

DNA Attachment and Hybridization at the Silicon (100) Surface

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The chemical modification of silicon surfaces for DNA attachment and subsequent hybridization has been investigated using X-ray photoelectron spectroscopy and contact angle measurements, in conjunction with fluorescence-based measurements of DNA hybridization. The feasibility of using small molecules to functionalize Si surfaces with primary amine groups for DNA attachment has been explored using the free amine 1-amino-3-cyclopentene (ACP) and a modified molecule with a protecting group on the amine, *N*-1-BOC-amino-3-cyclopentene (BACP). Direct attachment of amines leads to chemically heterogeneous surfaces, while the use of suitable protection and deprotection chemistry can produce a homogeneous surface with a high density of primary amine groups. The resulting amine-terminated surfaces were covalently coupled to thio-oligonucleotides using a heterobifunctional cross-linker. Hybridization experiments revealed that these DNA exhibit excellent stability to hybridization conditions, high specificity for recognition, and high density for hybridization. The ability to detect single-base mismatches is demonstrated. Factors controlling the stability, selectivity, and density of surface-bound DNA molecules are discussed.

(1) Introduction

Research on DNA-modified surfaces has become increasingly active in recent years and is anticipated to have a broad-based impact on a number of emerging biotechnology areas, such as DNA chip technologies and DNA computing.^{1–3} Although many different types of surfaces have been used as substrates for DNA attachment, the need for reproducible, stable surfaces has placed increased emphasis on the preparation of DNA-modified surfaces that are extremely homogeneous.

Recent studies have shown that crystalline silicon can be used as a starting point for preparing DNA layers exhibiting a high specificity and a high density of binding sites.^{4–6} Crystalline silicon has a number of potential advantages as a substrate for DNA immobilization. Starting with hydrogen-terminated silicon surfaces,^{7,8} it is possible to form well-defined organic films through direct Si–C covalent bond formation.^{9–12} Perhaps more impor-

tantly, the use of crystalline silicon substrates provides the potential for taking full advantage of existing microelectronic technologies associated with the silicon semiconductor industry, which can be used for highly parallel microfabrication and, in principle, direct electronic detection of biomolecular binding processes.^{3,13}

Recent experiments have shown that well-defined DNA layers can be prepared on the common (100) and (111) surfaces of silicon.^{4,5} In both cases, attachment was achieved by first using a long-chain alkene terminated with a reactive functional group. In one case, attachment began by attaching a long-chain ester to the surface which was then hydrolyzed to a carboxylic acid group, converted to an amine via electrostatic bonding with polylysine, and finally linked to DNA using the hetero-bifunctional cross-linker sulfo-succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SSMCC).⁴ A simpler scheme⁵ used direct attachment of a long-chain molecule terminated with a protected amine, which was then converted to a free amine and linked to DNA with SSMCC. In these and most other studies of DNA-functionalized surfaces, the first step has been attachment of a long-chain organic molecule to the surface. While such long-chain molecules might provide improved stability against oxidation or other undesired reactions at the interface, it is also possible that such long-chain molecules might decrease the quality of the interface through entanglement. In emerging applications involving direct electronic detection of DNA hybridization processes, however, the use of a shorter linking layer may be beneficial.^{13,14}

Ideally, one would like to identify the simplest chemical scheme for linking DNA to silicon substrates in a way that achieves the highest selectivity, sensitivity, and stability in subsequent hybridization steps. We report here an integrated study in which core-level photoelectron

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(1) Wang, L. M.; Liu, Q. H.; Corn, R. M.; Condon, A. E.; Smith, L. M. *J. Am. Chem. Soc.* **2000**, *122*, 7435–7440.

(2) Liu, Q.; Wang, L.; Frutos, A. G.; Condon, A. E.; Corn, R. M.; Smith, L. M. *Nature* **2000**, *403*, 175–179.

(3) Vo-Dinh, T.; Cullum, B. *Fresenius J. Anal. Chem.* **2000**, *366*, 540–551.

(4) Strother, T.; Cai, W.; Zhao, X.; Hamers, R. J.; Smith, L. M. *J. Am. Chem. Soc.* **2000**, *122*, 1205–1209.

(5) Strother, T.; Hamers, R. J.; Smith, L. M. *Nucleic Acids Res.* **2000**, *28*, 3535–3541.

(6) Lenigk, R.; Carles, M.; Ip, N.; Sucher, J. *Langmuir* **2001**, *17*, 2497–2501.

(7) Kinoshita, S.; Ohta, T.; Kuroda, H. *Bull. Chem. Soc. Jpn.* **1976**, *49*, 1149.

(8) Higashi, G. S.; Chabal, Y. J.; Trucks, G. W.; Raghavachari, K. *Appl. Phys. Lett.* **1990**, *56*, 656–658.

(9) Wagner, P.; Nock, S.; Spudich, J. A.; Volkmuth, W. D.; Chu, S.; Cicero, R. C.; Wade, C. P.; Linford, M. R.; Chidsey, C. E. D. *J. Struct. Biol.* **1997**, *119*, 189–201.

(10) Sieval, A. B.; Demirel, A. L.; Nissink, J. W. M.; Linford, M. R.; Maas, J. H. v. d.; Jeu, W. H. d.; Zuillhof, H.; Sudhölter, E. R. J. *Langmuir* **1998**, *14*, 1759–1768.

(11) Linford, M. R.; Fenter, P.; Eisenberger, P. M.; Chidsey, C. E. D. *J. Am. Chem. Soc.* **1995**, *117*, 3145–3155.

(12) Terry, J.; Linford, M. R.; Wigren, C.; Cao, R.; Pianetta, P.; Chidsey, C. E. D. *J. Appl. Phys.* **1999**, *85*, 213–221.

(13) Vo-Dinh, T.; Alarie, J. P.; Isola, N.; Landis, D.; Wintenberg, A. L.; Ericson, M. N. *Anal. Chem.* **1999**, *71*, 358–363.

(14) Janata, J. *Analyst* **1994**, *119*, 2275.

