Protocols for generating protein arrays

Arrays of microscopic features comprising different proteins are extremely important for proteomics and cell research, pharmaceutical screening processes ([proteomics and cell research, pharmaceutical assays (1–4)], and panel immunoassays (5, 6). Many conventional patterning techniques, including photolithography (6, 7), microcontact printing (2, 8), and spot arraying (9), have been used for fabricating such arrays. Submicrometer spot arrays of proteins have been generated by finely focused ion beam lithography (FIB) (9), and individual micrometer and submicrometer features have been prepared by atomic force microscopy (AFM) techniques with varying degrees of success (10–14). One of the advantages of miniaturization is that when a feature composed of receptors is miniaturized to the scale of the biological analytes, new methods for screening reactions involving such receptors and analytes become available, because almost every physical property of the receptor feature is changed upon reaction with the analyte, including the height, hydrophobicity, roughness, and shape of each feature, which are all variables easily probed with a conventional AFM. One disadvantage of receptor feature miniaturization in such an array is that nonspecific binding of the protein array can become a major problem leading to large background signals. For example, it could be difficult to differentiate inactive areas of the substrate that experience such binding from the active receptor features, thereby complicating the analysis of the nanoscale libraries. Indeed, others who have attempted to study reactions involving antibodies and antigens on a surface with an AFM have noted the importance of eliminating nonspecific binding when studying individual nano- and micrometer-scale protein features (10–13). Here, we describe how a high-resolution patterning technique, dip-pen nanolithography (DPN), can be used to construct nanorobots of proteins. Moreover, we demonstrate that these arrays can be fabricated with almost no detectable nonspecific binding of proteins to the passivated portions of the array, even in solutions containing multiple proteins, and that reactions involving the protein features and antigens can be screened by AFM. Finally, we show how such nanorobots can be implemented in the study of cellular interactions with nanopatterned surfaces.

A typical protein array was fabricated by initially patterning 16-mercaptop hexadecanoic acid (MHA) on a gold thin-film substrate in the form of dots or grids. The features studied thus far, both lines and dots, have been as large as 350 nm (line width and dot diameter, respectively) and as small as 100 nm (Fig. 1). The areas surrounding these features were passivated with 11-mercapto undecyl-tri(ethylene glycol) by placing a droplet of a 10 mM ethanolic solution of the surfactant on the patterned area for 45 min followed by copious rinsing with ethanol and then, NANOpure water (NANOpure, Barnstead/Thermolyne Corp.). As proof-of-concept experiments, the proteins were absorbed on the preformed MHA patterns (Fig. 2) by immersing the substrate in a solution containing the desired protein (10 μg/ml) for 1 hour. The substrate was then removed and rinsed with 10 mM Tris buffer [Tris-(hydroxymethyl) aminomethane], Tween-20 solution (0.05%) and, finally, NANOpure water. Such proteins have a high affinity for carboxylic acid–terminated monolayers at pH 7 (10–13) and a relatively weak affinity for surfaces coated with 11-mercaptop undecyl-tri(ethylene glycol) (15, 16). The protein arrays were then characterized by AFM. In the case of arrays composed of immunoglobulin G (IgG) patterns, the reaction of the array with rabbit antibody to mixtures of proteins was studied by AFM. Lysozyme, an ellipsoidal shaped protein (4.5 nm by 3.0 nm by 3.0 nm), (17) cleanly assembled on the MHA nanopatterns, as evidenced by contact and tapping mode AFM (Fig. 1, B to D), respectively (18). Note that there is almost no evidence of nonspecific geometry and materials property, which can be calculated from the equation shown in the work cited in this reference, and \( P_0 \) is the critical load at fracture (4). \( \alpha \) was measured to be 1.1 \times 10^{13} \text{ m}^{-1/2}. The fracture energy \( (G_c) \) value can be calculated according to the literature (7), \( G_c = \frac{1}{V} \int K^\alpha_{\text{III}} \delta \theta \).
protein adsorption on the array and that height profiles suggest that between one and two layers of protein adsorb at each MHA site (19). The height distribution observed in the image is likely due to the different orientations the protein can adopt on the MHA-coated surface (Fig. 1C, inset). Finally, the protein can be assembled in almost any array configuration, including lines and grids (Fig. 1D).

IgG, which has substantially different dimensions (Y-shape, height = 14.5 nm, width = 8.5 nm, thickness = 4.0 nm) (20, 21), exhibited qualitatively similar adsorption characteristics (19) (Fig. 3). The height profile of an IgG nanoarray shows that each IgG feature is 6.5 ± 0.9 nm (n = 10) high, which is consistent with a single monolayer of the protein adsorbed onto the MHA features and is comparable to what others have seen for macroscopic features (10–13).

