Protein Nanostructures

Direct-Write Dip-Pen Nanolithography of Proteins on Modified Silicon Oxide Surfaces**

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The development of direct-write patterning methods for protein-based nanostructures is important for researchers working in the areas of proteomics, diagnostics, and materials science. Such methods could allow one to fabricate patterns of

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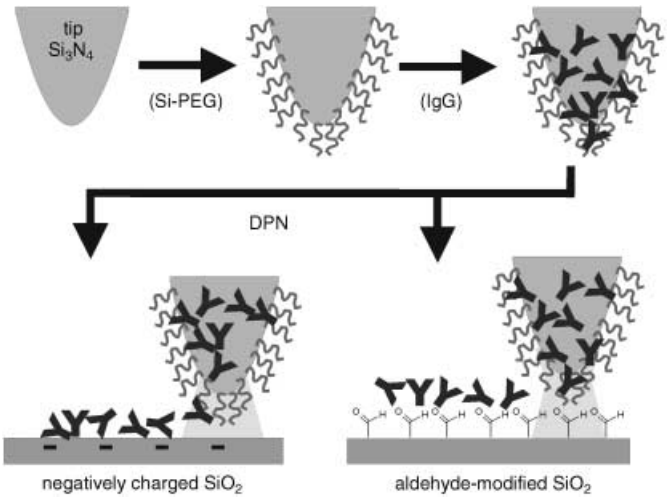
nanostructures of extraordinary complexity, thus offering routes to important tools in the life sciences such as gene chips and proteomic arrays, as well as templates that could be used by chemists and materials scientists to build ordered two- and three-dimensional functional architectures.[1] Although direct-patterning approaches offer many potential advantages over indirect methods (such as facile processing and fabrication procedures, and elimination of the need for resists), they pose several challenges. Methods must be developed for facilitating the transport of the high-molecular-weight biomolecules from a coated tip to a substrate without sacrificing sub-100-nm resolution and patterning speed. Dip-pen nanolithography (DPN)[2] is a promising tool in this respect. However, in the area of protein arrays it has thus far been used primarily as a tool for making affinity arrays out of small organic molecules that can subsequently direct the assembly of proteins from solution. This indirect approach does not allow one to generate arrays made of more than one protein.

Three approaches have been taken in the direct patterning of proteins by DPN. The first involves the use of a chemically tailored form of collagen that has surface-binding groups built into it to help facilitate nanostructure adsorption.[3] This designer-protein approach, although powerful, can not be used generally by researchers interested in proteomic-array research. The second method involves the use of glass pretreated with 3-glycidoxypropyltrimethoxysilane;[4] with this technique, human chorionic gonadotropin could be patterned at the micrometer scale. However, the biological activity of the resulting patterns has not been demonstrated. The third approach involves the use of gold substrates and modified tips.[5] No general methods have been developed for using DPN to generate arrays of protein nanostructures through direct deposition on oxide substrates, the materials of choice in the microarray industry, while preserving the biological activity of the proteins. Herein, we present two strategies that offer patterning capabilities from the 50- to the 550-nm scale. This method complements other approaches to the indirect patterning of proteins on surfaces through techniques such as nanografting[6] and electron-beam lithography[7], as well as larger-scale patterning techniques such as microcontact printing.[8]

The approach described herein relies on the modification of an AFM tip with 2-[methoxypoly(ethyleneoxy)propyl]trimethoxysilane (Si-PEG), which forms a biocompatible and hydrophilic surface layer. This layer inhibits protein adsorption and reduces the activation energy required for protein transport from tip to surface. It also protects the proteins from denaturation on the tip surface.[9] In the absence of this tip coating, the protein inking solutions (500 $\mu g\,mL^{-1}$ in phosphate-buffered saline (PBS) at pH 7.3) do not wet the silicon nitride cantilevers. We have found that untreated cantilevers often produce inconsistent or low-density protein patterns. In addition to cantilever modification, we have developed two other strategies to facilitate ink transport and nanostructure formation, both of which involve silicon oxide surfaces. The first such strategy is the creation of a negatively charged surface through treatment with base[10] and the other is the use of an aldehyde-modified surface[11] (Scheme 1). After the formation of the desired surface the slides were rinsed with ethanol and cured under flowing nitrogen at 90–100 °C for 15 min. Proteins can be attached to such surfaces through electrostatic interactions between the positively charged parts of the protein and the negatively charged substrate surface in the first case,[12] or through covalent bonding between the aldehyde-modified surface and amine groups on the protein molecule in the second case.[11] The use of antirabbit immunoglobulin G (IgG, developed in goat, Sigma) is described herein as a representative example.