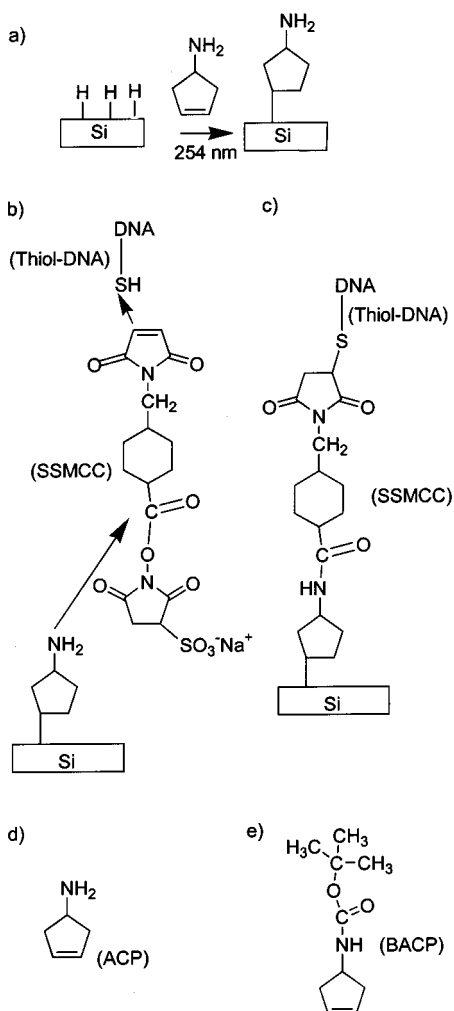


Figure 1. Overview illustrating the entire attachment process on Si(100) surfaces.

spectroscopy is used to optimize the formation of chemically modified Si(100) surfaces, and fluorescence measurements are used to assess the selectivity, stability, and sensitivity in subsequent hybridization studies. Our results show that, provided the surface chemistry is well-controlled, short, rigid linkers can also be used for DNA immobilization with good sensitivity, selectivity, and reversibility in subsequent hybridization processes. However, protection and deprotection of the amine group is critical to form chemically homogeneous layers. With optimized chemistry, we demonstrate the ability to detect even single-base mismatches. Our results also provide insight into the factors controlling these important variables.

(2) Experimental Section

(A) Overview. Figure 1 provides an overview of the overall scheme for attachment of DNA to the crystalline silicon surface. The procedure is based upon the fact that bare silicon surfaces will react readily with C=C double bonds,¹⁵ while silicon surfaces that have their exposed Si atoms bonded to hydrogen atoms (hydrogen-terminated Si surfaces) are unreactive. Hydrogen termination was accomplished by dipping the silicon wafers in a solution of 1% hydrofluoric acid, which removes any oxidized silicon from the surface and bonds H atoms to the exposed unsaturated Si "dangling bonds". Starting with a (unreactive)

hydrogen-terminated surface, local sites of reaction are defined by locally removing the H-passivation using ultraviolet light (here, at 254 nm), which then links the functionalized molecule of interest at the illuminated site. Because the bare silicon surface can also potentially react with other parts of the molecule (such as the unprotected amine group), we investigated attachment of "protected" and "unprotected" amines. Covalent linking with thiol-terminated DNA is then achieved using a hetero-bifunctional cross-linker such as SSMCC.

All reagents were purchased from commercial sources at the highest grade available. 1-Amino-3-cyclopentene (ACP), which has both an alkene group and a primary amine functionality, was purchased from Asta Tech. A protected version, in which one of the H atoms attached to the NH₂ group is replaced with a *tert*-butyl oxycarbonyl protecting group to yield the protected amine *N*-1-BOC-amino-3-cyclopentene (BACP), was also purchased from Asta Tech. Sulfo-succinimidyl 4-(*N*-maleimido-methyl) cyclohexane-1-carboxylate (SSMCC), used to link the amine-terminated surface to thiol-terminated DNA, was also purchased from Pierce Chemical. Silicon samples oriented to expose the (100) crystal face were purchased from Virginia Semiconductor; n-type (arsenic-doped) samples were used for the studies reported here. 18 M Ω -cm water from a Millipore system was used for rinsing the samples.

Oligonucleotides were synthesized by the University of Wisconsin Biotechnology Center and purified by high-pressure liquid chromatography immediately before use. Oligonucleotides to be covalently attached to the surfaces were thiol-modified at the 5'-end using the reagent 5'-thiol-modifier C6 (HSC6, Glen Research). The four sequences employed were 5'-HSC6-T₁₅AA CGA TCG AGC TGC AA-3' (S1), 5'-HSC6-T₁₅AA CGA TGC AGG AGC AA-3' (S2), 5'-HSC6-T₁₅AG GAA TGC CGG TTA T-3' (S3), and 5'-HSC6-T₁₅AG GAT TGC CGG TTA T-3' (S4). Complementary oligonucleotides used for hybridization were modified with fluorescein on the 5'-end using 6-FAM phosphoramidite (Applied Biosystems, Inc.). The three sequences employed were 5'-FAM-TT GCA GCT CGA TCG TT-3' (F1, complementary to S1), 5'-FAM-TT GCT CCT GCA TCG TT-3' (F2, complementary to S2), and 5'-FAM-AT AAC CGG CAT TCC T -3' (F3, complementary to S3 and with a single-based mismatch to S4). Immediately before use, the thiol-oligonucleotides were deprotected according to guidelines from Glen Research¹⁶ and then purified by reverse-phase HPLC. The thiol-oligonucleotides (for surface functionalization) were used at a concentration of approximately 1 mM while their fluorescent complements (for hybridization) were used at a concentration of 2 μ M. Hybridization and rinsing buffer used during the studies was referred to as 2 \times SSPE/0.2% SDS, which consists of 2 mM EDTA, 7 mM sodium dodecyl sulfate, 300 mM NaCl, and 20 mM NaH₂PO₄ with pH = 7.4.

(B) Optimized Preparation of DNA-Modified Silicon Surfaces. Si wafers were ultrasonically cleaned in acetone and methanol for 5 min each, followed by oxidation in a solution prepared by mixing 30% hydrogen peroxide, 28–30% ammonium hydroxide, and water in a 1:1:4 volumetric ratio. After soaking for 5 min at 75 $^{\circ}$ C, the Si samples were dipped briefly in a 1% HF solution to remove the oxidized layer and were rinsed in water to remove any residual fluorine, producing hydrogen-terminated Si surfaces.^{4,9} Because H-terminated Si surfaces will oxidize slowly at atmospheric pressure, they were used as soon as possible after preparation and kept under a nitrogen atmosphere whenever possible.

The BACP molecule is a solid at room temperature. To prepare a uniform solid layer on the Si-H surface, 50 μ L of BACP in methanol (50 mg/mL) was spread on a surface area of approximately 1 cm², and a thin film formed by evaporation of the solvent. The sample was then irradiated with 254-nm ultraviolet light to induce the desired alkene attachment reaction; a period of 1.5–2 h was the optimal reaction time. Physically adsorbed BACP molecules were removed by sonicating the samples twice in CH₃Cl and once in methanol, for 5 min each.

