Characterization of DNA-Functionalized Surfaces by Fluorescence Microscopy

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Abstract

In the field of clinical diagnostics, biosensors are used to identify genetic disorders by hybridizing a surface-bound DNA sequence with the patient's DNA. To further improve biosensor design, two nonlinear optical techniques, sum frequency generation (SFG) and second harmonic generation (SHG), were used to probe the orientation, chirality, and surface charge density of the surface-bound DNA strands. In this system, short DNA oligonucleotides were attached to a N-hydroxysuccinimide-ester (NHS) functionalized fused-quartz surface via an amide bond. However, the reaction steps involved in preparing these surfaces have not been fully optimized for the SHG and SFG experiments. In this current study, fluorescence confocal microscopy was used to image fluorescently labeled single-strand DNA (ssDNA) and its labeled complementary sequence. The optimum time duration required for hybridization was found to be 2 hr. Using contact angle, the minimum NHS linker concentration required for high surface coverage was found to be 1 mg/mL NHS linker in toluene, and an optimal rinsing method to minimize aggregates and remove unreacted silane was also determined. These optimizations of the experimental procedure will help to conserve valuable time and materials during the DNA surface-functionalization process to prepare substrates for SHG and SFG experiments.

Introduction

Medical applications of biodiagnostics have become increasingly important in recent years.[1] One simple and economical application used in clinical diagnostics is a biosensor.[2,3] Early disease recognition and proper treatment help prolong a patient's life. Biosensors as small as a dime are already being used to identify genetic disorders like Alzheimer's,[4,5] anthrax,[6] some cancers,[7] and HIV.[8] Some of these biosensors detect genetic diseases through the interaction of a patient's DNA with a corresponding DNA sequence immobilized at the biosensor's surface.[9] To further improve the diagnostic biosensor, it is necessary to have an excellent understanding of the chemistry of the DNA attached to the biosensor surface.

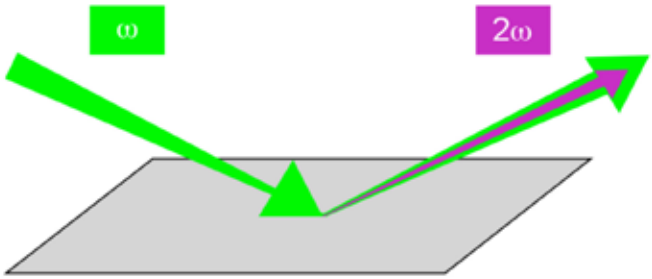


Figure 1. Second harmonic generation description. SHG is a nonlinear optical technique used to characterize surface-bound DNA. Photons of a certain wavelength are directed at a nonlinear material, such as a silica/water interface, at which point their frequencies are doubled.

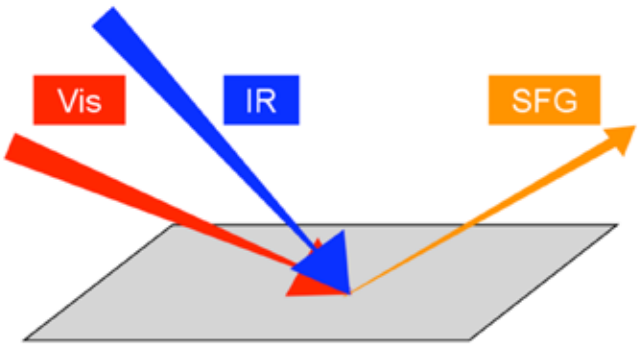


Figure 2. Sum frequency generation description. SFG is a nonlinear optical technique used to characterize DNA. Visible and IR beams are directed at the surface-bound DNA to produce a beam at the sum of their frequencies that contains vibrational information about the identity and orientation of the molecules at the surface.

To better understand the unique properties of DNA at surfaces, many researchers have studied various aspects of surface-bound DNA such as surface coverage,[3,10,11] chemical stability,[11] hybridization,[3,11–13] immobilization,[14] and recognition of base mismatch.[13,15] Methods to study DNA at surfaces include surface plasmon resonance spectroscopy (SPR), radioactive labeling, 3 X-ray photoelectron spectroscopy (XPS),[3,10,14,16] and atomic force microscopy (AFM).[16] Recently, two noninvasive, label-free nonlinear optical techniques — second harmonic generation (SHG) and sum frequency generation (SFG) — were applied to investigate short strands of DNA attached to a silica surface by an organosilane linker. The wealth of information from these methods helps engineers to characterize DNA at the biosensor surface in order to improve biosensor technology.[17,19]

