Synthesis and Characterization of DNA-Modified Silicon (111) Surfaces

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Abstract: Hydrogen-terminated Si(111) surfaces are modified by attachment of oligodeoxynucleotides and characterized with respect to DNA surface density, chemical stability, and DNA hybridization binding specificity. Surface functionalization employs the reaction of ω-unsaturated alkyl esters with the Si(111) surface using UV irradiation. Cleavage of the ester using potassium *tert*-butoxide yields a carboxyl-modified surface, which serves as a substrate for the attachment of DNA by means of an electrostatically adsorbed layer of polylysine and attachment of thiol-modified DNA using a heterobifunctional cross-linker. The resultant DNA-modified surfaces are shown to exhibit excellent specificity and chemical stability under the conditions of DNA hybridization. This work provides an avenue for the development of devices in which the exquisite binding specificity of biomolecular recognition is directly coupled to semiconductor devices.

Introduction

DNA-modified surfaces are the subject of considerable current activity in the field of biotechnology.[1−25] Despite their growing importance, several aspects of the performance of these novel composite materials is far from optimum, and their surface chemistry remains poorly characterized. Desired attributes of DNA-modified surfaces include the following: (a) surface flatness and chemical homogeneity; (b) ability to control surface chemical properties such as polarity or hydrophobicity, which impact strongly upon nonspecific binding properties; (c) amenability to DNA hybridization (duplex formation) and enzymatic manipulation with DNA-modifying enzymes such as ligase, polymerase, and restriction enzymes; (d) ability to control DNA surface density; (e) thermal and chemical stability; and (f) reproducibility of preparation. Few if any of these criteria are met by the surface chemistries presently in use.

Remarkable advances have been made in microelectronics technology over the last 20 years, primarily due to increasingly powerful capabilities for the parallel fabrication of transistors and other microelectronic devices on small length scales. A similar trend has become evident in the fields of biology/biotechnology, where arrays of tens of thousands of distinct DNA molecules on planar substrates have proven useful for the parallel analysis of genetic variation and gene expression levels. The development of robust, well-characterized surfaces and surface attachment strategies for biological analyses could benefit greatly from the well-developed infrastructure that exists in microelectronics. Previous researchers have successfully attached DNA to substrates such as latex beads,[13] polystyrene,[12] optical fibers,[24,26] carbon electrodes,[19,22,23,27] gold,[3,20−22,28] and oxidized silicon.[2,11,25,29] Although largely unexplored to date,

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crystalline silicon is a particularly attractive alternative substrate for DNA immobilization because of its purity and well-defined structure, and because crystalline silicon substrates provide the opportunity to take full advantage of existing technologies in microelectronics processing.

Chemical functionalization of silicon is complicated by the fact that silicon readily oxidizes in air to produce an oxide that is chemically similar to glass. Unfortunately, the use of glass or oxidized silicon substrates for highly parallel DNA attachment presents some problems that are a direct consequence of the fact that glass and oxidized silicon surfaces are amorphous, and that the relative number of Si−O−Si and Si−OH linkages exposed at the surface is highly dependent on the past history of the sample. This irreproducibility in surface chemistry leads to difficulties in the control, reproducibility, homogeneity, and stability of subsequent DNA attachment. Planar glass and oxidized silicon substrates can be modified using methoxysilane or chlorosilane reagents[2,11,30−33] to attach organic functional groups such as amines or thiols to serve as DNA attachment sites. Silane reagents containing only a single surface reactive functionality (e.g. monochloro- or monomethoxysilanes) produce poorly stable films.[34] The stability can be improved by using multiple reactive groups (e.g. trichloro- or trimethoxysilanes) to form more Si−O−Si linkages, but such polyfunctional reagents also lead to poorly controlled surface polymerization reactions, compromising surface homogeneity and reproducibility.[35]

A solution to this problem would be to develop strategies for direct attachment of DNA to silicon surfaces without an intervening oxide layer. In recent years, new attachment methods for the organic functionalization of silicon surfaces through formation of direct silicon−carbon bonds have been reported.[1,5,9,36−38] In this report we utilize these reactions as a route for the attachment and hybridization of DNA to crystalline silicon substrates. The resultant DNA-modified silicon surfaces are reproducibly prepared, stable to the conditions of DNA hybridization, and show no detectable nonspecific binding. The high fidelity of these surfaces promises substantial benefit to the emerging technology of large-scale biological analysis using nucleic acid arrays.

