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Biologically Active Protein Nanoarrays Generated Using Parallel Dip-Pen Nanolithography**

By Seung Woo Lee, Byung-Keun Oh, Raymond G. Sanedrin, Khalid Salaita, Tsuyohiko Fujigaya, and Chad A. Mirkin*

Proteins immobilized on surfaces have been extensively investigated because of their importance in drug screening, protein analysis, and medical diagnostics.^[1–3] Several methods for protein immobilization on various surfaces have been reported, including photolithography,^[4] microcontact printing (μ -CP),^[5] electron-beam lithography,^[6] and certain SPM-based lithographies (SPM: scanning probe microscopy).^[7] Of these techniques, SPM-based lithography methods provide access to the smallest features which, in principle, allow for a smaller chip size with many more reactive sites than conventional microscale techniques. Among the SPM-based lithographies, dip-pen nanolithography (DPN), in particular, is the only one capable of generating arrays with hundreds of features. Importantly, biomolecular arrays with extremely small features open the door for single-particle (protein, virus, and cell) studies in biology.^[8]

Unfortunately, most single-probe methods are limited with respect to scaling (typically $90\text{ }\mu\text{m} \times 90\text{ }\mu\text{m}$) or the ability to directly deposit soft matter, two capabilities essential for realizing highly miniaturized biomolecular nanoarrays.^[7,9] DPN, however, can be used to deliver many types of soft-matter reagents to nanoscopic regions of a target substrate with high resolution and registration.^[10] Importantly, DPN has been used to immobilize biomolecules such as proteins,^[9a–d] DNA,^[9e] and more recently single viruses^[9f] on a variety of substrates with indirect- or direct-write methods. To address the $90\text{ }\mu\text{m} \times 90\text{ }\mu\text{m}$ patterning-area limitation of DPN, centimeter-scale and sub-100 nm-resolution patterning through the use of multiple-pen cantilever arrays has been reported by

our group in the context of small molecules used as a resist to fabricate highly miniaturized solid-state structures.^[11] This capability allows one to make many similar structures that span macroscopic distances (centimeters) in a relatively high-throughput manner.^[12]

Using DPN, proteins have been deposited on gold, nickel oxide, and pretreated-glass substrates from chemically modified single cantilever tips.^[9] A variety of protein–surface interactions have been used in DPN, such as chemisorption^[9c,d] or electrostatic attraction.^[9b] However, adsorption often leads to full or partial denaturation of the proteins and therefore a loss of function.

To generate biologically active immobilized antibodies, protein A, protein G, and protein A/G have been used to optimize antibody orientation and minimize denaturation.^[13] It is well known that protein A and protein G have an affinity to bind to the Fc (fragment-crystallizable) region of immunoglobulin G (IgG), which is the tail region of a Y-shaped antibody.^[13] Their binding affinities, however, are relatively weaker compared to that of monoclonal antibodies and their respective antigens. Protein A/G, on the other hand, is a genetically engineered protein that is designed to contain four Fc-binding domains from protein A and two from protein G; which allow for an enhanced binding affinity.^[13] By using this property and coupling it with the parallel DPN patterning technique, we demonstrate centimeter-scale protein patterning, which exhibits enhanced binding properties. In particular, we present an indirect approach for the fabrication of features with biologically active antibodies using templates made by the covalent attachment of protein A/G to arrays of DPN-generated 11-mercaptoundecanoyl-*N*-hydroxysuccinimide ester (NHSC₁₁SH, ProChimia, Co., Gdansk, Poland) on gold surfaces. This is the first example of the use of parallel SPM to generate arrays of protein structures.

