Direct Immobilization of Cholesteryl-TEG-Modified Oligonucleotides onto Hydrophobic SU-8 Surfaces

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We introduce a rapid, simple one-step procedure for the high-yield immobilization of cholesteryl-tetraethyleneglycol-modified oligonucleotides (chol-DNA) at hydrophobic sites made of SU-8 photoresist. Topographic structures of SU-8 were microfabricated on microscope glass coverslips sputtered with a Ti/Au layer. Upon application, chol-DNA adsorbed to the SU-8 structures from solution, leaving the surrounding gold surface free of chol-DNA. chol-DNA immobilization is complete within 15 min and yields a surface coverage in the range of 20−95 pmol/cm^2, which corresponds to a film density of 10^{12}−10^{13} molecules/cm^2. chol-DNA immobilization is stable and can be sustained despite rinsing, drying, dry storage for several hours, and rehydration of chips. Furthermore, complementary DNA in solution hybridizes efficiently to immobilized chol-DNA.

Introduction

Many applications in biotechnology and bioanalysis are based on surface-assisted DNA hybridization.[1] Efficient immobilization protocols yielding high surface coverage and functional accessibility of single-stranded DNA (ssDNA) on different substrates are therefore of great importance. DNA has been covalently attached to glass,[2−5] silicon,[6] fused silica,[7−9] Si_3N_4,[10] gold,[11−13] SU-8,[14] PDMS,[15] PVA,[16] and PMMA.[17] In all of these cases, either the substrate or the oligonucleotide needs to be chemically modified. DNA is located at predefined locations on the solid support either by on-chip synthesis or by immobilization of presynthesized DNA. On-chip synthesis offers high-density arrays[18] but has practical limitations in terms of DNA sequence length, synthesis reliability, and affordability. Conversely, methods based on the immobilization of DNA are generally simpler, cheaper, and more versatile.[17,19] Most immobilization techniques involve incubation times of several hours,[2,10,11,17] several rinsing steps, and harsh chemical treatments. Noncovalent surface adsorption of DNA is the simplest and easiest method to automate because activation/modification of the substrate and subsequent immobilization procedures that are tedious, expensive, and time-consuming are generally not needed.[20]

Here we use SU-8, a negative epoxy UV resist with strong hydrophobic character for the adsorption of chol-DNA. SU-8 is inexpensive and easily produced by methods of microfabrication with the possibility to create feature sizes down to less than 30 nm.[21] Its thermal and chemical stability as well as light transparency above 360 nm make SU-8 ideal for device manufacturing, for example, MEMS/bio-MEMS and microfluidic structures.[22−24] The choice of the DNA anchoring moiety is crucial in the optimization of the coupling between the substrate and target. Cholesterol has a polar hydroxyl group and a nonpolar hydrophobic steroid ring system and a hydrocarbon tail. The assembly of cholesterol on hydrophilic and hydrophobic substrates such as Langmuir−Blodgett films has been reported,[25,26] and cholesteryl-modified oligonucleotides have, for example, been used as linkers between vesicles and supported lipid membranes.[27]

It is demonstrated that cholesteryl-TEG-modified oligonucleotides adsorb efficiently on highly hydrophobic SU-8 surfaces whereas nonmodified oligonucleotides stay in solution. The coupling of chol-DNA to SU-8 involves a strong hydrophobic interaction, and it is presumed that no covalent bonds are formed. The presented immobilization route grants an advantage over

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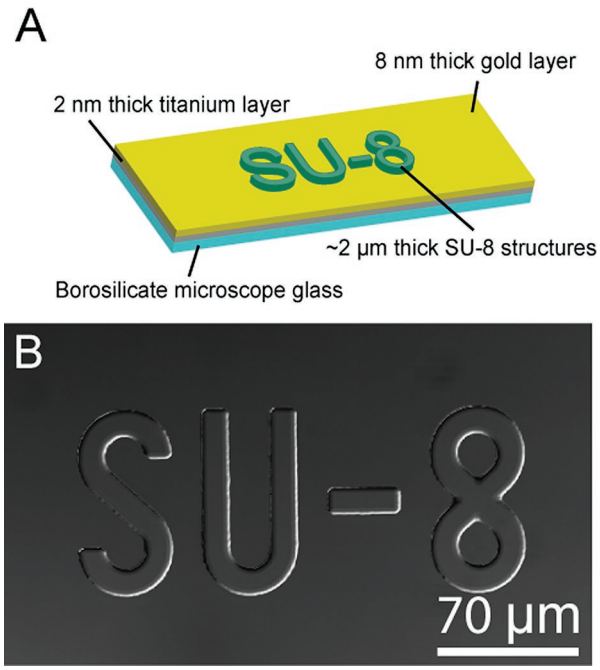

