Anal. Chem. **2004,** *76,* 6116−6121

Palladium as a Substrate for Self-Assembled Monolayers Used in Biotechnology

Xingyu Jiang, Derek A. Bruzewicz, Mamie M. Thant, and George M. Whitesides*

Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138

This paper describes self-assembled monolayers (SAMs) on palladium that resist the nonspecific adsorption of proteins and the adhesion of mammalian cells. These SAMs form when thin films of palladium are exposed to solutions of alkanethiol with the general structure $HS(CH_2)_m(OCH_2CH_2)_nOH$ (m = 2, 11; n = 3, 6, 7). Ellipsometry and surface plasmon resonance spectroscopy (using a palladium-on-gold substrate) showed that these SAMs resist adsorption of all proteins present in bovine serum. Microislands of SAMs of octadecanethiol on palladium allowed patterned adhesion and growth of mammalian cells (in a "sea" of oligo(ethyleneglycol)-terminated SAM). The oligo(ethyleneglycol)-terminated SAM resisted the invasion of cells for over four weeks under standard conditions of cell culture; similar SAMs on gold remained patterned for only two weeks.

This report describes self-assembled monolayers (SAMs) formed from oligo(ethyleneglycol) (OEG)-terminated alkanethiols on palladium and demonstrates that these SAMs resist nonspecific adsorption of proteins and adhesion of mammalian cells. (We call surfaces that resist adsorption of proteins and adhesion of cells "inert surfaces".) Most OEG-terminated SAMs on gold and silver are inert.[1−3] We and others have used these inert surfaces to study the interactions between ligands and receptors,[4−7] to investigate adsorption of proteins on surfaces,[8,9] to control the adhesion of mammalian cells,[10−14] and to fabricate surfaces that resist bacterial adhesion.[15,16] Tools based on inert SAMs allow control of adhesion of cells and modulation of the shape and size of cells using well-defined patterns of ligands on surfaces. These tools facilitate the investigation of interactions between cells and surfaces and the study of cell biology.[3,10,17−20]

Thiols also form well-ordered SAMs on thin films of palladium.[21] In this paper, we show that OEG-terminated SAMs on palladium provide inert surfaces useful for culturing mammalian cells. One specific OEG-terminated SAM on palladium performed better than any OEG-terminated SAM on gold. This SAM on palladium remained inert for at least 4 weeks under conditions of cell culture; analogous SAMs on gold lasted for 2 weeks under the same conditions. The long-term inertness of OEG-terminated SAMs and the low toxicity of palladium toward cells make SAMs on palladium excellent candidates for use in long-term culture of patterned cells.

EXPERIMENTAL SECTION

Reagents and Materials. All antibodies and reagents were obtained from Sigma (www.sigmaaldrich.com) unless otherwise specified. $HS(CH_2)_{11}(OCH_2CH_2)_3OH$, **1**, and $HS(CH_2)_{11}(OCH_2CH_2)_6OH$, **2**, were from Prochimia (www.prochimia.com), and $HS(CH_2)_2(OCH_2CH_2)_7OH$, **3**, was from LLC engineering (www.chemsupply.ch). All reagents were used as received. The molecular structures of these thiols were confirmed by NMR and mass spectrometry.

Preparation of Substrates and Ellipsometry. We evaporated 10−200-nm-thick films of palladium onto titanium- or chromium-primed substrates of glass or silicon wafers. Immersing the palladium films in 2 mM solutions of thiol in ethanol for 2−55 h yielded the SAMs.[21] After incubating the substrates in fibroblast growth medium (Invitrogen, www.invitrogen.com) at 25 °C for 12 h, we used ellipsometry (using a VB-400 ellipsometer and a HS 190 monochromator from J. A. Woollam Co., www.jawoollam.com) to determine the thickness of the dielectric layer (and, hence, the amount of adsorbed proteins) on each type of

* To whom correspondence should be addressed. E-mail: gwhitesides@gmwgroup.harvard.edu.

