Articles

Patterned Protein Films on Poly(lipid) Bilayers by Microcontact Printing

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The use of polymerized lipid bilayers as substrates for microcontact printing (μCP) of protein films was investigated. We have previously shown that vesicle fusion of bis-SorbPC, a dienoate lipid, on glass and silica substrates, followed by redox-initiated radical polymerization, produces a planar supported lipid bilayer (PSLB) that is ultrastable[1a] [Ross, E. E.; Rozanski, L. J.; Spratt, T.; Liu, S.; O'Brien, D. F.; Saavedra, S. S. *Langmuir* **2003**, *19*, 1752] and highly resistant to nonspecific adsorption of dissolved proteins [Ross, E. E.; Spratt, T.; Liu, S.; Rozanski, L. J.; O'Brien, D. F.; Saavedra, S. S. *Langmuir* **2003**, *19*, 1766].[1b] Here we demonstrate that μCP of bovine serum albumin (BSA) onto a dried poly(bis-SorbPC) PSLB from a poly(dimethylsiloxane) (PDMS) stamp produces a layer of strongly adsorbed protein, comparable in surface coverage to films printed on glass surfaces. Immobilization of proteins on poly(PSLB)s has potential applications in biosensing, and this work shows that direct μCP of proteins is a technically simple approach to create immobilized monolayers, as well as multilayers of different proteins.

Introduction

Recently we reported development of ultrathin (<50 Å), highly stable polymer films, based on reactive phospholipids, that have a lipid bilayer type architecture; that is, the surface of the polymer presents a uniform array of zwitterionic phosphatidylcholine (PC) headgroups while the interior of the polymer layer is cross-linked alkane.[1a] Nonspecific adsorption of dissolved proteins to these polymerized, planar supported lipid bilayers (poly(PSLBs)) is minimal,[1b] comparable to that on a fluid PC lipid bilayer, a feature that makes them potentially useful as substrate coatings in biosensor applications.[2] However, the inherent protein adsorption resistance implies that covalent tethering or biospecific binding strategies are required to conjugate to proteins to the bilayer for use in immuno- and enzymatic assays.

Planar arrays of spatially addressable biomolecules such as lipid films and proteins are being created to develop high throughput screening assays and multianalyte biosensors.[3,4] Approaches for patterning biomolecules introduced within the past decade include those based on soft lithography or microcontact printing (μCP).[5] In its original inception, μCP stamps cast from poly(dimethylsiloxane) (PDMS) were "inked" with reactive molecules such as alkanethiols which were subsequently transferred to an appropriate substrate via direct contact, resulting in patterned self-assembled monolayers (SAMs).[6] More recently, the surface chemistry of metal, oxide and polymer surfaces has been modified using μCP methods in a manner that promotes spatially selective protein adsorption or binding.[7] However, the simplest μCP approach for protein patterning is direct transfer of a protein layer adsorbed on a PDMS stamp to a target substrate surface by conformal contact. Many proteins readily adsorb to PDMS and can subsequently be transferred to target substrates, resulting in high surface coverage that often exceeds the saturation coverage observed when the dissolved protein nonspecifically adsorbs to the substrate.[8] Protein deposition by μCP has been demonstrated on a number of different types of substrates including oxides and metals, in spatially defined patterns with submicron resolution.[9]

The specific forces responsible for protein transfer from stamp to substrate during μCP are speculative, although a recent study by Tan et al. has demonstrated that both stamp and substrate wettability are crucial aspects.[10] Their data indicate that a critical difference between the wettabilities of the two surfaces must exist for efficient transfer to occur. This finding suggests that μCP may be a convenient route for creation of protein arrays on substrate surfaces that are inherently protein resistant, e.g., surfaces that are typically hydrophilic and uncharged. When used in bioassays, these surfaces should minimize or eliminate interactions with nontarget sample proteins that could produce false positive signals and/or loss in device sensitivity. A PSLB composed of polymerized bis-Sorbylphosphatidylcholine (poly-(bis-SorbPC)) is an example of a hydrophilic surface coating that is resistant to nonspecific adsorption of dissolved proteins.[1b] In this report, we investigate the suitability of poly(bis-SorbPC) PSLBs for microcontact printing of proteins.

