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Nanolithography

Facile Preparation of Complex Protein Architectures with Sub-100-nm Resolution on Surfaces**

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Proteins on surfaces play a ubiquitous and central role in host responses to implanted biomedical devices and in biotechnological applications,[1] including in vitro surface-based diagnostic assays[2] and cell-culture supports.[3] In many cases, complex biological functionality results from the interplay of multiple types of proteins: for example, in immune responses involving antigen-presenting cells,[4] in bone regeneration,[5] and in cell adhesion.[6] The activity of these systems is particularly dependent on a spatial organization that occurs primarily on the nanoscale. This has spurred the development of novel bioinspired materials and of nanofabrication routes.[7–9] The ability to control the patterning of proteins is, therefore, not only important for gaining insight into biological phenomena,[10] but is also a prerequisite for high-performance biosensors[11,12] and novel fabrication paradigms.[13]

Many approaches have been pursued for patterning proteins on surfaces with high resolution, including dip–pen lithography,[14] microcontact printing,[15–19] self-assembly,[20] ablation of patterns into monolayers of proteins or organic molecules using various techniques,[21] and nanografting based on scanning-probe methods.[22] Despite these efforts, no single technique has been widely applied to investigate the role of proteins on surfaces in biological phenomena because of practical limitations. These limitations include the time required for the high-throughput production of samples with nanoscale features over large areas, the need for specific surface chemistry to adsorb proteins from solution onto selected areas of a surface, and the challenge of molding high-resolution features in elastomers that are mechanically stable.

Herein, we present a method that combines the advantages of virtually any high-resolution lithographic method and microcontact printing by transferring a pattern of proteins from a nanotemplate to a substrate using a planar elastomer as the transfer vehicle. This method can be used to generate patterns with sub-100-nm resolution and arbitrary geometries consisting of one or multiple types of proteins. Moreover, co-aligning proteins into complementary patterns is simply accomplished. The intrinsic design of this method allows the production of a wide variety of complex protein architectures using easily accessible techniques and equipment.

The main steps of this method are to ink (I) a planar, hydrophobic elastomer using the spontaneous adsorption of proteins from solution onto hydrophobic surfaces (Figure 1a), to subtract (S) proteins from the elastomer using a nanotemplate during a brief contact step (Figure 1b), and to print (P) the remaining protein pattern from the elastomer onto a final substrate (Figure 1c). These three steps are combined to form the "ISP" strategy. The robustness of the ISP strategy is demonstrated by protein patterns with micrometer and submicrometer features (Figure 1d). The only requirement for this method is to use a nanotemplate and final substrate having a higher work of adhesion for water than the elastomer,[23] thereby yielding complete protein transfer. This requirement was easily accomplished in the following experiments using silicon nanotemplates and silicon or glass substrates by treating these surfaces with oxygen plasma to clean them and to increase their surface hydrophilicity. Many other surfaces that are less hydrophobic than the elastomer can be used for this purpose.[17,23] Poly(dimethylsiloxane) (PDMS) was used as the elastomer material because of its higher hydrophobicity over glass and silicon and its conformability to surface topographies.[24]

We assessed the resolution and contrast of the ISP method by using atomic force microscopy (AFM) to analyze patterns of isolated micrometer squares and nanoscale lines (Figure 2). Efficient printing from elastomer to substrate resulted in high-contrast patterns of homogeneous layers of proteins (Figure 2a). The profile of the patterns shows small variation from a height consistent with that of a monolayer of the protein (Figure 2b).[22] Patterns consisting of right-angle meshes exhibit no visible distortion along the lines or in the corners (Figure 2c), suggesting that, once inked on the elastomer, proteins retain their positions during subtraction and printing, without diffusing laterally.[25] High-resolution patterns (of lines with widths of less than 100 nm) were achieved after only a few optimization cycles of the nanotemplate preparation (Figure 2d). These patterns are repre-

