Fabrication and Imaging of Protein Crossover Structures

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ABSTRACT

Proteins often deform, dehydrate or otherwise denature when adsorbed or patterned directly onto an inorganic substrate, thus losing specificity and biofunctionality. One method used to maintain function is to pattern the protein of interest directly onto another underlying protein or polypeptide that acts as a buffer layer between the substrate and the desired protein. We have used microcontact printing (μcp) to cross-stamp orthogonal linear arrays of two different proteins (e.g., IgG, poly-lysine, protein A) onto glass substrates. This created three separate types of protein-substrate microenvironments, including crossover structures of protein one on protein two. We report preliminary fluorescent microscopy and scanning force microscopy characterization of these structures, including commonly encountered structural defects.

INTRODUCTION

Mono-molecular layers of proteins patterned onto surfaces at the micron and nanoscale can potentially serve in a number of useful capacities such as active components of biosensors [1], regulators of directed cell growth [2], and diagnostic microarrays or protein chips [3]. For example, protein chips are being developed in an effort to miniaturize biological assays by placing patterned arrays of multiple types of proteins onto surfaces [3]. Such protein chips will allow simultaneous detection and analysis of multiple species, require smaller quantities of expensive reagents, and have more rapid biochemical reactions due to short mass transport distances [3]. Biomedical industry will also benefit from improved design and patterning of protein chips for high-throughput detection and profiling of various interactions such as drug-protein, protein-protein, and disease antigen-antibody [3]. Patterning a chip containing hundreds if not thousands of different proteins—each with its own distinctive protein-substrate interaction forces—and having all proteins retain their unique three-dimensional shape and biological function is a formidable challenge. Although substantial investments are being poured into the commercial development of protein chips, reliability and reproducibility remain elusive owing primarily to an incomplete understanding and control of fundamental protein-substrate interactions during patterning.

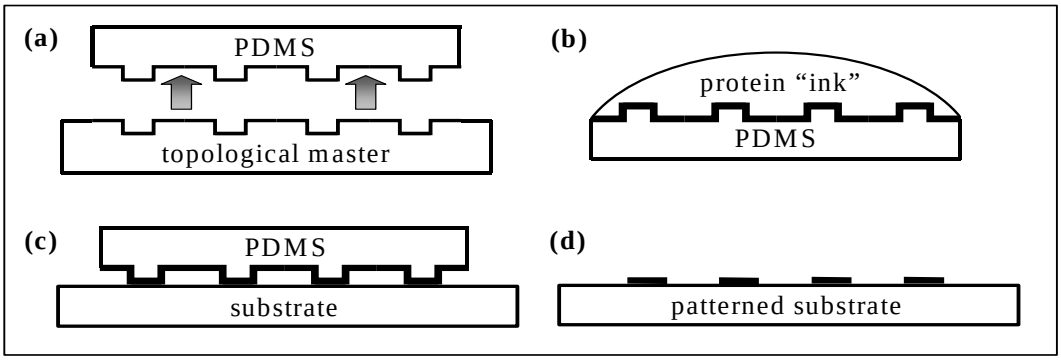

Figure 1. Microcontact printing procedure for patterning proteins on surfaces. (a) Patterned polydimethylsiloxane (PDMS) elastomeric stamp is molded from a topological master, which can be prepared by various microfabrication techniques. (b) Stamp is "inked" with a protein solution, (c) brought into contact with substrate, and (d) removed to leave protein pattern on substrate.

One method for patterning small molecules [4-7] and proteins [2,7-9] onto materials surfaces is microcontact printing (μcp)—a versatile, simple, and inexpensive lithographic process that uses a micro- or nano-patterned elastomeric stamp to transfer proteins to a surface (Figure 1). The quality of protein pattern transfer and maintenance of function will strongly depend on how the stamp and substrate surfaces are each chemically, physically, or biochemically modified (Figure 1). For effective pattern transfer, the protein of interest must not only have a higher affinity for the substrate than the stamp, but it must not bind too strongly to the substrate such that it undergoes conformational change resulting in loss-of-function [10]. Also, for maximum activity, the protein molecules must attach to the substrate with their active (or binding) sites accessible to the desired target biomolecules. Several groups have developed their own working μcp protocols for protein patterning, however, there is little mechanistic understanding of the entire process particularly at the molecular-level [9].

