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Published on Web 01/28/2006

Rewritable DNA Microarrays

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Abstract: Thiol-terminated single-stranded deoxyribonucleic acids (ssDNA) can be immobilized onto pulsed plasma deposited poly(allylmercaptan) surfaces via disulfide bridge chemistry and are found to readily undergo nucleic acid hybridization. Unlike other methods for oligonucleotide attachment to solid surfaces, this approach is shown to be independent of substrate material or geometry, and amenable to highly efficient rewriting.

1. Introduction

Surface immobilized deoxyribonucleic acid (DNA) has attracted tremendous attention due to its base pairing mechanism, which can store hereditary information and regulate gene expression.[1] Potential applications include DNA strands acting as a principal structural component or as a mediator for tethering ligands or particles[2,3] in the fields of biosensing,[4,5] nanotechnology,[6,7] DNA sequencing,[8] anti-sense agents,[9] molecular beacons,[10] DNA computing,[11] and probes for measuring gene expression in the form of oligonucleotide microarrays (DNA chips[12,13]). The latter can provide a highly parallel, addressable, miniaturized array format, which offers significant advantages over traditional gel-based methods in terms of reagent cost, labor, speed of throughput, and operational simplicity. In most DNA chip applications, the oligonucleotide arrays effectively capture the target sequences and/or detect probes via hybridization reactions.[14,15] The development of efficient surface chemistries for the manufacture of spatially resolvable microscale DNA arrays onto solid-supports has become essential for the realization of DNA chip technology. Parameters that govern the development of useful and reliable chemistries for DNA microarray fabrication include the accessibility and functionality of surface-bound DNA strands, density of attachment, and the stability of the array.

So far, two generic approaches have evolved for making DNA arrays: first, base-by-base attachment to build different DNA strands at different sites in the array,[16,17] or, alternatively, the attachment of different complete strands to individual array sites.[18] In the former case, the methods currently available for DNA strand synthesis on the surface are limited in terms of oligonucleotide purification following synthesis (this being necessary to eliminate incomplete strands or ones with an excess number of bases). On the other hand, attachment of complete DNA strands to a surface offers a number of distinct advantages, of which the most important is the fact that the oligonucleotides can be rigorously purified prior to surface immobilization.

Several methods exist for immobilizing single-stranded (ss) DNA oligonucleotides onto solid surfaces. These are predominantly multistep wet chemical reactions for silicon,[19−21] silica,[19,22] and gold[23−27] substrates. The two most promising approaches have been direct assembly of thiol-terminated ssDNA molecules onto gold[28,29] (this tends to suffer from the nonspecific adsorp-

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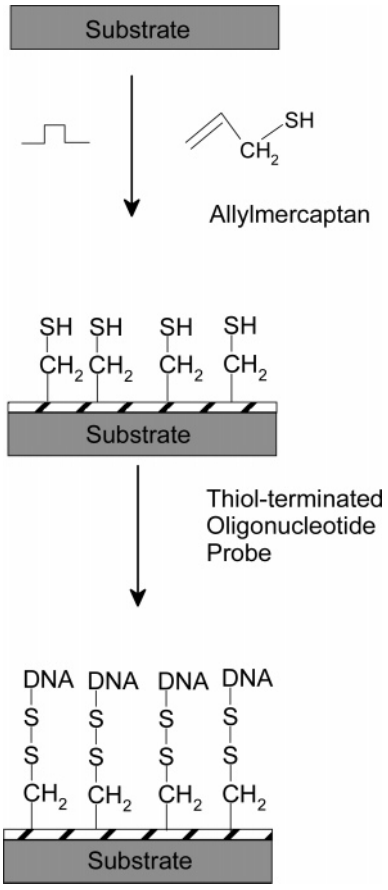
10.1021/ja056367r CCC: $33.50

tion of ssDNA onto the bare gold surface), and linking of thiol-DNA via the heterobifunctional linker sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SSMCC) to an 11-mercaptoundecanoic acid/poly-L-lysine bilayer.[30−33] To position the ssDNA sufficiently far away from the surface so that steric hindrance does not interfere with the hybridization adsorption process, a spacer region is prerequisite between the 5′-thiol modifier and the variable base sequence. However, the substrate-specific nature of such chemistries[34,35] prohibits their widespread applicability to other materials.