We addressed the issue of nonspecific binding by incubating a nanoarray of rabbit IgG in a four-component aqueous protein solution (10 mM Tris buffer, pH 7.3) containing lysozyme, Retronectin, goat/sheep antibody to IgG (anti-IgG), and human anti-IgG (each at 10 μg/ml) for 1 hour. A comparison of the AFM images of the array before and after treatment with this solution shows no evidence of protein binding to the array and almost no detectable nonspecific binding to the passivated, inactive areas of the substrate (compare Fig. 3, A and B). When a nanoarray of rabbit IgG was treated with a solution containing lysozyme, goat/sheep anti-IgG, human anti-IgG, and rabbit anti-IgG (concentration of each was 10 μg/ml) for 1 hour, each of the active features of the array increase in height from 6.5 ± 0.9 nm (n = 10) to 12.1 ± 1.3 nm (n = 10) (compare Fig. 3, C and D). This almost doubling in height is consistent with a 1:1 reaction between the two protein structures. These experiments show that regardless of the orientations of the IgG within the nano-}

Fig. 1. AFM images and height profiles of lysozyme nanosheets. (A) Lateral force image of an 8 μm by 8 μm square lattice of MHA dots deposited onto an Au substrate. The array was imaged with an uncoated tip at 42% relative humidity (scan rate = 4 Hz). (B) Topography image (contact mode) and height profile of the nanoarray after lysozyme adsorption. A tip-substrate contact force of 0.2 nN was used to avoid damaging the protein patterns with the tip. (C) A tapping mode image (silicon cantilever, spring constant = 30 N/m) and height profile of a hexagonal lysozyme nanoarray. The image was taken at a scan rate of 0.5 Hz to obtain high resolution. (D) Three-dimensional topographic image of a lysozyme nanoarray, consisting of a line grid and dots with intentionally varied feature dimensions. Imaging was done in contact mode as described in (B).

Fig. 2. Diagram of proof-of-concept experiments, in which proteins were absorbed on preformed MHA patterns. The resulting protein arrays were then characterized by AFM.
ependent signaling processes and also play an important mechanical role by connecting the actin cytoskeleton structure to the substrate. An understanding of how adhesion and signaling of cells depend on the sizes and distributions of focal adhesions has been limited in patterning studies of immobilizing proteins to 1-μm patches, which do not have the resolution necessary for characterizing the sizes of focal adhesions that are necessary for normal cell adhesion (4). Whereas current studies reveal that focal adhesions have sizes on the micrometer length scale, the lack of higher resolution patterning methods has prevented direct examination of the range of sizes that constitute active focal adhesions (23).

To test whether cells can adhere to protein nanoarrays with features well below 1 μm, we used DPN to pattern MHA into a square array of dots that are 200 nm in diameter and separated by 700 nm (Fig. 4A) (24). Like the other proteins, AFM images (19) showed that the Retronectin adsorbed almost exclusively on the MHA pattern (Fig. 4B). The protein nanoarray was then immersed for 12 hours in a Dulbecco’s Modified Eagle Medium (DMEM) solution with 10% bovine calf serum, which contained the 3T3 Swiss fibroblast cells (20,000 cells/ml). The arrays were then gently rinsed with phosphate buffered saline (PBS) and studied by optical microscopy. Significantly, cells only attach to the patterned region of the substrate and spread, but not completely, into a more flattened morphology. This morphology is a direct consequence of the sizes and spacings of the patterned protein, because cells placed on a nonpatterned substrate were well spread. Thus, submicrometer features can support cell adhesion and point toward the importance of a way of systematically identifying the relation between nanofeature size and composition in studies of cell adhesion and other related processes.

The ability to make protein nanoarrays on a surface with well-defined feature size, shape, and spacing should increase the capabilities of researchers studying the fundamental interactions between biological structures (cells, complementary proteins, and viruses) and surfaces patterned with proteins. Although we have explored only three proof-of-concept systems, we expect that it can be extended, with straightforward modifications of the aforementioned protocols, to a wide range of biomolecular structures and that the resolution of the technique, once optimized, should compare well with that of conventional DPN (10 nm) (25–27).

Fig. 3. AFM tapping mode image and height profile of rabbit IgG assembled onto an MHA dot array generated by DPN before (A) and after (B) exposure to a solution containing lysozyme, Retronectin, goat/sheep anti-IgG, and human anti-IgG. An IgG nanoarray before (C) and after (D) treatment with a solution containing lysozyme, goat/sheep anti-IgG, human anti-IgG, and rabbit anti-IgG. All images were taken at a 0.5-Hz scan rate in tapping mode.

Fig. 4. (A) Diagram describing the cell adhesion experiment on the DPN-generated pattern. The total patterned area is 6400 μm². The alignment marks were generated by scratching a circle into the backside of the Au-coated glass substrate. (B) Topography image (contact mode) of the Retronectin protein array. Imaging conditions were the same as in Fig. 1B. (C) Large-scale optical microscope image showing the localization of cells in the nanopatterned area. (D) Higher resolution optical image of the nanopatterned area, showing intact cells.

References and Notes
Niobium-Zirconium Chronometry and Early Solar System Development

Maria Schönbächler,1* Mark Rehkämper,1 Alex N. Halliday,1 Der-Chuen Lee,1 Michèle Bourot-Denise,3 Brigitte Zanda,3,4 Bodo Hattendorf,2 Deftef Günther2

Niobium-92 (92Nb) decays to zirconium-92 (92Zr) with a half-life of 36 million years and can be used to place constraints on the site of p-process nucleosynthesis and the timing of early solar system processes. Recent results have suggested that the initial 92Nb/93Nb of the solar system was high (>10−3). We report Nb-Zr internal isochrons for the ordinary chondrite Estacado (H6) and a clast of the mesosiderite Vaca Muerta, both of which define an initial 92Nb/93Nb ratio of ~10−2. Therefore, the solar system appears to have started with a ratio of <3×10−5, which implies that Earth’s initial differentiation need not have been as protracted as recently suggested.
Bioactive Protein Nanoarrays on Nickel Oxide Surfaces Formed by Dip-Pen Nanolithography**