Experimental Section

Poly(bis-SorbPC) Bilayers. PSLBs composed of polymerized bis-SorbPC were prepared by vesicle fusion as described in ref 1a. Briefly, bis-SorbPC vesicles (1 mg/mL) in deionized (DI) water (18 MΩ·cm) were fused on clean oxidized silicon wafers, microscope slides, or

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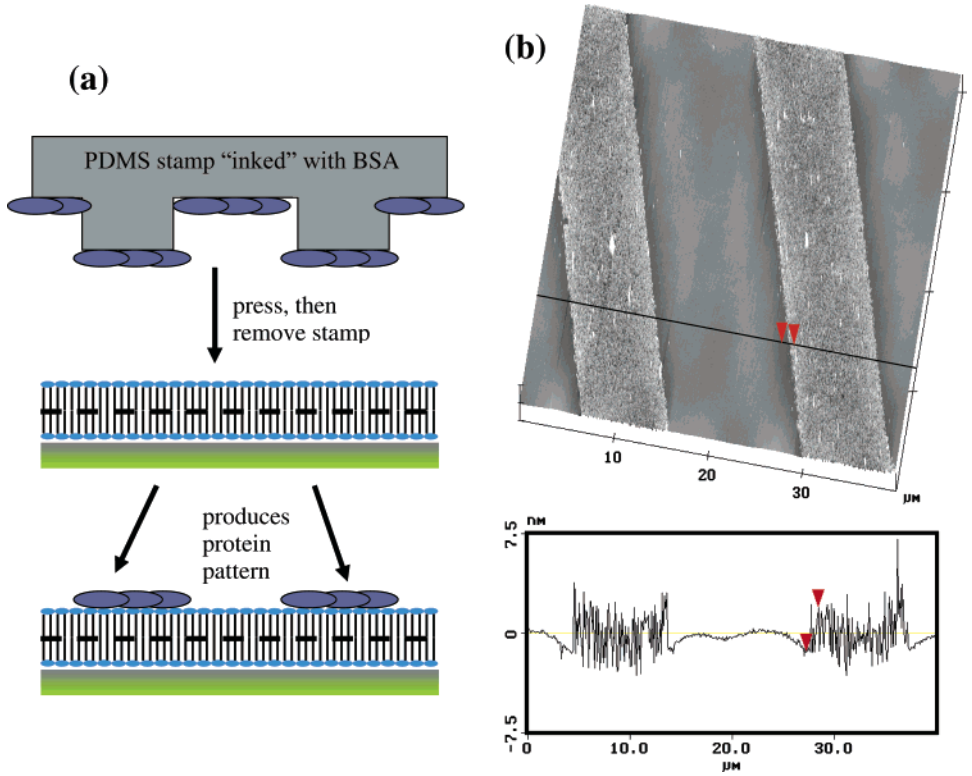

Figure 1. (a) Schematic of μCP of BSA on a poly(bis-SorbPC) PSLB. (b) A representative AFM image and linescan of BSA printed on a poly(bis-SorbPC) PSLB from a PDMS stamp. The image size is 40 μm × 40 μm, and the height scale is 10 nm. The dark line across the image indicates the position of the linescan. The height difference between the arrows in the linescan is 3.3 nm. In the linescan, the topography of the bilayer surface appears to oscillate between the regions of printed protein. This "waviness" is an artifact that occurs when scanning relatively rapidly, in tapping mode, over features that vary significantly in height and applying a processing algorithm to flatten the image.

coverslips to form PSLBs which were then transferred to polymerization solutions composed of freshly prepared redox initiators (bisulfite/persulfate). Following rinsing and drying, structural characterization by ellipsometry and AFM revealed a highly uniform, nearly defect-free film of approximate bilayer thickness (45 Å).[1a] The resistance of these PSLBs to nonspecific adsorption of dissolved proteins was equivalent to that of a POPC bilayer (see ref 1b), providing additional evidence that the structure of a poly(bis-SorbPC) PSLB is maintained throughout redox polymerization, rinsing and drying, and subsequent rehydration.

