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Fabrication of patterned DNA surfaces

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ABSTRACT

Two photolithographic methods are described for the formation of patterned single or multiple DNA species on SiO_2 substrates. In the first approach, substrates are treated with a photochemically labile organosilane monolayer film. Irradiation of these surfaces with patterned deep UV (193 nm) light results in patterned chemically reactive groups which are then reacted with heterobifunctional crosslinking molecules. Covalent attachment of modified synthetic DNA oligomers to the crosslinker results in stable DNA patterns. Alternatively, a photoresist is spin-coated over a silane film which had been previously modified with the hetero-bifunctional crosslinker. Upon patterned irradiation and subsequent development, the underlying cross-linker-modified layer is revealed, and is then reacted with a chemically modified DNA. Feature dimensions to 1 micron are observed when a single fluorescent DNA is attached to the surface. By performing sequential exposures, we have successfully immobilized two distinguishable DNA oligomers on a single surface. Synthetic DNA immobilized in this manner retains the ability to hybridize to its complementary strand, suggesting that these approaches may find utility in the development of miniaturized DNA-based biosensors.

INTRODUCTION

The notion of utilizing 'biochip arrays' for multiplexing biological reactions has become commonplace over the past several years. This concept evolved from the notion of using combinatorial synthetic approaches to create very large scale libraries of receptor molecules and to then screen the population for enhanced binding activity of individual members to a target of interest (1,2). Possible applications for large scale arrays of biomolecules include DNA sequencing-by-hybridization (3), DNA-based sensors, enzyme or antibody-based sensors, and peptide or nucleic acid oligomer libraries for screening ligand binding (1,2). Various schemes for the fabrication of such arrays have emerged, including the light-directed spatially resolved modification of surfaces using monolayer silane films (1,4). Silane films bearing a wide variety of functional groups may be formed on numerous types of surfaces (5). Certain photolabile silanes can be irradiated using a lithographic mask, resulting in a patterned reactive surface (5). UV patterning of self-assembled monolayer (SAM) silane films has been demonstrated to sub-micron geometries (5) and can also be extended to nanometer-scale dimensions with proximal probe exposure tools (6). A patterned silane film can consist of spatially resolved regions bearing different functional groups or physical properties (5) or may be silanized again to obtain a coplanar assembly of selected functional groups on the surface (7). This approach has been exploited to create patterned biomolecular surfaces modified with nucleic acids (4,8), proteins and antibodies (9–11) and living cells (12,13).

High resolution (100 micron scale), parallel synthesis using novel photoactivatable chemistries has been used to create high-density arrays of nucleic acids and peptides on surfaces (1,14,15). Low resolution (millimeter scale) oligonucleotide arrays have been prepared using standard phosphoramidite chemistry to synthesize different oligomers directly on glass substrates (16) and polypropylene membranes (17,18). These approaches suffer from an inability to purify the individual array members which, having been directly synthesized onto surfaces, may include failure sequences. Microdroplet deposition of chemically modified, full-length nucleic acid oligomers onto epoxy silane-treated silicon (or glass) surfaces (19–21) or amine-silanized SiO_2 surfaces (22), and microcontact printing of alkanethiol monolayers on Au surfaces (23) are alternative approaches to producing arrays without *de novo* synthesis.

Factors which are important for the construction of biomolecular arrays on surfaces are: the use of immobilization chemistries which result in retention of bioactivity or fidelity of biorecognition properties, ability to fabricate arrays with multiple components, durability/stability of the completed array, ability to create high resolution patterns (for certain applications), and minimization of crosstalk between array elements. We have developed a simple approach to fabricating surfaces patterned with nucleic acid oligomers which satisfies these criteria. The general scheme involves treatment of a hydroxyl-bearing surface such as SiO_2 with an aminosilane which under specific conditions will spontaneously form a SAM film. The SAM presents many alternatives for creating chemically reactive, patterned templates for immobilization of biomolecules (5). For the studies described here, the aminosilane films were treated with a heterobifunctional crosslinker which can then react with a thiol group specifically incorporated at the terminus of synthetic DNA oligomers

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(24–26). This is similar to a method of DNA immobilization which attached amino-modified DNA oligomers (delivered by micropipet) to an aminosilane film, via the use of a homobifunctional crosslinker. The use of the homobifunctional crosslinker is a disadvantage as it increases the likelihood of aminosilane–aminosilane crosslinks, which may diminish the resulting surface density of DNA (24).

Using direct laser irradiation or photoresist masking of silane monolayers, we have created patterned surfaces for the attachment of activated DNA oligomers. These approaches offers high feature resolution, access to any feature geometry using a broad range of irradiation wavelengths (e.g., 193, 254, 365 or 405 nm), ability to immobilize multiple species in discrete locations, and retention of biological activity. Direct exposure of silane films offers great flexibility for two-dimensional patterning of DNA and other biomolecules, as both the chemical reactivity and other surface properties (e.g., hydrophobicity, substrate choice) can be tailored to meet the needs of specific applications (5).