Figure 1. Schematic picture of a microfabricated chip and its transmission image. (A) SU-8 patterned chip. (B) Transmission image of an SU-8 patterned chip.

other methods that involve functionalized surfaces by eliminating the need for surface activation. Furthermore, we obtain high, reproducible yields of hybridization of complementary strands to immobilized chol-DNA.

Experimental Section

Chemicals and DNA Probes Used. All experiments were performed in phosphate buffer (adjusted with KOH (Sigma) to pH 7.8) containing 5 mM TRIZMA base (Sigma), 30 mM K_3PO_4 (Sigma), 30 mM KH_2PO_4 (Merck), 1 mM $MgSO_4$ (Merck), and 0.5 mM EDTA (Fluka) in deionized water. Oligonucleotides (20-mer) were purchased from Medprobe (Lund, Sweden). Before conducting the experiments, DNA concentrations were set by absorbance measurements with a Cary 4000 UV-visible spectrophotometer from Varian (Victoria, Australia).

Chip Microfabrication. Microscope coverslips (25 mm × 50 mm) from Menzel Gläser (Braunschweig, Germany) were used as substrates. The coverslips were thoroughly cleaned by 5 min of sonication in deionized water, followed by a plasma cleaning step in a Tepla Plasma Batch System 300, a microwave plasma system from AMO GmbH (Aachen, Germany) with oxygen plasma at 250 W for 2 min.

Before applying the SU-8 photoresist, an MS 150 sputter system from FHR Anlagenbau GmbH (Ottendorf-Okrilla, Germany) with a base pressure of 5×10^{-7} mbar in the main chamber is used for the deposition of the Ti/Au film onto the cleaned coverslips. A titanium adhesion layer (2 nm) and a gold layer (8 nm) were deposited onto the coverslips with dc magnetron sputtering at deposition rates of 5 and 20 Å/s, respectively, at 5×10^{-3} mbar process pressure.

The dark-field photomask for the SU-8 process was prepared on a JEOL JBX-9300FS electron beam lithography system. A UV-5/0.6 resist (Shipley Co., 455 Forest St., Marlborough, MA) coated Cr/soda-lime mask blank (3 in. size) was exposed, developed, and etched using a common process for micrometer resolution. Pattern files were prepared on the CADopia Intellicad platform.

Prior to applying the resist, gold-coated coverslips were rinsed with deionized water and blown dry with nitrogen. Then, commercially available SU-8 2002 from MicroChem (Newton, MA) was spin-coated at 3000 rpm onto the sputtered Ti/Au film. After applying the photoresist, soft baking at 65 and 95 °C for 6 min, UV-light exposure through a mask at 400 nm, 6 mW/cm^2 in a Karl Süss MJB3-UV 400 mask aligner for 15 s, postexposure bake at 65 and 95 °C for 1 min, and development in SU-8 developer bath from Microresist Technology GmbH (Berlin, Germany) were carried out. Finally, coverslips were thoroughly rinsed with deionized water, blown dry with nitrogen, and hard baked in a Venticell oven from MMM Medcenter Einrichtungen GmbH (Gräfelfing, Germany) at 200 °C for 30 min. Except for the hard-baking step, all fabrication procedures were executed in a cleanroom atmosphere (class 3-6 according to ISO 14644-1).

Contact Angle Measurements. Dynamic contact angle measurements on SU-8 and gold surfaces were carried out with Milli-Q water in a 10Mk2 drop shape analyzing system from Krüss GmbH (Hamburg, Germany).