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10.1021/ac049152t CCC: $27.50
Published on Web 09/22/2004

SAM.[2] The nominal thicknesses of the organic films adsorbed to each of the SAMs were calculated using an index of refraction of 1.45.[2]

Surface Plasmon Resonance. SPR on palladium alone did not offer high enough sensitivity for the detection of a small change in the index of refraction on the metal−dielectric surface at any visible wavelength.[23] We used a thermal evaporator to deposit metals onto glass substrates (Fisher Scientific, www.fishersci.com, sonicated in soapy water, rinsed with Millipore water): chromium (∼2 nm), gold (∼40 nm), and palladium (∼10 nm). The substrates were cut into ∼1 cm^2 pieces with a diamond cutter and mounted onto plastic cartridges (the same type used to mount the commercial chip by Biacore) with an epoxy glue (Devcon, www.devcon.com). We used either a Biacore 1000 or 3000 instrument (Biacore) for the SPR experiments.

Cell Culture. Micropatterning experiments followed published procedures.[18] NIH 3T3 fibroblasts (ATCC, www.atcc.org) were cultured in Dubecco's modified essential medium containing 10% heat-inactivated fetal bovine serum and supplemented with 1% penicillin/streptomycin cocktail (Invitrogen, www.invitrogen.com). Primary HUAECs were obtained from Cambrex (cambrex.com) and cultured as instructed using the media provided. Only cells from passages 2−8 were used. Immunostaining was carried out as described elsewhere.[60] Briefly, the cells were fixed with 4% glutaraldehyde in phosphate buffer saline (PBS) for 10 min, permeablized with 0.3% Triton-X in PBS, and blocked with 5% bovine serum albumin in PBS (wash buffer) for 1 h. Primary antibodies were diluted with wash buffer according to the manufacturer's specification (anti-Fn, 1:400 dilution; anti-Vinculin, 1:400 dilution) and used to incubate the samples for 4 h. The samples were rinsed with the wash buffer and incubated with the secondary antibodies for 2 h.

RESULTS AND DISCUSSION

Analysis of the Surface. We used four commercially available thiols in this work: $HS(CH_2)_{11}(OCH_2CH_2)_3OH$, **1**; $HS(CH_2)_{11}(OCH_2CH_2)_6OH$, **2**; $HS(CH_2)_2(OCH_2CH_2)_7OH$, **3**; and $HS(CH_2)_{17}CH_3$, $\mathbf{C_{18}}$. Thiols **1**, **2**, and **3** form inert SAMs on gold; SAMs formed from $\mathbf{C_{18}}$ on both gold and palladium adsorb proteins strongly.[1,18]

After incubating substrates that bore SAMs in fibroblast growth medium at 25 °C for 12 h, we used ellipsometry to determine the quantity of adsorbed proteins on each type of SAM.[2] By definition, inert surfaces resist adsorption of *all* proteins from the growth medium.[18,22] Ellipsometry experiments indicated an increase of ∼2 nm in the thickness of the dielectric layer on SAMs formed from $\mathbf{C_{18}}$ after incubation with fibroblast growth medium. There was essentially no change in the thickness of the dielectric layer on SAMs formed from thiols that terminate in OEG groups (**1**, **2**, and **3**; Table 1). We conclude that the SAMs formed from $\mathbf{C_{18}}$ on palladium or gold strongly promote the adsorption of proteins, while those formed from thiols that terminate in OEG groups (**1**, **2**, and **3**) on palladium or gold resist the adsorption of proteins.

We also used SPR to quantify the amount of protein adsorbed on the surface of SAMs on palladium. We could not use a commercial instrument (Biacore) with thin films of palladium directly because the sensitivity of detection in this instrument is 10−100 times lower on palladium than on gold.[23] Instead, we formed SAMs on a thin layer of Pd (∼10 nm)[24] deposited on a layer of gold (∼40 nm). Proteins in the fibroblast growth medium did not adsorb onto the surfaces of OEG-terminated SAMs on palladium, but they adsorbed strongly onto SAMs of $\mathbf{C_{18}}$ on palladium (Figure 1).

(22) The growth medium contains a mixture of proteins, including albumin, ∼4 mg/mL; immunoglobulins ∼2 mg/mL; transferrin ∼0.3 mg/mL; antitrypsin, ∼0.3 mg/mL; and liproteins, ∼0.5 mg/mL. See Putnam, F. W. In *The Plasma Proteins: Structure, Function, and Genetric Control*; Putnam, F. W., Ed.; Academic Press: New York, 1975; Vol. 1, p 58.

