5′-Thiolated Oligonucleotides on (3-Mercaptopropyl)trimethoxysilane−Mica: Surface Topography and Coverage

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DNA (deoxyribonucleic acid) microarrays are used as a tool in high-throughput methods to obtain genetic information. Using atomic force microscopy, X-ray photoelectron spectroscopy (XPS), time-of-flight secondary ion mass spectrometry, and confocal scanning fluorescence microscopy, we investigated how the surface properties and topography of (3-mercaptopropyl)trimethoxysilane (MPTS)-coated mica influence the immobilization of 5′-thiolated oligonucleotides. Mica surfaces were grafted with concentrations of MPTS varied over 3 orders of magnitude (5.38 mM to 5.38 M). At low concentration (5.38 mM), condensed MPTS appeared to form "island-like" structures with an average surface area of 100 nm^2. The MPTS islands grew in size to 2400 nm^2 as the concentration of MPTS was increased, suggesting the formation of an MPTS monolayer. XPS analysis indicated a corresponding increase in the elemental concentration of sulfur. At high MPTS concentrations (107.6 mM and 5.38 M), the island structures disappeared, suggesting the formation of a multilayered film. Fluorescence-labeled oligonucleotides were attached onto the MPTS−mica by covalent bonding via disulfide formation. Confocal scanning fluorescence microscopy was used to quantify the coverage of oligonucleotides. The data demonstrated a bell-shaped curve, in which the coverage of oligonucleotides on diluted MPTS incubated mica was ∼5 times higher than the coverage on mica incubated with pure MPTS. The maximum mean oligonucleotide density was 8.95×10^{13} probes/cm^2. The highest immobilization density was achieved when the MPTS formed a near monolayer. Increased MPTS concentration led to the formation of a multilayer. The reduced number of thiol groups and the increased presence of silanol groups on the surface led to a significant reduction of oligonucleotide surface density.

Introduction

In the past decade, devices such as DNA biosensors, gene chips, and miniaturized DNA analyzers have been developed for high-throughput analysis to aid gene discovery, disease diagnosis, drug screening, and other applications.[1−11] The fabrication of small, high-performance and low-cost DNA devices remains a major challenge.[12]

The surface properties of substrates influence the immobilization of the DNA probe molecules.[13−15] To improve the performance of the DNA-based analytical devices, it is important to have a thorough understanding of the nature of DNA−surface interactions. For DNA immobilization, support substrates, for example, silicon wafers or glass, are generally coated with another chemical layer, for example, amino- or mercaptosilanes, to which DNA is bound covalently via free amino or thiol groups. Thus, it is important to investigate not only the conditions influencing the interaction of the DNA with the coating layer but also the interaction of the coating substrate with the underlying supporting material. Radioisotopic measurements, electrochemical methods, and two-color surface plasmon resonance (SPR) spectroscopy have been employed in studies of DNA surface density.[9,15−19] Alternative methods such as fluorescent imaging could provide qualitative and quantitative measures of surface DNA coverage. Atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS), and time-of-flight secondary ion mass spectrometry (TOF-SIMS) are surface charac-

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terization techniques useful for elucidating the surface properties and DNA−surface interaction phenomena. The versatility and high resolution of AFM make it an ideal tool for the study of surface interactions between the support substrate, the grafted chemical moieties, and DNA probes. In addition to the surface properties of the substrate, other factors that affect DNA immobilization include the ionic strength, pH, and temperature of the immobilization buffer.

We undertook the present study in order to investigate the relationship between the surface topography of (3-mercaptopropyl)trimethoxysilane (MPTS) layers on mica and the immobilization of 5′-thiol-modified DNA oligonucleotides by disulfide bond formation between the DNA and the MPTS. We chose mica for these experiments because it provided an atomically flat crystalline surface. We employed surface-sensitive analytical methods such as contact angle measurement, AFM, XPS, TOF-SIMS, and confocal scanning fluorescence microscopy to characterize the MPTS-modified mica surface and the influence of the topography of the MPTS layer on the immobilization of oligonucleotides.

