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Covalent attachment of synthetic DNA to self-assembled monolayer films

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ABSTRACT

The covalent attachment of thiol-modified DNA oligomers to self-assembled monolayer silane films on fused silica and oxidized silicon substrates is described. A heterobifunctional crosslinking molecule bearing both thiol- and amino-reactive moieties was used to tether a DNA oligomer (modified at its terminus with a thiol group) to an aminosilane film formed on silica surfaces. A variety of aminosilanes, crosslinkers and treatment conditions have been tested to identify optimal conditions for DNA immobilization using this approach. The DNA films which result have been characterized using UV spectroscopy, water contact angle measurement, radiolabeling and hybridization methods.

INTRODUCTION

In recent years, standard molecular biology tools such as DNA hybridization have begun the transition to the development of devices such as DNA array-based sensors. DNA arrays have found applications in genetic mutational analysis (1,2) and sequencing by hybridization of unknown DNA segments (3,4). The key to these advances was the development of chemistry for the spatially resolved attachment or synthesis of DNAs on durable surfaces. Another field in which immobilization chemistry has increased in importance is proximal probe microscopy, where strong binding to the surface and accessibility of the molecule to a ligand or complementary molecule is essential (5,6). Numerous methods for the attachment (1,2,5–9) or direct synthesis (4,10,11) of DNA on surfaces have been described. For most applications, immobilization of a readily detectable quantity of functional DNA, film stability and fidelity of the immobilized sequence(s) are the key to developing useful DNA-based test devices. The ability to create high resolution DNA features concurrent with or subsequent to deposition is an additional feature required for certain applications (1,7,9,10,12).

Earlier we described the use of aminosilane films to non-covalently anchor DNA oligomers to surfaces (9) and demonstrated that DNA attached in this manner retained its ability to hybridize to a complementary strand. An additional benefit derived from the use of silane films for DNA attachment is that they can be photolithographically patterned using various approaches; this feature has been exploited to fabricate high resolution patterned DNA surfaces (1,9,12). However, DNA films which were formed using the non-covalent attachment method were susceptible to removal from the surface under high salt conditions (9). For applications where high ionic strength conditions are desirable, such as under physiological conditions, it is generally believed that a covalent attachment strategy will prove superior to one in which the nucleic acid is chemisorbed to the surface. We have thus developed a method for covalent attachment of thiol-modified DNA oligomers to self-assembled aminosilane monolayer films, via the use of heterobifunctional crosslinkers. The crosslinkers employed possess groups reactive toward NH_2 (such as *N*-hydroxysuccinimidyl esters) and -SH (such as maleimide or α-haloacetyl) moieties, for attachment to the aminosilane film and thiol-DNA oligomers respectively. The DNA immobilization process we have developed has several components: silanization, crosslinker attachment, reaction with thiol-DNA and removal of non-covalently bound DNA. Our attempts to optimize this process have focused on the selection of the silane, choice of crosslinker (and solvent) and thiolated DNA treatment conditions. The resulting DNA films have been evaluated using UV and contact angle methods and surface density and hybridization performance of the DNA films were studied using radiolabeled oligomers.

MATERIALS AND METHODS

Preparation and silanization of substrates

Substrates used were 1″ × 1″ fused silica slides (Dell Optics, Fairhaven, NJ) or *n*-type Si (100) wafers (Wafernet, San Jose, CA). For direct measurement of DNA on slides using UV spectroscopy, Suprasil grade fused silica is required (significant problems resulted when lesser grades of fused silica were employed due to the presence of UV absorbing impurities). All references in the text to H_2O refer to water obtained from a Nanopure™ purification system, >18 MΩ cm and 0.22 μm filtered. Substrates were cleaned by immersion in 1:1 concentrated HCl:MeOH for 30 min, rinsed in deionized H_2O, immersed in concentrated H_2SO_4 for 30 min and rinsed in H_2O before boiling in deionized H_2O for several minutes prior to silanization.

Silanization of substrates was performed using 1% solutions of distilled trimethoxysilylpropyldiethylenetriamine [DETA; United Chemical Technologies (UCT), Piscataway, NJ, or Gelest Inc., Tullytown, PA] or *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane (EDA; UCT) in 1 mM acetic acid in deionized (dI) H_2O for 20 min at room temperature. EDA and DETA slides were

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rinsed three times with dI H_2O, dried under N_2 and baked at 120°C for 3–4 min on a hotplate. In addition, PEDA [*m,p*-(aminoethyl-aminomethyl)phenethyltrimethoxysilane; Gelest] was used to treat substrates essentially as described (13). Briefly, a 1% solution of PEDA in 95:5 MeOH:1 mM aqueous acetic acid was used to treat acid-cleaned slides for 20 min at room temperature. The slides were then rinsed in MeOH, dried under N_2 and then baked at 120°C for 3–4 min. Optimum results were obtained when silanized substrates were treated promptly with the heterobifunctional crosslinker solution.

Modification of silanized substrates with heterobifunctional crosslinkers

The heterobifunctional crosslinkers succinimidyl 4-[malemidophenyl]butyrate (SMPB), *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS), succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), *N*-(γ-maleimidobutryloxy) succinimide ester (GMBS), *m*-maleimidopropionic acid-*N*-hydroxysuccinimide ester (MPS) and *N*-succinimidyl(4-iodoacetyl) aminobenzoate (SIAB) were obtained from Pierce (Rockford, IL) or Sigma Chemical Co. (St Louis, MO) and prepared as 1 mM solutions. Crosslinkers were dissolved in 100 µl DMSO and diluted to 30 ml in DMF, DMSO, 80:20 EtOH:DMSO or 80:20 MeOH:DMSO. Silanized substrates were immersed in the crosslinker solution for 2 h at room temperature, then rinsed with the solvent used for dilution and dried under N_2. At this point, the maleimide portion of the crosslinker is available for reaction with thiolated DNA or other thiols; prompt treatment with thiolated DNA is essential for best results.