Stamp Fabrication. PDMS stamps were fabricated using Sylgard 182 (Dow Corning) as directed by the manufacturer. Patterned stamps were cast on a silicon master (provided by Neal Armstrong, Department of Chemistry, University of Arizona) that had 10 micron wide line features separated by 15 micron spaces. After casting, stamps were cured for 2 h at 100 °C in an atmospheric oven. Excess PDMS was removed with a razor blade, and the stamp was glued to a SEM stub and allowed to cure overnight. The patterned region of the stamp was approximately 1 cm^2. Featureless stamps were created by curing the polymer on an oxidized silicon wafer. Prior to use, stamps were rinsed with ethanol and water extensively (>50 mL from a direct stream). Oxidized stamps were created by placing hydrophobic (as-cast) PDMS stamps in a plasma cleaner (Harrick model PDC-3XG) at 30 W for 1 min (slight air bleed, although this was not critical), followed by rinsing with deionized water and drying with a nitrogen stream.

μCP. Stamps used in protein inking and transfer experiments were incubated for 10 min with 1 mg/mL solution of protein, either bovine serum albumin (BSA, Sigma) or rhodamine-labeled BSA (Sigma) in 10 mM, pH 7.4 phosphate buffer (hereafter referred to as PBS). Stamps were then rinsed (approximately 100 mL from a directed stream) with buffer and then DI water before being dried briefly in a nitrogen stream and used immediately. Applying pressure by hand, stamps were brought into contact with poly(bis-SorbPC) films or bare substrates (glass slide or oxidized silicon wafer) so that conformal contact was made either in air or under water for 5 s. After stamping, substrates were rinsed with water and dried with nitrogen before analysis.

Fluorescence Microscopy. A Nikon Diaphot inverted epifluorescence microscope was used to collect fluorescence images of patterned protein films and substrates. Images were acquired using a liquid-nitrogen-cooled CCD camera and processed using the background fluorescence intensity outside of the illuminated area for normalization. An average of three line scans from fluorescence images was used to compare fluorescence intensities from different surfaces.

Atomic Force Microscopy. AFM was performed in tapping mode with a NanoScope III (Veeco). Forces between tip and sample were minimized as described in ref 1a.

X-ray Photoelectron Spectroscopy. A Kratos Axis-Ultra X-ray photoelectron spectrometer was used with a monochromatic Al Kα source at 1486.6 eV and a takeoff angle of 20 degrees. Peaks were fit using Kratos Vision2 software. An electron-emitting filament was used to reduce sample charging, and all spectra were corrected for charging effects by adjusting the C1s peak to 284.6 eV.

Results and Discussion

Characterization of BSA Deposited by μCP on Poly(bis-SorbPC). BSA adsorbs readily from solution to hydrophobic surfaces,[11] including PDMS, and μCP of BSA from PDMS to oxide surfaces has been previously reported.[9b] Here dried poly(bis-SorbPC) bilayers were contacted with PDMS stamps inked with BSA, rinsed with buffer, and examined for evidence of protein transfer. A representative AFM image of BSA printed on a poly(bisSorbPC) PSLB in air is shown in Figure 1. Transfer occurred uniformly in the printed regions; a continuous layer of protein approximately 1.5−3 nm thick is observed, consisting of parallel lines approximately 12 μm wide separated by 15 μm gaps. The dimensions of the features correspond well to

