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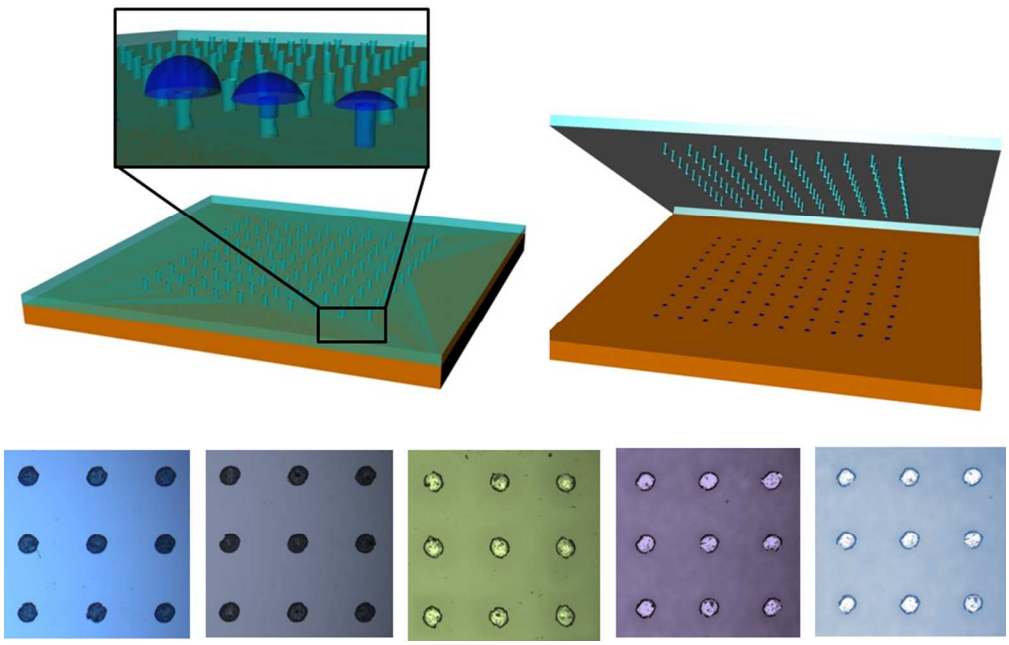

Holed-designed PDMS membrane is proposed as innovative microarray spotter exploiting vacuum procedure and verifying a good level of reproducibility spotting fluorescent proteins over large areas.
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TECHNICAL INNOVATION

Flow-through holed PDMS membrane as reusable microarray spotter for biomedical assays†

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We propose the exploitation of holed-designed poly(dimethyl)siloxane (PDMS) membrane as innovative microarray spotter. The membrane is fabricated by simple technological approach and can be reused several times. A good level of reproducibility is found upon spotting fluorescent proteins at different concentrations over large areas.

The possibility to dispense small amounts of liquid, down to the nanoliter scale, has allowed the discretization of the functionalized surfaces to perform biological assays.[1] It is therefore possible to obtain microarray of spots so as to reduce the number of substrates required for the assay implementation, while increasing the reproducibility of measurements and result statistics.[2] These microarrays can be applied in several biological fields such as proteomics, genomics and lipidomics. In addition, the development of robust and effective technologies for microarrays printing is strongly required in order to allow large scale production.[3]

Generally, the most common technology for surface micro-spotting consists in a complex robotic system armed with needles or cusps that dispense droplets according to predefined layouts.[4] The principle of operation can be either passive, in case the drops are loaded on the cusp tips due the surface tension of the liquid, or active, when piezoelectric actuation or micropistons are integrated within the needles (like in inkjet printing systems).[5] These approaches have gained popularity to become commercial products. Unfortunately, almost all of them are affected by some technological limitations. In passive devices, the quality of the spotting exhibits a strong dependence on the surface properties of liquids and substrates[6]. In active systems, the clogging of the nozzles may occur.[7] In the past, several alternatives have been developed in order to overcome the above mentioned limitations.[8-13]

