Fabrication of DNA Microarrays on Nanoengineered Polymeric Ultrathin Film Prepared by Self-Assembly of Polyelectrolyte Multilayers

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Microarray-based technology is in need of flexible and cost-effective chemistry for fabrication of oligonucleotide microarrays. We have developed a novel method for the fabrication of oligonucleotide microarrays with unmodified oligonucleotide probes on nanoengineered three-dimensional thin films that are deposited on glass slides by consecutive layer-to-layer adsorption of polyelectrolytes. Unmodified oligonucleotide probes were spotted and immobilized on these multilayered polyelectrolyte thin films (PET) by electrostatic adsorption and entrapment on the porous structure of the PET film. The PET provides higher probe binding capacity and thus higher hybridization signal than that of the traditional two-dimensional aminosilane and poly-L-lysine coated slides. Immobilized probe densities of 3.4×10^{12}/cm^2 were observed for microarray spots on PET with unmodified 50-mer oligonucleotide probes, which is comparable to the immobilized probe densities of alkyamine-modified 50-mer probes end-tethered on an aldehyde-functionalized slide. The study of hybridization efficiency showed that 90% of immobilized probes on PET film are accessible to target DNA to form duplex format in hybridization. The DNA microarray fabricated on PET film has wider dynamic range (about 3 orders of magnitude) and lower detection limit (0.5 nM) than the conventional amino- and aldehyde-functionlized slides. Oligonucleotide microarrays fabricated on these PET-coated slides also had consistent spot morphology. In addition, discrimination of single nucleotide polymorphism of 16S rRNA genes was achieved with the PET-based oligonucleotide microarrays. The PET microarrays constructed by our self-assembly process is cost-effective, versatile, and well suited for immobilizing many types of biological active molecules so that a wide variety of microarray formats can be developed.

The use of microarray-based technology is growing rapidly and has had considerable impact in genomic and proteomic research.[1,2] One crucial component of microarray technology is the surface chemistry of the substrate. The chemistry should be suitable for spotting and immobilizing a variety of biological active molecules (DNA, proteins, and cells) such that their biomolecular interactions may be evaluated. Therefore, strong emphasis is placed on developing innovative chemistries that provide high binding capacity, efficient hybridization, low background, good spot uniformity, and stability.

A variety of surface chemistries have been described for DNA microarray fabrication. These include in situ synthesis of DNA directly on glass substrates by photolithography or ink-jet printing technology[3−5] and the immobilization of presynthesized DNA to the substrate surface by chemical or physical attachment.[6−13] The chemical attachment requires activation of the substrate surface with cross-linking reagents and modification of DNA probes with reactive groups.[6−11] While the covalent bonding of DNA on the slide surface usually provides good stability and reproducibility, surface derivatization and the use of cross-linker reagents involves the use of toxic chemicals. The modifications of DNA probes with active groups also add considerable expense.

Physical attachment occurs through noncovalent interactions (i.e., hydrophobic interactions, electrostatic interactions, and entrapment in porous structures) between the DNA and the surface coatings of the substrate used for fabrication of DNA microarrays. The use of poly-L-lysine (PLL) and aminosilane coatings are examples of this approach.[12−14] These methods do not require terminal modifications of DNA probes and are easy to handle. However, it has been reported that these methods have low binding capacity and result in experimental inconsistencies that beget inconclusive data interpretation.[13]

The thickness of the coating film deposited on the slide substrate is also an important factor for microarray performance. Two-dimensional (2-D) and three-dimensional (3-D) films have been used thus far for microarray fabrication. The 2-D coatings are usually monolayer of organic molecules containing active groups, such as thiol,[6] amine,[11−14] aldehyde, and epoxy[7−9,15] which bind DNA

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(1) Lockhart, D. J. and Winterer, E. A. *Nature* **2000**, *405*, 827−836.
(2) Lander, E. S. *Nat. Genet. Suppl.* **1999**, *21*, 3−4.
(3) Fodor, S. P. A.; Read, J. L.; Pirrung, M. C.; Stryer, L.; Lu, A. T.; Solas, D. *Science* **1991**, *251*, 767−773.
(4) Hacia, J. G.; Fan, J. B.; Ryder, O.; Jin, L.; Edgemon, K.; Ghandour, G.; Mayer, R. A.; Sun, B.; Hsie, L.; Robbins, C. M.; Brody, L. C.; Wang, D.; Lander, E. S.; Lipshutz, R.; Fodor, S. P. A. Collins, F. S. *Nat. Genet.* **1999**, *22* (2), 164−167.
(5) Lipshutz, R. J.; Fodor, S. P. A.; Gingeras, T. R.; Lockhart, D. J. *Nat. Genet.* **1999**, *21*, 20−24.
(6) Rogers, Y. H.; Baucom, P. J.; Huang, Z. J.; Bogdanov, V.; Anderson, S.; Boyce-Jacino, M. T. *Anal. Biochem.* **1999**, *266*, 23−30.
(7) Dolan, P. L.; Wu, Y.; Ista, L. K.; Metzenberg, R. L.; Nelson, M. A.; Lopez, G. P. *Nucleic Acids Res.* **2001**, *29*, e107.
(8) Lindroos, K.; Liljedahl, U.; Raitio, M.; Syvänen, A. *Nucleic Acids Res.* **2001**, *29*, e69.
(9) Guo, Z.; Guilfoyle, R. A.; Thiel, A. J.; Wang, R.; Smith, L. M. *Nucleic Acids Res.* **1994**, *22*, 5456−5465.
(10) Lee, P. H.; Sawan, S. P.; Modrusan, Z.; Arnold, L. J.; Reynolds, M. A. *Bioconjugate Chem.* **2002**, *13*, 97−103.
(11) Podyminogin, M. A.; Lukhtanov, E. A.; Reed, M. W. *Nucleic Acids Res.* **2002**, *29*, 5090−5098.
(12) Belosludtsev, Y.; Iverson, B.; Lemeshko, S.; Eggers, R.; Wiese, R.; Lee, S.; Powdrill, T.; Hogan, M. *Anal. Biochem.* **2001**, *292*, 250−256.
(13) Lemeshko, S. V.; Powdrill, T.; Belosludtsev, Y. Y.; Hogan, M. *Nucleic Acids Res.* **2001**, *29*, 3051−3058.
(14) Benters, R.; Niemeyer, C. M.; Drutschmann, D.; Blohm, D.; Wöhrle, D. *Nucleic Acids Res.* **2002**, *30*, e10.

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probes. These 2-D coatings are usually less than 10 nm thick. Thus, long spacer arms of C12, C16, or poly(dT) are necessary in the oligonucleotide probes in order to improve the accessibility of target DNA.[7,9,15] The DNA microarrays fabricated using 2-D monolayer coatings have the advantages of good reproducibility and low background signal under fluorescent detection but have the disadvantages of low binding capacity and hybridization efficiency as well as narrow dynamic ranges.

The 3-D coatings are usually constructed by depositing thick polymer films on slide supports. The 3-D platforms for microarray fabrication include acrylamide gel pads or gelatin pads structured by photolithography,[16,17] aldehyde activated agarose film,[18] hydrogel polymer,[19] and nitrocellulose film.[20] The thickness of these 3-D coatings is usually above the micrometers level. The thick polymeric films increase the number of coupling sites by introducing additional reactive groups through branched linker molecules, which can provide higher probe binding capacity, and thus give higher signal intensity and wider dynamic ranges. However, compared to 2-D coatings, the 3-D coatings have lower reproducibility and a higher background signal caused by autofluorescence of the polymer materials.

