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# Porous Multilayer-Coated AFM Tips for Dip-Pen Nanolithography of Proteins

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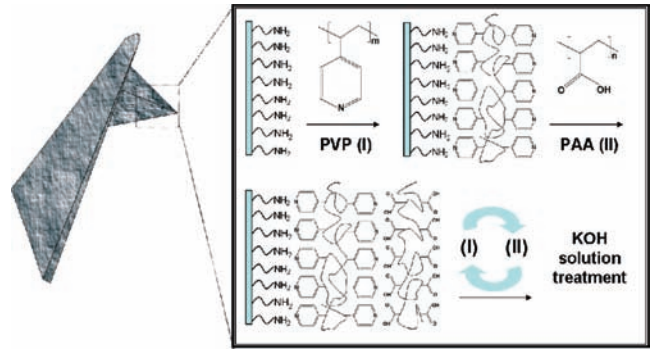
Dip-pen nanolithography (DPN) is a high-resolution patterning technique that enables the preparation of patterns at length scales from <100 nm to several micrometers.[1] Ink molecules coated on a tip are transported to a substrate via a water meniscus formed between the tip and the substrate.[1] Since the invention of DPN, many kinds of ink, including alkylthiols,[1,2] proteins,[3,4] DNA,[5] particles,[6] collagens,[7] and others,[8] have been deposited on gold, glass, and silicon oxide surfaces, enabling a range of applications, including direct fabrication of micro- and nanopatterns of biomolecules on surfaces in a controlled manner.[9] Although the DPN method provides a simple and straightforward way to fabricate biomolecular patterns, this approach is restricted by some drawbacks, such as the slow diffusion of high molecular weight molecules, the limited amount of ink, and the difficulty of maintaining functional biological properties of molecules on a dried tip.[10] Several modifications of tip surfaces[3,10] and tips[11] in order to overcome these restrictions have been reported. Here we present a new and simple approach that uses layer-by-layer (LbL) self-assembly techniques to form hydrophilic porous structures on the surface of $Si_3N_4$ AFM tips; this increases the volume of the ink reservoir and maintains the biomolecules' functionalities.

LbL self-assembly has attracted increasing attention because of its simplicity, versatility, and systematic control over the thickness and underlying structure of the resulting layer.[12] By immersion of the LbL multilayer in solutions with different pHs or ionic strengths, porous structures at the micrometer and nanometer length scales have been formed.[13,14] An attractive feature of this method is that the pore size and the thickness of the porous structure can easily be tuned by altering parameters such as the polymer concentration, the pH of the alkaline solution, and the duration of base treatment. Such porous structures have been shown to function as a reservoir for inks to be transferred to a substrate by microcontact printing.[15]

The preparation of the porous LbL tip is shown in Scheme 1.[13] Bare $Si_3N_4$ cantilevers were first functionalized with an *N*-[3-(trimethoxysilyl)propyl]ethylenediamine self-assembled monolayer (TPEDA−SAM) to allow interaction with the polymer via hydrogen bonding. The TPEDA−SAM-functionalized AFM cantilevers were alternately incubated in poly(4-vinylpyridine) (PVP) and poly(acrylic acid) (PAA) methanol solutions to assemble LbL15 films based on hydrogen bonding between the pyridine and carboxylic acid groups ("15" in the label LbL15 denotes the number of PVP−PAA bilayers). These LbL15-functionalized cantilevers were subsequently immersed in a KOH solution (pH 13) for 5, 10, or 15 min to dissolve the PAA in the multilayer. Because of its poor solubility in the alkaline solution, the PVP remained, forming a porous structure on the cantilever surfaces.

***Scheme 1.*** Procedure for Preparation of a Porous LbL AFM Tip




The porous LbL tips fabricated by varying the duration of the treatment in KOH solution were examined by scanning electron microscopy (SEM) (Figure 1a−c) and atomic force microscopy (AFM) in tapping mode (Figure 1d−f). Larger pore sizes and more apparent porous network structures were obtained upon longer treatment with alkaline solution. The pore sizes ranged from ∼50 to a few hundreds of nanometers as determined by AFM. The thickness of the porous film was 20−30 nm. These observations are similar to those for the porous structures fabricated on silicon substrates reported by Fu et al.[13] The SEM images also show that the LbL structures make the tip more blunt than a bare $Si_3N_4$ tip. Nevertheless, the radius of the porous tip was ∼100 nm, which is within the acceptable length scale for performing DPN experiments.

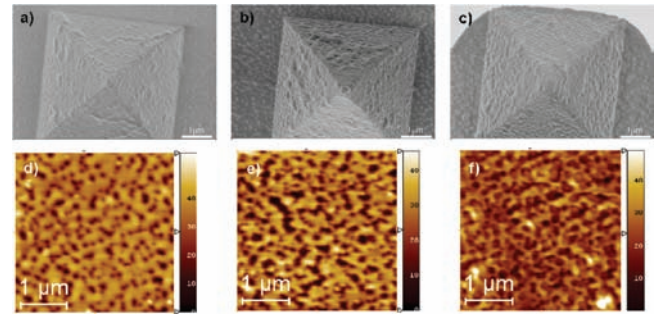



***Figure 1.*** (a−c) SEM images and (d−f) tapping-mode AFM height images of LbL15-functionalized tips incubated in KOH solution for (a, d) 5, (b, e) 10, and (c, f) 15 min (see the SI for more details).

