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Writing Droplets of Molecularly Imprinted Polymers by Nano Fountain Pen and Detecting Their Molecular Interactions by Surface-Enhanced Raman Scattering

Keren Kantarovich,[†] Inbal Tsarfati,[‡] Levi A. Gheber,[‡] Karsten Haupt,[§] and Ilana Bar*[,†]

Department of Physics, Department of Biotechnology Engineering, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel, and Compiègne University of Technology, UMR CNRS 6022, France

Molecularly imprinted polymer (MIP) droplets were printed using a pipet or a nano fountain pen on surface-enhanced Raman scattering (SERS)-active surfaces, to directly monitor the uptake and release of a template molecule, the *β*-blocking drug propranolol, by SERS. The monitored SERS bands can be related to the template, allowing for its detection but also identification in the MIP. This is an advantage if the technique is to be used during the development phase of MIPs as microstructures, but equally for the readout of MIP-based biochips.

Microbiochips or micrometer dot arrays of biological molecules are drawing considerable interest[1] for their possible use in clinical diagnostics, food analysis, and production monitoring as well as in the fields of biological, pharmaceutical, and environmental detection. In these biochips the target analyte is initially bound by a recognition element, being in most cases a biomacromolecule like an enzyme or an antibody. However, for some applications, biomacromolecules are not appropriate since they tend to be unstable out of their native environment. Moreover, for certain target analytes a natural receptor may not exist or may be difficult to obtain in pure form.

One of the most appealing approaches to make synthetic hosts is that of molecular imprinting of polymers, which enables generation of synthetic macromolecular receptors.[2−5] In this technique, monomer units assemble around or are attached to a template (imprint) molecule and subsequently are linked together using a cross-linking agent. Template removal, ideally, leaves cavities within the molecularly imprinted polymer (MIP), possessing a shape and functional group complementarity to the imprint molecule and offering its tight and selective uptake. For use in biochips, these MIPs have to be patterned on surfaces and interfaced with optical or electrochemical sensors platforms. It is thus of marked interest to develop methods for MIP patterning and for their direct measurement.

We have previously demonstrated that the nano fountain pen (NFP)[6] can be employed to write arrays of functional MIP droplets (∼4 μm in diameter). The NFP method uses a cantilevered nanopipette, mounted as a probe of an atomic force microscope (AFM), delivering minute volumes of solution in selected positions. In that case we chose a fluorescent target molecule (fluorescein) for fluorescence-based readout.[7] Although this study provided a proof of concept for the approach, it should be noted that it is limited to detection of fluorescent targets.

Another optical method that might be considered for MIP readout is Raman scattering[8,9] and, even more so, surface-enhanced Raman scattering (SERS),[10−17] which provides higher levels of sensitivity. These techniques have the advantage that they do not require labeling and that they produce specific vibrational spectra characteristic of the compound adsorbed in the MIP dot. In consequence, they can be used for its identification by comparison to reference spectra.

We have very recently[18] demonstrated the feasibility of using Raman spectroscopy to detect and quantify binding of a target molecule to a MIP with high accuracy, while measuring Raman signal signatures from ground bulk MIP, without surface enhancement. Here, we describe the use of the NFP for the deposition of

* To whom correspondence should be addressed.
[†] Department of Physics, Ben-Gurion University of the Negev.
[‡] Department of Biotechnology Engineering, Ben-Gurion University of the Negev.
[§] Compiègne University of Technology.

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MIP droplets and the detection of target binding to the droplets by SERS. We have chosen the β-blocking drug (*S*)-propranolol as a model target for which we have prepared a MIP. The polymer is identical to the one used in our earlier publication[18] describing Raman measurements on MIPs and nonimprinted control polymers. The MIP was deposited on the gold-coated patterned surface of a SERS-active substrate. This deposition, combined with Raman and SERS for detection and characterization of the template and of the template molecule bound to the MIP, offers the advantage of testing these droplets for application in sensors on a microscale and for promoting the concept of miniaturized arrayed sensors.

