Bioactive Protein Nanoarrays

Bioactive Protein Nanoarrays on Nickel Oxide Surfaces Formed by Dip-Pen Nanolithography**

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Biologically functional protein arrays are important for chip-based protein detection assays and proteomic profiling experiments.[1–3] Nanoscale arrays allow for smaller chips with more reaction sites, smaller test sample volumes, potentially higher sensitivity and speed, and direct feature analysis with a scanning probe instrument.[4–11] Several promising routes to protein nanoarrays with submicrometer and even sub-100-nm features have been reported.[9–11] The activity of the immobilized proteins in some of the arrays generated by dip-pen nanolithography (DPN)[4] has been confirmed by fluorescence labeling studies and direct imaging by atomic force microscopy (AFM).[9–11]

Nickel is a commonly used substrate for biological arrays because the oxidized Ni surface has a high affinity for polyhistidine residues, and this specific interaction, in principle, can provide control over the uniformity of protein binding and presentation to the analyte solution. The histidine tag allows for protein adsorption without direct contact between the active area of the protein and the substrate surface.[2,12–14]

The deposition of histidine-tagged peptides and proteins on Ni substrates by using electrochemical DPN[7] was recently reported; however, it was concluded that peptide and protein transport could not be effected without an applied field, and the biological activities of the generated nanofeatures were not studied.[15,16] The requirement of an applied field is limiting with respect to chemical compatibility of the protein inks and protein denaturation under such conditions (−2 to −3 V) and the complexity of the hardware used to effect such a process. Herein, we report a methodology based upon DPN and conditions that allow one to generate biologically active protein nanoarrays with feature sizes as small as approximately 80 nm on Ni surfaces without the need for an applied field (Figure 1).

To facilitate ink wetting and transport, AFM tips were coated with a thin layer of Ni (ca. 5 nm) by thermal

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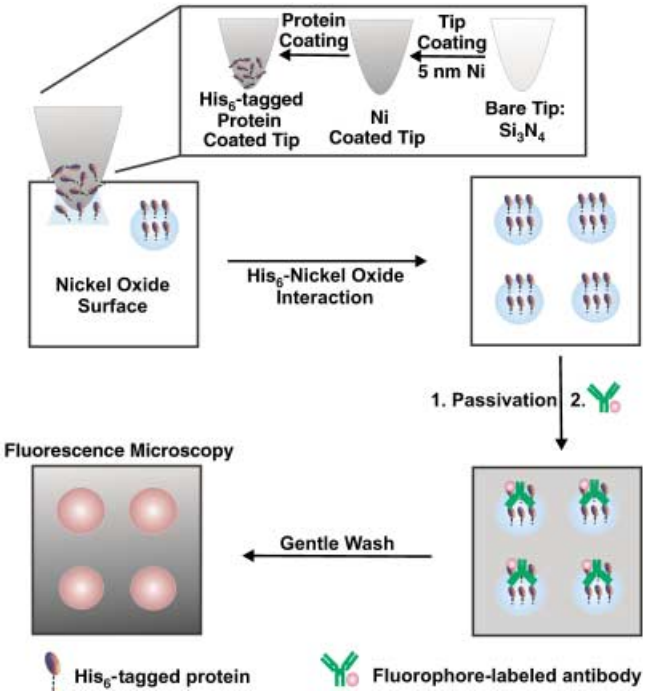

Figure 1. Tip-coating and direct-write DPN protocols.

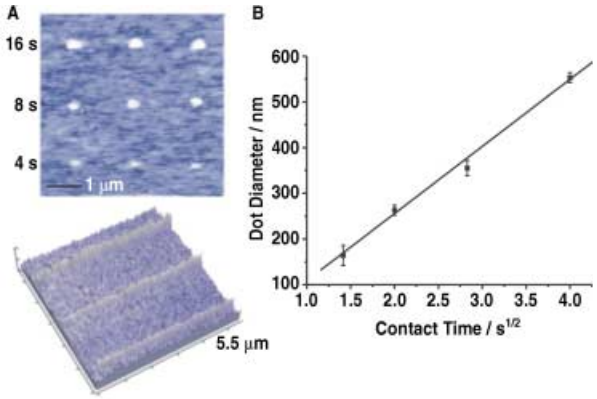

Figure 2. A) Dot and line patterns of ubiquitin generated by direct-write DPN and B) ubiquitin dot size as a function of contact time at pH 7.4.