The great demand for new chemistry which provides reliable attachment of DNA for various functional analyses, motivated us to explore using a multilayered, polyelectrolyte coating with a thickness ~100 nm on slide substrates for fabrication of DNA microarrays. We report here, a simple procedure to coat glass slides with ultrathin films by self-assembly of polyelectrolyte multilayer films (PET) and the protocols for fabricating DNA microarrays with unmodified oligonucleotide probes. We then compared the features of DNA microarrays fabricated on the PET-coated slide, with aminosilane and aldehyde-functionalized slides. Our results show that the utilization of PET film as a DNA microarray platform combines the advantages of the 2-D monolayer and the 3-D thick film coatings and eliminates their disadvantages.

Materials and Methods

Reagents. Microscope glass slides (76 mm × 26 mm × 1 mm) and glass cover slips were obtained from Sigma-Aldrich. Aldehyde-modified slides (SuperAldehyde) were purchased from TeleChem International (Sunnyvale, CA) and PLL-coated slides were purchased from Cell Associates (Houston, TX). Cy3-NHS ester was purchased from Amersham Biosciences Corp. (Piscataway, NJ). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Oligonucleotides. Oligonucleotides ranging from 11-mer to 50-mer derived from a sequence region of 16S rRNA genes (see Table 1) were synthesized at Michigan State University's Macromolecular Center. Oligonucleotide probes without alkylamino modification were used to fabricate DNA microarrays on PET-, PLL-, and (aminopropyl)trimethoxylsilane (APTS)-coated slides, while oligonucleotides with alkylamino modification at the 3′-end were used to fabricate microarrays on aldehyde-activated slides. Probes labeled with Cy3 at the 3′-terminal were used to determine the binding capacities of the slides. A Cy3-labeled 50-mer having partial sequence complementary to the 16S probes was used as a target template.

Slide Preparation. Glass slides were cleaned with hot Piranha solution (1:3 ratio of 30% H_2O_2 and H_2SO_4) and then thoroughly rinsed with distilled water and ethanol. Cleaned slides were immersed into 1 mM of APTS/ethanol solution for 30 min to form an APTS monolayer coating on the glass surface with amino functional groups toward the outside. The APTS-modified glass slides were then immersed in ca. 50 mL of 3 mg/mL polysodium styrenesulfonate solution (PSS; MW 70 000), 0.5 M NaCl at pH ~ 2.0 for 5 min, followed by washing with distilled water, and air-drying. The PSS-coated slide was then exposed to ca. 50 mL of 3 mg/mL polyallylamine hydrochloride solution (PAAH; MW 50000−65000), 0.5 M NaCl at pH 8.0 for 5 min. The surface was then washed again with distilled water. This procedure was repeated until the desired number of polyelectrolyte pair layers $(PSS/PAAH)_n$ were deposited on the slide with the positively charged PAAH on the outermost layer. The positively charged slides were then ready for fabrication of DNA microarrays.

For comparison purposes, a new type of dextran-coated slide with aldehyde active groups was prepared as described elsewhere.[21,22] Briefly, Dextran (M_w 70 kDa) was oxidized to produce aldehyde groups via standard periodate methods.[22] The APTS-coated slide was treated with 0.02 g/mL aldehyde−dextran solution in 0.2 M sodium phosphate buffer at pH 9.0 for 16 h. The slide was then incubated with 0.1 M sodium borohydride solution to reduce the Schiff bases formed between the glass surface and the dextran chain. The slide was then incubated in 0.1 M sodium periodate solution to produce aldehyde groups. After 2 h of reaction, the activated slide was washed with an excess of distilled water and stored at 4 °C.

Microarray Fabrication. Oligonucleotide microarrays were fabricated on five types of glass slides with different surface chemistries as summarized in Table 2. The 5′-terminal alkylamine-modified oligonucleotides were attached to the aldehyde and aldehyde−dextran functionalized slides while oligonucleotides without amino modifications were immobilized on PET, PLL, and APTS slides. Oligonucleotide printing solutions were prepared in a solution of DMSO/H_2O = 1/1 (for PET, PLL, and APTS slides) or 1× TeleChem spotting solution (for SuperAldehyde and aldehyde−dextran-functionalized slides). DNA probe samples were arrayed using a PixSys 5500 robotic printer (Cartesian Technologies, Inc., Irvine, CA) in 40% relative humidity. The printed slides were incubated overnight at room temperature. Oligonucleotides that were not bound after spotting were removed by washing the slides twice in a solution of 10 mM NaOH and 50 mM Na_2CO_3 for 2 min each and in distilled water for 2 min.

Blocking. To optimize blocking protocols, several physical and chemical blocking methods were tested on the microarrays with 16S-P and 16S-M probes fabricated on PET-, PLL-, and APTS-functionalized slides by evaluating the hybridization performance. The blocking protocols were as follows: (1) 0.5% BSA, 0.1% SDS in 100 mM PBS buffer for 30 min; (2) 5× Denhardt's solution (containing 0.1 mg/mL each of Ficoll, poly(vinylpyrrolidone), and bovine serum albumin) for 30 min; (3) 0.5 mg/mL sodium poly(styrenesulfonate) (PSS, MW 70 000) in 10 mM sodium acetate buffer at pH 7.0 for 10 min; (4) 0.5 M solution of succinic anhydride in *N*,*N*-dimethylformamide (DMF) overnight, the slides were carefully washed with DMF three times; (5) 0.5 M of glutaric anhydride (GA) in DMF overnight, then the slides were carefully washed with DMF three times; and (6) 100 mM solution of 5-formyl-1,3-benzenedisulfonic acid disodium salt in 100 mM sodium acetate buffer at pH 7.0 for 1 h at room temperature.

For the oligonucleotide microarrays fabricated on aldehyde and aldehyde−dextran slides, the microarrays were passivated by immersing them in a solution containing 0.25 g of Na_2BH_4 dissolved in 75 mL of 1× PBS and 25 mL of EtOH for 5 min, followed by washing three times in 0.2% SDS for 1 min and then in distilled water for 1 min.

(15) Zhao, X. D.; Nampalli, S.; Serino, A. J.; Kumar, S. *Nucleic Acids Res.* **2001**, *29*, 955−959.
(16) Proudnikov, D.; Timofeev, E. and Mirzabekov, A. D. *Anal. Biochem.* **1998**, *159*, 34−41.
(17) Ermantraut, E.; Wohlfart, K.; Woelfl, S.; Schulz, T. and Koehler, M. In *Miroreaction Technology- Proceedings of the First International Conference on Microreaction Technology*; Ehrfeld, W., Ed.; Spring Verlag: Heidelberg, 1997; pp 332−339.
(18) Afanassiev, V.; Hanemann, V.; Wölfl, S. *Nucleic Acids Res.* **2000**, *28* (12), e66.
(19) Kodadek, T. *Chem. Biol.* **2001**, *8*, 105−115.
(20) Stillman, B. A.; Tonkinson, J. L. *Biotechniques* **2000**, *29*, 630−635.
(21) Massia, S. P.; Stark, J. *J. Biomed. Mater. Res.* **2001**, *56*, 390−399.
(22) Penzol, G.; Armisen, P.; Fernandez-Lafuente, R.; Rodes, L. Guisan, J. M. *Biotechnol. Bioeng.* **1998**, *60*, 518−523.