In this paper, we report preliminary data regarding a method to help maintain protein function on materials surfaces using a two-step μcp procedure. This method created crossover array patterns of two different proteins (or polypeptides) in which one protein acted as a mono-molecular buffer layer between the substrate and functional protein of interest [11]. Protein functionality and structural features of these protein crossover arrays were characterized using fluorescent antibody labeling, scanning force microscopy (SFM), and fluorescence microscopy.

EXPERIMENTAL METHODS

Details of the microcontact printing of proteins and polypeptides have been described elsewhere [e.g., Ref. 2]. Briefly, the μcp procedure consisted of taking a cleaned elastomeric stamp with an array of micron scale lines (25 μm or 10 μm widths), coating the stamp with protein solution, removing excess solution with nitrogen gas, placing the stamp in contact with an air plasma-cleaned glass substrate or coverslip, and then removing the stamp (Figure 1). Protein crossover arrays were created by repeating this process with a second stamp inked with a

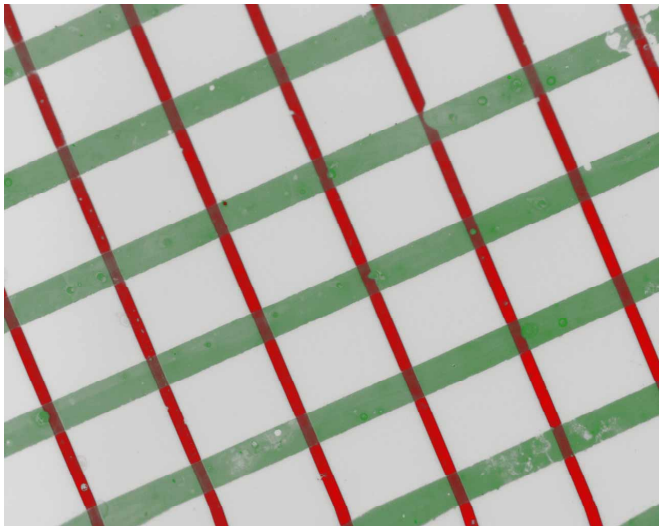

Figure 2. Composite fluorescent microscopy image of protein crossover structures on glass prepared by μcp. IgG lines (red) on poly-lysine lines (green) are 10 and 25 microns wide, respectively (Ref. [11]).

different protein and rotated approximately 90 degrees to the first pattern (Figure 2). The substrate was rinsed several times after each μcp step with a phosphate buffer saline (PBS) solution to remove weakly bound biomolecules and stored in PBS. FITC conjugated (green) poly-lysine hydrobromide, $(C_6H_{12}N_2OHBr)_n$; a polypeptide with an average M_w = 50,200 g/mol and Protein A were obtained from Sigma Chemical Co. Alexa 568 conjugated (red) IgG proteins were obtained from Molecular Probes. Fractional coverage of active unconjugated IgG on the substrate or on the poly-lysine crossovers was determined by fluorescent labeling with the appropriate Alexa 594, Alexa 488, or Alexa 568 conjugated antigen (Molecular Probes, Inc.).

Non-contact scanning force microscopy (NC-SFM) images of patterned proteins were obtained using an AutoProbe CP microscope (TM Microscopes, Veeco Metrology Group). NC-SFM images surfaces with very low tip-sample forces (ca. 10^{-12} N), which are due primarily to long-range van der Waal's interactions between tip and sample. Even upon repeated scanning, no apparent surface degradation effects were observed as commonly seen when imaging soft surfaces using contact mode SFM. Optical micrographs were obtained from a wide-field fluorescent microscope (Olympus BX41, Olympus America Inc., Melville, NY) using an Olympus Magnafire CCD camera and Magnafire software. Patterned samples were rinsed in distilled water prior to imaging with NC-SFM, however, no effort was made to completely dry the sample.

