

Microcontact Printing of Proteins on Mixed Self-Assembled Monolayers

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Microcontact printing of proteins from an elastomeric stamp has been demonstrated on a limited number of substrates. This work explores the generality of this method of patterning proteins by examining the role of surface wettability of both the substrate and the stamp in microcontact printing. The substrates used in this study consisted of two-component, mixed self-assembled monolayers (SAMs) of alkanethiols on gold presenting $-\text{CH}_3$ and polar groups $-\text{COOH}$, $-\text{OH}$, or $-(\text{OCH}_2\text{CH}_2)_6\text{OH}$. We found that protein adsorbed on a stamp successfully transfers onto a mixed SAM only when the mole fraction of polar functionality on the SAM exceeded a particular threshold. Although the mole fraction of polar groups required was different for each of the three types of mixed SAMs, the advancing water contact angles on these surfaces nearly coincided. Moreover, the minimum wettability of the SAM needed for the transfer of proteins decreased when the wettability of the stamp was decreased. Our findings suggest that the difference in wettability between the surfaces of the stamp and substrate is the dominant parameter that determines the successful microcontact printing of proteins.

I. Introduction

Micrometer-scale patterns of proteins may function as elements of biosensors,^{1–3} tissue constructs,⁴ and protein microarrays⁵ and as substrates for addressing fundamental questions in cell biology.^{6–9} Previous work has used microcontact printing (μCP) to directly pattern proteins on glass without significant loss in biological activity of the printed proteins.^{10–15} Because the substrates used in previous studies were selected empirically, the generality of the approach to produce patterns of protein by μCP is unknown. In this study, we have examined the role of the wettability of the substrate and the stamp on the μCP of proteins. This work enables the rational design of surfaces to allow successful μCP and greatly expands

the types of surfaces that can be easily patterned with proteins using μCP .

μCP uses an elastomeric stamp to print a variety of molecules in submicrometer resolution patterns without the need for dust-free environments or harsh chemical treatments.^{16–18} An elastomeric stamp is made by curing poly(dimethylsiloxane) (PDMS) against a microfabricated silicon master, acting as a mold, to allow the surface topology of the stamp to form a negative replica of the master.¹⁸ The stamp is coated with the desired molecules, and those residing in the raised regions of the stamp are brought into contact with the host substrate when the stamp is printed. Presumably, the molecules transfer from the stamp to the substrate if they can interact more strongly with the substrate than with the stamp. For instance, in μCP of alkanethiols on gold,¹⁸ alkanethiols coordinate tightly with gold; likewise, in μCP of alkylsilanes on glass,¹⁹ alkylsilanes bond covalently with Si–OH groups. In μCP of proteins, it is unclear what causes protein adsorbed on a stamp to transfer or why this process is successful only on certain surfaces.

In this study, we printed proteins onto two-component self-assembled monolayers (SAMs) composed of aliphatic and polar alkanethiols. The SAM serves as a molecularly well-defined, model substrate, the wettability of which can be independently controlled by the mole fraction of alkanethiol terminated with polar groups in the two-component SAM. We have found that the SAMs need to possess a minimum wettability in order for μCP to be successful, and this minimum wettability is influenced by the wettability of the stamp.

II. Materials and Methods

Materials. Dodecanethiol (98%), 11-mercapto-1-undecanol (97%), and 11-mercaptoundecanoic acid (95%) were used as received from Aldrich. We refer to these compounds as CH_3 -

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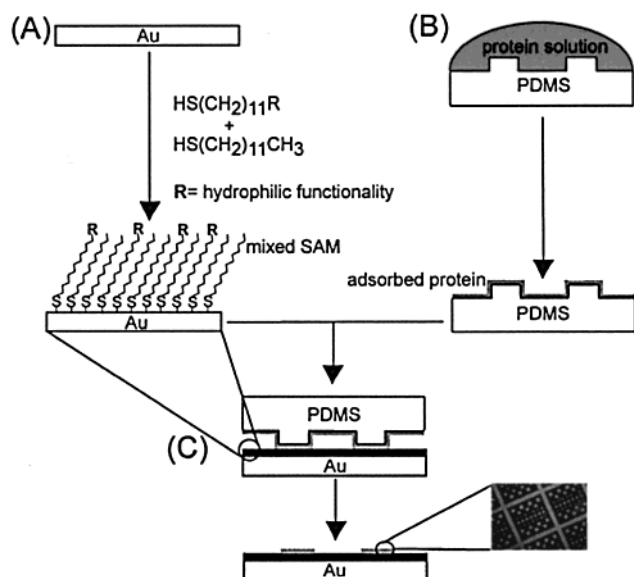