Table 1. Probes and Target Template Used[a]

length	name	sequence (5′ → 3′)
11-mer	16S-11P	G AGG TCT TGC G
	16S-11P-NH_2	NH_2-(C6)-G AGG TCT TGC G
	16S-11P-Cy3	G AGG TCT TGC G-Cy3
20-mer	16S-20P	AC GCG AGG TCT TGC GAT CCC
	16S-20P-Cy3	AC GCG AGG TCT TGC GAT CCC-Cy3
	16S-20P-NH_2	NH_2-(C6)-AC GCG AGG TCT TGC GAT CCC
	16S-20M	AC GCG AGG T***A***T TGC GAT CCC
30-mer	16S-30P	CC AAT CAC GCG AGG TCT TGC GAT CCC CCG C
	16S-30P NH_2	NH_2-(C6)-CC AAT CAC GCG AGG TCT TGC GAT CCC CCG C
	16S-30P-Cy3	CC AAT CAC GCG AGG TCT TGC GAT CCC CCG C-Cy3
40-mer	16S-40P	C CGC TCC AAT CAC GCG AGG TCT TGC GAT CCC CCG CTT ACC
	16S-40P-NH_2	NH_2-(C6)-C CGC TCC AAT CAC GCG AGG TCT TGC GAT CCC CCG CTT ACC
	16S-40P-Cy3	C CGC TCC AAT CAC GCG AGG TCT TGC GAT CCC CCG CTT AC-Cy3
50-mer	16S-50P	ATC GGC CGC TCC AAT CAC GCG AGG TCT TGC GAT CCC CCG CTT ACC CCC TC
	16S-50P- NH_2	NH_2-(C6)-ATC GGC CGC TCC AAT CAC GCG AGG TCT TGC GAT CCC CCG CTT ACC CCC TC-Cy3
	16S-50P-Cy3	ATC GGC CGC TCC AAT CAC GCG AGG TCT TGC GAT CCC CCG CTT ACC CCC TC-Cy3
19-mer	16S-P	ACG CGA GGT CTT GCG ATC C
	16S-P-NH_2	NH_2-(C6)-ACG CGA GGT **C**TT GCG ATC C
	16S-M1a	ACG CGA GGT ***G***TT GCG ATC C
	16S-M1a-NH_2	NH_2-(C6)-ACG CGA GGT ***G***TT GCG ATC C
	16S-M1b	ACG CGA GGT ***A***TT GCG ATC C
	16S-M1b-NH_2	NH_2-(C6)-ACG CGA GGT ***A***TT GCG ATC C
	16S-M1c	ACG CGA GGT ***T***TT GCG ATC C
	16S-M1c-NH_2	NH_2-(C6)-ACG CGA GGT ***T***TT GCG ATC C
	16S-M2a	ACG CGA GG***C G***TT GCG ATC C
	16S-M2a-NH_2	NH_2-(C6)-ACG CGA GG***C G***TT GCG ATC C
	16S-M2b	ACG CGA GG***G G***TT GCG ATC C
	16S-M2b-NH_2	NH_2-(C6)-ACG CGA GG***G G***TT GCG ATC C
	16S-M2c	ACG CGA GG***A A***TT GCG ATC C
	16S-M2c-NH_2	NH_2-(C6)-ACG CGA GG***A A***TT GCG ATC C
	16S-M2d	ACG CGA GG***C A***TT GCG ATC C
	16S-M2d-NH_2	NH_2-(C6)-ACG CGA GG***C A***TT GCG ATC C
	16S-M2e	ACG CGA GG***G A***TT GCG ATC C
	16S-M2e-NH_2	NH_2-(C6)-ACG CGA GG***G A***TT GCG ATC C
	16S-M2f	ACG CGA GG***A T***TT GCG ATC C
	16S-M2f-NH_2	NH_2-(C6)-ACG CGA GG***A T***TT GCG ATC C
	16S-M2g	ACG CGA GG***C T***TT GCG ATC C
	16S-M2g-NH_2	NH_2-(C6)-ACG CGA GG***C T***TT GCG ATC C
	16S-M2h	ACG CGA GG***G T***TT GCG ATC C
	16S-M2h-NH_2	NH_2-(C6)-ACG CGA GG***G T***TT GCG ATC C
	16S-M3	ACG CGA GG***C GA***T GCG ATC C
	16S-M3-NH_2	NH_2-(C6)-ACG CGA GG***C GA***T GCG ATC C
	16S-M4	ACG CGA G***CC GA***T GCG ATC C
	16S-M4-NH_2	NH_2-(C6)-ACG CGA G***CC GA***T GCG ATC C
	16S-M5	ACG CGA G***CC GAA*** GCG ATC C
	16S-M5-NH_2	NH_2-(C6)-ACG CGA G***CC GAA*** GCG ATC C
50-mer	target	GA GGG GGA AAG CGG GGG ATC GCA AGA CCT CGC GTG ATT GGA GCG GCC GAT-Cy3

[a] The 5′-terminus alkylamine modified probes were used for aldehyde-functionalized slides and unmodified probes were used for polyelectrolyte multilayer (PET), (aminopropyl)trimethoxylsilane (ATPS), and poly-L-lysine (PLL) slides. The mismatched base pair(s) in the probes are boldface and italic.

Table 2. Surface Chemistry for Probe Immobilization

slide type[a]	functional group on slide	5′-modification at probes
PET	long-chain hydrophilic polymer containing amine groups and pores	none
APTS	amine groups	none
PLL	amine groups	none
SuperAldehyde	aldehyde	alkylamine
aldehyde−dextran	aldehyde	alkylamine

[a] PET = polyelectrolyte multilayer film; ATPS = aminopropyltrimethoxylsilane; PLL = poly-L-lysine.

Hybridization. Hybridization was accomplished by initially dissolving the Cy3-labeled complementary target in hybridization buffer containing 3× SSC, 40% formamide, and 0.2% SDS. Next, 10 μL of hybridization solution was deposited on the DNA microarray and a glass cover slip was placed on the slide. Hybridization was carried out for 14 h at the 42 °C. Following hybridization, the arrays were washed with 1× SSC, 0.2% SDS and 0.1× SSC, 0.2% SDS for 5 min each and then with 0.1× SSC for 30 s at ambient temperature prior to being dried by centrifugation at 500*g*.

Signal Detection and Data Analysis. The microarrays were scanned at 523 nm using a scanning laser confocal fluorescence (ScanArray 5000 System, Packed Biochip Technologies, Boston, MA) microscope at 10 μm resolution. For all microarray experiments, the laser power was 80% and the PMT gain was 70%. The images were processed and analyzed using ImaGene 3.0 (Biodiscovery, Inc., Los Angeles, CA). Mean signal intensity of each spot was used for data analysis. The local background signal was subtracted automatically from the hybridization signal of each spot. Statistical analysis was performed using SigmaPlot 5.0 (Jandel Scientific, San Rafael, CA) or by Microsoft Excel.

Quantification of Immobilized Probe DNA and Hybridized Target DNA. A standard curve of fluorescent intensity versus the Cy3 concentration was generated by detection of the fluorescent signal of Cy3-NHS spots at different concentrations printed on bare glass slide. Cy3-NHS was diluted with printing

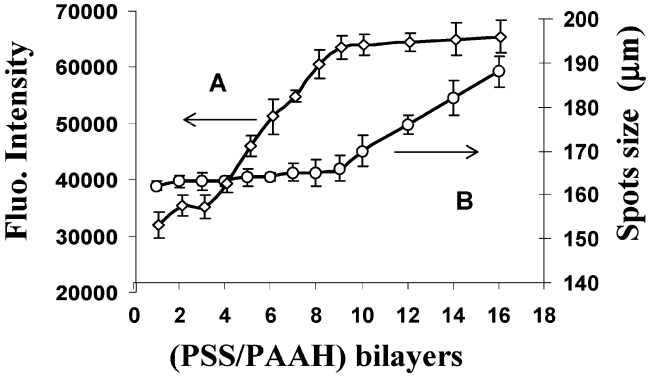

Figure 1. Effect of polyelectrolyte thin film (PET) thickness on binding capacity **(**A**)** and spot size **(**B**)**. A Cy3-labeled 20-mer oligonucleotide at 25 μM was spotted onto the glass slides coated with different thicknesses of PET. After washing with a solution of 10 mM NaOH and 50 mM Na_2CO_3, the microarray was scanned and analyzed as described in Materials and Methods. Data are mean ± standard deviation of 48 replicates from 3 slides.

buffer using a 2-fold dilution series from 50 to 0.00185 μM, and 10 replicate spots were printed for each dilution at ~1 nL in volume. The fluorescent intensities of the spots were examined and plotted against the Cy3 concentration. To detect the binding capacity of each slide, a series of diluted Cy3-labeled 11- to 50-mer oligonucleotides were arrayed on the slide, and the fluorescent intensities of the spots were quantified after washing, and the amount of attached oligonucleotides were then deduced from the standard curve and converted to binding coverage of DNA (in molecules/cm^2). To determine the amount of hybridized DNA, microarrays were prepared using unlabeled probes and then treated for hybridization with Cy3-labeled target. The fluorescent intensities of the spots were quantified and the amount of hybridized target DNA was then deduced from the standard curve. Hybridization efficiencies were calculated as the fraction of hybridized target coverage divided by the immobilized probe coverage.