DNA synthesis and purification

Trityl-on DNA oligonucleotides were synthesized using an Applied Biosystems Inc. (ABI, Foster City, CA) model 394 DNA synthesizer with conventional phosphoramidite chemistry. Thiol-modifier C3-S-S-CPG columns (Glen Research, Sterling, VA) were used to introduce a protected 3′-thiol onto oligomers to be immobilized. Thiolated oligomers were synthesized using an oxidizer, 0.02 M I_2 (instead of the usual 0.1 M), to preserve the protected thiol moiety. Following cleavage of the oligomer from the CPG, the 3′-thiol group is obtained as a protected disulfide, which remains intact through the purification and detritylation steps described below. The oligomer is maintained in disulfide form until immediately before use, as described below. Purification and detritylation of oligomers was accomplished using C-18 SPE cartridges (Supelco, Bellefonte, PA). The cartridges were washed with MeOH, then equilibrated in 0.1 M triethylammonium acetate, pH 7.0 (TEAAc buffer). Crude DNA samples were diluted with TEAAc buffer, then applied to the equilibrated columns. Failure sequences were removed by washing with 7% CH_3CN, 93% H_2O; trityl-on DNA was retained on the column during this wash. The trityl group was removed by treatment of the column with 2% trifluoroacetic acid, 98% H_2O (indicated by formation of an orange band on the column). The cartridge was neutralized with TEAAc, then desalted with H_2O. Full-length, detriylated DNA oligomers were eluted in 50% MeOH, 50% H_2O. MeOH was removed by evaporation, then the DNA samples were extracted twice with ethyl acetate to remove the free trityl group from the DNA oligomer. The DNA was dried completely, then reconstituted in H_2O.

Bulk solution concentrations were determined spectroscopically (Beckman DU-650 UV-Vis spectrophotometer; Beckman Instruments, Columbia, MD) using $A_{260\ nm}$ values obtained in 10 mM Tris, 1 mM EDTA, pH 7.0, and extinction coefficients calculated with Oligo 4.1 software (National Biosciences Inc., Plymouth, MN). DNA (with protected thiol group) was divided into portions and stored at 0°C until needed. The sequence used for most of the immobilization studies described here was 5′-d(ACTG)$_5$-SH-3′, a sequence designed to be non-self-complementary and unable to form a hairpin. Other sequences prepared included 5′-d(ACTG)$_5$-3′, 5′-d(CAGT)$_5$-3′, 5′-d(CCCC)$_5$-SH-3′, 5′-d(GGGG)$_5$-3′ and 5′-d(IIII)$_5$-3′.

Treatment of crosslinker-modified substrates with thiolated DNA oligomers

The thiol group was deprotected overnight with 0.04 M DTT, 0.17 M phosphate buffer, pH 8.0, as directed by the 3′-S-S-CPG column manufacturer (Glen Research). Repeated extraction with an equivalent volume of ethyl acetate to remove excess DTT was performed immediately before preparing a solution of thiol-DNA oligomer in deaerated 10 mM HEPES, 5 mM EDTA buffer, pH 6.6 (HEPES buffer). Concentrations of the DNA solution varied from 0.1 to 5 µM but were typically 1 µM. Crosslinker-treated silanized substrates were immersed in the thiolated-DNA solution for 5 min–8 h (typically 2 h) at room temperature, then rinsed and dried under N_2. This step was performed either in a glovebag under N_2 or in air. Control experiments lacking the crosslinker or using non-thiolated DNA were also performed to verify that covalent coupling was occurring. Fused silica slides were examined using UV spectroscopy (2400 UV-VIS-NIR spectrophotometer; Cary, Sugarland, TX) for the presence of the characteristic DNA A_{260} peak before and after treatment with 50 mM sodium phosphate, 1 M NaCl, pH 6.5 (SPSC buffer), to remove non-covalently bound DNA. Note that as the slides are fully immersed in each of the solutions listed (silane, crosslinker and thiol-DNA), both sides of the slide are modified with DNA and that the absorbances recorded are for the two DNA films. Average values for contributions to the absorbance at 260 nm were determined for the silane films and crosslinker-modified silane films using a minimum of two slides for these determinations. Numerical A_{260} values reported in this paper for DNA film absorbance have been corrected for silane and crosslinker absorbances at 260 nm by mathematical subtraction. Special slide holders were fabricated to position slides in front of the beams. It should be emphasized that the Cary 2400 is an instrument possessing extremely high sensitivity with a detection limit of 0.0004 AU and repeatability of 0.0003 AU, a feature critical for the direct measurement and characterization of DNA thin films. Reference slides were acid cleaned at least once per week and were taken from the same lot of slides as the sample slides.

Determination of surface density and hybridization of immobilized oligomers

For the determination of surface density and hybridization assays, 5′-d(ACTG)$_5$-SH-3′, 5′-d(CCCC)$_5$-SH-3′, 5′-d(CAGT)$_5$-3′, 5′-d(CCCC)$_5$-SH-3′, 5′-d(GGGG)$_5$-3′ and 5′-d(ACTG)$_5$-3′ were ^{32}P-5′-labeled using [γ-^{32}P]dATP (3000 Ci/mMol; DuPont-New England Nuclear, Boston, MA) and T4 polynucleotide kinase (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. Thiolated oligomers were subjected to labeling as the protected disulfides and were then deprotected immediately