Experimental Section

Materials. All chemicals and solvents were purchased from Fluka and Sigma-Aldrich (Milwaukee, WI). DNA oligonucleotides were purchased from Synthetic Genetics (San Diego, CA). For the immobilization experiments, the oligonucleotides used were 5′-thiol modified and 3′ labeled with Texas Red (TX), with the following sequence: 5′-HS-spacer-GTGCTTGGGCGA-TX-3′. For the hybridization experiments, the probe oligonucleotide used was 5′-HS-spacer-CACAAAACGGGGGCGG-3′ and the labeled target oligonucleotide was 5′-TX-CCGCCCCCGTTTTGTG-3′. Mica was purchased from New York Mica Co. (New York, NY).

Preparation of Substrates. Ruby muscovite mica was freshly cleaved before use. Silicon wafers were subjected to a thorough cleaning procedure before processing with CMOS (complementary metal oxide semiconductor)-compatible processes. The wafer was cut into 5 mm × 3 mm dies and immersed in a solution of ammonium hydroxide, 2-propanol, and water (1:1:1) overnight at room temperature. After washing with 2-propanol and double-distilled H_2O (ddH_2O) and drying with nitrogen gas, substrates were treated with either pure (3-mercaptopropyl)trimethoxysilane (5.38 M) or one of the following MPTS dilutions in 2-propanol (v/v): 1:50 MPTS (106.7 mM), 1:100 MPTS (53.8 mM), 1:1000 MPTS (5.38 mM). The control solution consisted of 2-propanol. At least two pieces of each substrate were used for each experimental condition. Substrates were immersed for 1 h at 80 °C in 5 mL of the solutions described above. They were then rinsed with 2-propanol and ddH_2O and dried with nitrogen gas.

Immobilization of Oligonucleotides. The probe oligonucleotides were diluted to 20 μM with 2X saline−sodium citrate buffer (SSC), which contained 0.3 M NaCl and 0.03 M sodium citrate adjusted to pH 7.3 with 1 M HCl unless specified. One microliter of buffer containing 3′-labeled and 5′-thiolated fluorescent oligonucleotides and 1 μL of non-labeled 5′-thiolated oligonucleotides for hybridization experiments were placed on the substrate surface and incubated at room temperature for 3 h inside a humidified chamber. Substrates were then rinsed with 2X SSC solution containing 1% sodium dodecyl sulfate (SDS), rinsed with ddH_2O, and dried with nitrogen gas.

Hybridization of Oligonucleotides. Oligonucleotide targets labeled with TX were diluted in hybridization buffer (consisting of 6X SSC pH 7, 5X Denhardt's solution (1% BSA (bovine serum albumin), 2% Ficoll 400 and 2% poly(vinylpyrrolidone) (PVP), 0.5% SDS, and sheared salmon sperm DNA (55 μg/mL)) to a final concentration of 20 μM. Substrates were incubated with 6 μL of target solution inside a humidified sealed Petri dish for 2 h at 53 °C in a vacuum oven. They were washed with 2X SSC solution containing 1% SDS, washed with ddH_2O, and dried with nitrogen gas. The fluorescent intensity of the oligonucleotides was measured and quantified as described below.