Definition of Discrimination Factor F_m/F_p. To evaluate the specificity of oligonucleotide microarray, the discrimination factor, which indicates the ability to differentiate the nucleotide polymorphisms, was calculated by using the ratio of hybridization intensity of mismatched probes (F_m) to the signal intensity of perfectly matched probe (F_p).

Results

Oligonucleotide Immobilization on Nanoengineered PET. To optimize the PET film thickness for the construction of DNA microarrays, we spotted the 16S-20P-Cy3 probe onto glass slides that were coated with different bilayers of PSS/PAAH. The effects of film thickness (presented as the number *n* of bilayers of PSS/PAAH) on probe binding capacity and spot size are shown in Figure 1. The fluorescent intensity increased with an increase in the number of bilayers and reached a saturation level when the bilayer number (*n*) was ~10 (Figure 1A). This correlated with an increase in the number of binding sites (the positively charged amino group and the porous network) on the 3-D PET. The fact that the binding capacity of the PET began to be saturated when the bilayer number was ~10 indicated that the DNA probes only penetrate several external polyelectrolyte layers. The use of a contact printing pin may facilitate the direct delivery of DNA probes into the inner layers of the PET. The spot size was constant when the number of bilayers was <9 but increased rapidly when the film thickness was >9 bilayers (Figure 1B). This could be because with >9 bilayers of PSS/PAAH, the external polyelectrolyte layers became loose and caused the spotted probe solution to spread. Thus, the optimized PET thickness was obtained

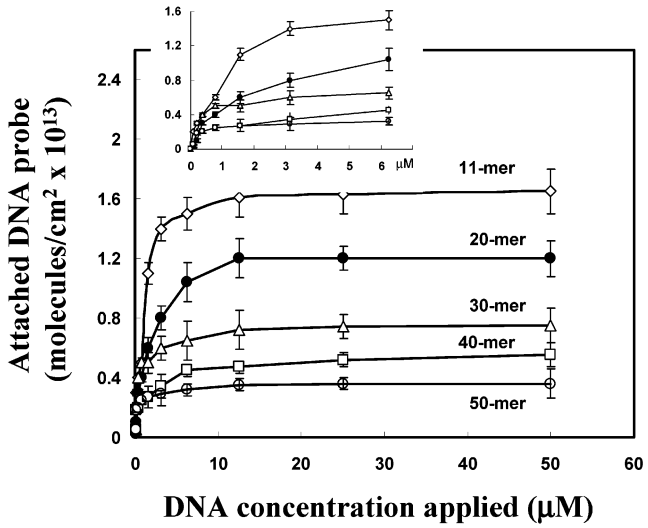

Figure 2. Effect of DNA probe concentration and length to the immobilization of DNA probes on polyelectrolyte multilayer film (PET) coated slides. The insert graph shows the effects of DNA probes at low concentration. Data are mean ± standard deviation of 48 replicates from 3 slides.

with 9 bilayers of PSS/PAAH. AFM analysis reveals that the $(PSS/PAAH)_9$ has the film thickness 80−100 nm. Glass slides coated with 9 bilayers of PSS/PAAH were therefore used for further study.

The binding capacities (i.e., the surface coverage) of oligonucleotide probes of different lengths on the $(PSS/PAAH)_9$ film after washing were further examined by analyzing the signal intensities of microarray spots printed from serial dilutions of each Cy3-labeled oligonucleotide probe. Quantitative data (molecules/cm^2) of binding capacities were calibrated from the standard curve. Figure 2 shows that the coupling efficiency increased as the oligonucleotide concentration increased and reached a plateau at 12.5 μM for all of the oligonucleotide probes. The saturated probe density of the 11- and 50-mer oligonucleotide was 1.7×10^{13} molecules/cm^2 and 3.4×10^{12} molecules/cm^2, respectively, on the PET. The surface coverage was also related to probe length. The surface coverage decreased with an increase in probe length from 11- to 50-mer, dropping by about an order of magnitude from 1.7×10^{13} to 3.4×10^{12} molecules/cm^2. A decreasing trend in surface coverage with increase of probe length is expected, as it takes fewer large probes to cover a unit area of substrate. The results displayed in Figure 2 also indicated that synthetic oligonucleotides without modifications as short as 11-mer can be effectively immobilized on the PET.

Optimization of Blocking Protocol for PET Slide. The PET films were composed of polyanions comprised of sulfonate groups and polycations comprised of amino groups. The spotted oligonucleotides were bound onto the PET through a combination of noncovalent interaction based on the electrostatic interaction and retention in porous network structures. Although the negatively charged groups on the slide surfaces lead to reduced background signal,[14] nonspecific adsorption of target nucleic acids may be caused by the positively charged amino groups and pores of the PET film. We therefore tested several physical and chemical blocking methods on the microarrays fabricated with a perfect match probe (16S-20P) and a probe having a single base-pair mismatch (16S-20M) by evaluating the hybridization performance after blocking. The physical blocking methods cap the unused positively charged groups on the microarray

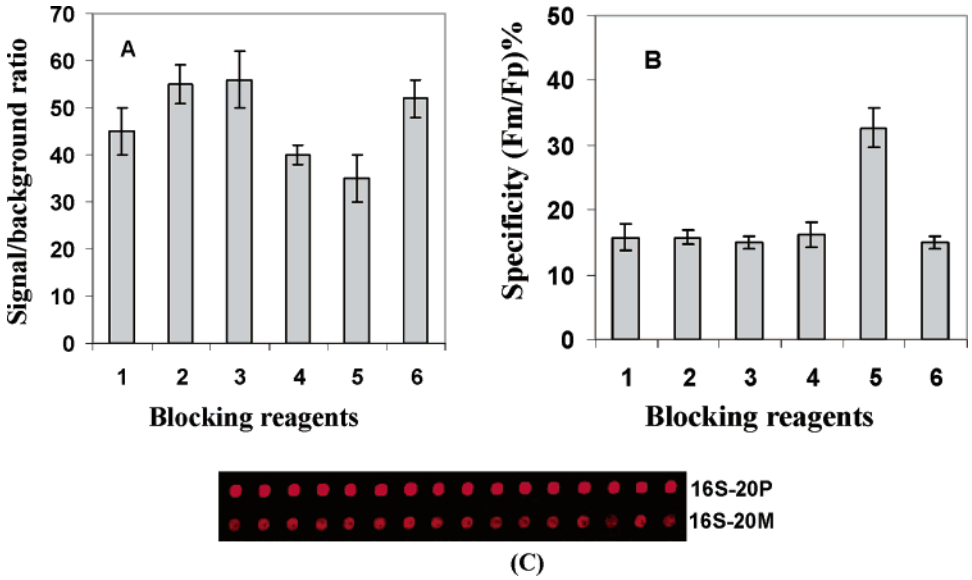

Figure 3. Effects of blocking reagents on microarray performance. 16S-20P and 16S-20M probes were spotted in parallel on 18 PET-coated slides. Before the glass slides were hybridized with the target DNA at 42 °C, the slides were passaviated with one of the reagents described in Materials and Methods (for each blocking experiments, three slides were tested). The data shown are mean ± standard deviation of 48 replicates. **(**A**)** Comparison of hybridization signal-to-background ratio of 16S-20P probe after treatment with different blocking reagents. **(**B**)** Effect of blocking reagents to the specificity of the F_m/F_p. **(**C**)** Display of hybridization image of microarray with 16S-20P and 16S-20M probes obtained after blocking with 5× Denhardt's solution and hybridization with 50-mer target DNA.