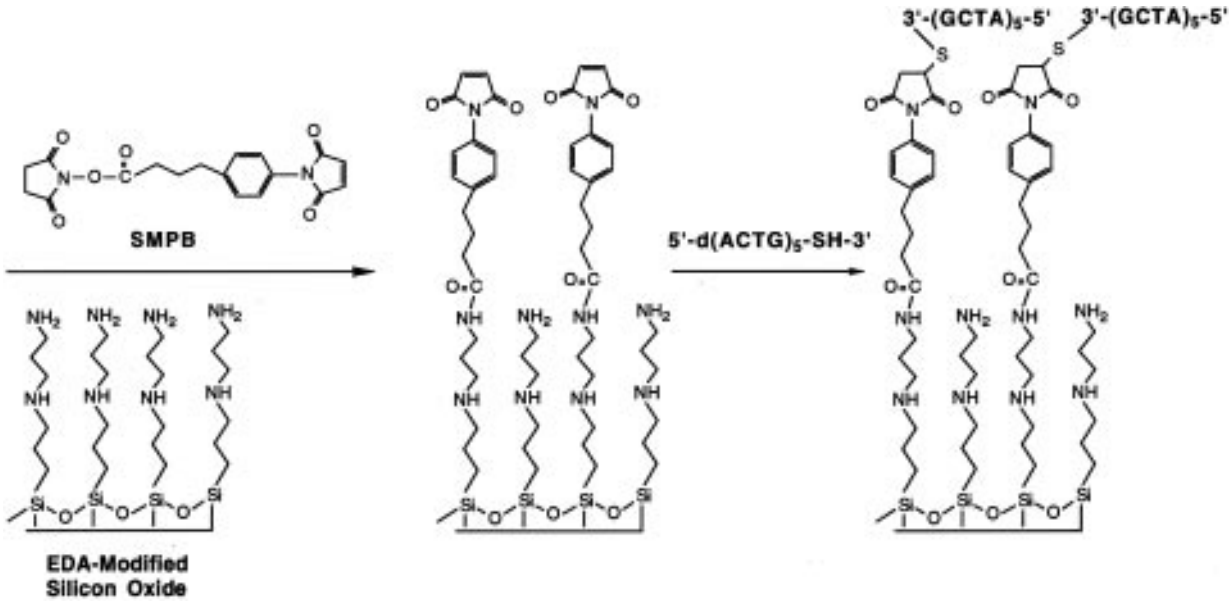

Figure 1. Chemistry used to covalently attach thiol-modified DNA oligomers to aminosilane-treated surfaces. In this example, an EDA-modified silicon oxide substrate is treated with the heterobifunctional crosslinker SMPB, whose succinimide ester moiety reacts with the primary amino group of EDA. The thiol-DNA oligomer subsequently reacts with the maleimide portion of the SMPB crosslinker, to yield the covalently bound species shown on the far right of the illustration.

before immobilization. Labeled oligomer was separated from unincorporated [γ-^{32}P]dATP using NENSORB columns as directed by the manufacturer (DuPont NEN). Specific activities of the radiolabeled oligomers were determined from bulk solutions using UV spectroscopy and liquid scintillation counting (LSC) (Packard Model 1500 Liquid Scintillation Analyzer, Meriden, CT; ScintiVerse IV scintillant, Fisher Scientific, Pittsburgh, PA).

Prior to immobilization on surfaces, 1.32 µM solutions of unlabeled 5′-d(ACTG)$_5$-SH-3′ and 5′-d(CCCC)$_5$-SH-3′ in HEPES buffer were spiked with radiolabeled oligomer to yield solutions with specific activities of 381 and 239 c.p.m./pmol, respectively. Si (100) wafer pieces (~2.5 × 1.2 cm) which had been silanized with EDA and then modified with SMPB were immersed in the thiolated DNA solution for 2 h at room temperature to achieve crosslinking, then rinsed briefly with dH_2O. The quantity of DNA bound was determined by placing the substrates in ScintiVerse IV scintillant (Fisher Scientific Products) and performing LSC. To determine the quantity of covalently attached DNA (versus that which was non-specifically bound to the surfaces), an identical set of wafers was further treated for 24 h in 1 M NaCl, briefly rinsed with H_2O, then surface-bound radioactivity determined using LSC. (It has been previously shown that treatment of a non-covalently bound DNA film with a high ionic strength solution resulted in nearly complete removal of the DNA from the substrate; 11.)

For hybridization experiments, unlabeled 5′-d(ACTG)$_5$-SH-3′ or 5′-d(CCCC)$_5$-SH-3′ were immobilized on Si pieces as described above. Note that these substrates were not treated with SPSC to remove non-covalently attached DNA before hybridization, in an attempt to minimize the non-specific binding of the radiolabeled oligomers. Substrates bearing 5′-d(ACTG)$_5$-SH-3′ were then hybridized with a 1.0 µM solution of radiolabeled 5′-d(CAGT)$_5$-3′ (504 c.p.m./pmol) and Si pieces modified with 5′-d(CCCC)$_5$-SH-3′ were hybridized with 5′-d(IIII)$_5$-3′ (752 c.p.m./pmol) or 5′-d(GGGG)$_5$-3′ (263 c.p.m./pmol). The hybridizations were performed in 1 M NaCl for 1 h at room temperature, followed by a 5 min rinse in 0.1 M NaCl and brief immersion in H_2O. Surface-bound radioactivity on the wafers was then determined using LSC as described above.

Contact angle and ellipsometry measurements

Sessile drop water contact angle measurements were obtained with an NRL Zisman-type contact angle goniometer under ambient conditions. A micropipettor was used to dispense 10 µl drops of dI H_2O at multiple points across the substrate surface. The precision of measurements taken on substrates averaged~3°; this small variation in contact angle suggests film coverage is likely to be homogenous. Measurements were taken promptly after completion of a treatment and drying under N_2.

Optical ellipsometry (Gaertner Model L115C equipped with Gaertner Waferscan software and 638 nm HeNe laser) was used to verify that the film thicknesses obtained for selected silanized samples were consistent with that reported for well-characterized silane monolayers (≈6 Å for DETA, ≈4 Å for EDA and ≈10 Å for PEDA; 14). Optical constants were determined for freshly cleaned silicon wafers, then these wafers were treated with EDA, DETA or PEDA as described above and 9–27 points per wafer sampled. Optical ellipsometry has a resolution of 2 Å and, while the method may be used to determine thicknesses of multilayer films on a layer by layer basis, the software used prohibited us from reliably characterizing the crosslinker–silane and DNA–crosslinker–silane film thicknesses.

Thermal stability experiments

DNA films were prepared using DETA, SMPB (20:80 DMSO:MeOH) and 1 µM 5′-d(ACTG)$_5$-SH-3′ as described, incubated overnight in SPSC buffer, then spectra recorded before exposure to heat. Pairs of slides were then incubated for 10 min in 1× phosphate-buffered saline (PBS, 8.2 mM Na_2HPO_4,

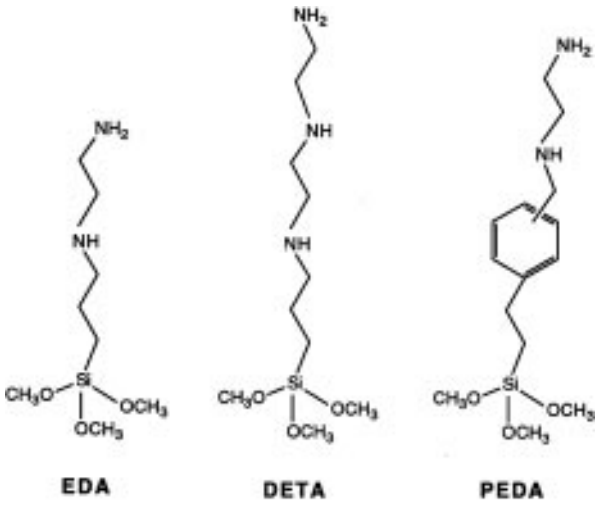

Figure 2. Structures of the three aminosilanes examined for their ability to support crosslinker modification and subsequent DNA attachment.