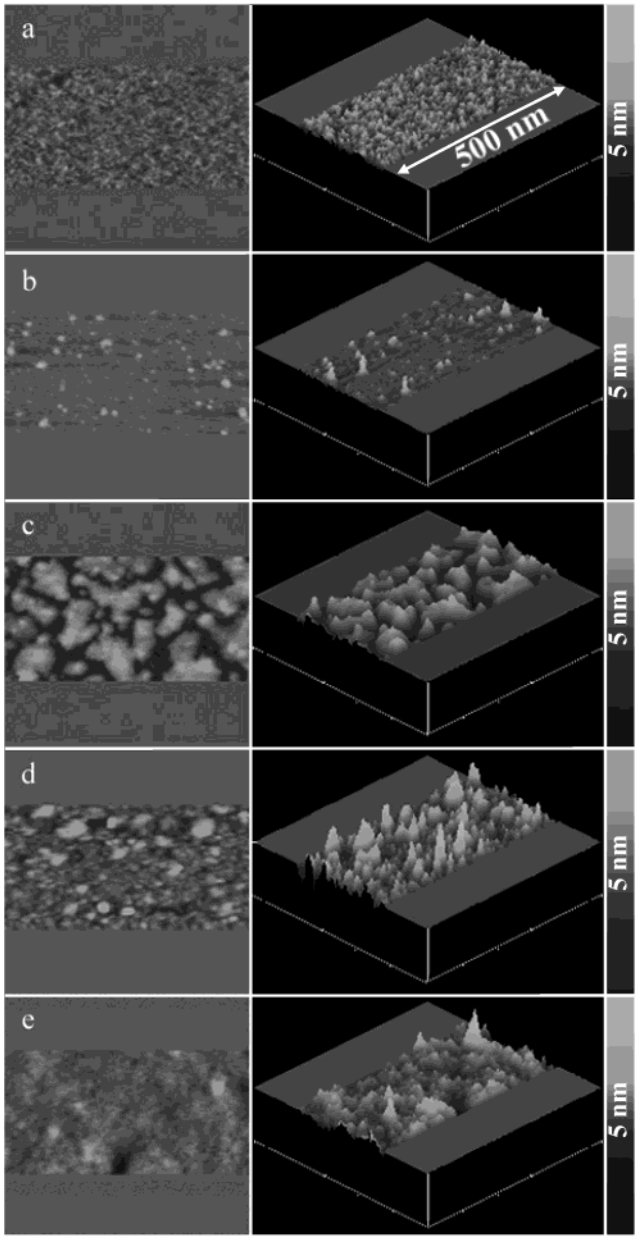

Figure 1. AFM images of chemically modified mica surfaces. The left panel shows the top surface view, and the right panel shows the three-dimensional surface plot. (a) Fresh mica surface; (b) incubated with 1:1000 diluted MPTS (5.4 mM); (c) incubated with 1:100 diluted MPTS (53.8 mM); (d) incubated with 1:50 diluted MPTS (107.6 mM); (e) incubated with pure MPTS (5.38 M).

Fluorescence Detection. A confocal laser scanner (ScanArray 5000, Packard Biosciences, CT) was used to scan the immobilized oligonucleotide spots. The photomultiplier tube (PMT) gain and laser power were set at 60 arbitrary units for all measurements using TX-compatible laser and filter settings (excitation, 594 nm; emission, 614 nm). Mean spot intensities were measured and evaluated with QuantArray (Packard Biosciences) imaging software. Calibration was conducted by measuring the fluorescent intensity corresponding to known concentrations of labeled oligonucleotides. The oligonucleotide solutions were diluted in 2X SSC to 500 μM, 300 μM, 100 μM, 50 μM, 20 μM, 2 μM, 200 nM, and 20 nM. Five microliters of each dilution was spotted onto a clean microscopic slide. The spots were dried off inside a clean Petri dish in the absence of light.

Surface Characterization. A tapping-mode atomic force microscope (Nanoscope 3a, Digital Instruments, Santa Barbara, CA) was used to image the surface of the mica substrates after the MPTS was grafted onto the support. Data with a resolution