surface by physical adsorption of neutral molecules, while chemical blocking methods convert surface amino groups into negatively charged carboxyl groups or sulfonic group. Figure 3 shows the array performance obtained from the six blocking experiments. Overall, the physical blocking with 5× Denhardt's solution and the chemical blocking with 10 mM solution of 5-formyl-1,3-benzenedisulfonic acid disodium gave the highest signal-to-noise ratio after hybridization (Figure 3A). The use of succinic anhydride and glutaric anhydride (GA) as blocking reagents yielded negatively charged surfaces that could not support hybridization (lower hybridization signal) although the background noise of the blocked surface was relatively lower. Moreover, the discrimination ability to identify single nucleotide mismatches (F_m/F_p) remained consistent except with the use of GA as a blocking reagent for which an unusual loss of specificity was observed (Figure 3B). This is probably due to the reaction of GA with part of the nucleoside of the DNA probe. Due to its simplicity, 5× Denhardt's solution was therefore used as blocking reagent for PET slides.

Comparison of Binding Capacities and Hybridization Efficiencies with Other Types of Slides. PET slides and other four chemically modified glass surfaces were studied for their characteristics relating to the immobilization of oligonucleotides, hybridization efficiency, resulting slide background signal after hybridization, spotting uniformity, and specificity to nucleotide polymorphisms. Table 2 lists the slide surface chemistries and the functional modifications of the oligonucleotide probes. These surfaces were selected because they are commonly used in microarray fabrication laboratories.

Parts A and B of Figure 4 show the comparison of the binding capacities of the 50-mer oligonucleotide probes and their hybridization on the PET slide and four other types of slides. One significant observation was that the probe binding capacity on PET was about 2-fold higher than that of the APTS and PLL slides where the probes were all immobilized on the surface by noncovalent interaction (Figure 4A). The binding capacity of the unmodified 50-mer probes on PET (3.4×10^{12} molecules/cm^2) is comparable to the binding capacity on the SuperAldehyde slide (3.6×10^{12} molecules/cm^2) and the aldehyde−dextran slide (3.8×10^{12} molecules/cm^2) where the alkylamine modified probes are end-tethered. It is also noteworthy that the PET slides provided high binding capacity even at a low concentration of probe spotting solution. For example, the binding capacity of the unmodified 50-mer probe immobilized on the PET slide was about two times greater than the alkylamine-modified 50-mer on the SuperAldehyde slide when the concentrations of both the probe spotting solutions were 12.5 μM. Thus, higher concentration probes of 25 μM must be used on aldehyde slides in order to achieve binding saturation.

To compare the hybridization efficiency, unlabeled 50-mer probe at 50 μM was spotted on five types of slides and then hybridized with different concentrations of Cy3-labeled target DNA under the same conditions. The amount of target DNA hybridized on the slides was quantified from the standard curve and plotted against the target concentrations. As shown in Figure 4B, the amount of target DNA hybridized on the slides with PET was 2.7×10^{12} molecules/cm^2, which is about 2-fold higher than the APTS- and PLL-coated slides. Unmodified probe immobilized on PET slides is also more accessible to target DNA in hybridization than the alkylamine-modified probe immobilized on the aldehyde-functionalized slides. The hybridization efficiency of the unmodified 50-mer probe on the PET slide was 90%, whereas it was 70% for the alkylamine modified 50-mer probe on the SuperAldehyde slide. The aldehyde−dextran-functionalized slide showed higher binding capacity and hybridization efficiency (∼82%) than the SuperAldehyde slide (∼70%). It is also noteworthy that the DNA microarray on PET film has wider dynamic range (about 3 orders of magnitude) and lower detection limit than the other four types of slides. The lowest concentration of target DNA that can be statistically distinguished from background (>backgrounds + 3 × STD) is 0.5 nM for oligonucleotide microarrays fabricated on PET slide. Displayed in Figure

