Reactive Microcontact Printing of DNA Probes on (DMA-NAS-MAPS) Copolymer-Coated Substrates for Efficient Hybridization Platforms

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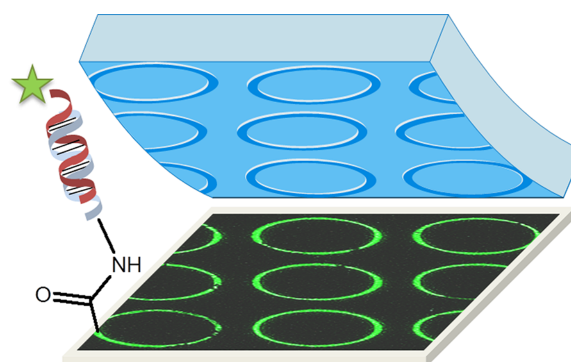
ABSTRACT: High-performing hybridization platforms fabricated by reactive microcontact printing of DNA probes are presented. Multishaped PDMS molds are used to covalently bind oligonucleotides over a functional copolymer (DMA-NAS-MAPS) surface. Printed structures with minimum width of about 1.5 μm, spaced by 10 μm, are demonstrated, with edge corrugation lower than 300 nm. The quantification of the immobilized surface probes via fluorescence imaging gives a remarkable concentration of 3.3×10^3 oligonucleotides/μm^2, almost totally active when used as probes in DNA-DNA hybridization assays. Indeed, fluorescence and atomic force microscopy show a 95% efficiency in target binding and uniform DNA hybridization over printed areas.



INTRODUCTION

Among the techniques implemented to immobilize biomolecules on functional platforms, microcontact printing (μCP) has emerged for the rapidity, robustness, and feasible scale-up of the whole process. The μCP technique was first proposed by Kumar and Whitesides for printing alkanethiols on gold[1] and then fast established in different scientific fields ranging from microfabrication,[2] sensing,[3] and nanobiotechnology.[4] The main feature of this technique is the possibility to pattern defined areas with a good spatial resolution, transferring the ink adsorbed onto a polymeric stamp, usually made of polydimethylsiloxane (PDMS), to a substrate. Monolayers of alkanethiols on a noble metal surface have been obtained with an ultrashort contact time,[5] down to 1 ms, with a structure of the self-assembled monolayer (SAM) which can be different from the one obtained by slow solvent evaporation and dependent on the ink concentration on the PDMS surface.[6] Not only thiols but also a variety of molecules, polymers, and biomolecules such as DNA,[7] proteins, and even cells[8] can be successfully patterned via μCP. A growing number of reviews on this topic can be found in the literature.[9−12] Recently, a humidified μCP (HμCP) was introduced for the protein printing, on any smooth surface, assisted by water vapor diffusion.[13]

A step forward to achieve well-defined surface functionalization and patterning has been indeed the approach based on the ligation of molecules to the surface with a covalent bond. This technique is called reactive microcontact printing (RμCP), and it was first proposed by Whiteside's group, which demonstrated the patterning of reactive SAM through amide bond formation.[14] This was a relevant improvement of the technique, allowing for the covalent binding of molecules[15] such as biotin and ligands,[16] polymers such as poly(ethylenimine),[17] peptide nucleic acids (PNA),[18] DNA macromolecules, and proteins[19] onto a surface. The combination of RμCP with photochemistry (photochemical-μCP) allows for the patterning of thiol−ene on SAM surface, taking advantage of the optical transparency of PDMS stamps.[20] Moreover, this reactive-nanoscale confined approach has been exploited to perform a peptide synthesis through amide bond formation.[21]

In particular, the RμCP of oligonucleotides can be used to covalently immobilize selected probes of DNA to build microarrays for genomics, proteomics, and biosensors.[22,23] Recently, it has been also demonstrated that "click" chemistry

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can be applied to tether oligonucleotides onto glass substrates with a good lateral resolution without the need for a catalyst.[24] However, a quantitative study revealing the efficacy of the reactive microcontact strategy is still missing.

