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Protein Nanostructures Formed via Direct-Write Dip-Pen Nanolithography

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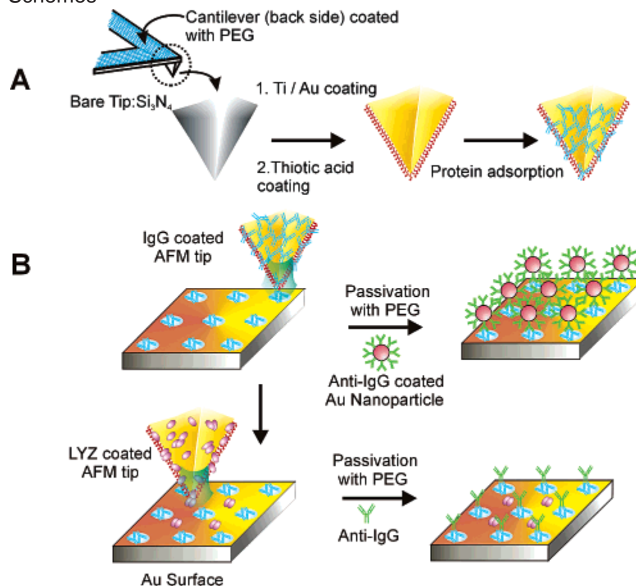
Microarrays of biomolecules such as DNA and proteins have proven useful as high-throughput screening tools in proteomics, genomics, and the identification of new pharmaceutical compounds.[1,2] For example, DNA microarrays can be used to probe gene expression and in panel assays for research- and clinical-based diagnostics.[2] Arrays of proteins have been used to ask and answer important questions regarding the interactions of cells with underlying substrates.[3] As the complexity of these arrays and corresponding number of features increase, the ability to reduce feature size becomes more important, especially since the area occupied by an array will affect the amount and volume of a sample that can be used with a particular chip. Therefore, arrays with smaller and more densely packed features are becoming increasingly attractive. In addition, if one can fabricate such structures with features that have nano- rather than macroscopic dimensions, one can enable new screening technologies and begin to address important fundamental questions regarding biomolecular recognition[3b,4] that are not addressable with microarrays. Indeed, biorecognition is inherently a *nano*- rather than a *micro*scopic phenomenon.

Promising advances have been made in making DNA and protein patterns with features with nanoscopic dimensions (<200 nm).[3b,5] However, except in the case of chemically modified collagen[6] and small peptides,[7] protein nanopatterns all have been made by *indirect* methods that either involve resists,[8] or prefabricated chemical affinity templates[3b] that direct the assembly of a single protein structure from solution onto a set of nanoscopic features on a surface of interest. Note that others have reported the generation of a 600 nm feature of HCG antibody on glass, but the biorecognition properties of this structure and control over feature size on the sub-200 nm scale were not demonstrated.[9]

To be able to generate nanoarrays of *multicomponent* systems, a requisite for many of the anticipated applications of nanoarrays, it is imperative that new surface analytical tools as well as the complementary chemistry be developed for directly placing a set of different protein structures on a surface of interest with nanoscale resolution, high-registration alignment capabilities, and control over the biological activity of the resulting structures. Herein, we show how one can use chemically modified AFM tips and dip-pen nanolithography (DPN) to generate two-component nanoarrays of native proteins that are biologically active[1,4b] and capable of recognizing a biological complement in solution, Scheme 1.

To use DPN to direct-write protein nanoarrays, we chemically modified the surface of a conventional, commercially available AFM tip (ThermoMicroscopes sharpened Si_3N_4 Microlever A, force constant = 0.05 N/m). The modification procedure involves immersing the gold-coated cantilever in a 1 mM ethanolic solution of a symmetric 11-mercapto-undecylpenta(ethylene glycol)disulfide (PEG). This results in the formation of a monolayer of PEG that prevents adsorption of protein[3b,10] on the reflective Au surface of the cantilever (backside). Tips treated in this manner were rinsed with ethanol, dried, and coated with gold (7 nm, with a 3 nm Ti adhesion layer) on the tip side by thermal evaporation methods. The cantilevers with the gold-coated tips were immersed in 0.1 mM thiotic acid in ethanol for 1 h, rinsed with ethanol, and then dried with N_2 at room temperature. To prepare tips for DPN experiments, they were immersed in solutions of the desired protein (500 μg/mL, 10 mM phosphate-buffered saline (PBS), pH 7.1) for 1 h and then used immediately. The hydrophilic tips with the carboxylic acid-terminated SAMs facilitate protein adsorption on the tip surface.[4a,11] Humidity is a critical variable, and optimum patterning results were achieved when the experiments were carried out in an environmentally controlled glovebox at a relative humidity of 80−90% at room temperature. Humidity values below 70% resulted in inconsistent transport properties. All DPN patterning was done with a ThermoMicroscopes CP AFM interfaced with DPN Write (NanoInk, Chicago, IL). Tapping mode images were taken with a Nanoscope IIIa and MultiMode microscope from Digital Instruments. Au substrates, prepared via literature methods,[12] were chosen for two reasons. First, the interaction between the cysteine residues of proteins and the Au surface provides a strong driving force for protein adsorption.[6,13] Second, they allow one to use PEG as a passivating layer in the areas not occupied by the proteins to resist nonspecific adsorption of proteins from solution.