The optimal reaction conditions for deprotection of the amine group were established using contact angle measurements to

(15) Hamers, R. J.; Coulter, S. K.; Ellison, M. D.; Hovis, J. S.; Padowitz, D. F.; Schwartz, M. P.; Greenleaf, C. M.; Russell, J. N., Jr. *Acc. Chem. Res.* **2000**, *33*, 617–624.

(16) *User Guide to DNA Modification*; Glen Research Corporation: 1996.

rapidly assess the chemical changes, in conjunction with more detailed XPS measurements. The optimized procedure for deprotection was determined to be immersion into 25% trifluoroacetic acid in CH_2Cl_2 for 2 h, followed by 10% NH_4OH for 5–7 min.⁵ This procedure is similar to that previously optimized for deprotection of a *t*-BOC-protected alkeneamine.⁵ The resulting amine-terminated surfaces were then allowed to react with a hetero-bifunctional cross-linker.

Although we compared several linkers, SSMCC was used most extensively. SSMCC was linked to the amine-modified Si(100) surface by covering the surface with 50 μL of a solution of SSMCC (1.5 mM in 150 mM triethanolamine buffer, pH 7) for 20 min. The DNA was then linked to the SSMCC-terminated surface by applying 0.4–0.8 μL droplets of ~ 1 mM thiol-oligonucleotides to the surface and allowing the reaction to continue overnight. Two other linkers, *N*-succinimidyl iodoacetate (SIA) and sulfosuccinimidyl(4-iodoacetyl) aminobenzoate (SSIAB) were also evaluated.

To use these linkers, 1.4 mg of SIA or 1.7 mg of sulfo-SIAB was dissolved into 1 mL of PBS buffer solution (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2).¹⁷ The resulting SIA or SIAB solution was dripped onto the amine-terminated surface and kept in the dark, producing surfaces terminated with the thiol-reactive iodoacetyl group. Thiol-modified oligonucleotides were then deposited onto the surface and allowed to couple with the iodoacetyl group in the dark for at least 20 h.

Chemical characterization of the surface modification chemistry was performed using X-ray photoelectron spectroscopy (XPS) measurements and water contact angle measurements. XPS spectra were obtained using an Al K α source (1486.9 eV photon energy) and a 16-channel detector. XPS spectra were analyzed using standard curve-fitting procedures in which the quality of fit was evaluated through a "quality-of-fit" parameter, based on a reduced χ^2 . A smaller value for the reduced χ^2 represents a better fit, with values of less than 1 indicating that the fit and the data are indistinguishable within the experimental noise. The static water contact angle measurements were carried out on an NRL goniometer (Ramé-hart 100) with an error of $\pm 2^\circ$.

The detailed procedures for hybridizing the fluorescent complements to the surface-immobilized thiol-oligonucleotides have been described previously.^{4,18} Briefly, the thiol-oligonucleotide modified surfaces were placed in 5–10 μL of 2 μM 5'-fluorescein-labeled complement and allowed to hybridize for 20 min in a humid chamber at room temperature. These hybridized surfaces were soaked twice in $2\times\text{SSPE}/0.2\%$ SDS buffer for a total of 10 min to rinse off any physically adsorbed oligonucleotides. A Molecular Dynamics FluorImager 575 was then used to visualize and quantify the fluorescence intensity from the modified silicon surfaces. These images were scanned after putting the silicon wafers face down in a droplet of $2\times\text{SSPE}/0.2\%$ SDS buffer on the FluorImager tray. Subsequent denaturation was performed by placing the surfaces in a 8.3 M urea solution at 37 $^\circ\text{C}$ for 5–10 min. The surfaces were then rinsed with water and rescanned with the FluorImager. After ensuring a complete denaturation, subsequent hybridizations could be redone using the same procedure.

(3) Results

(A) Chemical Attachment of Protected and Non-protected Amines to the Si(100) Surface. The studies presented here focus primarily on two methods of attaching amine groups to silicon surfaces. Figure 1d depicts the molecule 1-amino-3-cyclopentene (ACP). This molecule contains a reactive alkene group and an unprotected, primary amine group. Because of the possibility that a primary amine group might react directly with the Si surface, we also investigated protecting the amine with the *tert*-butyloxycarbonyl (*t*-BOC) group. For simplicity, we refer to the resulting protected molecule *N*-1-BOC-amino-3-cyclopentene as BACP; the structure of the BACP

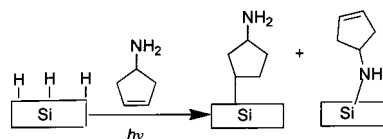


Figure 2. Reaction pathways for ACP interacting with the silicon substrate.

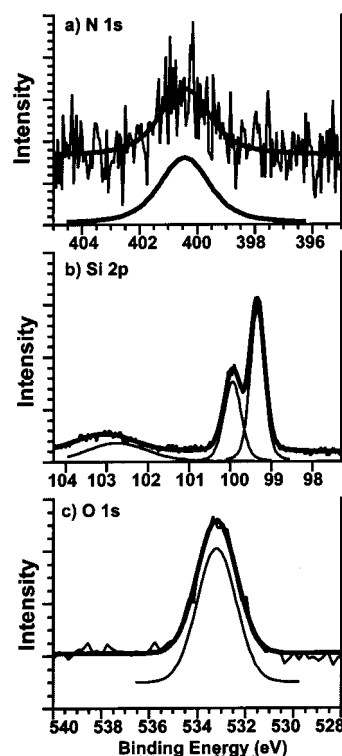


Figure 3. X-ray photoelectron spectra of the nitrogen (1s), silicon (2p), and oxygen (1s) core levels from the Si(100) surface modified by ACP.

molecule is shown in Figure 1e. Both the ACP and BACP molecules contain a reactive alkene ($\text{C}=\text{C}$ group). Since alkene groups are known to react with the Si(100) surface via the photochemical process used here,⁵ we anticipate that both molecules can, in principle, bind to the surface via the alkene group. However, as shown in Figure 2, since amines might also react with the surface, a comparison of the protected and unprotected amines was carried out to help identify how to best protect against such undesired side reactions.