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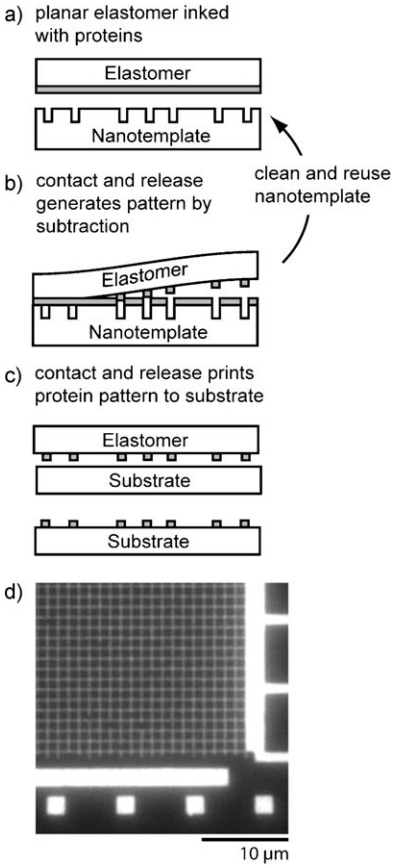

Figure 1. The ISP method for producing protein patterns. a) A planar elastomer is homogeneously inked with a monolayer of protein. b) Contacting the inked elastomer with a nanotemplate results in the selective subtraction of proteins from the elastomer. c) Proteins remaining on the elastomer subsequently transfer onto a target substrate during a printing step. d) Fluorescence micrograph of a pattern of tetramethylrhodamine isothiocyanate (TRITC) labeled antibodies on glass. The pattern, which includes micrometer (squares) and submicrometer (mesh) features, was produced using the ISP strategy.

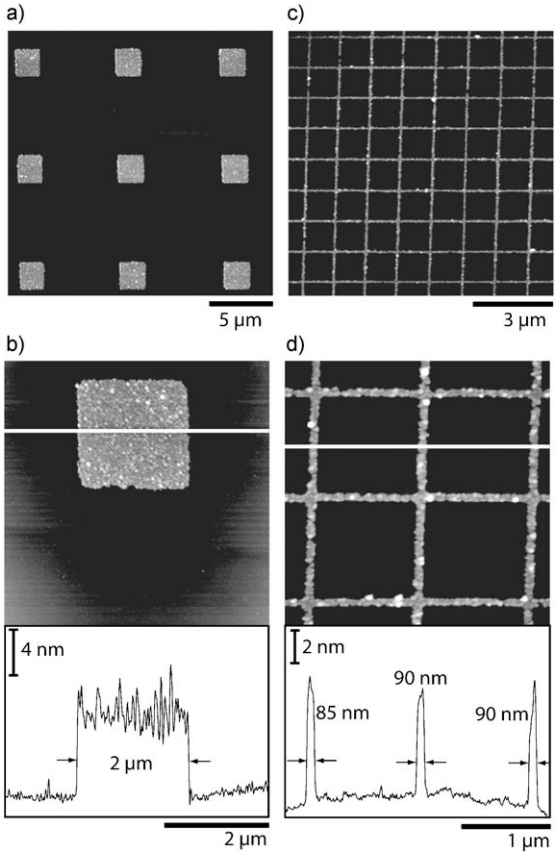

Figure 2. AFM images (noncontact mode) of high-resolution, high-contrast protein patterns of a,b) micrometer squares or c,d) sub-100-nm lines. A planar PDMS (Sylgard 184) elastomer was inked with antibodies. Proteins were selectively (and completely) subtracted from the elastomer using a nanotemplate produced by electron-beam lithography. The remaining proteins were printed onto a silicon substrate.

sentative of those obtained over large areas (0.25 mm^2). The mechanical stability of the nanotemplate suggests that uniform patterning of proteins might be completed over much larger areas. Such patterns can be used to array large numbers of individual biological elements on a surface, with the advantages of enabling the simultaneous study of individual elements and the collection of data on statistically meaningful populations.[26]

The controlled adhesion of single cells onto surfaces patterned with user-defined protein architectures provides a unique experimental system for cell-biology studies.[27–30] We therefore generated protein patterns having features of a range of sizes and spacings (Figure 3). Arrays of linelets having an average width of 260 nm (Figure 3c) and an inter-row spacing of 1–64 μm (Figure 3a,b) were obtained. Such protein patterns with nanoscale features separated by many micrometers would be very difficult to produce using microcontact printing stamps made from commercially available materials (or even from advanced polymer compositions) because of collapse or buckling of the features on the stamp.[31] Squares with an average minimum size of 280 nm (Figure 3f) were printed in clusters of one, two, or four (Figure 3d,e). These arbitrary patterns were simultaneously completed in less than 1 h, a time period which included inking of the elastomer (30 min), subtraction with the nanotemplate (1 min), and patterning to the final substrate (1 min).