RESULTS AND DISCUSSION

μcp was routinely used to cross-stamp orthogonal linear arrays of two different proteins onto glass substrates (Figures 2-5). Figure 2 is a composite fluorescent microscopy image of a protein cross-over array of Alexa 568 conjugated IgG (red) on FITC conjugated poly-lysine (green), which created three distinct types of protein-substrate microenvironments: IgG on glass; poly-lysine on glass, and crossover structures of IgG on poly-lysine on glass [11].

Protein function was assessed through various biochemical assays including fluorescent antibody labeling in which bath applications of both the target antigen and control antigens, tested for specific and non-specific binding, respectively. Quantifying the fluorescent signal versus protein coverage allowed one to determine the fraction of functional proteins successfully transferred to either the substrate or on top of the protein buffer layer by μcp. Initial results indicated both specific and non-specific binding to IgG and are not discussed further here [11].

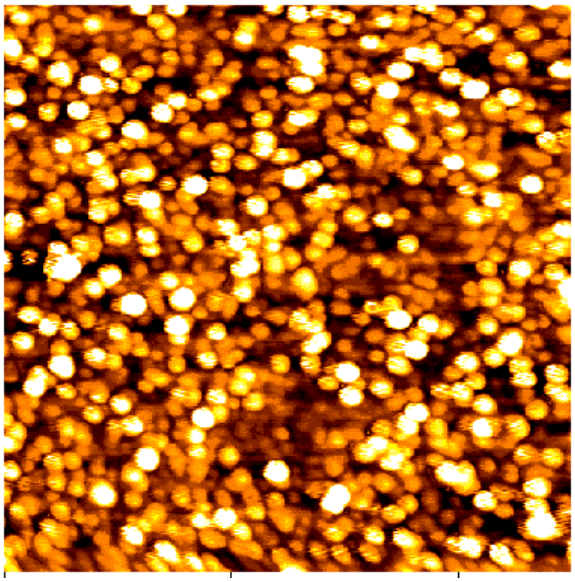

Figure 3. 1 μm by 1 μm SFM image of poly-lysine biomolecules on glass RMS roughness, 1.4 nm, and molecule size, 60 nm [Ref. 11]

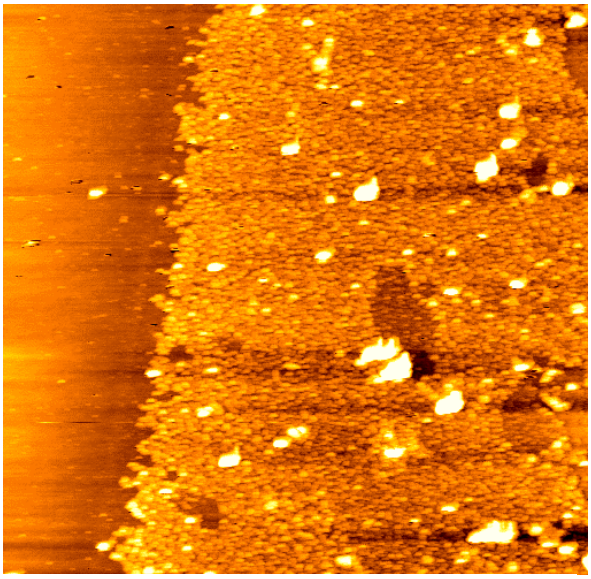

Figure 4. 10 μm by 10 μm SFM image of patterned poly-lysine line edge as prepared by μcp. The edge fine structure was stable over repeated scans [Ref. 11].

