

# Nanopatterning the Chemospecific Immobilization of Cowpea Mosaic Virus Capsid

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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,<sup>1</sup> proteins,<sup>2</sup> or carbohydrates.<sup>3</sup> In related themes, the use of patterned substrates for studies of cell adhesion<sup>4,5</sup> as scaffolds in tissue engineering<sup>6</sup> and as components of microfluidic systems for bioanalysis is progressing rapidly.<sup>7</sup> 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 proteins. We apply this strategy to the immobilization of cysteine mutant cowpea mosaic virus (CPMV) particles.<sup>8,9</sup>

DPN has been used previously to create affinity templates for constructing arrays consisting of nanoscopic features of

biologically active proteins.<sup>5</sup> 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 Si<sub>3</sub>N<sub>4</sub> 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- $\mu$ m 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  $\mu$ g/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

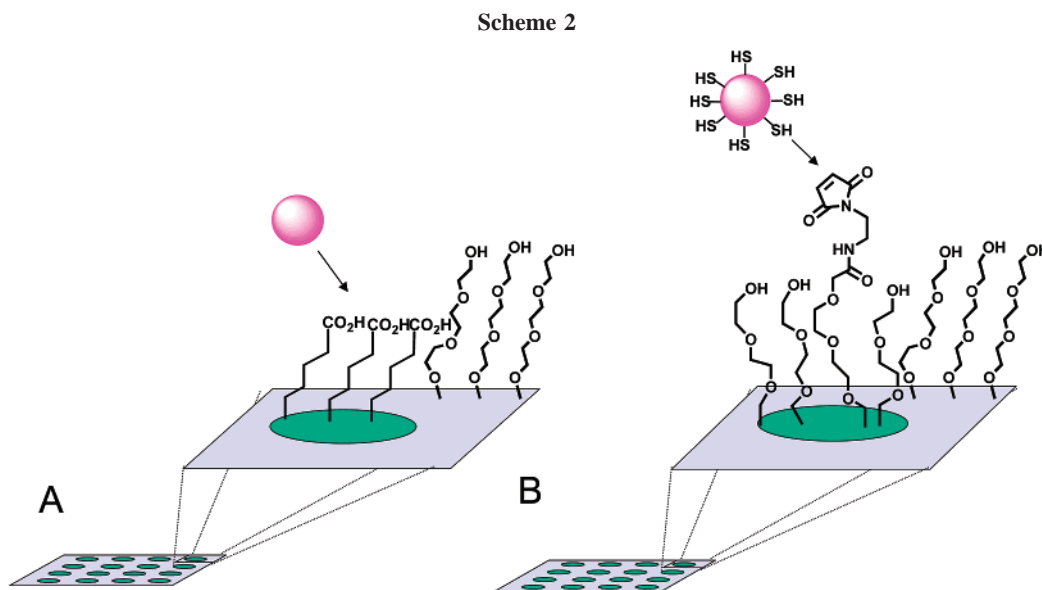
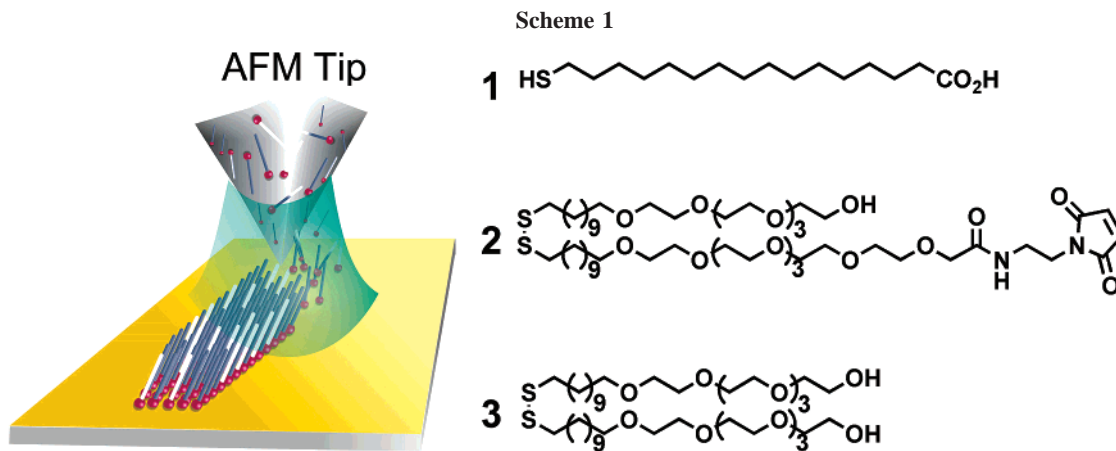
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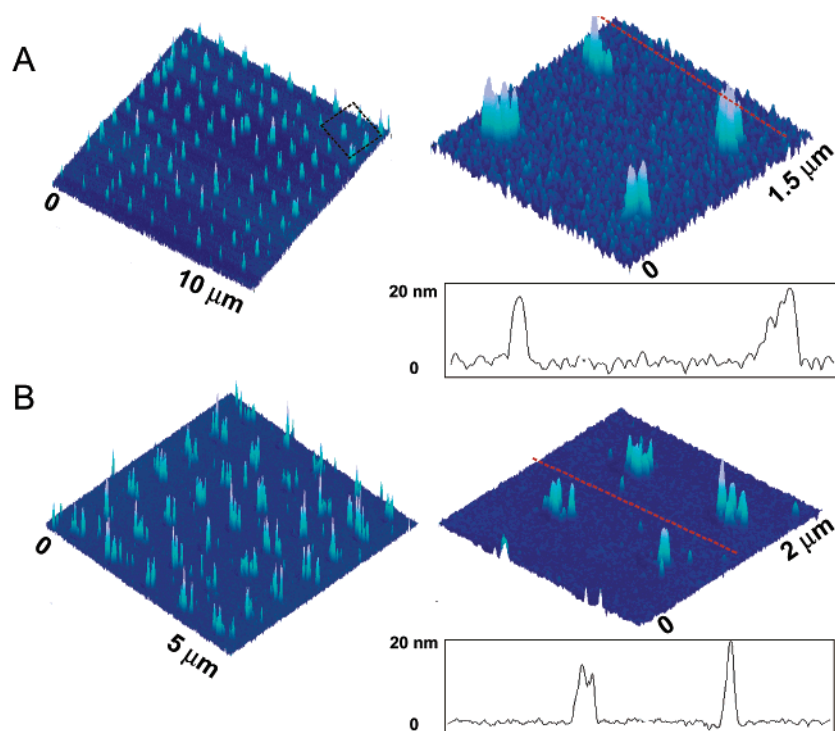
Nanopure water (Scheme 2A). The resulting arrays of wild-type virions were characterized by tapping-mode atomic force microscopy (TM-AFM).