In this Article, we describe a substrate-independent methodology for the fabrication of ssDNA microarrays based on disulfide bridge formation between thiol-terminated DNA strands and pulsed plasma deposited poly(allylmercaptan) surfaces. This plasmachemical functionalization technique entails modulating the electrical discharge on the millisecond−microsecond time scales.[36] For gaseous precursors containing polymerizable carbon−carbon double bonds, there are two distinct reaction regimes corresponding to the plasma duty cycle on- and off-periods (microsecond and millisecond, respectively),[37] where the short on-time generates active sites in the gas phase and at the substrate surface (via UV irradiation, ion, or electron bombardment), while conventional polymerization occurs during the subsequent prolonged off-time (in the absence of any UV-, ion-, or electron-induced damage). This can yield extremely high levels of structural retention and incorporation of specific functionalities at the surface. Furthermore, by programming the pulse duty cycle, it is possible to control (i.e., tailor) the surface density of desired chemical groups (in contrast to conventional grafting techniques). The functional films are covalently attached to the substrate via free radical sites created at the interface during the initial stages of plasma exposure. Examples devised in the past include anhydride,[37] epoxide,[38] carboxylic acid,[39] cyano,[40] hydroxyl,[41] furfuryl,[42] and perfluoroalkyl groups.[43] Effectively, any surface which relies on a specific functionality for its performance can, in principle, be generated by the aforementioned pulsed plasmachemical methodology. In the present study, thiol functional groups are generated for disulfide bridge formation with oligonucleotides, Scheme 1. Furthermore, the ability to successfully use and reuse the same thiol functionalized substrate via stripping and hybridization/denaturization mechanisms is demonstrated.

Scheme 1. Attachment of Thiol-Terminated Oligonucleotide to Pulsed Plasma Deposited Poly(allylmercaptan) Layer via Disulfide Bridge Formation



2. Experimental Section

Plasma polymerization of allylmercaptan (Aldrich, +80%, further purified using freeze−pump−thaw cycles) was carried out using a cylindrical glass reactor evacuated by a rotary pump via a liquid nitrogen cold trap (base pressure = 1×10^{-3} mbar and leak rate = 9.9×10^{-9} mol s^{-1}). A copper coil wrapped around the reactor was connected to a 13.56 MHz radio frequency power supply via an LC matching network. The whole system was enclosed in a Faraday cage. Prior to each experiment, the chamber was cleaned by scrubbing with detergent, rinsing in water, and then running an air plasma at 50 W power and 0.2 mbar pressure. Next, the substrate of interest was placed into the center of the reactor (i.e., cotton, polystyrene microspheres, paper, silk, glass slides, or polished silicon (100) wafer). Allylmercaptan vapor was then introduced into the chamber via a fine needle control valve at a pressure of 0.2 mbar and 1.7×10^{-7} mol s^{-1} flow rate, followed by ignition of the electrical discharge. Film deposition was allowed to proceed for 10 min, and then the power supply was switched off while maintaining allylmercaptan flow through the reactor for 5 min to quench any remaining active sites. In the case of pulsed plasma polymerization, a signal generator was used to trigger the RF supply at 40 W peak power in conjunction with pulse on- and off-periods set to 100 μs and 4 ms, respectively, following optimization. The pulse shape was monitored with an oscilloscope. Film thickness could be controlled down to 5 nm by simply reducing the deposition period.

Thiol-terminated 15 base oligonucleotide Probes I and IV (Table 1) were diluted in sodium chloride/sodium citrate (SSC) buffer (3 M NaCl, 0.3 M Na citrate−$2H_2O$, pH 4.5, Aldrich) to a final concentration of between 50 and 400 nM. The oligonucleotide buffered solution was dropped onto freshly deposited pulsed plasma poly(allylmercaptan) films using a pipet. These surfaces were subsequently incubated for

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Table 1. Oligonucleotide Sequences (Sigma-Genosys Ltd.) Employed

oligonucleotide label	base sequence (5′ end to 3′ end)	fluorophore used	linker
Probe I	GCTTATCGAGCTTTC	N/A	5′-HS−$(CH_2)_6$−
Probe II	GCTTATCGAGCTTTC	3′-[CY5]−	5′-HS−$(CH_2)_6$−
Probe III	GAAAGCTCGATAAGC	5′-[CY5]−	N/A
Probe IV	CGAATAGCTCGAAAG	N/A	5′-HS−$(CH_2)_6$−

16 h at 20 °C in a humidified chamber (64% relative humidity) to prevent spot drying (which can lead to uneven distribution) and then washed three times with water and buffer solution. Finally, the surfaces were dried with nitrogen gas.