To attach the ACP molecule to the surface, 250 μL of ACP was deposited onto a 1 cm^2 region of a hydrogen-terminated Si(100) surface. The surface and the thin liquid film were then illuminated with UV light (254 nm) for 1.5 h under a dry nitrogen atmosphere. Figure 3 shows the resulting X-ray photoelectron spectra in the regions corresponding to the nitrogen (1s), silicon (2p), and oxygen (1s) core levels. The N(1s) spectrum in Figure 3a shows a weak peak with a binding energy of 400.4 eV and a breadth of 2.2 eV, measured as the full-width at half-maximum (fwhm). The Si(2p) spectrum in Figure 3b shows two sharp peaks at 99.4 eV and 100.0 eV and a much broader peak at higher binding energy, extending from 102 to 104 eV. Finally, Figure 3c shows the oxygen 1s spectrum of this surface, revealing a peak at a binding energy of 533.2 eV. More quantitative information can be obtained from analysis of the peak areas, using the Si(2p) bulk as an internal standard. In Figure 3, the area of the N(1s) peak is 0.85 times the total area of the Si(2p) spectrum, while the area of the O(1s) peak is 3.9 times the total area of

(17) Hermanson, G. T. *Bioconjugate Techniques*; Academic Press: San Diego, 1996.

(18) Frutos, A. G.; Smith, L. M.; Corn, R. M. *J. Am. Chem. Soc.* **1998**, *120*, 10277–10282.

the Si(2p) region. The broad 102–104 eV Si peak constitutes 25% of the total area of the Si(2p) spectrum.

We consider first the Si spectrum. In XPS measurements, spin–orbit splitting causes the 2p levels from each Si atom to split into 2p_{1/2} and 2p_{3/2} components, in a 1:2 intensity ratio. The two peaks at 99.4 and 100.0 eV arise from a single chemical form of silicon and correspond to the well-known binding energy of unreacted, bulk silicon. The broader peak at 102–104 eV, however, can be attributed to silicon atoms in more positive oxidation states than those in the bulk. Silicon oxidized by O₂ and/or H₂O gives rise to peaks at 102–104 eV due to SiO₂, SiO, and SiOH groups.^{19–23} Likewise, we attribute the broad features in this region to oxidized Si atoms. Since the total area in the 102–104 eV range is approximately 0.25 times the total Si(2p) area, this suggests that ~25% of the silicon atoms within the sampling volume are oxidized.

The 400.4 eV N(1s) peak is higher in energy and also broader than those observed for other amines. For example, in studies of primary and secondary amines, we have found N(1s) binding energies near 398.8 eV for C–NH–Si moieties and 399.4 eV for C–NH–C moieties, with a fwhm near 1.0 eV.²⁴ Similarly, ammonia (NH₃) produces a N(1s) peak at 399.1 eV from Si–NH₂ species. The comparatively high energy and broad width of the N(1s) peak from ACP suggest an inhomogeneous distribution of binding sites, with the N atoms in comparatively electron-deficient environments. More importantly, analysis of the peak intensities shows that the N groups are present in low concentration. The absolute N coverage can be determined quantitatively by comparison with XPS measurements performed on this same instrument under conditions identical to those for the NH₃ molecule, which is known to dissociate into a single NH₂ fragment and an adsorbed H atom on each dimer of the Si(100) surface.²⁵ In most experiments reported here the N and Si signals are recorded using slightly different conditions (specifically, different pass energies) to provide the best balance between sensitivity and resolution. By performing similar measurements (not shown) on NH₃ and ACP under identical instrumental conditions, we find that the N/Si ratio of ACP is approximately 0.4 times the N/Si ratio produced by NH₃ adsorption. This then corresponds to an N coverage of approximately 0.2 nitrogen atom per surface Si atom. A similar value can be crudely determined from the known atomic sensitivity factors for N and Si and correcting for the finite escape depth of the photoemitted electrons. Finally, the amount of oxidation can be approximately determined. On the basis of the 1486 eV excitation, the relative sensitivities in the XPS measurements for Si, N, and O are 0.27, 0.42, and 0.66, respectively. The O(1s) spectrum shows a single, fairly strong peak at 533.1 eV. This binding energy is intermediate between the value ~533.7 eV usually associated with Si–O linkages and the value ~532.3–532.6 eV associated with Si–O–Si linkages.^{20,23} While physisorbed water can also give rise to a peak at this energy, the existence of the 102–104 eV peaks

in the silicon XPS spectrum indicates that silicon oxidation is substantial. Integrating the area under the peak yields an O/Si area ratio of 3.9; this corresponds to approximately 4.5 O atom per N atom, or approximately one oxygen atom per surface Si atom.

The XPS data presented in Figure 3 show that direct attachment of ACP via the photochemical scheme results in extensive surface oxidation, with relatively poor attachment of ACP. Similar results (not shown) have also been obtained for attachment of 10-aminodec-1-ene, a long-chain alkeneamine. Thus, we conclude that molecules containing unprotected amine groups cause extensive oxidation and poor attachment. The use of protecting groups is limited by the fact that many protecting groups can only be deprotected under harsh conditions that can also significantly alter the silicon substrate. Since silicon surfaces can be etched under both acidic and basic aqueous solutions,²⁶ neither acid-labile nor base-labile protecting groups are clearly preferable. In a previous study we found that protecting groups that require strongly basic conditions tend to cause surface oxidation unless a physically large base, such as potassium *tert*-butoxide, is used, and we obtained the best results using the acid-labile, *t*-BOC protecting group.⁴ Consequently, we investigated the attachment and subsequent modification of the Si(100) surface with BACP.

Following the experimental procedures outlined above, Figure 4 shows XPS data for the nitrogen (1s), silicon (2p), and carbon (1s) core levels of the Si (100) surface at various stages of chemical modification. We first present two control experiments. The first panel (Figure 4a) shows the spectra of a control sample that was H-terminated. The H-terminated surface shows no N contamination, and only a small C signal that is presumably due to physically adsorbed hydrocarbons, as this peak decreases in intensity upon mild heating. Panel 4b shows data from a sample that was H-terminated and then covered with BACP as described above, but *not* subjected to UV illumination. In this case, the N(1s) spectrum shows a weak peak with a binding energy of 400.5 eV and a fwhm of 1.50 eV. The carbon (1s) spectrum shows two weak peaks with binding energies of 284.9 and 286.6 eV. The Si(2p) spectrum in Figure 4b shows two sharp peaks at 99.4 and 100.0 eV but shows no significant intensity at the higher binding energies of 102–104 eV that are characteristic of oxidized silicon. Measurement of the N and Si peak areas yields a N/Si peak area ratio of 0.24.

