Topographical and Functional Characterization of the ssDNA Probe Layer Generated Through EDC-Mediated Covalent Attachment to Nanocrystalline Diamond Using Fluorescence Microscopy

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The covalent attachment method for DNA on nanocrystalline diamond (NCD), involving the introduction of COOH functionalities on the surface by photoattachment of 10-undecenoic acid (10-UDA), followed by the 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)-mediated coupling to NH_2-labeled ssDNA, is evaluated in terms of stability, density, and functionality of the resulting biological interface. This is of crucial importance in DNA biosensor development. The covalent nature of DNA attachment will infer the necessary stability and favorable orientation to the ssDNA probe molecules. Using confocal fluorescence microscopy, the influence of buffer type for the removal of excess 10-UDA and ssDNA, the probe ssDNA length, the probe ssDNA concentration, and the presence of the COOH-linker on the density and functionality of the ssDNA probe layer were investigated. It was determined that the most homogeneously dense and functional DNA layer was obtained when 300 pmol of short ssDNA was applied to COOH-modified NCD samples, while H-terminated NCD was resistant for DNA attachment. Exploiting this surface functionality dependence of the DNA attachment efficiency, a shadow mask was applied during the photochemical introduction of the COOH-functionalities, leaving certain regions on the NCD H-terminated. The subsequent DNA attachment resulted in a fluorescence pattern corresponding to the negative of the shadow mask. Finally, NCD surfaces covered with mixtures of the 10-UDA linker molecule and a similar molecule lacking the COOH functionality, functioning as a lateral spacer, were examined for their suitability in preventing nonspecific adsorption to the surface and in decreasing steric hindrance. However, purely COOH-modified NCD samples, patterned with H-terminated regions and treated with a controlled amount of probe DNA, proved the most efficient in fulfilling these tasks.

1. Introduction

Increasing interest in the combination of molecular biology and microelectronics has led to the dawn of a new field called bioelectronics. An important cornerstone in the development of this field is the design of interfaces that are compatible with both microelectronic processing methods and modification with biomolecules. This way, the biological reaction, such as a target recognition event, and the associated sensing and signal processing can be integrated into a single platform. A successful combination of biomolecules with solid substrates was first established in Southern blotting[1] and has since become the basis of the widely used and commercialized microarrays, or gene chips, used for expression profiling or mutational analysis.[2] Probe ssDNA molecules are covalently attached to silicon (Si) or glass, which are materials that are compatible with microelectronics. Hybridization of the probe ssDNA molecules with target ssDNA is usually detected fluorimetrically. However, degradation of these interfaces in aqueous solutions limits their use to that of disposable biosensors.[3,4]

Chemically vapor deposited (CVD) diamond has become an attractive alternative substrate for biosensors because of several very appealing physical, optical, chemical, and electrical characteristics.[5,6] It is transparent in a wide spectrum of optical wavelengths, is chemically inert, has a wide electrochemical window, and can be made semiconductive by doping.[7,8] At relatively low temperatures, diamond can also be deposited on substrates such as Si and quartz that are compatible with microelectronics processing. In addition, various procedures have been developed to covalently attach biomolecules to diamond, such as chemical,[9] photochemical,[3,10] and electrochemical methods.[11,12] These linkages were proven to be more stable than

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those with other commonly used semiconductors such as Si, allowing for reusable diamond-based biosensors. Furthermore, the biocompatibility of diamond permits *in vivo* applications.[13]

We developed a simple but efficient two-step process to covalently bind amino (NH_2)-modified DNA to carboxylic acid (COOH)-modified thin nanocrystalline diamond (NCD) films using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC).[14] It was shown that the attached DNA remained functionally active. However, since DNA of 250 bp was used, the probe density was rather low, and some physisorbed DNA was present. These results clearly demonstrated that several reaction parameters in the covalent attachment procedure are of critical importance in order to achieve biological activity combined with high density and specificity of the sensor surface. Elucidating these parameters is crucial for the fabrication of functional biosensors. First of all, covalency is to be preferred as a means of biomolecule attachment because of the associated stability of the resulting molecular interface, the favorable probe orientation that is brought about by a terminal attachment, and the ability to predetermine the location of the probes on the surface. Second, a high probe density should be attained in order to achieve a high sensitivity. However, the dense probe layer must remain biologically active; that is, the DNA molecules must still be accessible for hybridization. Finally, the amount of nonspecifically adsorbed DNA should be minimized, especially in the case of DNA attachment in an array format.

The current study was performed to address these issues. Several reaction parameters associated with the EDC-mediated covalent DNA attachment were evaluated in terms of their effect on probe density and functionality, such as the washing buffers after 10-undecenoic acid (10-UDA) and DNA attachment, DNA probe length, DNA probe concentration, surface functionality, and sample type. The conditions that yielded the highest probe density were used in a subsequent part of the study to investigate hybridization efficiency with complementary and 1-mismatch ssDNA. In a final subsection, the use of mixed functionalities at the diamond surface were evaluated for their suitability in controlling probe density and decreasing nonspecific adsorption of DNA. These experiments served to offer a detailed understanding of the process of attachment and to establish the conditions for optimal functionality of the DNA sensor surface.

2. Materials and Methods

2.1. Materials. The probe ssDNA molecules were purchased from Eurogentec (Seraing, Belgium) and were modified at the 5′ end with an NH_2 modifier (36 b: 5′-NH_2-C_6H_{12}-AAA-AAA-ACC-CCT-GCA-GCC-CAT-GTA-TAC-CCC-CGA-ACC-3′). The target ssDNA molecules bought from Invitrogen (Merelbeke, Belgium) were modified at the 5′ end with an Alexa 488 label, allowing us to monitor hybridization and denaturation using confocal fluorescence microscopy. Target ssDNA contains a sequence that either is completely complementary to the probe ssDNA (29 b: 5′-Alexa 488-GGT-TCG-GGG-GTA-TAC-ATG-GGC-T**G**C-AGG-GG-3′), carries a 1-base mismatch to the probe ssDNA (29 b: 5′-Alexa 488-GGT-TCG-GGG-GTA-TAC-ATG-GGC-T**C**C-AGG-GG-3′), or is completely noncomplementary (29 b: 5′-Alexa 488-TCA-AAT-TGC-CAG-AAC-AAC-TAC-TGA-CTG-AA-3′).

Sulfuric acid (H_2SO_4) and sodium dodecyl sulfate (SDS) were obtained from VWR International (Zaventem, Belgium). 10-UDA was bought from Sigma-Aldrich (Bornem, Belgium). EDC and 2-[*N*-morpholino]-ethanesulfonic acid (MES) were purchased from Perbio Science (Erembodegem, Belgium). MicroHyb hybridization buffer was synthesized by Invitrogen (Merelbeke, Belgium). Polymerase chain reaction (PCR) buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$) was obtained from Roche Diagnostics (Vilvoorde, Belgium). Sodium hydroxide (NaOH) and potassium nitrate (KNO_3) were acquired from Merck (Overijse, Belgium). Phosphate buffered saline (PBS) (1.29 M NaCl, 0.05 M $Na_2HPO_4.2H_2O$, 0.015 M KH_2PO_4, pH 7.2) and sodium chloride/sodium citrate (SSC) buffer (3 M NaCl, 0.3 M $C_6H_8O_7 \cdot 3Na$, pH 7.5) were homemade.