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Table 1. Nominal Thicknesses of the Films of Protein Adsorbed on SAMs Formed from Various Thiols on Pd and Au

thiols	thickness[a] (nm) palladium	gold
C_{18}	2.0 ± 0.3	2.2 ± 0.5
1	0.0 ± 0.01	0.1 ± 0.02
2	0.0 ± 0.01	0.1 ± 0.01
3	0.0 ± 0.01	0.1 ± 0.03

[a] Thicknesses are the averages of at least four measurements. Errors include the range of data.

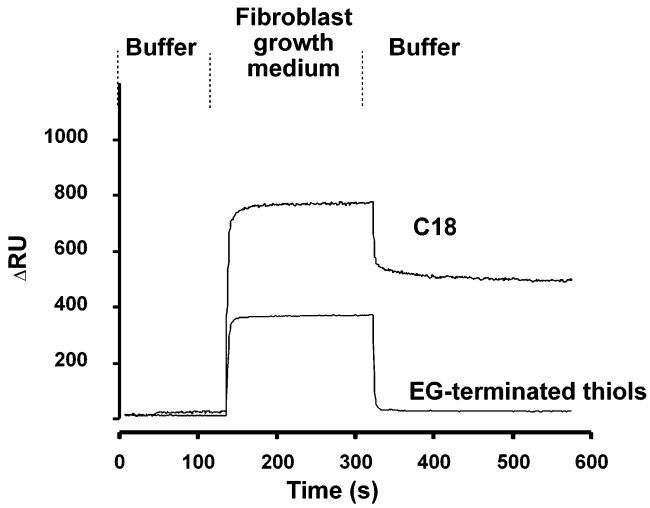

Figure 1. Sensorgrams of SPR experiments of SAMs on Pd-on-Au. The change in response (ΔRU) from experiments on SAMs formed from thiols **1**, **2**, and **3** is <5% of that on C_{18}-derived SAMs.

To prove that these SPR experiments measured the properties of SAMs on palladium (that is, the signals were not due to SAMs on gold or to SAMs on an alloy of gold and palladium, either of which could result when using such a thin layer of palladium on gold), we performed X-ray photoelectron spectroscopy (XPS) on the substrates used in SPR. XPS data showed that a 10-nm layer of palladium evaporated onto a gold-coated substrate had no gold on the top surface of the substrate (Figure 2). This result shows that a full layer of palladium formed on the surface of the gold. We conclude that these substrates had little or no gold exposed on the surface and that the results obtained by SPR indeed indicate that the SAMs on palladium resist the adsorption of proteins.

Similar experiments carried out on a thin film (~10 nm, the maximum thickness required to obtain signals on Biacore) of palladium on silver (~40 nm) showed that its surface composition (as sampled by XPS) was ~30% silver (see Supporting Information Figure 1). SPR experiments on the surface of OEG-terminated SAMs (**1**, **2**, **3**) on such films (Au-on-Ag) also showed no adsorbed proteins. These data indicate that OEG-terminated SAMs on both palladium and silver (or on Pd/Ag alloys) are inert. Separate SPR experiments on OEG-terminated SAMs (**1**, **2** and **3**) on thin films of pure silver confirmed that these SAMs on silver are inert (Supporting Information Table 1).[25]

Compatibility with Cell Culture. To test the ability of SAMs on palladium to confine the adhesion and growth of cells to micropatterns, we patterned SAMs by microcontact printing.[11] Like