4.2 mM NaH_2PO_4, 0.2 M NaCl, 2.7 mM KCl, 1.1 mM K_2HPO_4, pH 7.2) at 37, 55 or 80°C and spectra recorded again and A_{260} values determined.

RESULTS AND DISCUSSION

Silanization of substrates

Self-assembly of silane films has been demonstrated on a wide variety of hydroxylated surfaces, including glass, SiO_2, metals and metal oxides, plastics, polymers (15,16) and diamond (17). We have focused our study of DNA immobilization on the use of fused silica and silicon wafers; use of fused silica for DNA film deposition permitted direct observation of the UV absorbance characteristic of DNA (λ_{max} = 260 nm). We had determined earlier that non-covalent attachment of DNA to aminosilane monolayer films occurred and that the DNA film could be removed under high salt conditions (11). Building on these early findings we have developed the covalent attachment method described here, which also utilizes self-assembled films formed from aminosilanes (Fig. 1). Covalent attachment of the thiolated DNA was verified by evaluating the A_{260} of slides which had been silanized with DETA and either treated directly with 5′-$(ACTG)_5$-SH-3′ (i.e., no crosslinker) or treated with SMPB crosslinker and non-thiolated DNA [5′-d$(ACTG)_5$-3′]. These slides were compared to slides which had been silanized, treated with SMPB and 5′-$(ACTG)_5$-SH-3′, the treatment which results in covalent attachment of the oligomer. All slides were placed in high salt (SPSC) buffer overnight to remove non-specifically bound DNA, before determining the A_{260}. Slides to which DNA was covalently bound yielded an A_{260} of 0.0035 ± 0.0003, while the crosslinker-free slides (A_{260} = 0.0001 ± $7e^{-5}$) and non-thiolated DNA slides (A_{260} = 0.0005 ± $7e^{-5}$) clearly lacked an absorbance attributable to the presence of DNA.

The silanes EDA, DETA and PEDA were compared for their ability to mediate covalent DNA attachment. The structures of these silanes are shown in Figure 2. We observed tremendous variability in the quantity of DNA immobilized depending on the source and condition of these aminosilanes. Ideally, EDA and DETA should be used shortly after vacuum distillation (PEDA, unfortunately, cannot be easily purified). Storage in the dark to avoid generation of photoproducts and removal of silane from the stock bottle with glass pipettes under anhydrous conditions were the key to preserving optimum activity. Silanes which have deteriorated due to photodecomposition or polymerization may have a strong yellow color or precipitates present and may yield poor DNA attachment.

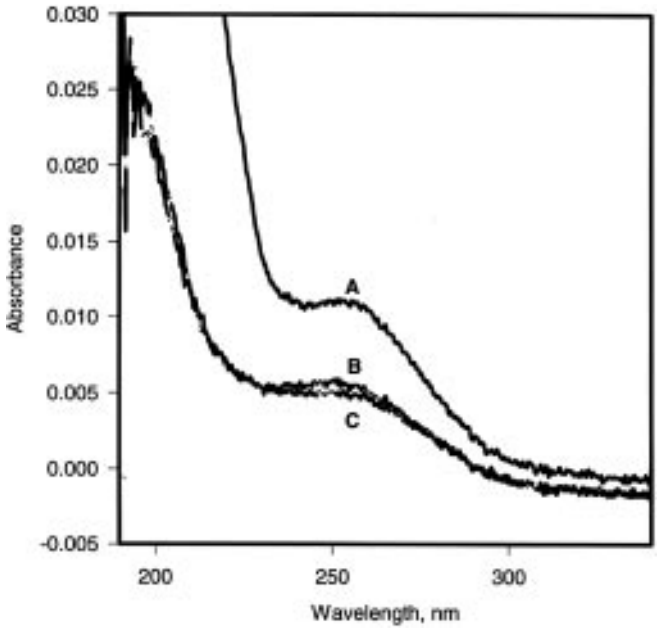

Figure 3. UV spectra taken of three DNA films formed on fused silica (quartz) slides. Three silanes, PEDA, EDA and DETA, were used to form self-assembled films on the slides as described in Materials and Methods. All slides were treated in SMPB (80:20 MeOH:DMSO) for 2 h, then with a 1 µM aqueous solution of 5′-d$(ACTG)_5$-3′-SH for 2 h. Spectra were acquired after a 15 h treatment of slides with SPSC, a high salt buffer for removal of non-covalently bound DNA. All numerical A_{260} values extracted from spectra were corrected for absorbance at 260 nm by films lacking DNA. (**A**) Spectrum of DNA on PEDA + SMPB. (**B**) Spectrum of DNA on EDA + SMPB. (**C**) Spectrum of DNA on DETA+ SMPB.

Fused silica slides were treated with the indicated silane, then with the crosslinker SMPB (MeOH:DMSO, 2 h) and subsequently with the DNA oligomer 5′-d$(ACTG)_5$-SH-3′ (1 µM, 2 h at room temperature). The slides were then incubated in SPSC buffer overnight at room temperature to remove chemisorbed DNA. Under these experimental conditions, PEDA yielded the highest A_{260} value (0.0081) (note that this PEDA was purchased from Gelest, however, PEDA purchased from UCT gave comparable results to distilled EDA and DETA; DETA and EDA were from UCT), while EDA (0.0052) and DETA (0.0045) were comparable (Fig. 3). We elected to use the significantly more economical silanes EDA and DETA for the purpose of studying other parameters of the immobilization process. PEDA was the silane utilized for experiments involving deep UV laser patterning(12), as this silane has a lower photochemical dose requirement for cleavage from the surface than either EDA or DETA.