Since the first report of Delamarche and coworkers,[14] microfluidics have been largely investigated to locally guide protein solutions onto substrates.[15-21] Also in this case, microchannels can be passive (exploting self-filling by capillarity)[17,18] or active (when pumping system was used).[16] Several configurations have been proposed starting from parallel channels[17] and chessboard-like pattern (by 90° re-alignment of parallel channels)[16,19] to three-dimensional microfluidic networks[20,21] in order to obtain complex and discontinuous pattern. Among them, an interesting approach lies in the fabrication of continuous-flow microspotters. This method, firstly proposed by Sjolander and Urbaniczky[22] and subsequently deeply investigated by Natarajan and co-workers,[23,24] allows to sensibly improve the individual spot quality and increase the sensitivity to low protein concentrations. Nevertheless also this approach suffers from the complexity of the fabrication process (several lithographic, alignment and bonding steps are implemented) and from the control system required to manage the fluidic channels.

We propose an alternative spotting method based on the vacuum filling of vertical microchannels that are regularly distributed in a PDMS membrane, to obtained confined biological solution in contact with the target surface. Our idea introduces a remarkable novelty, since the production of the spots is performed exploiting a slight vacuum. PDMS is the peerless silicone most employed in microfluidics, lab on chip, micro total analysis systems, MEMS, optical and electro-optical devices.[25-28] The elastomeric membrane can be fabricated by a simple technological approach and reused several times. The fabrication process and the working mechanism is schematically represented in Figure 1.

A commercial PMMA foil (5 mm thick) is used to fabricate the mould. The substrates are micro-machined by numerical controlled milling cutter (head diameter 300 μm) in order to obtain an uniformly distributed array of pillars (Figure 1a). The thickness of the dig is fixed to 200 μm, that is the minimum value to allow an easy handling of the PDMS membrane. The choice of a pillar diameter equal to 100 μm is a good solution allowing both a dense packing of spots and enough mechanical strength for PDMS casting and replica removal without incurring in damaging. The pillar inter-distance is imposed by the cutter head diameter but all the process can be easily substituted by single step SU8 lithographic approach on silicon allowing to increase the density of the spots.[11]

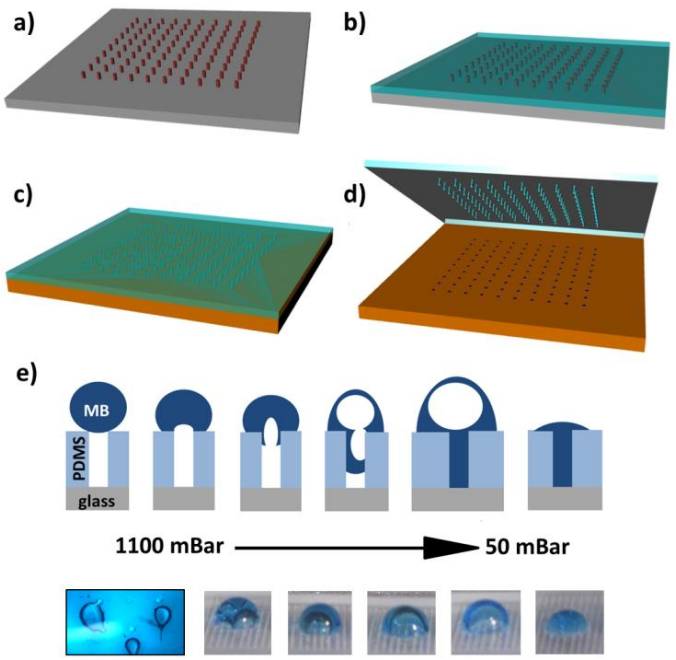

Figure 1. Schematic illustration showing the technological process and working mechanism: mould fabrication (a), casting and crosslinking of PDMS (b), replica removal and attachment to the target substrate (c). After dispensing a drop of solution on the holed membrane and applying a low vacuum the membrane can be detached from the patterned substrate (d). In e) a 3D scheme and relative photographs of the working principle are shown.

PDMS samples are prepared by mixing the polymer base and the curing agent with different weight ratios (5:1, 10:1). The mixture is poured into the moulds and thermally cross-linked (Figure 1b). Further details about the fabrication process are collected in the ESI. The 5:1 composition is chosen because its viscosity is low enough to allow the easy filling of the dig among the pillars. The tacking properties of PDMS membrane are affected by the reduction of the mixing ratio between prepolymer and curing agent[25] since the elastic modulus increases.[26] However also the thickness of the sample affects the elastic modulus, providing a balance of the compositional effect and allowing a sufficient adhesion on the substrate.