Figure 1. (A) Schematic of the formation of a two-component mixed SAM, (B) adsorption of proteins onto a PDMS stamp, and (C) μ CP of protein from the stamp onto the mixed SAM with a sample micrograph of fluorescently labeled protein printed onto a 100% COOH SAM substrate.

terminated alkanethiol, OH-terminated alkanethiol, and COOH-terminated alkanethiol, respectively. The hexadecanethiol was used as received from Fluka. Hexa(ethylene glycol)-terminated alkanethiol HS(CH₂)₁₁(OCH₂CH₂)₆OH, referred to as EG₆OH-terminated alkanethiol, was synthesized as previously described.²⁰ 3-Aminopropyl-trimethoxysilane was used as received from Aldrich. (Tridecafluoro-1,1,2,2,-tetrahydrooctyl)-1-trichlorosilane was obtained from United Chemical Technologies (Bristol, PA). Deionized water was used to rinse stamps and for contact angle measurements, and ethanol was used to dilute alkanethiol solutions and to rinse gold substrates.

Preparation of Mixed SAMs. Figure 1A depicts the formation of two-component mixed SAMs on gold. We coated (100) silicon wafers (Silicon Sense, Nashua, NH) with 3 nm of Ti (99.99%) to promote adhesion, followed by 50 nm of Au (99.999%) by electron-beam evaporation. Immersion of the gold-coated substrates in an ethanolic solution of HS(CH₂)₁₁CH₃ and HS(CH₂)₁₁R (R = -COOH, -OH, or -EG₆OH; total concentration of alkanethiol = 2 mM) for >20 h at 4 °C functionalized the gold surface with a mixed SAM. The substrates were removed from the alkanethiol solution, thoroughly rinsed with ethanol, and dried just prior to μ CP. Patterned SAMs were prepared by μ CP of HS(CH₂)₁₅CH₃ on a gold-coated surface, followed by immersion in a 2 mM solution of HS(CH₂)₁₁COOH for 1 h to fill the unprinted regions.

μ CP of Proteins. Figure 1B,C depicts the adsorption of proteins onto the stamp surface, followed by the μ CP process. PDMS stamps were made by casting Sylgard 184 (Dow Corning, Midland, MI) on a silicon master with 2 μ m thick features made by photolithography. The master was silanized with (tridecafluoro-1,1,2,2,-tetrahydrooctyl)-1-trichlorosilane vapor overnight under vacuum prior to casting of the PDMS to aid subsequent release. Upon curing at 60 °C overnight, the elastomeric stamp bearing the negative pattern of the master was peeled off, washed with ethanol, and dried under nitrogen. The stamp was used as cast, and the surface chemistry of the stamp was not modified unless stated otherwise.

To functionalize the surface of a stamp with -CF₃ groups, the surface of the stamp was oxidized in air plasma (~200 mTorr, 1 min; Plasma Prep II, SPI Supplies, West Chester, PA) and silanized with (tridecafluoro-1,1,2,2,-tetrahydrooctyl)-1-trichlorosilane vapor overnight under vacuum. To functionalize the surface of a stamp with -NH₂ groups, the surface of the stamp was oxidized in an air plasma (~200 mTorr, 1 min), immersed

in a solution of 25 mM aminopropyl-trimethoxysilane and 50 mM HCl in 98% ethanol for 2.5 h, rinsed thoroughly with ethanol, and dried under nitrogen.

To allow adsorption of proteins, we immersed the stamps for 1 h in an aqueous solution of fluorescently labeled protein (goat-derived immunoglobulinG conjugated to Alexa Fluor 594, Molecular Probes, Eugene, OR; 10 μ g/mL in phosphate buffered saline). The stamps were rinsed thoroughly with deionized water, blown dry under nitrogen, and placed in conformal contact with a freshly prepared SAM substrate for >10 s before being peeled off.

Imaging of Fluorescently Labeled Protein. Images of labeled proteins printed onto SAM substrates were acquired with an inverted fluorescence microscope (Eclipse TE200, Nikon) fitted with a SPOT RT digital camera system (Diagnostic Instruments, Sterling Heights, MI). All images were obtained with identical exposures unless otherwise noted. The fluorescence emission of the labeled protein was partially quenched by the gold substrate, and thus relatively long exposure times were used. Each image represents experiments repeated a minimum of three times.