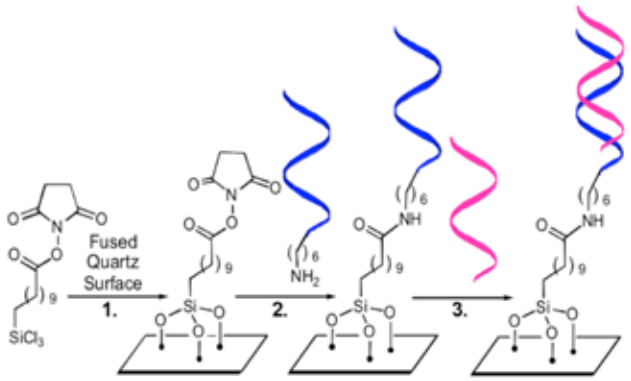


Figure 3. Silica functionalization. The -$SiCl_3$ functional group of the organosilane NHS linker reacted with the hydroxyl groups of the silica to form covalent bonds. The succinimide of the NHS linker was replaced by an amine-terminated ssDNA (T_{25}), forming an amide bond. The complementary strand (A_{25}) hybridized to the ssDNA, forming a dsDNA. Figure taken from Boman et al.[17]

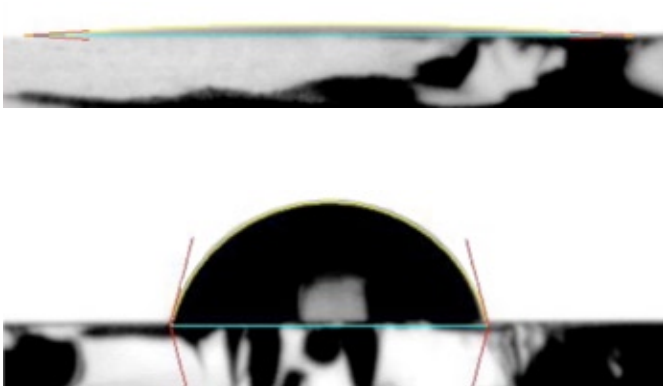


Figure 4. Contact-angle measurements of a silica surface and a NHS-functionalized silica surface. Two images taken of a water droplet of the same sessile volume (~2 µL) on two different surfaces using a contact-angle goniometer. The first surface is the hydrophilic glass slide. The contact angle measured for this surface was 3.9°. The second surface, the NHS linker surface, exhibited a high contact angle of 75°.

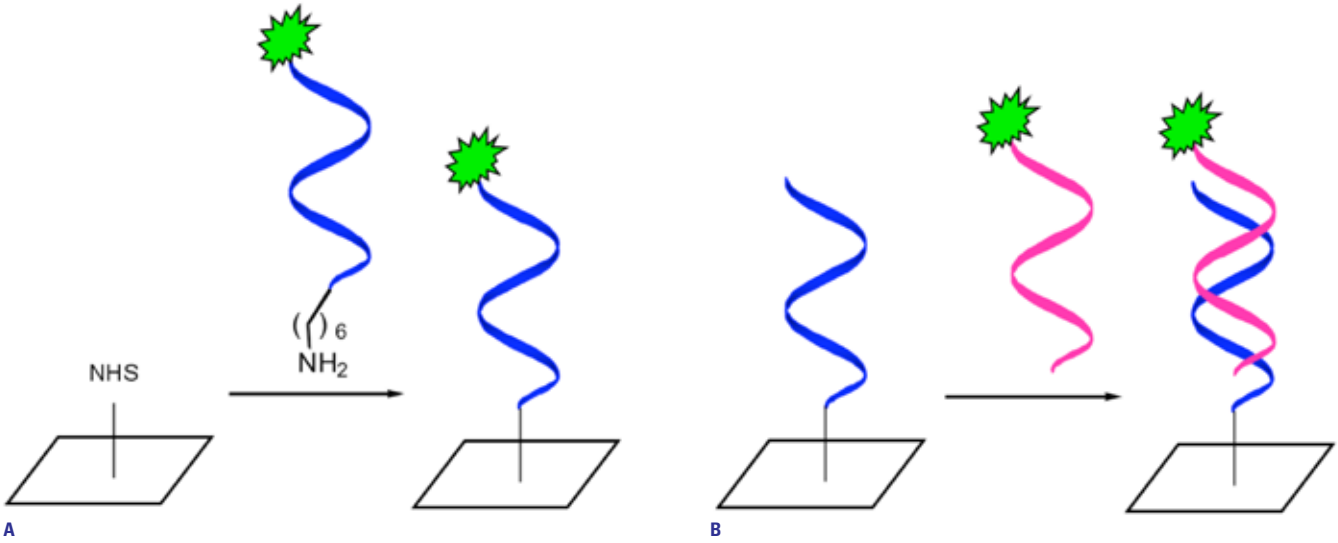


Figure 5. Fluorescent tagging of both the surface-bound ssDNA and its complement. (A) Fluorescein-tagged amine-terminated T_{25} ssDNA reacted with the N-hydroxysuccinimide of the linker to form an amide bond. (B) A_{25} fluorescein-tagged ssDNA hybridized with the amine-terminated T_{25} ssDNA. These fluorescent surface-bound DNA surfaces were imaged with confocal microscopy to observe surface coverage of the ssDNA and dsDNA, as well as hybridization times.