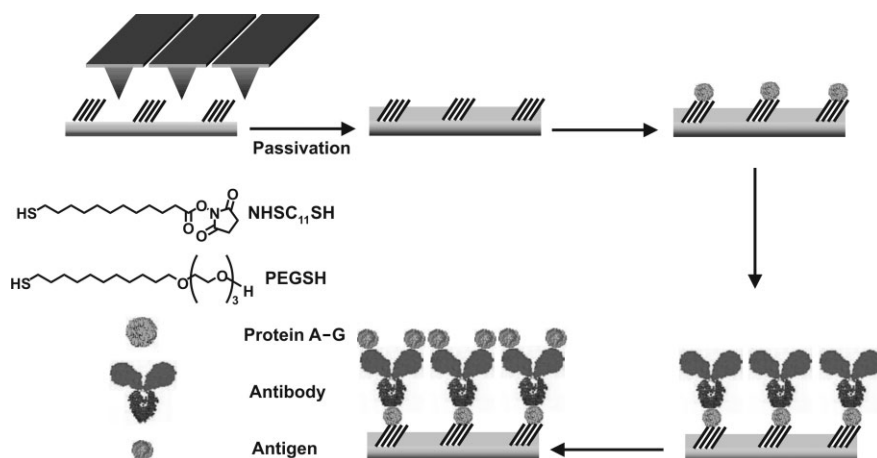
To generate nanoscale arrays of amine-reactive dot features on a gold substrate, which was prepared using literature methods,^[9] a 26-pen array (A-26) (see Supporting Information, Fig. S1) was coated with NHSC₁₁SH by immersing it in a 10 mM NHSC₁₁SH acetonitrile solution for 10 s. The tips were then dried under a stream of nitrogen (Scheme 1) and used in all DPN experiments, which were performed with an Nsciptor microscope under ambient conditions (23–26 °C, 30–36 % relative humidity). The substrates containing the DPN-generated dot features were then immersed in a 10 mM ethanol solution of 11-mercaptoundecyl tri(ethylene glycol) (PEG, ProChimia, Co., Gdansk, Poland) for 20 min, rinsed with eth-

[*] Prof. C. A. Mirkin, Dr. S. W. Lee,^[+] Dr. B.-K. Oh,^[++] R. G. Sanedrin, K. Salaita, T. Fujigaya
Department of Chemistry and Institute for Nanotechnology
Northwestern University
2145 Sheridan Road, Evanston, IL 60208-3113 (USA)
E-mail: chadnano@northwestern.edu

[+] Current Address: School of Chemical Engineering and Technology, Yeungnam University, Kyongsan 712-469, Korea.

[++] Current Address: Department of Chemical and Biomolecular Engineering, Sogang University, CPO Box 1142, Seoul, Korea.

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Scheme 1. Schematic representation of the process used to obtain biologically active antibodies on the protein A/G, which is covalently bound to NHSC₁₁SH nanoscale features patterned using DPN.

anol, and then dried with nitrogen. PEG has previously been used to prevent the nonspecific adsorption of proteins onto unpatterned gold areas.^[14]

The DPN-generated NHSC₁₁SH dot arrays served as a template for the immobilization of protein A/G. Self-assembled monolayers (SAMs) of *N*-hydroxysuccinimide (NHS) ester have been widely used for the immobilization of biomolecules, such as nucleic acids, enzymes, and proteins, because of their high reactivity with the primary amine groups of the biomolecules.^[15] However, direct patterning of molecules containing NHS ester moieties has not been explored using μ -CP or DPN. Because of the abundance of lysine side chains and terminal amine groups in the protein A/G, attachment of this protein onto the DPN-patterned SAMs could be achieved through covalent coupling. This was accomplished by immersing the NHSC₁₁SH-patterned substrates in an aqueous protein A/G solution (15 $\mu\text{g mL}^{-1}$, phosphate-buffered saline (PBS), pH 7.4) for 1 h, rinsing the substrates with 10 mM PBS, Tween-20 (0.05 %), and NanoPure water, and drying them with a stream of nitrogen. Nanoscale arrays of the covalently attached protein A/G were imaged by tapping-mode atomic force microscopy (TMAFM). Although the height difference between NHSC₁₁SH features coupled to protein A/G and the as-prepared NHSC₁₁SH features is very small (approximately 0.5 nm), the phase image shows a large contrast between the protein-covered features and the PEG-passivated nonpatterned areas (Fig. 1). This can be attributed to the difference in stiffness and viscoelasticity of the passivating molecule and the covalently attached protein.^[16]