As reactive substrate for biomolecules immobilization, not only thiols or silanes are good platforms to work with but also polymers bearing a reactive moiety can be successfully exploited.[25] In the work of Feng et al.[26] the immobilization of amino-functionalized DNA was conducted from solution on a poly(*N*-hydroxysuccinimidyl methacrylate) substrate after the passivation of the undesired area with a layer of PEG-NH_2 done by reactive microcontact printing using a patterned PDMS stamp. In particular, the use of a polymer substrate reveals to be the winning strategy with respect to a self-assembled monolayer (SAM) due to the higher robustness, stability, high reactivity, and high molecular loading.[26] Moreover, a comprehensive comparative study by Shovsky's group highlights how the exposed surface of a reactive ultrathin polymer film is approximately 5 times larger than a SAM bearing the same reactive group.[27]

Following this strategy, in this work we report on the patterning of functional oligonucleotide platforms for hybridization assays, using a reactive microcontact printing technique over a substrate coated with a reactive polymer. At variance with the work in ref 26, however, here we directly pattern the desired molecule, so that immobilization is assisted by mechanical pressure. In particular, a layer of copolymer (DMA-NAS-MAPS), arising from the radical copolymerization of *N*,*N*-dimethylacrylamide (DMA), *N*-acryloyloxysuccinimide (NAS), and 3-(trimethoxysilyl)propyl methacrylate (MAPS) is used.[28] This functional layer exposes *N*-hydroxysuccinimide ester groups (NHS) able to covalently bind amino-modified oligonucleotide single strands. The quality and the pattern with respect to the PDMS stamp are investigated by printing fluorescent DNA strands. A calibration procedure of the fluorescence signal allows for a quantification of the printed probes on the functional platform. Using the same approach, a quantification of active-printed probes is also given, after a hybridization assay with a fluorescent DNA strand to assess the efficiency of the printed platform. A major benefit of our reactive microcontact printing strategy arises from the covalent immobilization of probes to the reactive surface, as the amino-modified probes are covalently bound to the copolymer surface via the formation of an amide linker. This binding strategy can be straightforwardly extended to the binding of other amine-functionalized biomolecules such as PNA, peptide sequences, or even proteins and glucosamine involved in biological platforms.

In this framework, the use of amino-modified probes and *N*-hydroxysuccinimide (NHS)-modified substrate reveals to be more convenient than the alkyne−azide couple required for a click-chemistry reaction.[24]

EXPERIMENTAL SECTION

Materials. Polydimethylsiloxane SYLGARD 184 (PDMS elastomer kit) was purchased from Dow Corning (Midland, MI). SU-8 2035 and SU-8 2005 were supplied by MicroChem Corp (Westborough, MA). Hydrochloric acid, hydrogen peroxide, sodium bicarbonate, ethanolamine, tris(hydroxymethyl)aminomethane, isopropanol, SSC (saline sodium citrate), SDS (sodium dodecyl sulfate), BSA (bovine serum albumin), and coverslip for hybridization (60 mm × 24 mm × 0.25 mm) were supplied by Sigma-Aldrich. DNA strands were supplied by Metabion (Germany).

PDMS Stamp Preparation. PDMS is fabricated by mixing the elastomer and the curing agent in a 10:1 ratio. The mixture is poured on the master substrate in order to cover the whole surface and placed in a vacuum chamber for few minutes to remove all the air bubbles. The stamps are heated in an oven at 65 °C for 2 h to increase the curing reaction kinetics. The samples are then cooled down, and the PDMS is carefully peeled off the master. Before using it for the printing process, the patterned stamps are washed with isopropanol. The mold for the PDMS stamp is fabricated by conventional photolithography techniques, using the SU-8 photoresist over a silicon substrate (see Supporting Information for details).

PDMS Stamp Inking and Printing Procedures. The PDMS stamp is dipped for 1 h in an activation solution of hydrogen peroxide and hydrochloric acid to reduce its hydrophobicity (see Supporting Information for details). After careful washing in water and drying with nitrogen flow, the stamp is inked with DNA strands. For the pattern definition study and immobilized probes quantification an amino-modified ssDNA labeled with a cyanine dye (Cy3-ssDNA1: 5′-Cy3-TCACTTTTACCTTATAGGTGGGC-NH_2-3′) is used as ink. A few drops of 5 μM DNA solution (bicarbonate buffer 100 mM, pH 8.5) are spread onto a glass slide, and the freshly activated PDMS stamp is pressed onto the drops for 5 min in order to let the DNA be adsorbed on it. Before printing, the excess of DNA solution, which can cause some smears in the printing process, is removed by a gentle nitrogen flow. The PDMS-inked stamp is placed for 1 h over the copolymer-coated glass slide (refer to the literature for coating procedure[28]) applying a uniform pressure of about 650 Pa. After the printing procedure the excess of physically absorbed DNA molecules are washed away in a water bath.