Measurement of Contact Angles. Contact angles of water under ambient room temperature and humidity were determined using a goniometer (model 100-00, Ramé-Hart, Mountain Lakes, NJ). Reported values are an average of a minimum of three measurements taken on two separately prepared sets of SAMs.

III. Results and Discussion

Effect of the Density of Hydrophilic Functionalities on μ CP. We first examined whether the hydrophilic content of a substrate influences the μ CP of proteins. For this purpose, we formed two-component mixed SAMs on flat gold surfaces from the coadsorption of CH₃-terminated alkanethiol and alkanethiol functionalized with carboxylic acid (COOH).²¹ We refer to the SAMs according to the percentage of the alkanethiol terminated with polar groups in the coating solution; for instance, a "60% COOH SAM" refers to a two-component mixed SAM formed from a solution containing 1.2 mM COOH-terminated alkanethiol and 0.8 mM CH₃-terminated alkanethiol. It has previously been shown for the mixed SAMs used in this study that the density of the functionalized groups on the SAM surface is roughly proportional to the percentage of functionalized alkanethiols in the coating solution used to form the SAM.²¹⁻²³ In addition, the functionalized alkanethiols distribute uniformly in the SAM surface without aggregating into domains of more than a few tens of angstroms across.^{21,24}

We printed fluorescently labeled proteins onto mixed SAM substrates containing an increasing density of carboxylic acid groups and evaluated the transfer of protein by fluorescence microscopy. No protein transfer occurred on surfaces containing \leq 50% COOH SAM, while the protein pattern transferred completely from the stamp on surfaces containing \geq 65% COOH SAM (Figure 2 and Figure 3A). Following μ CP onto the \geq 65% COOH SAM, the fluorescence intensity of the contacted region of the stamp dropped to background levels, and μ CP repeated with the same stamp did not result in any additional observable transfer of proteins. These observations suggest that the monolayer of protein initially adsorbed onto the stamp had transferred completely onto the substrate during μ CP, and they agree with previous reports showing that the amount of protein transfer from the stamp exceeds 99%.¹¹ The protein pattern transferred partially onto

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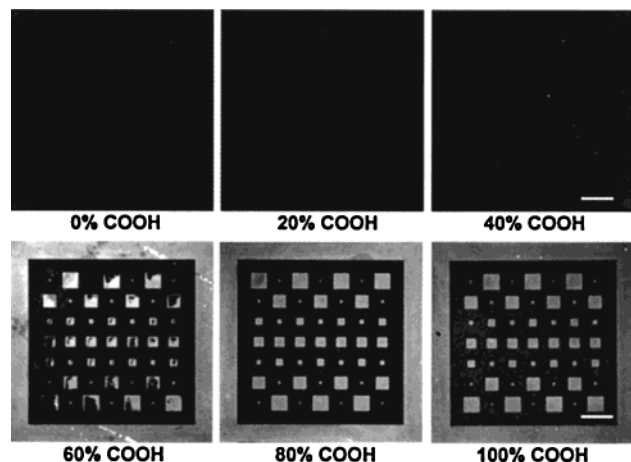


Figure 2. Micrographs of fluorescently labeled protein that was printed onto SAMs of various $-\text{COOH}/-\text{CH}_3$ compositions. Each image is a representative image from experiments repeated a minimum of three times with similar results. The scale bar represents $100 \mu\text{m}$.

COOH SAMs between 50% and 65%. Within this transition regime, increasing the density of COOH functionalities caused a higher fraction of the protein to transfer onto the SAM. Rather than transferring homogeneously, these fractions of protein transferred in a nonhomogeneous piecewise fashion; protein in some regions of the stamp transferred completely as a monolayer, while proteins in other regions did not transfer at all. In accordance with this observation, the fluorescence intensity of the protein transferred in incomplete patches on $<65\%$ COOH SAM was similar to the intensities of the protein printed on $\geq 65\%$ COOH SAM and at the level expected for a protein monolayer.