Finally, Figure 4c shows a sample that was H-terminated, covered with BACP, and then exposed to UV for 2 h. The N(1s) spectrum has a single peak with a binding energy of 400.5 eV and a width of 1.6 eV fwhm. This peak is much narrower than the 2.2 eV width observed from attachment of the unprotected ACP molecule to the Si(100) surface. This narrower width indicates that the chemical homogeneity of the amine groups is significantly improved. In fact, the 1.6 eV width is identical to that observed from NH₃ and other amines when prepared under pristine ultrahigh vacuum conditions on silicon surfaces.²⁴ Quantitative analysis of the peak areas yields a ratio of N to Si peak areas of 3.0. This can be compared with the value of 0.85 observed for the unprotected ACP molecule under identical instrumental operating conditions. Again, on the basis of comparison of the N/Si ratio for BACP layers with similar measurements for NH₃ adsorption,²⁵ the surface N density of the BACP-modified surface is approximately 1 N atom per surface Si atom, or approximately 6×10^{14}

(19) Himpsel, F. J.; McFeely, F. R.; Taleb-Ibrahimi, A.; Yarmoff, J. A.; Hollinger, G. *Phys. Rev. B* **1988**, *38*, 6084–6096.

(20) Prabhakaran, K.; Kobayashi, Y.; Ogino, T. *Surf. Sci.* **1993**, *290*, 239–244.

(21) Hollinger, G.; Himpsel, F. J.; Hughes, G.; Jordan, J. L. *Surf. Sci.* **1986**, *168*, 609–616.

(22) Chambers, J. J.; Parsons, G. N. *Appl. Phys. Lett.* **2000**, *77*, 2385–2387.

(23) Namiki, A.; Tanimoto, K.; Nakamura, T.; Ohtake, N.; Suzuki, T. *Surf. Sci.* **1989**, *222*, 530–534.

(24) Cao, X.; Coulter, S. K.; Ellison, M. D.; Liu, H.; Liu, J.; Hamers, R. J. *J. Phys. Chem. B* **2001**, *105*, 3759–3768.

(25) Dresser, M. J.; Taylor, P. A.; Wallace, R. M.; Choyke, W. J.; Yates, J. T. *J. Surf. Sci.* **1989**, *218*, 75–107.

(26) Yang, S. K.; Peters, S.; Takoudis, C. G. *J. Appl. Phys.* **1994**, *76*, 4107–4112.

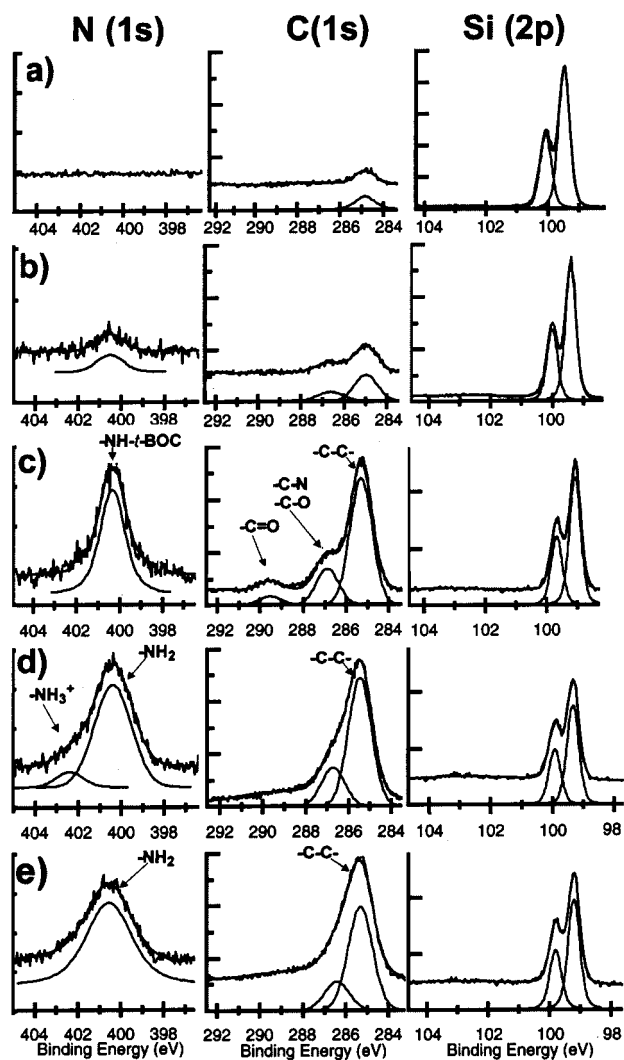


Figure 4. XPS spectra at various stages of chemical modification: (a) H-terminated Si(100) sample; (b) H-terminated Si(100) sample that was covered with BACP but not exposed to UV light; (c) H-terminated Si(100) sample that was covered with BACP and illuminated for 2 h with UV light; (d) Si(100) surface modified by BACP and then exposed to 25% trifluoroacetic acid (TFA) in CH_2Cl_2 for 2 h; (e) identical sample to that for part d, after subsequent treatment with 10% NH_4OH for 7 min.

N atoms/ cm^2 . We also note that the N/Si peak area ratio obtained on the sample that was covered with BACP and illuminated with UV is more than 12 times larger than that from the sample that was not illuminated with UV. This demonstrates that UV illumination greatly facilitates the attachment process.

Since later stages in the attachment process require chemically accessible amine groups, understanding the chemical nature of these N atoms is also important. Previous studies have shown that bonding of saturated primary amines such as 1-hexylamine leads to direct Si–N bond formation, with N(1s) binding energies of 398.8–399.0 eV.^{24,27} In contrast, previous experiments of free NH_2 groups have reported N(1s) binding energies of 399.5–400.5 eV.²⁸ The value of 400.5 eV we observe for BACP therefore suggests that the amine group is not bonded directly to the Si surface, suggesting that the protected

amine group is unaffected by the surface attachment. Analysis of the other core level leads to similar conclusions. The C(1s) spectrum in Figure 4b shows a large peak at 285.3 eV and two smaller peaks at 287.0 and 289.7 eV. In a previous study of a surface-bonded trifluoroethyl ester,⁴ we found that the carbon in the C=O group gave rise to a peak at 289.3 eV, while the ether-like carbon (C–O) had a binding energy of 287.5 eV. Similarly, for BACP we attribute the peak at 289.7 eV to the carbon atom in the carbonyl group, the peak at 287.0 eV to the carbon atom in the ether linkage (C–O) and possibly the C atoms bonded to N, and the large peak at 285.3 eV to the alkane-like carbon atoms. Thus, the C(1s) spectrum strongly suggests that the attachment of BACP to the Si surface leaves the *t*-BOC protecting group intact.