Hybridization Platform Preparation. For hybridization platform preparation of a nonfluorescent amino modified ssDNA2 sequence (5′-NH_2-GCCCACCTATAAGGTAAAAGTGA-3′) is patterned on the copolymer platform. Before hybridization tests the residual active sites of the copolymer are neutralized with surface blocking solution of ethanolamine (50 mM in 0.1 M Tris buffer, pH 9) at 50 °C for 15 min, to prevent any unwanted interaction of the fluorescent target ssDNA with the substrate surface. Blocking solution is discarded, and glass slides are rinsed with deionized water twice. Glass slides are placed in a washing solution with 4X SSC (saline−sodium citrate) and 0.1% of sodium dodecyl sulfate (SDS, prewarmed at 50 °C) for 15 min. Washing solution is discarded, and substrates are washed twice with deionized water and dried with nitrogen flow. After the surface blocking procedure, binding or interaction of DNA strands is prevented, and no further printing of oligonucleotides can be performed.

For the hybridization test, a full match complementary labeled fluorescent target oligonucleotide (Cy3-ssDNA3: 5′-Cy3-TCACTTTTACCTTATAGGTGGGC-3′) is diluted with hybridization buffer (2× SSC, 0.1% SDS, 0.2 mg/mL bovine serum albumin) to a final concentration of 1 μM. One drop of 5 μL of the target solution is dispensed onto the printed microarray, and the area is covered with a coverslip for hybridization. Slides are then incubated in a humidity chamber at 37 °C for 90 min. After that the coverslip is removed, and the slides are washed with different washing solutions: twice with 2X SSC, 0.1% SDS for 5 min each at 37 °C, once with 0.2X SSC at room temperature for 1 min, and once with 0.1X SSC at room temperature for 1 min. Finally the glass slides are dried with nitrogen flow.

Quantification of Printed ssDNA Molecules and Hybridization Efficiency. Quantification of immobilized Cy3-ssDNA1 molecules is performed by quantitative analysis of the fluorescence signal from printed areas, using a calibration curve built measuring the intensity from round spots obtained from standard concentrations of the oligonucleotide ink solutions. The curve has been obtained using oligonucleotide concentrations in the 2−100 nM range, and each concentration was then associated with the corresponding surface number of DNA molecules.

The same method has been used to extract the number of active probes immobilized on the hybridization platform via quantification of the fluorescence intensity upon hybridization of nonfluorescent DNA

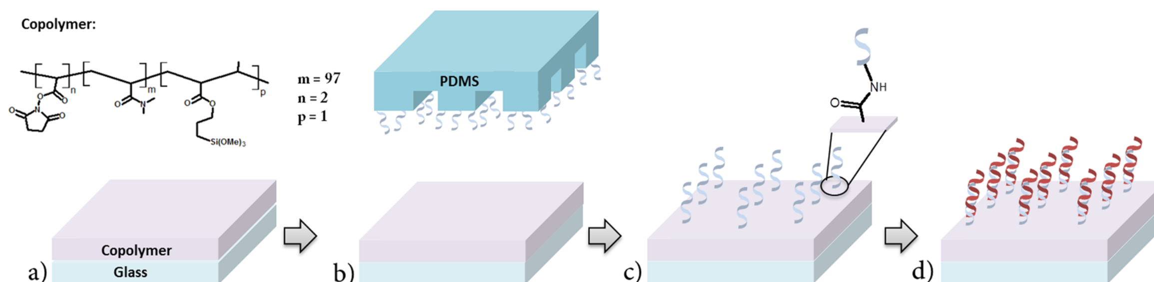

Figure 1. Reactive microcontact printing of oligonucleotide probes for hybridization assay. (a) Chemical structure of functional copolymer (DMA-NAS-MAPS) used to dip-coating a substrate glass slide. (b) Patterned PDMS stamp is inked with ssDNA and pressed onto copolymer surface to ensure the transfer of ssDNA. (c) Oligonucleotide probes are transferred onto the copolymer surface by means of reactive contact printing which enables the formation of a stable amide linker between the probe and the surface. (d) The patterned platform can be used for hybridization assays with full match oligonucleotide sequences.

oligonucleotides with Cy3-ssDNA3. Refer to the Supporting Information for further details and the experimental calibration curve.