MATERIALS AND METHODS

Preparation of self-assembled monolayer aminosilane films

Acid-cleaning of substrates and silane film formation on $1'' \times 1''$ fused silica slides (Dell Optics, NJ) or $3'' \times 1''$ glass microscope slides was carried out as described previously (27). Silanization of slides was performed in a glovebag under N_2 using either 1% trimethoxysilylpropyldiethylenetriamine (DETA, United Chemical Tech.), in 18 MΩ deionized (dI) water from a NANOPURE· still, acidified with 1 mM acetic acid; or 1% [*m,p*(amino-ethylaminomethyl)phenethyltrimethoxysilane], (PEDA, Gelest, Inc.) in 95:5 methanol–1 mM acetic acid in dI water. Slides were further processed and dried as described (24). Contact angles were generally determined using the sessile water drop method as described in reference 24, and were used to judge the quality of the silane film. On fused silica, values of $16° \pm 2°$ for DETA and $42° \pm 3°$ for PEDA were considered acceptable. Optical ellipsometry (Gaertner Model L115C equipped with Gaertner Waferscan software) was also used to verify that the film thicknesses obtained for selected samples were consistent with that reported for well-characterized silane monolayers [≈6 Å for DETA and ≈10 Å for PEDA, (28)]. The resolution of the technique is 2 Å. 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.

Modification of the silane films with the crosslinker succinimidyl 4-[malemidophenyl]-butyrate (SMPB, SIGMA, Inc., St Louis, MO) was effected by treatment for 2 h at room temperature with a 1 mM solution of the crosslinker prepared in 80:20 MeOH–DMSO (24).

DNA synthesis

DNA oligomers modified at the 3′-terminus with a thiol group (24) were synthesized using standard phosphoramidite chemistry and the thiol-modifier DNA synthesis support C3-S-S CPG (Glen Research, Sterling, VA). 3′-thiol, 5′-biotin-labeled congeners were prepared using the Bio-Teg phosphoramidite (Glen Research) in conjunction with the thiol-CPG. For preparation of fluorophore-modified, 3′-thiolated oligomers, an amine group was introduced at the 5′-terminus using amino modifier C6 phosphoramidite (Glen Research) in addition to the use of the thiol-CPG. All oligomers were purified and detritylated using C18 solid phase extraction columns then divided into portions which were stored at 0°C.

Fluorescent derivatives of the oligomers were prepared by reaction of purified 5′-amino-, 3′-protected thiol-modified DNAs with the succinimide esters of two cyanine dyes, Fluorolink CY-3.0 or Fluorolink CY-3.5 (Biological Detection Systems, Pittsburgh, PA), or tetramethylrhodamine-NHS ester (Pierce) by following manufacturer's directions. The fluorescent derivatives were purified to remove excess dye and unmodified oligo using two consecutive NAP-10 size exclusion columns (Pharmacia) with first ethanol (column 1) and then phosphate buffered saline [PBS (29), column 2] as the eluent. A long wavelength UV light (365 nm) was used to monitor the progress of the fluorescent material down the cartridge. A CY3.0-cyanoethyl phosphoramidite (Glen Research) was also used to label the 5′-terminus of 5′-d$(CAGT)_5$-3′, for use in hybridization studies. Following synthesis, this oligomer was purified and detritylated using C18 SPE cartridges as described (24).

The concentration of each oligonucleotide was determined spectrophotometrically using extinction coefficients calculated with Oligo 4.1 software (National Biosciences Inc., Plymouth, MN), and using a Beckman DU-650 UV-VIS spectrophotometer (Beckman Instruments, Columbia, MD). Ratios of dye-to-oligo in the fluorescent conjugates were determined in 10 mM Tris, 1 mM EDTA, pH 8.0 buffer following the manufacturer's instructions, and were found to be ~1:1 for the rhodamine, CY-3.0 and CY-3.5 conjugates. For the patterning experiments described below, 5′-d$(ACTG)_5$-SH-3′, an oligomer designed to lack both self-complementarity and the ability to form hairpins, was used.

DNA oligomers bearing a protected thiol were deprotected immediately before use and immobilized onto crosslinker modified silane films as described (24). Deprotected DNA was prepared as a 1 μM solution in HEPES buffer {10 mM *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid], 5 mM ethylenediaminetetraacetic acid (EDTA), pH 6.5}.

Laser patterning of silane films

Direct photochemical patterning of PEDA silane films was performed using deep UV (193 nm, ArF) laser exposure (30). A lithographic mask (chrome-on-quartz) with minimum feature sizes of 2 μM was clamped to the fused silica slide, then the assembly was exposed to a total dose of ≈400 mJ/cm^2 laser irradiation (0.81 mJ/pulse/cm^2, 10 Hz, 500 pulses per slide). The irradiated slide was rinsed in 1 M NaCl for 30 s to remove photolysis by-products created by the exposure (30). The patterned PEDA slide was immersed in the SMPB crosslinker solution, and treated as detailed above. Slides were then immediately treated by pipetting a freshly deprotected solution of thiolated DNA (1 μM in degassed 10 mM HEPES, 5 mM EDTA, pH 6.6) onto the patterned surface of the slide. The slides were incubated for 2 h at room temperature, with best results obtained if this step was performed under N_2 in a glovebag (24). SMPB-modified PEDA films could also be patterned using the same method and exposure dose as for PEDA-only films; after treatment with thiol-DNA these films yielded identical patterned DNA surfaces. Patterns were typically viewed after the DNA-modified slide was treated with a 50 mM Na-phosphate, 1 M NaCl (pH 6.5) buffer (SPSC buffer), to remove non-covalently bound DNA (24).