To generate nanoscale antibody-based arrays, highly dense DPN-generated dot arrays (23 400 dots with a 1 μm dot-to-dot spacing) of NHSC₁₁SH covalently coupled to protein A/G were incubated in a solution of Alexa Fluor 594-labeled human IgG solution (15 $\mu\text{g mL}^{-1}$, PBS, pH 7.4) for 1 h. The substrates were subsequently rinsed with PBS, Tween-20, and NanoPure water in an ultrasonic bath for 10 s to remove loosely adsorbed human IgG. The human IgG nanoarrays ob-

tained were then investigated using TMAFM and fluorescence microscopy (Fig. 2). Importantly, the feature size of the IgG nanoarrays could be adjusted from 100 to 650 nm by simply adjusting the tip–surface contact time. Regardless of the dot diameter, the patterned human IgG nanoarrays were 8.0 nm in height, which is comparable to values previously reported in the literature (see Supporting Information, Fig. S2).^[7a,c,13f,17] Furthermore, fluorescence microscopy images confirmed the adsorption of Alexa Fluor 594-labeled human IgG immobilized on the generated protein A/G arrays (Fig. 2b). Both the height profile and the fluorescence image suggests that the antibodies were adsorbed onto the DPN-generated protein-array templates.

To demonstrate the generality of this approach for protein patterning, anti- β -galactosidase and anti-ubiquitin (see Supporting Information, Fig. S3) nanoarrays (14 000 dots with a 2 μm dot-to-dot spacing) were prepared in a similar manner to that used for human IgG. The patterned anti- β -galactosi-

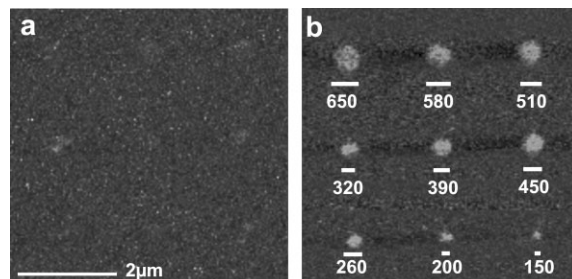


Figure 1. a) Topography and b) phase TMAFM images of nine protein A/G dots with successively decreasing diameters, generated by using different contact times between an AFM cantilever tip and a gold substrate (650 nm:8 s, 580 nm:7 s, 510 nm:6 s, 450 nm:5 s, 390 nm:4 s, 320 nm:3 s, 260 nm:2 s, 200 nm:1 s, 150 nm:0.5 s).

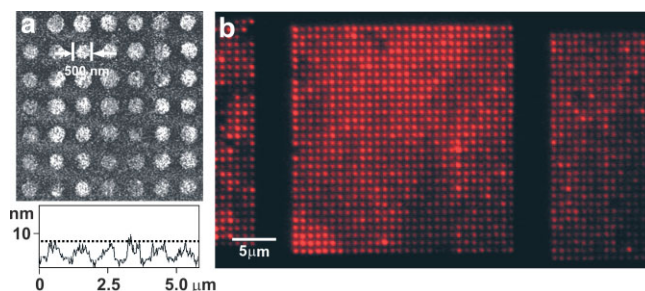


Figure 2. a) Topographical TMAFM image and its corresponding height profile of fluorescein isothiocyanate Alexa Fluor 594-labeled human IgG nanoarrays immobilized onto protein A/G templates. b) Representative fluorescence microscopy image of Alexa Fluor 594-labeled antibody nanoarray patterns. The patterns span a distance of 1 cm.

dase IgG nanoarrays were approximately 6.8 nm high (Fig. 3a). The biological activity of the patterned antibodies on the protein A/G was evaluated by incubating the substrates in a solution containing either Alexa Fluor 594-labeled β -galactosidase ($15 \mu\text{g mL}^{-1}$) or Alexa Fluor 488-labeled ubi-

many important biological structures (e.g., viruses, spores, and cells). This work will challenge researchers in nanoscience and biology to consider new ways of using such patterned interfaces to probe biological systems.