Scheme 1. Schematic representation of dip-pen nanolithography of proteins on two different modified surfaces.

In a typical DPN transport experiment, a Si-PEG-modified AFM tip was coated by immersing the tip in a solution containing the desired protein (500 $\mu g\,mL^{-1}$ in PBS buffer at pH 7.3) with glycerol (5 %) for 1 min. The tip was then mounted in the AFM and brought into contact (1.5 nN) with the substrate surface. Patterning of the proteins was performed at room temperature in a glove box with a controlled environment at a relative humidity of 60–90 %.[13] For probing of the biorecognition properties of the protein nanoarrays, the unpatterned region of the aldehyde-modified surface was passivated by immersing the sample in a 5 mM aqueous solution of amine-terminated oligo(ethylene glycol) ($n = 7$) for 20 min to impede nonspecific adsorption of proteins from the solution. The synthesis of amine-terminated oligo(ethylene glycol) was performed according to literature methods.[15]

By using these experimental conditions and the base-treated surfaces, we generated nanostructures composed of rabbit IgG and (fluorophore-labeled) antirabbit IgG in a direct-write fashion (Figures 1 and 2). The latter could be imaged by fluorescence microscopy as well as by AFM, thus allowing us to confirm the chemical identity of the transported material. The height profile of an antirabbit IgG pattern consisting of two parallel lines and three dots that was generated by direct-write DPN showed that each IgG feature was 8–10 nm high (Figure 1 a), which is consistent with the transfer of a monolayer of the protein to the negatively charged surface.[12,16] Protein arrays can be fabricated by repeating the procedure for forming a single dot feature

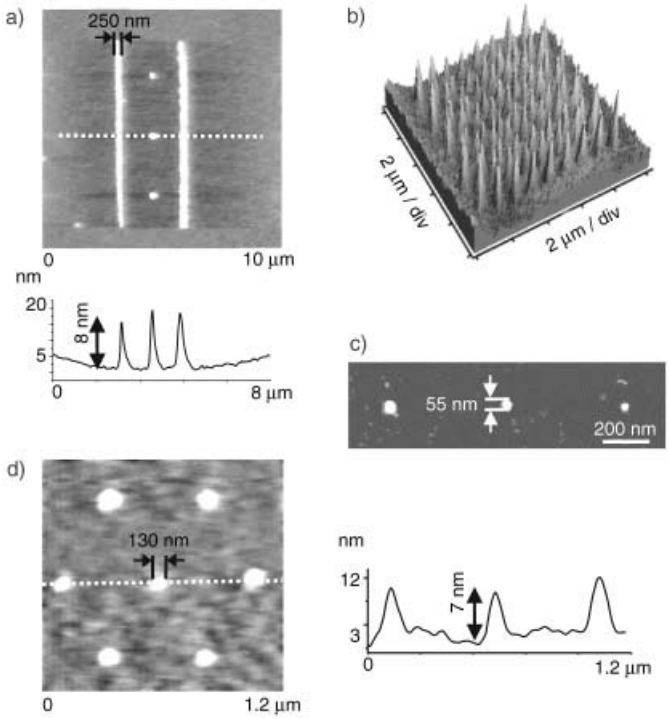


Figure 1. AFM topography images of protein nanostructures. a) Antirabbit IgG protein nanostructures on a negatively charged SiO_2 substrate written at 0.08 μm s^{-1} and corresponding line profile; b) 3D topography image of antirabbit IgG protein dot arrays (contact time = 3 s; div = division); c) tapping-mode image of antirabbit IgG protein dots (contact time = 0.5 s, set point = 0.5 nN); d) rabbit IgG nanostructures on an aldehyde-modified SiO_2 substrate (contact time = 2 s). AFM images were collected with an uncoated tip at a scan rate of 2 Hz.