Photoresist patterning

Microposit® S1400-27 positive-tone photoresist (1 ml; Shipley Corp, Marlborough, MA) was coated onto a DETA– or SMPB–DETA-modified slide by spinning at 5000 r.p.m. for 30 s on a Headway Research (Garland, TX) spincoater. The negative-tone photoresist, Microposit® SAL 601-ER2 (Shipley Corp.) was spincoated onto DETA– or SMPB–DETA-modified slides at 3000 r.p.m. for 30 s. The resist-coated slides were then soft-baked at 90°C in a convection oven for 30 min. All handling of the photoresist was performed under safe (yellow) light conditions until positioned onto the contact aligner. The S1400-27 resist was exposed (50 mJ/cm^2 total dose) using a Karl Süss standard UV (365–405 nm output) Hg lamp contact aligner and the SAL 601-ER2 resist with a Süss deep UV (220–300 nm) Hg lamp contact aligner (40 mJ/cm^2, at 254 nm).

Contact aligner exposures were performed using chrome-on-quartz lithographic masks having minimum feature sizes of 1µm. After exposure, S1400-27-treated slides were immersed in Microposit® Concentrate or Microposit® MF-319 developers (1:1 solution in dI water) for 90 s, then rinsed in dI water (60 s) and dried under a stream of N_2. After exposure, SAL 601-ER2-treated slides were baked on a hot plate for 1 min at 120°C, then immersed in Microposit® MF-312 developer (1:1 dilution in dI water, Shipley Corp.) for 90 s at room temperature. Slides were rinsed for 60 s in dI water and dried under a stream of N_2. At this point, slides which had not previously been treated with SMPB were modified with the crosslinker as described above. Covalent attachment of dye- or biotin-labeled thiolated DNA to the areas in which DETA/SMPB was displayed was carried out by pipetting a solution of freshly deprotected thiolated DNA onto the patterned surface of the slide, as described above. Prior to detection of the labeled DNA, the remaining photoresist was removed by agitation in acetone for 1 min, rinsing in dI water and drying under N_2. Slides were typically viewed after the DNA-modified slide was treated with a 50 mM Na-phosphate, 1 M NaCl (pH 6.5) buffer, to remove non-covalently bound DNA (24).

Effect of exposure of DNA films to alkaline photoresist developers

The UV absorbance of DNA films covalently bound to DETA/SMPB films was recorded directly from the slides using a Varian Cary 2400 UV-VIS-NIR spectrophotometer (Sugarland, TX), before and after each of four 90 s cycles of treatment with MF-312, MF-319 or Microposit concentrate developers (1:1 dilution with dI water) to observe the effects of developer on the integrity of the DNA film. The magnitude of the DNA absorbance peak at 260 nm remaining on the slide was used to establish the percent DNA retained by the film after each cycle of developer treatment. The ability of the developer-treated DNA film to hybridize to a complementary oligomer was tested by placing 5 µl drops of 5 µM 5′-biotin-d(CAGT)$_5$-3′ oligomer (in water) on an MF322-treated 5′-d(ACTG)$_5$-SH-3′ film (chosen at random). After 20 min at room temperature, the slide was washed vigorously in water, and dried under N_2. The slide was blocked, treated with streptavidin–horseradish peroxidase conjugate, and visualized as described below for ‘Detection of DNA patterns’.

Patterning with two discrete DNA oligomers

DETA/SMPB-modified glass slides were treated with the positive-tone, S1400-27 resist. The mask was positioned on the slide, and exposed and developed (with MF-319) as described above. The slide was treated with 1 µM 5′-biotin-d(ACTG)$_5$- SH-3′ in HEPES buffer, under safelight conditions. After a water rinse and drying under N_2, the slide was then spin-coated with a second layer of S1400-27 resist. The slide was then aligned with the mask in an orientation 180° relative to the first exposure, exposed with 100 mJ/cm^2, developed, rinsed, and treated with a second DNA oligomer [5′-rhodamine-d(ACTG)$_5$-SH-3′, in HEPES buffer] under safelights. The slide was then rinsed in HEPES buffer, rinsed twice in dI water and dried under a stream of N_2. Photoresist was stripped from the slide by treating with acetone (1 min), leaving the patterned DNA surface intact. Patterns were typically visualized prior to, and following, treatment with 1 M NaCl, 50 mM Na-phosphate (pH 6.5), which was used to remove non-covalently bound DNA. Slides were then rinsed three times in water and dried under a stream of N_2.

Typically, slides were evaluated first for the presence of the fluorescent DNA oligomer (using epifluorescent or confocal laser fluorescence microscopy), then treated using a streptavidin–peroxidase conjugate to visualize the biotinylated DNA oligomer. These detection methods are described below.

Hybridization to patterned DNA surfaces

Fused silica substrates were patterned using the direct photopatterning and photoresist patterning methods as described above. For the direct patterned slide, 5′-d(ACTG)-SH-3′ was used to modify an SMPB–PEDA patterned surface. For the photoresist patterned slide, S1400 resist was used to coat an SMPB–DETA treated slide, exposed, developed with MF319, then reacted with 5′-d(ACTG)-SH-3′. Both slides were then treated overnight in SPSC buffer to remove non-specifically bound DNA. The two slides were then blocked with prehybridization buffer [6× SSC (0.09 M Na-citrate, 0.9 M NaCl), 0.01 M Na-phosphate, 1 mM EDTA, 10 µg/ml salmon sperm DNA, pH 7.0] overnight, then hybridized with a solution of 5′-CY3.0-d(CAGT)$_5$-3′ (5 µM, in prehybridization buffer) for 1.5 h at 37°C. The slides were then washed in 1× SSC and then 0.1× SSC, each for 10 min at 37°C, and then rinsed briefly in water. Patterns were observed using confocal fluorescence microscopy as described below.

