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Short communication

A microcontact printing approach to the immobilization of oligonucleotide brushes

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ABSTRACT

Solution hybridized oligonucleotides were immobilized onto surfaces via micro-contact printing. Besides micro-patterning of the substrate, sequential dehybridization and rehybridization were monitored via laser scanning microscopy, which assess the surface tethering of the oligonucleotides into a brush.

1. Introduction

In comparison to the solution process, the study of hybridization of oligonucleotides or DNA onto surfaces has not reached yet a comprehensive understanding. However, investigations of this mechanism represent current fundamental, experimental and technological challenges. Surface hybridization is indeed the basic process ruling DNA chip technology and has been addressed theoretically very recently [1,2].

To enable physical chemistry studies of the mechanism of hybridization onto surfaces, facile, homogeneous and especially reusable surface immobilization of oligonucleotide brushes is a prerequisite.

As microstructures can be visualized via optical microscopy, spotting and microcontact printing have proven to be elegant approaches to assess the immobilization and subsequent hybridization of fluorescently labeled oligonucleotides [3,4]. In their early work, Vieu and co-workers demonstrated that uniformity and edge definition of spots could be obtained on glass slides functionalized with nanometer sized spherical dendrimers bearing aldehyde reactive groups at the periphery for the covalent attachment of a 5′-amino-modified sequence [3]. Recently, Reinhoudt and coworkers assessed an efficient method for transferring DNA to a substrate and facilitating the positioning of DNA with high lateral resolution by microcontact printing (μCP) based on the modification of the stamp with dendrimers [4].

However, major hindering to hybridization arises from steric effects due to a too high density of surface tethered oligonucleotides. We thus made use of the specific property of hybridization of complementary nucleotide sequences in an iterative fashion. Complementary oligonucleotides were first hybridized in solution prior to their immobilization onto a glutaraldehyde modified silicon surface via μCP. The use of hybridized sequences ensures that grafting of the oligonucleotides occurs at a single point, i.e. at the amino-modified 5′-terminus of the oligonucleotide to be surface-tethered. Hence, immobilization of the hybridized sequences prevents interaction between the aldehyde reactive groups on the substrate and the amino groups along the oligonucleotide backbones, which are involved in the assembly of the double stranded oligonucleotides [5]. Nevertheless, only one of the two complementary oligonucleotides is covalently immobilized onto the solid support. Sequential dehybridization and rehybridization of oligonucleotides labeled with fluorescent dyes emitting at different wavelengths could thus be followed with laser scanning microscopy to demonstrate, for the first time, the immobilization of hybridized oligonucleotides into brushes prior to dehybridization followed by efficient and selective rehybridization.

2. Results and discussion

The poly(dimethylsiloxane) (PDMS) mask, which is most commonly used for microcontact printing, is a soft polymer stamp, which allows a conformal contact between the stamp and the surface [6,7]. The scanning electron microscopy (SEM) image of the used PDMS structure is shown in Fig. 1 (straps of 10 μm width distant of 30 μm). Prior to the immobilization of oligonucleotides, modification of the surface was performed according to a widely used method of silanization of silicon oxide wafers with aminopropyltriethoxysilane (APTES) followed by coupling with amine-modified biomolecules through glutaraldehyde [8,9].

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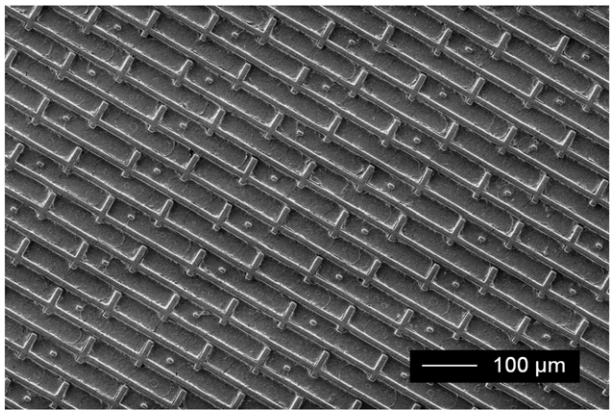


Fig. 1. Scanning electron micrograph of the PDMS mask.