Jwa-Min Nam, Sang Woo Han, Ki-Bum Lee, Xiaogang Liu, Mark A. Ratner, and Chad A. Mirkin*

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
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$_3$Ni$_4$ 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 2 A). 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 x 4.3 x 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 x 2.7 x 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 nano-patterned 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)
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, behaviors 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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The Use of Nanoarrays for Highly Sensitive and Selective Detection of Human Immunodeficiency Virus Type 1 in Plasma

Ki-Bum Lee,† Eun-Young Kim,‡ Chad A. Mirkin,*† and Steven M. Wolinsky*†‡

Department of Chemistry and The Institute for Nanotechnology, Northwestern University, Evanston, Illinois, and Division of Infectious Diseases, The Feinberg School of Medicine, Northwestern University, Chicago, Illinois

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ABSTRACT

Arrays of antibodies with well-defined feature size and spacing are necessary for developing highly sensitive and selective immunoassays to detect macromolecules in complex solutions. Here we report the application of nanometer-scale antibody array-based analysis to determine the presence of the human immunodeficiency virus type 1 (HIV-1) in blood samples. Dip-pen nanolithography (DPN) was used to generate nanoscale patterns of antibodies against the HIV-1 p24 antigen on a gold surface. Feature sizes were less than 100-nanometers, and the activity of the antibody was preserved. HIV-1 p24 antigen in plasma obtained directly from HIV-1-infected patients was hybridized to the antibody array in situ, and the bound protein was hybridized to a gold antibody-functionalized nanoparticle probe for signal enhancement. The nanoarray features in the three-component sandwich assay were confirmed by atomic force microscopy (AFM). Demonstration of measurable amounts of HIV-1 p24 antigen in plasma obtained from men with less than 50 copies of RNA per ml of plasma (corresponding to 0.025 pg per ml) illustrates that the nanoarray-based assay can exceed the limit of detection of conventional enzyme-linked immunosorbent assay (ELISA)-based immunoassays (5 pg per ml of plasma) by more than 1000-fold.

Arrays of proteins with well-defined feature size and spacing are important for studying surface-cellular interactions and detecting specific biomacromolecules. Recently, there have been significant developments in the use of nanolithography techniques for patterning surfaces with proteins on the submicrometer length scale. Dip-pen nanolithography (DPN) is one technique that has shown particular promise in this area, allowing one to prepare standardized multi-component arrays of biomolecules that can retain their biorecognition properties once transferred to a surface. A key issue pertains to the potential of such nanostructures in medical diagnostics, and at present it is unclear what advantages such structures will offer for clinical applications. In principle, one can use smaller sample volumes and achieve higher sensitivity due to the small size of the entire array and the individual features that comprise the array. Such improved detection systems would enable the diagnosis of infection with HIV-1 in the setting of mother-to-child transmission, for example, where small sample volumes and the presence of immune complexes consisting of passively transferred maternal antibodies and HIV-1 antigen present diagnostic challenges. Polymerase chain reaction (PCR) and other forms of target amplification have enabled the development of powerful tools for detecting and quantifying HIV-1 nucleic acid targets for clinical diagnosis and prognosis. Though simpler to perform, conventional immunoassays for HIV-1 Gag p24 cannot achieve this level of sensitivity. Herein, we show how DPN-fabricated nanoarrays of modified monoclonal antibodies against HIV-1 p24 can be used to detect the protein in plasma samples using gold nanoparticles modified with polyclonal anti-p24 IgG as probes in a three-component sandwich (Scheme 1). These data illustrate our capability to detect and measure HIV-1 p24 antigen by a nanoarray-based assay that exceeds the limit of detection of conventional ELISA-based immunoassays and provides a level of sensitivity comparable to a PCR-based assay without target amplification.

In a typical experiment, a nanoarray for the HIV-1 immunoassay was fabricated by initially patterning 16-mercaptohexadecanoic acid (MHA) into dot features as small as 60 nm (10 × 10 spot array) on a gold thin film using DPN. The large spacing between features improved our ability to locate the original pattern after reaction with biomolecules or gold nanoparticle probes. At pH 7.4, the
MHA is deprotonated\(^{24}\) and therefore the nanofeatures are negatively charged. To minimize nonspecific binding of proteins on the inactive portions of the array, the areas surrounding the MHA patterned features were passivated with PEG-alkythiol (11- mercaptoundecyl-tri(ethylene glycol)) by placing a droplet of a 1 mM ethanolic solution of the surfactant on the patterned area for 2 h followed by copious rinsing with ethanol and, then, Nanopure water. After passivation, mouse monoclonal antibodies to the HIV-1 p24 antigen (anti-p24, 200 \(\mu\)g/mL, 10 mM PBS (phosphate buffered saline), Abcam, Cambridge, UK) were immobilized on the patterned MHA dot features by immersing the template in a solution containing the anti-p24 IgG for 1 h. The substrate was then vigorously rinsed with 10 mM PBS and Tween-20 solution (0.05%). It is well known that IgG will adhere to the negatively charged deprotonated MHA surface features through electrostatic interactions (Scheme 1 and Figure 1A), thus retaining its biological activity toward its target antigen.\(^{25,26}\) Unmodified MHA features were passivated with BSA (10% solution in 10 mM PBS) to prevent unwanted binding from plasma samples.

