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Virus Patterning

Polymer-Coated Tips for Patterning of Viruses by Dip-Pen Nanolithography**

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Methods have recently been developed to allow the study of protein–protein and cell–surface interactions on a molecular level.[1] These techniques rely on nanoscale arrays for high-throughput analysis of biological functions.[2] Researchers working in areas including biosensors, biodiagnostics, genomics, proteomics, cytology, materials science, and general chemistry have benefited from these advances.[3] To further drive this technology, high-precision patterning techniques are necessary to allow a wider range of biomolecules to be deposited onto a substrate. A variety of methods including UV lithography,[4] electron-beam lithography,[5] contact printing,[6] nanoimprinting,[7] and scanning probe lithography[8] have been used to deposit biomolecules on various surfaces with nanometer accuracy. One such technique is dip-pen nanolithography (DPN).[9] DPN is a scanning probe lithography method based on atomic force microscopy (AFM). Using DPN, nanostructure patterns of extraordinary complexity can be created. This technique has potential applications for gene chips and multiple biomolecular arrays because one can create patterns with numerous molecules in a direct-write fashion.[10]

The driving force of DPN for transporting materials is molecular diffusion through the water meniscus formed between the AFM tip and the surface.[9] Although DPN provides a simple and efficient method to deposit DNA, peptides, and proteins on surfaces at nanoscale resolution, most DPN methods struggle with higher-molecular-weight biomolecules.[11] Molecules larger than 15 nm (the maximum size of most immunoglobulin G proteins) are not efficiently transported through this water meniscus. Recently, many strategies have been employed to solve this problem by modifying tip surfaces,[12] and by adding carrier materials to ink solutions.[13] For example, Mirkin and co-workers published a description of a polymer-pen lithography (PPL) method,[14b] in which elastomeric tips without cantilevers are used to deliver ink solutions. These tips, which are made entirely of poly(dimethylsiloxane) (PDMS), make it possible to control feature sizes by varying the tip–substrate contact time and contact force.

However, no general methods have been developed that would allow the direct-write DPN technique to create patterns of large-sized biomaterials such as viruses. Herein, we describe a novel and effective method for fabricating porous polymer-coated AFM tips, which overcome the shortcomings of DPN, such as the difficulty of transporting large bioparticles and the limited amount of ink solution that can be delivered.[12] Using this tip, we have generated patterns of adeno-associated viruses (AAVs).

Our approach uses a nanoporous poly(2-methyl-2-oxazoline) (PMeOx)-coated tip (Scheme 1).[11] A bare SiO_2 AFM tip was first modified with 11-iodoundecyltrichlorosilane as an initiator through a silanization reaction. This active layer was then used to initiate the ring-opening polymerization of 2-methyl-2-oxazoline monomer, which resulted in the formation of nanopore network structures. A typical scanning electron microscopy (SEM) image of the fabricated PMeOx-coated tip shows that the pore sizes ranged from 50 nm to a few hundred nanometers (Figure 1a). The AFM image of PMeOx, taken under ambient conditions, showed similar nanopore structures (Figure S1 in the Supporting Information).

PMeOx is a well-characterized, hydrophilic, biocompatible, and water-soluble nonionic polymer that is often used for drug delivery.[15] A variety of PMeOx hydrogels can be formed by chemically cross-linking a hydrophilic polymer matrix through covalent bonds or by physically cross-linking with hydrogen bonds.[16] In our method, when the PMeOx-coated AFM tip is brought into contact with an aqueous ink solution, the surface-immobilized porous polymer layer absorbs the ink solution and forms a swollen hydrogel. Because the AAV used