EXPERIMENTAL SECTION

Materials. The materials used for MIP preparation include trimethylolpropane trimethacrylate (TRIM), methacrylic acid (MAA), diethyleneglycol dimethylether (diglyme), poly(vinyl acetate) (PVAc MW 140 000), the template (*S*)-propranolol, and 2,2-dimethoxy-2-phenyl acetophenone (DPAP). All these materials were purchased from Sigma-Aldrich, except DPAP which was from Fluka.

After establishing the best conditions for preparation, bulk MIPs were synthesized by UV photopolymerization from a mixture containing TRIM (0.0964 μmol), MAA (0.0964 μmol), (*S*)-propranolol (0.0192 μmol), DPAP (5 mg), and 0.168 μL of diglyme containing 2% PVAc. The polymers were deposited by pipettes (500 μm diameter) or NFPs (see below) on the border between the smooth gold-coated surface and on the patterned surface of SERS substrates, respectively. These SERS substrates are engineered on the basis of a photonic crystal, namely, Klarite (D3 Technologies Ltd.), comprising grids of pyramidal wells with 2×2 μm^2 aperture and $\sim$2 μm depth.

Following deposition, the drops were polymerized in a closed compartment under argon atmosphere using a 6 W low-pressure UV lamp (254 nm, Vilber Lourmat) at a 3 cm distance from the light source, for 30 min. The template was eluted by incubation in ethanol/acetic acid 9:1 for 1 h, followed by a brief dipping in ethanol. The MIP samples were then incubated in 300 μL of rebinding solution (0.03 g/L) for 40 min prior to redipping in ethanol.

NFP Printing. Droplet deposition by NFP was performed under ambient conditions using a near-field scanning optical microscope/AFM (NSOM/AFM) 100 system (Nanonics) with a flat scanner and an optical microscope, enabling the examination of the sample and precise positioning of the nanopipette on the Klarite surface. NFP probes were Cr/Au-covered cantilevers of 500−600 μm length and 300 nm aperture diameter (Nanonics). The nanopipette was filled from the back with the imprinting mixture. Following deposition, drops were polymerized as mentioned above. The polymer dots were characterized by the AFM, using "Ultrasharp" gold-covered silicon contact cantilevers (Mikromasch, CSC12/CR-Au/15).

Raman Microspectroscopy. Raman and SERS spectra of (*S*)-propranolol and MIPs deposited over the gold-coated smooth border and the SERS-active patterned grid area of the Klarite substrates were collected with a micro-Raman spectrometer (LabRam UV HR, Jobin-Yvon). The 784.9 nm excitation wavelength of a diode laser was focused onto the sample with an ×50/0.75 numerical-aperture microscope objective, with $\sim$10 mW intensity. The scattered light was redirected from the microscope through a sharp edge long wavepass filter, that rejected the excitation laser line and the elastically scattered light, and through a confocal pinhole for increased axial resolution. The scattered light was then focused into a 0.8 m dispersive spectrometer, equipped with a 600 groove/mm grating, and the scattered Raman light was detected with an air-cooled charge-coupled device (CCD), consisting of 1024×256 pixels. The fingerprint region in the spectra was monitored while scanning the spectrum across the CCD and moving the grating three times. The spectral acquisition was performed following adjustment of the zero-order position of the grating and control of the Rayleigh line position of a (100) polished single-crystal silicon wafer.