In a typical assay, an anti-p24 nanoarray was immersed in a plasma sample containing HIV-1 p24 from the AIDS Clinical Trials Group Virology Laboratories Quality Assurance Program (VQA, serially diluted to yield from 200 pg/mL to 0.2 pg/mL, in 0.5% Triton X-100 in RPMI 1640 media) for 1 h. The binding of the protein to the nanoarray of anti-p24 IgG was confirmed by AFM, which showed a modest height increase (2.3 \(\pm\) 0.6 nm (\(n = 10\))) for each of the features within the array (Figure 1B). To amplify the signal associated with p24 antigen binding to the array, gold nanoparticle probes, which were heavily functionalized with polyclonal antibodies to the p24 antigen, were reacted with the nanoarray by soaking the array in a solution containing anti-p24 IgG coated gold nanoparticles (20 nm, 10 nM in 10 mM PBS) for 1 h. We used AFM to interrogate the antibody chips by measuring height differences between features that have reacted with the HIV-1 p24 and gold nanoparticles and features that have not. A significant topography change in (20.3 \(\pm\) 1.9 nm (\(n = 10\))) accompanied the nanoparticle binding to the captured p24 molecules (Figure 1C). This increase in height was consistent with a 1:1:1 reaction between monoclonal anti-p24 IgG absorbed onto the MHA features on the substrate, HIV-1 p24, and polyclonal anti-p24 IgG functionalized gold nanoparticles.

To validate selectivity, anti-p24 nanoarrays incubated in the presence of plasma samples without HIV-1 p24 and then queried with the anti-p24 IgG gold nanoparticle probes showed no height increase for the anti-p24 features.

**Scheme 1.** Schematic Representation of the Immunoassay Format Used to Detect HIV-1 p24 Antigen with anti-p24 Antibody Nanoarray

**Figure 1.** HIV-1 p24 antigen (0.2 pg/mL) detection with a nanoarray. (A) Anti-p24 IgG protein nanoarray. Topography trace of adsorbed anti-p24 IgG (6.4 \(\pm\) 0.9 nm (\(n = 10\))) on MHA, showing a height profile consistent with a monolayer of anti-p24 IgG. (B) After p24 binding to anti-p24 IgG, an average height increase of 2.3 \(\pm\) 0.6 nm (\(n = 10\)) for the IgG features is observed. (C) p24 detection after amplification with anti-p24 IgG coated gold nanoparticles (20 nm). An average topographic change of 20.3 \(\pm\) 1.9 nm (\(n = 10\)) is observed.
To assess the applicability of this new approach, the nanoarray-based immunoassay was used to screen plasma samples from HIV-1-infected (n = 8) and uninfected (n = 10) men enrolled in the Chicago component of the Multimember AIDS Cohort Study (MACS). The study participants infected with HIV-1 were selected based on their levels of HIV-1 RNA in plasma measured by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). The levels of plasma HIV-1 RNA ranged between 3,500 copies per ml of plasma to less than 50 copies per ml of plasma. Because the HIV-1 p24 Gag antigen is bound by p24-specific antibodies soon after primary infection and becomes undetectable in the majority of people with disease, we treated 90 μL of each sample with glycine hydrochloride (90 μL, 1.5 M, pH 2.4, PerkinElmer, Inc.) to dissociate the immune complex followed by neutralization with TRIS-hydrochloric acid (1.5 M, pH = 8.3, PerkinElmer, Inc.) and permit detection of the protein with the nanoarray.27 A topographic signal, consistent with nanoparticle binding, for more than 60 of the 100 spots was considered positive for HIV-1 p24, and an array that showed less than 10% binding (10 of 100 spots) was considered nonspecific and therefore negative.

After immune-complex dissociation to disrupt the HIV-1 antigen–antibody complexes, we could detect HIV-1 p24 antigen in a 1 μL sample of plasma from all eight men infected with HIV-1 and none of the ten uninfected controls in less than six hours. Duplicate assays showed reproducible results. HIV-1 p24 was not detected in samples from the uninfected controls or from men infected with HIV-1 who had less than 50 copies of RNA per ml of plasma by a conventional ELISA-based immunoassay (Supporting Information). The height profile in AFM topography verified p24 Gag protein binding to anti-p24 IgG features and the anti-p24 IgG functionalized gold nanoparticles (Figure 2 A, B, and C). Because gold nanoparticles are good electron microscopy labels, we imaged them by field-emission scanning electron microscopy (FE SEM) to verify their selective complexation to the captured target protein (Figure 2D). Measurable amounts of HIV-1 p24 found in plasma from men with less than 50 copies of RNA per mL (corresponding to 0.025 pg per mL) show that the nanoarray-based assay exceeds the limits of detection of conventional ELISA-based immunoassays (5 pg per mL of plasma).27,28

Thus, the nanoarray-based assay successfully achieved highly...
sensitive and selective detection of HIV-1 in microliter-scale volumes of plasma.

The availability of sensitive and specific detection methods to identify HIV-1 in clinical specimens is highly desirable. This is the first example of a clinical application of a nanoarray in biodetection with real patient samples. In its present format, the assay is qualitative, with a level of sensitivity that rivals the current generation of RT-PCR-based assays for detection of HIV-1 in plasma. Furthermore, the assay only requires one microliter of input sample to get the reported results, a particular advantage for repeated testing with the blood volumes obtained from infants and children. Although these data show proof-of-concept, we expect that this platform can be extended to the detection of other pathogenic microbes in microliter-scale volumes of clinical sample in parallel. The nanoarray-based assay has the desired sensitivity, whereas antibodies of matched avidity and affinity will achieve the desired biological analyte selectivity. When coupled to a simple detection method with a high probe-to-target ratio, the application of an immunoassay using nanotechnology to detect HIV-1 p24 antigen in small volumes of peripheral blood from people at-risk for or infected with HIV-1 could have significant impact as a medical diagnostic.