The resulting PDMS samples are then manually removed from the mould and subsequently attached on the desired target surface (Figure 1c) thus causing the formation of a reversible sealing. Different substrates are tested, such as a polished silicon wafer, a glass slice, metallic thin films, polymeric substrates, so as to assess the wide exploitability of the proposed spotting system.

A 3D scheme of the micropores filling mechanism is reported in Figure 1e. In order to produce the microspots a microliter drop of solution is dispensed onto the holed surface and a slight vacuum is applied (for example placing the sample into a vacuum oven in order to control temperature and pressure – see Figure S1 in ESI). As the pressure decreases, the air trapped into the vertical microchannels begins to leak in bubbles within the drop of solution released on the surface. When the pressure falls below about 50 mbar, the pressure difference is sufficiently high to completely extract the air trapped inside the microchannels. At this point the surface tension of the liquid is no longer sufficient to retain the trapped air and the bubble explodes. Upon this operation, microchannels are entirely filled with the solution. The optical microscope image (on top) and digital photographs (side view) collected in Figure 1e clearly illustrate the air bubbles evolution decreasing the pressure.

In ESI Figure S2 a photograph of the PDMS membrane is presented, showing the possibility of a large area patterning with microarray spots.

Figure 2 a-e) collects exemplary optical images of methylene blue (MB) aqueous solution spotted on different surfaces (silicon (a), Pt thin film on silicon (b), PMMA (c), PET (d), PDMS (e)). The spots obtained show a well define morphology and no evidence of lateral spreading, thanks to the PDMS vertical channel confinement. All these tests are done using the same PDMS membrane ultra-sonicated in acetone and ethanol after every spotting. In order to evaluate the dependence from the solvent nature, a commercial solution of iodine into acetonitrile (Iodolyte AN50, Solaronix) is used in place of water to fabricate the same pattern onto glass surface (figure 2f).

Different membrane areas are fabricated in order to evidence the possibility to scale up the spottable area. In figure 2 g) an image of a 4 cm^2 glass slice fully covered by microspots is presented. A zoomed microscope image of the same surface is reported in the inset.

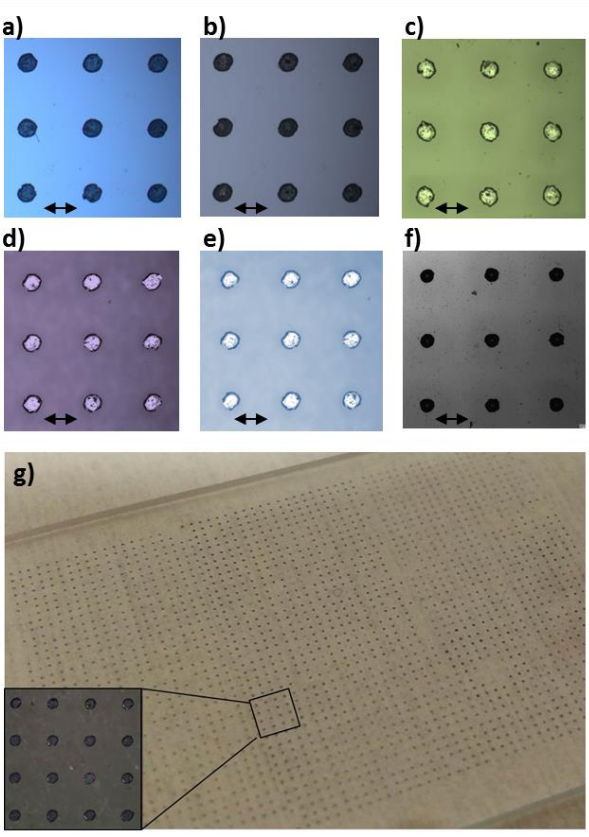

Figure 2. Optical microscope images of MB aqueous solution spotted on silicon (a), Pt thin film on silicon (b), PMMA (c), PET (d), PDMS (e) while in f) an acetonitrile dye solution is used onto glass surface (the scale arrows correspond to 200 μm). In g) a digital photograph of a microscope glass slice fully patterned by microspots is shown.