Crosslinker effects on DNA immobilization

We compared six different heterobifunctional crosslinkers for their relative effectiveness at linking a thiolated DNA oligomer

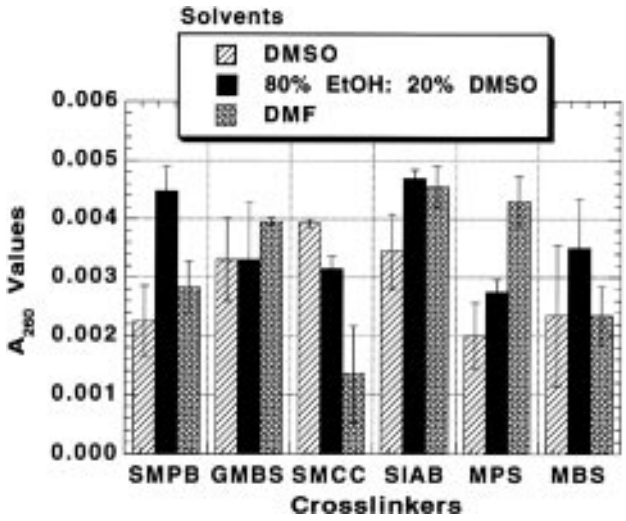

Figure 4. A_{260} values obtained for DNA films formed on fused silica slides treated with DETA silane (2 h) and each of six heterobifunctional crosslinkers. The six crosslinkers were dissolved in three different solvent systems: after initial dissolution of crosslinkers in DMSO, neat DMF, neat DMSO or an 80:20 EtOH:DMSO mixture were used to dilute the crosslinkers to their final 1 mM concentration. The DNA oligomer 5′-d(ACTG)$_5$-SH-3′ (1 µM in HEPES buffer) was used to treat crosslinker-modified slides for 2 h, then slides were immersed in SPSC buffer overnight before acquisition of spectra. Duplicate slides were analyzed, so the error bars indicate the upper and lower values obtained. The A_{260} values were extracted from the spectra after correction for absorbance at this wavelength by the crosslinker-modified silane films (prior to DNA treatment).

to the surface. The term 'heterobifunctional' derives from the fact that the linkers possess functional groups capable of reaction with two chemically distinct functional groups, e.g. amines and thiols (18). Unlike homobifunctional crosslinkers, the use of a heterobifunctional crosslinker diminishes the possibility of dimer formation or intramolecular reactions.

The linkers serve two purposes: to covalently bind two distinct chemical entities which otherwise would remain unreactive toward each other and as a physical spacer which provides greater accessibility and/or freedom to each of the linked biomolecules. This last feature is especially important for the reaction we describe here, where one of the reactants is the substrate-bound aminosilane film and the other reactant is a DNA molecule, which needs to remain functional (e.g., able to form a duplex) despite the constraints of attachment. This strategy differs from that employed by others, who utilized amino-modified DNA for direct or homobifunctional crosslinker-mediated attachment to silanized surfaces (2,7,8,19). Note that we have focused our thiol linking chemistry on the use of reactive groups (maleimide and iodoacteyl) that do not yield disulfide bonds, as this moiety is undesirable due to the potential for reductive cleavage of the linked molecules. An alternate attachment strategy could consist of an amino-modified DNA which, following reaction with the crosslinker, could be covalently attached to a thiol-silane surface. However, thiol-silane monolayer films are especially susceptible to reaction with ambient thiols and under certain irradiation conditions are converted to a sulfonate form (20), thus we avoided their use by adopting the strategy described. Although the thiol-DNA used is also susceptible to reaction with thiols, we considered that as a bulk solution it should be more robust under ambient conditions than would a thiol-silane monolayer film.

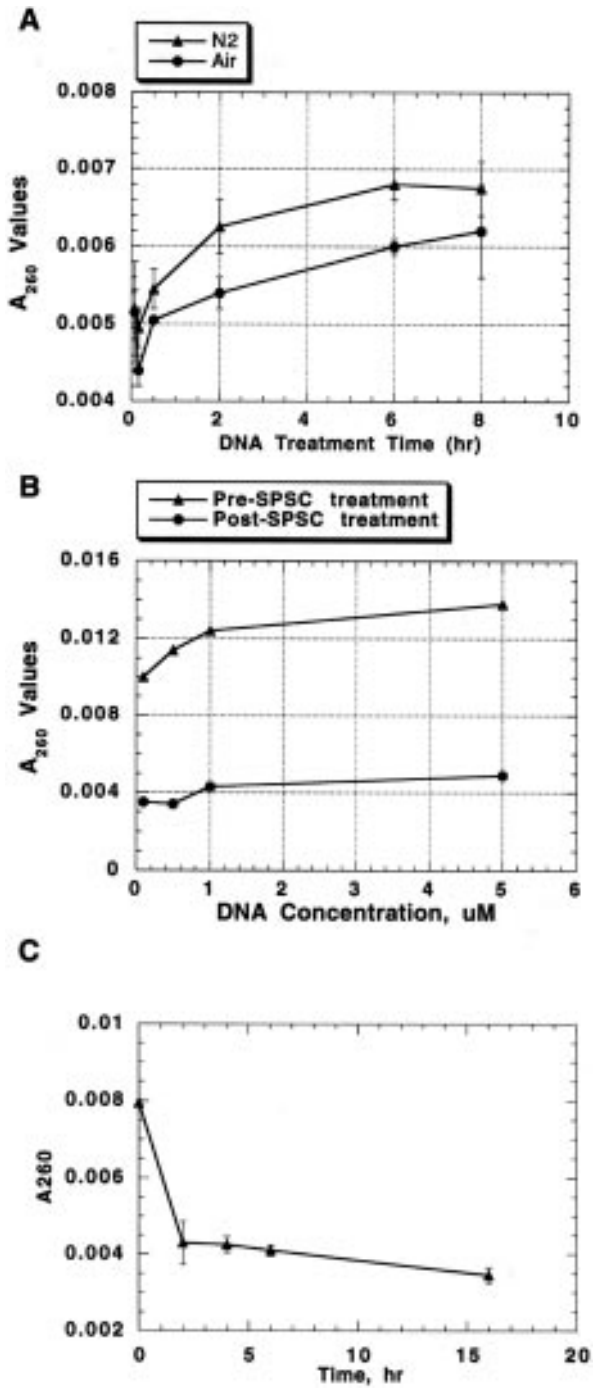

Figure 5. Effects of varying different aspects of the attachment chemistry on the density of immobilized DNA. DETA silane and SMPB crosslinker (80:20 MeOH:DMSO, 2 h) on fused silica slides were used for all experiments. Unless otherwise noted, the concentration of the 5′-(ACTG)$_5$-3′-SH solution was 1 µM and the A_{260} values were acquired after an overnight SPSC treatment. Duplicate slides were tested to provide data for (a) and (c), so error bars indicate upper and lower values obtained, while (b) was the result of a single experiment. (**a**) Effects of varying the DNA treatment time from 5 min to 8 h and of performing the DNA step under N_2 versus in air. (**b**) Effect of varying the thiolated DNA concentration from 0.1 to 5.0 µM. Data are shown for DNA spectra acquired before and after an overnight treatment with SPSC buffer. (**c**) Removal of non-covalently attached DNA from the surface as a function of incubation time in SPSC buffer.

