

DNA-Modified Diamond Surfaces

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Preparation and hybridization of DNA-modified polycrystalline diamond substrates with fluorescently labeled complementary and noncomplementary oligonucleotide sequences were investigated. Hydrogen-terminated, free-standing diamond substrates were photochemically modified to produce amine-terminated surfaces. X-ray photoelectron spectroscopy and Fourier transform infrared spectroscopy were used to characterize the initial attachment of a protected amine and the subsequent deprotection chemistry. Thiol-terminated DNA oligonucleotides were then linked to the amine-terminated diamond surfaces using a heterobifunctional linker. It is shown that hybridization on DNA-modified polycrystalline diamond is specific, with strong binding of perfectly matched 16-mer complements and little or no binding to 16-mers with 4 mismatched nucleotides. A direct comparison of DNA hybridization on DNA-modified diamond and DNA-modified surfaces of crystalline silicon shows that the diamond surfaces exhibit superior chemical stability under the conditions employed to hybridize and denature the DNA-modified surfaces.

Recent advances in biotechnology and molecular electronics have fueled an interest in fabrication of well-defined, highly stable interfaces between biomolecules and solid supports. Much attention has been given to the attachment of biomolecules to polymers^{1,2} and glass substrates.^{3–5} However, the desire to characterize the electrical properties of biomolecules for applications such as direct electronic biosensing systems is leading to increased interest in alternative substrates such as gold,^{6–8} silicon,^{9–15} and diamond.^{16–20} Diamond is a particularly attractive substrate material because of its chemical

stability and biocompatibility²¹ and because it can be doped and deposited in very thin films on a variety of substrates even at comparatively low temperatures.^{22,23} Diamond is used in applications such as internal-reflection elements for infrared spectroscopy because of its good optical properties and chemical stability,²⁴ but typical diamond surfaces do not have well-defined chemical or biochemical groups on the surface and therefore lack chemical *specificity*. Since diamond can be made conductive by doping, it is also of interest for a variety of electrically based chemical and biological sensing applications that would achieve improved performance by selective biological modification.^{25,26}

Because only a small number of schemes have been proposed previously for chemical modification of diamond,^{16,18,20,27,28} performing studies on free-standing polycrystalline films provides a way of studying the chemistry of diamond in a way that can be extended to other morphologies, such as thin films.²⁰ Recent studies have shown the ability to photochemically modify diamond surfaces with molecules containing an olefin (C=C) group.¹⁶ Here we show that this chemistry can be extended to permit the attachment of DNA to polycrystalline diamond surfaces, and we show that the DNA-modified

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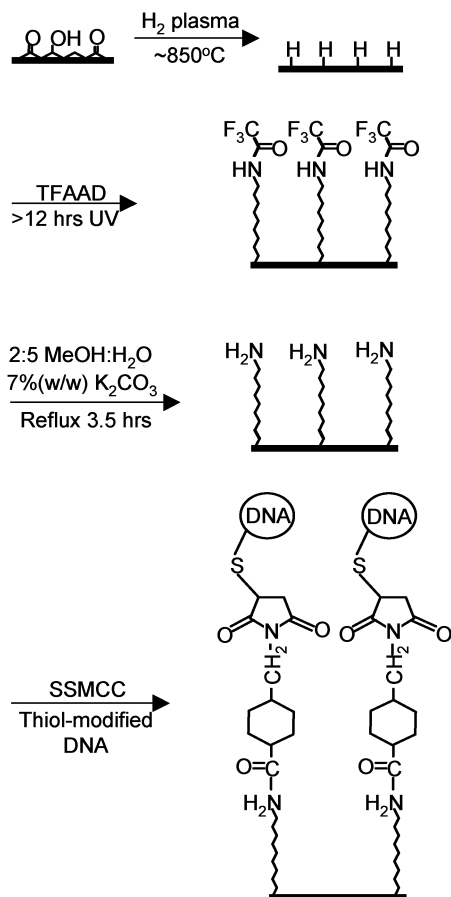


Figure 1. Reaction scheme for attachment of DNA to diamond surfaces.

diamond surfaces exhibit extremely good selectivity and stability in subsequent hybridization studies.

