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Spore-Terminated Cantilevers for Chemical Patterning on Complex Architectures

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S Supporting Information W Web-Enhanced

ABSTRACT: Atomic force microscope tips terminated with spore cells are used to directly pattern onto glass and tissue surfaces. The spore cells act as sponges and eliminate the need to use microfabricated ink reservoirs during lithography.

Scanning probe-based lithography has been widely used to explore and manipulate micro- and nanoscale surfaces.[1] Many efforts have been directed toward engineering microfabricated devices that can function as ink cartridges and well systems.[2] Here we present a simple modification scheme that allows one to place ink reservoirs on cantilevers for chemical patterning by utilizing living spores of *Bacillus subtilis*. The structure and morphogenesis of spore cells have been studied since 1976.[3] Generally speaking, spore cells are considered one of the most durable cell types. Studies have shown that they can survive low and high humidity, starvation, radiation, and many chemical agents.[4] *B. subtilis* is composed of a mother cell surrounded by a coat.[5,6] The structures that comprise the spore are responsible for the high resistivity against environmental fluctuations. The three main substructures of the spore are the core, the cortex, and the coat. Historically, the coat and the cortex have been thought to act as protective layers.[5] Work has been performed to characterize spores with scanning probe[7] and thermogravimetric analysis (TGA).[8] Our strategy is founded on the spore's ability to respond to a set of environmental or physical changes. We utilize the spore's ability to adsorb water and small molecules in a hydrated environment[9] and its resilience to heating or drying.[10] Studies have examined spore cells as part of sensor platforms,[11] but to the best of our knowledge no one has used them directly as an active component of a lithographic strategy such as dip-pen nanolithography (DPN).[12]

The spore surfaces are negatively charged and can be placed on tipless cantilevers. Prior to placement, one needs to silanize the cantilevers in order to terminate the surface on protonated amines, a procedure commonly done on many oxide surfaces (see Supporting Information (SI)).[13] Our approach is novel and incredibly cost effective because it does not require any microfabrication but simply the use of extremely durable bacteria that can be handled with no setup. To localize the spores to primarily one side of the cantilever, the tips were flattened against a silicon oxide surface. The natural self-assembly of the spores facilitated the sheet-like attachment of the spores. By varying the length of the tip over the edge of the surface, the size of the spore layer at the end of the tip can fluctuate (Figure 1). Using this method, it is conceivable that different orientations and placements of the spore layer could easily be achieved by simply altering the amount of spore solution, the tip placement, or the tip orientation. The SI displays the drying edge of the spores. This drying edge varies in width depending upon the amount of spores present. By placing the edge of the drop of solution over the end of the cantilever, one can easily place a layer of spores on a single side of the flattened cantilever. Thus, by continually increasing the concentration, volume, or layering of spores, one can produce a wider and denser layer of spores. Figure 1 displays two different tips with a large or small spore layer. The tips can also be completely coated with spores. The spores' tight attachment to the cantilever makes them incredibly difficult to break free. Only the spore located at the lowest point is used for patterning, as shown in Figure 1. The tips we fabricated remained intact after repeated cycles of patterning.

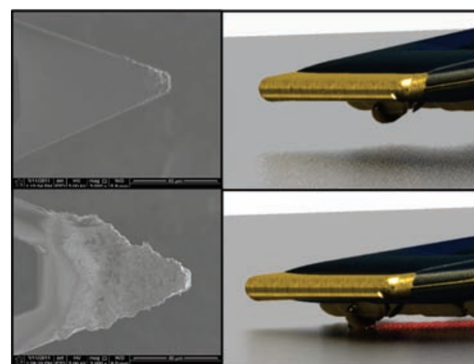

Figure 1. (Left) Spore-terminated tips: (top) small versus (bottom) large amount of spores. (Right) DPN where the lowest spore on the cantilever will be the primary contact point.

Prior to tip immobilization, we verified the spores' response (swell, adsorb water) to changes in the environmental humidity using TGA (Q500 V6.7 Build 203 instrument). Experiments were done with *B. subtilus*, 10^7 CFU (Raven Labs, Omaha, NE). TGA measures the change in sample mass with heating over time. Spore samples were exposed to relative humidity of 23% up to 60%. Immediately following this, the samples were heated, and the mass loss was measured. The runs were repeated for each condition, and the standard deviations are presented. Figure 2 demonstrates that the spores lose more water (mass) as they are heated with higher environmental humidity. The utility of the spore-terminated tips for chemical patterning was tested by performing proof-of-concept experiments using a fluorescent

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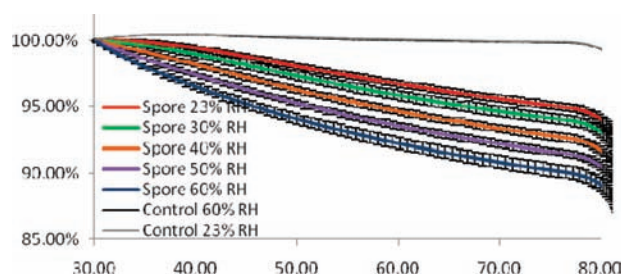

Figure 2. Percent mass loss vs temperature. Samples were heated after being exposed to increased humidity in the environment.