evaporation prior to DPN deposition experiments. The Ni-coated tips were immersed in solutions of His-tagged (His_6) proteins (ubiquitin ($300\,\mu g\,mL^{-1}$) or thioredoxin ($250\,\mu g\,mL^{-1}$) in 0.1 M phosphate-buffered saline (PBS) at pH 7.4) for 1–2 min. Ubiquitin and thioredoxin were chosen as initial ink candidates because they are biologically important. (The attachment of ubiquitin to a lysine residue of a protein tags the protein for intracellular proteolytic destruction by a proteasome, and thioredoxin mediates the reduction of disulfide bonds in proteins.[21,22]) The Ni-coated tip presumably adsorbs His-tagged protein molecules as a result of the interaction between the nickel oxide surface and the polyhistidine tag. Bare Si_3Ni_4 AFM tips could not be homogeneously coated with proteins under the conditions employed, and this resulted in inconsistent transport and non-uniform protein patterns on the nickel oxide surfaces, an outcome consistent with previous observations by Stone and co-workers.[19,20]

Ni substrates were prepared by thermal evaporation of Ni (30 nm) on Si(100) wafers. The Ni substrates were oxidized by exposing them to air (ambient conditions) for 24 h prior to use. All DPN experiments were done with a ThermoMicroscopes CP AFM apparatus interfaced with customized software (DPNWrite, Nanoink, Inc., Chicago, IL). Protein patterning was performed in a closed environment with 80% humidity at 24 °C. High humidity was used to effect uniform and rapid protein diffusion from the tip to the surface and to prevent the denaturation of the protein structures on the Ni substrate. Patterning could be effected down to 50% relative humidity, but in general, lower quality results were obtained with humidity values below 80%. N-terminal His-tagged ubiquitin nanoarrays were constructed in direct-write fashion in the form of dots and lines (Figure 2A). The height profile of the nanofeatures shows that each ubiquitin structure is approximately 5 nm tall, which is consistent with a monolayer of ubiquitin proteins being attached to the underlying nickel oxide surface (the size of ubiquitin $= 5.1 \times 4.3 \times 2.9\ nm^3$).[21] Patterns could not be generated under comparable conditions when ubiquitin proteins without histidine tags were used as inks. This suggests that the interaction between the oxidized nickel substrate and the polyhistidine residues is critical for the patterning process.

An important attribute of DPN is the ability to pattern various molecules (small organic molecules, polymers, DNA, and proteins) with control over feature size (micrometer to sub-100-nm length scale) and shapes. One of the major obstacles of direct-write DPN of proteins has been the diffusion of protein molecules on a surface. Proteins on both modified Si and Au surfaces diffuse very slowly.[10,11] On nickel oxide surfaces, however, His-tagged proteins show tip to substrate diffusion behavior similar to that observed for small alkanethiol molecules diffusing from the tip to an Au substrate (Figure 2B), but different from the stamping behavior observed for protein transport to other substrates.[5–7,10,11] The transport process is facilitated on Ni because of the high hydrophilicity of the oxidized substrate and its ability to support a meniscus, in addition to the strong binding interaction between the histidine-tagged ink and the nickel oxide substrate (see the Supporting Information).

Nanoarrays of ubiquitin and thioredoxin (Sigma–Aldich) were patterned with DPN (Figure 3). Ubiquitin and thioredoxin ($6.8 \times 2.7 \times 5.2\ nm^3$) have similar dimensions.[21,22] Regularly spaced arrays with feature sizes as large as 500 nm and as small as approximately 80 nm could be easily constructed (Figure 3). To address the biological activity of the nanopatterned proteins the nanoarrays were treated with fluorophore-labeled antibodies (Figures 1 and 4). The area surrounding the ubiquitin pattern was passivated with His-tagged polypeptides (ASASHH, $10\,\mu g\,mL^{-1}$ in PBS, pH 7.4; Sigma–Genosys) for 30 min, and this was followed by copious gentle rinsing with buffer solution (0.1 M PBS solution, pH 7.4) and NANOpure water (18 megohm, Barnstead International, Dubuque, IA). The ubiquitin nanoarray was then incubated in a solution containing fluorophore-labeled (Alexa Fluor 594)