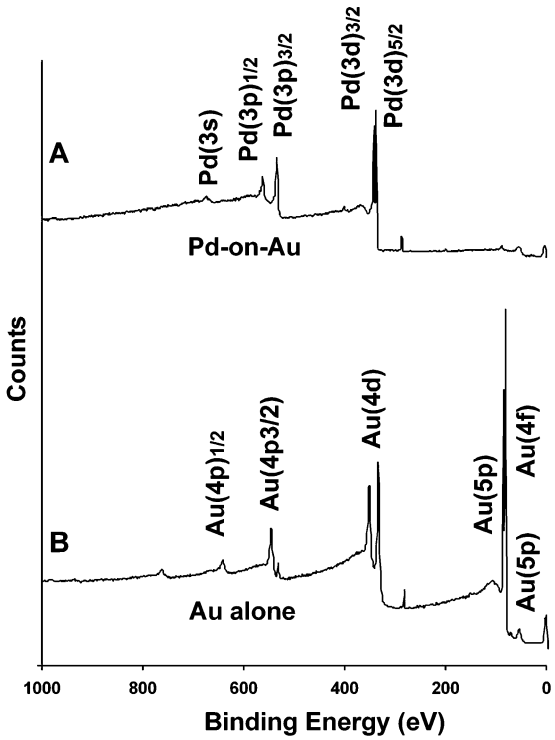

Figure 2. Results from XPS of (A) a thin film of palladium (~10 nm) on gold (~40 nm). (B) A thin film of gold (~40 nm) for comparison. Note that no peaks due to Au are visible in the top curve.

micropatterned surfaces on gold, micropatterned surfaces on palladium confined NIH 3T3 fibroblast cells: the regions of the SAMs formed from C_{18} supported the adhesion of cells, while the regions of SAMs formed from thiols **1**, **2**, or **3** resisted the adhesion of cells (Figure 3).

Regions of OEG-terminated SAMs ceased to be inert after 2 days to 6 weeks, depending on the combination of the thiol and the metal substrate (Figure 3): on gold, the SAMs that remained inert for the longest period of time formed from thiols **1** or **2** (these SAMs remained inert for ~2 weeks); by contrast, on palladium, the best SAM formed from thiol **3**; this SAM remained inert for 4−6 weeks. When OEG-terminated SAMs failed, cells originally confined to the microislands migrated and proliferated to form a confluent layer of cells on the metal substrate. It is not clear whether invasion of cells into previously inert regions resulted from degradation and desorption of the OEG-terminated SAMs or from a change in the structures of the OEG moiety of these SAMs.[26,27]

Groups of human umbilical artery endothelial cells (HUAECs) also remained confined on SAMs on palladium for longer periods of time than they did on SAMs on gold. Table 2 summarizes the performance of SAMs in confining both NIH 3T3 fibroblasts and HUAECs for all combinations of thiols and metal substrates.

We can define the geometry of single cells with SAMs on palladium, just as we can with SAMs on gold. Figure 4 shows that HUAECs conformed to different geometric shapes on the surface. These shapes are useful in probing the behavior of single cells: for instance, we observed that secreted fibronectin localized at the corners of these patterns; this finding corroborates our earlier results.[28]

Using features much smaller than a spread cell (5-μm-diameter dots separated by an edge-to-edge distance of 5 μm), we showed