DNA Absorbance Measurements. Solutions of DNA1 and DNA2 of 1, 2, 3, and 4 μM were prepared in PBS buffer. Concentrations of stock solutions were determined with a UV−vis spectrophotometer. Thereafter, a droplet of stock solution was applied to an SU-8 surface of a defined area. After 15 min of incubation at room temperature, the supernatant was removed, and its absorbance spectrum was

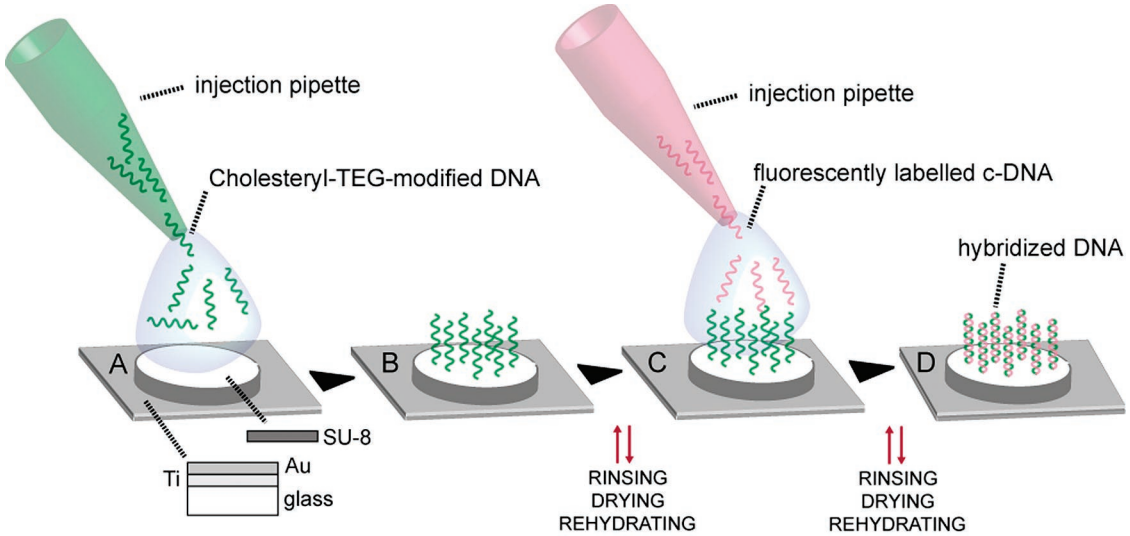

Figure 2. Schematic picture of DNA immobilization and the hybridization procedure. (A) chol-DNA-containing solution is pipetted manually onto the SU-8 structured chip. (B) Incubation at room temperature. (C) Following incubation, the chip is rinsed, dried, and rehydrated with c-DNA-containing solution. (D) Following hybridization of c-DNA to the surface-bound chol-DNA, the chip is again rinsed, dried, and rehydrated with buffer solution.

Table 1. List of Oligonucleotides Used[a]

name	5′mod	3′mod	sequence written 5′ to 3′
DNA1	chol-TEG	Cy5	GCGAGTTTCG
DNA2	chol-TEG		GCGAGTTTCG
DNA3	6-FAM	chol-TEG	GCCAGTTTCGTCTAAGCACG
DNA4		chol-TEG	GCCAGTTTCGTCTAAGCACG
c-DNA1/2		Cy3	CGAAACTCGC
c-DNA3/4	Cy3		CGTGCTTAGACGAAACTGGC

[a] chol-TEG referes to cholesteryl-tetraethyleneglycol, and c-DNAX/X refers to the oligonucleotide complementary to DNAX.