2.2. NCD Synthesis and Hydrogenation. At the Institute for Materials Research (IMO), CVD diamond layers were deposited as follows. Doped (1−20 Ω·cm) p-type Si(100) was seeded with ultradispersed detonation diamond powder in an ultrasonic bath.[15] Next, undoped NCD films with a thickness of ∼300 nm and a grain size of 50−150 nm were grown on this pretreated substrate using microwave plasma enhanced (MWPE) chemical vapor deposition in an ASTeX "AX6550" reactor (Seki Technotron Corp., Tokyo, Japan) equipped with a 2.45 GHz microwave generator. The NCD thin films were deposited from a standard mixture of 15 sccm (standard cubic centimeter per minute at standard temperature and pressure) CH_4 and 485 sccm H_2. Microwave power was set at 3500 W, process pressure was around 33 Torr, and substrate temperature was around 710 °C. The growth rate was approximately 600 nm/h.

After growth, the wafer was cut in 1 cm^2 pieces using a diamond scribe and cleaned for 30 min in an oxidizing mixture of hot H_2SO_4 and KNO_3. This procedure was followed by washing in an ultrasonic bath with distilled, ultrapure water at room temperature. Afterward, the samples were thoroughly rinsed with heated distilled, ultrapure water and dried with nitrogen gas. The final hydrogenation was performed at 700 °C during 60 s at 3000 W, 35 Torr, and 1000 sccm H_2.

To investigate the robustness of the EDC-mediated DNA attachment approach, also commercially available diamond samples grown by hot filament chemical vapor deposition (HFCVD) from rho-BeSt (Innsbruck, Austria) were used. The latter samples consisted of a 2−4 μm thick film with a grain size of 5−15 nm, grown on p-type silicon.

2.3. Synthesis of NH_2-Modified and Alexa 488-Labeled dsDNA. A 250 bp amplicon of the small tandem repeat (STR)-region of the phenylalanine hydroxylase (PAH) gene was obtained using a MyCycler personal thermal cycler (Bio-Rad Laboratories, Eke, Belgium). Alexa Fluor 488-labeled dsDNA was generated using an Alexa Fluor 488-labeled forward primer (22 b: 5′-Alexa Fluor 488-C_6H_{12}-TCA-AAT-TGC-CAG-AAC-AAC-TAC-T-3′) and an NH_2-labeled reverse primer (26 b: 5′-NH_2-C_6H_{12}-CTT-CTC-ACA-GTA-ATC-ATA-AGT-GTT-CC-3′).

Standard 100 μL PCR reactions contained 200 nmol of each dNTP, 50 pmol of each primer, 5 U Taq DNA polymerase, genomic template DNA, and PCR buffer. The PCR consisted of a preliminary denaturation for 5 min at 95 °C, followed by 35 cycles of a subsequent three-step reaction: denaturation for 20 s at 95 °C, annealing for 20 s at 52 °C, and extension for 30 s at 72 °C. Finally, the PCR amplicons were subjected to a final extension for 6 min at 72 °C.

Amplified dsDNA was purified using phenol−chloroform extraction and concentrated to ∼100 ng·μL^{-1} (∼2 pmol·μL^{-1}) through ethanol precipitation.

2.4. NCD Functionalization. The surfaces of the IMO and rho-BeSt NCD samples were covered with either pure 10-UDA, containing a carboxylic acid (COOH) functionality, or a mixture of 10-UDA and 9-decenol (9-DO), containing an hydroxyl (OH) functionality. To prevent evaporation and to obtain more reproducible liquid film thicknesses, a quartz glass of 1 mm thickness was placed on top of the samples. The samples were subsequently illuminated for 20 h with 254 nm light from a Philips TUV G4T4 4W lamp (>2.5 mW·cm^{-2}), mounted in an EPROM eraser box from Lawtronics (ME5, Edenbridge, U.K.) under a protective nitrogen atmosphere inside a glovebox, as described previously.[14] The samples were subsequently rinsed either with 10% SDS and distilled water or with hot acetic acid and distilled water.

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To establish the effect of different washing buffers on the DNA binding, IMO NCD samples were treated with 5 μmol (0.1 M) of EDC and 30 pmol of NH_2-modified and Alexa 488-labeled probe ssDNA (8 b) in a 25 mM MES buffer (pH 6) for 2 h at 4 °C.

To investigate the effect of DNA length on the attachment efficiency, photochemically modified rho-BeSt surfaces were incubated with a mixture of 5 μmol (0.1 M) of EDC and ~60 pmol of either NH_2-modified and Alexa 488-labeled dsDNA (250 bp) or NH_2-modified and Alexa 488-labeled probe ssDNA (8 b) in a 25 mM MES buffer (pH 6) for 2 h at 4 °C.

For the determination of the most efficient probe DNA concentration, rho-BeSt samples were covalently modified with different amounts of NH_2-modified and Alexa 488-labeled probe ssDNA (8 b) (300 pmol, 30 pmol, 3 pmol, 300 fmol, 30 fmol, or 3 fmol) using 5 μmol (0.1 M) of EDC in a 25 mM MES buffer (pH 6) for 2 h at 4 °C.

To investigate whether the EDC-mediated covalent attachment proceeded through the COOH functionalities, the reaction was also executed on alternative H-terminated IMO NCD and oxidized IMO NCD. H-termination occurred inside the ASTeX reactor at 700 °C for 60 s at 3000 W, 35 Torr, and 1000 sccm H_2. Oxidation was achieved by incubation of the samples in heated H_2SO_4/KNO_3, yielding a surface covered with various oxygen-containing groups.

For the mixed functionality experiments, 300 pmol of Alexa 488-labeled probe ssDNA (8 b) was covalently bound to rho-BeSt samples using 5 μmol (0.1 M) of EDC in a 25 mM MES buffer (pH 6) for 2 h at 4 °C.

For the hybridization experiments, 300 pmol of NH_2-modified unlabeled probe ssDNA (36 b) was attached to rho-BeSt samples with 5 μmol (0.1 M) of EDC in a 25 mM MES buffer (pH 6) for 2 h at 4 °C.

As a negative control, photochemically modified IMO and rho-BeSt surfaces were treated with similar mixtures in which EDC was replaced by an equal volume of 25 mM MES (pH 6). Noncovalently bound DNA was removed from the IMO and rho-BeSt samples either by thoroughly washing with 1× PBS buffer, as described previously,[14] or by washing with 1× PBS buffer followed by 2× SSC buffer containing 0.5% SDS.

2.5. DNA Hybridization. Hybridization of the ssDNA molecules (36 b) attached to the NCD was performed by incubating ssDNA-modified IMO NCD samples for 2 h at different temperatures with 600 pmol of Alexa 488-labeled target ssDNA (29 b), either complementary to the probe ssDNA or containing a 1-base mismatch, in MicroHyb hybridization buffer. After hybridization, the samples were rinsed in 2× SSC buffer containing 0.5% SDS for 30 min at room temperature, followed by two 5 min rinsing steps in 0.2× SSC buffer, once at a few degrees below the hybridization temperature and once at room temperature.

To determine the effect of various surface functionalities on the hybridization efficiency, the ssDNA molecules (36 b) attached to rho-BeSt surfaces were hybridized with 600 pmol of Alexa 488-labeled target ssDNA (29 b), either complementary or noncomplementary to the probe ssDNA, for 2 h at 70 °C. Next, the samples were rinsed in 2× SSC buffer containing 0.5% SDS for 30 min at room temperature, followed by two 5 min rinsing steps in 0.2 × SSC buffer: once at 65 °C and once at room temperature.