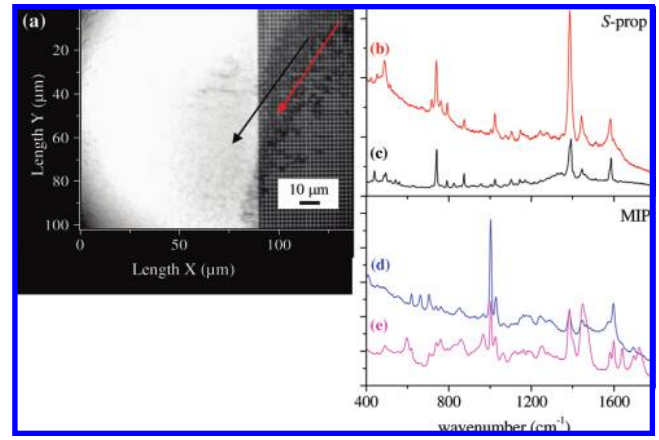

Figure 1. Micrograph of the gold-coated Klarite substrate showing the SERS-active pattern grid area (right side) and the smooth border (left side) with a macrodroplet of (*S*)-propranolol (a). The black and red arrows represent the measurement points for the Raman and SERS spectra. Representative SERS and Raman spectra of the free template (*S*)-propranolol (b and c) and of the molecularly imprinted polymer (MIP) (d and e), respectively, without background subtraction. The integration times used for the measurement of traces b, c, d, and e were 15, 120, 7, and 30 s, respectively.

To ensure the correct position of the sample relative to the objective, an adjustment was made, based on the information observed by a TV camera monitor. This camera also allowed obtaining measured micrographs of the samples. A representative SERS line map was created by scans over the droplet, using a computer-controlled *x*/*y* motorized stage (Mertzhauser) with step size of 1.5 μm. Successive recordings of Raman spectra from the samples allowed monitoring of the characteristic spectral features of (*S*)-propranolol and of the MIP following synthesis, template removal, and propranolol rebinding. The definition of the measurement parameters and measuring control was done by the LabSpec 4.04 software. The SERS spectra contained a varying background overlaying the SERS signal. In most of the spectra, this background was removed by fitting it to a high-order polynomial and subsequently subtracting this polynomial from the spectra.

RESULTS AND DISCUSSION

For initial investigation, large drops of (*S*)-propranolol (the template molecule) and MIP were applied to the Klarite substrate, resulting in droplets extending over both the smooth gold and the patterned SERS grid, as seen in the micrograph of an (*S*)-propranolol droplet (Figure 1a). Therefore, the resulting Raman and SERS signals could be measured in both regions, at the points marked by the black and red arrows, respectively, under identical

conditions. Spectra of (*S*)-propranolol (trace b of Figure 1) and MIP (trace d of Figure 1) on patterned grid (SERS) and of (*S*)-propranolol (trace c of Figure 1) and MIP (trace e of Figure 1) on smooth gold (Raman) surfaces are shown. Despite the significantly shorter integration time used for the spectra acquired on the SERS-active region (7 vs 15 s for (*S*)-propranolol and 30 vs 120 s for the MIP) the signals in the SERS spectra are considerably higher than those collected on the smooth gold area. The backgrounds are also higher and likely the result of fluorescence but also of the emission continuum typically appearing in SERS.[15,19] The signal enhancement in SERS is related to the localized plasmon characteristics of the SERS substrates, which lead to electromagnetic enhancement.[12−16] For the Klarite surfaces, it was predicted by theoretical modeling that a localized electromagnetic field confined at the edges and in the pit induces the enhancement.[20] This rises from the initial excitation of the localized surface plasmons by the light, which then induces electromagnetic radiation into the molecules, enhancing the Raman scattering by many orders of magnitude.

Also, by comparing the Raman to the corresponding SERS spectra, it is immediately apparent that the latter are quite similar to the non-surface-enhanced spectra, but some differences are encountered. These include changes in wavenumbers, bandwidths, and relative intensities of the bands and even appearance as well as disappearance of bands in the SERS spectra. This behavior could be attributed to the interaction of the samples with the substrate, leading to allowable vibrational modes in the SERS spectra, although they are normally forbidden in the Raman spectra. Furthermore, when the molecules are adsorbed to the surface, slight modification of the symmetry of the molecule might occur, which may lead to differences in mode selection.[21] The orientation of the molecules on the substrate might also affect the spectra leading to strong SERS bands for vibrations with molecular polarizability components perpendicular to the metal substrate. Although the Raman and SERS spectra differ somewhat, the signature of the detected molecules in the latter is specific, allowing their identification with higher sensitivity.