Results and Discussion

Overview of Surface Attachment Chemistry. The chemistry employed for DNA attachment to Si(111) surfaces is diagrammed in Figure 1. A hydrogen-terminated Si(111) surface is reacted with an ω-undecylenic acid methyl or trifluoroethyl ester by UV irradiation of a thin film of the ester applied to the surface.[5,37] Similar reactions of ω-alkenes have previously been described, mediated either by diacyl peroxides[1] or by direct thermal activation.[9] Hydrolysis of the ester by treatment with potassium *tert*-butoxide in DMSO[39,40] yields a carboxylic acid-modified surface. Subsequent addition of poly-L-lysine (PL) and reaction of the lysine ϵ-amino groups with the heterobifunctional cross-linker SSMCC results in a maleimide-activated surface that may then be coupled in aqueous solution with a thiol-modified oligodeoxynucleotide to yield the DNA-modified surface.[3]

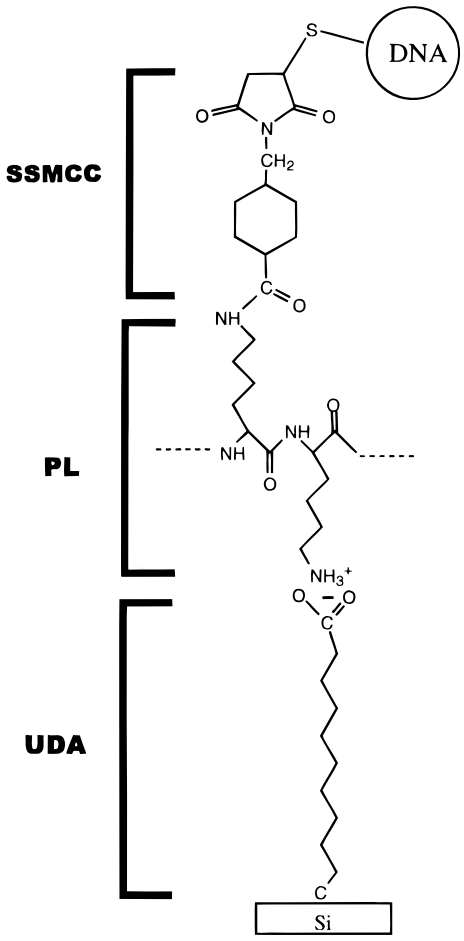

Figure 1. Drawings illustrating the chemistry employed for preparation of the modified Si(111) surface. A layer of 10-undecylenic acid (UDA) is bound to the surface by attachment and subsequent hydrolysis of the ester. A layer of electrostatically bound polylysine (PL) and a layer of sulfosuccinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SSMCC) follow the UDA. Thiol-modified DNA is subsequently bound to the SSMCC.

Surface Characterization by XPS. X-ray photoelectron spectroscopy (XPS) was used to follow the surface coupling reaction and ester hydrolysis, as well as providing a means of monitoring the degree of oxidation occurring on the silicon surface. In initial work the methyl ester of ω-undecylenic acid was employed; however, it was found that the XPS signal from the methyl group was too poorly resolved from the signal corresponding to the undecylenic acid alkyl chain to permit hydrolysis of the ester to be monitored. To address this, the trifluoroethyl ester of ω-undecylenic acid was synthesized and reacted with the surface; the fluorine 1s signal from the trifluoroethyl group is strong and well resolved from other XPS