DNA microarrays were fabricated using a robotic microarrayer (Genetix Inc) equipped with 1 nL delivery micro-machined pins. 200 nM buffered ssDNA solution was spotted onto pulsed plasma poly(allylmercaptan) coated glass slides (18 × 18 × 0.17 mm, BDH) and commercial mercaptosilane-coated glass slides (CEL Associates Inc., Texas) for comparison. Typical circular spots with diameters ranging from 100 to 125 μm and with a minimum print pitch of 110 μm could be routinely obtained. After spotting, the oligonucleotide immobilized slides were kept in a humidity chamber (64% relative humidity) for 24 h at 20 °C. Finally, the slides were removed and washed with buffer solution and copious amounts of water (pH = 7 to remove salt species for XPS analysis and also ensure that the fluorescence signal was not due to DNA trapped in salt species). Probes I, II, and IV were used in the spotting process, Table 1.

DNA microarrays and fully immobilized surfaces of Probes I and IV were subsequently exposed to a solution of Probe III in hybridization buffer (consisting of 1.8 M SSC pH 7, Denhardt's solution (1% w/v BSA (bovine serum albumin), 2% Ficoll 400, and 2% polyvinylpyrollidone (PVP)), 0.5% sodium dodecyl sulfate (SDS), and sheared salmon sperm DNA (55 μg/mL) to a final concentration of 400 nM). Two small strips of adhesive tape were affixed along the rim of the DNA oligonucleotide Probes I and IV slides and then covered with a cleaned microscope slide cover glass. The cavity that formed between the chip and the cover glass was slowly filled by loading 10 μL of the hybridization solution by capillary action. The slide was then incubated in a hybridization chamber (100 μL of water was placed next to the chip). Afterward, the cover glass was removed and the chip washed three times with buffer solution, followed by copious rinsing in water, and blow-drying with nitrogen gas.

Stripping of the bound oligonucleotides from the pulsed plasma poly(allylmercaptan) and mercaptosilane-coated slides was investigated by placing the chips in a boiling solution of 200 nM TrisCl pH 7.0, 0.03 M SSC and 0.1% (w/v) SDS for 10 min. Alternatively, denaturization of hybridized DNA strands to leave behind the original surface-bound oligonucleotide entailed placing the substrate in 0.1% (w/v) SDS for 10 min.

Film thickness measurements were carried out using an nkd-6000 spectrophotometer (Aquila Instruments Ltd.). Transmittance−reflectance curves (over the 350−1000 nm wavelength range) were fitted to the Cauchy model for dielectric materials using a modified Levenburg−Marquardt method.[44]

A VG Escalab spectrometer equipped with an unmonochromated Mg Kα X-ray source (1253.6 eV) and a concentric hemispherical analyzer was used for X-ray photoelectron spectroscopy (XPS) analysis of the functionalized surfaces. Elemental compositions were calculated using sensitivity (multiplication) factors derived from chemical standards, C(1s):P(2p):S(2p):O(1s):N(1s) equals 1.00:0.66:0.52:0.63:0.45. All binding energies were referenced to the C(1s) hydrocarbon peak at 285.0 eV. A Marquardt minimization computer program was used to fit core level envelopes with fixed width Gaussian peak shapes.[45]

Table 2. XPS Atomic Percentage for Deposited Allylmercaptan Pulsed Plasma Polymer Layers

conditions	composition (%) C	N	O	P	S
allylmercaptan pulsed plasma	76 ± 1.0	0	0	0	24 ± 1.0
allylmercaptan (theoretical)	75.0	0	0	0	25.0
oligonucleotide Probe I on allylmercaptan plasma polymer	46 ± 1.0	10 ± 1.0	24 ± 1.0	4 ± 1.0	16 ± 1.0
oligonucleotide Probe I (theoretical)	49.4	13.3	32.3	4.8	0.16

Fourier transform infrared (FTIR) analysis of the films at each stage of reaction was carried out using a Perkin-Elmer Spectrum One spectrometer equipped with a liquid nitrogen cooled MCT detector operating across the 700−4000 cm^{-1} range. Reflection−absorption (RAIRS) measurements were performed using a variable angle accessory (Specac) set at 66° in conjunction with a KRS-5 polarizer fitted to remove the s-polarized component. All spectra were averaged over 5000 scans at a resolution of 1 cm^{-1}.