Detection of DNA patterns

Biotinylated DNA patterns were visualized using a streptavidin–horseradish peroxidase conjugate (SA-HRP, Kierkegaard and Perry, Gaithersburg, MD). Slides were first blocked to prevent non-specific binding of proteins by immersion into glycine blocking buffer (10 mM glycine, 5 mg/ml salmon sperm DNA in 1× PBS buffer, pH 7.0) for 30 min, then washed briefly in two changes of 1× PBS, and once in PBS + 0.05% Tween 80. SA-HRP (initial dilution of 0.5 mg/ml in PBS, then 1:500 dilution in glycine blocking buffer) was pipetted onto the patterned surface of the slides, and incubated at room temperature for 30 min. Slides were washed briefly in two changes of 1× PBS, and once in PBS + 0.05% Tween 80. The slides were then immersed in 1-step tetramethylbenzidine (TMB) membrane blotting substrate (Kierkegaard and Perry) to induce color development. Patterns

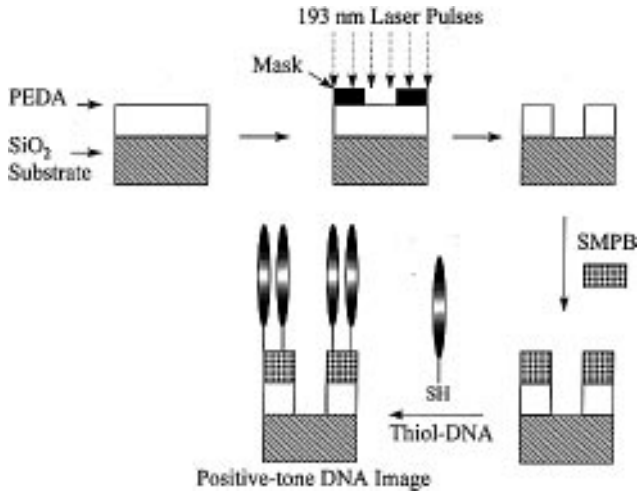

Figure 1. Schematic of the direct, deep UV (193 nm) method for patterning of PEDA films. The patterned PEDA films can subsequently be modified with the crosslinker SMPB, and then a thiolated DNA to form patterned DNA surfaces. An alternate configuration of the process involves deep UV irradiation of a SMPB-modified PEDA film to produce a patterned surface which is then treated with the thiolated DNA oligomer.

Figure 2. Epifluorescent photomicrograph of a line-space pattern formed by reaction of 5′-CY-3.0-d(ACTG)$_5$-SH-3′ with a deep UV patterned SMPB-modified PEDA film formed on fused silica. The photo was taken at 400× magnification, and depicts 2 µm bars of fluorescent DNA separated by 1 µm spaces. The various steps involved in the process are outlined in Figure 1.

were observed/photographed using an optical microscope (Nikon Optiphot) in brightfield mode.

Photomicrographs of fluorescent patterns were obtained on a Ninon Optiphot equipped for epifluorescence with a G-1B 'green' filter block, consisting of an EX546/10 excitation filter, DM580 dichroic mirror and 590 nm barrier filter. Kodak Ektachrome (EPN-135) 1600 ASA film captured the image which was exposed and push processed at 3200 ASA using broad area automatic exposure. Confocal images were collected on a Molecular Dynamics (San Jose, CA) Sarastro 2000 confocal scanning laser microscope. The excitation of CY-3.0, CY-3.5 and rhodamine-labeled DNA at 514 nm was obtained by a 5 mW Ar-ion laser through a 514 nm line filter. The resultant emission between 535 and 570 nm was collected by a photomultiplier after passing through a 535 nm beam splitter and 570 nm barrier filter. Images were collected in 512 × 512 pixel arrays and averaged over three to five scans. Data were corrected for the sample conditions (air mount/air immersion) before analysis. Data were collected, stored and analyzed on a Silicon Graphics (Mt View, CA) Indy graphics workstation. Contrast ratios were determined by comparing the fluorescence intensity of single pixel points within the patterned area to the surrounding region (of similar area). At least 10 random points were selected within a given area and the average signal intensity determined.

RESULTS AND DISCUSSION

Formation of DNA patterns using deep UV laser irradiation of silane films

193 nm ArF laser irradiation was used to alter the PEDA monolayer film on one side of a substrate to define spatially resolved regions of different chemical reactivity on the exposed surface. Photolysis of the Si–C bond of the PEDA molecules results in the exposed regions, and an oxidized Si surface is the by-product (30). The surface at this point may be described as spatially resolved regions of aminosilane film and oxidized substrate. The patterned PEDA substrates were then treated with a heterobifunctional crosslinker which contains two active moieties: a succinimide ester (NH_2-reactive) and a maleimide group (-SH reactive). Neither the crosslinker nor the thiolated DNA oligomer react to a significant extent with the oxidized Si regions produced by film photolysis, so DNA deposition is apparently confined to the unexposed PEDA regions. The sequence of steps employed is outlined in Figure 1. Alternatively, a PEDA film was treated with the crosslinker SMPB prior to laser exposure and the combined PEDA–SMPB film is then irradiated. Using either approach, an identically patterned DNA surface is obtained.

Use of a fluorescently-tagged DNA oligomer allowed the patterns to be viewed by epifluorescent or confocal laser microscopy. In general, patterns were bright enough to be seen with the epifluorescent microscope, but the confocal microscope was used to determine the fluorescence intensity of dye-labeled versus background areas (contrast ratios). Figure 2 illustrates an example of a line–space pattern obtained by depositing a CY3.0-derivatized DNA oligomer onto a laser patterned PEDA–SMPB film. The direct patterning method provides a 'positive-tone image' of the mask, that is, the area of substrate that is protected from irradiation by the mask features is where the PEDA is retained, and hence where the DNA is bound.