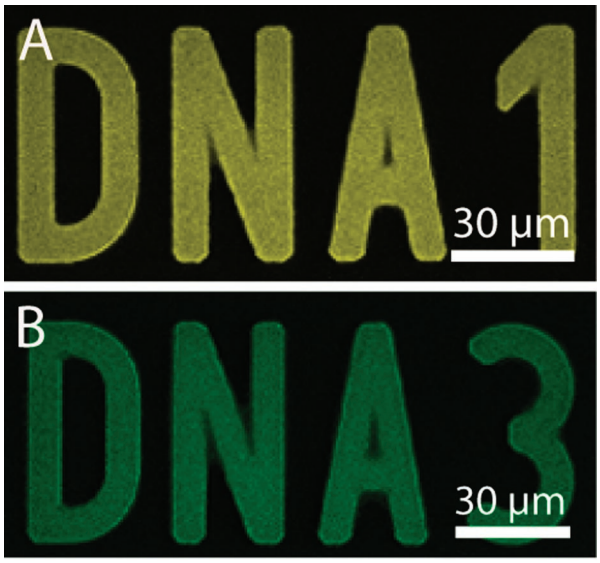

Figure 3. Immobilization detection of fluorescently labeled chol-DNA molecules. Images are artificially colored. (A) Fluorescence of DNA1 immobilized on SU-8 in buffer solution after 15 min of incubation (λ_{exc} = 633 nm, λ_{em} = 660−750 nm). (B) Fluorescence of DNA3 immobilized on SU-8 in buffer solution after 25 min of incubation (λ_{exc} = 488 nm, λ_{em} = 500−540 nm).

recorded. The concentration difference between the stock solution and the supernatant yielded the number of adsorbed DNA molecules on the SU-8 surface, from which the density of immobilized DNA was calculated.

Immobilization and Hybridization Detection. Fluorescently labeled oligonucleotides were scanned with a Leica IRE2 confocal microscope equipped with a Leica TCS SP2 scanner (Wetzlar, Germany). Immobilization and hybridization experiments were carried out at room temperature and in the open atmosphere.

For immobilization experiments, an SU-8 structured chip was placed on the stage of the confocal microscope, and a 2 μM, 250 μL solution containing chol-DNA molecules was manually pipetted onto the chip. After incubation times of 15 min for DNA1 and DNA2 and 25 min for DNA3 and DNA4, the chip was rinsed with Milli-Q water and then dried gently in a nitrogen stream. Thereafter, the chip was rehydrated with buffer, and fluorescence micrographs were recorded for fluorescently labeled chol-DNA molecules. The same procedure was also repeated for hybridization experiments, differing only in the rehydration step. Instead of rehydrating with buffer solution, a chip containing immobilized chol-DNA was rehydrated with a 2 μM, 250 μL solution containing complementary DNA. After the defined incubation period of 15 min for c-DNA1/2 and 25 min for c-DNA3/4, the chip was rinsed with Milli-Q water and then dried gently in a nitrogen stream. Thereafter, the chip was rehydrated with buffer, and fluorescence micrographs were recorded. For the DNA4+c-DNA3/4 and DNA2+c-DNA1/2 probe couples, fluorescence recovery after photobleaching (FRAP) experiments were carried out. A region of interest was bleached using a high-intensity laser, whereafter the fluorescence recovery was monitored.

