Direct writing of molecularly imprinted microstructures using a nanofountain pen

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Molecularly imprinted polymers (MIPs) constitute a very appealing avenue to parallel sensors of a multitude of small target molecules due to their stability, relative ease of preparation, and their ability to recognize targets for which natural capture molecules do not exist. The authors present here a way of arraying MIP structures with micrometer dimensions, using nanofountain pen, and demonstrate their functionality using a fluorescent template molecule. © *2007 American Institute of Physics*. [DOI: 10.1063/1.2730753]

Micro- and nanobiochips are drawing recent interest, as miniaturization is expected to increase their portability, thus expanding the use of these arrayed biosensors to point-of-care clinical testing, environmental monitoring, security, etc. The recognition elements in biosensors are usually enzymes or antibodies. However, these molecules are usually unstable and moreover, a natural receptor for the particular target analyte of interest may not always exist. Thus, researchers have long sought the creation of *de novo*, tailor-made receptors for a desired molecular target. One surprisingly simple way of generating artificial receptors is through the molecular imprinting of synthetic polymers.[1–3] Here, the target molecule (or a derivative thereof) acts as a molecular template around which interacting and cross-linking monomers are arranged and copolymerized to form a castlike shell. After polymerization and removal of the template, binding sites complementary to the target molecule in size, shape, and position of functional groups are exposed and their conformation is preserved by the cross-linked structure. Thus, the polymer can selectively rebinding the target. For multisensors and biochips, molecularly imprinted polymers (MIPs) have to be patterned on surfaces and interfaced with a transducer. For this purpose, two different strategies can be considered. The prepolymerization mixture can be deposited in a precise pattern on the transducer surface and then polymerized *in situ* or prepolymerized MIP nanoelements are deposited. Patterning methods that may be used with MIPs are soft lithography, microspotting techniques, or localized polymerization. There has been a report on the use of soft lithography in combination with MIPs, using poly(dimethylsiloxane) (PDMS) stamps.[4] However, current imprinting recipes are not always compatible with the PDMS stamps used, which tend to swell in certain organic solvents. There is no report as yet on localized, *in situ* polymerization for the preparation of MIP arrays. One possible approach could be an extension of laser polymerization, used by Conrad *et al.* for microstereolithography of MIPs.[5]

Alternatively, it should be possible to apply standard microspotting techniques such as ink jetting[6] or mechanical microspotting[7] to the deposition of MIP arrays on a surface. Arrays of silicon microcantilevers have been used to deposit biomolecules onto glass slides.[8] The diameter of the dots can vary but is normally in the higher micrometer range. In order to obtain smaller dots, it should be possible to use techniques such as dip-pen nanolithography (DPN) or the nanofountain pen (NFP), which have been shown to be very useful in creating nanoscale patterns of biomolecules due to their high spatial precision. DPN consists of the dipping of an atomic force microscope (AFM) probe in an "ink." The ink is transferred to the substrate by capillary transport.[9] Inks such as proteins,[10] polymers,[11] DNA,[12] and active enzymes[13] have been used to create nanometric patterns. With NFP,[14] AFM tips are replaced by cantilevered nanopipettes. The pipettes are filled with the solution to be deposited and the liquid flows to the substrate when the pipette is placed in contact with the surface. Thus creating structures of below micrometer size. NFP has been used to deposit proteins,[15] active enzymes,[16] DNA,[17] and polymers.[18]

Here we describe a proof of principle showing the use of NFP for the deposition of MIP structures with micrometer dimensions on flat surfaces. Commonly employed transduction methods, such as surface plasmon resonance or quartz crystal microbalance, are not sensitive enough to detect such minute amounts of analyte. However, the development of ultrasensitive transduction methods is beyond the scope of this work. Therefore, in order to show the feasibility of this approach, we used a model system, where we have chosen a fluorescent target analyte—fluorescein. This enabled us to directly observe its recognition by MIP structures in fluorescence microscopy and avoid further complications imposed by a transduction system.