Acknowledgment. The authors thank Sam Wu for assistance with the RT-PCR assay to detect HIV-1 RNA in plasma. The polyclonal antibody anti-p24 was obtained from infants and children. Although these data show proof-of-concept, we expect that this platform can be extended to the detection of other pathogenic microbes in microliter-scale volumes of clinical sample in parallel. The nanoarray-based assay has the desired sensitivity, whereas antibodies of matched avidity and affinity will achieve the desired biological analyte selectivity. When coupled to a simple detection method with a high probe-to-target ratio, the application of an immunoassay using nanotechnology to detect HIV-1 p24 antigen in small volumes of peripheral blood from people at-risk for or infected with HIV-1 could have significant impact as a medical diagnostic.

Supporting Information Available: Table of immunologic data for the eight men infected with HIV-1, the HIV-1 patients’ sample information, and the method to prepare antibody-functionalized Au nanoparticles. This material is available free of charge via the Internet at http://pubs.acs.org.

References


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Nanopatterning the Chemospecific Immobilization of Cowpea Mosaic Virus Capsid

Jennifer C. Smith,† Ki-Bum Lee,‡ Qian Wang,§ M. G. Finn,§ John E. Johnson,§ Milan Mrksich,*† and Chad A. Mirkin*,‡

Department of Chemistry and The Institute for Biophysical Dynamics, The University of Chicago, 5735 South Ellis Avenue, Chicago, Illinois 60637, Department of Chemistry and The Institute for Nanotechnology, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208-3113, and Departments of Chemistry and Molecular Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

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ABSTRACT

This paper presents a flexible approach for using Dip Pen Nanolithography (DPN) to nanopattern mixed monolayers for the selective immobilization of bioassemblies. DPN was used with a binary ink—consisting of a symmetric 11-mercaptoundecyl-penta(ethylene glycol) disulfide and a mixed disulfide substituted with one maleimide group—to pattern nanoscale features that present functional groups for the chemospecific immobilization of cysteine-labeled biomolecules. This strategy was applied to the chemospecific immobilization of cysteine mutant cowpea mosaic virus capsid particles (cys-VCPs). The combination of DPN for defining nanopatterns and surface chemistries for controlling the immobilization of ligands will be broadly useful in basic and applied biology.

The development of methods for patterning and immobilizing biologically active moieties with micrometer- and nanometer-scale control has proven integral to a range of applications in basic research, diagnostics, and drug discovery. Some of the most important advances have been in the development of biochip arrays that present either DNA,1 proteins,2 or carbohydrates.3 In related themes, the use of patterned substrates for studies of cell adhesion4,5 as scaffolds in tissue engineering6 and as components of microfluidic systems for bioanalysis is progressing rapidly.7 A remaining challenge for many applications is the development of patterning methods that combine nanoscale feature sizes with surface chemistries that facilitate both selective immobilization and preservation of the activities of patterned biomolecules. In this letter, we report a method that uses dip-pen nanolithography (DPN) to pattern self-assembled monolayers into nanoscale pattern features that present functional groups for the chemospecific immobilization of cysteine-labeled biomolecules. We apply this strategy to the immobilization of cysteine mutant cowpea mosaic virus (CPMV) particles.8,9

DPN has been used previously to create affinity templates for constructing arrays consisting of nanoscopic features of biologically active proteins.5 In a first demonstration, we used a variant of this method to create nanoarrays of wild-type CPMV. DPN was used to initially pattern a gold substrate (40-nm Au and 10-nm Ti on a silicon wafer, Silicon Sense, Inc) with an array of 150-nm circular features consisting of monolayers of 16-mercaptohexadecanoic acid (MHA, 1, Aldrich, Milwaukee, WI) (Scheme 1). All DPN patterning was done with a ThermoMicroscopes CP AFM interfaced with DPN Write (NanoInk, Chicago, IL) and conventional Si3 N4 cantilevers (Thermo Microscopes sharpened Microlever A, force constant

0.05 N/m). Tapping-mode images were taken with a Nanoscope IIIa and a MultiMode microscope from Digital Instruments. Unless noted otherwise, all DPN patterning experiments were conducted at 40% relative humidity and 24 °C with a tip–substrate contact force of 0.5 nN. A 90-µm scanner with closed-loop scan control was used to minimize piezo tube drift and alignment problems. After the circular features were written, the remaining regions surrounding those features were passivated with a monolayer of 11-mercaptoundecyl-tri(ethylene glycol) by applying a drop of the alkanethiol (1 mM in ethanol) on the patterned area for 24 h followed by rinsing with ethanol and Nanopure water (NANOpure, Barnstead/Thermolyne Corp.). The substrate was immersed in a solution containing the virus (30 µg/mL, phosphate buffered saline (PBS), pH = 7.0) for 1 h, removed, and rinsed with 10 mM PBS, Tween-20 (Sigma, St. Louis, MO, 0.05% in water), and then
Nanopure water (Scheme 2A). The resulting arrays of wild-type virions were characterized by tapping-mode atomic force microscopy (TM-AFM).