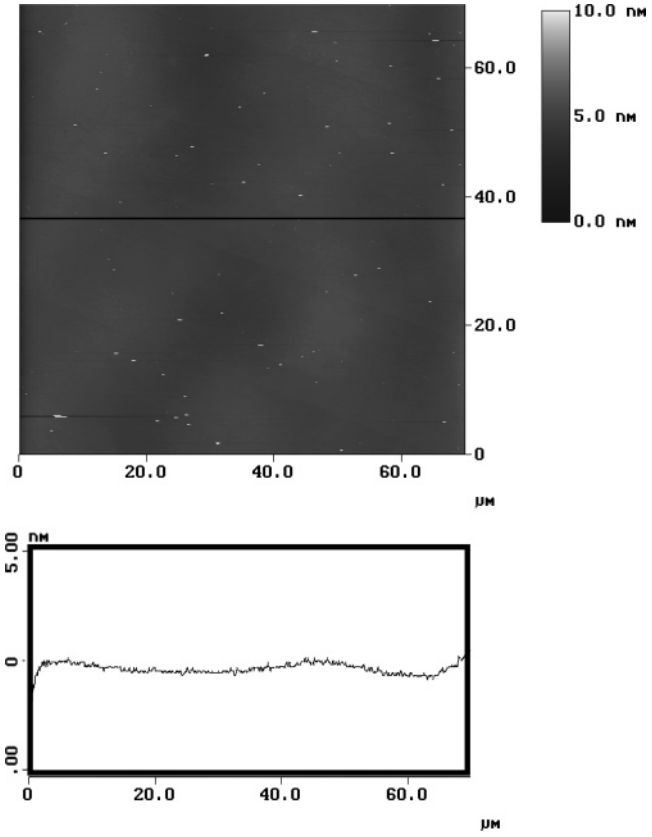

Figure 2. AFM image and linescan of a poly(bis-SorbPC) PSLB that was stamped with uninked PDMS. The PDMS stamp had the same features as that used to stamp the PSLB in Figure 1. The image size is 70 μm × 70 μm, and the height scale is 10 nm. The dark line across the image indicates the position of the linescan. No discernible topographical features are observed, showing that the PSLB structure is not altered by stamping with PDMS.

those of the master from which the stamp was cast. The protein layer is sufficiently dense such that individual BSA molecules are not discernible, and no proteins are observed outside the printed region. AFM scans taken over very large areas (greater than 100 μm^2) show that the transferred protein films are very uniform with very few defective areas.

Previously, Boxer and co-workers have shown that unpolymerized lipid bilayers can be structurally altered by contact with PDMS stamps.[5c] Thus, the possibility must be considered that during μCP the PSLB is removed (or partially removed) and replaced with a BSA film that is adsorbed directly to the underlying glass substrate (or to the remnants of the PSLB). However, we have previously shown that poly(bis-SorbPC) PSLBs are not structurally altered when sonicated in surfactant solutions or immersed in common organic solvents, demonstrating the extreme chemical stability of these polymer films and suggesting that displacement by BSA during μCP is unlikely.[1a] The AFM data presented in Figure 2 provides further evidence that PDMS contact does not alter the poly(PSLB) morphology. μCP was performed as before, except that the stamp was not inked with BSA. No changes in bilayer surface topography are apparent, showing that the PSLB structure is not macroscopically altered by μCP. In another experiment (see the Supporting Information), a μCP BSA film on poly(bis-SorbPC) was sonicated in a surfactant solution, which desorbed most of the BSA. AFM imaging showed that after BSA removal, the underlying PSLB was still intact.

The amount of BSA deposited by μCP on poly(bis-SorbPC) PSLBs was compared to that on clean glass surfaces using AFM.

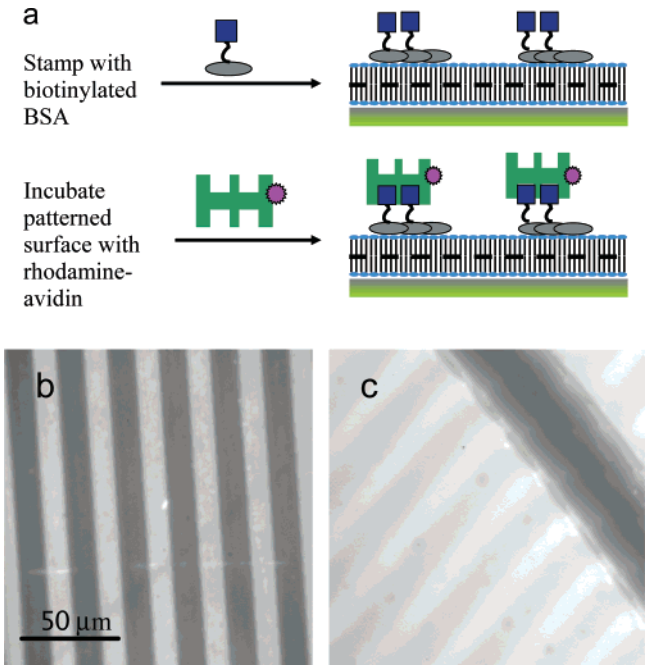

Figure 3. (a) Schematic of an immobilization method in which a film of biotinylated BSA, deposited by μCP on a poly(bis-SorbPC) bilayer, is used to immobilize avidin. The unfilled biotin binding sites on avidin provide a means for subsequent attachment of a soluble biotinylated protein (not shown). Epifluorescence was used to image a poly(bis-SorbPC) PSLB (b) and a glass substrate (c) that were printed with biotinylated BSA and then exposed to dissolved rhodamine-labeled avidin for 30 min. The avidin bound to the biotinylated BSA in the printed regions. On glass (c), a high degree of nonspecific adsorption of dissolved avidin to the unprinted regions greatly reduced the contrast between the printed and unprinted regions. The dark area in (c) is a region of the substrate that was scratched to remove adsorbed protein and illustrate the minimal contrast elsewhere. Nonspecific adsorption of dissolved avidin was minimal on the PSLB surface (b), as indicated by the lack of fluorescence emitted from the regions between the lines of printed biotinylated BSA.