During hybridization, the samples were placed in a closed container under a saturated water vapor atmosphere to avoid evaporation of the reaction fluid.

2.6. Confocal Fluorescence Microscopy and Software. The EDC-mediated covalent attachment of the Alexa 488-labeled ssDNA (8 b) and the hybridization efficiency were evaluated by measuring the fluorescence of the sample surfaces in 1× PBS buffer solution with a Zeiss LSM 510 META Axiovert 200 M laser scanning confocal fluorescence microscope (Zeiss, NV/SA, Zaventem, Belgium), using 488 nm argon-ion laser excitation with a maximum intensity at the sample surface of 1.00 ± 0.05 mW. Confocal fluorescence microscopy has the benefit of being able to restrict the volume of observation and to perform local photobleaching, to distinguish between actual fluorescence and mere reflection from the surface. Apart from better refractive index matching resulting in a significantly brighter fluorescence signal, measuring biological layers in buffer solution allows to one study them under physiological conditions. To obtain this, a 1 mm thick glass microscope slide, with a 1 cm wide hole, sealed at the bottom with a thin cover glass was placed on the stage of the inverted microscope. The samples were positioned over this hole, with the functionalized side facing downward. The hole then formed a sealed compartment and was entirely filled with buffer solution, making sure that no air bubbles were present at the sample surface. Spurious 514 nm light passing through the acousto-optic tuning filter (AOTF) was blocked by a 488/10 nm interference filter placed in front of the coupling fiber optic. Confocal optics included a 490 nm dichroic and BP 500−550 nm emission filter.

All images were collected with a 10×/0.3 Plan Neofluar air objective with a working distance of 5.6 mm. Image size was 512 × 512 pixels corresponding with ~900 μm^2, unless stated otherwise. Pinhole size was 150 μm. Pixel dwell time was 25.6 μs. The settings that were varied in different measurements were the laser illumination (2% or 10% of the previously mentioned maximum, to avoid detector saturation between various measurements) and the detector gain (800, 850, 931, or 1200). The latter is a measure for the photomultiplier voltage in arbitrary units. For photobleaching experiments, laser illumination was set to 100% for 3 min. Fluorescence images were processed, and the average fluorescence intensities were retrieved using the ImageJ software version 1.37c (National Institute of Health, Bethesda, MD).

3. Results and Discussion

3.1. Optimization of EDC-Mediated Covalent DNA Attachment. In our previous work, we introduced an efficient, two-step procedure to covalently bind 250 bp dsDNA to NCD. In a first step, the NCD samples were covered with 10-UDA and illuminated with UV (254 nm) for 20 h, yielding a COOH-modified NCD surface. Subsequently, NH_2-modified DNA was attached to these COOH groups through the formation of a peptide bond, using the zero-length cross-linker EDC.[14] However, the DNA binding capacity and the adsorption of nonspecific DNA remains a major concern in biosensor development. Both of these aspects are addressed in this study. The parameters that were evaluated in this respect were the washing buffers used to remove the excess 10-UDA and probe DNA, the length of the probe DNA, the most efficient probe concentration to be immobilized, and the effect of 10-UDA as a functionalization layer on the NCD.

3.1.1. Effect of Washing Buffers. A particular problem encountered in this procedure is a less than optimal removal of excess, noncovalently attached 10-UDA appearing as a vertically stacked multilayer of molecules, held firmly in place by strong hydrogen bonding between COOH dimers.[16] Only the top layer will present available COOH functionalities for the attachment of NH_2-modified ssDNA. However, since many of the 10-UDA molecules in this top layer will not be covalently attached to the underlying NCD surface, they will gradually desorb in the subsequent processing steps, leading to bright fluorescent patches, corresponding to regions of covalently attached ssDNA through bound 10-UDA molecules, alternating with less intense regions where most of the 10-UDA and the associated DNA were lost (Figure 1A and B).

Hot acetic acid was described to efficiently remove COOH-terminated alkyl monolayers from H−Si(111) surfaces by breaking the hydrogen bonds between the COOH dimers.[17] To verify if this could also be transferred to NCD surfaces, four

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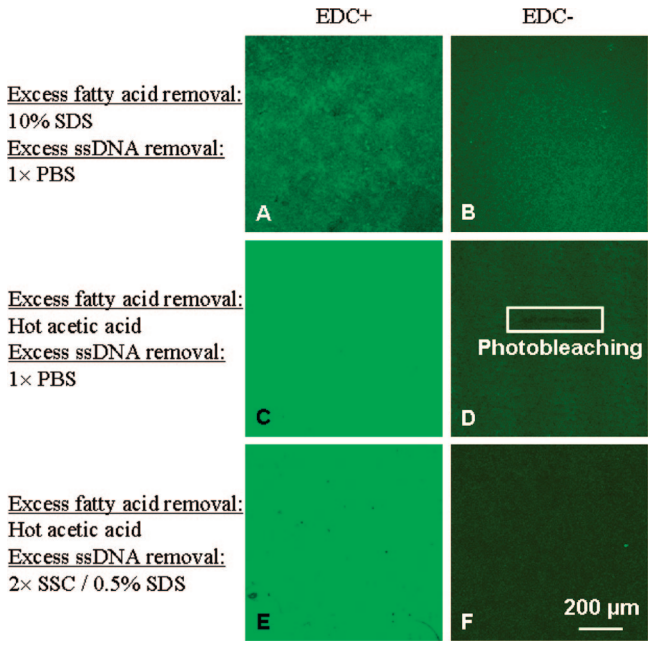

Figure 1. Comparison of fluorescence intensity of IMO NCD samples after treatment with 30 pmol of NH_2-modified and Alexa 488-labeled ssDNA (8 b) using different washing steps. (A) Washed with 10% SDS after photochemical modification and with 1× PBS after covalent attachment. (B) Negative control sample treated under the same conditions as (A) but having omitted EDC in the DNA attachment procedure. (C) Washed with hot acetic acid after photochemical modification and with 1× PBS after covalent attachment. (D) Negative control sample treated under the same conditions as (C) but having omitted EDC in the DNA attachment procedure. A photobleached area, indicating the presence of adsorbed ssDNA, is highlighted. (E) Washed with hot acetic acid after photochemical modification and with 2× SSC/0.5% SDS after covalent attachment. (F) Negative control sample treated under the same conditions as (E) but having omitted EDC in the DNA attachment procedure.

IMO NCD samples were photochemically modified with 10-UDA, of which two were subjected to washing steps in 10% SDS and distilled water. The two remaining samples underwent washing steps in heated acetic acid and distilled water. One sample of each condition was subsequently modified with 30 pmol of NH_2-modified Alexa 488-labeled ssDNA (8 b) using EDC, while the other served as a negative control, in which EDC was omitted from the reaction and replaced with an equal volume of 25 mM MES buffer (pH 6). Removal of excess ssDNA still occurred by thoroughly rinsing in 1× PBS. Figure 1C and D show the remaining fluorescence. The positive control sample (Figure 1C) showed a dramatic increase in fluorescence intensity as compared to Figure 1A. Rinsing with hot acetic acid after photochemical modification thus succeeded in removing most of the noncovalently attached 10-UDA molecules, leaving only the covalently bound monolayer of COOH functionalities that are all available for ssDNA binding.