Trimethylolpropane trimethacrylate (TRIM), 4-vinylpyridine (4-VPy), diethyleneglycol dimethylether (diglyme),

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dimethylsulfoxide (DMSO), and poly(vinyl acetate) (PVAc, molecular weight=140 000 g/mole) were from Sigma-Aldrich. 4-VPy was vacuum distilled before use. 2,2-dimethoxy-2-phenylacetophenone (DPAP) and fluorescein were from Fluka. N-doped silicon wafers were from Pi-Kem. Polished glass microscope slides were from Hellma.

For dot deposition by NFP, a prepolymerization mixture was prepared as follows: 6 mg of fluorescein was first solubilized in 100 μl of DMSO. After sonication, 30 μl of this mixture (5.42×10^{-6} mol of fluorescein) was pipetted into 50 μl of porogen (pure diglyme, diglyme containing 5% PVAc, or DMSO containing 5% PVAc). 69 μl of TRIM (2.16×10^{-6} mol) was then added, followed by 11 μl of 4-VPy (1.02×10^{-6} mol) and 5 mg of DPAP. Untreated and silanized glass surfaces and silicon wafers as well as gold-coated glass surfaces were tested for dot deposition. For silanization, the surfaces were incubated in a 2% v/v solution of 3-trimethoxysilyl-propylmethacrylate in toluene for 12 h. Gold-coated glass surfaces were silanized in the same manner, after a first coating with mercaptopropyltrimethoxysilane (5 mM in acetonitrile for 12 h).

Dot deposition was performed under ambient conditions using a Nanonics NSOM/AFM 100 system (Nanonics, Jerusalem, Israel) with a flat scanner and two coaxial optical microscopes that allowed examination of the sample simultaneously from above and below. NFP probes were cantilevered nanopipettes, 300–600 nm aperture diameter, Cr/Au covered cantilevers of 500–600 μm length (Nanonics, Jerusalem, Israel). Following deposition, drops were polymerized in a closed compartment under argon atmosphere using a 6 W low-pressure UV lamp (254 nm, Vilber Lourmat) at a distance of 3 cm from the light source for 5 min. Polymer dots were characterized by AFM. AFM probes were "Ultrasharp" gold-covered silicon contact cantilevers (Mikromasch, CSC12/CR-Au/15).

In order to remove the imprinting template, the surfaces with MIP and control polymer dots were washed in DMSO followed by rinsing in acetone until the fluorescence was reduced below detection level. Rebinding experiments were performed by incubation in a 0.52 mg/ml solution of fluorescein in DMSO for 90 s, quick rinse with acetone, and drying. Fluorescein binding to the dots was quantified by fluorescence microscopy (Zeiss Axioplan2 microscope with a 20×, 0.5 numerical aperture (NA) objective or 40×, 1.3 NA oil objective, and a SPOT (Diagnostic Instruments Inc.) digital camera). Images were analyzed using the IMAGEJ software.[19] For the study of several sequential binding and unbinding steps, the 1×1 cm^2 substrates were mounted in a Teflon flow cell with a glass window and operations done *in situ* under the microscope.

To assess whether an imprinted polymer can be obtained with this choice of components, a bulk MIP and a corresponding nonimprinted control polymer (same composition as the MIP, but lacking the template molecule fluorescein) were first synthesized. Equilibrium binding assays with fluorescein revealed indeed an imprinting effect, that is, the MIP bound significantly more fluorescein than the control at polymer concentrations ranging from 0.1 to 30 mg/ml (data not shown).

A pipette with a 600 nm aperture was used in order to deposit large structures that are easily visible under an optical microscope onto silicon. Diglyme was first chosen as the main porogenic solvent. The solution tended to spread on the silicon surface following deposition and the shapes of the resulting dots were not well defined [Fig. 1(a)]. In order to increase the viscosity of the mixture and limit its spreading on the surface, we added a linear polymer (PVAc) to the porogenic solvent (diglyme). Small amounts of PVAc in the porogenic solvent had been previously used by our group to accelerate phase separation and thus improve pore formation during fast photopolymerization of molecularly imprinted thin films.[20] As shown in Fig. 1(b), the shape of the dot obtained with 5% PVAc is much more regular, but the dots are still rather large (40 μm in diameter). A further improvement was obtained when a more hydrophilic mixture was deposited, where diglyme was replaced by DMSO, resulting in smaller dots [Fig. 1(c)].