Figure 3 is a 1 μm by 1 μm SFM image of poly-lysine on glass revealing individual molecules with diameters of 60 ± 10 nm and an overall RMS roughness of 1.4 ± 0.5 nm. This image was stable over repeated scans. Fluorescent microscopy and longer range SFM inspection of the structures in Figure 2 also revealed some frequently encountered patterning defects associated with μcp. Figure 4 is a 10 μm by 10 μm SFM image of a μcp poly-lysine line edge, whose fine molecular-level structure was stable over repeated scans. The large and still unidentified bright features on the poly-lysine in Figure 4 are attributed to either dust, elastomer debris from stamp, or salt crystals from the PBS. This debris was not uniformly distributed across the surface, nor did it occur on every stamped surface.

Figure 5 is an SFM image of a single IgG/poly-lysine crossover junction which revealed common stamping defects, including incomplete transfer of IgG to both the substrate and poly-lysine line. Also, poly-lysine from the first set of stamped lines was often found to be removed by the second stamp during μcp of the IgG crossovers (Figure 5). This reverse transfer of poly-lysine was confirmed by fluorescent microscopy inspection of the IgG stamps *after* printing which revealed the telltale green fluorescent signal of FITC-conjugated poly-lysine (not shown).

Protein adsorption on surfaces from solutions is a complex process and can be mediated by a number of molecular-level interactions including, (1), electrostatic interactions between the protein and substrate which can be mediated by coadsorbed ions, (2), hydrogen bonding, (3), van der Waal's interactions, (4), changes in waters of hydration of the protein and the substrate, (5), conformational entropy changes due to structural rearrangement of the protein upon adsorption and, (6), lateral interactions between adsorbed protein molecules [12].

Microcontact printing adds another aspect of complexity to the above protein-substrate interactions, however, μcp may also assist in binding weakly adhering proteins by physically bringing the protein into intimate contact with the substrate. Efforts to understand the molecular-level structure and micron-scale defects and their relationship to the maintenance of

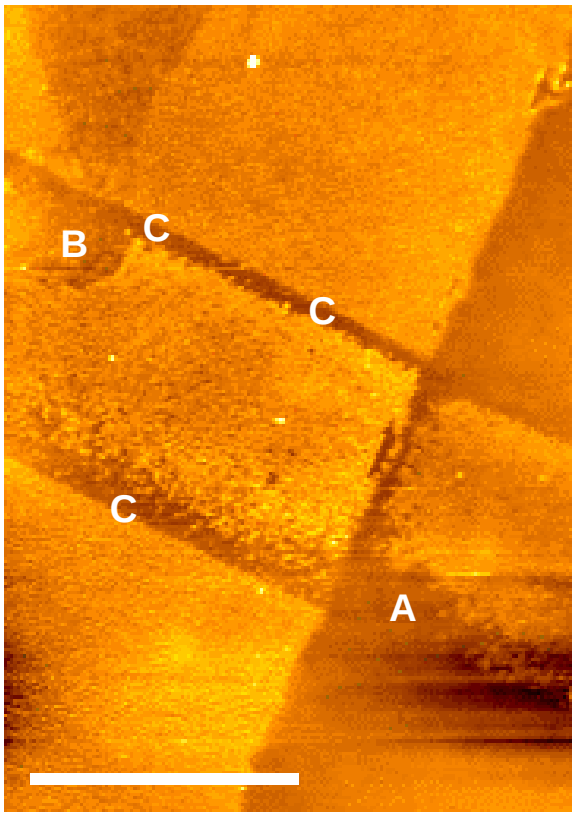

Figure 5. SFM image of protein crossover junction (10 μm wide IgG line on 25 μm poly-lysine line) from Figure 2 as prepared by μcp. Crossover array defects include regions of incomplete transfer of the IgG to the substrate (A), incomplete transfer on top of stamped poly-lysine line (B), and reverse transfer of the poly-lysine to the second stamp (C). Fiducial marker is 10 μm [Ref. 11].

function of microcontact printed proteins on surfaces will assist in developing a basic understanding of the μcp process, specifically, and protein adsorption, in general. Much work remains in correlating these types of defects with initial surface conditions of stamp and substrate, protein type, and other details of the μcp process, and then determining the impact of these defects on protein function, cell growth, biosensing capabilities, long-term stability of stamped protein features, etc.