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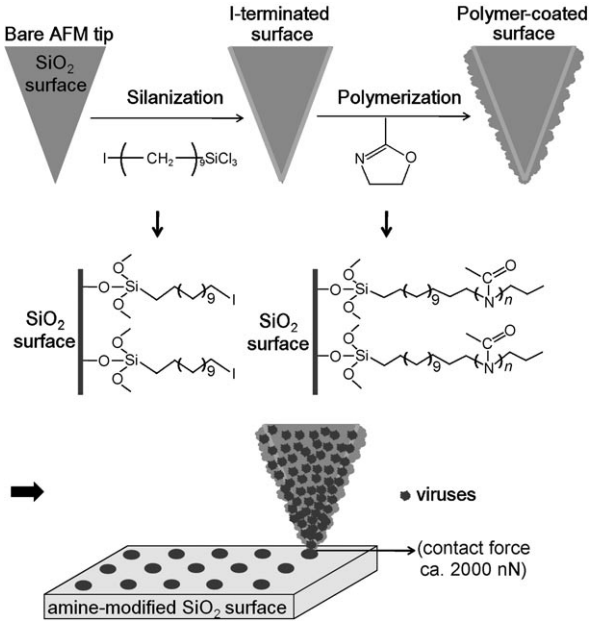

Scheme 1. Stepwise fabrication of a nanoporous PMeOx-coated tip. A bare SiO_2 AFM tip was modified with 11-iodoundecyltrichlorosilane by a silanization reaction, thus forming an I-functionalized monolayer. The I-terminated initial active layer was then used to initiate the ring-opening polymerization of 2-methyl-2-oxazoline monomer. The patterning illustration shows the DPN process.

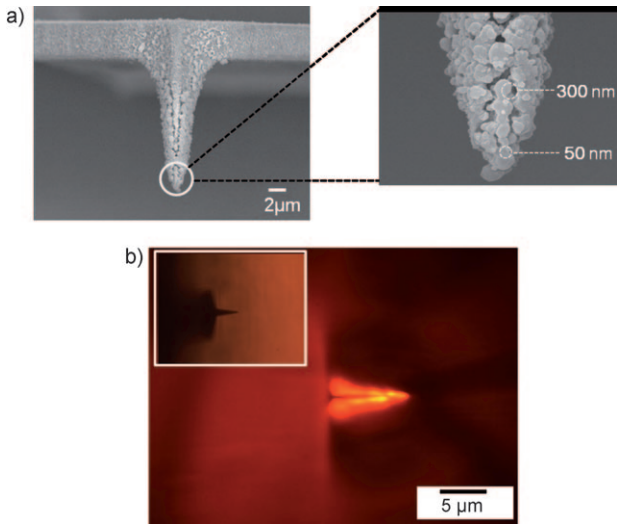

Figure 1. a) Typical SEM image of a nanoporous PMeOx-coated tip. b) Fluorescence microscopy image of a swollen hydrogel PMeOx-coated tip. The tip was placed in contact with a Cy-3-labeled AAV ink solution for 1 min using the AFM approaching method. Inset: optical microscopy image.

in these experiments is labeled with a fluorophore, we can visualize tip saturation by using fluorescence microscopy (Figure 1 b). In addition, because the hydrogel absorbs the ink solution, the ink and associated bioparticles are not surface-exposed and are thus less likely to dry out and become denatured.

The novelty of our approach comes from the use of this hydrogel PMeOx-coated tip for DPN. When the ink-absorbed tip is brought into contact with the amine-functionalized substrate surface with a high tip–substrate contact force, the ink solution diffuses out of the nanoporous gel and onto the surface. The diffusion barrier within this hydrogel is very small in comparison with the classical DPN (meniscus-mediated ink diffusion) method, which allows the transport of larger biomaterials. Importantly, diffusion from the hydrogel is enhanced by the PMeOx, which is known to exhibit biomolecule-repellent "stealth" behavior.[16] Furthermore, the diffusion out of the hydrogel is also driven by the electrostatic interactions of biomaterials with the substrate.

In a typical DPN experiment, a nanoporous PMeOx-coated tip was immersed in AAV solution (1.14×10^{12} particles mL^{-1} in phosphate-buffered saline at pH 7.3) with glycerol (5 %) for 1 min. The AFM-mounted tip was then brought into contact with a substrate surface. Patterning of AAV particles was performed at room temperature at a relative humidity of 15–30 % and a contact time of 1 s. Our system does not require extra environmental equipment such as a glove box, because the pattern size is not significantly influenced by humidity.