Finally, we note that the Si(2p) spectrum in Figure 4b shows only two peaks, with little or no intensity in the 102–104 eV region. The absence of peaks in the 102–104 eV region (cf. Figure 3b) also indicates that there is no significant oxidation of the underlying Si surface. Although interpretation of the O(1s) spectrum is often complicated by adsorption of trace amounts of water, the O(1s) spectrum (not shown) exhibits two O(1s) peaks, at 533.9 and 532.5 eV, with nearly identical integrated areas. The peak at lower binding energy is similar to that observed for carbonyl groups and is therefore attributed to the C=O of the *t*-BOC group; the peak at higher binding energy (533.9 eV) is attributed to the ether-like C–O group. These binding energies are similar to those in previous studies, which reported 531.5–532.2 eV for C=O groups and 533.2–534.0 eV for oxygen atoms in ether-like (C–O–C) linkages.^{29–31}

(B) Deprotection of BACP. The above experiments show that the protected amine BACP attaches to the Si surface with a much higher density and in a more homogeneous manner than the unprotected amine (ACP). While unprotected silicon surfaces oxidize rapidly in the presence of base,³² the monolayer film of attached BACP molecules acts as a protecting layer. Figure 4 shows the XPS spectra of the BACP-modified Si surface (Figure 4c), the same sample after exposure to 25% trifluoroacetic acid (TFA) in CH_2Cl_2 for 2 h (Figure 4d), and then the same sample after a subsequent exposure to 10% NH_4OH for 7 min (Figure 4e). Comparing the spectra in Figure 4c with those in Figure 4d shows that exposure to TFA reduces the N(1s) peak at 400.6 eV and introduces a peak at 402.4 eV, close to the binding energy of 403.2 eV associated with ammonium-like N atoms in compounds such as NH_4Cl .²⁴ Figure 4e shows that subsequent exposure to 10% NH_4OH leaves most of the peaks unchanged, except that the N(1s) peak at 402.4 eV is eliminated and replaced by a new peak at 400.6 eV, close to the binding energy observed for the molecule before deprotection. These trends in binding energy, including the 1.7 eV shift associated with the protonated amine, are nearly identical to those reported in a recent study with a *t*-BOC protected 10-aminodec-1-ene, a similar long-chain linear molecule.⁵ The chemical changes inferred from the N(1s) spectra during the deprotection steps are also reflected in the C(1s) spectra. In particular, we note that the C(1s) peak in Figure 4c at 289.7 eV, which we attributed above to the carbonyl carbon, disappears after exposure to TFA (Figure 4d). This disappearance is consistent with the loss of the *t*-BOC protecting group via acid hydrolysis in TFA. The

(27) Hovis, J. S.; Lee, S.; Liu, H.; Hamers, R. J. *J. Vac. Sci. Technol. B* **1997**, *15*, 1153–1158.

(28) Moulder, J. F. S. W. F.; Sobol, P. E.; Bomben, K. D. In *Handbook of X-ray Photoelectron Spectroscopy*; Chastain, J., King, R. C., Jr., Eds.; Physical Electronics, Inc.: Eden Prairie, 1995.

(29) Bubert, H.; Lambert, J.; Burba, P. *Fresenius J. Anal. Chem.* **2000**, *368*, 274–280.

(30) Patnaik, A.; Li, C. O. *J. Appl. Phys.* **1998**, *83*, 3049–3056.

(31) Hutt, D. A.; Leggett, G. J. *Langmuir* **1997**, *13*, 2740–2748.

(32) Wind, R. A.; Hines, M. A. *Surf. Sci.* **2000**, *460*, 21–38.

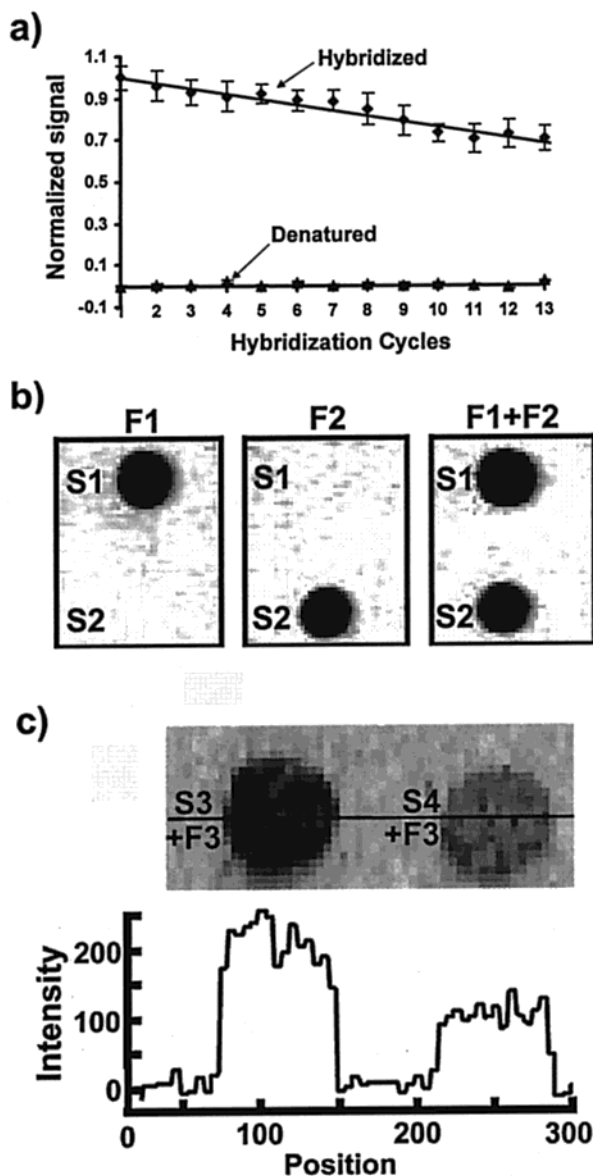


Figure 7. (a) Surface stability toward multiple hybridization and denaturation cycles. Error bars reflect error estimates from measurements on three replicate samples. (b) Fluorescence measurements (black = high intensity) of hybridization of surface-bound DNA with fluorescently labeled oligonucleotides. Two different oligonucleotides with a four-base difference (out of 16) were first attached to the surface at the spots indicated. (left) Fluorescence image of surface after hybridization with fluorescent oligonucleotides F1, complementary to sequence S1. (center) Same surface, after denaturing and hybridizing the surface with fluorescent oligonucleotides F2, complementary to sequence S2. (right) Same surface, after denaturing and hybridizing the surface with both fluorescent oligonucleotides simultaneously. (c) Fluorescence measurements of single-base mismatches in DNA hybridization. Two different 16-base oligonucleotides (S3 and S4) with a single-base difference were first attached to the surface. The image shows the intensity of fluorescence after oligonucleotide F3, complementary to S3, was hybridized on the surface.

signal. There is a small decrease in the intensity of the fluorescence in successive cycles. After 13 cycles the maximum fluorescence signal is approximately 75% of that obtained on the first cycle, corresponding to a decrease of approximately 2% per cycle.