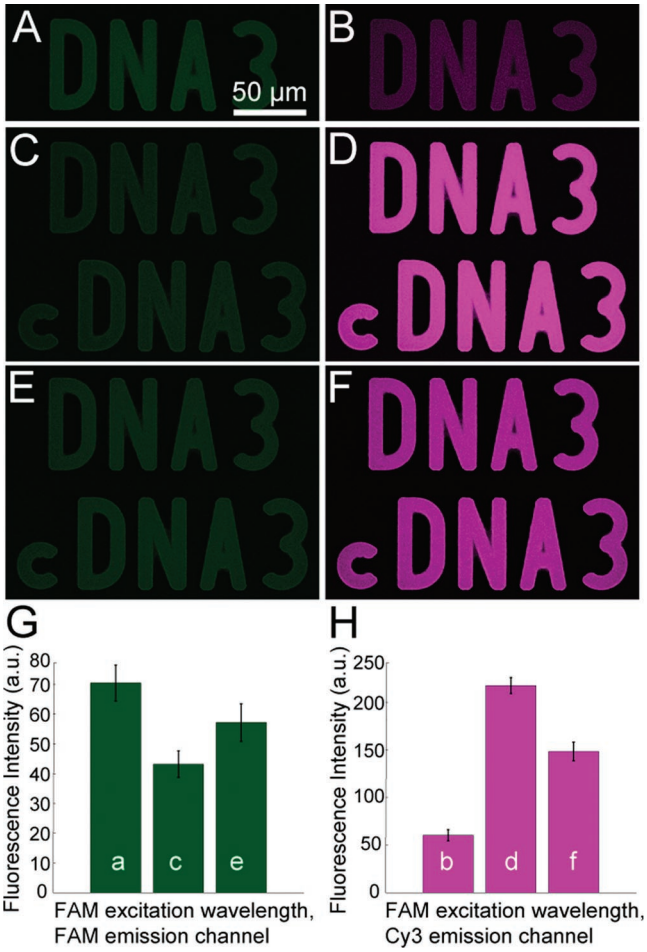

Figure 4. Hybridization detection by FRET using the DNA3+c-DNA3/4 probe couple. Images are artificially colored. The left column represents DNA3 fluorescence detection (λ_{exc} = 488 nm, λ_{em} = 500−540 nm), and the right column represents c-DNA3/4 fluorescence detection (λ_{exc} = 488 nm, λ_{em} = 550−620 nm). (A, B) After washing away the DNA3 solution and then drying and rehydrating the chip with buffer solution. (C, D) After washing away the buffer solution and then drying and rehydrating the chip with c-DNA3/4-containing solution. (E, F) After washing away the c-DNA3/4 solution and then drying and rehydrating the chip with buffer solution. (G, H) Fluorescence intensity of images shown in the left and right columns, respectively. Each column represents the averaged data of four lines from the fluorescence micrograph.

Results and Discussion

Chip Fabrication. For microchip fabrication, a Ti/Au layer was first sputtered on top of a microscope glass coverslip, followed by SU-8 spin coating. Micrometer-sized SU-8 structures were patterned using UV-light exposure through a mask. In the end, the chip was hard baked at 200 °C for 30 min. The final microfabricated chip (Figure 1) thus contained two layers with distinctive surface properties. The gold surface is hydrophilic (contact angle with water is 77.9 ± 3.2°), and the SU-8 structures are hydrophobic (contact angle with water is 91.4° ± 1.5°).

SU-8 Autofluorescence and Surface Coverage of ssDNA. To be able to distinguish the DNA probe fluorescence from the autofluorescence of SU-8, we scanned the patterned SU-8 surface at different excitation wavelengths. A decrease in the SU-8 layer thickness leads to lower autofluorescence.[14] The SU-8 layer in our experiments was approximately 2 μm thick, and its autofluorescence needs to be taken into account only when using an excitation wavelength of 488 nm. The surface coverage of immobilized chol-TEG-5′-GCGAGTTTCG-3′-Cy5, and chol-