We also evaluated the use of various solvents for reaction of the crosslinker with the aminosilane film. In order to avoid hydrolysis of the ester we focused on the use of non-aqueous polar solvents, such as MeOH:DMSO, EtOH:DMSO, DMSO and DMF, which are compatible with both the crosslinkers and the aminosilane film. The A_{260} values achieved using DETA-modified slides with the six crosslinkers, dissolved in DMF, DMSO or 80:20 EtOH:DMSO, are shown in Figure 4. Following crosslinker treatment, slides were placed in 1 μM 5-d(ACTG)$_5$ -SH-3′ for 2 h, then kept overnight in SPSC before UV spectra were examined. There was a nearly 3-fold difference in A_{260} values obtained depending on choice of crosslinker and solvent. SMPB (EtOH:DMSO), SIAB (EtOH:DMSO and DMF) and MPS (DMF) yielded the highest A_{260} levels. Overall, SIAB, the only linker which contains an iodoacetyl group for reaction with the DNA-thiol, appeared to yield the highest A_{260} values with all three solvents, while MBS performed the poorest. Many of the crosslinkers performed well in one or more solvents but poorly in others; in general there was no choice as to 'best' solvent. There was also no clear trend for increased A_{260} value as a function of crosslinker length.

Although the SIAB crosslinker performed well in the solvent comparison study, when SMPB and SIAB were directly compared (in 80:20 MeOH:DMSO) to determine which crosslinker should be utilized for optimizing DNA attachment, SMPB yielded significantly higher A_{260} values (0.0035 ± 0.0003 versus $0.0012 \pm 7e^{-5}$). This lack of reproducibility in DNA film formation using SIAB may be a consequence of the light sensitivity of this crosslinker. The majority of subsequent experiments utilized DETA with SMPB (EtOH:DMSO or MeOH:DMSO for 2 h), as this combination yielded DNA films with reproducible A_{260} values. The crosslinker treatment time was also evaluated using DETA silane films, with SMPB (MeOH:DMSO) treatment time varying from 30 min to 8 h followed by 1 μM 5′-d(ACTG)$_5$-SH-3′ (2 h). No significant increase in A_{260} values were observed by increasing the SMPB reaction time above 2 h, thus 2 h was the duration employed for subsequent experiments (data not shown). It should also be noted that after modification with the crosslinker, the films may be sensitive to ambient thiol concentrations and immersion in the thiol-DNA solution should be performed promptly.

Optimizing DNA immobilization onto crosslinker-modified aminosilane films

The effect of the environment (N_2 versus air) in which the treatment is performed, DNA concentration, incubation period with DNA and conditions for desorption of non-covalently bound DNA were studied. For these experiments, fused silica slides, DETA silane and the crosslinker SMPB (80:20 EtOH:DMSO, 2 h) were used. The trends and A_{260} values are for data acquired after a treatment with SPSC buffer to remove non-covalently bound DNA unless noted otherwise.

We examined the effect of performing the DNA treatment step under N_2 in a glovebag, as the DNA used bears a thiol which is susceptible to oxidation once DTT is extracted from the solution prior to immobilization. This experiment was coupled with one in which the length of time a 1 μM DNA solution [5′-d(ACTG)$_5$-SH-3′] was incubated with the slides was varied from 5 min to 8 h. The results indicated that thiol-DNA treatment times of 6 h or greater yielded the greatest A_{260} values, although even after a 5 min incubation a measurable A_{260} DNA peak is clearly evident following UV analysis (0.0052 in either N_2 or air; Fig. 5a). Performing the DNA immobilization step in an inert atmosphere (N_2) resulted in an average increase of 10% in A_{260} values, over experiments performed at the bench, at each time point tested (Fig. 5a). Ideally, the DNA treatment step should be performed for a minimum of 2 h under N_2 to minimize oxidation of the thiol DNA.

The consequence of varying the DNA concentration from 0.1 to 5 μM on the absorbance of the deposited film was also evaluated. Although the A_{260} did increase with the concentration of DNA solution used, the difference between 0.1 and 5.0 μM did not scale linearly and in fact only a 22% increase in A_{260} was observed over this 50-fold concentration range (Fig. 5b). This may be due to saturation of available crosslinking groups on the surface at low DNA concentrations. The large loss of DNA from the films observed following overnight treatment with the SPSC buffer is illustrated in Figure 5b. Typically, the A_{260} value falls by 50–70% from the initial value (obtained immediately after the DNA immobilization step). This effect appears to be independent of the concentration of DNA solution used. Figure 5c illustrates the time-dependent loss of DNA from 5′-d(ACTG)-SH-3′-treated, SMPB-modified DETA slides when incubated in SPSC buffer. It is clearly shown that the most significant drop in A_{260} occurs during the first 2 h and is apparently stabilized after this time.

For our standard DNA immobilization conditions we elected to use 1 μM DNA solutions, in order to keep experimental costs moderate while reproducibly making detectable films. Given the cumulative length of the cleaning, silanization, crosslinker modification and DNA immobilization protocol, an overnight SPSC treatment was typically implemented for convenience (although we have determined that the SPSC treatment could be for as little as 2 h). Our standard conditions of DETA silane, SMPB crosslinker (EtOH:DMSO, 2 h), 1 μM DNA (2 h, in air or under N_2) and overnight SPSC treatment were selected to yield the optimum reproducible DNA films while considering cost, time and convenience of film production.

Physical properties of the DNA films

Surface density and hybridization of immobilized DNA oligomers. Surface density of covalently bound DNA was determined using two different sequences [5′-d(ACTG)$_5$-SH-3′ and 5′-d(CCCC)$_5$-SH-3′] of ^{32}P-labeled DNA oligomers on SMPB/EDA-modified silicon wafer pieces using the protocols described. Liquid scintillation counting was utilized to determine the density of the surface-bound radiolabled DNA oligomer. The density of DNA acheived varied for the two sequences tested. Total DNA bound (covalent + chemisorbed DNA) was $\approx$47 pmol/cm^2 for 5′-d(ACTG)$_5$-SH-3′ and $\approx$32 pmol/cm^2 for 5′-d(CCCC)$_5$-SH-3′ (see Table 1). The density of DNA which remained bound to the surface after treatment overnight in a high salt solution, denoted as covalently bound DNA, was $\approx$16 pmol/cm^2 (34% of the total DNA) for 5′-d(ACTG)$_5$-SH-3′ and $\approx$23 pmol/cm^2 (73% of the total DNA) for 5′-d(CCCC)$_5$-SH-3′ (Table 1). Analysis of the surface density suggests the DNA is present at sub-monolayer coverage, which could result from either patchy surface coverage or a film composed of DNA molecules of variable orientation with respect to the surface (6).