Porous LbL tips were examined for their potential application in transferring biomolecules via DPN. Proof-of concept experiments were performed by generating His-tagged visible fluorescent protein (VFP) patterns on TPEDA−SAM- or nickel(II) nitrilotriacetic acid-

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terminated self-assembled monolayer (Ni-NTA−SAM)-functionalized glass substrates through electrostatic interactions or the formation of metal ion−imidazole bonds, respectively. Two proteins with different molecular weights, His-tagged enhanced green fluorescent protein (His-EGFP, 27 kDa) and His-tagged tetrameric reef coral fluorescent protein (His-DsRed, ∼100 kDa), were used. The intrinsic fluorescence of the VFPs can be used as an indicator of structural and functional integrity, since structurally damaged or misfolded proteins do not fluoresce. Comparison of the fluorescence of porous and bare tips loaded with VFP clearly shows the higher loading capacity of the former [see the Supporting Information (SI)].

A His-EGFP-impregnated porous tip was brought into contact with a TPEDA−SAM-functionalized surface to write a 25 $\mu$m wide square in one move with an atomic force fluorescence microscope (AFFM) at ∼60% relative humidity (RH) with a writing speed of 25 $\mu$m s$^{-1}$. The resulting fluorescent pattern was immediately imaged in situ by the AFFM. Without a reinking process, the same His-EGFP square pattern (Figure 2a) was generated more than 20

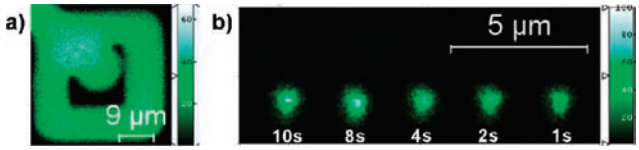


***Figure 2.*** (a) One of 21 fluorescence images acquired in situ after His-EGFP square patterns were created on a TPEDA−SAM-functionalized surface using DPN. (b) Fluorescence images of His-EGFP dot patterns generated on a Ni-NTA−SAM-functionalized surface. The dots were successively generated using different tip−surface contact times, as listed below each spot (see the SI for details).

times. These results successfully demonstrate that the porous LbL tip acts as a sponge-like reservoir for the aqueous protein solution,[15] maintaining the protein functionality and enabling ink release during the short tip−substrate contact period. Moreover, His-EGFP dot patterns with smaller feature sizes were successfully generated on Ni-NTA−SAM-functionalized surfaces by reducing the RH to ∼45% (Figure 2b). We observed that reducing the tip−surface contact times did not significantly influence the diameters of the deposited dots. This result suggests that the transport of ink molecules from the tip to the substrate cannot be explained simply by the diffusion model of DPN. The porous LbL tips were rinsed with Milli-Q water after use and re-examined by SEM to confirm that no apparent polymer transfer occurred during the DPN experiments (see the SI).

To further demonstrate the general applicability of the porous LbL tips, a higher molecular weight biomolecule, His-DsRed, was also used to create patterns on the Ni-NTA−SAM-functionalized glass substrates. A porous tip impregnated with His-DsRed was brought into contact with the surface for a contact time of 10 s at ∼35% RH. The inked porous tip was used to generate 42 spots and was retracted after depositing each dot. No reinking process was performed during the entire writing sequence. The topographical information about the protein patterns was revealed by tapping-mode AFM (Figure 3); to avoid contamination, a new $Si_3N_4$ AFM tip was used for imaging. The dot diameters of the His-DsRed array were 200−300 nm.

In summary, a simple method for fabricating surface-modified nanoporous AFM tips using the LbL technique has been presented.

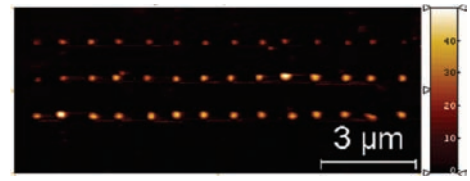


***Figure 3.*** Tapping−mode AFM image of His-DsRed dot patterns acquired immediately after DPN with a porous LbL tip. The dot patterns were successively generated from left to right and from top to bottom (zigzag) using a tip−surface contact time of 10 s (see the SI for details).

The thickness of the porous film and the pore sizes can easily be adjusted. We have successfully demonstrated that aqueous fluorescent protein solutions can easily be absorbed into the porous LbL tip while retaining the biological activity of the proteins for extended periods. Furthermore, the pore structures provide a large-volume ink reservoir for DPN experiments. His-EGFP and His-DsRed patterns at micrometer and submicrometer scales were successfully fabricated with these porous tips. Different parameters for tuning the pore sizes and properties are currently under investigation in order to study the mechanisms of DPN and writing with different inks, such as nanoparticles and high-molecular-weight biomolecules.

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**Supporting Information Available:** Experimental details and additional results. This material is available free of charge via the Internet at http://pubs.acs.org.

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