The transfer of protein failed abruptly within a narrow threshold range of COOH densities on the substrate surface. At or below the lower limit of the threshold range of $\sim 50\%$ COOH SAM, the protein did not transfer, while at or above the upper limit of the threshold range of $\sim 65\%$ COOH SAM, the protein transferred completely. Above 65% COOH SAM, we observed only minor variation in the transfer of proteins. It is likely that the CH_3 functionality interacts with protein primarily through van der Waals interactions, while the COOH functionality interacts additionally through the usually stronger dipole-dipole, hydrogen bond, and ionic interactions,²⁵ which we collectively refer to as polar interactions. These data suggest that during μCP , the strength of protein-substrate interaction increases as the density of polar COOH functionalities presented on the surface increases; the threshold level of interaction needed for μCP of protein begins to be reached at slightly above $\sim 50\%$ COOH SAM.

Effect of Substrate Wettability on μCP . To examine whether our observations were generally applicable or specifically restricted to the COOH functionality, we substituted the polar functionality in the SAM with hydroxyl (OH) or hexa(ethylene glycol) (EG_6OH). In comparison to the COOH functionality, the OH functionality is smaller and provides only one site for polar interaction with proteins. Nonetheless, OH mixed SAMs are similar in thickness, packing density, and adsorption profiles to COOH mixed SAMs.²⁶ In comparison to COOH groups, EG_6OH functional groups are much larger and

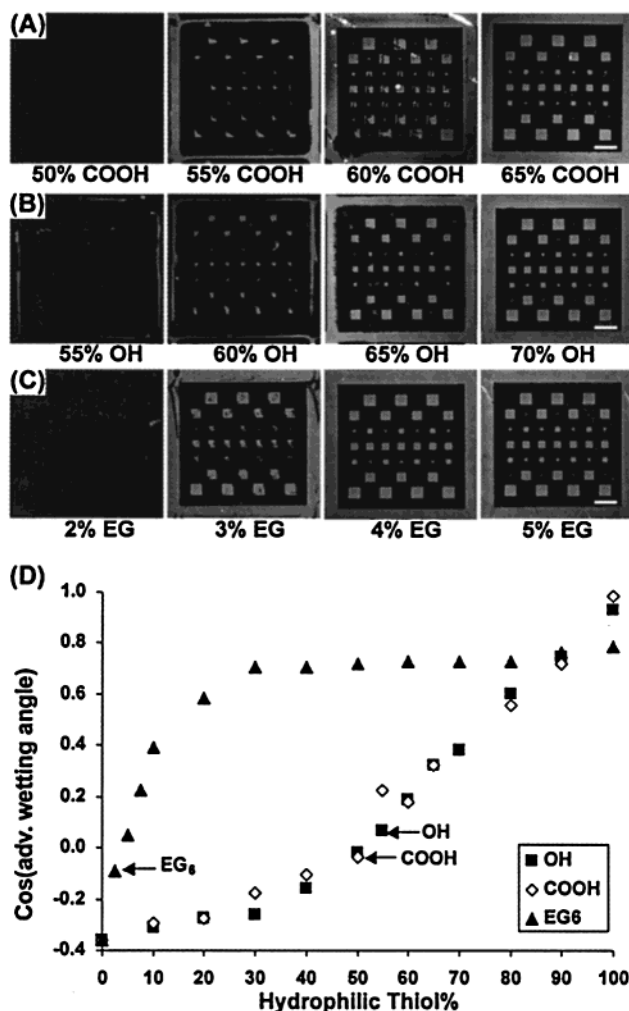


Figure 3. Printing of proteins on three different types of mixed SAMs. (A–C) Micrographs of fluorescently labeled protein printed onto mixed SAMs of alkanethiol presenting $-\text{CH}_3$ and (A) $-\text{COOH}$, (B) $-\text{OH}$, and (C) $-\text{EG}_6\text{OH}$ functionalities. Each image is a representative image from experiments repeated a minimum of three times with similar results. The scale bars represent $100 \mu\text{m}$. (D) Plot of the cosine of the advancing water contact angle against the percentage of polar functionalities for each of the three types of mixed SAMs. Each data point plotted is the average of a minimum of three measurements taken on two separately prepared SAM substrates. Labeled arrows mark the contact angles of the minimum percentage of the indicated SAM on which we observed any protein transfer onto each type of mixed SAMs.

provide up to seven sites for polar interactions with proteins. In addition, EG_6OH -terminated alkanethiol is considerably longer than COOH-terminated, OH-terminated, or CH_3 -terminated (unfunctionalized) alkanethiol. As a result, the EG_6OH mixed SAMs likely have an increased variation in surface topology in comparison to OH or COOH mixed SAMs. The differences in the SAM surfaces allowed us to explore the relationships between the chemical nature of the functionality, the wettability of the SAM, and the amount of protein transferred.