RESULTS AND DISCUSSION

In order to prepare hybridization platforms by reactive microcontact printing, a microfabricated PDMS stamp is used to pattern ssDNA oligonucleotides onto the substrate through the formation of stable covalent bonds with the copolymer coated slides. The main steps of the procedure are reported in Figure 1. First, the glass slide is coated with the copolymer[28] in order to enhance the binding capabilities of immobilized probes by producing an efficient 3D platform for the DNA hybridization.[30] The homogeneity of the copolymer coating is remarkable, as shown by atomic force microscopy (AFM) images of the glass-coated sample (Figure S2). The surface roughness (R_{RMS}) is 0.754 nm, to be compared with the nominal thickness of the copolymer layer (2.5 nm),[29] and there is no trace of uncovered areas.

To increase the affinity of the siloxane stamp with aqueous media where the ssDNA inks are prepared, the PDMS is activated in hydrogen peroxide−hydrochloric acid bath before the printing process. Activation in oxygen plasma is another suitable path, but the wet process resulted to be efficient enough for our purpose. The variation of the sessile water contact angle upon activation, from $111.2 \pm 0.6°$ to $77.9 \pm 5.7°$, confirms a sizable reduction in hydrophobicity (see Supporting Information). Then the PDMS stamp is inked and pressed against the substrate (Figure 1b). After the printing and washing procedure, the ssDNA probes result to be linked to the copolymer surface through a newly formed amide bond (Figure 1c): the platform is ready to be used for the hybridization assay (Figure 1d). X-ray photoelectron spectroscopy (XPS) has been used to obtain a compositional characterization of the surface of a (i) glass slides, (ii) a copolymer coated slide, and (iii) the same slide upon DNA printing (see Tables S1−S3 in the Supporting Information). After modification with copolymer (Table S2) and ssDNA probes (Table S3), both the C and N content increase, thus signaling a successful chemical deposition of the copolymer and DNA probes. This is confirmed by the fact that the C 1s peak upon copolymer casting and DNA immobilization displays the typical fingerprint of C−N bonds (see Figure S5 and related discussion), present in both the DNA and copolymer. Unfortunately, this prevents to assess the covalence of the amide bond between the DNA and the copolymer because its spectroscopic fingerprint is also present in the spectra from the copolymer, which indeed contains amide groups. Nevertheless, the stability of printed DNA probes definitely points to the formation of a robust covalent bond.

In order to evaluate the effectiveness of the printing process, Cy3-ssDNA1 is used as ink. This procedure allows for monitoring the quality of the printing step using fluorescence microscopy. A customized master was fabricated with patterns of different dimensions and spacing (i.e., the minimum distance between two adjacent features) ranging from 10 to 300 μm. This permits to assess the minimum feature, in terms of resolution, and to control the uniformity over large patterned areas. A remarkable quality of the μCP process emerges from Figure 2, showing optical microscopy images of the PDMS stamps with different shapes, compared to the corresponding fluorescence images of patterned features. As a matter of fact, the printed pattern faithfully replicates the PDMS master, with an average enlargement of less than 1 μm in each direction and edge corrugation which reflects the presence of defects in the

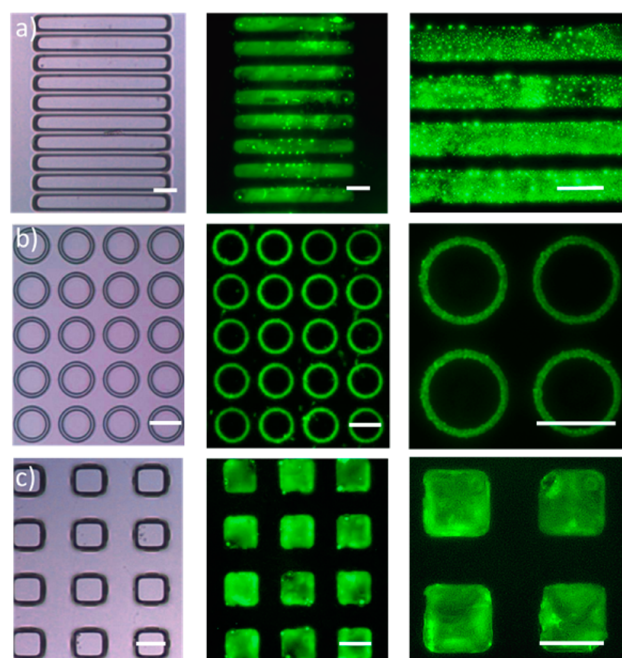

Figure 2. Comparison between fabricated PDMS stamps (left column) and corresponding fluorescence images of Cy3-ssDNA1 features printed onto copolymer-modified glass slides (middle and right columns) at different magnifications. (a) Rectangles 30 μm × 170 μm, (b) rings with 30 μm diameter and 3 μm line width, and (c) squares of 30 μm side. Scale bars: 30 μm.