The target surface structure is shown in Scheme 1. At each step of the preparation the samples were characterized by contact angle measurements for wettability monitoring, ellipsometry for thickness control and AFM for roughness control. The immobilization reaction was proved (data not shown) at each stage; layers of APTES and glutaraldehyde were homogeneous with thicknesses of about 1.8 nm and 1.7 nm, correspondingly. Theoretical calculations of the molecular length predicted a monolayer thickness of 0.8 nm for APTES and 1.4 nm for glutaraldehyde. Those measurements showed that APTES was deposited as a multilayer, and that the polymerization of glutaraldehyde was negligible. The samples were then further analyzed by laser scanning microscopy (LSM).

A solution of hybridized oligonucleotides, SeqA and SeqB was used for stamping (see Table 1). The complementary sequence (SeqB) of the surface-tethered oligonucleotide (SeqA) is fluorescently labeled. The fluorescence images of the solution hybridized oligonucleotides, deposited by µCP, are shown in Fig. 2. The detection of aggregation is associated to the resolution of this technique, which is approximately 250 nm ($\lambda/2NA$, with NA being the numerical aperture). The silicon wafer without APTES and glutaraldehyde modification were chosen as control samples. In Fig. 2A, the deposition of some oligonucleotides onto the control surface can be observed. However, the quality of µCP on the glutaraldehyde modified surfaces is conformal and demonstrates the possibility to immobilize solution hybridized oligonucleotides via µCP (Fig. 2B).

Dehybridization of the stamped oligonucleotides upon incubation with a solution of urea is expected to cleave the hydrogen bonds between the immobilized complementary oligonucleotides and, following rinsing, the fluorescently-labeled complementary oligonucleotides, SeqB, should be washed away, leaving a brush of single stranded oligonucleotides covalently grafted at their 5′-terminus onto the surface (Fig. 3). Because it is impossible to avoid completely non-specific interaction, the efficiency of dehybridization did not reach 100%. Fluorescent signal traces from remaining dyes on the surface can still be observed. However the intensity was significantly lower than prior to dehybridization.

Table 1
Oligonucleotide sequences

Name	Sequence	Modification
SeqA	5′-AGAGAGAGAGGGAGAGAGAGAGGG-3′	5′-amino modifier C6
SeqB	5′-CCCTCTCTCTCTCCCTCTCTCTCT-3′	5′-amino modifier C6-Alexa Fluor 488 (ester)
SeqC	5′-CCCTCTCTCTCTCCCTCTCTCTCT-3′	5′-amino modifier C6-Alexa Fluor 532 (ester)
SeqD	5′-GGGAGAGAGAGA-3′	5′-amino modifier C6-Alexa Fluor 488 (ester)

The fluorescence image of immobilized oligonucleotides via µCP after re-hybridization with SeqC, which is the fluorescently labeled complementary sequence of the grafted oligonucleotides onto the surface, is shown in Fig. 4. The control images of both channels were taken at every step of the µCP deposition; the signal in the red channel was negligible except upon efficient rehybridization of the immobilized oligonucleotides with their fully complementary sequence.

The stamping of hybridized oligonucleotides into brushes would not be fully assessed at this stage without essential control experiments. First, deposition of the fluorescently labeled single stranded oligonucleotide SeqC onto the surface by µCP shows that the

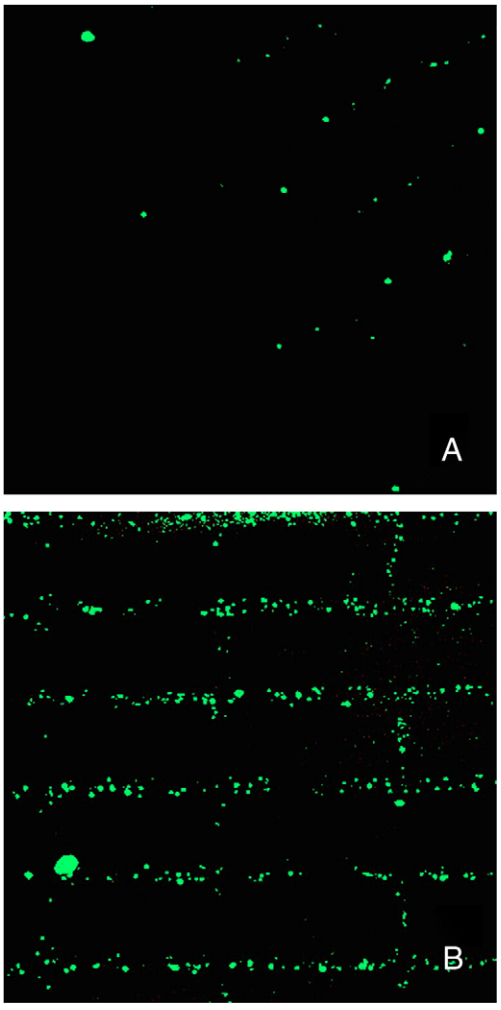
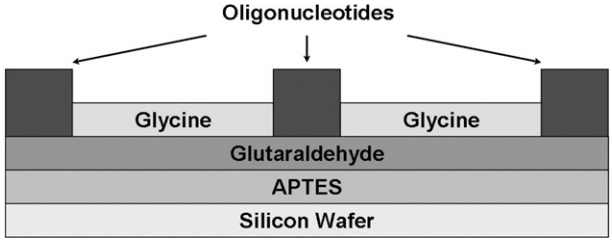