In SHG, photons are directed onto a nonlinear material, such as a surface, producing a photon with double the incident frequency (Figure 1).[20] These experiments probe the electronic resonance, surface charge density, and chirality of surface-bound DNA strands.[17] In SFG, visible and IR beams are directed to a surface, and a signal is produced at the sum of their frequencies (Figure 2).[21,22] These experiments illuminate the local chirality of the DNA bases, the supramolecular chirality of the helix, and the orientation of the DNA strands through the vibrational modes of the carbon-hydrogen stretches of the DNA.[18] These two techniques are called "nonlinear" due to the mathematical relationship between the frequencies of the incident and the generated photon.[23]

The surfaces used in the SFG and SHG experiments modeled a simple diagnostic biosensor and involved three steps to attach DNA to a surface.[17,18] The first step in the surface preparation was covalent attachment of a hydrocarbon linker molecule to a fused quartz surface.[24] The second step was the coupling of an amine-terminated DNA strand to the linker surface. The third step involved the hybridization of a complementary DNA strand to the single-stranded DNA (ssDNA) on the surface (Figure 3).[17]

This investigation is the first time these functionalized surfaces were imaged, giving further evidence that indeed there is DNA attached to these silica surfaces. The goal of these experiments was to make the functionalization of the quartz lenses a more efficient procedure and produce surfaces with higher, more even coverage. Parameters such as

reaction times and reactant concentrations were not fully optimized. This investigation not only helps others who are studying DNA at surfaces functionalize surfaces more efficiently but also gives insight about surface-bound DNA hybridization. These experiments provide a better understanding of medical biosensors that utilize DNA hybridization.

Background

Two techniques, contact-angle goniometry and fluorescence confocal microscopy, were used in this investigation. The results from these methods complemented SHG and SFG experiments, which use the same surface-functionalization procedures.

Contact-Angle Goniometry

A goniometer measures the contact angle that a droplet of water forms on a surface, thereby determining the hydrophobicity or hydrophilicity of the surface.[25,26] A water droplet brought into contact with a hydrophilic polar surface will spread out and have a low contact angle. However, a water droplet brought into contact with a hydrophobic or nonpolar surface will bead up, resulting in a higher contact angle (Figure 4). This technique has been used to observe the increase in water droplet angle as the surface changes from hydrophilic to hydrophobic and thus to determine whether a monolayer of nonpolar molecules has completely covered a surface.[27] The surface measured in these experiments with the use of contact-angle goniometry consisted of a hydrophobic monolayer of a hydrocarbon chain covalently attached to a hydrophilic glass slide.

Fluorescence Confocal Microscopy

Fluorescence confocal microscopy involves tagging a sample with a fluorescent dye and then exciting the dye with light of a particular wavelength so that the dye emits light of another wavelength for a prolonged period of time.[28] The emission that occurs can then be imaged. A wide variety of fluorescent tags are used for detection. Fluorescein is a commonly exploited dye, and its small size makes it an optimal tag for this investigation.[29,30]

Fluorescence-based techniques have been used for an optimization study where the surface coverage and hybridization efficiency of DNA strands attached to gold films and nanoparticles were determined.[31] The goals of previous investigations were similar to those of the current study, but a different surface and functionalization process were used. Fluorescence confocal microscopy was used to detect tagged DNA strands at very small concentrations, with a lower limit of 0.8 fM, when using a nanoparticle-based sandwich assay.[15] Thus, due to its high resolution capabilities, the confocal microscope allowed for the first true image of tagged single- and double-stranded DNA (ssDNA and dsDNA) attached to silica by an organosilane linker.[28]

Approach

Single-Stranded DNA Synthesis

The DNA needed for the second and third steps of surface functionalization was synthesized in house. A 25-base-long thymine strand (T_{25}) with a 3'-amine terminated end and its complementary 25-base-long adenine strand (A_{25}) with a 3' fluorescent tag were synthesized using Expedite, an instrument that builds ssDNA strands by stringing nucleotides together one base at a time. Due to time constraints, a T_{25} strand with a 5' fluorescein tag and additional A_{25} strands with a 3' fluorescein tag were purchased from Integrated DNA Technologies, Inc. (Coralville, Iowa).