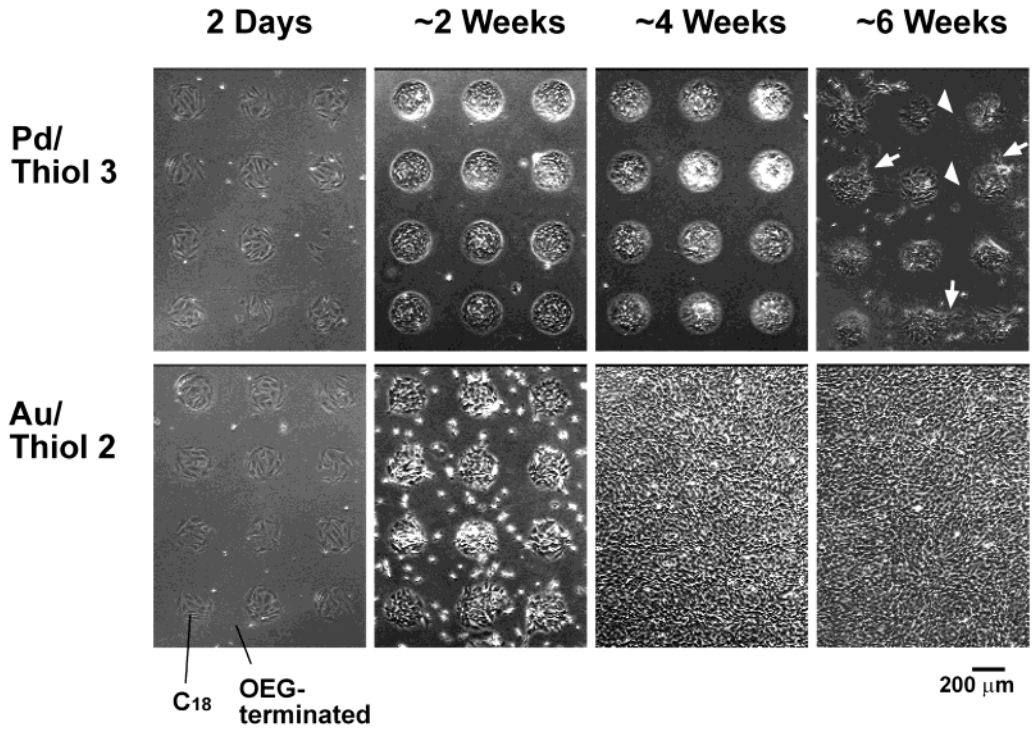

Figure 3. A comparison of long-term culturing of 3T3 fibroblast cells on the SAMs formed on Pd and Au. Thiol **3** was the best on Pd, while thiol **2** was the best on Au. SAMs from thiol **2** on Au failed after 2 weeks, and SAMs of thiol **3** on Pd remained inert for at least 4 weeks. After 6 weeks, the surfaces on Pd began to fail in certain areas (arrows) but remained inert in others (arrowhead).

Table 2. Statistical Summary of the Duration of Inertness of Micropatterned SAMs for 3T3 Fibroblasts and HUAECs[a]

thiols	gold	palladium
	NIH 3T3	
1	16 ± 4	5 ± 2
2	15 ± 3	6 ± 2
3	3 ± 2	40 ± 6
	HUAEC	
1	12 ± 4	5 ± 2
2	11 ± 4	6 ± 2
3	3 ± 2	35 ± 7

[a] The inert regions formed from thiol **1**, **2** or **3**. The data indicate the number of days until the density of cells outside the patterns exceeded 5% of that inside the patterns. The errors represent one standard deviation from the mean, as calculated from seven sets of experiments.

that it is possible to cause the focal adhesions (FAs, the subcellular structures that cells use to adhere to surfaces) to localize (Figure 5). FAs are complexes of macromolecules that tether the cytoskeleton of a cell to the extracellular matrix. We used immunostaining for vinculin, an indispensable component, to identify the FAs.[29] It is possible to visualize the FAs when the density of cells is low or when a full layer of cells is removed from the surface by vigorous pipetting or mechanical scraping. The top panels of Figure 5 show the FAs of intact NIH 3T3 fibroblasts,; the bottom panels of Figure 5 show the FAs on substrates from which we sheared off the layer of cells. In either case, no FAs formed on the OEG-terminated SAMs. This experiment shows that SAMs of OEG-terminated thiols on palladium can be useful in investigating subcellular events.[11]

SAMs on palladium have additional, practical advantages, as compared to those on gold: (i) Thin films of palladium have a longer shelf life than gold. After four months of storage in ambient conditions (sealed with Parafilm at atmospheric pressure and room temperature), we successfully patterned SAMs and cells on substrates of thin films of palladium. Experiments using substrates of gold film stored under the same conditions for over a month failed. (ii) Thin films of palladium prepared by thermal evaporation with a chromium adhesion layer successfully confined cells. SAMs formed on films of gold prepared by the same method failed to confine cells (we typically used thin films of gold deposited by electron-beam evaporation to confine cells). (iii) Palladium currently costs approximately ~25% less than gold by weight.

Mechanisms of Inertness of SAMs. We do not understand, mechanistically, why the best OEG-terminated SAMs on palladium are inert for longer periods of time than the best OEG-terminated SAMs on gold. Despite extensive theoretical and experimental investigation, the mechanisms by which surfaces covered with OEG-terminal groups resist the adsorption of proteins remain incompletely defined.[26,30−37] One hypothesis involves the density and conformation of the OEG moiety: a certain density of coverage of the OEG-group seems to be necessary for the surface to be inert. For mixed SAMs of OEG-terminated groups and methyl-terminated groups, for example, the density of OEG on SAMs must reach ~50% for the surface to be inert.[2,38] Surprisingly, when the organic chains of thiolate molecules pack *too* densely on the surface (for example, in the case of the SAM formed from $HS(CH_2)_{11}(OCH_2CH_2)_3OCH_3$ on Ag), the surface is not inert, perhaps because the OEG moiety adopts a conformation that has a high level of order and crystallinity.[26,39] Thus, if the density of