stamp. Few intense spots are seen in the case of the large printed rectangles, which can be caused by small droplets of DNA ink remaining on the platform surface after the removal of the PDMS stamp.

The limit of resolution of our reactive μCP process has been assessed by printing rings with 20 μm diameter and nominal line width of 1 μm. Figure 3 shows fluorescent images that clearly demonstrate the good uniformity and continuity of individual rings over large areas, apart from some defects arising from the stamp irregularity. The actual line width of the circles is 1.55 μm, only slightly larger than the nominal one, thus testifying that the deformability of the stamps and the chemistry of printing do not produce a significant enlargement of the features. As expected for stamps fabricated with conventional photolithography, the resolution limit is about 1 μm.[10] It is also observed that the main limitation for the printing resolution arises from the quality of the master (see Supporting Information); this suggests that an even higher definition could be obtained by this technique if different, but slower, fabrication methods for the stamp[31] are employed.

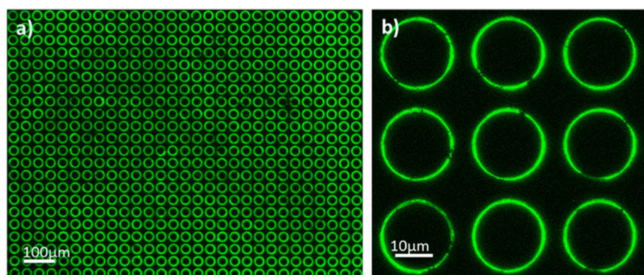

Figure 3. (a) Wide area fluorescence image of Cy3-ssDNA1 rings of 30 μm diameter, printed onto copolymer modified glass slides. (b) High magnification image of Cy3-ssDNA1 rings of 20 μm diameter from which an average line width of 1.55 μm for the patterned rings has been measured.

Quantification of immobilized Cy3-ssDNA1 molecules is performed by means of a calibration curve, and in this way, a remarkable surface concentration of $3.3 \times 10^3 \pm 0.6 \times 10^3$ probes/μm^2 is measured. This value is similar to the one reported for nonreactive microcontact printing techniques (0.9×10^4 molecules/μm^2) by Lange et al.[7] Note, however, that in the paper by Lange at al. DNA strands are just adsorbed on the surface, while in our case the DNA probes are chemically attached to the substrate, thus allowing a reliable use of patterned areas for biosensing. This good result arises from the combination of the copolymer functional layer with the use of the microcontact printing strategy. The copolymer allows for sizably increase of the concentration of binding sites with respect to the bare substrate, while μCP ensures a good binding efficiency due to the application of a mechanical pressure at the interface between the substrate and the stamp. We sampled 10 printed areas and recorded their raw fluorescence intensities, in order to estimate the relative standard deviation (RSD%) value and to use it as figure of merit of the reproducibility and homogeneity of the printing process. We estimated a value of RSD = 13%, which points out a very low deviation from the mean value and thus assesses the good performance of our technique.

The functionality of the hybridization platform has been assessed in a conventional surface DNA hybridization assay. Printing of ssDNA2 onto a copolymer coated slide is followed by blocking of the residual active sites of the polymer to prevent any unwanted interaction of the fluorescent target and the substrate. The platform is then incubated with a solution of the fluorescent target Cy3-ssDNA3, a careful washing of the platform to remove unreacted strands and drying is done before pattern characterization. As shown in Figure 4a, a uniform fluorescence signal comes from the whole printed area, thus indicating a uniform hybridization of the target with the printed platform. The efficiency of the hybridization platform is evaluated by quantifying the surface concentration of fluorescent-target molecules on the substrate after hybridization test, using a fluorescence calibration curve. A value of $3.2 \times 10^3 \pm 0.7 \times 10^3$ target molecules/μm^2 is measured. If compared with the probe density, this means that about 95% of the probes molecules is active in recognizing the target strand. This definitely demonstrates that printed DNA probes preserve their functional properties once the platform is functionalized.