Fluorescence quantification was achieved using a Dilor Labram microscope by irradiating a 20 mW He−Ne laser at a wavelength of 633 nm (which corresponds to the excitation frequency of the Cy5 fluorophore) and a polarization of 500:1 through the microscope objective (×10). The corresponding fluorescence signal was collected through the same objective via a backscattering configuration in combination with a cooled CCD detector. The diffraction grating was set at 1800 lines mm^{-1} with the laser filter at 100% transmission.

The microarrays were scanned using a fluorescence imager, Gene Tac LS IV Biochip Analyzer (Genomic Solutions Inc., Huntingdon, Cambridgeshire, UK). The excitation (633 nm) and detection wavelengths (650 nm) selected were based on the dye label used. All images were recorded at a resolution of 10 μm, and scans were acquired from 10 repeat acquisitions of data per point.

3. Results

Evidence for the viability of the disulfide bridge chemistry employed for oligonucleotide immobilization onto solid supports was obtained using XPS and FTIR analysis. XPS characterization of the pulsed plasma deposited poly(allylmercaptan) layers (200 nm) confirmed the presence of only carbon and sulfur at the surface, with no Si(2p) signal from the underlying silicon substrate showing through, Table 2.

The S(2p) peak for the pulsed plasma polymer film is centered at a binding energy of 163.7 eV, which is consistent with thiol as the sulfur species.[46,47] In contrast, pulsed plasma deposited poly(allylmercaptan) film, exposed to the oligonucleotide Probe I, indicated the presence of a range of elements pertaining to DNA attachment, including carbon, nitrogen, oxygen, sulfur (as sulfide or disulfide centers, 161.4 eV[46,47]), and phosphorus centers, Table 2. The packing density of DNA at the surface could be varied by diluting the solution with buffer, Figure 1.

For the ssDNA Probe I oligonucleotide under investigation, the surface concentration of nitrogen (%N) was found to correlate with the degree of dilution. Surface saturation levels corresponded to dilutions above 150 nM. There was no evidence obtained by XPS of any sodium or chlorine remaining from

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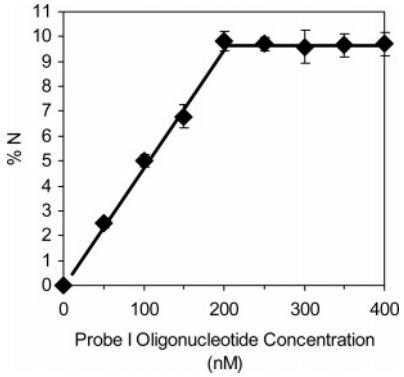

Figure 1. Surface nitrogen concentration (% N) for deposited pulsed plasma poly(allylmercaptan) layer following DNA Probe I immobilization as a function of solution dilution.

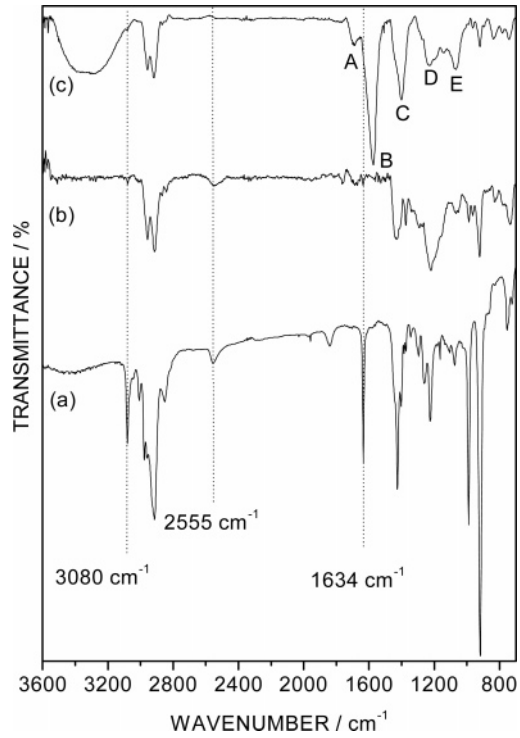

Figure 2. Infrared spectra of: (a) allylmercaptan monomer; (b) allylmercaptan pulsed plasma polymer layer ($P_p = 40$ W; $t_{on} = 100$ μs; $t_{off} = 4$ ms; deposition time = 10 min); and (c) DNA Probe I bound from 200 nM solution onto (b).