Fig. 2. Fluorescence microscopy of µCP deposited hybridized oligonucleotides (SeqA and SeqB) onto control surface (A) and APTES and glutaraldehyde-modified surface (B).



Scheme 1. Schematic representation of the sequential chemical modification of silicon wafers.

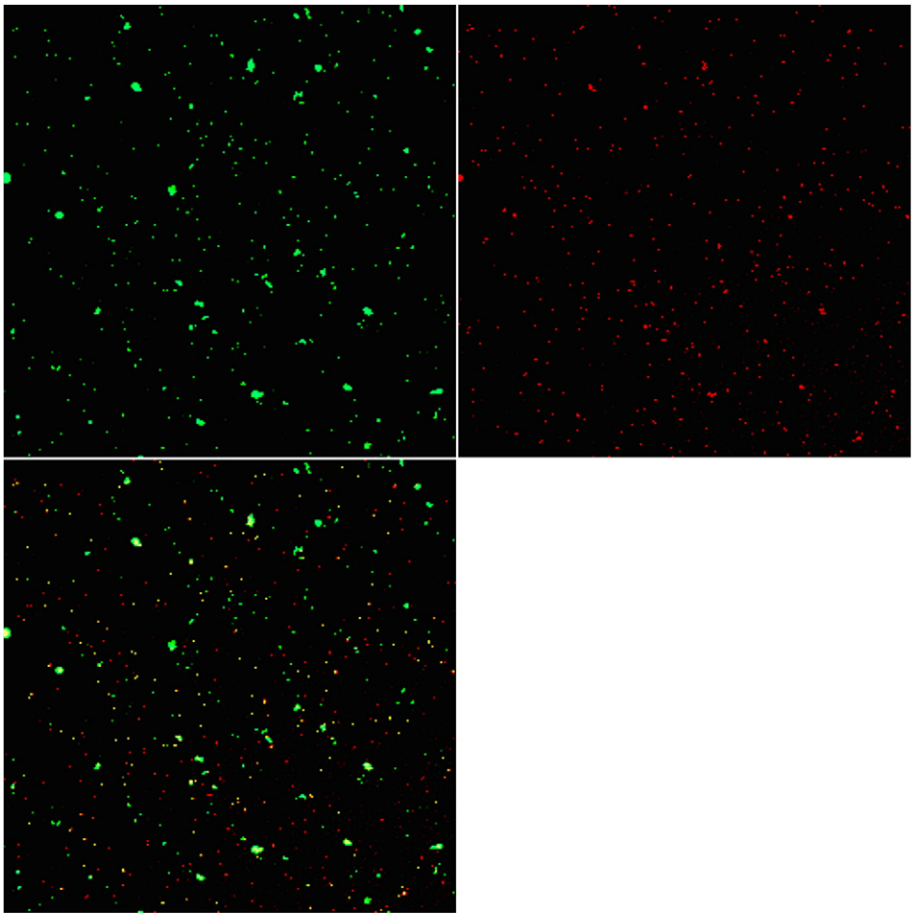


Fig. 3. Fluorescent images of μCP deposited oligonucleotides after dehybridization. Top right: Green fluorescence channel for detection of immobilized oligonucleotide; top left: Red fluorescence channel, in which only the signal from rehybridized oligonucleotides is detected; Bottom: Colocalization of both green and red fluorescence signals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

oligonucleotides bind to the surface. After printing we indeed observed the structure of the mask onto the surface, which assesses the oligonucleotide interaction with the aldehyde-modified surface. However, stamping of the single stranded SeqA, followed by hybridization with its fully complementary sequence, SeqB does not enable the LSM detection of any features originating from the PDMS mask. Those observations demonstrate that for successful hybridization onto the surface the oligonucleotides should be tethered within a brush-like structured layer. If the amino groups present along the oligonucleotide backbone interact with the surface, which is the case upon immobilization of the single stranded sequence, the chain "cross-linkage" onto the surface prevents efficient hybridization. However, upon immobilization of the hybridized sequences, the amino groups along the oligonucleotides are protected within the double strand except the function at the 5′-terminus of the oligonucleotide to be grafted onto the solid support, enabling immobilization of the oligonucleotides into a brush-like layer.