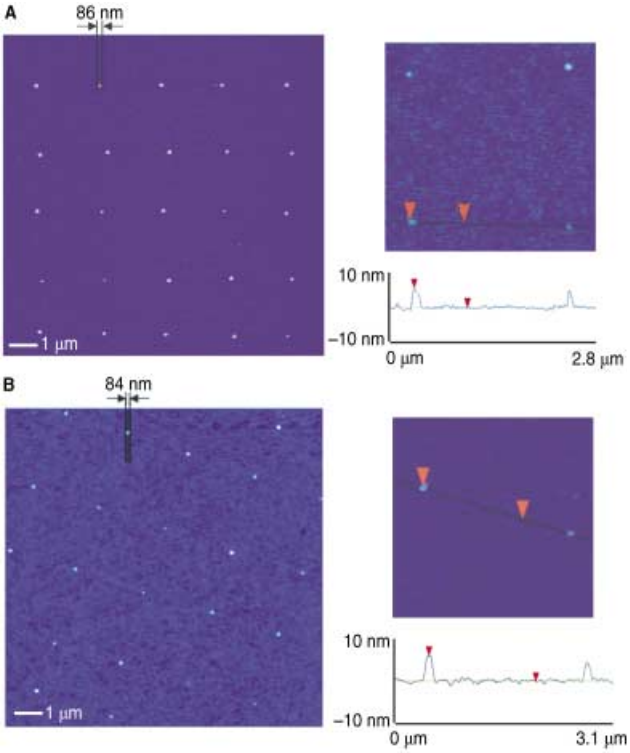

Figure 3. Protein nanoarrays of: A) ubiquitin and B) thioredoxin. Images were taken at 0.5 Hz in tapping mode (Nanoscope IIIa and multimode microscope from Digital Instruments). The contact time was 3 s for the ubiquitin array and 5 s for the thioredoxin array.

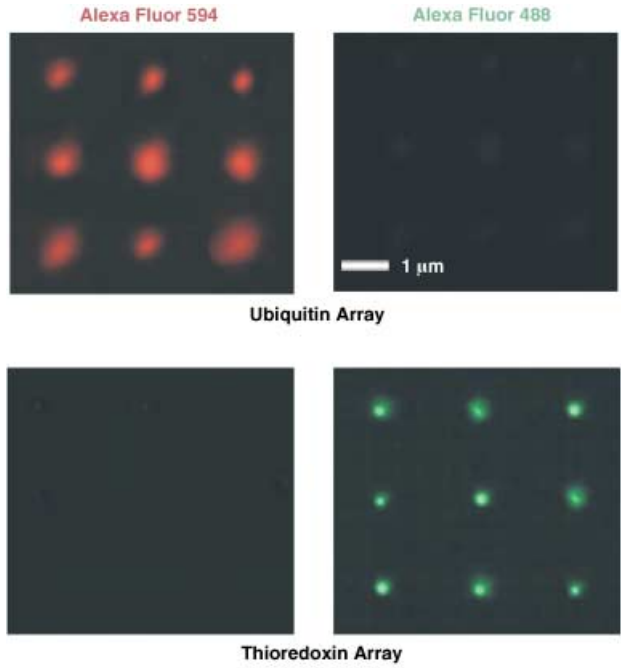

Figure 4. Probing the biorecognition properties of the ubiquitin and thioredoxin nanoarrays with dye-labeled anti-ubiquitin (red) and anti-thioredoxin (green).

anti-ubiquitin (50 $\mu g\,mL^{-1}$) mixed with anti-thioredoxin labeled with Alexa Fluor 488 (50 $\mu g\,mL^{-1}$) in PBS buffer for 2 h. After gentle rinsing of the substrate with PBS buffer solution and NANOpure water, labeled anti-ubiquitin molecules were bound to the ubiquitin-immobilized nanofeatures (red in Figure 4), while no detectable nonspecific binding of anti-thioredoxin (green) was found within the ubiquitin pattern region (Zeiss Axiovert 100 microscope). A similar result was obtained with the comparable labeling studies involving the thioredoxin arrays (that is, fluorophore-labeled anti-thioredoxin only attaches to the thioredoxin features, Figure 4).