The topography of the printed area after hybridization assay is investigated by means of atomic force microscopy (AFM), allowing for a more precise assessment of the uniformity, due to the higher spatial resolution of the scanning probe method with

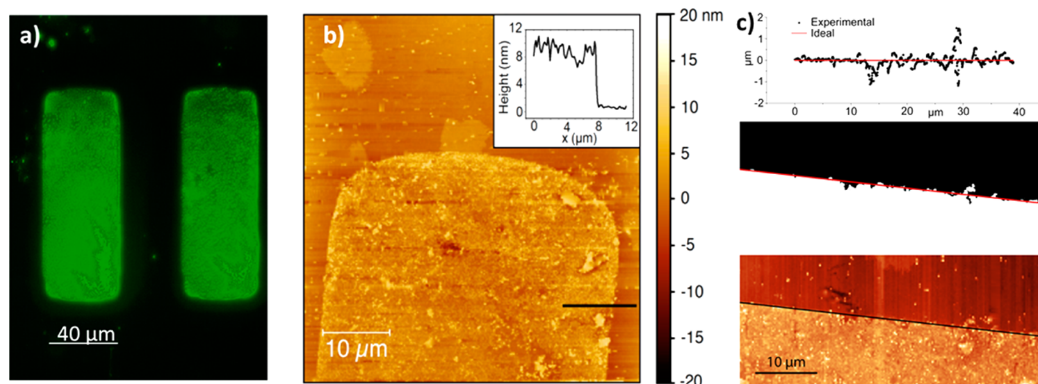

Figure 4. Optical and topographical characterization of hybridization assay. (a) Fluorescence microscopy of two rectangular patterns. Fluorescence signal is due to Cy3-ssDNA3 full match of the probes ssDNA2 printed on the copolymer surface. (b) AFM topography image of a hybridized surface within a 50 μm × 50 μm scan area. Inset: height profile of the patterned hybridized area taken along the black line in the main panel. (c) On top: plot of the printed pattern edge profile coordinates (black dots) evaluated as displacement from the ideal features profile (red line). Center and bottom: The patter profile is extrapolated from the analysis of the AFM image of the hybridized sample. The mean-squared displacement calculated from the ideal pattern profile is 0.32 μm.

respect to fluorescence microscopy. From Figure 4b it is clear that the coverage is highly uniform over the printed area after hybridization, corresponding to a layer thickness of about 8 nm,[32] in agreement with the expected extension of 23-mer DNA targets and probes (see inset of Figure 4b reporting a profile taken across the edge of the patterned area). The AFM images have been used also for investigating the corrugation of the edges of printed features, as shown in Figure 4c. To this scope the topographic image at the edge of a patterned area (Figure 4c, bottom) has been converted into a binary image and the corrugation studied by means of the software MATLAB. A corrugation of the edge profile on the order of 0.32 μm (Figure 4c, center) has been evaluated by calculating the mean-square displacement from the ideal profile observed in the PDMS stamp (see the straight red line in Figure 4c on top). This sets the limit of resolution of the whole patterning technique: decreasing the pattern size below 1 μm is critical because the structure can display some discontinuities arising from corrugation.

OUTLOOK

A reactive microcontact printing method for selective immobilization of ssDNA probes on the surface of a copolymer (DMA-NAS-MAPS)-coated substrate is proved. The method is successfully exploited to transfer oligonucleotides with a good reaction yield, leading to a probe density immobilization of about 3.3×10^3 probes/μm^2 with 95% active probes. The minimum feature size is on the order of 1.5 μm, which is of high relevance in view of possibly functionalizing the surface of compact and very small devices for hybridization assays, suitable for point-of-care diagnostics.[33] These results demonstrate a simple and straightforward technique, at the intersection between surface functionalization and materials engineering, to pattern suitable platforms for on-chip molecular recognition.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.5b04669.

Information on experimental details on master and PDMS preparation, surface functionalization and hybridization assay, sample characterization (contact angle, optical microscopy, atomic force microscopy, quantification of printed probes and x-ray photoelectron microscopy) (PDF)

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Notes

The authors declare no competing financial interest.

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