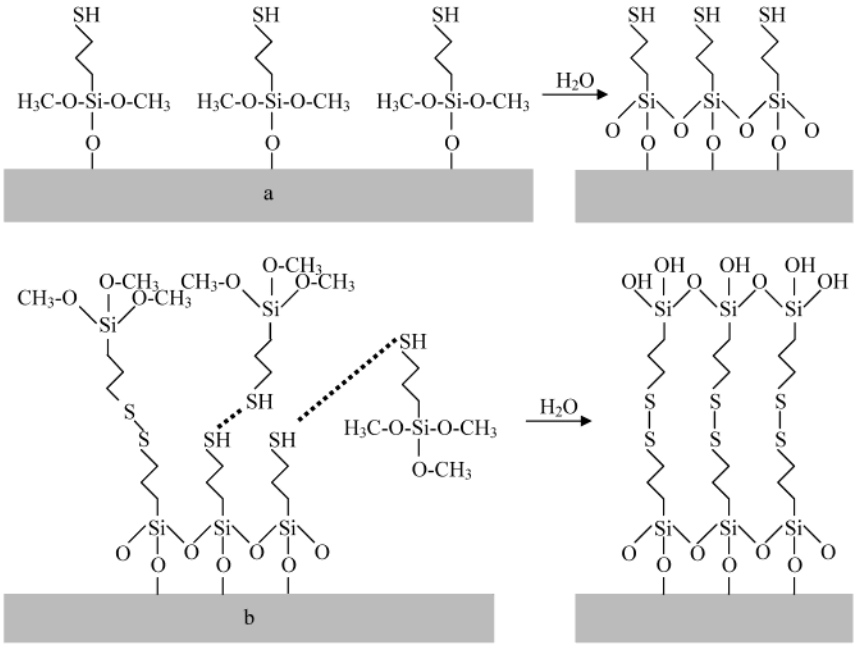

Figure 2. Schematic of the formation of the MPTS monolayer (a) in the presence of H_2O molecules and MPTS multilayers (b) in the presence of H_2O molecules and the interaction between their thiol groups.

better than 5 nm were obtained for each sample and analyzed for their surface roughness and topography with the aid of imaging software from Digital Instruments. Although the AFM provided us with a detailed picture of the surface, it lacks information on the composition and chemistry of the surface. The surface chemical properties were analyzed by X-ray photoelectron spectroscopy (Physical Electronics, PHI 5600). The analyses were conducted at both 45° and 25° angles between the incident beam and the sample to obtain a sampling depth of 5 and 2 nm, respectively. The wettability of the surface is an important parameter for DNA immobilization that is directly affected by both the surface roughness and chemistry of the substrate. A Ramé-Hart goniometer model no. 100-00 was used for the contact angle measurement. A 5-μL droplet of ddH_2O was deposited onto the surface using a pipet. Three readings were taken for each sample after 1 min of deposition.

Results and Discussion

Surface Properties of MPTS−Mica. Mica provided an atomically flat crystalline surface, onto which the MPTS was grafted. Micas grafted with different concentrations of MPTS were prepared to investigate how surface topography and chemistry affected the immobilization of the oligonucleotides. The 5′-thiolated 3′ fluorescence labeled oligonucleotides were subsequently attached onto the MPTS−mica by covalent bonding with the grafted MPTS serving as a bifunctional linker.

AFM images of the freshly cleaved mica surface (Figure 1a) displayed a mean surface roughness of about 0.09 nm. MPTS molecules were grafted onto the mica by condensation of silanol groups onto the hydroxylated surface. Figure 1b shows the appearance of grafted molecular groups on the mica surface following treatment with dilute MPTS solution (5.38 mM). Condensed MPTS appeared to form "island-like" structures with an average surface area of 100 nm^2. Formation of similar islands has been observed previously in a study reporting the morphological characterization of MPTS layers on silicone oxide.[20] Both XPS and TOF-SIMS analyses were conducted on the sample. XPS yielded quantitative data on the surface composition, and TOF-SIMS provided a precise chemical map of the surface. Both XPS and TOF-SIMS analyses indicated the appearance of sulfur-containing compounds on the surface of the MPTS-treated mica. Grazing angle XPS confirmed that the sulfur was exposed on the surface of mica as thiol groups.

The MPTS islands grew in size as more concentrated MPTS solutions (53.8 mM) were used (Figure 1c). The average surface area of these islands was 2440 nm^2, and their growth was encouraged by condensation between neighboring silanol groups grafted onto the mica surface. The appearance of the islands was accompanied by an increase in the surface roughness to 0.48 nm. The size and coverage of the MPTS islands in Figure 1c suggest that there was a near monolayer MPTS coverage (0.8 layer) on the mica. Figure 2a illustrates the schematic of the formation of the MPTS monolayer by the presence of H_2O molecules. XPS analysis indicated a corresponding increase in the elemental concentration of sulfur from 0.63 to 0.91 atomic percent for these samples (Table 1).