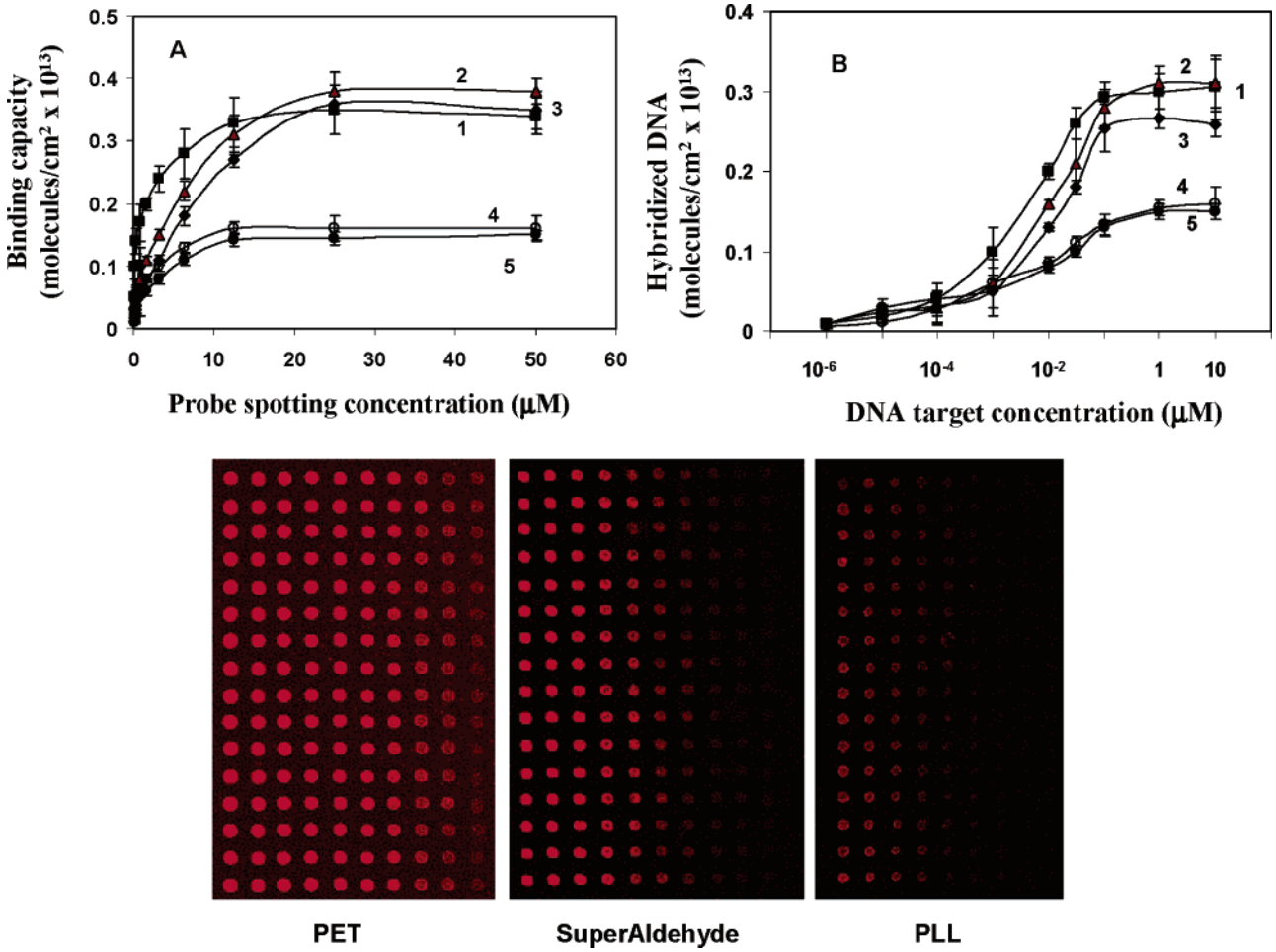

Figure 4. Comparison of binding capacities and hybridization efficiencies on different slides. Dilutions of Cy3-labeled and unlabeled 50-mer probes in printing buffer were immobilized on two separate batches on five types of slides. Slides with Cy3-labeled probes were used to determine immobilization efficiencies after extensive washing, while the slides with unlabeled probes were hybridized with Cy3-labeled target DNA for 16 h. **(**A**)** The binding capacities and **(**B**)** hybridized amount of target DNA on: 1, PET slide; 2, aldehyde−dextran slide; 3, SuperAldehyde slide; 4, (aminopropyl)trimethoxylsilane (APTS) slide; and 5, poly-L-lysine (PLL) slide. Values are mean ± standard deviation from 16 spots. **(**C**)** Side-by-side comparison of hybridization image on PET, SuperAldehyde, and PLL slides.

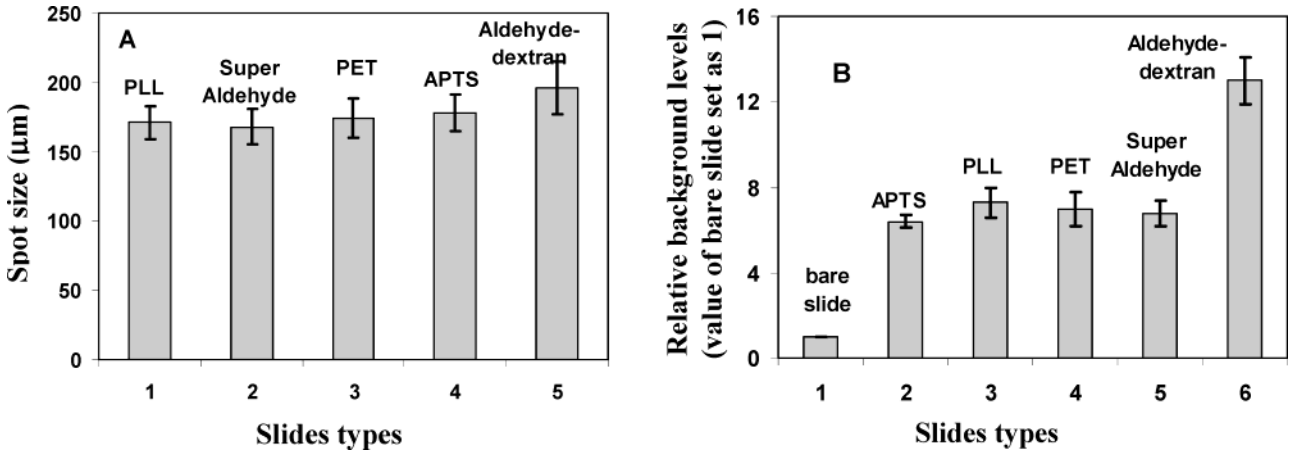

Figure 5. Comparison of spot size **(**A**)** and background signal **(**B**)** on slides immobilized with 20-mer probes after blocking and hybridization with Cy3-labeled target. Values are mean ± standard deviation from 160 spots.

4C are the hybridization images obtained on the PET, aldehyde and PLL slides.

Comparison of background signal and spot size among different slides. Figure 5 compares the spot size and background signal on different slides after hybridization. The size of the spots on the PET slide was 173 ± 10 μm, which was similar to the SuperAldehyde slide (168 ± 10 μm) and the PLL slide (171 ± 10 μm). The background signal of the PET slide was also at the same level as the SuperAldehyde, PLL, and APTS slides but with remarkably high and homogeneous signal distributions with the individual spots as evidenced by the small standard deviation. This allows for minimized signal deviations of the data and, thus, minimizes experimental errors. The

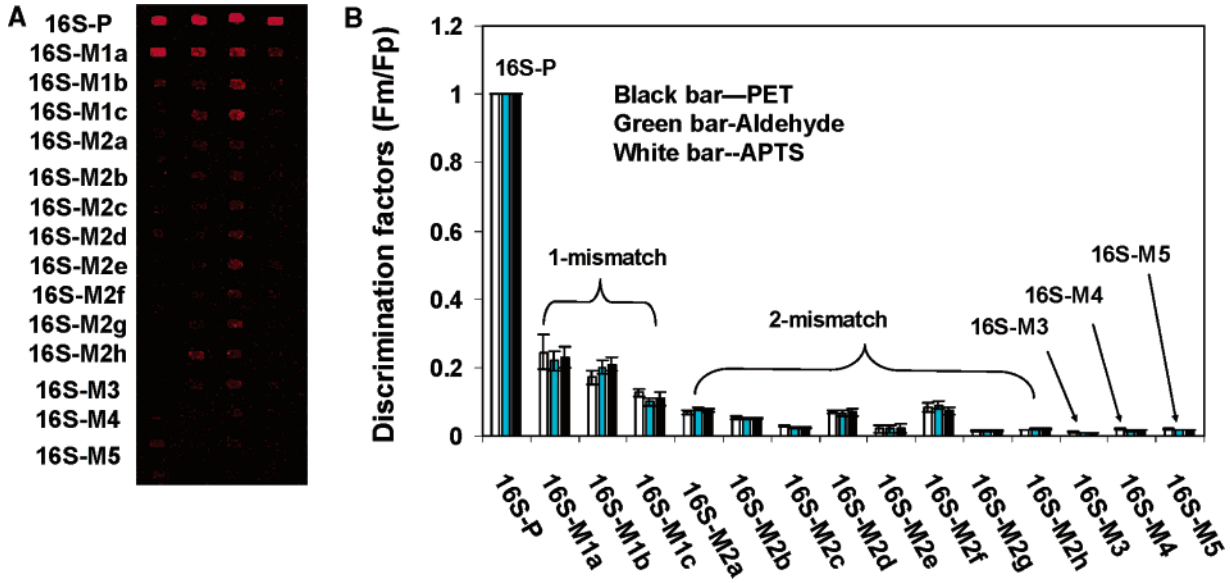

Figure 6. Detection of nucleotide polymorphisms on oligonucleotides microarrays. **(**A**)** Layout of hybridization image on PET slide. **(**B**)** Comparison of discrimination of nucleotide mismatches on PET, aminopropyltrimethoxylsilane (APTS), and SuperAldehyde slides. Data are mean ± standard deviation of 12 replicates from three slides.

relative high background signal of the aldehyde−dextran slide was probably due to the multistep synthesis conducted using this type of slide.