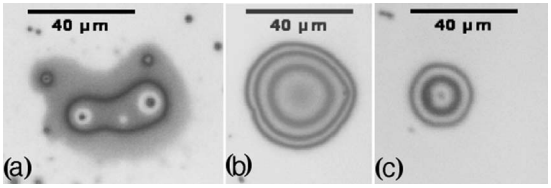

FIG. 1. Influence of the viscosity and of the wetting properties of the mixture on the shapes of dots deposited onto a silicon wafer using prepolymerization mixtures containing different solvent systems: (a) diglyme, (b) diglyme containing 5% PVAc, and (c) DMSO containing 5% PVAC.

Studying a dot like the one in Fig. 1(b) with AFM reveals the dimensions of the dot are 40 μm diameter and 5 μm thickness [Fig. 2(a)] and also the porous morphology of the dots [Fig. 2(b)] due to the presence of PVAc in the porogen, as expected. The pores are between 150 and 200 nm in diameter, as determined from the AFM image. As shown in Figs. 2(c) and 2(d), dots lacking PVAc are not porous.

Following optimization, we found that 5% PVAc in DMSO as porogen with TRIM, 4-VPy, and fluorescein was a perfectly balanced mixture in terms of viscosity and surface wetting, which permits the accurate depositions of smaller dots. As we have previously reported, the contact time between the pipette and the surface can be used to precisely control the size of the features.[18] Indeed, using the above mixture and a shorter contact time (2 s instead of 8 s), we

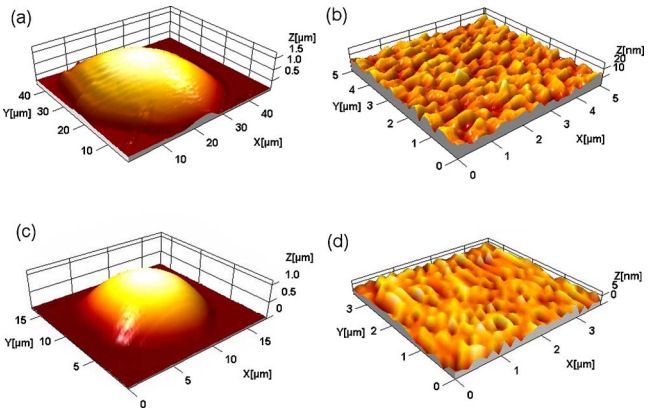

FIG. 2. (Color online) (a) Contact-mode AFM image of a dot deposited using 5% PVAc in diglyme; (b) zoom onto the dot surface, *Z* range is 20 nm. (c) A control dot, lacking PVAc in diglyme; (d) zoom onto the surface of the dot in (c), *Z* range is 5 nm. The porosity induced by the presence of PVAc in the porogenic solvent is clearly observed: average rougness of (d) is 0.9 nm while that of (b) is 2.75 nm [vertical range of (b) is 20 nm and that of (d) is 5 nm].