CONCLUSIONS

In summary, micron-scale crossover array structures consisting of two different mono-molecular layers of protein were successfully created on glass substrates using a two-step microcontact printing procedure. Several types of stamping defects particular to the fabrication of crossover arrays were identified including, (1), incomplete transfer of the second protein to the substrate adjacent to the crossovers, (2), incomplete transfer on top of the first protein and, (3), reverse transfer of the first protein (poly-lysine) to the edges and centers of raised features on the second stamp. These crossover array structures yielded three different protein microenvironments for further structural and functional studies.

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References

[1] Nanofabrication and Biosystems: Integrating Materials Science, Engineering, and Biology, Eds. H.C. Hoch, L.W. Jelinski, and H.G. Craighead, Cambridge University Press, New York (1996); Commercial Biosensors: Applications to Clinical, Bioprocess, and Environmental Samples, Ed. G. Ramsay, Wiley-Interscience, New York (1998); Biological Performance of Materials: Fundamentals of Biocompatibility, J. Black, Marcel Dekker, New York (1999).

[2] C.D. James, R.C. Davis, M. Meyer, A. Turner, S. Turner, G. Withers, L. Kam, G. Banker, H.G. Craighead, M. Isaacson, J.N. Turner, and W. Shain, **IEEE Trans. Biomed. Eng.** *47*, 17 (2000).

[3] For example, B.T. Houseman, J.H. Huh, S.J. Kron, and M. Mrksich, **Nature Biotech**. *20*, 270 (2002); P. Mitchell, **Nature Biotech**. *20*, 225 (2002).

[4] A. Kumar and G.M. Whitesides, **Appl. Phys. Lett**. *63*, 2002 (1993).

[5] Y. Xia and G.M. Whitesides, **Angew. Chem. Int. Ed**. *37*, 550 (1998).

[6] D.J. Graham, D.D. Price, and B.D. Ratner, **Langmuir** *18*, 1518 (2002).

[7] B. Michel, A. Bernard, A. Bietsch, E. Delamarche, M. Geissler, D. Junker, H. Kind, J.P. Renault, H. Rothuizen, H. Schmid, P. Schmidt-Winkel, R. Stutz, and H. Wolf, **IBM J. Res. & Dev**. *45*, 697 (2001).

[8] C.D. James, R.C. Davis, L. Kam, H.G. Craighead, M. Isaacson, J.N. Turner, and W. Shain, **Langmuir** *14*, 741 (1998); A. Bernard, E. Delamarche, H. Schmid, B. Michel, H.R. Bosshard, and H. Biebuyck, **Langmuir** *14*, 2225 (1998); M. Geissler, A. Bernard, A. Bietsch, H. Schmid, B. Michel, and E. Delamarche, **J. Am. Chem. Soc.** *122*, 6303 (2000); A. Bernard, J.P. Renault, B. Michel, H.R. Bosshard, and E. Delamarche, **Adv. Mater.** *12*, 1067 (2000); N. Patel, R. Bhandari, K.M. Shakesheff, S.M. Cannizzaro, M.C. Davies, R. Langer, C.J. Roberts, S.J. Tendler, and P. Williams, **J. Biomater. Sci. Polym. Ed.** *11*, 319 (2000); J. Hyun, Y. Zhu, A. Liebmann-Vinson, T.P. Beebe, and A. Chilkoti, **Langmuir** *17*, 6358 (2001).

[9] J.L. Tan, J. Tien, and C.S. Chen, **Langmuir** *18*, 519 (2002).

[10] K. Wilson, S.J. Stuart, A.J. Garcia, and R.A. Latour, **Soc. Biomat. 28th Annual Meeting Transactions**, 25, 455 (2002).

[11] J.R. LaGraff, D.J. Graber, Y.-P. Zhao, G.-C. Wang, T.-M. Lu, and J.N. Turner (unpublished work).

[12] Biopolymers at Interfaces, Surfactant Science Series, Ed. M. Malmsten, Marcel Dekker, Inc., New York (1998).