As a proof-of-concept experiment, we generated AAV nanostructures on amine-terminated silicon oxide surfaces through a general electrostatic immobilization approach under the above experimental conditions (Figure 2 a).[10a] The height profile of AAV dots showed that each AAV feature was 30 nm high, consistent with the monolayer size of AAV particles. Monolayer formation (as opposed to a multilayered pattern) was most likely because the AAV features were rinsed with deionized water after patterning. To confirm these results, we performed the same ink-loading and DPN experiments with bare and amine-functionalized tips.

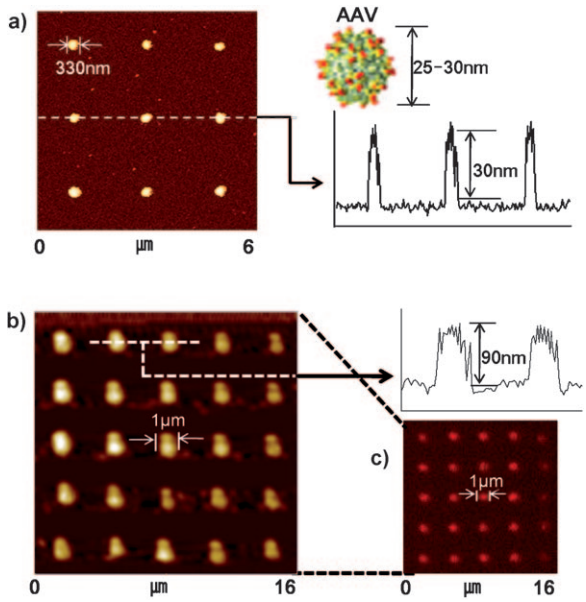

Figure 2. a) AFM topographic image and corresponding height profile of an AAV array created by DPN with a PMeOx-coated tip. The tip–substrate contact force and contact time were 2000 nN and 1 s, respectively. b,c) AFM and fluorescence microscopy images, respectively, of a Cy-3-labeled AAV dot array deposited by a stamp tip with 1300 nm end size (contact time 5 s).

No patterns were produced with these tips, even though virus particles adhered to the tip surface (Figure S2 in the Supporting Information).

During the AAV patterning study, we found the most important factor controlling the transport of AAV particles was the tip–substrate contact force. Following initial trials, the contact force was set at 2000 nN. Experiments with contact forces lower than 100 nN produced very low density virus patterns or no patterns at all. This force-dependent ink transport process is analogous to PDMS PPL.[14]

Another finding was that the pattern size was almost independent of the contact time of the tip on the surface for AAVs. This is similar to a study by Velders and co-workers who obtained the same results with higher-molecular-weight proteins using a porous layer-by-layer polymer tip.[12]

To confirm that AAV particles were successfully transported from the tip onto the surface, we labeled the AAV particles with a fluorophore (Cy-3) and generated a 25-dot AAV array in the same way. To allow the AAV particles to be visualized by fluorescence microscopy, larger dots were necessary. To this end, we made a larger PMeOx-coated tip with a tip-end size of approximately 1300 nm (tip-end size of SiO_2 bare tip: 1000 nm) and created patterns of AAVs with this tip at a contact time of 5 s (Figure 2b,c,). The AAV features generated with a tip contact time of 5 s (without rinsing) were about 90 nm, which corresponded to three layers of AAV particles and thus to an increased AAV density (not the dot size). An AAV array consisting of 25 1-μm-diameter dots spaced 3 μm apart was visualized by fluorescence microscopy. In contrast to conventional DPN with small ink molecules, many of the dots patterned in this study did not have uniform edges. An explanation may be that the transport in our case seems to be initiated by physical stamping with a high tip–substrate contact force. Surprisingly, we were able to generate more than 1000 AAV dots without a re-inking process (Figure S3 in the Supporting Information), which indicates that the PMeOx-coated hydrogel tip can act as an ink reservoir for the aqueous ink solution. From this result, we infer that AAV particles are transported to the emptied part of the tip end during DPN patterning.