A distinct change in surface morphology was observed on the mica substrates exposed to concentrated MPTS solutions (107.6 mM and 5.38 M). Images d and e of Figure 1 show the disappearance of the island structures observed at lower MPTS concentrations. Figure 1d shows that the island structure disappeared. This transformation was complete when pure MPTS solution was used. It is very interesting to note that the surface roughness diminished from 0.48 to 0.29 nm. XPS data on the sulfur content as well as the TOF-SIMS signal ratio for CH_3OSi/Si showed increased values as the concentration of the MPTS solutions used for surface treatment of mica was increased. However, the TOF-SIMS signal ratio for S/CH_3OSi and SO_3/CH_3OSi was maximal at 53.8 mM MPTS. At MPTS concentrations less than 53.8 mM, the thiol groups of the (3-mercaptopropyl) trimethoxysilanes grafted on the mica were exposed, whereas at higher concentrations the surface of the sample was enriched with silanol groups. These results are consistent with the formation of a multilayered film where the second layer of MPTS was coordinated to the first layer through interactions between their thiol groups. Figure 2b shows the schematic of formation of multilayered MPTS. This also means that

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Table 1. Surface Composition of the MPTS-Modified Mica Surface

	XPS analysis			TOF-SIMS analysis		
[MPTS] (mM)	S (at. %)	S/Al	S/Si	S/CH_3OSi	SO_3/CH_3OSi	CH_3OSi/Si
5.4[a]	0.63 (45°)	0.05 (45°)	0.03 (45°)	14.2	1.30	0.30
	0.55 (45°)	0.05 (45°)	0.04 (45°)			
	1.01 (25°)	0.10 (25°)	0.06 (25°)			
10.7	0.91	0.08	0.06	13.3		0.11
53.8	1.69	0.16	0.10	42.0	3.60	0.81
71.7	1.86	0.17	0.12	40.0	2.80	2.10
108	2.14	0.21	0.13	40.0		3.30
5380	2.49	0.26	0.16	27.0	1.10	

[a] XPS analysis was conducted using two different angles of the incident X-ray beam.

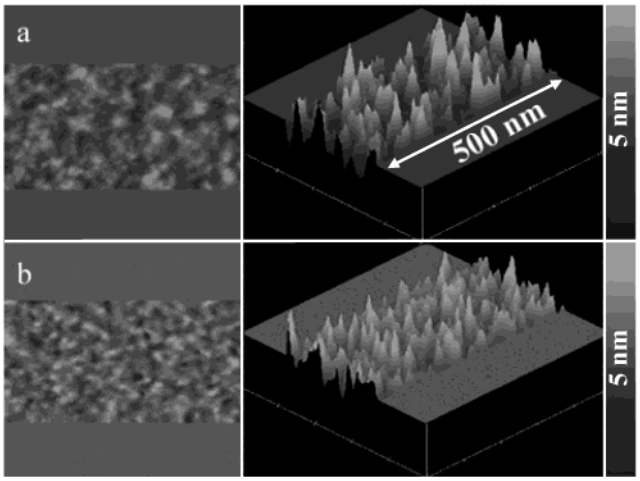

Figure 3. AFM images before (a) and after (b) the immobilization of DNA probes on MPTS-modified (1:50 diluted) mica surfaces. The left panel shows the top surface view, and the right panel shows the three-dimensional surface plot.