The result of μCP onto OH or EG_6OH mixed SAMs was similar in trend to that previously observed on COOH SAM, where the amount of the printed protein rapidly transitioned from none to all over a relatively narrow range of increasing functionality densities on the surface. The percentage of OH SAM required for printing was similar to that for COOH SAM; we observed no transfer of protein on $\leq 55\%$ OH SAM, partial transfer on 60% and 65% OH SAM, and complete transfer on $\geq 70\%$ OH SAM (Figure

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3B). On EG₆OH SAM, the transition in protein transfer occurred at a much lower density of polar functionalities and was particularly sharp; we observed no transfer on $\leq 2\%$ EG₆OH SAM, partial transfer on 3% EG₆OH SAM, and complete transfer on as low as $\geq 4\%$ EG₆OH SAM (Figure 3C). On all of the SAMs that resulted in partial transfer, the density of protein patches that transferred onto the substrate was similar to that of a complete monolayer.

Our findings suggest a difference in the mechanism of protein immobilization by physisorption versus μ CP. EG₆OH SAM resists adsorption²³ but allows printing; in contrast, CH₃ SAM allows adsorption but resists printing. The cause of this difference is unknown. One possibility is that μ CP physically delivers proteins to the EG₆OH SAM surface in a manner that disrupts the molecular conformation of EG groups thought to be responsible for protein resistance in oligo(ethylene glycol) SAMs.²⁷

Collectively, our findings suggest that neither the density of polar interaction sites nor the density of hydrophilic functionalities on the substrate surface can alone reliably predict the transition point in the amount of protein transferred during μ CP. However, both of these parameters and other factors combine to determine the surface wettability, a parameter that can be estimated through the measurement of contact angles. We measured dynamic and sessile water contact angles on the mixed SAM substrates under ambient conditions. The hysteresis between advancing and receding angles was between 5° and 15° with a median hysteresis of $\sim 10^\circ$; both the contact angles and hysteresis measurements were similar to previously reported values.^{21,28} The cosine of the advancing angle was plotted against substrate composition (Figure 3D). The Young–Dupr  equation (eq 1)^{29,30} relates the cosine of the water contact angle with the reversible work of adhesion of the water to the SAM, which we refer to as wettability of the SAM, by

$$W_a = \gamma_{vi}(1 + \cos \theta) \quad (1)$$

where W_a is the work of adhesion and γ_{vi} is the interfacial surface energy of the air–water interface. As expected, for each set of the mixed SAMs, the wettability increased as the density of polar functionalities increased (Figure 3D). More importantly, we observed that the contact angles on SAMs where protein transfer initially occurred were comparably similar. For the three types of mixed SAMs, the lowest percentage of polar functionality at which any transfer of protein occurred was approximately 50% for COOH, 55% for OH, and 2.5% for EG₆OH SAMs. The corresponding cosines of the advancing contact angle were $-0.035 = \cos 92^\circ$, $+0.070 = \cos 86^\circ$, and $-0.087 = \cos 95^\circ$, respectively (indicated by arrows in Figure 3D).

These results consistently demonstrate the general trend that more hydrophilic substrates allow better protein transfer; furthermore, they suggest that the wettability of the substrate can assess the outcome of μ CP onto a given substrate. Consequently, it is reasonable to infer that the same parameters that influence wettability increase the strength of protein–substrate interactions required for μ CP. Measuring contact angle appears to be a simple method for assessing the strength of protein interactions with a substrate during μ CP.

Table 1. Advancing Contact Angles of Deionized Water on the Silanized Surface of the Stamp and on Transition Substrates^a

surface functionality of PDMS stamp	θ_{stamp} (deg)	%COOH ₁	θ_1 (deg)	%COOH ₂	θ_2 (deg)
untreated	112	55	92	65	71
–CF ₃	120	0	111	20	106
–NH ₂	28	80	56	90	44

^a θ_{stamp} refers to advancing contact angles of water on the indicated PDMS surface. %COOH₁ indicates the minimum percentage of COOH SAM where any transfer of protein was observed from the corresponding stamp, and θ_1 refers to the advancing angle on the SAM. %COOH₂ indicates the minimum percentage of COOH SAM where the complete transfer of protein was observed, and θ_2 refers to the advancing angle on the SAM. Measurements were taken using deionized water in ambient conditions. For each contact angle listed, $n \geq 2$ and the standard deviation is $< 3^\circ$.