Combining the I, S, and P steps provides a variety of avenues for creating complex architectures consisting of multiple proteins (Figure 4). In one strategy, two different types of proteins are individually patterned onto separate elastomers by subtraction, prior to being printed onto one substrate (2 × ISP; Figure 4a). By varying the in-plane orientation of the elastomers during printing to the final substrate, patterns having regions of overlapping antibodies were produced (Figure 4b). Non-overlapping patterns of proteins can also be produced with various spatial organizations (Figure 4c). This robust method might be used to produce patterns of two proteins whose functionality results from their interaction to investigate the roles of spatial orientation and density on the activity of the two proteins.

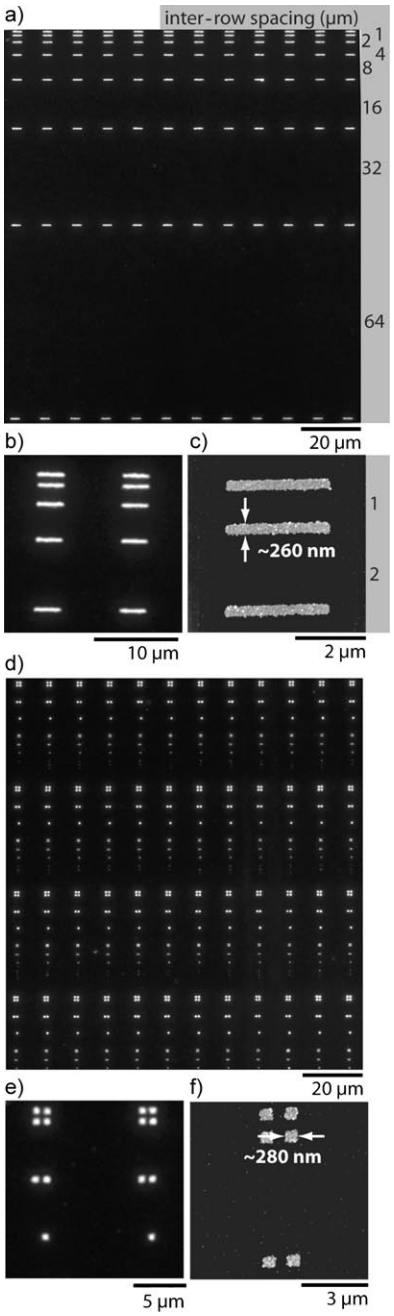

Figure 3. High-resolution patterns of TRITC-labeled antibodies on glass (fluorescence) or silicon (AFM). a,b) Fluorescence micrographs and c) AFM image of arrays of linelets (with an average width of 260 nm). d,e) Fluorescence micrographs and f) AFM image of arrays of squares (with an average minimum size of 280 nm) in clusters of one, two, or four. The low fill factor of the patterns in (a–c) and the geometric variability in (d–f) were eadily achieved using the ISP strategy.