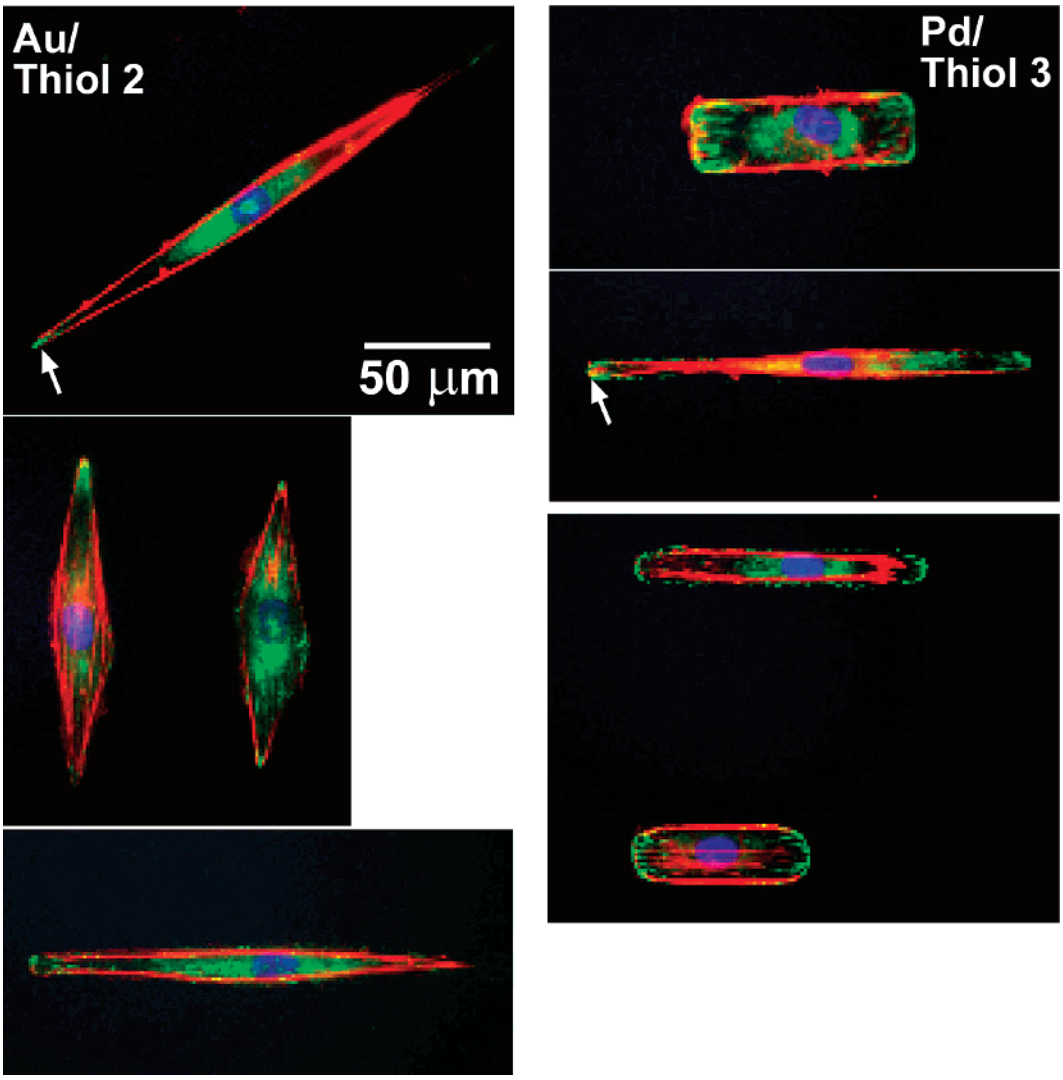

Figure 4. Single human umbilical artery endothelial cells immobilized on various types of patterns. SAMs on Pd (right panels) confined single cells to micropatterns, just as SAMs on Au (left panels). Anti-fibronectin (green), phalloidin−Texas red (red), and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, blue) were used to visualize fibronectin (Fn), actin, and the nucleus, respectively. Secreted Fn is concentrated at the corners of the microislands (arrows). The perinuclear stain of Fn is most likely from newly synthesized cytosolic proteins. The scale bar applies to all figures.