The surface coverages appeared to be equivalent (see images in Supporting Information). However, determination of surface coverage by AFM is compromised by tip effects which make the apparent size of small features comparable to those of the AFM tip; thus, this method is not accurate when applied to films of high surface coverage.[12] Epifluorescence microscopy was used to perform a more quantitative comparison. The results are listed in Table 1. The surface coverage of rhodamine-labeled BSA printed on poly(bis-SorbPC) PSLBs relative to that printed on glass substrates is approximately 90%, showing that μCP of BSA on these two substrates is of comparable efficiency. This finding is consistent with a study by Tan et al.,[10] who showed that the wettabilities of the stamp and the substrate must be significantly different for efficient transfer in protein μCP. The contact angle of water on a poly(bis-SorbPC) bilayer is 32 degrees,[1a] whereas the surface of as-cast PDMS is very hydrophobic (contact angle of 112°[13]). Based on the data reported in ref 10, this difference is sufficient to predict that efficient transfer should be observed.

When the PDMS stamp was oxidized in a plasma (a process that temporarily renders PDMS highly hydrophilic, with a water contact angle <5°[13]) then inked with BSA, the surface coverage of μCP BSA films on both glass and poly(bis-SorbPC) was reduced to less than 5% of that obtained using a hydrophobic PDMS stamp (Table 1). The results of these experiments are also consistent with those of Tan and co-workers[10] (i.e., in these experiments, the difference in stamp and substrate wettability

Table 1. Relative Surface Coverage of Rhodamine-Labeled BSA on Glass Substrates and Poly(lipid) Bilayers after Protein Immobilization by μCP or Nonspecific Adsorption from Solution[a]

substrate	μCP from PDMS in air[b]	μCP from ox-PDMS in air[c]	nonspecific adsorption from solution[d]	μCP from PDMS under water[b]	nonspecific adsorption after PDMS stamping[b,e]
poly(bis-SorbPC)	91 ± 28^f	4 ± 0.6^f	6 ± 2	14 ± 4^g	34 ± 10^f
glass	100 ± 41^f	3 ± 0.3^f	64 ± 2	4 ± 3^f	115 ± 8^f

[a] The values are given as the fluorescence intensity of the film relative to that of μCP BSA films on glass, expressed as a percentage. [b] Hydrophobic (as-cast), featureless PDMS stamps were used. [c] Featureless PDMS stamps were oxidized in a plasma prior to protein inking and stamping. [d] Data from ref 1b. Substrates were exposed to 1 mg/mL rhodamine-BSA and then rinsed. [e] Substrates were stamped with unlinked, featureless PDMS prior to exposure to 1 mg/mL rhodamine-BSA, then rinsed. [f] $n = 3$. [g] $n = 2$.

Table 2. Stability of μCP BSA Films to Rinsing

rinse treatment	% of printed BSA removed
PBS	1 ± 3^a
BSA (10 mg/mL)	5 ± 9^a
salt (1 M NaCl)	29 ± 12^a
surfactant (20 mg/mL Triton X-100)	67 ± 11^a

[a] $n = 3$.

is too small for efficient transfer). However, in other situations, the use of hydrophilic PDMS can enhance protein transfer to a hydrophilic substrate. Runge and Saavedra observed that, in the case of cytochrome *c* on indium−tin oxide (water contact angle of $<5°$), both the total surface coverage and fraction of the protein film that was electrochemically addressable were significantly higher when μCP was performed using an oxidized stamp.[13] This discrepancy illustrates that the mechanism of protein transfer during μCP is not well understood, although clearly the surface chemistry of the stamp and the substrate are important factors.