Second, the specificity of interaction between the oligonucleotides was investigated. For this purpose SeqD, which is a non-complementary sequence of the surface-grafted SeqA was used at the rehybridization step. LSM measurements did not show any mask-like structure on the surface, indicating that specific hybridization only occurs between the complementary SeqA and SeqC.

In this paper we demonstrated that an oligonucleotide brush can be obtained by covalent interactions between the surface and the terminus of the nucleotide sequence by μCP of hybridized oligonucleotides. The fact that the oligonucleotide patterns, immobilized onto the surface, can undergo sequential hybridization and dehybridization proves the stability of the immobilization of the oligonucleotides within a brush. Since LSM does not enable satisfying quantification, investigations of the hybridization kinetics and efficiency with label-free detection techniques are under progress.

As several recent reports highlight cell recognition of oligonucleotides, micro-patterned surfaces are being investigated further to study cell response onto surfaces [10,11]. Being the minimal modified surface square area of interest of the oligonucleotide stamped surfaces large enough, i.e. between 10 and 30 μm, to allow the spreading of various cell types with normal functionality, oligonucleotide micro-contact printing appears to have fundamental and experimental appeal and a high potential to design not only biosensors or biochips but also implants and cell-growth promoting or antifouling surfaces for tissue engineering.

3. Experimental section

3.1. Surface modification

The silicon wafers were chosen for those experiments due to their low roughness and high chemical homogeneity. Silicon wafers were cut in 1×1 cm^2 pieces. The samples were treated with a 1% (v/v) solution of APTES in water-free toluene for 2 h at room temperature to bring amino groups on the surface. Use of water-free toluene is necessary to avoid any precipitation of APTES and to reduce multilayer formation, which was shown by ellipsometrical measurements (data not shown). To remove most of the non-covalently bonded silane, the surfaces were sonicated 3 times over 5 min in toluene and rinsed 3