Differentiation of Nucleotide Polymorphisms. Further studies were performed to evaluate the ability to discriminate single nucleotide polymorphisms using oligonucleotide microarrays fabricated on PET and APTS slides with unmodified probes, and an oligonucleotide microarray aldehyde slides with alkylamine-modified probes. The microarrays were comprised of 15- of 19-mer oligonucleotide probes. Oligonucleotides 16S-P and 16S-P-NH_2 were fully complementary to the part of the Cy3-labeled target present in the hybridization buffer, while oligomers 16S-M1a (and 16S-M1a-NH_2) to 16S-M1c (and 16S-M1c-NH_2) contained a single mismatched nucleotide in the middle with different nucleoside types. Oligonucleotide 16S-M2a to oligonucleotides 16S-M2h contained two mismatches in the middle and oligonucleotides 16S-M3, 16S-M4, and 16S-M5 contained three, four, and five mismatches, respectively (Table 1). After hybridization under identical conditions, the microarrays on PET, APTS, and SuperAldehyde slides were analyzed and the ratios of signal intensities of mismatched probes to perfectly matched probes, F_m/F_p, were determined. Figure 6A displays the image obtained on the PET slide, and Figure 6B shows the F_m/F_p of each probe determined on the three types of slides. As shown in Figure 6B, the signal intensities of the oligonucleotides having single mismatched base pairs were discriminated at a signal intensity of 15−25% of the perfectly matched probe, varying with the nucleoside type. The signal intensities of probes with two mismatched nucleosides in the middle were about ∼5−15% of the perfectly matched probe, whereas oligonucleotide probes containing three, four, and five mismatches showed no detectable signal for the target DNA (hybridization signals smaller than ∼5% of the perfect matched probe, which is within the standard variation of the statistical analysis), due to the centralized position of three additional mismatches. Overall, the discrimination factor of each probe obtained from the microarrays on the three types of slides was similar. This indicates that the discrimination of nucleotide polymorphisms on an oligonucleotide microarray is independent of the surface chemistry used to immobilize the oligonucleotide probes, although the surface chemistry affects the hybridization signal intensity.

Discussion

Polyelectrolyte thin films constructed by the sequential layer-to-layer adsorption of cationic and anionic polyelectrolyte layers constitute a novel and promising technique to modify surfaces in a controlled way.[23,27−34] Multilayered polyelectrolytes can be easily engineered on a glass substrate to form uniformly thin films with plurality of internal pores with charged surfaces which has been evidenced by atomic force microscopy studies.[27] PET films have been used as substrates for the immobilization of biomolecules in DNA biosensors and protein biosensors because of their higher binding capacity,[23,28−31] However, the utilization of PET as a platform for the fabrication of biomolecular microarrays has not been explored.

Lemeshko et al. recently reported that the negatively charged phosphate groups of each nucleic acid strand are available for interaction with cationic groups.[13] They also demonstrated that DNA microarrays can be fabricated on 2-D amine surfaces, such as APTS and PLL monolayer coated glass. Compared with the monolayer of amine

(23) Santos, J. P.; Welsh, E. R.; Gaber, B. P.; Singh, A. *Langmuir* **2001**, *17*, 5361−5367.
(24) Record, M. T. Jr.; Anderson, C. F.; Lohman, T. M. *Q. Rev. Biophys.* **1978**, *11*, 103−178.
(25) Steel, A. B.; Levicky, R. L.; Herne, T. M.; Tarlov, M. J. *Biophys. J.* **2000**, *79*, 975−981.
(26) Balladur, V.; Theretz, A.; Mandrand, B. *J. Colloid Interface Sci.* **1997**, *194*, 408−418.
(27) McAloney, R. A.; Sinyor, M.; Dudnik, V. Goh, M. C. *Langmuir* **2001**, *17* (21), 6655−6663.
(28) Caruso, F.; Niikura, K.; Furlong, D. N.; Okahata, Y. *Langmuir* **1997**, *13*, 3427−3433.
(29) Lvov, Y.; Ariga, K.; Ichinose, I.; Kunitake, T. *J. Am. Chem. Soc.* **1995**, *117*, 6117−6123.
(30) Caruso, F.; Rodda, E.; Furlong, D. N.; Niikura, K.; Okahata, Y. *Anal. Chem.* **1997**, *69*, 2043−2049.
(31) Zhou, X. C.; Huang, L. Q.; Li, S. F. Y. *Biosens. Bioelectron.* **2001**, *16* (1−2), 85−95.
(32) Ladam, G.; Schaad, P.; Voegel, J. C.; Schaaf, P.; Decher, G.; Cuisinier, F. *Langmuir* **2000**, *16*, 1249−1255.
(33) Klitzing, R. v.; Möhwald, H. *Langmuir* **1995**, *11*, 3554−3559.
(34) Steitz, R.; Leiner, V.; Siebrecht, R.; von Klitzing, R. *Colloids Surf., A* **2000**, *163*, 63−70.

groups on APTS- and PLL-coated slides, PET film contains multilayers of amine functional groups and therefore increased binding sites for immobilizing DNA probes. Moreover, the multilayered polymer film on the slide produced porous structures, which in turn increased the surface area. Besides the tight electrostatic interaction of the negatively charged phosphate backbone of nucleic acids with the positively charged amine groups of the PET film, the porous structure on the PET also helped to retain the DNA probes spotted on the surface.

Although the polyelectrolyte thin films on solid substrate fabricated by sequential electrostatic adsorption of oppositely charged polyelectrolytes have been reported previously, these methods disclosed to date, however, are not to create large-area uniform coatings. We found that 0.5 M NaCl in polyelectrolyte solutions can create uniform thin film coatings over large areas of microscopic glass slides (76 mm × 26 mm) with nine bilayers of PSS/PAAH. The thickness of the $(PSS/PAAH)_9$ multilayer film is about 80−100 nm under the depositing condition with high salt concentration of 0.5 M NaCl, which is higher than the previous values that using low salt concentration (<0.1 M NaCl) in the deposition of polyelectrolyte multilayers.[28−30] It has been established that film thickness increases with an increase in concentration of added salt. Several publications reported that when deposited from solution with high salt concentration (>0.3 M NaCl), the thickness of the multilayer films varies over the first few layers before reaching constant incremental increases per bilayer.[27,32−34] The salt effects on polyelectrolyte multilayer film thickness and morphology have been well studied by McAloney et al. with atomic force microscopy (AFM).[27] With 1.0 M NaCl in deposition of multilayers, the AFM study by McAloney showed that the first three bilayers were featureless and had a thickness of ∼6 nm/bilayer, and the formation of vermiculate morphology began at the fourth bilayer with the average thickness of ∼46 nm/bilayer.[27]

We used a fluorescent-based method to quantify the immobilization of Cy3-labeled oligonucleotide probes on PET after extensive washing. The binding capacity was estimated from a standard curve (see Materials and Methods). Because the fluorescent scanner provides spot intensity as the average fluorescence intensity per unit pixel, the measured value was independent of spot size. As shown in Figure 2, the saturated probe density of an 11-mer oligonucleotide was observed at 1.7×10^{13} molecules/cm^2, which was 1.6 times higher than that of the maximum amount of the 11-mer oligonucleotides forming a closely packed monolayer on a planar surface (assuming 1 nm width for an oligonucleotide strand, 0.7 nm for the phosphate backbone per base at full extension, and 1 nm^2 for the appended dye label). The saturated probe density for a 50-mer oligonucleotide on PET was 3.4×10^{12} molecules/cm^2, which is 1.3 times greater than the formation of a closely packed monolayer of 50-mer probes on a planar surface. Experimentally, formation of a closely packed oligonucleotide monolayer by adsorption is impractical because of the additional steric requirements of counterions and water of hydration.[24,25] The coverage of 50-mer oligonucleotide obtained at the jamming limit on a planar surface, corresponding to a relative surface filling of 50%,[26] is roughly 1.3×10^{12} molecules/cm^2. The comparison of experimental coverage with this reasonably calculated coverage suggests that at adsorptive binding saturation, the abundance of amine groups on the multilayered polyelectrolytes and the 3-D porous structure contributed to the immobilization of oligonucleotide probes.

Effective blocking of the microarray on PET is needed to protect nonspecific adsorption of target samples, caused by the positively charged amino groups and pores of the PET. Some blocking protocols have been tested to give lower background signal on positively charged surfaces.[12,13] However, achieving minimally detected fluorescence of nonspecific adsorption is only one practical criterion to measure the blocking effect. The more important merit for a blocking protocol is to achieve and maintain high specificity in hybridization. In this work, we optimized the blocking procedures not only by the signal-to-noise ratio but also by evaluating the discrimination of single nucleotide polymorphism under the blocking procedures. We found that 5× Denhardt's solution provided a simple and effective method for blocking the PET slide, which also provided a higher signal-to-noise ratio and did not affect the specificity (Figure 3).