(Figure 1b). For example, an array consisting of 85450-nm-diameter dots spaced 350 nm apart was fabricated from antirabbit IgG. The array was fabricated in less than 5 min with a holding time of 3 s for each dot.

The protein features studied thus far have been as large as 550 nm and as small as 55 nm (Figure 1c). Sub-100-nm protein features could be patterned successfully by using appropriate conditions (contact time = 0.5 s, contact force = 0.5 nN). We have demonstrated the generality of the electrostatic approach by patterning fluorophore-labeled antirabbit, -mouse, -goat, and -human IgG as well as unlabeled rabbit, mouse, goat, and human IgG. Certain proteins can also be patterned by DPN on aldehyde-derivatized surfaces (Figure 1d) by using the chemical approach adopted by MacBeath and Schreiber[11] for microarrays. In this approach, a reaction between aldehyde groups on the surface with primary amines (lysine and α-amines at their N termini) on the proteins results in the formation of Schiff bases and the immobilization of the proteins.[11]

Since the antirabbit IgG used in these experiments is labeled with a fluorophore, we can visualize patterns generated with it by fluorescence microscopy (Figure 2). Protein nanostructures with 550-nm line widths (Figure 2a) and dot arrays (450-nm dots, 6-μm spacing) can be visualized easily in this way (Figure 2b). A fluorescence image of the array represented by the 3D AFM image in Figure 1b is shown in Figure 2c. To demonstrate multiple protein-ink capabilities, two different fluorophore-labeled proteins, antirabbit IgG (Alexa Fluor 594) and antihuman IgG (Alexa Fluor 488) were deposited on aldehyde-derivatized surfaces by DPN in a serial fashion (Figure 2d). The line widths were determined by AFM rather than by fluorescence microscopy because of the resolution limitations of the optical technique. Finally, unlike many of the other inks studied thus far,[2] the transport of the protein structures described herein are not highly humidity dependent (over a 60–90% relative humidity). The most important factor controlling their transport seems to be set-point (large set-points lead to larger features and contact area). Many of the features generated in this study do not exhibit the uniform edges associated with the DPN transport of small ink molecules and DNA.[2,14] Indeed, transport in this case seems to be dominated by physical stamping rather than by diffusion from the tip to the surface.

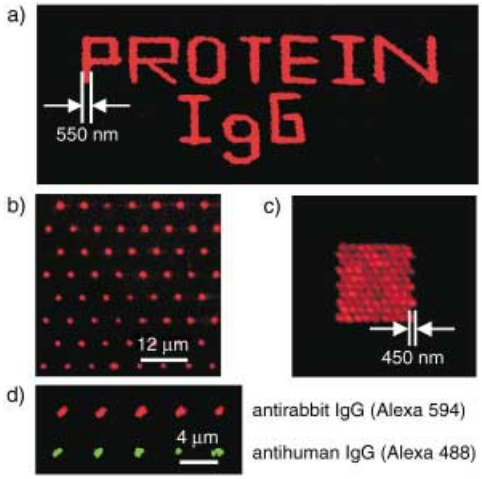


Figure 2. Fluorescence images of DPN-generated antirabbit IgG (labeled with Alexa 594) structures on a negatively charged SiO_2 surface (a–c): a) words (writing speed = 0.06 μm s^{-1}); b) dot arrays (contact time = 5 s); c) dot arrays (contact time = 3 s); d) two-component protein patterns formed by DPN on an aldehyde-derivatized SiO_2 surface (contact time = 5 s).