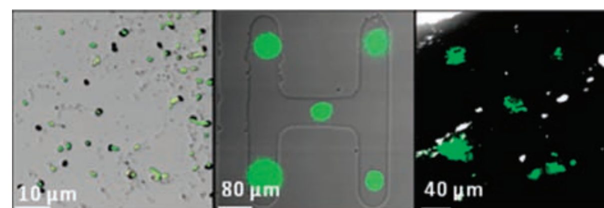

Figure 3. (Left) Spore cells soaked with dye solution. (Middle) Representative patterning on glass. (Right) Representative patterning on a tissue surface.

Ⓦ A video related to this figure is available in the HTML version.

dye, 12-NBD stearate (12-*N*-methyl-(7-nitrobenz-2-oxa-1,3-diazo)-aminostearic acid, Avanti Polar Lipids, Alabaster, AL). Others have patterned lipid dyes using conventional AFM tips.[14] Imaging of the spore tips prior to patterning reveals that the spores contain more ink than the surrounding surface, an initial indication that the spores can act as ink carriers. Gerhardt[15] demonstrated that spores are permeable to solutes with low molecular masses. After the tip was washed with copious amounts of chloroform, the dye in the spores remained (Figure S3a−c). The unique capabilities of the spore tips as a patterning tool for DPN are evident from photobleaching experiments that confirmed the protective nature of the spore tips (Figure S3d−f). Regular tips with no spores were coated with the dye and could be completely photobleached after 10 min of exposure. In contrast, tips or glass slides terminated with spores were photobleached under identical conditions and still maintained fluorescence from the 12-NBD stearate even after 30 min of exposure.

The spores were also dried on a glass coverslip and then inked with 12-NBD stearate and washed. Figure S2a,b shows spore samples which had been washed with water and chloroform, respectively, and still highly fluoresce. Indeed, the SI displays two spore samples, one with and one without inking, showing clearly the spore's internal fluorescence due to the dye. Spores can also adsorb the dye while in solution, as shown in Figure 3 (left). Here spores mixed in a 50:50 dye:spore solution for 1 h were dried on a slide and immediately washed with chloroform. Following washing, the spores produced readily observable fluorescence, and similar results were observed for spores treated by either inking method. The same experiments were performed with spores already mounted on the AFM cantilever (see SI).

DPN[16] was carried out on glass and tissue surfaces. The need to perform DPN on a variety of surfaces has been previously recognized.[17] First, a clean glass coverslip (Electron Microscopy Sciences, Hatfield, PA) was patterned with 12-NBD stearate (Figure 3, middle). The patterning rate and size can be adjusted by changing the humidity, scan size, frequency, and force as with regular DPN. Small submicrometer line patterns are also easily produced (Figure S4). In Figure 3 (middle), a five-dot pattern was produced on top of the letter "H" on a photoetched glass coverslip. We have produced lithographic patterns on both simple and complex surfaces to demonstrate the utility of the spore-terminated cantilevers. The tissue onto which we patterned directly (Figure 3, right) was derived from the inner collagenous membrane of the Bruch's membrane in eye samples from pig donors. Our group has demonstrated the use of DPN on this complex surface,[18] and the SI provides details. Patterning becomes more formidable when one considers tissues' uneven and chemically heterogeneous nature on both local (Figure S5, 10 μm image of the tissue) and general (10−100 μm) length scales. The 10 μm patterns produced in Figure 3 show the versatility of DPN for a variety of fields, including tissue engineering. It is becoming more and more apparent that true tissue engineering will require the ability to place desired molecules on engineered scaffolds.[19] The uneven nature of these tissues interferes with obtaining clear images of all planes of the surface. Figure 3 demonstrates the best plane for imaging of the pattern; however, a 3D image is provided in the SI (Figure S6) which shows the complete pattern. The spores' properties can also be utilized to adsorb a solute from a surface. In such an application, the tip would function as a soaking sponge to form patterns similar to ones produced by nanografting or the AFM stamp tip.[20] Indeed, this may explain the formation of hollow patterns we observed in some of our exploratory experiments (Figure S7). Diffusion across cell membranes is controlled by a number of mechanisms, including active diffusion. One can envision relying on active diffusion through the cell wall to initiate or terminate a patterning procedure in future efforts.

In conclusion, we report a lithographic procedure that relies on the spores' ability to adsorb water and small molecules under variable humidity conditions.[21] We have presented a single potential function of these tips, namely DPN. The ease of production, variability, and durability of these tips means they can be utilized by many groups with diverse needs. Setlow et al. have shown that small molecules are released in significant amounts during stage II of germination.[21] They have identified glutamic acid and arginine among the amino acids released. We have shown that such amino acids can be patterned by DPN.[22] In future efforts one can monitor their release while the spores are swelling and taking up water during the germination process if AFM tips terminated with the spores are used in a DPN diffusion study.

■ ASSOCIATED CONTENT

Ⓢ **Supporting Information.** Additional data and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

Ⓦ **Web Enhanced Feature.** A video related to Figure 3 is available in the HTML version.

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