Table 1. Surface density of immobilized oligomers 5′-d(ACTG)$_5$-SH-3′ and 5′-d(CCCC)$_5$-SH-3′

Oligomer sequence	Total DNA: covalently + non-covalently bound	Covalently bound DNA	
	Surface density (pmol/cm^2)	Surface density (pmol/cm^2)	Percent of total DNA
d(ACTG)$_5$	46.9 ± 8.7	16.2 ± 1.7	34
d(CCCC)$_5$	31.9 ± 1.8	23.4 ± 1.9	73

The experimental details are given in Materials and Methods. Briefly, EDA and SMPB-modified silicon wafer pieces (2.5 × 1.25 cm) were treated with 5′-radiolabeled, 3′-thiolated DNA oligomers, then 'Total DNA' bound was determined from specific activities of the oligomers and LSC of the substrates. A parallel set of substrates was further treated for 24 h in 1 M NaCl to remove non-specifically bound DNA and quantitated by LSC to yield the density of 'Covalently bound DNA'. Percent total DNA = (Covalently bound DNA/Total DNA)× 100. Values reported result from experiments using two substrates for each experimental condition.

Table 2. Density and hybridization efficiency for complementary oligomers with immobilized 5′-d(ACTG)$_5$-SH-3′ and 5′-d(CCCC)$_5$-SH-3′

	Oligonucleotide hybridization	
	Density of complementary oligomer (pmol/cm^2)	Efficiency (% covalently immobilized DNA engaged in duplex formation)
[^{32}P]d(CAGT)$_5$ on d(ACTG)$_5$-SH surface	1.5 ± 0.1	9.3
[^{32}P]d(IIII)$_5$ on d(CCCC)$_5$-SH surface	2.9 ± 0.4	12.4
[^{32}P]d(GGGG)$_5$ on d(CCCC)$_5$-SH surface	17.8 ± 4.8	76.1

Surfaces modified with cold 5′-d(ACTG)$_5$-SH-3′ and 5′-d(CCCC)$_5$-SH-3′ were hybridized as described in Materials and Methods with 1.0 μM solutions of ^{32}P-5′-d(CAGT)$_5$-3′ and/or ^{32}P-5′-d(GGGG)$_5$-3′ respectively. 'Efficiency' of hybridization was calculated as the fraction of covalently immobilized DNA which participated in duplex (or triple helix) formation with the indicated complementary strand. For 5′-d(ACTG)$_5$-SH-3′ surfaces, the covalently immobilized DNA was assumed to be 16.2 pmol/cm^2 and for the 5′-d(CCCC)$_5$-SH-3′ surfaces, 23.4 pmol/cm^2 (taken from Table 1). Values reported result from experiments using two substrates for each experimental condition.

The density of covalently attached DNA achieved was significantly greater than that reported for DNA films formed on similar substrates (e.g., glass or SiO_2) prepared using aminosilane films with a diisothiocyanate crosslinker (2) or epoxysilane films (7,8). In addition, these other methods typically required 5–100-fold greater DNA concentrations for the immobilization step than the 1–1.3 μM we employed. As expected, the DNA surface density we determined on planar SiO_2 surfaces falls short of densities reported for high surface area substrates such as porous polypropylene membranes (10). It is difficult to correlate our surface density to coupling strategies which involve attachment to solid supports such as porous or solid beads, as these densities are typically reported on a concentration to mass basis rather than area (21). Overall, in comparision to other methods for DNA attachment to glass or similar substrates, that described here appears to be superior both in the density of DNA achieved as well as the reduced concentration of DNA required to achieve this density.

Hybridization of a complementary oligomer to an oligomer which was covalently attached to Si using the chemistry described was also studied. It should be noted that slides to which unlabeled thiolated DNA had been covalently attached were not treated with SPSC to remove the non-covalently bound fraction prior to hybridization with a radiolabeled complementary oligomer. It was assumed that under the conditions used for hybridization (1 M NaCl at room temperature), it was likely that *any* non-covalently attached DNA (i.e., unlabeled thiolated and radiolabeled oligomers) was removed from the surface, and that only covalently attached DNA (as well as any hybridized radiolabeled DNA) would remain on the substrates. After allowing hybridization to occur, the quantity of radiolabeled complementary oligomer (pmol) detected on the slides was used to calculate the percent of covalently bound oligomer which can particpate in duplex formation (Table 2). The surface densities determined for covalently attached DNA (see Table 1) were used to perform this calculation.

The aptitude of immobilized DNA to form hybrids was extremely dependent on the sequence of the immobilized strand and ranged from 9.3 to 76.1% of the covalently immobilized DNA (1.5–18 pmol/cm^2; see Table 2). Immobilized oligomer 5′-d(ACTG)$_5$-SH-3′ was able to hybridize with radiolabeled 5′-d(CAGT)$_5$-3′, however, under our experimental conditions only ≈9% of surface-bound 5′-d(ACTG)$_5$-SH-3′ strands participated in hybrid formation. The homopolymeric oligomer 5′-d(CCCC)$_5$-SH hybridized to varying extent with both 5′-d(IIII)$_5$-3′ and 5′-d(GGGG)$_5$-3′; for the 5′-d(GGGG)$_5$-3′ oligomer, over 75% of surface-bound 5′-d(CCCC)$_5$-SH strands apparently form hybrids. The great variation in hybridization efficiency between the sequences tested may stem from the greater hydrogen bonding capability of the 5′-d(CCCC)$_5$-SH-3′:5′-d(GGGG)$_5$-3′ system (i.e., shorter stretches of the oligomers can bond and form duplexes stable under our assay conditions) and/or the propensity of this particular system to form triple-helical structures (5,6). It is also possible that access to the immobilized oligomer for hybridization is sterically restricted due to its attachment to the surface. The higher efficiency of hybridization observed with the 5′-d(CCCC)$_5$-SH-3′:5′-d(GGGG)-3′ system could be explained in this context, as it has the ability to form a greater number of stable partial duplexes under the hybridization conditions used. Under the room temperature, 1 M NaCl hybridization conditions used, dG:dC duplexes of as few as six bases may be stable, whereas for the (ACTG):(CAGT) system, a minimum of eight to nine bases is required.