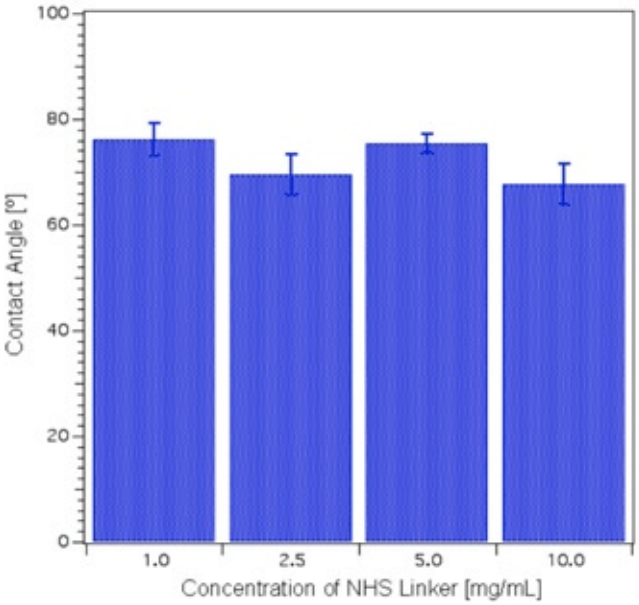


Figure 6. NHS linker concentration minimization study. Contact-angle measurements were taken after the NHS linker reaction at various NHS linker concentrations in toluene.

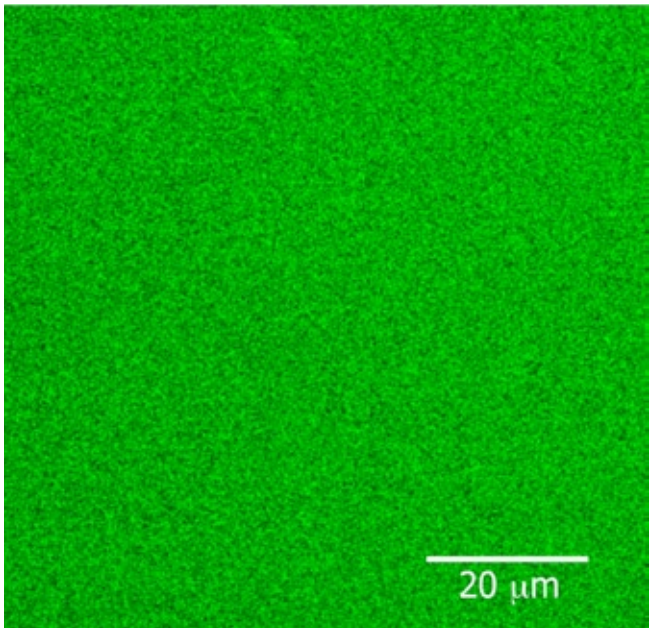


Figure 7. Fluorescently imaged–tagged surface-bound ssDNA strands. A fluorescein-tagged ssDNA surface was rinsed with water to remove nonspecific binding of the ssDNA to other parts of the surface and imaged using the 488 nm argon laser of the fluorescence confocal microscope.

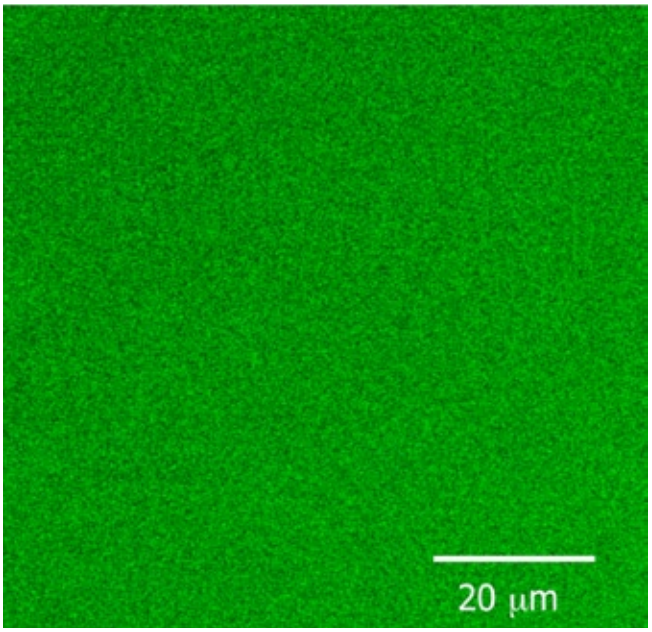


Figure 8. Fluorescently imaged–tagged hybridized complementary DNA strands. A fluorescein-tagged A_{25} strand was hybridized to the surface-bond T_{25} ssDNA surface. It was then rinsed with 0.25 M NaCl to remove any nonspecific binding of the complementary strand to other parts of the surface. The tagged, surface-bound DNA was excited to emit in the green by using the 488 nm argon laser of the fluorescence confocal microscope.