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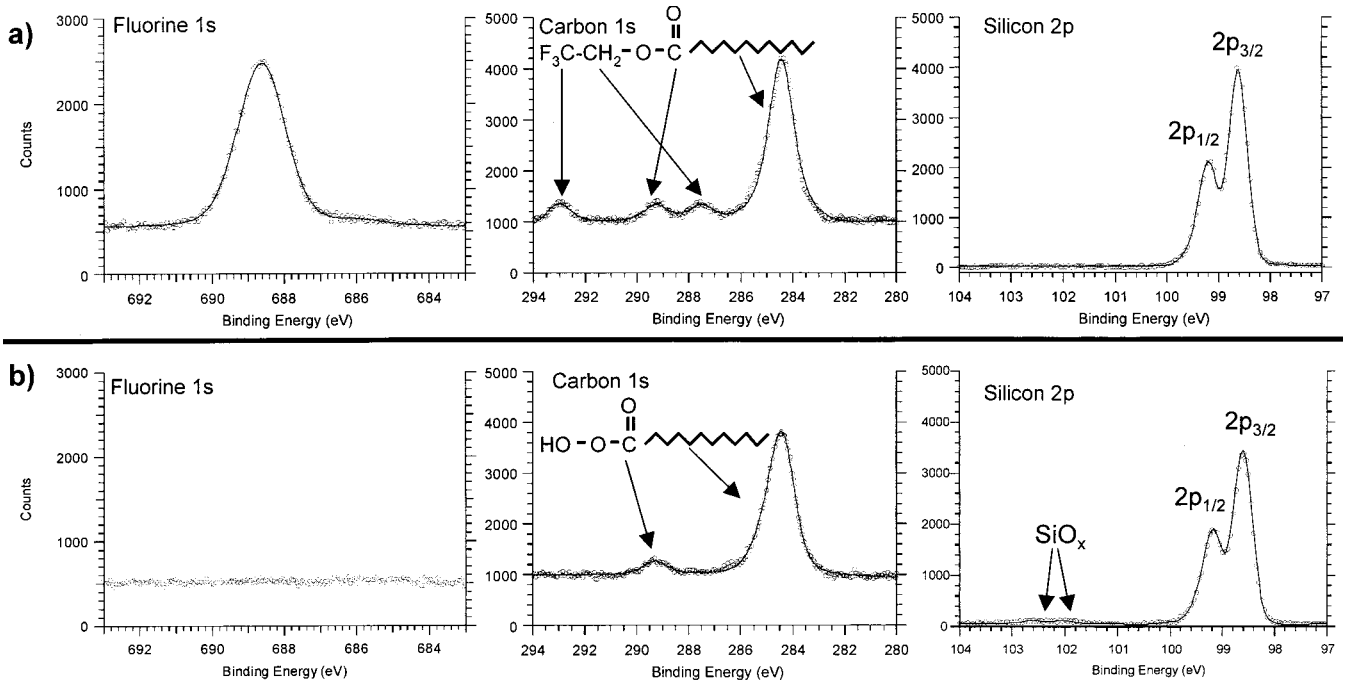

Figure 2. X-ray photoelectron spectra of the trifluoroethyl ester (a). The fluorine spectrum shows a strong peak at 688.5 eV. The carbon spectra shows peaks at 287.5 and 293.0 eV assigned to the carbons found in the trifluoroethyl ester moiety. The carbonyl carbon and the bulk carbons have been assigned the peaks at 289.3 and 284.4 eV, respectively. Additionally, the silicon spectrum shows no oxidation of the surfaces as evidenced by no signal in the 101−103 eV region. After treatment in 250 mM potassium *tert*-butoxide in DMSO for 30 s (b) the fluorine peak disappears, as do the peaks at 287.5 and 293.0 eV in the carbon spectrum. After treatment the silicon spectrum shows little oxidation.

signals, providing a clear means of following the hydrolysis reaction. Typical XPS results for this system are shown in Figure 2. The upper (panel a) three spectra show the fluorine 1s, carbon 1s, and silicon 2p signals from the Si(111) surface modified by attachment of the ω-undecylenic acid ester. The lower three spectra (panel b) show the same surface after hydrolysis of the ester by treatment with potassium *tert*-butoxide in DMSO. A comparison of the upper and lower panels reveals several important points. First, the fluorine 1s signal is completely removed by the hydrolysis procedure, proving that the ester was efficiently cleaved from the surface. Second, four carbon 1s signals are evident in the upper panel, corresponding to the alkyl chain carbons, the carbonyl carbon, and the two trifluorethyl group carbons. The two signals associated with the trifluoroethyl ester are absent in the lower panel, again confirming the complete hydrolysis of the ester; in contrast, the XPS signals corresponding to the alkyl chain and carboxylic acid group remain, demonstrating that the linkage between the alkyl chain and the surface was not disrupted by the hydrolysis reaction. Finally, comparison of panels a and b for the silicon 2p signal shows very little signal corresponding to oxidized silicon atoms on the surface, indicating that the overall integrity of the Si(111) surface is not adversely affected by the hydrolysis process.