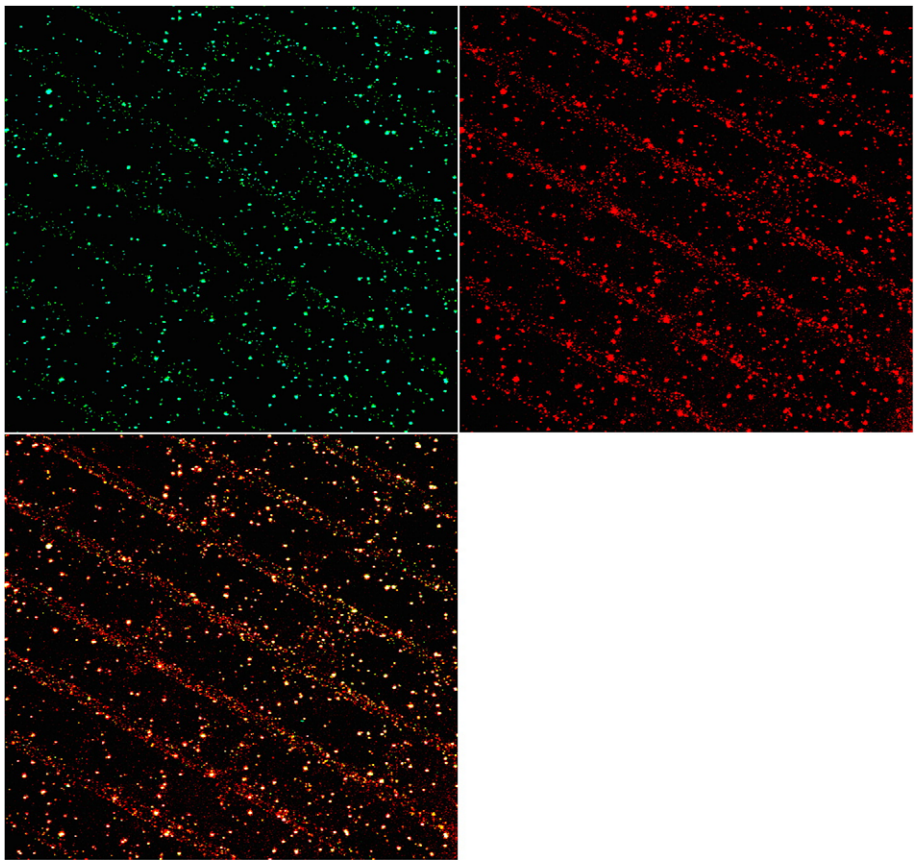


Fig. 4. Fluorescent images of μCP deposited oligonucleotides after re-hybridization with SeqC. Top right: Green fluorescence channel for detection of immobilized oligonucleotides; Top left: Red fluorescence channel corresponding to the detection of rehybridized oligonucleotide. Bottom: superposition of both green and red channel signals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

times with toluene afterwards. Subsequent rinsing steps with a suitable buffer after every deposition step are very important to avoid as much as possible contamination with non-covalently bonded molecules. The samples were immersed in 1% (v/v) glutaraldehyde solution in water for 1 h, rinsed 3 times with water and stored in water until use for stamping the same day. The result of the reaction between APTES and glutaraldehyde is a so-called protonated Schiff base [12]. Schiff bases, which form at neutral pH, are usually unstable bonds at a pH higher than 5, but could be stabilized by immersion of the samples in a solution of 50 μM sodium cyanoborohydride in water, which will reduce the Schiff base into an amine bond. Since in the course of all experimental stages described here the pH of all solutions was kept at 5, the reduction step can be done at the very last stage of the surface preparation.

3.2. Oligonucleotide immobilization

24 bases long oligonucleotides (HPLC, purity 99%) were purchased from Operon (Köln, Germany). Probe, SeqA and its fully complementary sequences target, SeqB and SeqC, Alexa 532 and Alexa 488 modified were chosen as linear non self-hybridizing sequences, SeqD Alexa 488 modified was chosen as the non-complementary sequence of SeqA (Table 1). Concentrations of the oligonucleotide solutions were controlled by measurement of the optical density of the solution at 260 nm. Hybridization of the complementary sequences SeqA and SeqB at concentrations of 300 nM was performed in a 50 mM NaCl solution during 1 h at 4 °C. Thus only amino groups at the 5′-terminus were available for reaction with the aldehyde groups present on the surface. This reaction hence prevails over non-specific binding. A drop of 30 μL of hybridized oligonucleotide solution was deposited onto the PDMS stamp and slightly spread during 3 min; the excess of solution was soaked out by pipetting. The stamp was placed into uniform conformal contact with the flat substrate for 3 min and rinsed afterwards. To quench free aldehyde groups on the surface after deposition by μCP, the samples were immersed for 1 h in a 20 mM solution of aminoacetic acid (glycine) prepared in water. Afterwards the reduction step was done. After these manipulations we obtain microstructured surfaces with covalently bound hybridized oligonucleotide brushes. As control experiments the samples without APTES and glutaraldehyde modifications were used for the stamping procedure. To de-hybridize the immobilized oligonucleotides, the samples were immersed in a 4 M urea solution for 1 h. Re-hybridization between SeqC or SeqD and the remaining oligonucleotides immobilized onto the surface, SeqA, was done by immersion of the surfaces in 300 nM solution of SeqC or Seq D in 50 mM NaCl for 1 h in dark condition. After rinsing, the samples were analyzed by laser scanning microscopy, LSM.

For control experiments single stranded SeqA was printed on glutaraldehyde modified silicon, prior to immersion in glycine and incubation with a solution of SeqB.

3.3. Fluorescent microscopy

A Zeiss LSM 510 laser confocal microscope was used to study the fluorescence signal from modified silicon surfaces in the epi-fluorescence detection mode. Fluorescence imaging of μCP stamping of Alexa-labeled oligonucleotides was performed relative to a background corresponding to the fluorescence of the sample modified with APTES and glutaraldehyde. The fluorescence signal from Alexa 488-labeled oligonucleotide, was registered upon emission at 488 nm wavelength with 505–550 nm emission filters (green channel)

whereas the fluorescence of Alexa 532-labeled oligonucleotides, was recorded at 543 nm and 560–615 nm excitation and emission wavelengths respectively (red channel). The use of long-pass filters is not expedient in this study case since, over a wide range of wavelengths, the signal to background ratio is lowered with band-pass filters due to the background fluorescence of aldehyde and silane over the whole wavelength range of interest. All samples were investigated in the multi-track mode to avoid the cross-talk of the various dyes used for these investigations.

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