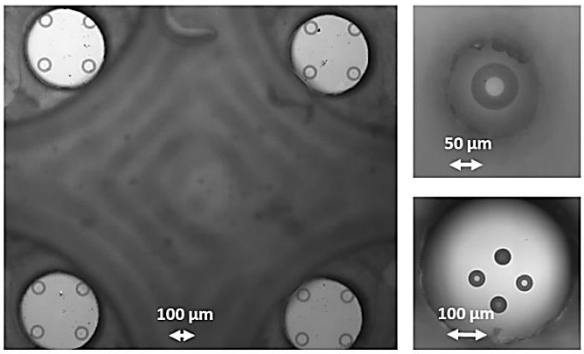

Figure 3. Optical microscope images collecting examples of PDMS membrane alignment on nanoscale features on 1D PCs using different layouts.

No undesired phenomena, such as comet tailing or coffee ring (typically affecting the drop dispensing method) are evidenced thanks to the confinement of the solution within the vertical microchannel and to the good adhesion of the PDMS membranes to the substrates investigated.

The proposed PDMS membrane allows the eventual alignment to any substrate features by exploiting the transparency of PDMS. In this case, a common microscope or a mask aligner is required. Figure 3 shows an example of PDMS membrane alignment on circular nanogratings recently proposed to improve the fluorescence extraction on one-dimensional photonic crystals (1D PCs).[29,30]

In order to check the applicability of this technical innovation for biological assay, a proof of principle biological binding experiment is performed on functionalized glass substrates. Details on the functionalization and activation of the surfaces are reported in ESI and in a previous work.[31]

These pre-activated samples are incubated with 10µl Protein A-Alexa 546 solution, spotted using the PDMS membrane, for 20 minutes at room temperature (only 2 minutes are required to obtain the microchannel filling by vacuum). Working standard solutions at six different concentrations 50 – 10 – 5 – 1 – 0.5 – 0.1 µg/mL in Phosphate-Buffered Saline (PBS "Dulbecco") are prepared by serial dilution. After the incubation, during which proteins diffuse and bind to the target surface, substrates are deeply washed in PBS-Tween 20 (0,05%), rinsed with deionised water for three times and dried in a nitrogen stream.

The samples are characterized by fluorescence microscope using a customized setup (see ESI Figure S3) described in detail in ESI. Briefly, fluorescence is excited by means of a collimated Nd:YAG laser beam impinging on an area larger than the spot size and collected on a CMOS RGB camera through a NA= 0.07 optics.

The resulting images (Figure 4a) are analysed by dedicated software in order to quantify the intensity of the signals. Sets of different spots are collected for each concentration. For each image, the red and green channels are merged in order to have a single intensity matrix and two areas of interest are identified, the first one included in the spot and the second one outside the spot to evaluate the background level. The intensity is evaluated over these two areas by extracting the average values and the standard deviations. The limit of detection has been set as three times the standard deviation of the background noise (i.e. equal to 0.0581). All the values are normalized to the maximum intensity level. To a first approximation the fluorescence intensity depends on the percentage of area covered by the protein. A simple and common way to model such situation is the Langmuir adsorption model.[32]