the buffer solution. The binding energy shift of the S(2p) peak from 163.7 to 161.4 eV can be attributed to successful oligonucleotide Probe I immobilization onto the surface, Scheme 1. The relatively high surface concentration of sulfur can be attributed to the XPS probe depth exceeding the length of the DNA strands, and therefore sampling the underlying sulfur-rich pulsed plasma polymer layer as well (this was confirmed by angle-resolved XPS studies).

The following infrared band assignments can be made for the allylmercaptan monomer:[48] allyl C−H stretch (3080 cm^{-1}); allyl CH_2 stretch (3031 cm^{-1}); alkyl CH_2 stretch (2891 cm^{-1}); thiol S−H stretch (2555 cm^{-1}); and allyl C═C stretch (1634 cm^{-1}), Figure 2. All of the bands associated with the allylmercaptan monomer are clearly discernible following pulsed plasma polymerization, except for the allyl carbon−carbon double bond features, which disappear during polymerization. Retention of the thiol feature confirmed structural retention had occurred during pulsed plasma deposition conditions.

Immobilization of oligonucleotide Probe I onto the pulsed plasma poly(allylmercaptan) film surface was confirmed by FTIR studies showing attenuation of the thiol S−H stretch (2555 cm^{-1}), and the inclusion of several nucleic acid peaks:[49,50] amide carbonyl stretching C═O at 1694 cm^{-1} (A), nucleic acid base amine N−H stretching at ca. 3360 cm^{-1}, in-plane ring vibration mode of C−H and C═N, and the bending vibration mode of C−H and N−H at 1574 cm^{-1} (B), in-plane imidazole ring vibration at 1415 cm^{-1} (C), pyrimidine (mainly thymine) ring vibration at 1278 cm^{-1} (D), and the symmetric stretching vibration of PO_2^- at 1022 cm^{-1} (E), Figure 2. No signals from the sodium citrate buffer solution were detected.

To determine the density and specificity of covalent oligonucleotide surface immobilization via the disulfide bridge linkages, fluorescently labeled oligonucleotide Probe II was attached to allylmercaptan pulsed plasma polymer layers and also a commercially available thiol-modified glass surface. Reactions were performed with oligonucleotide concentrations ranging from 50 to 400 nM. Quantitative measurements of the attachment density were obtained via direct fluorescent counting and show that, at lower oligonucleotide concentrations, the attachment efficiency is directly proportional to the concentration of the oligonucleotide solution, Figure 3. The attachment density reaches a plateau at an oligonucleotide concentration of approximately 200 nM. This fits in well with the XPS measurements, Figure 1. It is also evident that the density of oligonucleotide attachment onto the commercial slide is lower as compared to the pulsed plasma polymer layer at the same solution concentration of oligonucleotide.

A major factor in oligonucleotide array preparation is the accessibility and specificity of the surface-bound probe toward hybridization. Overloading the surface with probes can cause crowding effects, which can lower the accessibility of the surface-bound probes for hybridization with the target probes. To evaluate the accessibility and specificity of the surface-bound oligonucleotides for hybridization as a function of surface-bound probe density, the fluorophore labeled oligonucleotide Probe III was hybridized to different densities of capture probe oligonucleotide surfaces, Probe I. The signal intensity of the fluorophore as measured by laser excitation (at 633 nm) indicated that the hybridization efficiency correlated directly with the immobilized probe attachment density, Figure 3. It was found that maximum hybridization to the target template occurred when using a 150 nM immobilizing solution of Probe I. An overcrowding effect exceeding 150 nM concentrations is evident for the pulsed plasma deposited poly(allylmercaptan) when comparing Figures 1 and 3a versus Figure 3b. In the case of the commercial slide, a maximum hybridization to the target template was observed at 200 nM solutions; however, the optimum hybridization efficiency value measured for the pulsed plasma poly(allylmercaptan) coated slides was approximately 67% higher as determined by fluorescence measurements.