For strands synthesized at Northwestern University, high-pressure liquid chromatography (HPLC) was used to purify correctly synthesized strands from failure strands by identifying a dimethoxytrityl (DMT) group that the Expedite attached only to full-length ssDNA. After the DMT group served its purpose in both the Expedite and HPLC, it was removed with acetic acid, leaving DNA strands with only the desired base pairs and modifications. The DNA was also desalted using a Naphodex desalting column to ensure no reagents of the DNA synthesis process remained in the final sample. By measuring the UV-Vis absorbance of the DNA using UV-Vis and applying the Beer-Lambert law, the DNA concentration was calculated. Typical amounts of DNA synthesized ranged between 10 and 50 nmol.

Glass-Slide Functionalization

Functionalized glass slides were used as a model biosensor surface due to their low cost and consistency with the fused silica hemispheres used in the SHG and SFG experiments. Numerous slides were cut approximately 2 cm × 1 cm, sonicated in methanol for 15 min with Aquasonic Model 75T, and O_2 plasma cleaned in a Harrick plasma cleaner/sterilizer PDC 32G to remove impurities. The first reaction in the DNA attachment procedure (surface functionalization) involved reacting a trichlorosilane linker terminated with an N-hydroxysuccinimide (NHS) ester with the hydroxy groups of the glass surfaces, forming covalent bonds. The glass slides were covered with a solution of 10 mg/mL NHS and toluene for 1 hr in a glove box with an N_2 atmosphere. The glove box prevented the trichlorosilane of the linker from self-

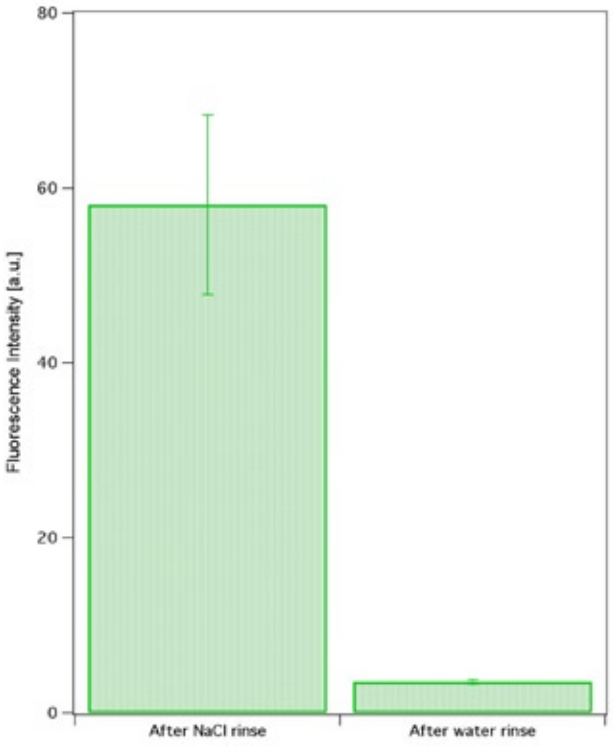


Figure 9. Nonspecific binding study of the hybridized DNA surfaces. The tagged A_{25} strand was rinsed with 0.25 M NaCl, pH 7 to remove any nonspecific binding. The surfaces were rinsed with water, separating the two surface strands and rinsing away any previously hybridized complementary strand. After the water rinse, no fluorescence was detected.