OEG is either higher or lower than that in the intermediate inert regime, the surface allows the adsorption of proteins and adhesion of cells.

A number of groups carried out theoretical studies of the mechanisms of inert, OEG-grafted surfaces.[30,35,40−42] Although the details of their explanations of the mechanisms vary, most researchers agree that a layer of tightly bound water exists on OEG-grafted surfaces and has a structure different from that of bulk water. This layer of water might be important in preventing proteins from approaching and adsorbing onto the surface.[25,42−44]

To study how the chemical properties of the surface affect the inertness of the surface, our group has tested the inertness of the surfaces by grafting a variety of chemical functional groups onto SAMs on gold.[33,45] The chemical groups that make surfaces inert have the following shared characteristics: (i) overall electric neutrality (no net positive or negative charge), (ii) a polar structure that contains heteroatoms (such as O, N, S, and P), (iii) the presence of hydrogen-bond acceptors, and (iv) the absence of hydrogen-bond donors.[3,34,46] We correlated molecular structures of the terminal groups of each SAM to the amount of proteins that the surface adsorbs.[3] Comparisons of SAMs on different metal substrates will also yield valuable information about the mechanisms underlying the ability of inert surfaces to resist the adsorption of proteins, because the structures and organizations of the organic chains of SAMs vary among different metals. Inert surfaces on palladium, therefore, provide another set of data relevant to understanding inert surfaces.

Long-Term Behavior of SAMs. Over time, all inert SAMs degrade and cease to resist the adhesion of proteins and cells, but the mechanisms of degradation remain unclear. The degradation of SAMs on gold probably proceeds by oxidation of the alkanethiolate to an alkanesulfinate or alkanesulfonate and subsequent desorption of the organic chain.[47] Our data, and those of Langer and co-workers, confirm that inert SAMs on gold remain inert in cell culture for about 2 weeks.[48] Addition of reagents that reduce oxidation prolongs the inertness of these SAMs.[48] Chen and colleagues found that cells that secrete metabolic oxidants tend to degrade SAMs faster than those that do not.[49] Surfaces