TM-AFM images reveal that the CPMV particles—which are 27-nm-diameter icosahedral structures composed of 60 heterodimeric protein subunits<sup>10</sup>—are adsorbed predominantly on the MHA features, demonstrating that the 11-mercaptoundecyl-tri(ethylene glycol) monolayer is inert to interaction with the virus (Figure 1A).<sup>11</sup> The height profile of the patterned surface shows that each raised feature has an apparent height of  $20.0 \pm 3.5$  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.<sup>12</sup>

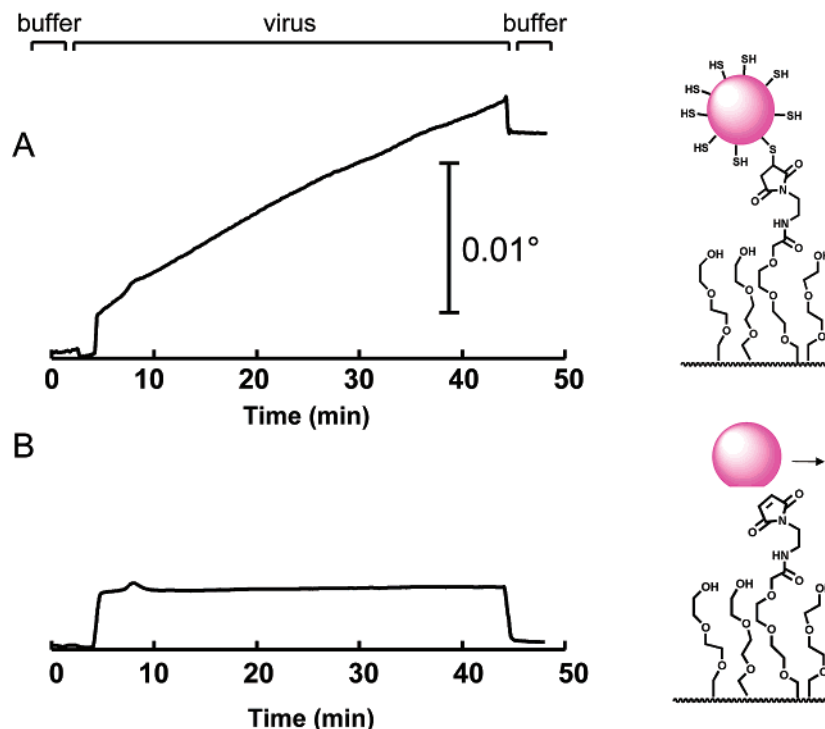
Patterning methods that rely on the nonspecific adsorption of proteins to hydrophobic or electrostatic substrates often result in substantial denaturation and hence loss of biological activity. Although the loss of activity is acceptable for many immobilized format applications, it can be to be a considerable 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-mercaptoundecyl-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.<sup>13</sup>

Mutant CPMV particles have been engineered with cysteine inserts on the outer surface of the coat protein (capsid), having robust chemical reactivity toward maleimides.<sup>9</sup> We employed one of these (designated Cys-CPMV), bearing an exposed cysteine residue at each  $\beta E-\beta F$  loop of the capsid structure and therefore at 60 widely spaced positions in all,<sup>9</sup>



**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 ( $60 \mu\text{g}/\text{mL}$ ) 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.

for attachment to maleimide-functionalized surfaces. Surface plasmon resonance (SPR) spectroscopy<sup>14</sup> 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 immers-

ing 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  $2 \mu\text{L}/\text{min}$  at

25 °C with potassium phosphate buffer (pH = 7.0). Cys-CPMV buffer solutions also contained 2 mM tris(carboxyethyl)phosphine hydrochloride (TCEP) 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  $20.0 \pm 4.3$  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.<sup>15</sup> 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).<sup>16</sup> 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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