High-quality polycrystalline free-standing diamond films were deposited in a microwave plasma reactor using 1% CH₄ in H₂.²⁹ These films contain almost no sp²-hybridized carbon. The 1 mm thick films were cut into 5 mm × 10 mm samples that were cleaned by exposure to a series of acid baths designed to remove metals, amorphous carbon, and silicates.²⁹ The diamond films were then heated to approximately 850 °C in a 13.56 MHz inductively coupled hydrogen plasma (15 Torr H₂) for 20 min and subsequently cooled in the hydrogen plasma to terminate the surface with hydrogen.^{30,31}

Chemical functionalization of the diamond surface (Figure 1) was accomplished by placing 5–10 μL of trifluoroacetamide-protected 10-aminodec-1-ene (TFAAD) directly onto the hydrogen-terminated diamond sample in a nitrogen-purged Teflon reaction chamber covered with a quartz window. The sample was then exposed to ultraviolet light from a low-pressure mercury vapor quartz grid lamp ($\lambda_{\max} = 254$ nm, 0.35 mW/cm²) for ~ 12 h. In a previous study, it was shown that this process induces a reaction that terminates at a single monolayer.¹⁶ Although the reaction mechanism has not yet been established, we note that H-terminated polycrystalline films exhibit a number of unusual properties, including the ability to emit electrons from the valence band directly into a vacuum when illuminated with 254 nm light.³² These unusual properties make H-terminated diamond surfaces very

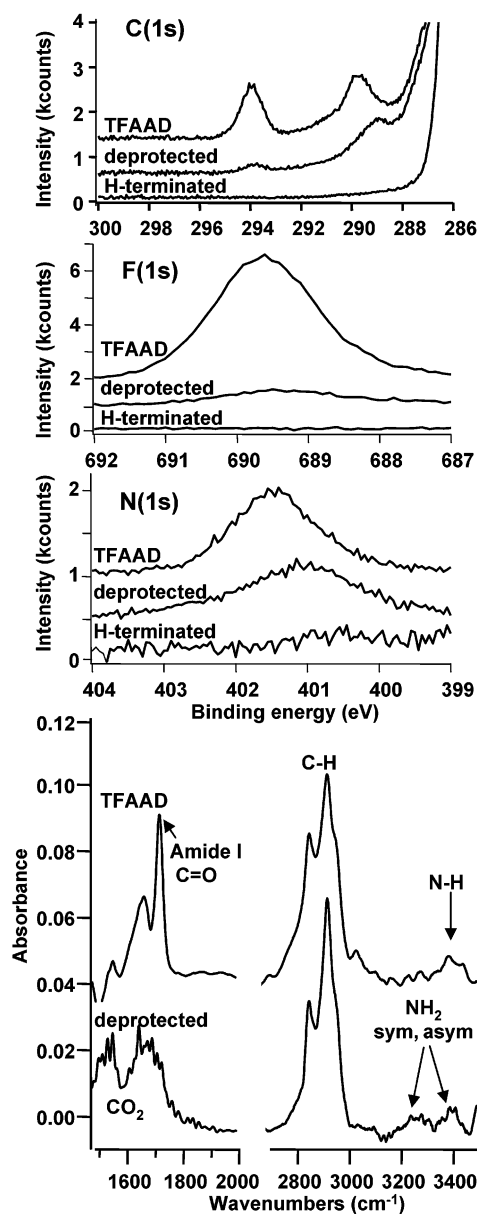


Figure 2. C(1s), F(1s), and N(1s) XPS spectra (top) and FTIR spectra (bottom) of TFAAD-modified diamond before and after deprotection. XPS spectra of hydrogen-terminated diamond are also shown for comparison. In the FTIR spectrum, deprotection leads to the loss of the amide I band at 1685 cm⁻¹ and the splitting of the NH line into two components arising from the symmetric and asymmetric vibrations of the -NH₂ group. The spectrum of deprotected TFAAD also shows two broad peaks at 1680 and 1540 cm⁻¹ from atmospheric CO₂.

different from hydrocarbons, even though both are comprised of carbon and hydrogen.