Laser irradiation of aminosilane films is an efficient way to produce patterns composed of a covalently-bound, single DNA species. A wide range of features such as lines, letters or numerals with sizes ranging from microns to millimeters can be produced with a labeled DNA molecule. The DNA is apparently confined to the desired areas, with little or no modification observed using fluorescence microscopy in the photodamaged areas. Using the laser patterning method and confocal laser fluorescence microscopy, we have calculated contrast ratios of nearly 10:1 for fluorescent DNA on PEDA regions over the surrounding photolyzed substrate (31).

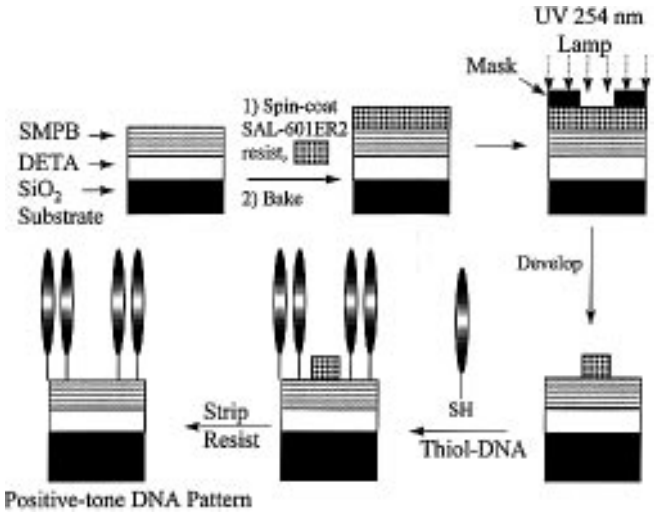

Figure 3. Schematic illustrating the UV lamp exposure of the photoresist SAL-601-ER to form DNA patterns on a DETA silane film. The SMPB crosslinker may be used to modify the photoresist-coated, patterned DETA surface as shown. For each of these approaches, the thiolated DNA reacts only where the crosslinker-modified silane domains are revealed to form positive-tone DNA patterns.

Figure 4. Epifluorescent photomicrograph of fluorescent DNA stripes formed by reaction of 5′-CY-3.5-d(ACTG)$_5$-SH-3′ with an SMPB-modified DETA film on glass. SAL-601-ER2 negative-tone photoresist was used to define the pattern in the SMPB–DETA layer to which the thiolated fluorescent DNA was attached. The various steps involved in the process are outlined in Figure 3. The photo was taken at 400× magnification, and depicts 20 μm (upper portion) and 2.5 μm bars (lower portion) of fluorescent DNA.

Use of photoresists to create patterned, single DNA surfaces

Both negative- and positive-tone photoresists were utilized to create DNA patterns. This is an adaptation of the use of photoresists for selective metallization of silane films with photoresist-defined channels (32). Typically, a glass substrate was treated with DETA, and was then modified with the crosslinker, SMPB. The selected photoresist was then spin-coated onto one side of the silanized substrate. Irradiation of the resist-coated surface through the appropriate mask, followed by development of the resist, permitted the underlying crosslinker-modified silane layer to be revealed. The resulting patterned surfaces were composed of areas bearing resist, and areas of uncovered SMPB which were receptive to covalent modification with a thiolated DNA. This was typically accomplished by pipetting the DNA solution onto the patterned resist/crosslinker-silane surface of the substrate. (Similar DNA patterns were obtained by spin-coating the resist over the DETA film and subsequently modifying the patterned DETA surface with SMPB after exposing and developing the resist.) An attractive feature of the use of the photoresist system to create DNA patterns is that even if significant non-specific attachment of the thiolated DNA to the resist occurs, the resist is stripped from the substrate in acetone, prior to visualization. Although we have not conducted rigorous stability studies, resist-coated SMPB–DETA-modified slides have been prepared and stored in the dark in a dessicator for days before use. Under these conditions, exposure and development of the resist and subsequent treatment with thiolated DNA resulted in DNA patterns which appeared similar to those which had been prepared without delay.

With the positive-tone resist, S1400-27, the DNA is ultimately bound wherever 405 nm light was allowed to impinge on the surface. For the negative-tone resist SAL 601-ER2, the DNA was immobilized wherever the substrate was protected from 254 nm irradiation. Figure 3 summarizes the steps involved in DNA pattern formation using the negative-tone photoresist. Figure 4 illustrates a DNA pattern obtained using SAL 601-ER2 with a CY-3.5-modified fluorescent DNA oligomer. Using the confocal laser fluorescence microscope, a signal-to-noise ratio of 2:1 was determined for the CY-3.5-DNA-modified domains over the SMPB-modified DETA film background.

The negative resist approach yielded low contrast ratios between fluorescent and non-fluorescent domains on the surface. In comparison, the use of the positive photoresist S1400-27 yielded contrast ratios ranging from 3:1 to 7.5:1, depending on the DNA employed. A similar series of steps to that shown in Figure 3 for the negative resist is adopted when the positive resist S1400-27 is employed to create a patterned surface composed of a single DNA species. The use of the S1400-27 resist for fabrication of surfaces composed of two patterned DNA species will be discussed below.