To test the applicability of SERS to detection of the template and of the template bound to MIP microstructures we further focused on measurements of droplets deposited by NFP. A representative SERS spectrum of the free (*S*)-propranolol template is shown in Figure 2a. The spectra are characterized by several distinct bands, with the most prominent being at 1384.9 ± 0.8 cm^{-1} and corresponding to the aromatic naphthalene moiety of (*S*)-propranolol. In addition, the spectra include the characteristic bands at 738.3 ± 0.5 and 1585.3 ± 4.9 cm^{-1}, along with several weaker bands. The SERS spectrum of the MIP, after elution and following rebinding of the template, is shown in Figure 2b−d, respectively. Each of the spectra consists of distinct bands and in general presents a somewhat different pattern. Nevertheless, comparison of these spectra to that of the pure template, Figure 2a, clearly shows that the intensities of the strongest bands originating from (*S*)-propranolol (marked by the arrows) vary, according to the amount of template present in the MIP. In particular, in the MIP, Figure 2b, these bands are quite pronounced; in the extracted MIP, Figure 2c, they were reduced extensively or disappeared (the 738 cm^{-1} band) and eventually increased again after rebinding of the template to the polymer matrix, Figure 2d.

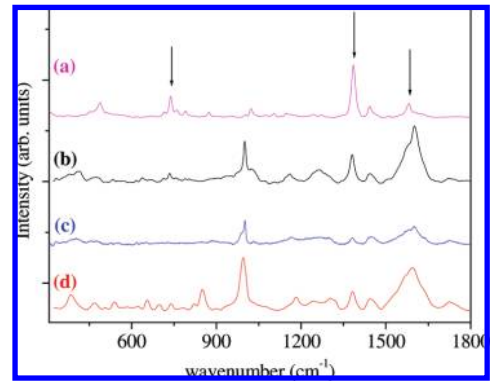

Figure 2. Representative SERS spectra of the (a) free template (*S*)-propranolol, (b) molecularly imprinted polymer (MIP), (c) extracted MIP, and (d) after rebinding of the template to the MIP. All the spectra are of samples deposited on the Klarite surface and were measured with integration times of 40 s. The background has been subtracted, and the spectra are plotted on the same scale and are shifted for clarity.

Furthermore, by comparing the spectral features originating from (*S*)-propranolol, in the spectrum of the free template, Figure 2a, to those appearing in the MIP, Figure 2b, and in the MIP after rebinding, Figure 2d, it is seen that there are small changes between them, expressed by the observed Raman shifts. Particularly, the bands related to (*S*)-propranolol, when incorporated into the polymer, are shifted to 735.0 ± 0.4, 1382.4 ± 2.3, and 1568.5 ± 2.7 cm^{-1} in the MIP and the last two bands to 1381.7 ± 0.6 and 1566.7 ± 2.3 cm^{-1} in the MIP after rebinding. The exact origin of these shifts in the SERS spectra is not clear yet; however, changes in the shifts should be anticipated if minor structural alterations in the vibrational modes of a molecule incorporated into a polymer matrix occur. It should be noted that a similar observation was encountered by McStay et al.[22] in the Raman spectra of the MIP that was prepared using triazine and BOC−phenylalanine as a template. It is thus very satisfying to note that the peaks related to (*S*)-propranolol in the SERS spectra provide an unambiguous means of identifying the presence of the template in the MIP and can be used for its quantification in template elution and rebinding experiments with MIP.

An additional aspect that has to be considered is how the extent of filling-up the Klarite microwells influences the resulting SERS signals. A three-dimensional (3D) representation of a NFP deposited MIP droplet, as obtained by AFM, is shown in Figure 3a. This 3D image shows that the droplet covers well the patterned grid surface with varying heights, extending from several micrometers above the plane of the surface (overfilled microwells) through empty microwells. In Figure 3b we show the AFM topographic image, and the aligned corresponding micrograph, Figure 3c, of the droplet with the marked lines, along which SERS spectral measurements were acquired. The droplet height profile