TM-AFM images reveal that the CPMV particles—which are 27-nm-diameter icosahedral structures composed of 60 heterodimeric protein subunits—are adsorbed predominantly on the MHA features, demonstrating that the 11-mercaptooundecyl-tri(ethylene glycol) monolayer is inert to interaction with the virus (Figure 1A).11 The height profile of the patterned surface shows that each raised feature has an apparent height of 20.0 ± 3.5 nm (determined from 10 particles). This is consistent with the size of a single virion, taking into account previous observations that tapping-mode images of soft materials such as proteins often give somewhat smaller vertical dimensions than would be expected on the basis of the solution-phase structure.12

Patterning methods that rely on the nonspecific adsorption of proteins to hydrophobic or electrostatic substrates often result in substantial denaturation and hence loss of biological activity. Although the loss of activity is acceptable for many immobilized format applications, it can be to a considerable drawback for proteins patterned on the nanoscale, where only a few of the biomolecules are present. To address this issue, we have integrated DPN and a functional dialkyl disulfide ink. With this ink, DPN writes monolayers that present maleimide groups among penta(ethylene glycol) groups. The latter prevent nonspecific interactions of proteins with the surface whereas the maleimide groups provide for the chemospecific immobilization of proteins that display a thiol group. We use a mixture of two dialkyl disulfides for the ink: a symmetric 11-mercaptopoundecyl-penta(ethylene glycol) disulfide (3) (98%) and a mixed disulfide substituted with one maleimide group (2) (2%) (Scheme 1). With this composition of ink, the density of the maleimide groups in the monolayer is approximately 1% among penta(ethylene glycol) groups. At these low surface densities, the maleimide groups provide for efficient and complete thiol capture through Michael addition of the thiol to the maleimide group and at the same time do not interfere with the inert properties of the ethylene glycol-terminated monolayer.13

Mutant CPMV particles have been engineered with cysteine inserts on the outer surface of the coat protein (capsid), having robust chemical reactivity toward maleimides.9 We employed one of these (designated Cys-CPMV), bearing an exposed cysteine residue at each βE–βF loop of the capsid structure and therefore at 60 widely spaced positions in all.9
for attachment to maleimide-functionalized surfaces. Surface plasmon resonance (SPR) spectroscopy\textsuperscript{14} was employed to characterize the specificity of the immobilization process. Gold films (80-nm Au and 8-nm Ti) were evaporated on microscope coverslips. Monolayers were formed by immersing substrates in an ethanolic solution of disulfides 2 and 3 for 18 h (ratio of 2:98, 1 mM total concentration). The substrates were subsequently rinsed with ethanol and dried in a nitrogen stream. SPR experiments were performed with a Biacore 1000 instrument using a flow rate of 2 $\mu$L/min at

Figure 1. (A) Tapping-mode image, zoom-in image, and height profile of a wt-VCP nanoarray (see dotted line). (B) Tapping-mode image, zoom-in image, and height profile (see dotted line) of a cys-VCP nanoarray. See text for TM-AFM conditions.

Figure 2. SPR spectroscopy data showing the chemospecific irreversible immobilization of the Cys-VCP. Experimental conditions are described in the text. The change in resonance angle, $\Delta \theta$, is plotted on the vertical axis. The scale bar applies to all data. (A) Cys-CPMV (60 $\mu$g/mL) was immobilized by the maleimide groups presented at the monolayer interface. The immobilization did not proceed to completion, and hence the linear rise represents the initial rate for the immobilization. (B) Under identical experimental conditions, wt-VCP did not bind.
25 °C with potassium phosphate buffer (pH = 7.0). Cys-CPMV buffer solutions also contained 2 mM tris(carboxyethyl)phosphine hydrochloride (TECP) to prevent aggregation through disulfide bridging.

In the SPR experiment, we first flowed phosphate buffer across the monolayer, followed by a solution of Cys-CPMV in the same buffer for 40 min and then in the original buffer for 4 min. Irreversible attachment of the virions was demonstrated by the persistence of the adsorbed SPR signal throughout the final wash with buffer (Figure 2A). A control experiment with CPMV—which does not display the reactive cysteine thiol groups—gave no immobilization and establishes the chemospecificity intrinsic to this strategy (Figure 2B). As a further control experiment, we found that SAMs were inert when exposed to concentrated solutions of fibrinogen (1 mg/mL, Sigma, St. Louis, MO), a large protein with a tendency to adsorb nonspecifically to surfaces. Taken together, these observations demonstrate the specific cysteine-mediated immobilization of Cys-VCp to the monolayers.

We have also demonstrated that DPN can be combined with the surface chemistry for the specific immobilization of Cys-CPMV to create patterned surfaces. We used DPN to generate a pattern of circular features, 150 nm in diameter, that presented maleimide groups at low density among penta(ethylene glycol) groups. A mixture of disulfides 3 and 2 (molar ratio 98:2, saturated ethanol solution) was used as the ink for DPN. The region surrounding the features was modified with a monolayer of 3 by immersing the substrate in a solution of 3 (1 mM in ethanol) for 24 h (Scheme 2B). After treatment with Cys-CPMV as described above, TM-AFM revealed that the virus particles attached almost exclusively to the circular features (Figure 1B). As before, the height profile of the patterned substrate shows that immobilized virions are 20.0 ± 4.3 nm in diameter (determined from 10 particles). Repetition of this experiment with wt-CPMV resulted in no immobilization. These results demonstrate that DPN can be used to pattern a mixed SAM that presents a functional group for chemospecific immobilization of biomolecules and at the same time maintains inertness to nonspecific protein adsorption.