Although this approach successfully demonstrated the patterning of high-molecular-weight virus particles, it revealed that feature sizes could not be easily adjusted during the patterning process. Conventional DPN allows the size of the pattern nanostructure to be adjusted by changing the contact time of the tip on the surface and the humidity level.[9] To solve this problem, we fabricated tips of different sizes by changing the reaction conditions of polymerization (Figure 3a–c).

Three I-functionalized silicon tips were placed in a 1 % (v/v) acetonitrile solution of 2-methyl-2-oxazoline monomer and incubated for 2 h, 30 min, or 10 min. SEM images showed that the corresponding tip-end diameters were 500, 300, and 100 nm, respectively. To demonstrate that the tips could create different dot sizes, we generated AAV patterns under the same conditions (contact time 1 s, contact force 2000 nN) using the three newly prepared tips. The resulting AFM images showed that the dot diameters of the AAV nanostructures corresponded to the tip-end sizes. Using the 500, 300, and 100 nm tips, AAV dots 400, 200, and 80 nm in diameter, respectively, were generated (Figure 3d–f). This indicates that it is possible to create variably sized patterns by altering the tip size and adjusting the polymerization reaction times of the tip surfaces.

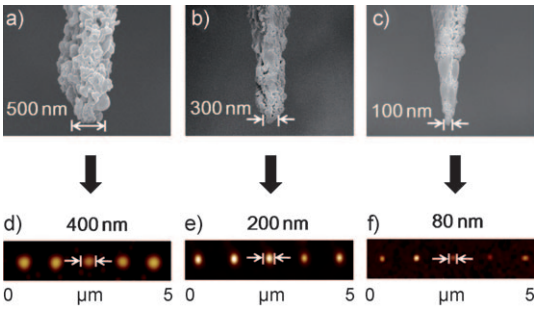

Figure 3. PMeOx-coated tips of different sizes and their corresponding dot patterns. a–c) SEM images of the PMeOx-coated tips prepared under different reaction conditions; incubation times were: a) 2 h, b) 30 min, and c) 10 min. d–f) AFM images of dot patterns of AAV molecules. The dots were generated by the corresponding tips with a tip–surface contact time of 1 s.

In conclusion, we have developed a straightforward and effective DPN method that uses a nanoporous PMeOx-coated tip to generate patterns of large-sized viruses. To the best of our knowledge, this is the first time a DPN direct-write technique has been used to pattern such large biomaterials. The size of the PMeOx-coated tip can be easily adjusted by changing the polymerization reaction time. This allows the size of the pattern features to be controlled. We demonstrated this by creating several nanoarrays of large virus particles, with dot sizes ranging from 80 to 400 nm in diameter. Using our technique, nanoarrays (or microarrays) of virus particles can be produced in a flexible, precise, and rapid manner.

Experimental Section

The surfaces of silicon oxide AFM tips (M2N, Inc., Korea; spring constant = 40 N m^{-1}, Model STP4) were cleaned with piranha solution, and then immersed for 1 h in a toluene solution of 10 mM 11-iodoundecyltrichlorosilane to form a surface-active monolayer for polymerization of 2-methyl-2-oxazoline monomer. PMeOx-coated tips were prepared according to previously reported procedures.[11] Amine-modified surfaces were prepared by cleaning oxidized silicon wafers with piranha solution for 10 min, washing them with pure water, and then treating them with 10 mM *N*-2-aminoethyl-11-amino-undecyltrimethoxysilane for 30 min.

AAVs were prepared according to previously reported procedures.[17] Cy-3 reactive dye pack (GE Healthcare, Waukesha, WI, USA) was used to label the AAVs.

All "stamp-on" DPN patterning was carried out with a XE-100 AFM system (Park systems, Inc., Korea). Tapping-mode AFM images of the generated patterns were obtained by using the same XE-100 AFM system, with conventional bare silicon oxide cantilevers (model STP30). Fluorescence images were obtained with a confocal laser scanning microscope system (LSM 510, Zeiss).

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