Experimental

All DPN patterning was done with an Nscriptor (NanoInk, Chicago, IL) driven by custom lithography software (NanoInk, Chicago, IL) and passive multiprobe arrays ($k=0.097 \text{ N m}^{-1}$, NanoInk, Chicago, IL). Gold substrates were prepared by means of thermal evaporation with a 10 nm titanium adhesion layer and subsequently coated with 50 nm of gold under vacuum (pressure $< 1 \times 10^{-7}$ mbar). The gold substrates obtained were used immediately for DPN experiments. The multiple-pen arrays were coated with $\text{NHSC}_{11}\text{SH}$ and were used to generate dot features with various sizes ranging from 100 to 1000 nm under ambient conditions ($22\text{--}24^\circ\text{C}$, $30\text{--}36\%$ relative humidity). These substrates were subsequently incubated in the protein A/G and antibody (human IgG or anti-ubiquitin). Tapping-mode images were obtained using Nanoscope IIIa and MultiMode microscopes (Digital Instruments, Santa Barbara, CA) and the fluorescence images were taken using a Zeiss Axiovert 100 microscope (Carl Zeiss, Thornwood, NY).

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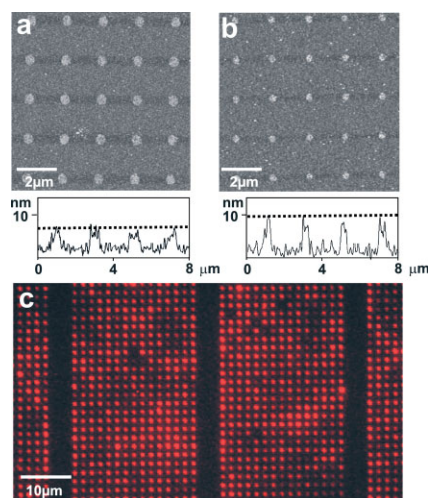


Figure 3. Topographical TMAFM images and their corresponding height profiles of anti- β -galactosidase nanoarrays immobilized on protein A/G templates a) before and b) after incubation in a β -galactosidase protein solution. c) Fluorescence microscopy image of the Alexa 594-labeled β -galactosidase complex nanoarrays.

quitin ($15 \mu\text{g mL}^{-1}$) in PBS with pH 7.4 for 2 h. After washing the substrates with PBS solution and Tween-20, they were sonicated for 10 s in an ultrasonic bath. The substrates were then imaged using TMAFM and fluorescence microscopy. The binding of the Alexa Fluor 594-labeled β -galactosidase molecules to anti- β -galactosidase increased the height of the patterned features from 6.8 to 9.5 nm; this result can be seen from a comparison of the height profiles in Figure 3a and b. Uniform β -galactosidase binding can be observed from the fluorescence image in Figure 3c. Importantly, no binding was observed when the arrays were exposed to fluorophore-labeled ubiquitin protein. This demonstrates that the arrays of immobilized antibodies retained their biological activity after patterning.^[9c,18]

In conclusion, we have demonstrated that the amine-reactive alkyl thiol molecule, $\text{NHSC}_{11}\text{SH}$, can be used as a template molecule for high-throughput DPN-based protein patterning. Through the use of the affinity binding of the antibodies on the protein A/G, biologically active antibody nanoarrays can be generated over macroscopic distances through parallel DPN, demonstrating the versatility of this approach for making many similar antibody structures in a relatively high-throughput manner. Although this approach is currently limited to the deposition of only one type of protein, this work is a crucial first step toward building massive biomolecular libraries with feature sizes that are unattainable by other methods and on a par with or smaller than the size of

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