The functionalization of the surface was assessed using X-ray photoelectron spectroscopy (XPS) with a monochromatic Al K α source (1486.6 eV photon energy). Figure 2 shows the XPS spectra for the C(1s), F(1s), and N(1s) areas before and after modification with TFAAD. Before modification, the carbon spectrum shows one major peak centered at 285.5 eV, corresponding to bulk carbon (off-scale on the right-hand side of the spectrum), which was used as an internal standard for comparison between spectra. After modification, the carbon spectrum shows two additional peaks at 293.9 and 289.8 eV attributed to

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the C atoms of the $-\text{CF}_3$ group and carbonyl ($\text{C}=\text{O}$) group, respectively. The F(1s) spectrum shows a peak at 689.6 eV from the fluorine atoms in the TFA protecting group with $\sim 35\%$ of the area of the bulk carbon peak. The N(1s) spectrum shows a peak at 401.4 eV from the N atom of the amide group with $\sim 6\%$ of the area of the bulk carbon peak. Correcting the $\text{F}/\text{C}_{\text{bulk}}$, $\text{N}/\text{C}_{\text{bulk}}$, and $\text{C}/\text{C}_{\text{bulk}}$ ratios for sensitivity factors ($\text{C} = 0.25$, $\text{N} = 0.42$, $\text{F} = 1$), electron escape depth (2 nm), and the angle of the sample normal with respect to the analyzer (45°) yielded an average number density of $\sim 7 \times 10^{14}$ TFAAD molecules/ cm^2 . This number is, within experimental error, the same as that anticipated from the area of the methyl groups determined from X-ray crystal structures (area = 18.4 \AA^2 implying a number density of $5.4 \times 10^{14} \text{ cm}^{-2}$)^{33,34} and is slightly smaller than the number density of 2×10^{15} C atoms/ cm^2 of the C(111) surface. These XPS results show that the TFAAD molecule attaches to the diamond surface to produce a dense monolayer film.

To provide chemically reactive amine groups, the trifluoroacetamide protecting group was removed by refluxing the TFAAD-modified sample in 2:5 MeOH/ H_2O with 7% (w/w) K_2CO_3 .^{35,36} Figure 2 also shows XPS spectra of the same sample after deprotection. The deprotected sample has a C(1s) peak at 285.5 eV with the peak area nearly identical to that of the unprotected sample. However, the other peaks change significantly. The most obvious change upon deprotection is the significant reduction in intensity of the peak at 293.9 eV that was attributed to the $-\text{CF}_3$ group. The deprotection process reduces this peak to only 15% of its original area. Additionally, the carbonyl peak at 289.8 eV shifts to 288.9 eV and is reduced in area. An F(1s) peak is observed at 689.6 eV, but with only 15% of its original area. Thus, the C(1s) and F(1s) data both indicate that $\sim 85\%$ of the $-\text{CF}_3$ groups are removed by the deprotection process. The deprotection process also leads to changes in the N(1s) spectrum. The peak originally at 401.4 eV before deprotection is broadened and shifted to a lower binding energy of 401.0 eV. The shift indicates an increase in electron density around the nitrogen atom, consistent with removal of the electron-withdrawing TFA protecting group. Comparison of the nitrogen peak areas before and after deprotection shows a small ($\sim 10\%$) loss of nitrogen during the deprotection step, possibly due to loss of residual physisorbed TFAAD.

The process outlined in Figure 1 would be expected to decrease the area under the O(1s) peak (not shown) and the C(1s) carbonyl peak. However, the area under the O(1s) peak increased slightly, while the C(1s) carbonyl peak shifted to lower binding energy. Subjecting dodecene-modified surfaces to the deprotection conditions also gave rise to a peak at 288.9 eV and an O(1s) peak. These results are attributed to the generation of surface carbonyl groups and likely arise from oxidation of unreacted surface C–H sites.

To confirm that deprotection does indeed occur as depicted in Figure 1, two additional types of experiments were conducted. First, Fourier transform infrared (FTIR) spectra were obtained of a diamond thin film deposited on a silicon substrate ($\sim 0.5 \mu\text{m}$ thick coating) before and after deprotection. Experiments on diamond thin films show that thin films have chemistry similar to that of the

substrates studied here.²⁰ Figure 2 shows FTIR spectra of a TFAAD-modified diamond surface before and after deprotection. Before deprotection, the sample showed a strong amide stretch at 1726 cm^{-1} . Deprotection led to the disappearance of the amide peak and the appearance of two new N–H stretching bands near 3400 and 3270 cm^{-1} , close to the values typically observed for the symmetric and asymmetric N–H stretches of a primary amine group.³⁷ Thus, the FTIR data indicate that the surface is terminated with primary amine groups.