Effects of repeated contact with developers on the DNA films

A potential concern about the utilization of the photoresist approach to create DNA patterns is the use of developers which remove soluble photoresist from the surface and reveal the underlying crosslinker-functionalized aminosilane layer. The resist developers used are aqueous and strongly alkaline (~0.3 N OH^-, pH ≈13.5). Although the developer step for each resist was brief (90 s), hydrolytic loss of DNA [or silane (32)] from the surface was a possibility. This becomes especially important when considering the use of photoresists for sequential modification of a surface with different DNA molecules (see below). To assess the stability of covalently-bound DNA to developer solutions, DETA/SMPB/DNA fused silica slides were treated with each of the developers used (MF-312, MF-319 or Concentrate) for four consecutive cycles of 90 s each. The UV spectrum was recorded before and after each cycle of developer treatment. Note that for these experiments, both sides of the slide are coated with both

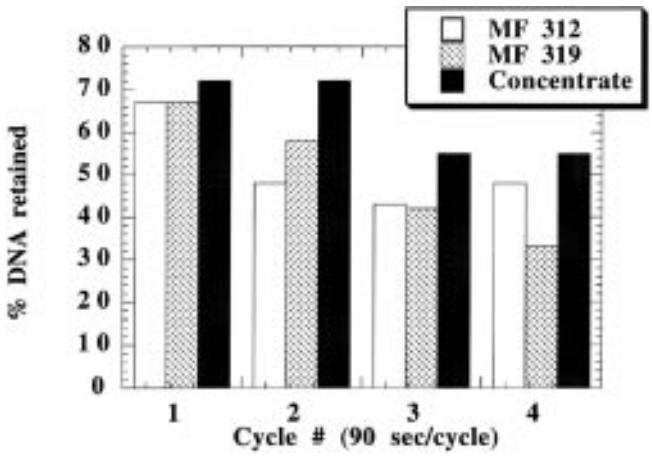

Figure 5. Effect of treatment with various photoresist developers on covalently bound DNA films. SMPB-modified DETA films were used to immobilize 5′-d(ACTG)$_5$-SH-3′. The A_{260} values determined after each cycle of developer treatment were divided by the initial A_{260} value and multiplied by 100 to yield the percent DNA retained on the slide.

crosslinker and DNA, thus the UV spectrum observed is for two DNA films, unlike the patterning experiments in which only one side of the slide is treated with DNA. Figure 5 illustrates that there is an initial loss of ~25–35% of the DNA from the surface after the first developer treatment step. Subsequent treatments (cycles 2–4) also resulted in some loss of DNA from the surface, although for MF-312 and Concentrate there was little difference between cycles 3 and 4. The developer which least affected the DNA film was the Microposit concentrate. With this developer ~55% of the original DNA was retained after four cycles, and the amount of DNA remaining appears to have leveled off. Microposit Concentrate therefore was the developer of choice when using the S1400-27 resist system. It should be noted however, that even films which had been treated with MF-322 developer for four 90 s cycles remained able to hybridize with a complementary, biotinylated oligomer (33). Aliquots of a 5′-biotin-d(CAGT)$_5$-3′ DNA were pipetted onto the surface of an 5′-d(ACTG)$_5$-SH-3′ slide following repetitive treatment with MF-322 developer, and permitted to hybridize (in water, at room temperature) in a humidified chamber to avoid drying of the aliquots. The slide was subsequently blocked, treated with a streptavidin–peroxidase (SA-HRP) conjugate, and visualized with a precipitable colorimetric substrate, which turns blue in the presence of the SA-HRP. Discrete blue spots were observed where the 5′-biotin-d(CAGT)$_5$-3′ had been permitted to hybridize to the 5′-d(ACTG)$_5$-SH-3′ film (data not shown).

Fabrication of patterned surfaces bearing two different DNA species

The creation of surfaces bearing patterns of more than one discrete DNA species is considerably more challenging than the patterned single species case. We sought to develop a method which was simple, did not involve *de novo* oligomer synthesis (14–18) or a micropipettor-based deposition step (3,19–22), and yet was flexible enough to permit the creation of a wide range of high resolution patterns. Once it was shown that the photoresist developers could be used repetitively while retaining most of DNA on the surface, the S1400-27 photoresist system was chosen for creating surfaces bearing patterns from two different DNA oligomers. To verify that both of the DNA oligomers were deposited in discrete patterns, and to assess the potential for