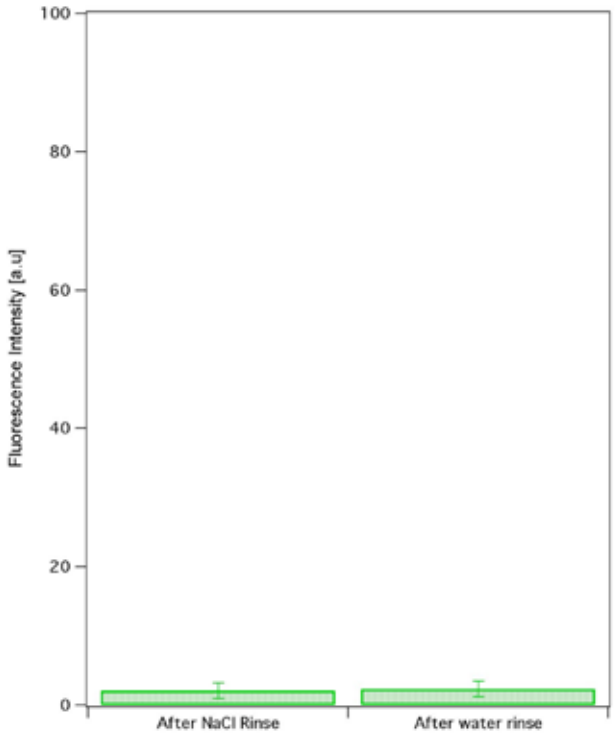
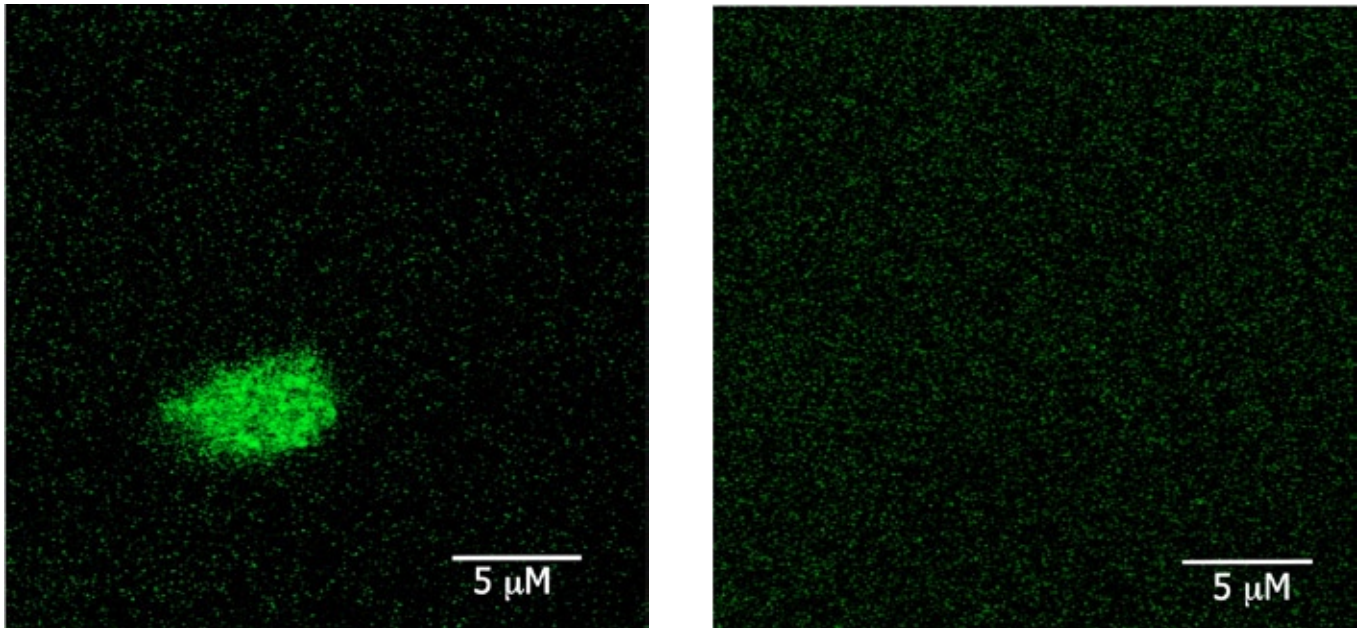


Figure 10. Binding study of the complementary strand to the NHS linker surfaces. The NHS linker surface was exposed to a solution of the tagged A_{25} to test if the complementary strand nonspecifically bound to the linker. No fluorescence was observed in the image after both a 0.25 M NaCl rinse and a water rinse.



A B

Figure 11. Sonication after NHS linker reaction removed any extreme DNA clumping. (A) A fully functionalized DNA surface was imaged without sonication in toluene after the NHS linker reaction. This resulted in large clumps of fluorescently tagged DNA. (B) A fully functionalized DNA surface was imaged with sonication in toluene after the NHS linker reaction, resulting in less clumping on the surface.

polymerizing in the presence of water. Afterwards, a solution of 10 μM ssDNA dissolved in a sodium tetraborate buffer at pH 9 was reacted to form an amide bond between the NHS ester of the linker and the amine of the ssDNA. Finally, the functionalized slides were covered with a solution of 10 μM of the complementary strand dissolved in 0.25 M NaCl solution to hybridize the DNA strands.

Contact-Angle Goniometry Measurements

The NHS linker must be synthesized in lab, a time-consuming process involving multiple synthesis steps. To conserve NHS linker reagent and time, contact-angle goniometry measurements were performed to minimize the concentration of linker needed for full surface coverage of the glass slides. Contact angles were measured for six different concentrations of NHS linker in toluene: 0.0, 1.0, 2.5, 5.0, 7.5, and 10.0 mg/mL. Samples at each concentration were made in duplicate, and the contact angles were found to be fully reproducible. The average contact angle measured for the clean glass slide without NHS linker was only 5°, due to its hydrophilicity. Previous to this experiment, the concentration of linker used for surface functionalization in SFG and SHG experiments was 10 mg/mL.