This work provides an early demonstration of integrating a nanoscale patterning methodology (DPN) with surface chemistries that are tailored for biological applications. By performing DPN with a dialkyl disulfide ink mixture of 2 and 3, nanoscale pattern features presenting cysteine-reactive maleimide groups can be constructed with a reasonable degree of control over their surface density. Importantly, these sites are presented within a biologically inert background. The preservation of the native structure and biological activity of patterned proteins has been an intractable challenge in initial micrometer-scale patterning schemes.15 Often, assays on these length scales can tolerate the inefficient presentation of immobilized protein so long as a sufficient fraction of proteins are active. On the nanoscale, however, it is critical to optimize the presentation of proteins to achieve reproducible activities. We note that the resolution of the technique described here has not been optimized, and it is reasonable to expect that it will rival that of conventional DPN (10 nm).16 The ability to generate patterned arrays of biomolecules and/or bioassemblies with well-defined nanoscale features while simultaneously maintaining bioactivity will have a significant impact in areas ranging from biosensors to engineering model substrates for studying receptor–ligand interactions at cell–substratum interfaces.

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References

Multicomponent Magnetic Nanorods for Biomolecular Separations**

Ki-Bum Lee, Sungho Park, and Chad A. Mirkin*

Nanomaterials have been used extensively in the development of high sensitivity and high selectivity biodetection schemes.[1–4] Designer particles, including noble-metal polyhedral structures, quantum dots, nanopatterns, and nanorods have found application in many forms of biological tagging schemes, including DNA and protein detection, cell sorting, and histochemical staining.[5–12] Significant advantages over conventional molecule-based fluorophore strategies have been identified for several of these structures.[13–15] Other applications for nanomaterials in biology, beyond diagnostics, include therapeutic agents and separations.[9] A key area for researchers working with proteins involves separation and purification. Traditionally, nickel columns have been used in conjunction with histidine tagged proteins to separate such structures from a matrix of other undesirable biological elements. We and others have been working with nanorod structures prepared by the porous template synthesis approach pioneered by Martin and the group of Moskovits.[16,17] This synthetic procedure allows one to prepare rods electrochemically with uniform diameters and with predefined block lengths of inorganic and organic materials with excellent control.[18,19] We hypothesized that if nickel were introduced as one of the blocks in a single or multicomponent nanorod structure, it could be used as a magnetic nanosized affinity template for histidine tagged proteins and, therefore, an appropriately applied magnetic field could be used to effect separation of the protein–rod complex from a multicomponent solution. Herein, we demonstrate how one can use two-component triblock ≈ 330 nm diameter rod structures with gold end blocks and a nickel interior block as materials that can very efficiently separate His-tagged proteins from non-his tagged structures. This work builds on our work with the transport of His-tagged structures by dippen nanolithography (DPN) to bulk solid-state nickel oxide substrates and the work of others involving the generation of microscopic arrays of His-tagged proteins on bulk nickel oxide substrates.[20,21]

Magnetic multisegment nanorods composed of nickel and gold blocks were synthesized by using the method of electrodeposition into a porous alumina membrane (experimental conditions are described in the Supporting Information). The gold portions of the three-component structure were used to prevent nickel domain etching during the silver dissolving procedure. Prior to use, the rods were repeatedly rinsed with distilled water until the pH of the solution was 7.

The multicomponent nanorods were washed with methanol and ethanol to remove contaminants from their surfaces. This was done by using a magnetic field (BioMag, Polysciences, Inc.) to pull the rods to the sidewalls of a plastic vial while rinsing them with the appropriate solvent. The gold portions of the nanorods were passivated with 11-mercaptoandecyl-tri(ethylene glycol)(PEG–SH) by incubating the rod samples in 1 mL, 10 mM ethanolic solution of the surfactant for 2 h followed by copious rinsing with ethanol and then nanopure water (Barnstead International, Dubuque, IA, USA). Others have shown that alkylthiols preferentially modify the gold surface in such two component structures.[25] The gold surface was modified with PEG–SH for two reasons. First, the PEG–SH minimizes nonspecific binding of proteins to the nanorod structures.[22,23] Second, it stabilizes the rods by minimizing bare gold surface–surface interactions.

The specific interaction of polyhistidine (His x 6) with bulk oxidized nickel surface is well known.[20,21] Similarly, fluorescein-tagged poly-His (His x 6) binds specifically to the Ni portions of the substrate as evidenced by confocal fluorescence microscopy. In a typical experiment, Au-Ni-Au nanorods (10^9–10^10) were incubated in a 63 μM fluorescein labeled poly-His solution (1 mL, 0.1× PBS (phosphate buffered saline), pH 7.4) for 12 h at room temperature (22°C). Then the nanorods were vigorously rinsed with phosphate buffered saline (PBS) solution followed by nanopure water. During each rinsing step, the rods were separated from the supernatant by using magnetic force. Fluorescence imaging shows that the fluorescein-tagged polyhistidines efficiently bind to the nanorod structures (Figure 1b). This reaction between the poly-His and Au-Ni-Au rods can be monitored with the naked eye simply by watching the color of the solution decrease in intensity as a function of reaction time (Figure 1c). A quantitative analysis of the efficiency of poly-His adsorption was performed by preparing a standard calibration curve from the fluorophore-labeled poly-His over a range of concentrations starting with the experimental poly-His initial concentration of 63 μM and going to 0.16 μM (inset Figure 2). The fluorescence emission intensity of supernatant solution isolated from the reacted nickel nanorods shows that ≈ 90% of the poly-His was captured by the rods from the starting solution (Figure 2). As a control experiment, pure Au nanorods (no Ni), passivated with PEG–SH, were incubated in the poly-His solution (63 μM in 0.1x PBS, pH 7.4) under nearly identical conditions, and little interaction between the poly-His molecules and PEG–SH modified Au particles was observed (i.e., no detectable change in the emission of the fluorescein as measured by fluorescence spectroscopy, data not shown).