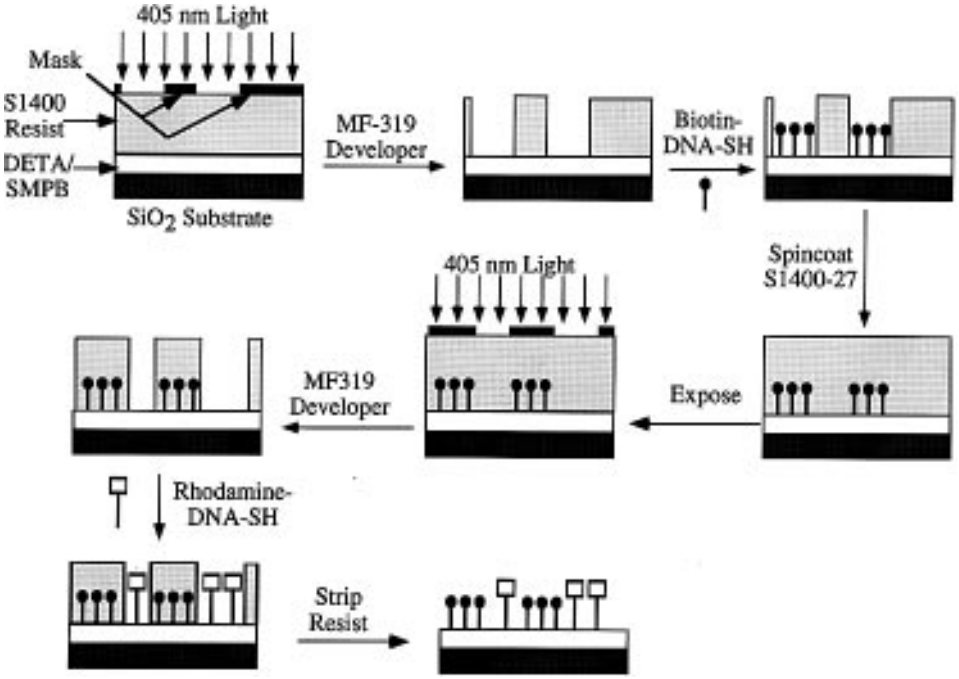

Figure 6. Schematic depicting the process whereby two DNAs are sequentially deposited onto a slide which had been treated first with DETA and SMPB, then spin-coated with the positive-tone photoresist S1400-27. 5′-biotin-d(ACTG)$_5$-SH-3′ is deposited after exposure of the assembly to a UV lamp. After spincoating a second layer of the photoresist, re-positioning the mask, exposure and development of the assembly is followed by treatment with 5′-rhodamine-d(ACTG)$_5$-SH-3′.

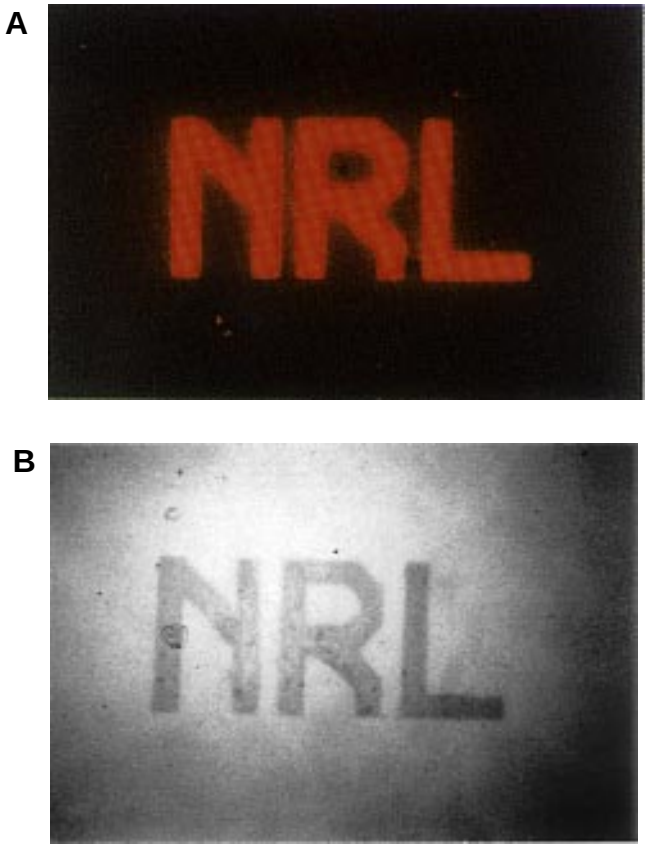

Figure 7. An example of a surface patterned with two differentially labeled DNA oligomers. The photoresist approach for pattern formation was employed as detailed in Figure 6. The patterned DETA/SMPB slide was immersed in a solution of 5′-biotin-d(ACTG)$_5$-SH-3′. Following the application, exposure and development of the second S1400-27 layer, the slide was treated with a solution of 5′-rhodamine-d(ACTG)$_5$-SH-3′. The remaining photoresist was stripped from the slide before viewing. The slide was visualized first using confocal laser fluorescence microscopy (**A**), then was subjected to streptavidin–peroxidase–TMB treatment to visualize the biotinylated DNA (**B**). The two depictions of the 'NRL' (one with rhodamine, and the other with biotin) were separated from each other by <200 µm on the slide, but could not be imaged simultaneously due to different detection methods required for the two labels. Letters are 250 µm in length.

crosstalk between patterned oligomers, two distinguishable oligomer labels were employed. The first oligomer deposited was biotin-labeled, and the second oligomer carried a fluorescent label (rhodamine). Figure 6 illustrates the sequence of steps required to immobilize two DNA species on a single surface. The first (biotinylated) DNA was immobilized as described for the S1400-27 system in the Materials and Methods section. Prior to exposing the slide a second time, the patterned surface of the slide was spin-coated with another layer of resist, effectively covering over the first DNA layer. The second exposure was performed after re-positioning the mask. The dose required to clear the resist for this second exposure was twice that needed for the first exposure, possibly due to chemical changes in the resist due to contact with the developer. The slide was then developed, treated with the rhodamine-labeled DNA, and the resist was removed from the entire slide. The location and contrast ratio of the fluorescent DNA was documented using confocal laser fluorescence microscopy, and then the position of the biotin DNA was determined by using a streptavidin–peroxidase conjugate. Figure 7 illustrates the high resolution deposition and confinement of different DNA species within discrete areas on the same surface. For the rhodamine–DNA domains, fluorescence contrast ratios of 7.5:1 were obtained. By comparision, the biotin–DNA domains (which are non-fluorescent) gave fluorescence signal-to-noise ratios of <1 (i.e., same as background). This demonstrates that no cross-reaction of the fluorescent DNA (which had been deposited second) occurred in areas occupied by the first (biotinylated) DNA. After the slide was treated with SA-HRP to visualize the biotinylated DNA regions, it was clear that the biotin DNA was located only in the discrete regions where it had been initially deposited. Immobilization of the two different DNAs without an intervening layer of photoresist led to significant cross-reaction. The fluorescent rhodamine–DNA (which had been deposited second) had infiltrated into the areas occupied by the biotin DNA (which had been deposited first). However, it may be possible to eliminate the second photoresist deposition step by either capping or blocking available reactive sites after depositing each DNA oligomer. Presumably, crossreactivity (by oligomer #2) occurs because there are maleimide groups still available in the site occupied by oligomer #1. It may be possible to quench the excess maleimide groups chemically with a thiol prior to exposing a through new region of the photoresist to deposit a new thiolated DNA. This approach and others are currently under investigation in our laboratory.