The 2% per cycle decrease we observed is comparable to that obtained in recent studies in which DNA was attached to the Si(100) surface by first preparing an ester-

terminated surface, hydrolyzing to the acid form, depositing polylysine to produce an amine-terminated surface, and then using SSMCC to link DNA to the surface.⁴ Thus, we can conclude that using BACP to form the amine-terminated surface leads to surfaces that are equally robust, while the BACP procedure involves fewer steps than the previous method. Since the presence of multiple charged groups in polylysine may increase its propensity for nonspecific adsorption, we believe that the BACP molecule may provide better specificity. However, we also note that the 2% decrease in fluorescence intensity using BACP chemistry is larger than that observed in another recent study in which the long-chain alkeneamine (*t*-BOC-10-aminodec-1-ene) was used, on the (111) surface of silicon.⁵ In that case the fluorescence intensity decreased by only 1% per cycle.

(ii) *Density of Active Sites Accessible to DNA Hybridization.* A useful surface requires bound oligonucleotides arranged on the surface with enough interstitial space to allow high hybridization efficiency. To quantitatively determine the number of surface-bound DNA molecules, we performed a calibration using gel electrophoresis and comparing the fluorescence intensities with known standards.^{4,18} In this calibration procedure, single-stranded DNA was attached to the Si(100) surface as described above; the fluorescently tagged complement was then hybridized with the surface-bound DNA, and the fluorescence was measured as described above. The hybridized surfaces were then placed into 1 mL of water at 90 °C for 15 min to denature the duplex, and the fluorescent complements were collected. The denatured solution containing the complements was then reduced to a 10 μ L volume using a vacuum spin-drier and loaded on one lane of a polyacrylamide gel. Fluorescent DNA standards were loaded into the other lanes. After electrophoresis, the gel was scanned and the total fluorescent signals were measured. Using this method, we determined that the density of hybridized molecules was approximately 6.0×10^{12} per cm^2 , close to the value reported previously on other substrates including silicon,⁴ self-assembled monolayer silane films on fused silica,³³ SiO_2 ,³⁴ and gold.³⁵

(iii) *Binding Specificity in DNA Hybridization.* To evaluate the binding specificity, we performed experiments comparing the binding of surface-bound DNA with perfect complements as well as controlled mismatches. To illustrate general hybridization specificity, two spots of 31-mer thiol-oligonucleotides (sequences S1 and S2) with a four-base difference were applied to the SSMCC modified surface to form two circular areas approximately 2 mm in diameter. The surface was then exposed to a solution containing the fluorescently tagged sequence F1, which is complementary to S1. After 20 min of hybridization, the surface was washed and the fluorescence signal from the surface was measured. The left panel of Figure 7b shows the fluorescence images (black = high fluorescence intensity), indicating binding of F1 to the region functionalized with the S1 sequence and not to the S2 sequence. After the surface was denatured with urea and then exposed to sequence F2, fluorescence was observed only on the spot where DNA with the S2 sequence was bonded (middle panel, Figure 7b). Finally, after the surface was denatured and then exposed to an equimolar mixture of

(33) Chrisey, L. A.; Lee, G. U.; O'Ferrall, E. C. *Nucleic Acids Res.* **1996**, *24*, 3031–3039.

(34) Lamture, J. B.; Beattie, K. L. B.; Eggers, M. D.; Ehrlich, D. J.; Fowler, R.; Hollis, M. A.; Kosicki, B. B.; Reich, R. K.; Smith, S. R.; Varma, R. S.; Hogan, M. E. *Nucleic Acids Res.* **1994**, *22*, 22121–22125.

(35) Jordan, C. E.; Frutos, A. G.; Thiel, A. J.; Corn, R. M. *Anal. Chem.* **1997**, 4939–4947.

F1 and F2, fluorescence was observed from both spots with equal intensity.

To test whether the chemistry is sufficiently selective to distinguish a mismatch of only a single base pair, experiments were conducted using the sequences S3 and S4 (which differ by only a single base) and their 15-mer complements, sequences F3 and F4. As above, sequences S3 and S4 were attached to the surface in two areas, and the DNA-modified surface was then exposed to a solution containing F3. After 20 min of hybridization, the surface was washed and the fluorescence was measured. Figure 7c shows the resulting fluorescence image and the intensity profile along the indicated line. Although some binding is observed on both spots, the fluorescence signal was much stronger on its complement (S3) than on the single base pair mismatch (S4). Quantitative analysis of the fluorescence intensities shows that the average intensity (in arbitrary units) in the mismatched spot S4 + F3 was 119 (standard deviation 19), the intensity in the perfect match S3 + F3 was 239 (standard deviation 19), and the background level of the surrounding region was 5.4 (standard deviation 6). The difference in intensity between the perfect match and the single-base mismatch is 120, more than 4 times the combined standard deviation of 27. Thus, we conclude that the chemistry described here is sufficient to be able to identify a single-base mismatch with excellent discrimination.

Our experiments are largely based upon the precept that improved surface chemistry can lead to improved sensitivity and selectivity in DNA hybridization. To illustrate this, we note that the XPS data indicate that use of the unprotected amine ACP leads to extensive surface oxidation, with relatively little attachment of ACP. To identify how this affects the subsequent hybridization chemistry, we also did a control experiment in which we attempted to link thiol-terminated DNA to the ACP-modified surface using the SSMCC linker using the same procedure described above. When the fluorescently labeled complementary sequence was applied, the resulting fluorescence intensity was 107 with a standard deviation of 66, while the background regions yielded an average intensity of 112 with a standard deviation of 83. Thus, we conclude that no detectable amount of DNA hybridizes to the surfaces prepared using the unprotected amine ACP.