Scheme 1. (A) Tip Modification Scheme; (B) Protein Patterning Schemes



As proof-of-concept experiments, lysozyme (Lyz) and rabbit immunoglobulin-gamma (IgG) nanodot arrays were constructed in direct-write fashion, Figure 1A and B. Feature size could be controlled over the range 45 nm to many micrometers by controlling the tip−substrate contact time. In general, longer contact times led to larger features, but the rate of transport is highly dependent upon protein composition, Figure 1 (caption). The areas surrounding the

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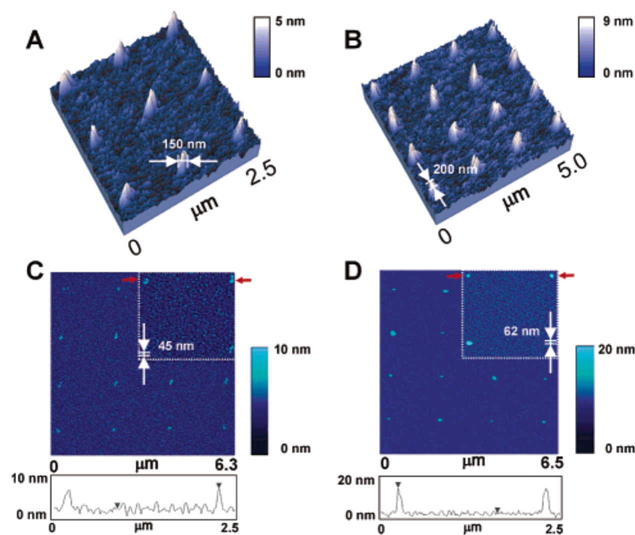

Figure 1. Protein nanoarrays prepared via direct-write DPN. (A) Contact mode image (contact force 0.1 nN) of lysozyme nanodot arrays. Each dot took 20 s to form. (B) Contact mode image (contact force 0.1 nN) of IgG nanodot arrays. Each dot took 30 s to form. An IgG nanodot array before (C) and after (D) treatment with a solution anti-IgG coated Au nanoparticles: Images were taken at 0.5-Hz scan rate in tapping mode. Each dot took 5 s to form.

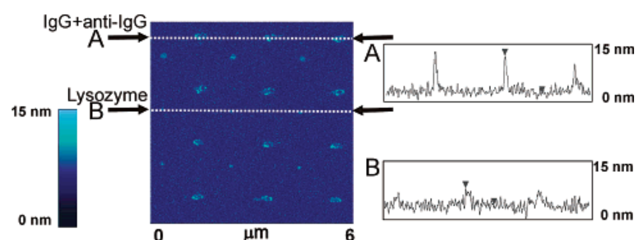

Figure 2. Two-component protein pattern after reaction with anti-IgG. (A) A height increase of 5.5 ± 0.9 nm (n =10) in the IgG features is observed by AFM. (B) No height increase is observed. The image was taken under the same conditions in Figure 1 (C) and (D).

patterns were then passivated with PEG by adding a droplet of 1 mM PEG in Nanopure H_2O (18.1 MΩ) directly on the patterned area for 45 min in a sealed vessel followed by copious rinsing with Nanopure H_2O. Nanopure H_2O was used as a solvent for the PEG to minimize denaturation of protein structures in the patterned area. Organic solvents such as ethanol, which are used often with PEG, have the potential to denature the protein structures and subsequently cause them to lose their biorecognition properties. In the case of IgG, to test the biorecognition properties of the nanoarray, it was incubated in a solution of gold nanoparticles (10 nm, diluted 1/10 in 10 mM PBS, obtained from Ted Pella) coated with anti-rabbit IgG for 3 h. A comparison of the AFM height profiles of the array before and after treatment with this solution shows a height increase of 9.6 ± 0.9 nm (n =10) in the active area of the array with little nonspecific binding to the passivated, inactive areas, Figure 1, C and D.

One of the advantages of direct-write DPN patterning over indirect methods is that one can fabricate complex multicomponent nanostructure assemblies with no cross-contamination. To demonstrate this concept, we first generated rabbit IgG nanoarrays as described above, and then lysozyme features were patterned between the IgG features, Scheme 1 and Figure 2. To confirm the biorecognition properties of the IgG in the two component array, the array was incubated in a solution containing anti-rabbit IgG (10 μg/mL, 10 mM PBS, pH 7.1) for 1 h. Significantly, a height increase (5.5 ± 0.9 nm (n =10)) due to anti-rabbit IgG binding can be observed only on the rabbit IgG features and not the area patterned with lysozyme, Figure 2. This near doubling of feature height is attributed to a 1:1 reaction between the two protein structures,[3b,7] and further demonstrates how probeless detection is possible with these nanoscale systems. Note that the density of the anti-IgG bound to the IgG features is nonuniform; this has been attributed in other studies to the random orientation of the IgG epitopes and their partial denaturation after adsorption on the Au surface.[13]

This work is important for several reasons. This is a convenient method, amendable to massive parallelization[14] for generating protein *nano*structures on a surface in direct-write fashion; in fact, these are some of the smallest protein structures generated by any lithographic technique. Second, the direct-write nature of DPN allows one to make and align multiple nanostructures made of different proteins on one surface, a prerequisite for generating functional multicomponent proteomic arrays for use in biochemical and molecular biology research. Third, these proteins are some of the largest structures ever transported by DPN, demonstrating the versatility of the approach. The slow diffusion rates of these large structures (seconds to minutes per feature, Figure 1) and the need for chemically modified tips underscore the importance of developing chemistry and physical processes that facilitate macromolecule transport in a DPN experiment.

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Supporting Information Available: High-resolution images of single and multiple protein nanodot arrays (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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