Fluorescence microscopy was used to assess the viability of producing DNA microarrays using pulsed plasma deposited

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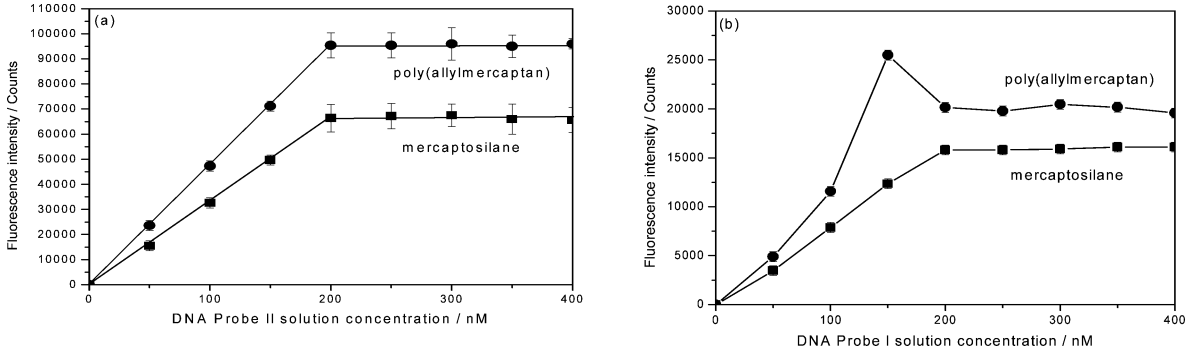

Figure 3. Fluorescence intensity at the surface as a function of oligonucleotide Probe solution concentration: (a) Probe II, and (b) Probe I followed by hybridization with Probe III (fixed concentration of 400 nM), where the poly(allylmercaptan) is pulsed plasma deposited and the mercaptosilane is a commercial slide.

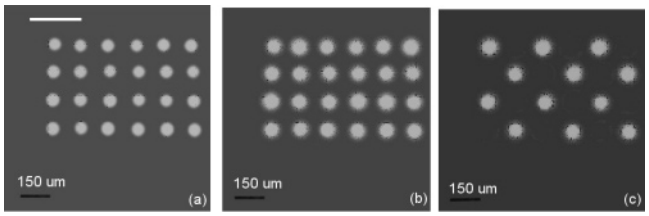

Figure 4. Fluorescence microscope images of micro-spotted arrays produced on allylmercaptan pulsed plasma polymer layer: (a) oligonucleotide Probe II (200 nM); (b) oligonucleotide Probe I (150 nM) subsequently hybridized with oligonucleotide Probe III (400 nM); and (c) oligonucleotides Probe I (150 nM) and IV (150 nM) spotted in an alternating pattern and subsequently hybridized with oligonucleotide Probe III (400 nM).

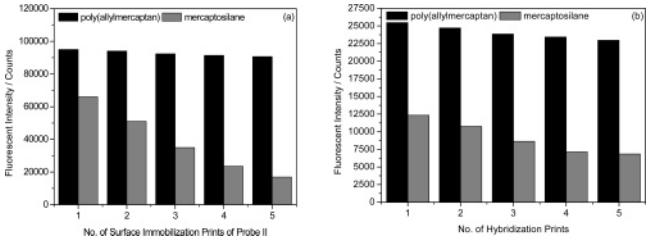

Figure 5. Fluorescence intensity at the surface: (a) after stripping and re-immobilization of oligonucleotide Probe II (150 nM); and (b) denaturization of oligonucleotide Probe III from surface-bound oligonucleotide Probe I (150 nM) and then re-exposure to a solution of Probe III (400 nM). The fluorescence signal dropped to the background level for each fully stripped/denatured sample between their rewriting.

poly(allylmercaptan) layers as the substrate. In the case of immobilization of oligonucleotide Probe II, a fluorescent image of an array of spots with an average diameter of 125 μm and a print pitch of 150 μm was consistent with the spotting parameters employed, Figure 4a. In the case of hybridization arrays, a similar pattern was obtained, thereby indicating that immobilization of thiol-labeled oligonucleotides is thiol group specific, and the nucleic acid backbone does not contribute to the attachment, Figure 4b. Additionally, no measurable hybridization signal from the noncomplementary control oligonucleotide (Probe IV) was detected, attributing the important faculty of base pair mismatching to these manufactured DNA microarrays/chips, Figure 4c.