DNA Hybridization Binding Specificity. The performance of these DNA-modified Si(111) surfaces in DNA hybridization was evaluated with respect to binding specificity, surface density, and chemical stability to the conditions of DNA hybridization. To evaluate binding specificity, two different thiol oligonucleotides were attached to a piece of a silicon wafer (approximately 2 cm × 2 cm) in approximately 2 mm diameter spots. A solution containing a fluorescent oligonucleotide complementary to one of the attached oligonucleotides was placed on the surface and hybridization was allowed to occur. After washing the fluorescence image was acquired, and revealed only a single spot (Figure 3a). Denaturation and

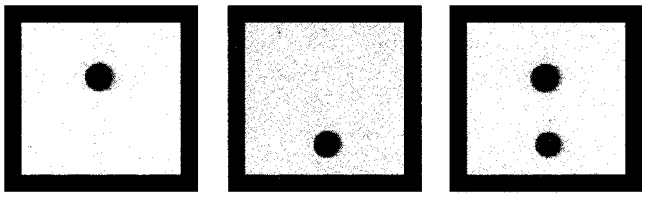

Figure 3. Images of DNA-modified Si(111) hybridized with fluorescent complements. Two spots, approximately 2 mm across, of different oligonucleotides are attached to the surface. Hybridization with fluorescent complement of the lower spot shows a clear image (a). Denaturation and hybridization to the upper spot with its fluorescent complement also shows a clear image (b). Denaturation and hybridization with both complements allow visualization of both spots (c).

subsequent hybridization using the alternate fluorescent oligonucleotide revealed only the second individual spot (Figure 3b). Denaturation and hybridization with both fluorescently tagged oligonucleotides showed two distinct spots, as expected (Figure 3c). These results indicate that the thiol oligonucleotides are indeed attached to the surface and are accessible to specific hybridization with their respective complements. In control experiments, a series of samples were prepared to determine if the thiol oligonucleotides could attach to the silicon surface in unexpected ways. Thiol oligonucleotides were spotted on the silicon surfaces in various stages of preparation. Only those samples with the hydrolyzed ester, polylysine, and SSMCC gave significant signal when hybridized with the fluorescent complements. Those samples with no ester, just hydrolyzed ester, or hydrolyzed ester with polylysine gave no signal. Similar controls with unhydrolyzed ester were also performed. Unexpectedly, significant fluorescent signal was seen with the unhydrolyzed ester that had been treated with polylysine and SSMCC. This

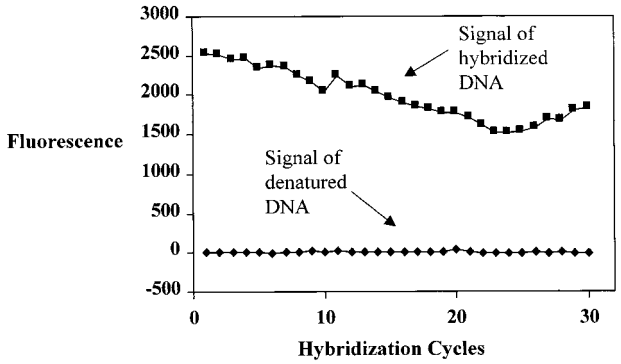

Figure 4. Long-term stability showing behavior of the silicon surface during 30 hybridization and denaturation cycles. Fluorescent signal from the hybridized DNA decreases slightly with each hybridization until the 23rd hybridization. A slight increase in fluorescent signal occurs with later hybridizations. The wafers were imaged after each denaturation to demonstrate the complete removal of the fluorescent complement. The fluorescent signal of the denatured DNA was negligible.

is consistent with binding of polylysine to the ester by polar interactions and subsequent binding of the SSMCC to the polylysine.

Number Density. The density of fluorescent oligonucleotides that hybridized to the surface was determined according to a previously described method.[28] In this procedure the surface area of the spots is determined by fluorescence imaging, and the amount of hybridized oligonucleotide is measured by quantitative elution from the surface followed by gel electrophoretic analysis (see the Experimental Section). The surface density determined in this manner was found to be approximately 5.3×10^{12} oligonucleotides per cm^2, comparable to densities reported on other substrates.[2,29,31,41] Control experiments performed on similarly modified gold surfaces[28] yielded comparable results.

Chemical Stability Under the Conditions of DNA Hybridization. The stability of these DNA-modified Si(111) surfaces to the conditions of DNA hybridization was determined by performing a series of 30 successive cycles of oligonucleotide hybridization, washing, fluorescence imaging, and denaturation to regenerate the original surface (see the Experimental Section for details). The fluorescence intensities obtained after hybridization in each cycle are plotted in Figure 4. The total fluorescence intensity of the hybridized DNA decreases after 30 cycles to approximately 60% of the initial value, corresponding to a loss of approximately 2% per cycle. Control experiments performed on similarly modified gold surfaces[3] showed comparable stability; however, the fluorescence background on the gold surfaces was roughly 75% higher than that on the Si surfaces (possibly due to the greater reflectivity of the gold surface), causing a higher fluorescence signal-to-background ratio for the Si surfaces. The fluorescence intensity of the DNA was measured after the denaturation step of each of the 30 cycles and was found to be negligible compared to background (Figure 4). This background fluorescence is also plotted in Figure 4. It may be noted that the fluorescence intensity actually appears to increase during the later hybridization cycles. The cause of this unexpected increase is currently under investigation.