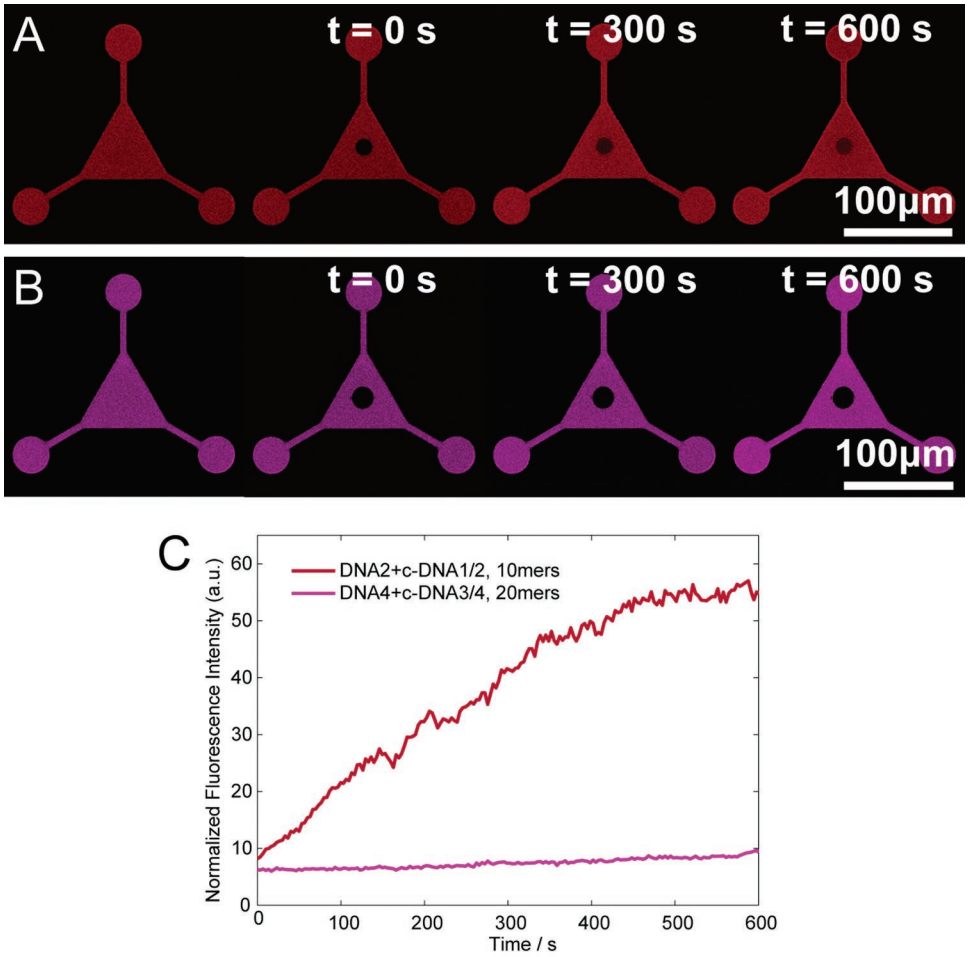

Figure 5. Fluorescence recovery after photobleaching time series with DNA4+c-DNA3/4 and DNA2+c-DNA1/2 probe couples. Images are artificially colored and are taken in buffer solution (λ_{exc} = 543 nm, λ_{em} = 550−620 nm). (A) DNA1+c-DNA1/2 before bleaching and at 0, 300, and 600 s. (B) DNA4+c-DNA3/4 before bleaching and at 0, 300, and 600 s. (C) Fluorescence recovery of bleached region vs time. Fluorescence intensity values are normalized to 100.

TEG-5′-GCGAGTTTCG-3′ was determined using a UV−vis spectrophotometer. Absorbance measurements of chol-DNA-containing stock solution and samples collected from the droplet applied to the chip were recorded. From the concentration difference, the adsorbed amount of chol-DNA was calculated. The surface density of chol-DNA was in the range of 20−95 pmol/cm^2, corresponding to 10^{12}−10^{13} molecules/cm^2. This result can be compared to 150 pmol/cm^2, which is the maximum immobilization density of a monolayer of ssDNA (where the molecules are considered to be cylinders with 20 Å diameter and are oriented perpendicularly to the plane of the surface).[17] The area covered by one ssDNA molecule was thus between 1250 and 333 Å^2. As a comparison, the theoretical area coverage of one ssDNA molecule in a closely packed full monolayer of ssDNA (without cholesteryl-TEG modification) is 111 Å^2.

Immobilization Detection and ssDNA Chip Stability. chol-DNA (Table 1) immobilization and hybridization monitoring were carried out by fluorescence detection via laser scanning confocal microscopy (LSCM). Solutions containing chol-DNA were pipetted and incubated on the chip (Figure 2). Upon application of the droplet, chol-DNA adsorbed to the SU-8 surface within seconds. Following rinsing, drying, and rehydration of the chip with buffer solution, fluorescence images were recorded. Figure 3a,b shows immobilized DNA1 and DNA3 after 15 and 25 min of incubation time, respectively. Control experiments performed using the cholesterol-free c-DNA3/4 clearly show that the SU-8 surface is virtually DNA-free (data not shown). These results strongly suggest that cholesterol plays a critical role in the adsorption of DNA to the SU-8 surface. Increasing incubation times and concentrations, both for the 10- and 20-mers of DNA, do not significantly change the fluorescence intensity (data not shown). This suggests that the SU-8 surface is easily saturated with chol-DNA. To investigate the stability of the SU-8 cholesterol interaction, chips with immobilized chol-DNA were kept dry on the shelf for 6 h and then rehydrated with buffer solution. From the fluorescence intensity data in Figures 3b and 4a, it was calculated that only ∼40% of the immobilized chol-DNA was lost after storage in air.