It was also observed that less than 5% of the disulfide bound oligonucleotides were detached from the solid support when treated with the hybridization salts used in this experiment for 48 h at room temperature.

The potential reusability of the chips was investigated by stripping using high-stringency washes and low-stringency washes to remove immobilized and hybridized oligonucleotides from the surface, respectively. In the first instance, the removal of the immobilized oligonucleotides by stripping was followed by another set of immobilization procedures, and the fluorescent intensity dropped by only 5% from the first experiment when allylmercaptan pulsed plasma polymer layers were used as the substrate, Figure 5a.

In fact, the chip could easily be used five times without significant loss of immobilization efficiencies. This was not the case for the mercaptosilane commercial slides. A similar trend was found for the reusability of the chips with regard to denaturing/rehybridization experiments, Figure 5b. This confirms that both the poly(allylmercaptan)−oligonucleotide covalent bond (immobilization) and the oligonucleotide−oligonucleotide hydrogen bonds (hybridization) are very durable. The low level of immobilization observed for the mercaptosilane commercial slides, and the subsequent degradation, can be attributed to the instability of silane coupling to glass at high pH in aqueous solutions.[51]

DNA was also immobilized in a similar fashion onto a variety of other substrates (silk, paper, cotton, nonwoven polypropylene, glass microspheres, and polymer beads) using the pulsed plasmachemical functionalization approach.

4. Discussion

The formation of robust, reproducible arrays of oligonucleotides tethered to solid substrates is an essential requirement for the development of large arrays of DNA molecules immobilized onto solid surfaces for the rapid screening of DNA sequences.[52,53] While many of the elementary procedures for DNA microchip technology are already established, quite some headway can still be made toward improved quality and regular application. The development of flexible rewritable plasma polymer substrate systems described here is a significant advancement in the field.

Mechanistically, pulsed plasmachemical surface functionalization using allylmercaptan precursor consists of two sequential reaction regimes within each duty cycle.[54] First, there is a short burst of plasma (microseconds) generating active sites (e.g., free radical centers) in the gas phase and also at the substrate surface

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produced by the UV, ion, and electron constituents of the electrical discharge. Subsequently, the prolonged extinction period (milliseconds) allows fresh allylmercaptan molecules to undergo conventional graft polymerization at these activated centers via their carbon−carbon double bond (in the absence of any UV-, ion-, or electron-induced damage). The excellent adhesion of these functional films to the substrate stems from the free radical sites generated at the plasma−solid interface during ignition of the electrical discharge, giving rise to covalent attachment.[55]

One imperative objective of this study was to investigate the reusability of the DNA arrays attached to these pulsed plasma poly(allylmercaptan) surfaces. By design, all DNA fragments were covalently bound to the surface, Table 2 and Figure 2. Reusability was confirmed by subjecting the chips to the conditions of standard hybridization and stripping procedures carried out at neutral pH, Figure 5. In every case, the DNA microarrays withstood consecutive cycles of stripping without significant loss of rewriting density. Therefore, multiple probes can be patterned simultaneously or consecutively onto the surface using a simple robotic liquid-delivering system, with no cross-contamination problems between different probes. An array-manufacturing process employing this chemistry could be easily automated and scaled up. The superior oligonucleotide immobilization surface density and compliance to rewriting for the plasma deposited coatings as compared to the state-of-the-art mercaptosilane slides can be attributed to the greater flexibility and penetrable three-dimensional nature of the plasma polymer nanolayer as compared to the rigid mercaptosilane derived surfaces. This should be of particular benefit for DNA computing devices.

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5. Conclusions

Pulsed plasma deposited poly(allylmercaptan) films can be employed for the immobilization of thiol-terminated single strand DNA oligonucleotides. A wide range of substrate materials and geometries can be functionalized using this approach. Furthermore, these thiol-functionalized surfaces are highly stable toward the stripping/rewriting of immobilized DNA oligonucleotides as well as denaturing/rehybridization cycling.

Acknowledgment. J.P.S.B. is grateful to the EPSRC for an Advanced Research Fellowship.

JA056367R