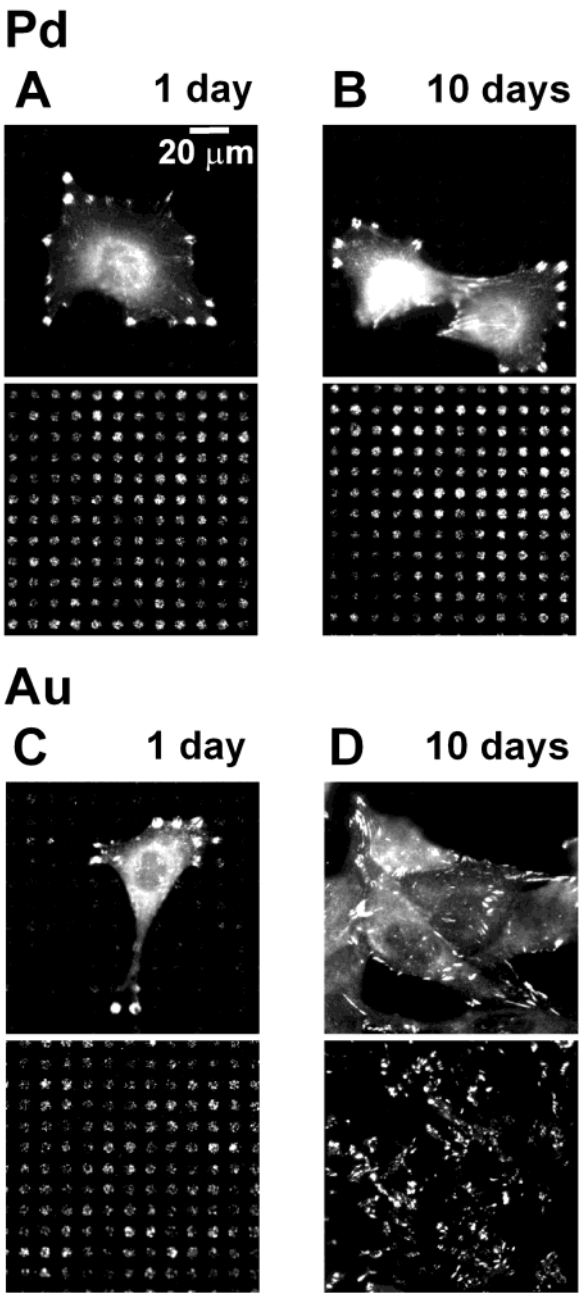

Figure 5. Localization of focal adhesions (FAs) in 3T3 cells on SAMs on palladium lasts longer than on gold. The top panels in each figure illustrate FAs of intact cells. The bottom panels illustrate the localization of FAs after a monolayer of cells was removed from the surfaces (see text). (A) and (B) are FAs on palladium. (C) and (D) are FAs on gold. Scale bar in A applies to (A−D). (A) and (C) were taken after 1 day in culture. (B) and (D) were taken after 10 days in culture.

that resist degradation for longer periods of time are suitable for studying cells in a strongly oxidizing environment.

A number of groups have studied the stability of SAMs on silver and gold in air.[47,50,51] Using XPS and Raman spectroscopy, Pemberton and colleagues reported that the conformations of the organic chains in SAMs formed from alkanethiols on silver and gold changed little after exposure to air, despite extensive oxidation of sulfur (up to 85% of all bound sulfur atoms). They also found that the organic layer did not desorb immediately after the oxidation of the sulfur. Electrochemical measurements showed that the ability of the SAMs to prevent electron transfer (an indication of the integrity and coverage of the SAM) remained constant over the span of 1 week (when the substrates were left in air).[47,52] These studies also showed that silver retains more of the organic chains of the SAM on the surface than does gold over a period of a week in air.[47]

In terms of structure, SAMs on palladium resemble SAMs on silver more than those on gold.[21] The cytotoxicity of silver ions precludes using SAMs on silver in most biological applications. Unlike silver, palladium does not form soluble, toxic ions in aqueous environments.[53,54] SAMs on palladium appear to combine the biocompatibility of gold with the resistance to desorption of SAMs on silver.

Mrksich and co-workers reported another method for extending the inertness of SAMs. They found that SAMs on gold formed from $HS(CH_2)_nR$ (n = 8, 11, R = mannitol) remained inert for 3−4 weeks in culture.[55] The fact that simply changing the terminal group of the SAMs from OEG to mannitol on gold can extend the inertness of the surface from 2 to 4 weeks suggests that SAMs on metals degrade by various mechanisms and should be examined in a case-by-case manner.

CONCLUSIONS

OEG-terminated SAMs on palladium are "inert", that is, resistant to the adsorption of proteins, and unable to support the adhesion and spreading of mammalian cells, for long periods of time (more than 4 weeks). Confining patterned cells to micropatterns for long intervals is useful in studies of certain cellular behaviors—morphogenesis of tissues, oncogenesis, and the effects of toxins on cells—that require long-term observation.[56−58] Except for complications in carrying out the SPR experiments on palladium using a Biacore instrument, inert SAMs on palladium offer features similar or superior to inert SAMs on gold.

Since palladium is more compatible with CMOS technologies than gold, SAMs on palladium could allow the integration of biological assays with devices fabricated by CMOS technologies.[21,59] The study of SAMs on palladium also provides another point of reference in efforts to understand the mechanisms of inert surfaces.[3]

ACKNOWLEDGMENT

Financial Support was provided by NIH (GM 065364) and DARPA/NSF (ECS-0004030). M.M.T. thanks the Harvard MRSEC for a summer fellowship. We thank Lu Yuan for XPS and the Center for Genomic Research for mass spectrometry and use of the Biacore 3000 instrument. We also thank Dr. Bobak Azamian for the culturing of HUAECs and Dr. Vincent Semetey for help with mass spectrometry.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review June 9, 2004. Accepted August 12, 2004.

AC049152T