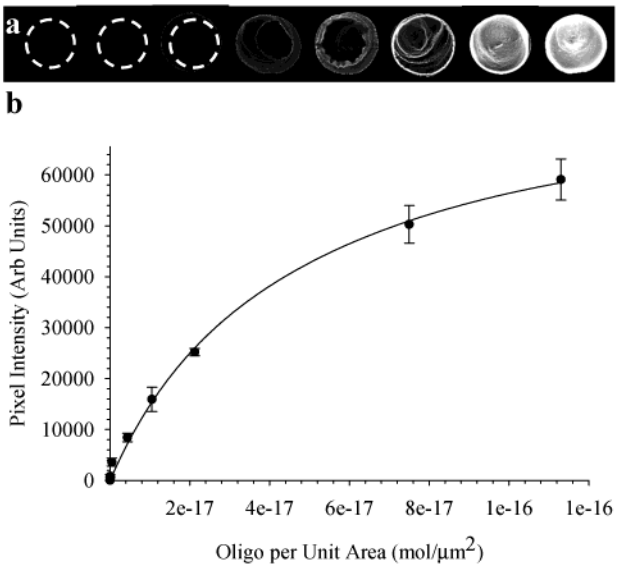

Figure 4. (a) Confocal laser scanner images of Texas Red labeled oligonucleotides. The concentrations of oligonucleotides are, from left to right, 20 nM, 200 nM, 2 μM, 20 μM, 50 μM, 100 μM, 300 μM, and 500 μM. (b) Calibration curve with the mean fluorescent intensity as a function of the mean of oligonucleotides per unit area.

the silanol groups were exposed on the surface of these samples. Exposure to moisture from the ambient air resulted in the condensation of the silanol group to form a silylated surface (Figure 1e). The wettability of the surface decreased with the contact angle increasing from 7° for bare mica to 52.7° ± 0.3° for mica incubated with 5.38 mM MPTS, 67.1° ± 0.4° for 53.8 mM MPTS, 71.8° ± 1.9° for 107.6 mM MPTS, and 85.2° ± 0.4° for pure MPTS (5.38 M).

Surface Coverage of Single-Stranded DNA (ssDNA) on MPTS-Incubated Mica. The AFM images in Figure 3a and b show examples of the surface of the substrate before and after immobilization of thiolated oligonucleotides on mica treated with 107.6 mM MPTS, respectively. The addition of the oligonucleotides resulted in a substantial change in the surface morphology. It can be seen from the AFM image that an additional layer was deposited onto the original MPTS-grafted mica. This layer consisted mostly of small globular particles (~4 nm) coated on top of the MPTS islands.

The AFM data suggested a mean size of the DNA particles of about 40 Å compared to the theoretically predicted length of 78 Å for a fully stretched 12-mer oligonucleotide. The difference in the length may be explained by a number of factors. Hansma and co-workers demonstrated that AFM imaging of well-extended ssDNA was difficult.[21] Even when denatured, single-stranded X-174 DNA could only be extended to a maximum length of only ~0.2 nm/base.[22,23] Ideally, the 5′-thiolated oligonucleotide should attach on the MPTS surface by disulfide bond formation, but the rough surface (Figure 3a) might affect how the oligonucleotides are coordinated to the surface. ssDNA is known to form secondary structures, hairpins or loops, and this may account for the globular shape of the oligonucleotides revealed in the AFM images. The annealing score for the oligonucleotides used in these experiments corresponded to a value of 6 (maximum score = 18; Omiga software). If there was a hairpin structure formed between bases 3−10 and 4−9, the expected height of the particle would be 2.72 nm, close to the 4 nm measured by AFM, accounting for $^2/_3$ of the size of the globular particles. However, the fluorophore linked to the oligonucleotides also contributes to the size of the globular particles seen on the surface.

An attempt to estimate the surface density of immobilized oligonucleotides on the modified mica surface by AFM was not successful due to the irregular pattern and high density of oligonucleotides making accurate measurements impossible.[24] Therefore, we used confocal fluorescent microscopy to determine the surface density of immobilized oligonucleotides on the modified mica surface.

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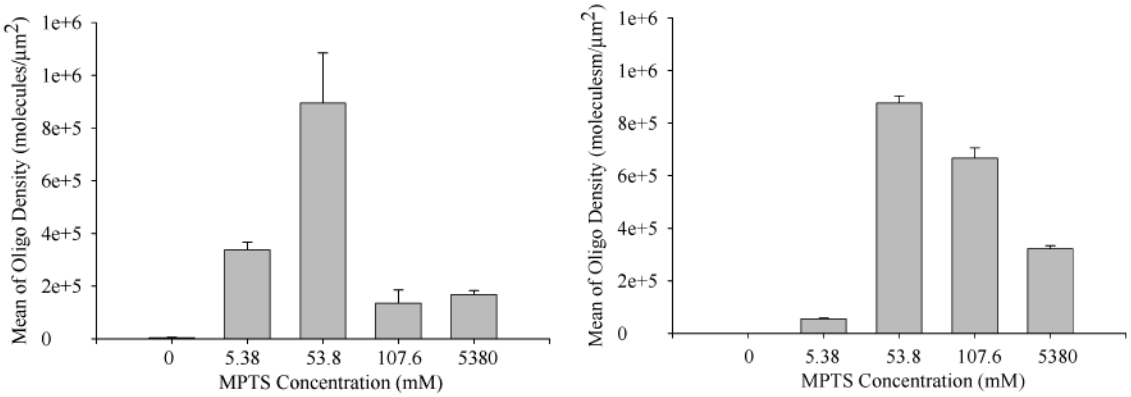