Experimental Section

Materials. All chemicals were reagent grade or higher and used as received unless otherwise specified. Ultrapure water was obtained from a Millipore system and used to rinse the surfaces when scrupulously clean conditions were required. Distilled water was used in other cases.

Other reagents were obtained as follows: 11-Mercaptoundecanoic acid (MUA) (Aldrich), 10-undecylenic acid (UDA) (Fisher), 2,2,2-trifluoroethanol (Aldrich), methanol (Fisher), potassium *tert*-butoxide (Fisher), poly(L-lysine) hydrobromide (PL) (Aldrich), sulfo-succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SSMCC) (Pierce), silicon (111) wafers (Virginia Semiconductor). Gold surfaces were obtained from Evaporated Metal Films (Ithica, NY) with 50 Å of chromium covered by 1000 Å of gold. Oligonucleotides were synthesized by the University of Wisconsin Biotechnology Center. The oligonucleotides were modified with either Glen Research 5′-Thiol-Modifier C6 or 5′-Fluorescein (6-FAM) modifier to produce thiol-modified oligonucleotides or the fluorescein-modified complements. The thiol-modified oligonucleotides were deprotected according to guidelines provided by Glen Research Corp[42] then purified using reverse-phase HPLC with a binary gradient elution. Fluorescent oligonucleotides were also purified using HPLC. The thiol oligonucleotides were used at approximately 1 mM concentration and their fluorescent complements were used at approximately 2 μM. The hybridization and rinsing buffer was 300 mM NaCl, 20 mM sodium phosphate, 2 mM EDTA, and 7 mM sodium dodecyl sulfate, pH 7.4, referred to as "2×SSPE/0.2% SDS."

Synthesis of Esters. (a) Methyl Ester. The methyl ester of 10-undecylenic acid was prepared similarly to the method used by Sieval.[9] The 10-undecylenic acid was dissolved in methanol and allowed to reflux for 3 h with a small amount of sulfuric acid. Excess methanol was removed by vacuum. The crude product was dissolved in ether, washed with a saturated sodium bicarbonate solution, water, then a saturated NaCl solution, and dried over magnesium sulfate. The identity of the vacuum distilled product was confirmed using proton NMR.

^{1}H NMR: δ 5.73−5.89 (m, 1H), 4.89−5.04 (m, 2H), 3.67 (s, 3H), 2.25−2.35 (t, 2H), 1.99−2.09 (m, 2H), 1.54−1.69 (m, 2H), 1.23−1.44 (m, 10H).

(b) 2,2,2-Trifluoroethyl Ester. The fluorinated ethyl ester of 10-undecylenic acid was prepared similarly to the methyl ester. To 30 g of 10-undecylenic acid was added 22 g of 2,2,2-trifluoroethanol dissolved in 40 mL of toluene. This mixture was allowed to reflux with a small amount of sulfuric acid for 3 h using a Dean−Stark apparatus. Workup similar to the methyl ester produced a crude product that was further purified by vacuum distillation. Proton, COSY, and fluorine NMR confirmed the identity of the product.

^{1}H NMR: δ 5.70−5.90 (m, 1H), 4.86−5.06 (m, 2H), 4.38−4.51 (q, 2H), 2.35−2.45 (t, 2H), 1.98−2.10 (m, 2H), 1.55−1.75 (m, 2H), 1.20−1.50 (m, 10H). Fluorine NMR: single triplet.