The stability of rhodamine-labeled BSA films deposited by μCP on poly(bis-SorbPC) PSLBs was also examined using epifluorescence microsopy; the results are listed in Table 2. No desorption of printed protein occurred with repeated drying and rinsing with PBS buffer. Likewise, very little desorption was observed when a printed BSA film was incubated for 2 h in a buffer solution containing 10 mg/mL unlabeled BSA.[14] Incubation in PBS containing 1 M NaCl for 2 h removed 29% of the printed protein, likely representing the fraction of proteins having predominantly electrostatic interactions with the poly(lipid) bilayer surface. Desorption of 67% of the protein film occurred during a 15 min incubation in a nonionic surfactant solution (Triton X-100 at 20 mg/mL).[15] This finding indicates that hydrophobic interactions are also a significant contributor to the forces that bind microcontact printed BSA to poly(bis-SorbPC) bilayers. AFM imaging of a μCP BSA film was performed after incubation in Triton X-100 and verified that this treatment removed a significant fraction of printed protein (see data in the Supporting Information). A discontinuous film structure was observed in which individual proteins or small clusters of proteins are resolved. Line scans from these images also show that the underlying poly(lipid) bilayer morphology is not measurably altered by μCP or Triton X-100 incubation, consistent with the results on structural stability presented above and in ref 1.

It is well established that dissolved proteins, including BSA, nonspecifically adsorb strongly to the surface of clean glass and silica[1b,11] (here nonspecific adsorption is used to refer to adsorption of dissolved proteins to a solid−liquid interface in the absence of a biospecific binding mechanism). Thus, it is not surprising that BSA and many other proteins can be efficiently transferred from hydrophobic PDMS to glass substrates. However, in contrast to glass, nonspecific adsorption of BSA is minimal on a poly(bis-SorbPC) PSLB (see data listed in Table 1). Thus, it is somewhat surprising that μCP of BSA on poly(bis-SorbPC) produces a strongly adsorbed protein film. This result suggests that hydration by immersion in bulk water is necessary to shield the attractive forces between BSA and the PC moieties on the PSLB surface. To explore this issue further, μCP was performed with both the stamp and the PSLB submerged in DI water. In these experiments, the force between the substrate and the stamp was increased considerably, relative to μCP in air, to ensure conformal contact. Regardless, significantly lower transfer was observed (14% relative to μCP on glass in air; see Table 1), and AFM imaging of the printed surface confirmed this result (see the Supporting Information). However, when the same experiment was performed on glass substrates, an even greater reduction in transfer efficiency was observed (4% relative surface coverage; see Table 1). These experiments show that for the two cases examined here, μCP under water is clearly inefficient, indicating that the forces responsible for protein transfer are disrupted when the stamp and substrate are hydrated. However, the results do not explain why μCP of BSA on poly(bis-SorbPC) in air produces a strongly adsorbed protein film

Poly(bis-SorbPC) Film Contamination from PDMS Contact. It is known that silicone material can be transferred from a hydrophobic (as-cast) PMDS stamp to a substrate during μCP;[16] thus, another possibility we considered is that μCP fouls the PSLB surface, increasing its adsorptive properties. X-ray photoelectron spectroscopy (XPS) was used to investigate this issue. Spectra of poly(bis-SorbPC) bilayers were acquired before and after stamping with an uninked, hydrophobic PDMS stamp (see spectra in the Supporting Information). Only one Si 2p peak, assigned to the substrate SiO_2, was observed in spectra of unstamped PSLBs, whereas a second, less oxidized Si2p peak appeared in the spectra of stamped PSLBs. This second peak, attributed to contamination by the hydrophobic stamp, occurred despite extensive rinsing and sonication of stamps in ethanol before use. Kasemo and co-workers observed that silicone contamination of substrates during μCP was reduced when PDMS was oxidized using a UV/ozone treatment.[16b] A similar result was observed here: XPS spectra of poly(bis-SorbPC) PSLBs that were stamped with plasma-oxidized PDMS were identical to spectra of unstamped PSLBs (see the Supporting Information).

After confirming the presence of contaminating material, we assessed the more important issue: Is the resistance to nonspecific protein adsorption characteristic of a poly(bis-SorbPC) PSLB compromised by stamping with uninked, hydrophobic PDMS? After stamping, the PSLB was incubated for 30 min in a 1 mg/mL solution of rhodamine-labeled BSA in PBS and then rinsed in DI water. The protein surface coverage, measured using fluorescence microscopy as described above, was 34% relative to that deposited by μCP on glass substrates (Table 1). The relative surface coverage of rhodamine-labeled BSA nonspe-

cifically adsorbed to a native (unstamped) PSLB is only 6%. Thus, stamping with hydrophobic PDMS significantly degrades the protein resistance of a native poly(bis-SorbPC) bilayer. Identical experiments were performed on glass substrates. After stamping, an increase in nonspecific adsorption of rhodamine-labeled BSA was also observed (data in Table 1), which implicates the PDMS stamp as the source of the difference in protein adsorption behavior. Comparative experiments were also performed using plasma-oxidized PDMS. In this case, stamping poly(bis-SorbPC) resulted in no measurable increase in nonspecific BSA adsorption relative to unstamped regions of film ($n = 2$).