Figure 5. Oligonucleotide probe (left panel) and target density (right panel) on mica treated with different concentrations of MPTS.

Estimation of the Surface Coverage of ssDNA. The surface density of 5′-thiolated fluorescent oligonucleotides on mica surfaces coated with different concentrations of MPTS was quantified in order to determine which coating gave the best surface coverage for 5′-thiolated oligonucleotides. The fluorescent images with known concentrations of TX-labeled oligonucleotides (500 μM, 300 μM, 100 μM, 50 μM, 20 μM, 2 μM, 200 nM, and 20 nM) were acquired with a confocal laser scanner (Figure 4a). The images were then analyzed and quantified using the software mentioned above. A calibration curve with the mean fluorescent intensity as a function of the mean of oligonucleotides per unit area (μm^2) was constructed (Figure 4b). Figure 5 (left panel) shows the results of fluorescence intensity of the labeled oligonucleotides immobilized on mica treated with MPTS at different concentrations. The data indicated that there was virtually no unspecific attachment of oligonucleotides to the bare mica surface, resulting in very low background fluorescence intensity. When the concentration of MPTS increased 10-fold (from 5.38 to 53.8 mM), the mean florescence intensity increased, reaching the maximum. The concentration of immobilized oligonucleotides was significantly lower when mica was coated with MPTS concentrations higher than 53.8 mM. These results are consistent with our inference about the formation of a second layer of MPTS exposing silanols on the surface instead of the thiols available for disulfide linkage affecting the immobilization efficiency. Furthermore, it is possible that in the presence of water the silanol condensed to form a silylated surface that is known to be a poor substrate for immobilization of oligonucleotides.

The density of oligonucleotide per unit area was determined to be 1.48×10^{-18} mol/μm^2, corresponding to 895 174 probes/μm^2 (8.95×10^{13} probes/cm^2). This density is similar to previously observed densities in the range of $10^{12}-10^{13}$ probes/cm^2 using either 5′- or 3′-thiol-modified probes immobilized on gold, glass, and silicon substrates.[13,16,18,19,24] These results are consistent with the notion that surface coverage is influenced by a number of factors such as the concentration of the oligonucleotides, the length of oligonucleotides, the pH of buffer, the ionic strength of the immobilization buffer, the temperature, the incubation time, and the rinsing steps. The length of carbon spacers may also contribute to the irregular oligonucleotide layer.

Hybridization of Target Oligonucleotides to Immobilized Probes. In parallel with the immobilization of fluorescently labeled oligonucleotide probes, we immobilized unlabeled oligonucleotide probes in order to investigate the relationship between probe density and hybridization signal (Figure 5, right panel). Probe and target density were in good agreement at low MPTS concentrations (5.38 and 53.8 mM). The increased probe density at higher MPTS concentrations suggested nonspecific interaction ("sticking") of unhybridized probe to the surface under the stringency conditions used in these experiments.

Conclusion

The aim of the present study was to investigate the relationship between the surface topography of (3-mercaptopropyl)trimethoxysilane layers and the immobilization efficiency of thiol-modified DNA oligonucleotides. Using surface-sensitive methods (AFM, XPS, TOF-SIMS, contact angle measurements), we determined that the highest immobilization density was achieved when the MPTS formed a near monolayer. Increased MPTS concentration led to the formation of a multilayer where the layers were coordinated by interaction between their thiol groups. The reduced number of thiol groups and the increased presence of silanol groups on the surface were accompanied by a reduction of the density of immobilized oligonucleotides.

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