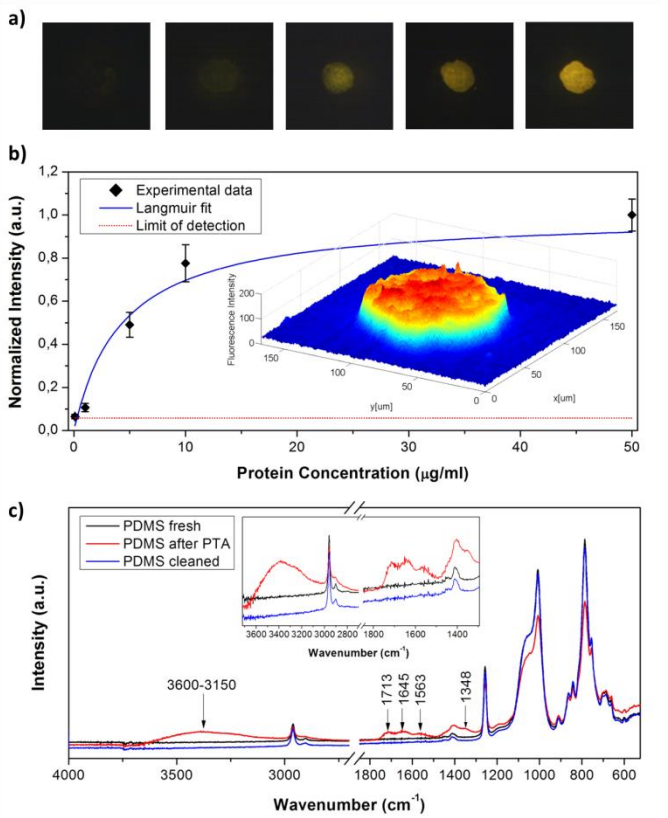

Figure 4. RGB images of protein spots at different concentrations are shown (a). Calibration curve of fluorescence intensity versus protein concentration (b). The diamonds with relative error bars are the experimental data, while the blue continuous line is the fitting curve. The red dashed line represents the limit of detection. The inset shows a 2D intensity map of a protein spot. In c) the FTIR spectra of PDMS membrane and a zoom of the range of interest is reported.

We fit the experimental data with a Langmuir equation, having the following expression:

$$I(c) = \frac{\alpha c}{1 + \alpha c}$$

where c is the protein concentration, I is the fluorescence intensity and α is a fit parameter (whose value is 0.23). The resulting curve, together with experimental data and corresponding error bars, is reported in Figure 4b. It can be seen from this figure that the intensity of the fluorescence images increases with increasing protein concentration from 0.1 µg/mL up to 50 µg/mL. The resulting calibration curve is linear below 5 µg/mL. When the protein concentration increases to a certain level, the fluorescence intensity starts to level off, indicating that the binding affinity reaches saturation.

Compared to other protein microarray experiments our results reveal that the lowest concentration detectable by fluorescence analysis is

lower than the minimum one (0.05 mg/ml) observed by Ho et al. using micro-contact printing approach.[33] Indeed a protein detection ability down to concentration of 0,1 μg/ml is demonstrated and this result is in accordance to the minimum protein concentration (0.12 μg/ml) detected by Natarajan et al. using continuous-flow microspotter.[23]

With the purpose of assessing the uniformity of the spotted proteins fluorescence images of the spots have been analysed by dedicated software in order to obtain the 2D profile of the fluorescence intensity spatial distribution. An exemplary image is reported as inset of Figure 4b showing a uniform intensity inside the spot area with a rapid fluorescence drop at the boundary of the spot.

In order to check the reusability of the PDMS membrane, the sample cleaning method (described in ESI) can be addressed by means of FTIR spectroscopy. Results are presented in Figure 4c. The spectrum of a freshly prepared PDMS displays its characteristic absorption peaks assigned elsewhere.[25,26] New absorption bands appear in the spectra of PDMS after PtA Alexa Fluor 546 incubation, indicated by arrows. In particular: OH stretching broad band at 3600-3150 cm^{-1} characteristic of carboxylic groups, C=O stretching mode at 1713 cm^{-1} and the typical amide I 1645 cm^{-1}, amide II 1563 cm^{-1} and amide III 1348 cm^{-1}.[34] After the cleaning procedure the protein characteristics bands have completely disappeared thus demonstrating the possibility to reuse the PDMS membrane for multiple processes.

In addition, the proposed approach could be exploited also for multiple protein microarray printing and cascade reactions by simply coupling the membrane or the spotted surface with ad-hoc designed microchannels array as depicted in Figure S4 and S5 in ESI.

Conclusions

Herein we have proposed for the first time the exploitation of holed-designed PDMS membrane as innovative passive microarray spotter. The elastomeric membrane is fabricated taking advantage of very simple technological approach and only slight vacuum is required to activate the procedure. Moreover the PDMS can be easy cleaned and reused several times. To demonstrate the effectiveness of this method aqueous and organic solvent based dye solutions are spotted onto several substrates and alignment ability on surface feature is demonstrated. The proposed approach is practically almost independent by the substrate of immobilization since PDMS presents a good self-adhesion to almost every kind of substrates usually employed in biological assay. In order to verify the employability of this technical innovation for biological assay, fluorescent proteins at different concentrations are patterned onto functionalized substrate and the calibration tests by fluorescence measurements confirm the high sensitivity (detection ability down to concentration of 0,1 μg/ml) and the good degree of reproducibility of this microarray spotting approach.

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Notes and references

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