(4) Discussion

Optimization of surface chemistry for surface-based analysis of biomolecules such as DNA requires careful attention to the chemistry of DNA attachment in order to maximize the density of DNA binding sites, while retaining high selectivity against sequences with small numbers of mismatches. Degradation of the interface may occur during the initial attachment chemistry or during the subsequent hybridization and denaturation processes. Our research is focused on identifying the specific chemical and structural factors that lead to robust DNA-functionalized surfaces, to develop attachment chemistries that are simple and reproducible.

One general scheme for attachment of DNA to surfaces has focused on the direct bonding of alkenes containing functional groups, such as esters, acids,^{4,10} and chlorides,¹¹ to hydrogen-terminated silicon surfaces. Previously, we have found that molecules with carboxylic acid groups will react directly with the surface but that carboxylic acid-terminated Si surfaces can be readily prepared by linking alkenes bearing an ester functionality first and then hybridizing this to the carboxylic acid after surface attachment. Similarly, the results presented here show

that the direct attachment of alkenes that also bear a primary amine group leads to poorly defined attachment. Indeed, amines are widely used in the microelectronics industry for etching of silicon, and primary amines are widely known to promote the reaction of silicon with water.³⁶ Although our amines do not have water present intentionally, most amines are hygroscopic and readily acquire more than sufficient water to permit formation of several monolayers of oxidation products. Thus, we believe that the extensive oxidation we observe using the unprotected amine likely arises from a similar amine-enhanced reaction with trace amounts of water. However, protecting the amine with the t-BOC group reduces the basicity of the amine and apparently also reduces or eliminates its oxidizing effect on the Si surface. By utilizing the protected amine, it is possible to prepare a surface with a high density of reactive amine groups.

Our results show that the number density of amine groups is much higher than the density of DNA molecules that can ultimately be hybridized to the surface. The density of amine groups formed using BACP is approximately 6×10^{14} molecules/cm². In comparison, recent studies of aminosilanes have shown densities of approximately 4×10^{14} amines/cm².³⁷ Studies of the attachment of functionalized alkenes to silicon via a thermal process have also yielded attachment densities of $\sim 4 \times 10^{14}$ amines/cm².¹⁰ Although these estimates indicate a slightly higher amine density using BACP than with alternative methods, this difference is not likely to be significant. Quantitative XPS analysis of the N(1s) levels shows that the SSMCC linkers are also present at comparable number densities, while hybridization and fluorescence measurements show no appreciable difference between the three different linkers employed. The number density of hybridized DNA molecules ($\sim 6 \times 10^{12}$ cm⁻²) is typically much smaller than the number of amine sites ($\sim 6 \times 10^{14}$ cm⁻²). This suggests that either (1) only a small number of DNA molecules are actually linked to the SSMCC molecule or (2) only a small fraction of the surface-bound DNA molecules hybridize with their complement. Our experimental data do not allow us to distinguish between these possibilities. However, it is clear that the density of DNA molecules that can ultimately hybridize with the surface is not limited by any of the initial stages of the surface modification chemistry.

The above studies show that the level of nonspecific adsorption on these silicon surfaces is sufficiently low that one can clearly distinguish mismatches of only a single base pair out of 16. Nonspecific adsorption arises from a number of possible factors, including van der Waals' forces, polarization forces created by charged groups or dipoles on the surface, and physical entanglement. Most previous studies have prepared amine-terminated surfaces via a polylysine layer that is electrostatically bonded to an acid-terminated surface, and have typically used long-chain molecules containing 12–18 carbon atoms as a "spacer" layer between the surface and the molecules of interest. Our results show that it is possible to prepare equally good layers using shorter molecules. The thickness of these functionalization layers and any subsequent linkers may help to minimize nonspecific adsorption by reducing the possibility of entanglement of DNA molecules. Additionally, it could be quite important for true integration of biomolecules with silicon to achieve (for example) direct electronic detection of hybridization processes. The po-

(36) Finne, R. M.; Klein, D. L. *J. Electrochem. Soc.* **1967**, *114*, 965–970.

(37) Moon, J. H.; Shin, J. W.; Kim, S. Y.; Park, J. W. *Langmuir* **1996**, *12*, 4621–4624.

tential drawback of the thin BACP layers is that the BACP layer is sufficiently thin that chemical degradation of the interface may be more facile. In the experiments reported here, we observed about 2% degradation in fluorescence intensity per cycle during multiple-hybridization experiments. This is slightly larger than the loss of only ~1% observed using the long-chain alkene amines such as *t*-BOC-10-aminodec-1-ene.⁵ Since the (111) surface of silicon is known to be microscopically flatter and more uniform than the (100) surface used in the study presented here, this difference may simply reflect the intrinsic differences in the microscopic structure and chemistry of these different crystal faces.³⁸ Further careful comparisons will be required to determine whether these differences in stability arise from intrinsic differences in the chemistry or in the different atomic structures of the (111) and (100) surfaces of silicon. Additionally, the N(1s) peak from BACP is somewhat broader than the similar peak using *t*-BOC-10-aminodec-1-ene, suggesting a possibly greater degree of chemical degradation for BACP. Further work still needs to be done to explore and develop optimized chemical pathways for surface attachment of short organic linkers and, particularly, to identify the chemical pathways involved in the interfacial degradation.

(5) Conclusions

Covalent bonding of DNA to crystalline silicon substrates yields interfacial layers showing high selectivity

(38) Dumas, P.; Chabal, Y. J.; Jakob, P. *Surf. Sci.* **1992**, *269/270*, 867–878.

in subsequent hybridization processes. Analysis of the chemical structure of the films via core-level photoelectron spectroscopy permits optimization of the chemical processing steps, yielding DNA-functionalized Si surfaces that are selective and stable toward multiple hybridization cycles. Our results show that initial attachment of protected amines, followed by subsequent deprotection of the surface-bound molecules, produces amine-terminated surfaces that are more homogeneous and have a higher density of free amine sites than surfaces produced by alternative methods involving direct attachment of unprotected amines. This improvement in homogeneity, combined with the use of a hetero-bifunctional cross-linker such as SSMCC, leads to formation of DNA-functionalized silicon surfaces exhibiting good selectivity and stability. Even short-chain amines can form DNA-functionalized surface layers exhibiting good selectivity, capable of identifying a single-base mismatch out of 16 bases, but the stability of the interface with repeated hybridizations is slightly less than that obtained using longer chain, protected alkeneamines. The residual degradation of the interface during repeated hybridization steps requires further investigation.

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