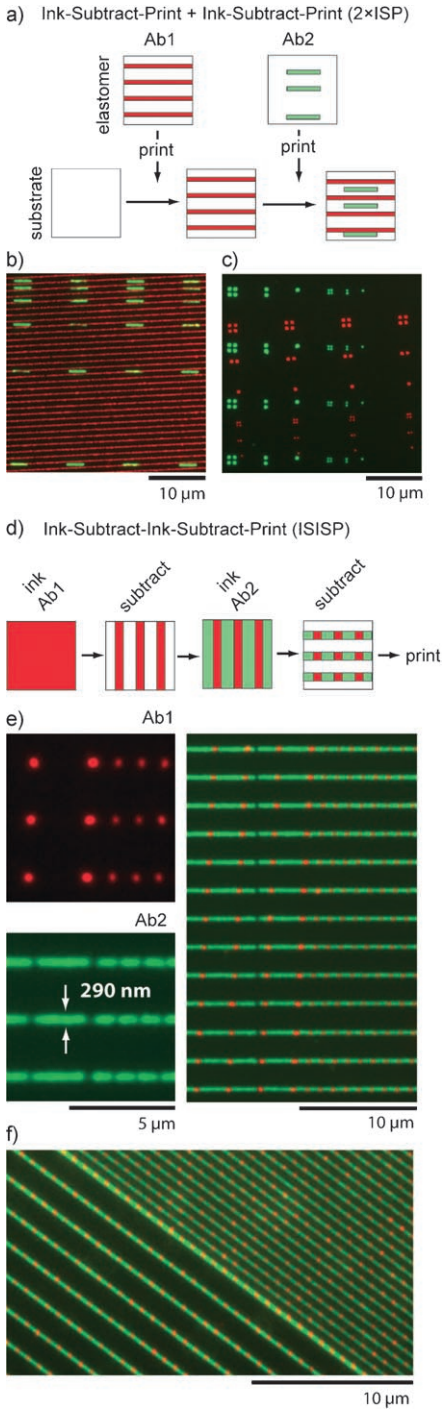

Figure 4. Combinatorial printing methods for producing complex protein architectures. a) Successive ISP patterning of two different types of antibodies onto a glass substrate (2×ISP). Fluorescence micrographs of patterns of b) overlapping or c) non-overlapping antibodies. d) Repeated inking and subtraction of two different types of antibodies followed by one printing step (ISISP). e) Fluorescence micrographs of a pattern of two antibodies (right) and the complementary patterns of each component antibody (left). f) Fluorescence micrograph of a pattern produced using a different angle between the nanotemplate features during the two subtraction steps. Intrinsically aligned patterns of proteins are produced irrespective of lateral shifts and angle variations. The positions of each type of antibody were recorded separately and then digitally recombined using, as encoding colors, red for TRITC-labeled IgG (Ab1) and green for Alexa Fluor 647 labeled IgG (Ab2).

In a second strategy, one elastomer is inked with the first antibody, subtracted using a nanotemplate, inked with a second antibody, subtracted again, and then contacted with the final substrate to print the proteins (ISISP; Figure 4d). In the final pattern, the two types of antibodies are intrinsically aligned because of the simultaneous patterning by subtraction using one nanotemplate during the second subtraction step

(Figure 4e). As proteins will not adsorb from solution onto previously patterned proteins, the final pattern consists of two complementary patterns of proteins that form boundaries at which the two proteins are adjacent. The flexibility of this technique enables a multitude of variations in the size, spacing, and orientation of features in the protein pattern through simple changes in the procedure. By rotating the second nanotemplate with respect to the pattern obtained by the first subtraction step, the spacing of the two proteins is varied between a minimum and maximum spacing that are defined by the layout of the nanotemplates (Figure 4f).

This method for patterning proteins on surfaces enables the production of arrays with high resolution, high contrast, and self-alignment and consisting of multiple types of proteins. In contrast to many techniques used for patterning surfaces at high resolution, a nanotemplate is the only key component needed to implement this method. The template does not have to be fabricated by means of electron-beam lithography, but can also be prepared at various scales and from many materials using in-house or commercially available sources. The template can be reused or made disposable by structuring polymers with molding, hot embossing, or nanoimprint techniques. The method presented herein enables researchers to easily pattern proteins on surfaces at very high resolution, after spending a minimum effort on generating a patterned template. As most proteins adsorb from solution onto hydrophobic surfaces and transfer by printing from a less wettable to a more wettable substrate, this method should be widely applicable to the patterning of a variety of proteins and substrates. The contiguous placement of multiple types of proteins on the nanometer scale creates complex architectures for which advanced functionalities can be expected. These functionalities include the selective anchoring of protein complexes, vesicles, or even cells with high specificity and orientational control.

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