then sonicated for 5 s in 10 mL of TSB containing 0.05% Tween. The resulting suspension of bacteria was diluted (10- or 0-fold) before being placed on agar plates at 37 $^\circ$ C overnight. This procedure was necessary to simplify counting the colonies that grew on the agar plates and determining the density of cfu's that were found in the suspension obtained from sonicating the gold-coated samples. Each SAM was tested in triplicate, and the bare gold surfaces were tested in quadruplicate; the experiments were repeated on three different days.

Culture of BCE Cells. Bovine adrenal capillary endothelial cells were cultured on Petri dishes (Falcon) coated with gelatin in Dulbecco's modified Eagle's medium (DMEM) under 10% CO₂. We supplemented the DMEM with 10% calf serum, 2 mM glutamine, 100 μ g/mL streptomycin, 100 μ g/mL penicillin, and 1 ng/mL basic fibroblast growth factor (bFGF).⁸⁹ The SAMs were

(87) Mrksich, M.; Sigal, G. B.; Whitesides, G. M. *Langmuir* **1995**, *11*, 4383–4385.

(88) Biacore now sells glass substrates coated with bare gold for SPR studies.

(89) Ingber, D. E.; Folkman, J. *J. Cell Biol.* **1989**, *109*, 317–330.

incubated with a solution of fibronectin (50 $\mu\text{g}/\text{mL}$ in PBS) before being exposed to a suspension of cells. Prior to incubation with the SAMs, cells were dissociated from culture plates with trypsin–EDTA and washed in DMEM containing 1% BSA (BSA/DMEM).⁸⁹ The substrates were exposed to a suspension of BCE cells in chemically defined medium (BSA/DMEM) containing 10 $\mu\text{g}/\text{mL}$ high-density lipoprotein, 5 $\mu\text{g}/\text{mL}$ transferrin, and 5 ng/mL bFGF and incubated in 10% CO_2 at 37 °C.^{85,89} Cells that adhered to substrates were fixed with 4% paraformaldehyde (v/v) in PBS for 20 min; after being washed once with PBS and being incubated with methanol for 1 min, the cells were stained with Coomassie blue (5 mg/mL in 40% v/v methanol, 10% v/v acetic acid, and 50% v/v water) for 30 s, rinsed with distilled water, and dried in air.

Adhesion Assay for BCE Cells. Following the methods described in the preceding section, the SAMs were exposed to 4 mL of a suspension of cells (15 000 cells/mL) for 24 h. After being fixed and stained, the attached cells were counted visually under a Zeiss Axiophot microscope; micrographs were obtained with a 35-mm camera connected to the microscope.

Patterning BCE Cells. Gold-coated substrates were patterned with hexadecanethiol as described previously.⁴⁰ The

remaining areas of the surfaces were then incubated with ethanolic solutions of each alkanethiol overnight. Following the procedures described in the previous two sections, the substrates were exposed to 4 mL of a suspension of cells (40 000 cells/mL). After a 24 h incubation, the substrates were rinsed, fixed, and stained.

Acknowledgment. We are grateful to the National Institutes of Health (GM30367) for support of this work. We used shared MRSEC facilities supported by NSF under Contract DMR-9809363. E.O. acknowledges a predoctoral fellowship in organic chemistry from Glaxo Wellcome, Inc. R.G.C. is grateful to the National Science and Engineering Research Council (Canada) for a postdoctoral fellowship. G.P. is grateful to NIH (1R01AI46706) for financial support.

LA010552A