Hybridization to patterned DNA surfaces

Patterned slides prepared using both the direct patterning and photoresist patterning methods were reacted with 5′-d(ACTG)$_5$-SH-3′, then blocked to prevent non-specific binding of the labeled complementary DNA oligomer. After prehybridization, the two slides were treated with 5′-CY3.0-d(CAGT)$_5$-SH-3′ oligomer in a 6× SSC buffer, then washed in buffers of lower ionic strength. Fluorescent patterns were detected using confocal laser microscopy on each substrate (Fig. 8). Slides were then heated at 37°C for 1 h in H_2O to reverse hybridization; following this treatment there was still fluorescent DNA patterns visible, but the signal was greatly decreased relative to before heating (not shown). After overnight treatment in SPSC buffer [to remove non-specifically bound DNA, (24)], no patterns were visible. These results confirm that the patterned DNA surfaces generated using the methods described are indeed functional for hybridization despite the various steps involved in pattern generation.

CONCLUSION

Two methods of creating patterned DNA surfaces were developed that involve either direct, deep UV irradiation of the photolabile silane film, or the use of photoresists to mask the underlying activated crosslinker-modified silane film. Sequential photoresist exposure and development, with treatment of the activated silane film revealed at the end of each cycle with differentially labeled nucleic acid oligomers, has permitted the fabrication of patterned surfaces bearing two different DNAs. Feature sizes on the micron scale have been achieved using standard contact printing, with both the direct deep UV patterning and photoresist patterning methods.

Compared with other DNA array fabrication techniques, the silane patterning methods described here offer many advantages, such as the ability to deposit purified oligomers, resolution at the micron scale, use of commercially available materials and simple lamp or laser-based contact lithography techniques (that are compatible with automated processing). Although we have at

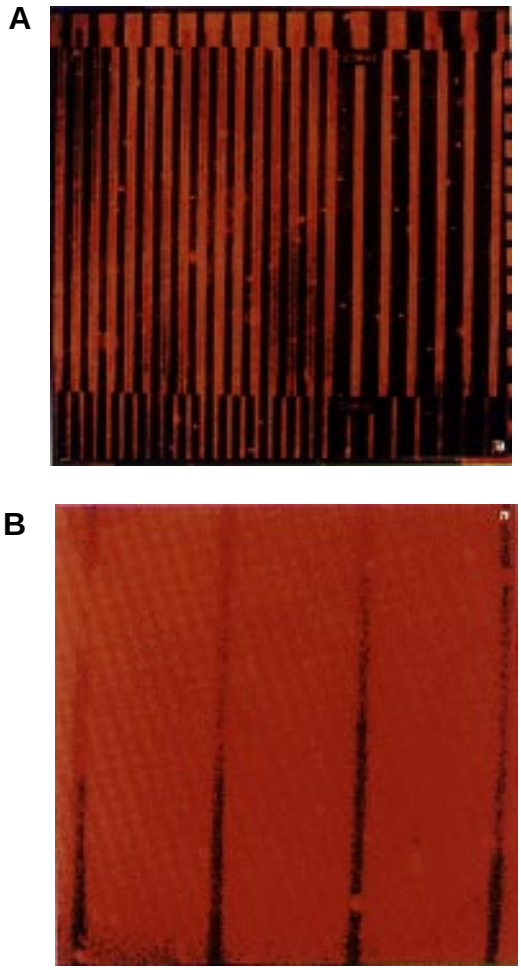

Figure 8. Examples of patterned DNA substrates which have been hybridized with a fluorescently labeled complementary DNA oligomer. Both a direct photopatterned slide (A) and a photoresist patterned slide (B) were treated with 5′-d(ACTG)$_5$-SH-3′ to effect covalent attachment. After prehybridization, slides were immersed in a 5 µM solution of 5′-CY3.0-d(CAGT)$_5$-3′, and hybridized at 37°C for 1.5 h. Slides were observed using confocal laser fluorescence microscopy. (**A**) Hybridization of 5′-CY3-d(CAGT)$_5$-3′ to a direct photopatterned 5′-d(ACTG)$_5$-SH-3′ DNA surface. Lines and spaces of varying dimensions. Center portion of image shows 20 µM lines separated by 20 µM (left) or 40 µM (right) spaces. (**B**) Hybridization of 5′-CY3-d(CAGT)$_5$-3′ to a photoresist photopatterned 5′-d(ACTG)$_5$-SH-3′ DNA surface. Bars are 290 µM wide, separated by 29 µM spaces.

present demonstrated immobilization of two discrete DNA species we are developing ways to extend these patterning methods to efficiently fabricate more complex DNA patterns or arrays. These approaches may be best suited for the spatially resolved immobilization of specific DNA oligomers in a low-to-medium density array for hybridization-capture based applications rather than for construction of very high density DNA arrays which are useful for sequencing-by-hybridization (3) or ligand screening applications (14).

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