The DNA-functionalized surfaces exhibited an average contact angle of 68° (Figure 4). Thus, the aim of these experiments was to determine the lowest concentration of NHS linker necessary to generate a functionalized surface, which exhibited average contact angles comparable to surfaces functionalized at higher concentrations. This information will help conserve valuable reagent in future experiments.

Fluorescence Confocal Microscopy

Previously, only SHG and SFG were used to study DNA for these particular functionalized surfaces. It was important to characterize these surfaces using an additional method to demonstrate that DNA was in fact attached to the surface. Thus, fluorescence confocal microscopy was used to image the NHS, tagged and nontagged ssDNA, and dsDNA surfaces. The SHG and SFG experiments relied on a DNA hybridization time of 2–6 hr and were carried out in situ, creating a large and interfering time commitment. Since the laser is stable and well aligned for only a few hours, unnecessarily long hybridization times were undesirable. Fluorescence confocal microscopy was used to determine the shortest possible reaction times needed for the complementary strand to completely bind to the surface-bound ssDNA.

In order to accomplish these three goals through imaging, both DNA strands were tagged with fluorescein. First, the complementary strand of DNA was fluorescently tagged with fluorescein on the 3' end. The 20x objective on the Zeiss fluorescence confocal microscope was used. Its xy resolution is 344 nm. Thus, every hybridized strand of DNA appeared as a bright spot on the image due to the emission of the complementary strand's fluorescent tag (Figure 5). Therefore, the mean brightness of an image of DNA is directly proportional to the amount of hybridized DNA. To compare the extent of hybridization due to a variation in hybridization reaction times, the mean brightness of a 100 μm × 100 μm section of the functionalized surface was measured for a time range of 0–4 hr. Additionally, the ssDNA was tagged with fluorescein to observe a general coverage of ssDNA reacted for 6 hr in buffer (Figure 5).

During this preliminary imaging of fluorescence emission intensity as a function of reaction time, it was observed that glass slides not sonicated in toluene after the linker reaction showed an uneven distribution of DNA. Consequently, the rinsing and sonicating procedure was reevaluated using confocal microscopy. These two different methods of treating the samples were imaged to determine which rinsing procedure resulted in a more even distribution of DNA and a more even distribution of linker as well. These images also added to the understanding of attached DNA dispersion.

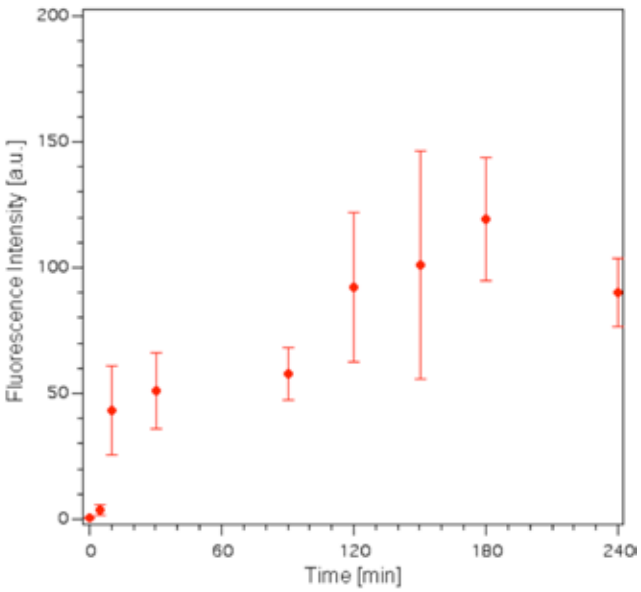


Figure 12. Hybridization reaction time determined through fluorescence confocal microscopy. Ten ssDNA samples were hybridized at various reaction times between 5 min and 4 hr and rinsed in 0.25 M NaCl at pH 7. Each surface was imaged in 10 spots, and a mean fluorescence brightness was calculated. The fluorescence brightness is directly proportional to the amount of dsDNA attached to the surface.