The Au-Ni-Au rods can be used in a novel scheme for separating His-tagged proteins from structures without His-
tags (Scheme 1a). For example, a 1 mL solution of two different proteins with different dye labels (anti-rabbit IgG without a His tag but labeled with Alexa 488 and His-tagged ubiquitin labeled with Alexa 568; concentration of each protein = 100 μg/mL⁻¹, in 0.1 M PBS, pH 7.4) were prepared (Figure 1d, far left, orange solution). The orange solution formed after release of the red-dye labeled His-tagged ubiquitin in the eluent buffer.

**Figure 1.** a) A field emission SEM image of Au-Ni-Au rods. b) A confocal fluorescence image of Au-Ni-Au rods after modification of Ni blocks with fluorescein-tagged poly-His. c) A picture of two cuvettes showing solutions of fluorescein-tagged poly-His before (left-hand side) and after (right-hand side) exposure to the Au-Ni-Au rods. d) Left: Orange solution containing dye-labeled His-tagged (Red, Alex 568-tagged ubiquitin)) and untagged (green, Alex 488-labeled anti-rabbit IgG) proteins before exposure to nanorods. Middle: After exposure to the Au-Ni-Au rods, the solution is green, indicating removal of the red-dye labeled His-tagged ubiquitin. Right: The red solution formed after release of the red-dye labeled His-tagged ubiquitin in the eluent buffer.

Biotech., Inc.) was added to the vessel containing the nanorods coated with His-tagged, Alexa 568-labeled ubiquitin. This addition results in the release of the His-tagged proteins from the nanorods and the formation of a red supernatant (Figure 1d, right). Each of the solutions discussed above was studied by UV/Vis and fluorescence spectroscopy and compared with each other. The spectrum of the mixture of proteins before the addition of nanorods shows two bands at λ_max = 516 nm and 600 nm that correspond to the two dye labels. After addition of the nanorods, there is an 86 % decrease in the signal (λ_max = 600 nm) for the dye label associated with the His-tagged protein and only 6 % for the protein without the His tag (λ_max = 516 nm). After adding the eluent buffer, which is at pH 2.8 and results in the release of proteins from the Ni surface, we see a strong signal at λ_max = 600 nm associated with the His tagged proteins. Fluorescence spectroscopy shows that 56 % of the original His-tagged ubiquitin has been effectively separated from the two component mixture in pure form (Figure 3).

**Figure 2.** Fluorescence spectra corresponding to Figure 1c, before and after separation of poly-His with Au-Ni-Au rods: Inset shows the standard curve and the arrows represent each concentration of poly-His before and after separation. NE is normalized emission, arbitrary units; A is the integrated area, arbitrary units.

**Figure 3.** Fluorescence spectra corresponding to Figure 1d. Solid lines represent a fluorescence spectrum of a mixture of dye labeled His-tagged and untagged proteins. Dashed traces show a spectrum of the supernatant after removal of His-tagged proteins with Au-Ni-Au rods. Dotted traces show a spectrum of the product generated by releasing the His-tagged proteins into eluent buffer.
Finally, Poly-His tagged Au-Ni-Au rods are bioactive and selectively react with antibodies for poly-His, Scheme 1b. Indeed, when a mixture of Alexa 488-labeled anti-human IgG and Alexa 568-labeled anti-poly-His IgG (100 μg mL⁻¹ in 0.1 M PBS, pH 7.4) was introduced to a PBS solution (pH 7.4) of the Poly-His tagged Au-Ni-Au rods, the dye-labeled anti-poly-His IgG was very efficiently removed from the solution as evidenced by fluorescence spectroscopy (see Supporting Information). After 24 h, 70% of the anti-poly-His was removed from the solution while only 4% of the signal associated with the anti-human IgG is lost. The rods can be attracted to the side of the reaction vessel with a magnetic field allowing one to remove the supernatant. The anti-poly-His can be released from the rods by adding eluent buffer at pH 2.8. Acidic conditions are known to decrease the specific interaction between the antibody and poly-His.[24]

We have demonstrated that Ni-containing nanorods can be used as novel materials for the efficient separation of mixtures of biomolecules by exploiting the chemical and physical properties of these nanostructures. The Ni portion of the nanostructure provides a docking site for His-tagged proteins and is ferromagnetic, allowing one to selectively and efficiently remove them from solution with an appropriately applied magnetic field. The gold ends are used to chemically protect the nickel interior and provide a second platform for functionalization. Histidine tagging of proteins has been broadly adopted throughout the molecular biology and biochemistry communities, and this novel and straightforward separation scheme provides an alternative to Ni chromatographic columns and potentially has a much greater degree of tailorability through choice of nanorod block compositions and surface coatings.

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