The dark region highlighted in Figure 1D was obtained by regional photobleaching. When an excitation wavelength hits a flat sample surface, part of it is absorbed by the fluorophores and the biggest part is reflected off the surface. Appropriate filters and beam splitters inside the microscope will allow only the fluorescence emission wavelength to pass to the detector. However, in spite of these filters and beam splitters, a fraction of very intense reflection will still reach the detector. Photobleaching is an efficient technique to distinguish between this residual reflection and real fluorescence due to the presence of a fluorescent label. Hitting the sample surface containing the fluorescent molecules with a high intensity excitation wavelength will photobleach these fluorescent molecules: their chemical structure will become permanently changed, and this structure cannot be fluorescently excited. In the image, the fluorescence in that region will have disappeared because of the bleached molecules. Reflection will not be reduced after this intense illumination: it does not bleach. The obvious destruction of fluorescence in Figure 1D indicated the presence of nonspecifically attached ssDNA.

To overcome this, the same two samples as in Figure 1C and D were subjected to different washing buffers, and a fluorescence image was taken after each rinse. Upon washing with 2× SSC buffer containing 0.5% of the anionic detergent SDS, there was no longer any evidence of nonspecifically bound ssDNA (Figure 1F), while the fluorescence intensity of the positive control remained unaffected (Figure 1E). These conditions yielded reproducibly superior attachment efficiencies. The mean fluorescence intensity value after EDC-mediated ssDNA attachment was 68.0 ± 5.5%, while that of the negative control amounted to only 9.5 ± 1.5% ($N = 3$). In all further experiments described below, the excess 10-UDA and ssDNA were therefore removed by washing with hot acetic acid and 2× SSC/0.5% SDS, respectively. Because the fluorescence intensities measured at gain 1200 now caused detrimental saturation of the detector, preventing certain fluorescence images from being distinguishable from each other in terms of their absolute intensities (Figure 1C and E), subsequent images were collected with the detector gain set at lower, nonsaturating values.

3.1.2. Effect of Probe ssDNA Length. Next, the effect of probe length on the attachment efficiency was evaluated. It has been published by Steel et al. that surface coverage of gold (Au) substrates with DNA is strongly dependent on DNA probe length.[18] For probes shorter than 24 bases, the attachment occurs mainly through the end anchoring group, yielding highly extended ssDNA configurations. For longer probes, the surface coverage begins to decrease notably. This decrease was presumed to be associated with a less ordered arrangement of the ssDNA chains, reflecting a more flexible and polymeric ssDNA configuration. Again, to examine if this effect is also valid under our experimental settings, four rho-BeSt samples were modified with two types of DNA. One sample was functionalized with ~60 pmol of NH_2-modified Alexa 488-labeled dsDNA with a length of 250 bp in an EDC-mediated reaction. A second sample was treated with ~60 pmol of NH_2-modified Alexa 488-labeled ssDNA with a length of 8 b. The two remaining samples served as negative controls, in which the EDC was omitted from the reaction. The confocal fluorescence images of these samples are shown in Figure 2.

It is clear that the density of the short ssDNA probes (Figure 2A) is much higher than that obtained with long dsDNA molecules (Figure 2C). The negative controls in both cases showed no fluorescence (Figure 2B and D). To verify that there was indeed some covalently attached dsDNA present in Figure 2C, the fluorescence was bleached, and the resulting image is shown in Figure 2E. For clarity, the latter image was collected at a higher gain. This indicates that the observations made by Steel et al.[18] are also applicable to NCD-bound DNA. The 8 b ssDNA remains more upright and highly ordered as compared to the 250 bp dsDNA. The latter will obtain the conformation of flexible coils, despite the additional straightening effect associated with a duplex.[19] In this manner, the dsDNA molecules attach themselves to the diamond surface on multiple locations along their length, thereby blocking large regions of adjacent surface space. In all

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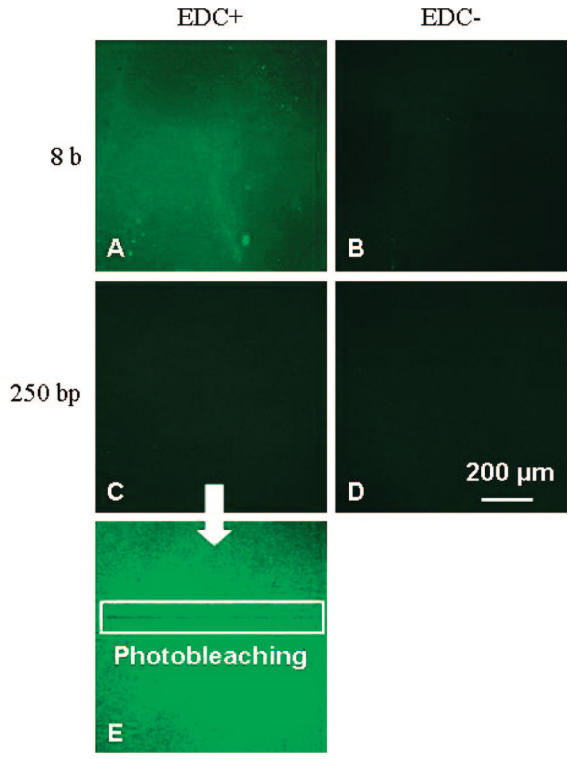

Figure 2. Comparison of fluorescence intensity of rho-BeSt NCD samples after covalent attachment of DNA molecules of different lengths. (A) Covalent attachment of ~60 pmol of NH_2-modified and Alexa 488-labeled ssDNA (8 b). (B) Negative control sample treated with ~60 pmol of NH_2-modified and Alexa 488-labeled ssDNA (8 b) but having omitted EDC in the DNA attachment procedure. (C) Covalent attachment of ~60 pmol of NH_2-modified and Alexa 488-labeled dsDNA (250 bp). (D) Negative control sample treated with ~60 pmol of NH_2-modified and Alexa 488-labeled dsDNA (250 bp) but having omitted EDC in the DNA attachment procedure. (E) The same image as in (C) but with a photobleached area. In (A−D), laser illumination = 10% and detector gain = 800. In (E), laser illumination = 10% and detector gain = 1200.

following experiments, short ssDNA probes of 8 and 36 b were used for the EDC-mediated covalent attachment.

3.1.3. Effect of Probe ssDNA Concentration. In order to determine the optimal ssDNA probe density on the surface of the NCD, eight rho-BeSt samples were modified with eight varying amounts of NH_2-modified Alexa 488-labeled ssDNA (8 b) (3 nmol, 300 pmol, 30 pmol, 3 pmol, 300 fmol, 30 fmol, and 3 fmol) using EDC. Figure 3 shows the resulting fluorescence images.

The fluorescence intensity becomes weakly visible at 300 fmol and rises until a plateau is reached for the sample treated with 300 pmol, which translates into 10^{14} molecules. The plateau is not due to saturation of the detector, since a lower gain gave the same results (data not shown). This amount yields the highest ssDNA probe density on a NCD sample of ~1 cm^2. Increasing the applied amount of DNA further has no effect on fluorescence intensity, which confirms the saturation of the NCD surface. Below 30 pmol, the homogeneity of the DNA layer becomes compromised, resulting in brightly fluorescing DNA clusters on a darker background. It should be noted that the EDC-mediated reaction does not bind 100% of the probe DNA that is offered to the NCD surface. It has been shown that the DNA is not perpendicular to the surface but present under an angle of ~45° to the surface normal.[20] Other groups report similar angles of 30°−36° for dsDNA of 16 bp in length covalently bound to single-crystalline diamond (SCD).[21−23] This will decrease the binding density slightly, likely resulting in the value most cited in the literature, 10^{12} molecules·cm^{-2}.