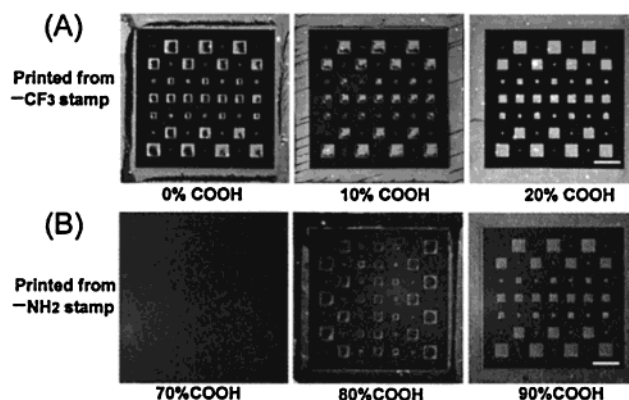


Figure 4. The role of the surface energy of the stamp on printing. Micrographs of fluorescently labeled protein printed onto the indicated percentage of COOH mixed SAM from (A) the –CF₃ functionalized stamp and (B) the –NH₂ functionalized stamp. We observed that less than a complete monolayer of protein adsorbed onto the –NH₂ functionalized stamp, which likely resulted from the reduced capacity of hydrophilic surfaces to adsorb protein (ref 27). To accommodate for the decreased fluorescence even in conditions that allow complete transfer of protein onto the substrate, we lengthened the exposure time for micrographs in (B) in comparison to all previously shown images. The scale bars represent 100 μm .

Effect of Stamp Wettability on μ CP. Having demonstrated that the wettability of the substrate can predict the outcome of μ CP, we next examined if the wettability of the stamp had any influence on the μ CP of proteins. We increased or decreased the wettability of the stamp by attaching –NH₂ or –CF₃ functionalities, respectively, to the surface of the stamp using silane chemistries. The expected changes in wettability were reflected by an increase in advancing water contact angle on the –CF₃ functionalized surface and a decrease in contact angle on the –NH₂ functionalized surface (Table 1). After surface modifications, the hysteresis of the contact angles increased, most likely as a result of increased surface roughness and heterogeneity. During μ CP, proteins adsorbed on –CF₃ functionalized stamps transferred partially on 0% COOH SAM and completely on 20% COOH SAM (Figure 4A). In contrast, protein adsorbed on –NH₂ functionalized stamps transferred partially on 80% COOH SAM and completely on $\geq 90\%$ COOH SAM (Figure 4B). Thus, the minimum wettability of the SAM needed to allow protein transfer was significantly increased or decreased when the wettability of the stamp was increased or decreased, respectively (Table 1). We cannot eliminate the possibility that these observed changes were due in part to changes in other surface properties of the stamp,

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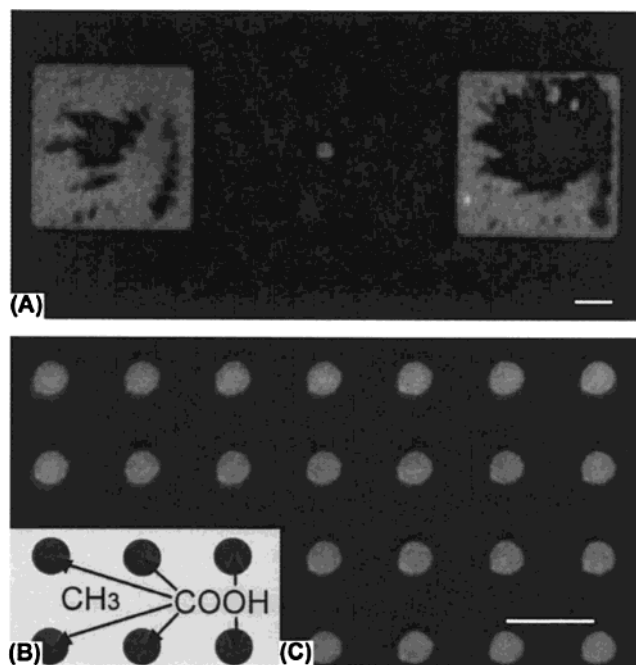