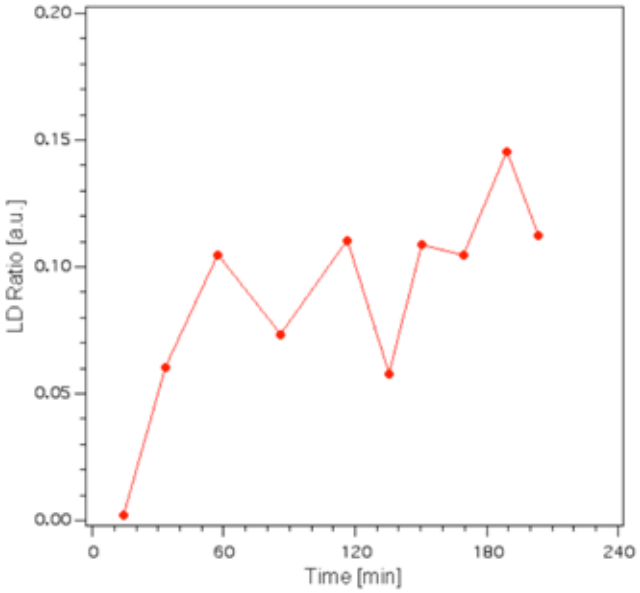


Figure 13. In situ hybridization reaction time determined by second harmonic generation. This is a second harmonic generation plot of linear dichroism ratio vs. hybridization time. On this plot the higher LD ratio means that more chiral-hybridized DNA has formed at the surface.

Results and Discussion

Contact-Angle Analysis

Figure 4 shows a contact angle of 68° (4) with the contact-angle measurements for a linker concentration of 10 mg/mL. The contact angle for glass surfaces reacted with 1 mg/mL linker concentration had a similar mean contact angle of 76° (3) (Figure 6). This result shows that the hydrocarbon linker concentration can be decreased by a factor of 10 and still maintain full surface coverage.

It was also shown that the contact angles remained the same between the first and second use of the same concentration solution of NHS linker on a surface. For the first use of 2.5 mg/ml of NHS linker in toluene, a contact angle was measured to be 69° (4). After the second use of the solution on different samples, the same contact angle of 69° (4) was measured. Even after the solution had reacted once for 1 hr, it was still possible to put this same solution at any of the varied concentrations on another sample and obtain full linker surface coverage.

Fluorescence Confocal Microscopy Imaging Analysis

Fluorescence confocal microscopy was used to image both the tagged ssDNA and the dsDNA surfaces (Figure 7 & 8), as well as to characterize the reaction times of DNA hybridization. First, control studies were conducted to verify that only specific binding of the tagged complementary strand to the surface bound ssDNA was observed. The tagged complementary strand was hybridized to the ssDNA and rinsed with 0.25 M NaCl to remove any nonspecific binding. In order to verify that the complementary strand was reversibly bound and could be removed by melting, the surfaces were rinsed with water. Water caused the negatively charged strands to become unstable and repel each other, separating the two strands. After the water rinse, the fluorescence from the tagged complementary DNA strand did not appear in the image of the surface (Figure 9).

Another experiment involved exposing the NHS linker surface to the complementary strand of DNA for 1 hr to test whether the nonspecifically bound complementary DNA would exhibit a fluorescence signal. This surface was then rinsed with 0.25 M NaCl and no fluorescence was detected, indicating that nonspecifically bound complementary DNA did not remain at the surface. These surfaces were rinsed again with water and also showed no fluorescence (Figure 10).

Two different rinsing techniques for the NHS linker reaction were also studied (Figure 11a and b). After the linker glove box reaction, the samples were rinsed in toluene and annealed in the oven or rinsed in toluene, sonicated in toluene for 5 min, rinsed in toluene again, rinsed in methanol and water, and annealed in the oven. Without sonication, large clumps of DNA appear in the images (Figure 11a). Sonicating the samples allowed for a more even distribution of DNA on the surface, removing any extremely large clumps due to polymerization (Figure 11b).

The complementary strand was tagged and imaged for a variety of hybridization reaction times from 10 different samples ranging from 5 min of hybridization to 4 hr. After 30 min, a significant amount of DNA was hybridized at the surface. The brightness increased gradually with longer reaction times of up to 2 hr, where it leveled off again. Therefore, after 2 hr the amount of DNA hybridized at the surface does not significantly increase (Figure 12). These data are important because hybridization of these functionalized surfaces was done during limited laser experiment time, and the reaction time necessary could then be confidently reduced. These results complement the hybridization efficiency study conducted using a SHG linear dichroism (LD) experiment, which measures the chirality of the duplex (Figure 13). After 2 hr, the LD signal ratio from the DNA was highest. This data will allow for more efficient and accurate organization of SHG and SFG in situ hybridization experiments.

Conclusion

In this investigation, optimizations were made to the sample functionalization process used in the SFG and SHG experiments. The NHS linker concentration was minimized tenfold from 10 mg/mL to 1 mg/mL NHS in toluene, saving time needed to synthesize the linker in lab. The minimal DNA hybridization time was determined to be 2 hr, allowing for easier and more efficient SFG and SHG experiments. Finally, both the ssDNA and dsDNA surfaces were imaged using fluorescence confocal microscopy. These optimizations will help future characterizations of these DNA-functionalized surfaces to be more efficient and accurate. Characterizing DNA with SHG and SFG provides valuable information to chemists and engineers wishing to make improvements on diagnostic biosensors.

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