To evaluate the dependency of the EDC-mediated attachment procedure of DNA on the origin of the NCD sample, 3 nmol and 300 pmol of NH_2-modified Alexa 488-labeled probe ssDNA were also covalently bound to IMO NCD samples. Figure 4 shows that the procedure yields comparable results when applied to a NCD substrate from a different source: Figure 4A shows a similar fluorescence intensity as Figure 3A, and Figure 4B is comparable to Figure 3B. In all subsequent experiments, probe amounts of 300 pmol DNA were used, and IMO and rho-BeSt samples were employed interchangeably.

3.1.4. Effect of 10-Undecenoic Acid (10-UDA). To examine if the attachment method was truly proceeding through the expected formation of a peptide bond between the NH_2 group of a ssDNA molecule and the COOH group of a 10-UDA molecule on the surface, the fluorescence intensities of H-terminated, oxidized, and COOH-terminated diamond samples were compared after EDC-mediated reactions with those of 300 pmol of NH_2-modified Alexa 488-labeled ssDNA (8 b). As previously mentioned, H-termination of the samples was obtained inside the ASTeX reactor at 700 °C during 60 s at 3000 W, 35 Torr and, 1000 sccm H_2. Oxidation of the samples was achieved by immersing the samples in a heated H_2SO_4/KNO_3 solution, resulting in a surface covered with various oxygen-containing groups. These are mostly −OH and =O and, to a lesser extent, −O− and −COOH.[24] These oxidized NCD samples are hereafter referred to as O-terminated. For each condition, a negative control was included, in which the EDC was omitted.

Figure 5 shows the fluorescence images of H-, COOH-, and O-terminated surfaces to investigate their suitability for functional ssDNA attachment. It is clearly visible that H-terminated diamond samples are resistant to EDC-mediated ssDNA attachment (Figure 5A and B). O-terminated samples, however, show a significant fluorescence intensity after EDC-mediated ssDNA attachment (Figure 5F and G). Nevertheless, when an analogous O-terminated sample was covalently modified with 300 pmol of unlabeled ssDNA (36 b) and subsequently hybridized with 600 pmol of Alexa 488-labeled complementary target ssDNA (29 b) under conditions that will be described in section 3.3, the ssDNA molecules attached during the EDC reaction appear to be nonfunctional (Figure 5H). In contrast, hybridization of the COOH-modified sample was successful (Figure 5E).

These results can be explained as follows. First of all, the preferred substrate for EDC is COOH, with the highest available amount being situated on the photochemically modified samples. However, DNA molecules also contain internal NH_2 groups along their length. Hence, to some extent, each EDC reaction will also cause cross-linking of DNA into a network of intertwined strands. As previously mentioned, oxidation of the NCD samples with hot H_2SO_4/KNO_3 will also generate a small amount of COOH

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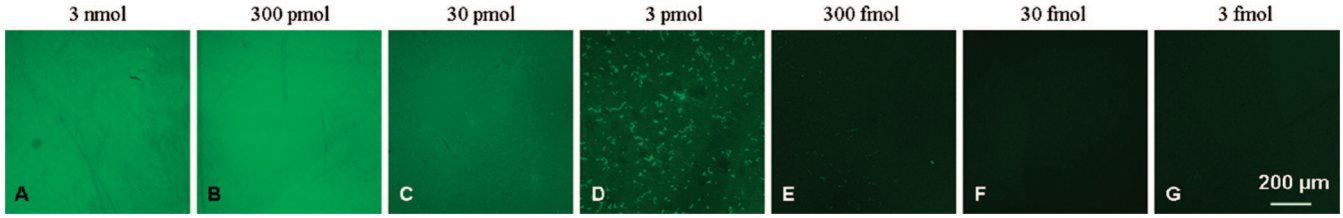

Figure 3. Comparison of fluorescence intensity between rho-BeSt NCD samples treated with varying amounts of NH_2-modified Alexa 488-labeled probe ssDNA. (A) 3 nmol, (B) 300 pmol, (C) 30 pmol, (D) 3 pmol, (E) 300 fmol, (F) 30 fmol, and (G) 3 fmol. Laser illumination = 10%, and detector gain = 931 (see Figure S.1, Supporting Information).

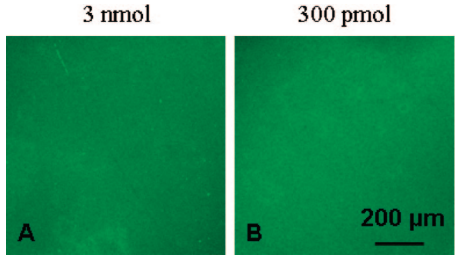

Figure 4. Comparison of fluorescence intensity between IMO NCD samples treated with varying amounts of NH_2-modified Alexa 488-labeled probe ssDNA. (A) 3 nmol and (B) 300 pmol. Laser illumination = 10%, and detector gain = 931.

groups on the surface, which can then serve as anchor points to covalently link the entangled and cross-linked DNA strands. Second, it is known that EDC can also catalyze esterification reactions, for instance, between OH groups on the NCD surface and the phosphate backbone of the DNA. Oxidation of the NCD surface produces a large amount of these OH groups that can lend themselves for these esterification reactions. These two processes cause the DNA molecules to bind to the surface through multiple sites along their lengths. The result is a surface attachment of entangled DNA molecules, making them largely unavailable for hybridization. These side effects of cross-linking and esterification of the DNA strands play a minor role when the amount of surface-associated COOH groups is significantly higher, as is the case with the 10-UDA-modified NCD samples, and when the DNA is modified with a terminal NH_2 group. The ssDNA molecules will be covalently attached to these COOH groups through their 5′ NH_2-terminus, taking preference over their internal functionalities. The H-terminated diamond surface contains no COOH- or other oxygen-containing groups. Even though the cross-linking between the DNA strands probably still occurs, these clusters find no anchor points on the sample surface. Hence, they will not be covalently attached to the surface and can be washed away easily.

It is noteworthy that the fluorescence intensity of the COOH-modified sample appears higher after hybridization than after covalent binding of ssDNA. This is the same effect as was observed by Rant et al., when comparing the fluorescent intensity of Cy3-labeled ssDNA and Cy3-labeled dsDNA. Time-resolved measurements indicated that cyanine dyes exist in two distinct stereoisomers: a *trans*-isomer with a relatively short lifetime and a *cis*-isomer with a longer lifetime. The relative contributions of these stereoisomers differed when comparing ssDNA with dsDNA. Cy3 appeared to adopt the *cis*-configuration with a higher probability when interacting with dsDNA, leading to a higher fluorescence quantum yield associated with this isomer.[25] We believe that the observations made by Rant et al. also apply in this case.

3.1.5. Patterning. Since H-termination appears to be an efficient barrier for EDC-mediated DNA attachment as compared to COOH groups, this phenomenon can be exploited for patterning purposes. Using shadow masks during photochemical attachment of 10-UDA that obstruct the UV light from reaching the surface in some areas but allow the passage of UV light in other regions, the location of the probe ssDNA can be easily controlled. This can prove to be a very useful way to array the sensor surface in the framework of high-throughput biosensor development.

A Cu transmission elecron microscopy (TEM) grid of ~3 mm in diameter and forming 45 μm^2 open squares, separated by 20 μm wide bars, was placed on top of a diamond sample during the photochemical modification. The UV light was only able to reach and covalently attach the 10-UDA molecules through the open squares. The subsequent NH_2-modified Alexa 488-labeled ssDNA (8 b) attachment could therefore only occur at these same regions (Figure 6).