Hybridization of Complementary DNA to ssDNA Bound to SU-8. We used two different techniques to verify hybridization. First, hybridization was shown by fluorescence resonance energy transfer (FRET) imaging (Figure 4) between the fluorescently labeled DNA3 and its complementary c-DNA3/4. In Figure 4, the right panels show the emission of the acceptor whereas the left panels show the emission of the donor, both being excited at the donor excitation wavelength. Prior to adding the complementary c-DNA3/4, the fluorescence micrograph shows the immobilized DNA3 on SU-8 after the chip was kept dry for 6 h (Figure 4a). By comparing Figure 4b and d, it was found that the addition of c-DNA3/4 increases the fluorescence signal of the Cy3-label significantly whereas it decreases for the FAM-label (compare Figure 4a and c). This proves FRET between the labeled couple and thus DNA hybridization. After the chip has been rinsed, dried, and rehydrated with buffer solution, the

immobilized and hybridized DNA3+c-DNA3/4 pair was still present on the SU-8 surface (Figure 4e,f). Hybridization was also verified by the detection of fluorescently labeled complementary DNA strands that were bound to unlabeled immobilized chol-DNA (Figure 5). As mentioned above, oligonucleotides lacking a cholesteryl moiety do not adsorb on SU-8 surfaces. We used the DNA4+c-DNA3/4 as well as the DNA2+c-DNA1/2 pairs, and fluorescence recovery after photobleaching (FRAP) was monitored. The immobilized DNA (DNA4 and DNA2) were not labeled, and evidence of hybridization comes from the detection of fluorescence from Cy3 in the complementary strands (c-DNA3/4 and c-DNA1/2). Using high laser light intensity, a defined region of interest was bleached, and the fluorescence recovery of the bleached spot was monitored. The kinetics of the exchange of bleached and unbleached double-stranded oligonucleotides (dsDNA) from the solution to the substrate is yet to be determined, but as the dsDNA-chip system equilibrates, the shorter oligonucleotides shows faster desorption/adsorption behavior compared to that of the longer oligonucleotides. However, the bleached spot was never fully recovered within the experimental time frame. In conclusion, the results from the hybridization experiments prove that cholesteryl-TEG-modified oligonucleotides are accessible to their complementary strands, even after the immobilized DNA has been kept dry for several hours.

Conclusions

We report a straightforward one-step process for immobilizing cholesteryl-modified oligonucleotides on hard-baked SU-8 surfaces. The attachment between the substrate and the DNA is presumably based on the hydrophobic nature of the SU-8 and the cholesteryl-TEG modification at the 5′ or 3′ position of the oligonucleotide. The immobilized DNA on SU-8 shows robust and efficient attachment and high surface coverage and is accessible to hybridization by complementary strands. Previous surface coverage values for DNA immobilization by covalent attachment are in the range of $10^{11}-10^{12}$ molecules/cm^2.[4] Here we achieve a 10 times higher surface coverage in a simple one-step immobilization protocol. Furthermore, the immobilized chol-DNA is still functional after being kept dry for several hours. Regarding chip fabrication, the essential step is how SU-8 is processed. Bare SU-8 surfaces without topographical features are very simple to produce and are adequate for chol-DNA immobilization. SU-8 can, for example, be deposited on glass using spin coating. This does not require any cleanroom procedures. For making structured devices, the deposition of metal layers is required. However, highly automated methods can be used such as sputtering or evaporation, and they can be substituted by simpler techniques such as electroplating, which again does not require cleanroom technology. DNA bound to surfaces has great importance in processing and analyzing different kinds of samples.[18] The techniques presented here for ssDNA and dsDNA immobilization represent a simple and highly efficient procedure with potential applications in DNA microarrays,[19] microfluidic devices,[1,15] and functionalized surfaces (i.e., in cantilevers,[28] QCM[29] (quartz crystal microbalance), and SPR[30] (surface plasmon resonance)).

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