To further verify the efficacy of the deprotection, we also reacted the deprotected surface with sulfo-succinimidyl-4-*O*-(4,4'-dimethoxytrityl)-butyrate (SDTB), which is an amine-reactive compound that can liberate trityl groups into solution; these trityl groups were then measured spectrophotometrically. Diamond samples prepared and deprotected as described above were immersed in a 0.1 mM solution of SDTB in 50 mM NaHCO_3 at pH 8.5 for 30 min. Under these conditions, the *N*-hydroxy-sulfosuccinimidyl ester reacts with the amine groups to form an amide bond.³⁸ The reacted surfaces were then removed from the SDTB solution, rinsed with distilled water to remove any excess reagent, and exposed to a solution of 4.14 M trifluoroacetic acid. This step releases into the solution the dimethoxytrityl groups, which were detected using absorption spectroscopy ($\lambda_{\text{max}} = 498 \text{ nm}$) and quantified by comparison with a series of standards. This method yielded a value of $(1.6 \pm 0.5) \times 10^{13}$ molecules/ cm^2 as the number of density of accessible primary amine groups. This value is smaller than that obtained from XPS measurements; this difference likely arises from steric effects associated with the large size of the SDTB molecule and implies that the number of free amine groups that are accessible to smaller reactants may be higher than $1.6 \times 10^{13} \text{ cm}^{-2}$. In control experiments with TFAAD-modified diamond that was not deprotected, no dimethoxytrityl groups were detected. The SDTB test, FTIR, and XPS data all indicate that the deprotection chemistry proceeds as expected.

The amine-terminated diamond surfaces were reacted with a 14 nM solution of the heterobifunctional cross-linker sulfo-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SSMCC) in 0.1 M pH 7 triethanolamine (TEA) buffer for 20 min at room temperature in a humid chamber. The NHS-ester group in this molecule reacts specifically with the $-\text{NH}_2$ groups of the surface to form an amide bond. The maleimide moiety was then reacted with thiol-modified DNA (250 μM thiol DNA in 0.1 M pH 7 TEA buffer) by placing the DNA directly onto the surface in a humid chamber and allowing it to react for $> 6 \text{ h}$ at room temperature, as depicted in Figure 1.^{12,39} To assess the efficacy of the DNA attachment, strand A (31 T's modified with a 3'-thiol C6 modifier and a 5' fluorescein phosphoramidite, University of Wisconsin–Madison Biotechnology Center) was attached to the diamond surface. Fluorescence imaging on a Molecular Dynamics Fluor-Imager 575 was used to detect the surface-bound oligonucleotides. Figure 3 shows a representative fluorescence image (black = high intensity). The appearance of a bright spot of fluorescence demonstrates that DNA can be attached to the surface.

To explore hybridization at DNA-modified diamond surfaces, DNA oligonucleotides with two sequences dif-

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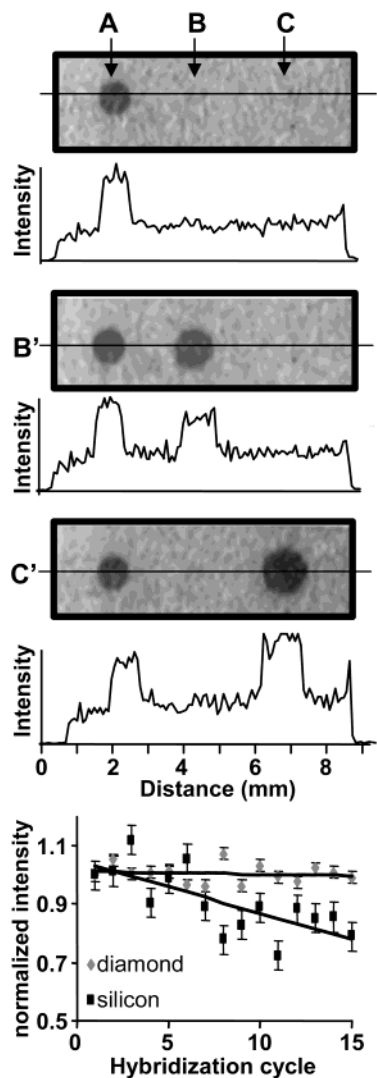


Figure 3. A diamond surface modified with sequences A, B, and C. Sequence A has been modified with a 5' fluorescein. Sequences B and C differ by 4 bases (see the text for a detailed description of base sequences). The top image shows the surface and its fluorescence intensity profile before exposure to any complement (sequence B' or sequence C'). The middle and bottom images show the surface and the fluorescence intensity profiles after exposure to sequences B' and C', respectively. The graph shows a direct comparison of the hybridization stability of DNA on diamond and silicon surfaces.