This surface functionality-dependent DNA attachment efficiency is an important observation. The H-terminated regions can suffer from oxidation in several processing steps, leading to an increase in the alternative, internal, attachment of DNA probes to O-terminated regions. However, as described in the previous section, this will not lead to an increased background signal, since these probes are not functionally active.

3.2. Optimization of DNA Hybridization. After the covalent attachment of short ssDNA molecules to COOH-modified NCD, the functional activity of these bound probes needed to be investigated. Different NCD samples covalently modified with 300 pmol of unlabeled probe ssDNA (36 b) were hybridized at different temperatures with 600 pmol of either complementary or 1-mismatch Alexa 488-labeled target ssDNA (29 b) in MicroHyb hybridization buffer. This buffer, containing bovine serum albumin (BSA) and nonspecific DNA for blocking purposes, is used traditionally in general hybridization experiments. After the hybridization reaction, stringency washings at different temperatures were evaluated.

For each condition, Figure 7 shows the relative fluorescence intensity values of the sample hybridized with complementary target ssDNA (filled circles) and the sample hybridized with 1-mismatch target ssDNA (open circles). Each fluorescence intensity value is the average intensity of an image of 512 × 512 pixels, corresponding to a surface of 900 μm^2, and is normalized to the intensity value of a fully saturated image, corresponding to 100% intensity. It can be seen that hybridization at 80 °C followed by two stringency washings in 0.2× SSC at 75 °C and at room temperature, respectively, gave the best contrast between the sample hybridized with complementary target ssDNA and the sample hybridized with 1-mismatch target ssDNA. This could be reliably reproduced. Hybridization with cDNA at 80 °C followed by stringency washing at 75 °C gave a mean fluorescence intensity of 98.2 ± 0.7% (N = 6). Hybridization with 1-mismatch

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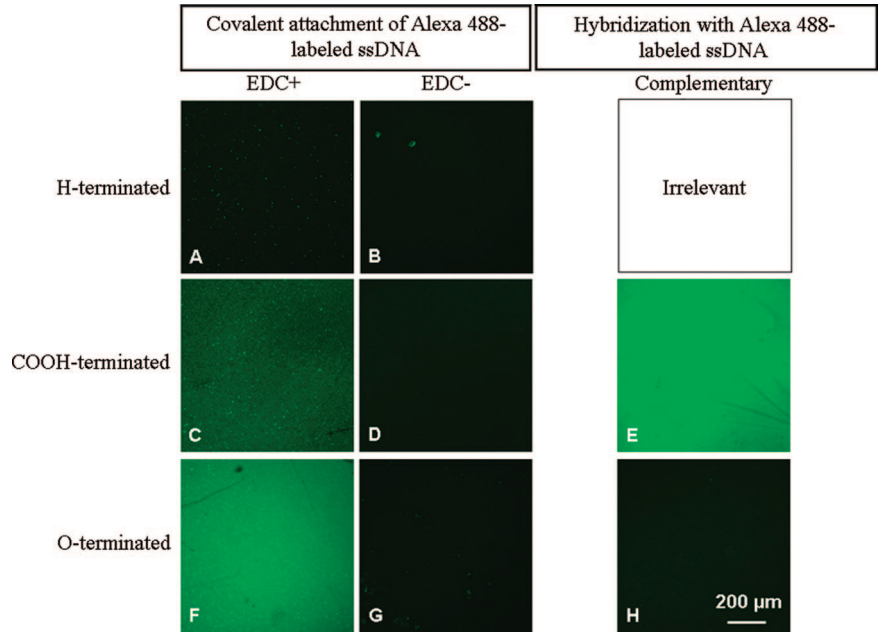

Figure 5. Comparison between differently functionalized diamond samples for their suitability for functional ssDNA attachment. In each condition, 300 pmol of ssDNA was used. (A) H-terminated sample after covalent attachment of NH_2-modified Alexa 488-labeled ssDNA (8 b). (B) Negative control sample treated under the same conditions as (A) but having omitted EDC in the DNA attachment procedure. (C) COOH-terminated sample after covalent attachment of NH_2-modified Alexa 488-labeled ssDNA (8 b). (D) Negative control sample treated under the same conditions as (C) but having omitted EDC in the DNA attachment procedure. (E) COOH-terminated sample after covalent attachment of NH_2-modified ssDNA (36 b) and hybridization with 600 pmol of complementary Alexa 488-labeled target ssDNA (29 b). (F) O-terminated sample after covalent attachment of NH_2-modified and Alexa 488-labeled ssDNA (8 b). (G) Negative control sample treated under the same conditions as (F) but having omitted EDC in the DNA attachment procedure. (H) O-terminated sample after covalent attachment of NH_2-modified ssDNA (36 b) and hybridization with 600 pmol of complementary Alexa 488-labeled target ssDNA (29 b). Laser illumination = 10%, and detector gain = 800.

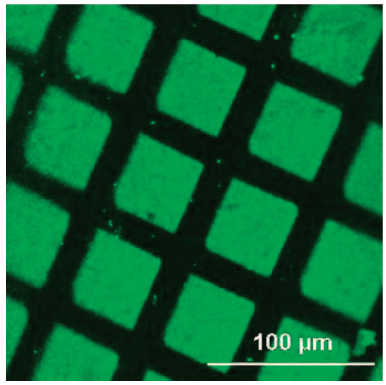

Figure 6. Fluorescence image of a diamond sample with covalently attached NH_2-modified and Alexa 488-labeled ssDNA (8 b) after patterning with a Cu TEM grid (~3 mm in diameter, 45 μm^2 open squares, 20 μm wide bars) during photochemical modification. Laser illumination = 10%, and detector gain = 850.

DNA at 80 °C followed by stringency washing at 75 °C gave a mean fluorescence intensity of 62.4 ± 19.7% (N = 3). The probe ssDNA molecules bound to the NCD surface via EDC thus retain their biological ability to selectively recognize and hybridize their targets.

3.3. Effect of COOH/OH Mixed Surface Functionalities. In the work of Huang et al., it was demonstrated that self-assembled monolayers (SAMs) of thiolated COOH- and OH-modified alkanes, yielding mixed functionalities on Au, enhanced the binding capacity of an antibody against prostate-specific antigen (anti-PSA) and its target recognition capability. Moreover, these mixed monolayers minimized nonspecific binding by distributing the suitable functional groups over the surface while covering the remaining surface with an inert functionality.[26] It was examined whether this phenomenon could be transferred to binding and hybridization of DNA molecules on NCD. This could present a simple and easy method to "dilute" the reactive 10-UDA molecules in order to control the ssDNA probe density on the surface.

The surfaces of 10 NCD samples were photochemically modified with five different ratios of 10-UDA and 9-DO: 100%−0%, 80%−20%, 50%−50%, 20%−80%, and 0%−100%. A total of 300 pmol of NH_2-modified Alexa 488-labeled ssDNA (8 b) was covalently attached via EDC. For each condition, a negative control was included, in which EDC was omitted.

Figure 8 shows the relative fluorescence intensity values of each sample after an EDC reaction in the presence of 300 pmol of NH_2-modified and Alexa 488-labeled ssDNA (8 b), normalized to the intensity value of a fully saturated image. It appears that all of the examined combinations of 10-UDA and 9-DO yield comparable fluorescence intensities to the reproducible 100% COOH-modified surface (mean fluorescence intensity of 68 ± 5.5% for EDC+ and 9.5 ± 1.5% for EDC−, both N = 3). This suggests that DNA attachment via internal esterifications becomes more important with increasing fraction of 9-DO.