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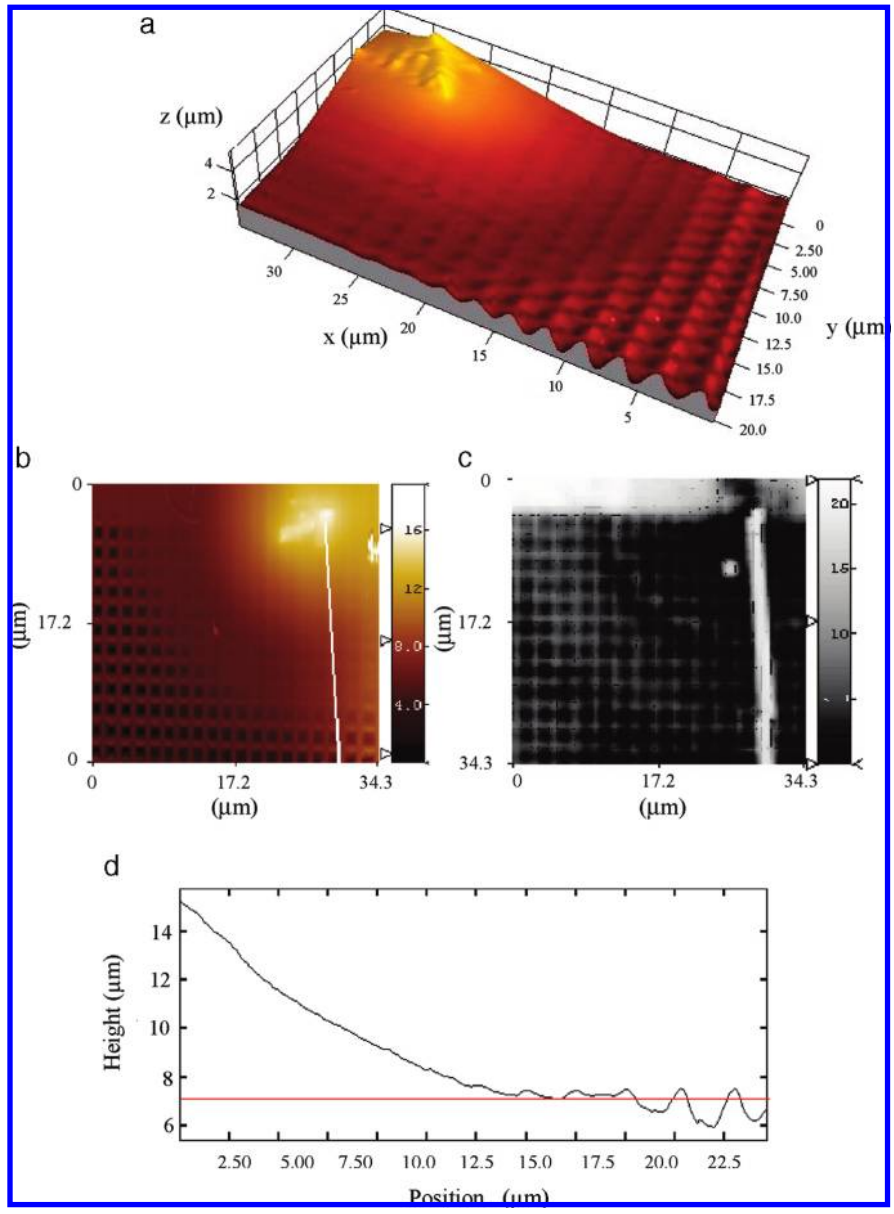

Figure 3. (a) Three-dimensional representation of a droplet of molecularly imprinted polymer (MIP) deposited on the Klarite surface, as characterized by the atomic force microscope (AFM). Aligned AFM (b) and optical (c) images of the droplet. A series of SERS spectra were collected along the line in panel c. The height profile along this line is extracted from the AFM image (b) and plotted in panel d. The red line in panel d indicates the height of the smooth gold surfaces.

along the line marked in Figure 3b is shown in Figure 3d. Considering the droplet height relative to the level of the smooth gold-coated surface, marked by the red line, it is seen that its height starts at 8 μm, decreases gradually until its disappearance, at a horizontal distance of approximately 16 μm.