The density of surface-bound oligonucleotide probes and their accessibility to targets during the hybridization process is one of the major concerns in fabrication of oligonucleotide microarray, because the hybridization signal depends on the immobilized DNA probes and on their availability for hybridization. Oligonucleotide probe density has been reported as a controlling factor for the efficiency of target capture as well as the kinetics of the target/probe hybridization.[35,36] We compared the binding capacity and hybridization efficiency of 50-mer probes on five types of slides, including PET-, APTS-, and PLL-coated slides and SuperAldehyde and aldehyde−dextran slides. As observed in the experiments (Figures 4 and 5), the 3-D PET showed higher binding efficiencies for unmodified 50-mer probes and greater dynamic signal range for hybridization than the 2-D APTS or PLL surfaces.

The binding capacity of oligonucleotide probes on slide surface relies on the surface chemistry and the modification of the oligonucleotide. The alkylamine modified oligonucleotide probes bound to aldehyde slide in a "stand up" configuration while unmodified oligonucleotide bound to PET slide in a flattened configuration. It was thought that the stand up configuration would provide much high binding capacity than the flattened configuration. However, the results indicated that the binding capacity of 50-mer probe on the PET slide was only slightly lower than that on the aldehyde slide (Figure 4A). It has been reported that long probes (> 24-mer) tend to be absorbed in flattened configurations with multiple contacts to the substrate surface even when they are end-tethered on a surface.[25] In other words, the long probes behave as flexible, coillike polymer chains that adsorb side-by-side on the surface even though they are immobilized by end-tethering (see Figure 7). Thus, the high binding capacity on PET is reasonable given that the probes approximate multilayers on the PET compared with the relatively flattened configuration of a monolayer immobilized on aldehyde slides.

Although the binding capacity of PET slide is slightly lower than that of the SuperAldehyde slide, the PET slide provides a higher hybridization efficiency, which resulted in comparable hybridization signal intensity to the SuperAldehyde. The high hybridization efficiency of PET slide indicated that the probes immobilized on PET slide are more accessible for target DNA because of the less steric hindrance of the probes in hybridization. The aldehyde−dextran functionalized slide showed higher hybridization

(35) Charles, P. T.; Vora, G. J.; Andreadis, J. D.; Fortney, A. J.; Meador, C. E.; Dulcey, C. S.; Stenger, D. A. *Langmuir* **2003**, *19* (5), 1586−1591.

(36) Shchepinov, M. S.; CaseGreen, S. C.; Southern, E. M. *Nucleic Acids Res.* **1997**, *25*, 1155−1161.

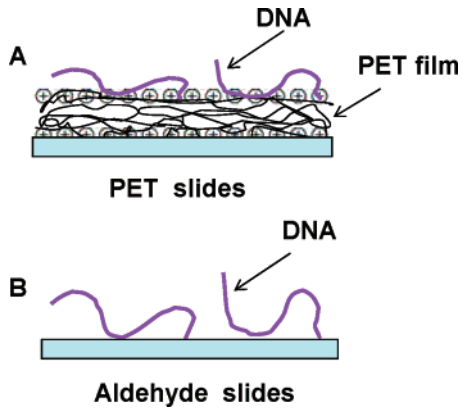

Figure 7. Diagram of configurations for long oligonucleotide probes immobilized on slide surface: **(**A**)** unmodified oligonucleotide probes adsorbed within polyelectrolyte thin film (PET); **(**B**)** alkylamino-modified oligonucleotides bound on aldehyde slide with a sticky end group but also in flattened configuration with multiple contacts to the slide surface.

efficiency (~82%) than the SuperAldehyde slide (~70%), indicating that a long space arm between the oligonucleotide probe and slide surface would improve the accessibility of the target DNA to the immobilized oligonucleotides. This is because the dextran in the dextran−aldehyde slide is a linear polymer and serves as long and hydrophilic spacer arms. This is consistent with the report that oligonucleotides probe modified with long spacer arms, such as C12 or C18, increase the hybridization efficiency for the oligonucleotide probes end-tethered on the slide.[9,15,37,38] However, these specific modifications for probes will significantly increase the cost for fabricating oligonucleotide microarrays. Preparing a slide using a functionalized linear polymer, such as dextran−aldehyde, is one way to reduce the cost of modifying oligonucleotide probes, but the dextran−aldehyde slides have a relatively high background signal owing to the multiple steps in organic synthesis.

The higher sensitivity and wide dynamic range on the PET slide is most likely due to the combination of high probe binding capacity of the PET due to its 3-D nature and the high level of probe accessibility of the PET. Unlike thick polymer film coated slides, such as the FAST slide,[20] on which the DNA probes were retained primarily by hydrophobic adsorption, short oligonucleotide probes (<30-mer) can be effectively immobilized on the PET slide, whereas only cDNA or PCR product can be immobilized on a FAST slide. The PET film also has a low fluorescence background compared to the thick nitrocellulose film. Although we only presented the fabrication of oligonucleotide microarrays on PET films at this work, the PET films can also be used for fabrication of cDNA microarrays. Ultraviolet or thermal cross-linking of cDNA to PET could be used to further stabilize the arrayed spots, which allows the cDNA on PET to be applied in vigorous denature and washing steps. The use of PET slides for fabricating cDNA microarrays is an ongoing study in our laboratory. Moreover, the binding capacity and hybridization sensitivity of the microarray on PET can be further increased by using dendrimeric polymers,[14] such as polyamindoamine starburst polymers as starting materials for preparing PET.

The use of oligonucleotide-based microarrays for the analysis of point mutations and single nucleotide polymorphisms (SNPs) in the genomic DNA of different organisms is more complicated and currently under intensive development. However, there is no report on the effect of surface chemistry to discriminate SNPs. We evaluated the ability to discriminate nucleotide polymorphisms using an oligonucleotide microarray fabricated on PET-, SuperAldehyde-, and APTS-coated slides. The results showed that although the hybridization signal intensity varied on different slide types, the discrimination factors (F_m/F_p) of each probe obtained from the microarrays on the three types of slides were similar (Figure 6). This indicates that the discrimination of nucleotide polymorphisms on an oligonucleotide microarray is independent of the surface chemistry used to immobilize the oligonucleotide probes.

Conclusions

In summary, we have demonstrated the use of PET as a highly stable platform for fabrication of oligonucleotide microarrays through spontaneous one-step direct immobilization of the unmodified oligonucleotide probes. The PET immobilization chemistry described here is distinguished from other immobilization chemistries such as the lower binding capacity of 2-D film (thickness less than 20 nm) and high background noise of 3-D thick polymer film (thickness larger than 1 μm). Our PET film presented the first example to fabricate DNA microarrays on ultrathin polymer films (i.e., film thickness around 100 nm). Indeed, the PET immobilization chemistry combines several advantages of a 2-D monolayer coating and a 3-D thick polymer coating. First, PET can be easily deposited on a solid substrate using a layer-to-layer adsorption process. No chemical synthesis steps or handling of toxic coupling reagents is required in preparing the coating film. Second, the PET chemistry provides an economic method to fabricate oligonucleotide microarrays because the oligonucleotide probes do not have to be modified with active functional groups prior to being immobilized. PET slides also do not require any special handling or storage precautions. This provides convenience for researchers to conduct microarray studies for multiple projects. In addition, compared with conventional aldehyde, APTS, or PLL slides usually employed, PET has significantly greater binding capacity and hybridization efficiency and reveals highly specific hybridization properties.

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(37) Southern, E.; Mir, K.; Shchepinov, M. *Nat. Genet.* **1999**, *21*, 5−9.
(38) Chrisey, L. A.; Lee, G. U.; OFerrall, C. E. *Nucleic Acids Res.* **1996**, *24*, 3031−3039.