This effect can be explored by investigating the functional activity of this bound ssDNA. Ten samples analogous to the ones in Figure 8 were produced but with 300 pmol of unlabeled ssDNA (36 b) instead. Hybridization occurred with 600 pmol of either Alexa 488-labeled complementary or noncomplementary target ssDNA (29 b) for 2 h at 70 °C, followed by two stringency washings in 0.2× SSC: one at 65 °C and one at room temperature. The greater degree of mismatch for the noncomplementary target ssDNA as compared to the 1-mismatch target ssDNA used in

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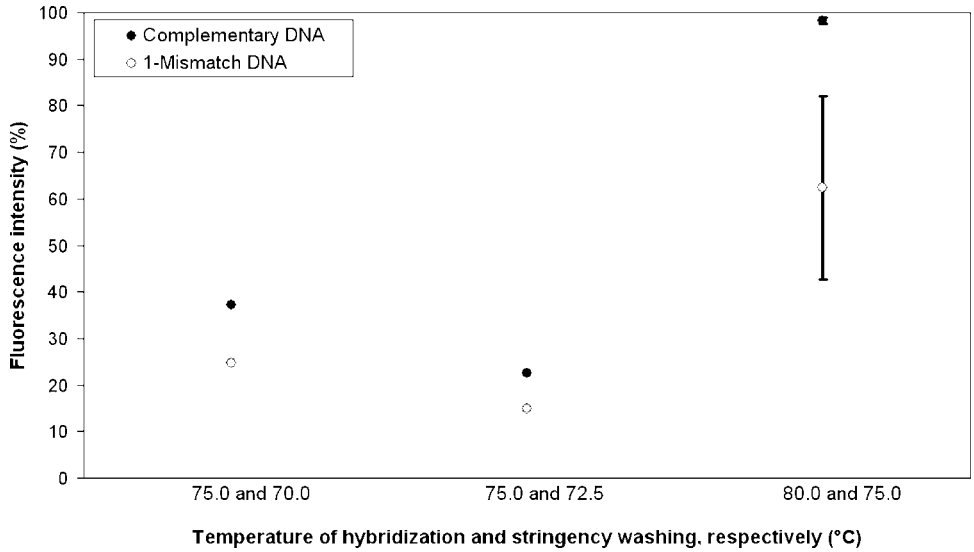

Figure 7. Relative fluorescence intensity values of ssDNA (36 b)-modified IMO NCD samples after hybridization with complementary (filled circles) and 1-mismatch (open circles) target ssDNA (29 b) at different hybridization and stringency washing temperatures. Laser illumination = 10%, and detector gain = 800.

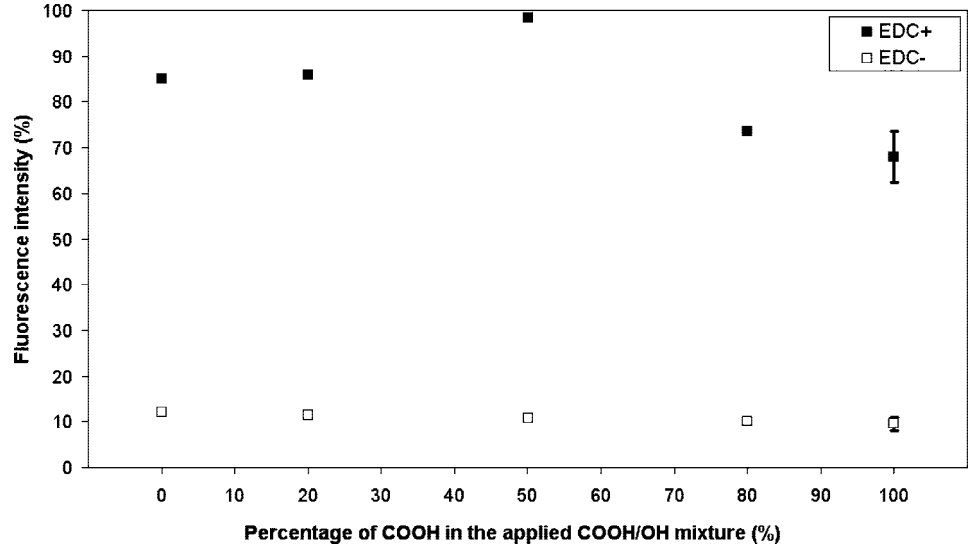

Figure 8. Relative fluorescence intensity values of NCD samples with different ratios of mixed COOH and OH functionalities after covalent attachment of 300 pmol of NH_2-modified and Alexa 488-labeled ssDNA (8 b) (filled squares) and their corresponding negative controls (open squares). Laser illumination = 10%, and detector gain = 931.

section 3.2 allowed the use of less stringent hybridization conditions in this experiment.

Figure 9 shows the relative fluorescence intensity values of each sample after hybridization. On the 0% 10-UDA sample, no functional ssDNA probes were present, in spite of the rather high fluorescence intensity after direct covalent attachment of Alexa 488-labeled ssDNA (Figure 8). This can be explained in the same way as for the O-terminated sample (Figure 5F−H). The ssDNA molecules become anchored at multiple locations along their lengths to the OH surface groups through esterification reactions by EDC, limiting their availability for hybridization in comparison to the ssDNA probes that are attached to the surface through their 5′ NH_2-terminus.

The NCD samples carrying the mixed functionalities did show some hybridization due to the presence of few functional probe ssDNA molecules attached to the dispersed COOH functionalities in the mixed layer. However, an explanation for the low variability in hybridization efficiency in all of these mixed samples as compared to the 100% COOH-modified sample could be that the ratio of 10-UDA/9-DO in the treatment mixture does not reflect the final ratio of COOH/OH on the NCD surface.[27] Because of the shorter length, 9-DO could be preferentially attached through photochemical modification, leading to less distinct surface compositions than expected. Ultimately, the highest hybridization efficiency was still obtained with samples modified with 100% COOH groups. This was again a reproducible result, with a mean fluorescence intensity for the complementary hybridization of 64.1 ± 7.3% ($N = 3$) and a mean fluorescence intensity for the noncomplementary hybridization of 10.7 ± 1.5% ($N = 3$).