This corresponds very well to the SERS spectra of the MIP droplet, along the line of Figure 3c, which were measured with a step size of 1.5 μm. These spectra are presented in Figure 4a−j corresponding to the droplet covering the first six pits of the Klarite surface. It is clearly seen that all the spectra show similar spectral features, matching to the SERS spectrum of the MIP, displayed in Figure 2b. The last two traces, k and l, of Figure 4, measured at 17.2 and 31.8 μm from the beginning of the line, are representative spectra from 22 additional spectra that were measured. It is clearly seen that these spectra show very different signatures compared to the upper traces in Figure 4a−j. In fact, when scanning across the line and departing from the droplet two very broad peaks appear with some small narrow bands, Figure 4k, and finally without narrow bands, Figure 4l. The narrow bands correspond to those observed in the MIP SERS spectra, whereas the broad features correspond to amorphous carbon cluster,[19] implying a contamination of carbon as far as 31.8 μm from the droplet center. The presence of this contamination can be due to some spread over of the droplet on the SERS substrate, during the NFP deposition.

In addition, the spatial variation of the most intense characteristic peaks (1385 and 1585 cm^{-1}) of (*S*)-propranolol in the MIP droplet was assessed. The computed SERS line imaging maps (Figure 5) show that there is some variation in the peak intensities as a function of the measurement position. The peak at 1585 cm^{-1}

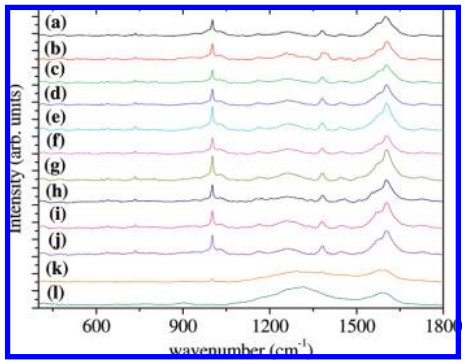

Figure 4. Spatial-dependent SERS spectra along the line indicated in Figure 3c, of the molecularly imprinted polymer (MIP) droplet, recorded with 14 s integration time. The spectra are of the MIP droplet shown in Figure 3. Spectra a–j cover the first six wells of the Klarite surface, at a 1.5 μm spacing. Spectra k and l, recorded at 17.2 and 31.8 μm from the beginning of the line, show characteristic amorphous carbon bands. The background has been subtracted, and the spectra are plotted on the same scale and shifted for clarity.

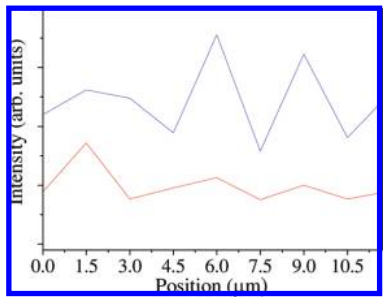

Figure 5. Line map of the molecularly imprinted polymer (MIP) droplet, displaying the intensities of the ~1385 and ~1585 cm^{-1} bands, shown in red and blue, respectively.

varies most extensively, probably because of the difficulty in resolving it from the near-lying peak at 1595 cm^{-1}. An additional reason for the variation is the mapping positions not being perfectly aligned with the SERS grid and also the laser spot at focus being of the order of the pit dimensions, implying that the laser will not necessarily excite all the localized surface plasmons at each step. Nevertheless, the SERS intensities follow the droplet morphology, found by the AFM, allowing identification of the template in the MIP. Also, it is clear that the precise positioning of the excitation spot is not a tight requirement for obtaining the characteristic fingerprint of the template molecule from the droplet.

To summarize, the study reported here shows that Raman spectroscopy, and particularly SERS, can be used to characterize the MIP, after elution and following template rebinding. We have also shown that SERS can be used to follow the morphology of a NFP-printed droplet, opening the possibility of using SERS to monitor MIP-based sensors on microscale. Further investigations should reduce the size of the droplets and set the limits of sensitivities provided by NFP and SERS in this microbiochip system.

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