fering by 4 bases were linked to the surface. These sequences are: 5' TTT TTT TTT TTT TTT GCT TAT CGA GCT TTC G 3' (sequence B) and 5' TTT TTT TTT TTT TTT GCT TAA GGA GCA ATC G 3' (sequence C). These DNA oligonucleotides were modified with the 5'-thiol C6 modifier and attached to the amine-functionalized diamond surfaces. We explored the hybridization of these two surface-bound sequences with two different sequences that were labeled with the fluorescein phosphoramidite at the 5' end. Sequence B' (5' FAM CGA AAG CTC GAT AAG C 3') is a perfect complementary match to sequence B, and sequence C' (5' FAM CGA TTG CTC CTT AAG C 3') is a perfect complementary match to sequence C, while the hybridization of sequence B with sequence C' and that of sequence C with sequence B' involve 4-base mismatches.

Figure 3 shows a diamond surface modified with sequences A, B, and C. The first image shows the surface as prepared, before exposure to any complementary DNA. Only sequence A appears here, as this sequence has been

directly (covalently) modified with a 5' fluorescein phosphoramidite. The surface was then placed facedown in 5 μ L of sequence B' (the complement of sequence B; 5 μ M in an aqueous solution of 0.3 M NaCl, 0.02 M NaPO₄, 0.002 M EDTA, and 0.2% sodium dodecyl sulfate (HB)) at room temperature in a humid chamber. After 20 min, the surface was removed, soaked twice (5 min) in HB to remove any excess sequence B', and then imaged again (second image). The second spot, which appears at sequence B, shows that hybridization occurs as anticipated. The intensity at spot A remained constant, indicating that there was no nonspecific binding of DNA strands having completely unrelated sequences. Also, spot C was indistinguishable from the background, which shows that no hybridization occurs at a sequence with a 4-base mismatch. After imaging, the surface was exposed to an aqueous solution of 8.3 M urea at room temperature for 5 min, rinsed thoroughly with distilled water, and imaged again (not shown) to ensure complete removal of sequence B'. The surface was then placed in 5 μ L of sequence C' (the complement of sequence C, 5 μ M in HB) at room temperature in a humid chamber. After 20 min, the surface was removed, soaked twice (5 min) in HB, and imaged again (third image). The appearance of the spot at sequence C shows that hybridization occurred as expected. Again, the intensity at spot A remained the same and the intensity at spot B was indistinguishable from the background, showing the expected specificity. Therefore, both sequences are indeed attached to the surface and accessible for hybridization, and hybridization occurs only at the correct complementary sequence as anticipated.

One of the potential benefits of covalent attachment of DNA to diamond substrates is increased long-term stability. The stability of the surface-bound oligonucleotides was investigated using fluorescence imaging in repetitive cycles of hybridization and denaturation. In each cycle, the surface-bound DNA was hybridized with its fluorescently labeled complement for 20 min at room temperature in a humid chamber, and the intensity of fluorescence was measured. The sample was then denatured in an aqueous solution of 8.3 M urea for 5 min at room temperature and rinsed with distilled water, and the fluorescence intensity was measured again. This hybridization/denaturation process, including imaging after both hybridization and denaturation, was repeated 15 times. The fluorescence intensity measured in relative fluorescence units shows no significant loss of DNA over the 15 hybridization cycles (Figure 3). In contrast, DNA-modified silicon surfaces prepared via direct Si-C bond formation using a similar method¹² showed a loss of $1.8 \pm 0.4\%$ fluorescence per hybridization cycle or $\sim 27\%$ loss of fluorescence intensity over the 15 hybridization cycles.

To determine the number density of DNA molecules hybridized to the surface, a diamond surface was modified with sequence B, hybridized with sequence B', and then denatured with 5 mL of an aqueous solution of 0.05 M KCl and 0.132 M KOH. The emission spectrum ($\lambda = 522$ nm) of the solution was measured on a fluorimeter and compared with a series of standards. These experiments yielded a concentration of $(3.1 \pm 0.7) \times 10^{12}$ DNA molecules/cm². Similar results have been observed on other surfaces such as silicon¹⁴ and gold.⁴⁰

The above results show that diamond surfaces can be functionalized with DNA, and the resulting surfaces exhibit high selectivity and stability in subsequent

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hybridization experiments. Our results show that DNA-modified diamond surfaces are significantly more stable than DNA-modified silicon surfaces. Diamond also exhibits very good electrical properties that may make it suitable for direct electronic detection of biological binding processes.

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