In summary, a straightforward method for the preparation of biologically active protein nanoarrays on nickel oxide surfaces is reported. Importantly, with this new method, an applied potential is not necessary to generate active biological structures with excellent control over the feature size. Unlike previous studies involving the transport of proteins,[10,11] the protein molecules in this system seem to diffuse from the Ni-coated tips to the Ni-coated substrate, behavior similar to that observed for the alkanethiol on gold system.[5] This technique could be combined with multiple-pen AFM techniques[23,24] to generate protein arrays with extraordinary complexity in massively parallel fashion.

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[1] G. MacBeath, S. L. Schreiber, *Science* **2000**, *289*, 1760.
[2] H. Zhu, M. Bilgin, R. Bangham, D. Hall, A. Casamayor, P. Bertone, N. Lan, R. Jansen, S. Bidlingmaier, T. Houfek, T. Mitchell, P. Miller, R. A. Dean, M. Gerstein, M. Snyder, *Science* **2001**, *293*, 2101.
[3] B. B. Haab, M. J. Dunham, P. O. Brown, *Genome Biol.* **2001**, *2*(2), research 0004.1.
[4] R. D. Piner, J. Zhu, F. Xu, S. Hong, C. A. Mirkin, *Science* **1999**, *283*, 661.
[5] A. Ivanisevic, C. A. Mirkin, *J. Am. Chem. Soc.* **2001**, *123*, 7887.
[6] P. E. Sheehan, L. J. Whitman, *Phys. Rev. Lett.* **2002**, *88*, 156104.
[7] Y. Li, B. W. Maynor, J. J. Liu, *J. Am. Chem. Soc.* **2001**, *123*, 2105.
[8] D. L. Wilson, R. Martin, S. Hong, M. Cronin-Golomb, C. A. Mirkin, D. L. Kaplan, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 13660.
[9] K.-B. Lee, S.-J. Park, C. A. Mirkin, J. C. Smith, M. Mrksich, *Science* **2002**, *295*, 1702.
[10] K.-B. Lee, J.-H. Lim, C. A. Mirkin, *J. Am. Chem. Soc.* **2003**, *125*, 5588.
[11] J.-H. Lim, D. S. Ginger, K.-B. Lee, J. Heo, J.-M. Nam, C. A. Mirkin, *Angew. Chem.* **2003**, *115*, 2411; *Angew. Chem. Int. Ed.* **2003**, *42*, 2309.
[12] G.-Y. Liu, S. Xu, Y. Qian, *Acc. Chem. Res.* **2000**, *33*, 457.
[13] J. R. Kenseth, J. A. Harnisch, V. W. Jones, M. D. Porter, *Langmuir* **2001**, *17*, 4105.
[14] M. A. Case, G. L. McLendon, Y. Hu, T. K. Vanderlick, G. Scoles, *Nano. Lett.* **2003**, *3*, 425.
[15] D. J. Zhou, X. Wang, L. Birch, T. Rayment, C. Abell, *Langmuir*, **2003**, *19*, 10557.
[16] R. Gentz, C.-H. Chen, C. A. Rosen, *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 821.
[17] G. B. Sigal, C. Bamdad, A. Barberis, J. Strominger, G. M. Whitesides, *Anal. Chem.* **1996**, *68*, 490.
[18] R. Yasuda, H. Noji, K. Kinosita, M. Yoshida, *Cell* **1998**, *93*, 1117.

[19] G. Agarwal, L. A. Sowards, R. R. Naik, M. O. Stone, *J. Am. Chem. Soc.* **2003**, *125*, 580.
[20] G. Agarwal, R. R. Naik, M. O. Stone, *J. Am. Chem. Soc.* **2003**, *125*, 7408.
[21] S. Vijay-Kumar, C. E. Bugg, W. J. Cook, *J. Mol. Biol.* **1987**, *194*, 531.
[22] J. F. Anderson, D. A. R. Sanders, J. R. Gasdaska, A. Weichsel, G. Powis, W. R. Montfort, *Biochemistry* **1997**, *36*, 13979.
[23] M. Zhang, D. Bullen, S.-W. Chung, S. Hong, K. S. Ryu, Z. Fan, C. A. Mirkin, C. Liu, *Nanotechnology* **2002**, *13*(2), 212.
[24] J. Zou, D. Bullen, X. Wang, C. Liu, C. A. Mirkin, *Appl. Phys. Lett.* **2003**, *83*, 581.