Because of the obvious reactivity of EDC toward the surface-bound OH moieties of 9-DO, controlling the ssDNA probe density with this alcohol is inadequate. Attempts were made to repeat this experiment using mixtures of 10-UDA and 1-undecene (1-UD) containing a simple methyl (CH_3) functionality. However,

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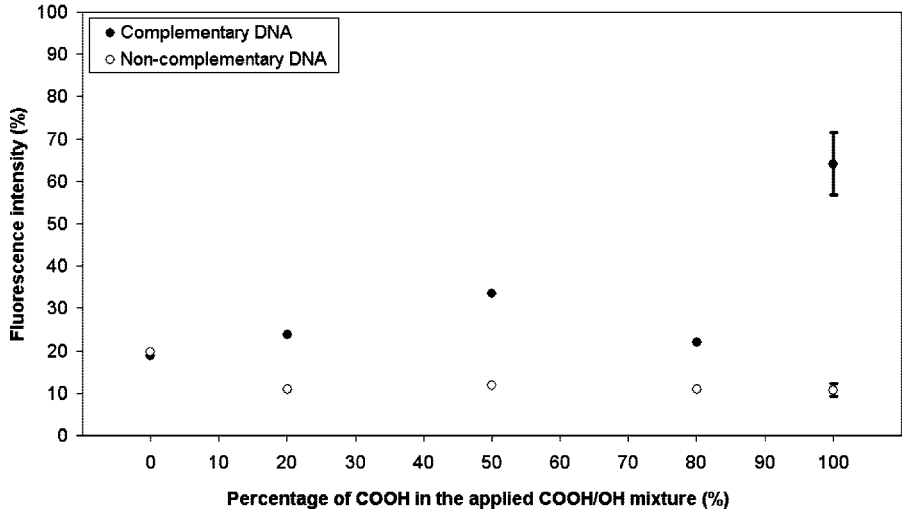

Figure 9. Relative fluorescence intensity values of NCD samples with different ratios of mixed COOH and OH functionalities after covalent attachment of 300 pmol of NH_2-modified ssDNA (36 b) and hybridization with 600 pmol of Alexa 488-labeled complementary (filled circles) and noncomplementary (open circles) target ssDNA (29 b). Laser illumination = 10%, and detector gain = 800.

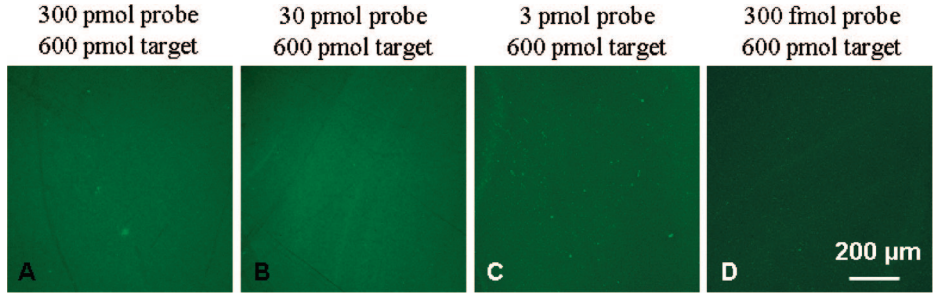

Figure 10. Comparison of hybridization efficiencies on NCD samples modified with different amounts of NH_2-modified unlabeled probe ssDNA. (A) 300 pmol, (B) 30 pmol, (C) 3 pmol, and (D) 300 fmol. Hybridization occurred with 600 pmol of Alexa 488-labeled complementary target ssDNA. Laser illumination = 10%, and detector gain = 931.

the latter substance led to difficulties with the UV illumination procedure due to its volatile nature. The 1-UD evaporated and adsorbed onto the lamp, reducing the UV output.

A much simpler way to control the surface probe density was achieved by varying the probe concentration, as shown in Figure 3. To investigate if a decrease in surface probe ssDNA improved the hybridization efficiency, four samples were covalently modified with 300 pmol, 30 pmol, 3 pmol, and 300 fmol of NH_2-modified and unlabeled ssDNA (36 b). Next, these samples were hybridized with 600 pmol of Alexa 488-labeled complementary target ssDNA (29 b) under the conditions as described above. The results are shown in Figure 10.

The hybridization efficiency increases from 300 fmol, 3 pmol, and 30 pmol probe ssDNA, but no strong additional improvement could be detected with 300 pmol probe ssDNA. Given that the area of a ssDNA molecule is $\sim$1 nm^2, the 10^{14} molecules (300 pmol) that were presented to the NCD surface during the EDC reaction yield a geometrical upper limit for the probe density on a 1 cm^2 sample. This would leave no room for hybridization. However, since the hybridization efficiency for this condition is significant, the final amount of probe ssDNA that was attached to the surface is lower than the amount present in the reaction mixture. Decreasing the probe DNA concentration in the reaction solution by a factor of 10 (30 pmol or 10^{13} molecules), which resulted in a clear reduction of the eventual probe density on the surface (see Figure 3), gave a comparable hybridization efficiency, indicating that the same amount of probe molecules were available for hybridization. The hybridization efficiency for even lower probe densities was significantly reduced. We therefore postulate that the highest hybridization efficiency would be obtained with a presented probe content of $10^{14}-10^{13}$ molecules. An efficiency lower than this optimal value will be achieved with more or less probe molecules, either because of the slight steric hindrance of a too tightly packed probe layer or because of a less than optimal probe density, respectively. However, the hybridization efficiency obtained with a DNA probe content in the $10^{13}-10^{14}$/cm^2 range was quite acceptable. These presented amounts will likely yield an eventual probe density on the surface close to the density value most often cited in the literature: 10^{12}/cm^2.

4. Conclusions

In this work, we evaluated the two-step EDC-mediated DNA attachment method that was introduced in a previous report in terms of stability, density, and functionality of the resulting DNA probe layer. In the first step, 10-UDA is covalently bound to the NCD through a photochemical reaction with UV (254 nm), resulting in COOH-functionalized NCD. The excess 10-UDA was most efficiently removed with hot acetic acid. In the second step, NH_2-modified DNA is attached to these COOH groups through the formation of a peptide bond. Efficient removal of nonspecifically attached DNA was accomplished with 2× SSC/0.5% SDS. Short, highly organized ssDNA was revealed to be more suitable for a maximal binding capacity to NCD as compared to long, dsDNA, which indicated that the results obtained by Steel et al. could be transferred from the Au to the NCD setting.[18] The highest ssDNA probe density was obtained with an applied

amount of ~300 pmol·cm^{-2}, correlating to a maximum of 10^{14} molecules·cm^{-2}. The thus obtained surface density was still suitable for efficient hybridization, and a clear distinction could be made between fully complementary and 1-mismatch target DNA. It was also determined that purely COOH-terminated NCD was the most suitable substrate for the functional end-mediated attachment of NH_2-modified DNA. The DNA that was covalently attached to O-terminated NCD samples and samples carrying mixed functionalities was nonfunctional. The EDC-mediated DNA attachment to H-terminated NCD samples was not possible. This result can be turned into a major advantage in straightforward photopatterning reactions. Hence, in this study, we succeeded in generating a stable, dense, functionally active and patterned biological interface on NCD. The influence of density-dependent self-quenching is unlikely, since the fluorescence intensity after direct attachment of 300 pmol ssDNA onto O-terminated NCD was higher than the intensity after attachment of 300 pmol of ssDNA onto COOH-terminated NCD (Figure 5). If self-quenching was an issue with high DNA densities, this phenomenon would certainly be obvious with O-terminated samples, since the binding of DNA to these surfaces causes a much higher amount of DNA molecules to become attached through additional cross-linking and esterification than binding to COOH-terminated NCD. The covalent attachment of DNA to CVD diamond surfaces creates a very stable bond, as was shown clearly by Yang et al. in 2002 and by Christiaens et al. in 2006,[3,14] allowing reusability through denaturation and rehybridization, thus influencing cost. Moreover, these hybridization events on top of DNA-modified diamond films can be electronically monitored in real time, reaching a sensitivity high enough to detect point mutations or single nucleotide polymorphisms (SNPs).[28] The combination of this stable DNA−diamond platform with photopatterning can thus be applied in the fabrication of high-throughput array-based electronic DNA biosensors.

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Supporting Information Available: Relative fluorescence intensity values of rho-BeSt NCD samples treated with varying amounts of NH_2-modified Alexa 488-labeled probe ssDNA. A numerical conversion of the fluorescence images in Figure 3. This material is available free of charge via the Internet at http://pubs.acs.org.

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