Preparation of Silicon Surfaces. The silicon (111) wafers were treated similar to the method described by Sieval with some modification.[9,37] The wafers were sonicated in acetone for 5 min then methanol for 5 min. They were then soaked in a hydrogen peroxide−ammonia−water bath (1:1:4) for 5 min at 75 °C. After a brief dip in 2% hydrofluoric acid to hydrogen terminate the silicon atoms, the wafers were placed in a chamber. The chamber had a quartz window to allow passage of ultraviolet light and was designed to have nitrogen continually passed through at atmospheric pressure. Thin layers of the esters were placed on the hydrofluoric acid etched wafers and they were sealed in the chamber. The wafers were then subjected to ultraviolet light from a low-pressure mercury vapor lamp for 2 h. The wafers were subsequently sonicated in chloroform for 10 min then in methanol for a further 10 min. The modified ester surface was then converted to the carboxylic acid. Sieval and others have used acid baths to hydrolyze the ester though there is evidence that such treatment does not adequately convert the ester to the carboxylic acid.[9,43] We found the neat ω-undecylenic esters did not completely hydrolyze even in boiling concentrated HCl for several hours when measured with thin-layer chromatography (TLC). An alternate method of hydrolysis using potassium *tert*-butoxide in DMSO was explored;[39,40] it was found that dipping the surfaces in a 250 mM solution of potassium *tert*-butoxide

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in DMSO for 3 min at room temperatures followed by rinsing in acidified water (100mM HCl) successfully hydrolyzed the esters. TLC analysis of hydrolysis products confirmed the effectiveness of this procedure. In addition, XPS measurements and contact angle measurements of the surfaces indicated successful surface hydrolysis. Contact angle measurements: methyl ester, 70.8 ± 1.6°, after hydrolysis, 52.2 ± 2.6°; fluorinated ethyl ester, 84.5 ± 1.2°, after hydrolysis, 58.2 ± 1.5°.

After being rinsed in water, the acid terminated surface was soaked for 40 min in a 0.15 mg/mL PL solution in a 5mM borate buffer at pH 8.5. The surfaces were then rinsed in ethanol and water. The final layer was added by soaking the surfaces for 15 min in 1.5 mM SSMCC in a 100 mM triethanolamine hydrochloride buffer at pH 7. A final rinse with water was performed and the thiol-modified oligonucleotides were spotted on the surface and left overnight in a humid chamber. The thiol DNA used was approximately 0.5 μL of a 1 mM solution. It may be noted that alternate methods of ester attachment have yielded similar results. These include heating the ester with the cleaned silicon surface at 200 °C for 2 h, use of a propyl ester, and use of silicon (001).[9]

Hybridization and Denaturation Conditions. The detailed procedures employed for hybridization of the fluorescein-labeled complements to the surface-immobilized DNA and their subsequent denaturation has been described elsewhere.[28] Imaging of the surface-bound oligonucleotides on silicon surfaces was done by scanning with a Molecular Dynamics FluorImager 575 after hybridizing the immobilized oligonucleotides with their fluorescent complements. Briefly, 5−10 μL of 2 μM 5′-fluorescein-labeled complement in 2×SSPE/0.2% SDS buffer was placed on a microscope cover slip in a humid chamber. The DNA-modified silicon surfaces were placed in this droplet of fluorescent complement for 20 min at room temperature to allow for hybridization. The surfaces were then removed and soaked twice for 5 min in 2×SSPE/0.2% SDS buffer to remove any unhybridized complement. The silicon wafers were then placed face down in a droplet of 2×SSPE/0.2% SDS buffer on the FluorImager tray and scanned. Denaturation was accomplished by placing the samples in an 8.3 M urea solution for 15 min at 37 °C followed by rinsing with water. Subsequent hybridizations could then be performed using the same procedure.

Determination of Number Density. The method used to determine the number of oligonucleotides that have hybridized to a gold surface was previously reported by Frutos.[28] A similar method was employed here. Thiol oligonucleotides are immobilized on the silicon wafer surface in a 5 mm diameter spot; complementary fluorescent oligonucleotides are hybridized, the wafer is washed thoroughly, and the fluorescent images are acquired, providing values for the exact surface area of the spots. The silicon wafer is then heated at 90 °C for 15 min in a microfuge tube with 800 μL of water. The wafer is removed and rinsed twice with 200 μL water, which was added to the original 800 μL. After the fluorescent complements are quantitatively eluted into the water, the surface is rescanned on the FluorImager to check for complete denaturation (removal of fluorescence signal). The volume of water containing the fluorescent complements is reduced by vacuum centrifugation to about 10 μL and loaded on one lane of an acrylamide gel, while known amounts of fluorescent oligonucleotide standards are loaded in the other gel lanes. A 20% polyacrylamide gel containing urea is prepared using standard techniques.[44] Electrophoresis is performed and the gel is imaged; analysis of the image permits quantification of the unknown by reference to a standard curve prepared from the known samples.

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