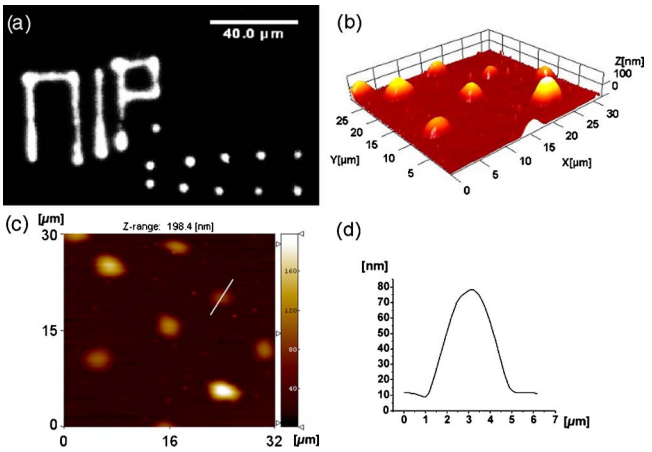

FIG. 3. (Color online) (a) Fluorescent image of polymerized dots and lines (the word "MIP") drawn with NFP filled with an imprinting mixture containing 5% PVAc in DMSO and fluorescein as the template on a nonsilanized silicon wafer; (b) contact-mode AFM image of the dots (surface plot); (c) cross section through one dot, showing its profile (4 μm diameter and 70 nm thickness).

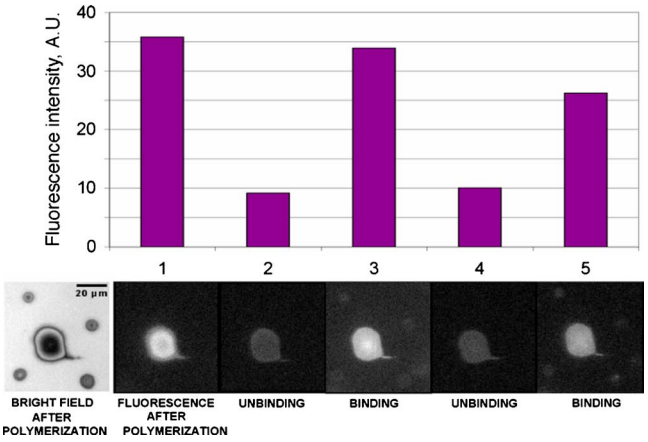

FIG. 4. (Color online) Lower panel: Bright field and fluorescence images of binding-unbinding cycles obtained with a MIP dot surrounded by four control dots. Upper panel: The height of the bars indicate the average fluorescence intensity (total intensity divided by the area of the dot), as extracted from the images.

were able to deposit arrays of dots with diameters in the micrometer range and thicknesses in the tens of nanometer range. It was also possible to draw lines and shapes that are visible under the fluorescence microscope. As can be seen in Fig. 3, the dots are approximately 4 μm in diameter and 50–80 nm thick. Smaller features, although easy to fabricate, are difficult to image and quantify in fluorescence microscopy due to the small amount of fluorescent molecules present in the dot.

Silicon wafers and gold surfaces provided more controllable features, compared with bare glass surfaces. Silanization of silicon or glass surfaces did not seem to have much impact on the shape of the dots or on their adherence to the surface. Therefore, nonfunctionalized surfaces were subsequently used.

The ability of the MIP dots to recognize the template was studied by writing imprinted and nonimprinted (control) dots next to each other. The control dots were made half the size of the imprinted ones in order to clearly identify them in light microscopy. MIP and control dots were exposed to the same solution in the same environment. The samples were imaged immediately after polymerization, then they were incubated in DMSO to elute the template. For rebinding studies, the surface was exposed to 0.52 mg/ml of fluorescein in DMSO for 90 s. A series of binding and unbinding cycles is shown in Fig. 4. The fluorescence intensity was quantified as the *average* count per pixel (sum of all counts divided by the number of pixels making up the dot), for both the imprinted and nonimprinted dots, thus eliminating the dependence of intensity on the size of the dot. The MIP dots showed bright fluorescence after each incubation in fluorescein, whereas the control dots showed only very little fluorescence (nonspecific adsorption). These data indicate that unlike the nonimprinted dots, the dots polymerized in the presence of the fluorescein template are able to bind fluorescein and thus behave like MIPs.

We have demonstrated the feasibility of creating structures of molecularly imprinted polymers, consisting of dots and lines, using nanofountain pen. The thickness of the dots, in the range of tens of nanometers, allows for quick access and releasing of the template due to short diffusion paths. It should be easily possible to further reduce the diameter of future MIP nanoarrays, upon design of more sensitive transduction systems. We believe that this technique has a strong potential for the fabrication of highly integrated biomimetic nanochips as well as other types of integrated biosensors.

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