To test the biorecognition properties of the IgG nanostructures generated by DPN, a rabbit IgG and a human IgG pattern were immersed in a 5-μg mL^{-1} solution (PBS buffer, pH 7.3) of fluorophore-labeled antirabbit IgG (Alexa 594, red) and antihuman IgG (Alexa 488, green) for 30 min. After the substrate had been rinsed with PBS buffer (10 mM), Tween-20 solution (0.05%), and Milli-Q water, fluorescence microscopy showed that the antirabbit IgG had bound selectively to the rabbit IgG patterns (Figure 3a), and the antihuman IgG had bound to the human IgG patterns (Figure 3b). Cross-reactivity between the rabbit IgG and the human IgG was estimated to be approximately 20% based on the two-color-reading approach (compare red and green signals in Figure 3a and b).[17] It is very important that the proteins remain hydrated throughout all experimental steps to prevent their denaturation by drying.[11] The protein patterns that were either generated with the protein ink itself

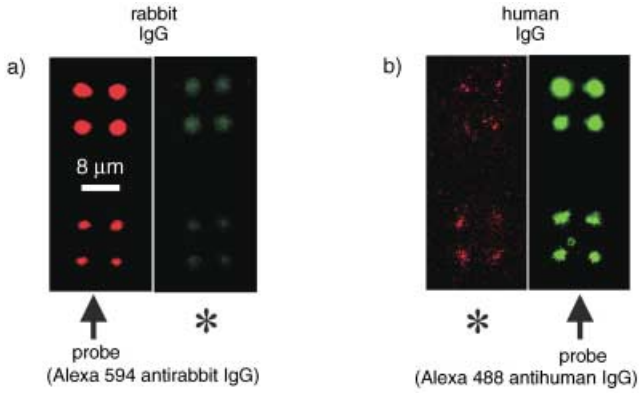


Figure 3. Probing biorecognition properties of the protein nanoarrays: a) a rabbit IgG slide; b) a human IgG slide. Both slides were incubated in a mixture of Alexa 594 labeled antirabbit IgG (red) and Alexa 488 labeled antihuman IgG (green). The images on the left probe the fluorescence of the red dye and those on the right probe the contributions from the green dye. A comparison of the two allows one to determine the extent of cross-reactivity. The images marked * are equivalent exposures and demonstrate the relatively low (< 20%) degree of nonspecific interaction between the patterned surfaces and the anti-IgGs.

(in the absence of glycerol) or exposed to air during experimental steps did not show selective protein–protein interactions. The glycerol apparently helps to hydrate the protein and inhibits denaturation.[11]

In conclusion, our studies show that DPN can be used to direct-write proteins on two different classes of SiO_2 substrates, which permits the use of both fluorescence and AFM detection methods. Importantly, the strategy we have developed provides a protocol for creating protein nanostructures on substrates while keeping their natural structures intact. When combined with multiple-pen AFM arrays,[2b,c,18] this approach could allow one to generate arrays of proteins rapidly with extraordinary complexity, almost at the resolution limit of physical-feature size (i.e. the size of the molecules).

Experimental Section

The negatively charged silicon wafers were prepared by literature methods.[19] Aldehyde-modified surfaces were prepared by cleaning oxidized silicon wafers with piranha solution (30% H_2O_2/H_2SO_4 (1:4); **Caution**: Piranha solutions are extremely dangerous and should be used with extreme caution) for 1 h, and then treating them with 3-aldehydepropyltrimethoxysilane (0.5% w/v solution in ethanol/water (95:5), adjusted to pH 5 with acetic acid, Bio-Connext, United Chemical Technologies) for 2 min.

DPN-patterning and AFM-imaging experiments were carried out with a ThermoMicroscopes CP atomic force microscope (AFM) driven by commercial lithography software (DPNWrite, DPN System-1, NanoInk Inc., Chicago, IL) and conventional silicon nitride cantilevers (force constant = 0.05 N m^{-1}). Tapping-mode images were taken with a Nanoscope IIIa and MultiMode microscope from Digital Instruments. Fluorescence images were obtained with a Zeiss Axiovert 100 microscope with a Hg lamp excitation source and standard filters.

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