The most probable explanation for these observations is that silicone material or oils on hydrophobic PDMS are transferred to the poly(bis-SorbPC) PSLB during stamping, and this contamination results in an increase in hydrophobically driven, nonspecific adsorption of dissolved BSA. The efficiency with which μCP BSA is desorbed when immersed in Triton X-100 solution is consistent with this explanation. Oxidizing the stamp reduces the amount of contaminating material on its surface to below a detectable level, and eliminating this contamination preserves the characteristic protein resistance of native poly(bis-SorbPC), albeit at the expense of eliminating the apparent mechanism by which efficient protein μCP is achieved.

Using μCP and Biospecific Binding to Create Protein Multilayers. A variety of different proteins have been deposited by μCP on oxide and metal substrates, and in most cases, at least some of the protein molecules in the film are bioactive (see ref 8e for a recent example). However, the specific activity of the film may be low, due to strong adsorptive interactions with the substrate which may alter the native protein structure.[8,11] Creation of a layered structure (depicted in Figure 3a), in which a biotinylated base protein is deposited by μCP, followed by biospecific adsorption of avidin or streptavidin, then a biotinylated probe protein (e.g., an antibody, not shown) offers a strategy to address this problem. This strategy may minimize adsorptive interactions between the probe protein and the substrate, as well as the contamination issues associated with hydrophobic PDMS described above. Furthermore, it may provide some control over the orientation of the immobilized probe protein.[17] To test this concept, line patterns of biotinylated BSA were deposited by μCP on poly(bis-SorbPC) PSLBs. These PSLBs were then incubated with a solution of rhodamine-labeled avidin and rinsed in PBS. The same procedure was also performed using glass substrates. The respective epifluorescence images are shown in Figure 3, panels b and c.

In theory, this procedure should generate an array of fluorescent stripes corresponding to specific binding of avidin to the line pattern of biotinylated BSA. The predicted contrast was observed on the PSLB. Avidin adsorption to nonprinted regions was less than 5% of that in the regions that were printed with biotinylated BSA. This result is consistent with the body of previous work showing that a poly(bis-SorbPC) PSLB is highly resistant to nonspecific adsorption of dissolved proteins (Table 1 and ref 1b). However on the glass substrate the predicted contrast was not observed. Nonspecific adsorption of avidin to nonprinted regions nearly eliminated the expected contrast; the signal intensity was approximately 85% relative to that in the printed regions. These results demonstrate the viability of a μCP strategy for constructing protein multilayers on poly(bis-SorbPC) and illustrate the superiority of the PSLB surface in discriminating between specific protein binding and nonspecific protein adsorption.

Conclusions

μCP is shown to be a viable approach for immobilization of proteins on poly(bis-SorbPC) supported bilayers. High protein surface coverages are achieved, and the method is technically much simpler than covalent tethering to lipid headgroups. The potential use of μCP protein films on poly(PSLBs) as bioactive coatings is also evident from the stability of the protein film during buffer rinsing and in the presence of excess dissolved protein, and the low nonspecific protein adsorption properties of the poly(lipid) in unstamped regions. However, if protein film stability in high salt or surfactant solutions is required for the intended application, a covalent tethering method is probably a better choice. The use of a layered deposition strategy, coupled with bioinert nature of poly(bis-SorbPC), may minimize denaturation of immobilized proteins relative to other polymeric or inorganic substrates. Although all of the μCP described herein was performed using BSA, we have also successfully printed antibodies and streptavidin on poly(bis-SorbPC) PSLBs.[18] However, the general applicability of the method to other proteins remains to be determined.

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Supporting Information Available. Additional AFM images of μCP BSA films formed under different conditions and XPS spectra of stamped poly(PSLBs) and hydrophobic PDMS. This material is available free of charge via the Internet at http://pubs.acs.org.

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