Table 3. Sessile drop water contact angles (θ°) for films formed on fused silica slides

Step in DNA immobilization process	Sessile drop contact angle (θ°)		
	EDA	DETA	PEDA
Silane only	30 ± 1°	17 ± 1°	42 ± 20°
Silane + SMPB	45 ± 1°	26 ± 1°	39 ± 2°
Silane + SMPB + DNA	37 ± 2°	31 ± 21°	28 ± 2°
Silane + SMPB + DNA + SPSC	41 ± 4°	37 ± 3°	43 ± 5°

Water drops of 10 µl were positioned at various points across the substrate surface and contact angles determined. A single substrate was used for the determination of contact angle for each experimental condition; three to five water drops were used to determine the homogeneity of the treated substrates.

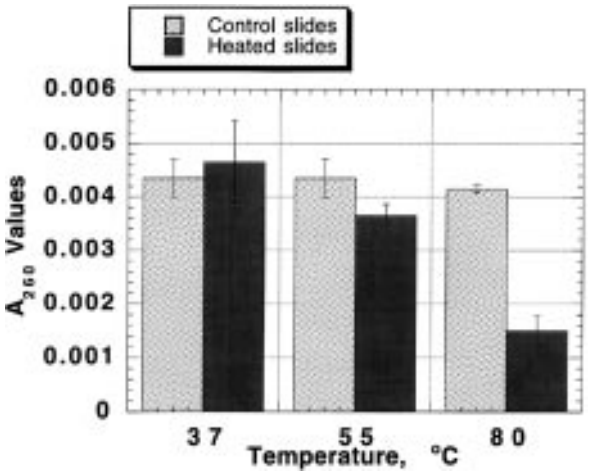

Figure 6. Thermal stability of the DNA films. DNA films were prepared on fused silica slides with DETA silane modified with SMPB crosslinker (80:20 EtOH:DMSO, 2 h). The DNA used was a 1 µM solution of 5′-d(ACTG)$_5$-3′-SH (2 h). Slides were treated overnight in SPSC buffer before heating for 10 min in 1× PBS at the indicated temperatures. The 'control' slide measurements are for A_{260} values obtained from those same slides before heating. Duplicate slides were tested so error bars indicate upper and lower values only.

Selectivity of duplex formation by immobilzed DNA was demonstrated by attempting to hybridize ^{32}P-5′-d(ACTG)$_5$-3 to a 5′-d(ACTG)$_5$-SH-modified surface. Negligible radioactivity was obtained, demonstrating that neither specific (e.g., duplex formation) nor non-specific binding of the radiolabeled DNA had occurred.

Thermal stability. We evaluated the thermal stability of films formed using 1 µM 5′-d(ACTG)$_5$-SH-3′ on SMPB/DETA substrates, by subjecting them to a 10 min treatment at 37, 55 or 80°C in 1× PBS. Only the films held at 80°C showed a significant loss of DNA, suggesting that this immobilization chemistry is stable to moderate heat (Fig. 6). This suggests that DNA films formed using the described chemistry may not be suitable for certain applications which involve thermal cycling to temperatures above 80°C, such as PCR, but are well-suited for applications involving brief exposures to temperatures up to 55°C.

Contact angle measurements. Sessile drop contact angle measurements with H_2O drops were performed on fused silica slides which had been silanized, treated with SMPB (2 h), 1 µM d(ACTG)$_5$-SH-3′ and SPSC buffer. Contact angle (θ) measurements were obtained after each step in the immobilization process. Self-assembled monolayer films formed from the three silanes tested, EDA, DETA and PEDA, varied in their degree of water wettability. Sessile drop θ values obtained are summarized in Table 3. The sessile drop θ values reported here for EDA (30°) and DETA (17°) are comparable to the *advancing* drop θ values reported for these monolayer films (22) (32° and 15° respectively) but we observe a significantly lower value for PEDA (42 versus 55°). This may be due to the use of PEDA silane obtained from different vendors, variation in film preparation conditions or contact angle measurement techniques.

The increasing hydrophobicity of PEDA > EDA > DETA is not maintained as these surfaces are modified with the hydrophobic crosslinker SMPB, as EDA emerges as the most hydrophobic surface at 45° and PEDA drops to 39°. This may reflect more extensive reaction of the SMPB crosslinker with EDA films. After treatment with the relatively hydrophilic thiolated DNA oligomer, the three silane surfaces exhibit θ values within 9° of each other; after the SPSC treatment, these values increase, yet fall within an even narrower range (<6°). The relationship of these contact angles to other properties, such as the A_{260} values, is unclear; for example, the nearly 2-fold increase in A_{260} seen with PEDA versus either EDA or DETA (Fig. 3) does not correlate with a dramatic increase in hydrophilicity of the PEDA surfaces.

CONCLUSION

In conclusion, we believe that the use of aminosilane monolayers to anchor DNA oligomers via the use of crosslinking molecules may be useful for certain applications where thin, covalently attached DNA films or patterned DNA surfaces are required. Covalently bound DNA surfaces are likely to be more useful than chemisorbed DNA films under conditions of high ionic strength or elevated temperature, which may be required for some applications. Covalent attachment was clearly demonstrated, as the A_{260} values obtained for slides on which either the crosslinker was omitted or a non-thiolated DNA oligomer was used were neglible compared to slides where all of the components required for covalent coupling (aminosilane, crosslinker and thiolated DNA) were present. This paper describes our attempts to optimize the DNA attachment process and presents physical data on the films produced, such as UV absorbance, thermal stability and sessile water drop contact angles. Variables tested included: the choice of aminosilane (three tested); selection of heterobifunctional crosslinker and its corresponding solvent (six linkers tested in three solvent systems); DNA concentration; incubation period for the DNA attachment step; and time required for

removal of non-covalently bound DNA from the surface in a high salt buffer. Our standard conditions (for fused silica sildes) utilized DETA silane, SMPB crosslinker (80:20 EtOH:DMSO or 80:20 MeOH:DMSO, 2 h), 1 μM thiolated DNA oligomer (2 h) with an overnight SPSC buffer treatment to remove non-specifically bound DNA. Some distinct advantages to the use of the described approach over other methods are apparent, such as a higher surface density, despite using a 5–100-fold lower concentration of DNA oligomer, and the ability to photopattern the DNA surface. It should be stressed, however, that for the chemistry described here to be successful, it is important to rigorously control the quality of the silanes used and to process films from one step to the next promptly, as they are susceptible to side reactions. Given these precautions, stable and reproducible covalently attached DNA films can be prepared using the attachment chemistry described.

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