Figure 5. (A) Digitally magnified image of protein printed onto 60% COOH mixed SAMs, (B) schematic of the localization of COOH⁻ and CH₃- terminated alkanethiols on a patterned SAM, and (C) representative image of proteins printed onto a patterned SAM from a flat unpatterned stamp. We observed similar results when a patterned stamp was used to μ CP protein onto a patterned SAM. This image was captured on a higher magnification objective with a shorter exposure time than other images. The scale bars represent 10 μ m.

such as roughness, heterogeneity, hardness, or degree of surface cross-linking, that may be introduced by the surface modification procedure. Nonetheless, these findings are consistent with a model of competing attractive forces between the stamp surface and substrate surface for the protein, where the polar functionalities on either surface act to increase the attractive force. The ultimate determinant of whether proteins will transfer may therefore be the difference in wettability between the stamp and substrate. Thus, lowering the hydrophilicity of the stamp may be a practical approach to μ CP protein onto some substrates that resist μ CP from typical, untreated stamps.

Patterning of Proteins by Patterning Wettability.

Our findings suggest that patterns of proteins may be produced by relying on differential surface wettabilities of the substrate. μ CP of proteins onto a surface containing regions of both high and low wettability may result in the selective transfer of protein only in the hydrophilic regions. This approach to pattern proteins, however, may be hindered by lateral interactions between protein molecules: Printing of proteins on transition substrates resulted in the transfer of incomplete patches of protein that are fractured and jagged in appearance (Figure 5A). Both the transferred patches and the untransferred patches remaining on the stamp have the fluorescence intensity of a complete protein monolayer. The piecemeal fracture of the monolayer may be caused by strong lateral protein-protein interactions; a region of protein that has stochastically transferred onto a substrate may pull adjacent protein molecules onto the substrate through lateral interactions, until the eventual catastrophic failure of the process results in the characteristic jagged appearance of the printed protein.

To test the ability to print defined regions of protein from a flat stamp, we used μ CP to form patterned SAM

surfaces¹⁸ consisting of COOH SAM localized in 5 μ m diameter islands and separated from neighboring islands by 10 μ m by CH₃-terminated SAM (schematically depicted in Figure 5B). Upon μ CP on the patterned SAM substrates using a flat, unpatterned stamp, a pattern mimicking the COOH SAM regions of the substrate emerged (Figure 5C). The transfer of protein ended sharply and smoothly at the boundary between high and low wettability regions, rather than in a jagged manner seen on transition substrates (Figure 5A). Our data suggest that for the purposes of printing proteins restrictively onto hydrophilic regions, lateral protein-protein interactions do not cause the protein monolayer to behave as a contiguously linked sheet of protein across micrometer dimensions.

Previous work had reported successful μ CP of proteins onto hydrophobic surfaces such as polystyrene.¹¹ In our experiments, μ CP onto polystyrene was only possible after either increasing the wettability of polystyrene by oxidation or decreasing the wettability of the stamp. This finding is consistent with our results on SAMs. We examined several additional proteins (albumin, streptavidin, fibronectin, and collagen IV) and found that in all cases, increased substrate wettability improved printing. In general, we have found that surface modifications that increase the wettability of the substrate or that decrease the wettability of the stamp expand the types of surfaces that can act as substrates for μ CP of proteins.

IV. Conclusion

Direct μ CP of proteins is an effective and relatively simple approach to spatially control the immobilization of proteins on surfaces. In this study, we demonstrated that a minimum wettability of the substrate is required for successful μ CP of proteins, and this minimum wettability can be decreased if the wettability of the stamp is decreased. Applying these findings, we generated patterns of proteins at micrometer resolution by μ CP onto substrates containing regions of differential wettability. From a practical perspective, the selective transfer of proteins onto hydrophilic regions may be used as a simple approach to detect and visualize microscopic variations in surface energies on surfaces.

Our findings reveal that the mechanism of μ CP of protein is different from protein adsorption: (1) Surfaces resistant to protein adsorption in aqueous conditions are susceptible to μ CP in ambient conditions. (2) The relationship between the amount of protein immobilized and the wettability of the substrate varies gradually for the adsorption process³¹ but transitions rapidly at a threshold wettability for the μ CP process. The mechanistic differences between adsorption and μ CP may be exploited for specific applications. For example, μ CP can be used as an effective method to immobilize proteins onto surfaces that do not readily adsorb protein from solution, such as glass or ethylene glycol.

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