Impact of surface chemistry and blocking strategies on DNA microarrays

Scott Taylor[1], Stephanie Smith[1], Brad Windle[2] and Anthony Guiseppi-Elie[1,3,*]

[1]Center for Bioelectronics, Biosensors and Biochips (C3B), [2]Department of Medicinal Chemistry and [3]Department of Chemical Engineering, Virginia Commonwealth University, PO Box 843038, 601 West Main Street, Richmond, VA 23284-3038, USA

ABSTRACT

The surfaces and immobilization chemistries of DNA microarrays are the foundation for high quality gene expression data. Four surface modification chemistries, poly-L-lysine (PLL), 3-glycidoxypropyl-trimethoxysilane (GPS), DAB-AM-poly(propyl-eminime hexadecaamine) dendrimer (DAB) and 3-aminopropyltrimethoxysilane (APS), were evaluated using cDNA and oligonucleotide sub-arrays. Two un-silanized glass surfaces, RCA-cleaned and immersed in Tris–EDTA buffer were also studied. DNA on amine-modified surfaces was fixed by UV (90 mJ/cm2), while DNA on GPS-modified surfaces was immobilized by covalent coupling. Arrays were blocked with either succinic anhydride (SA), bovine serum albumin (BSA) or left unblocked prior to hybridization with labeled PCR product. Quality factors evaluated were surface affinity for cDNA versus oligonucleotides, spot and background intensity, spotting concentration and blocking chemistry. Contact angle measurements and atomic force microscopy were preformed to characterize surface wettability and morphology. The GPS surface exhibited the lowest background intensity regardless of blocking method. Blocking the arrays did not affect raw spot intensity, but affected background intensity on amine surfaces, BSA blocking being the lowest. Oligonucleotides and cDNA on unblocked GPS-modified slides gave the best signal (spot-to-background intensity ratio). Under the conditions evaluated, the unblocked GPS surface along with amine covalent coupling was the most appropriate for both cDNA and oligonucleotide microarrays.

INTRODUCTION

The DNA microarray enables researchers to survey the entire transcriptome of virtually any cell population. This capability produces unprecedented quantities of raw data and enables the investigation of gene expression, functional genomics and genetic complexity with potentially many more applications (1–4). Although production capabilities and use of micro-arrays are becoming increasingly well established, significant differences exist with regard to fabrication techniques and end user protocols. Such differences make it difficult to compare results across platforms and present data management challenges for the integration of databases. Fabrication parameters that may vary include: surface chemistry of slides (5–9), type and length of printed DNA (2,9) and immobilization or fixing strategies for the spotted DNA. Various end user protocols include: pre-hybridization surface blocking (3), mRNA labeling protocols, hybridization protocols, post-hybridization wash stringency and data analysis techniques (4,10,11). An additional area of great concern is the implementation (placement and type) of appropriate controls aimed at quality assurance and quality control. The absence of approaches that are based on 'best practices' for design, fabrication, and end use of microarrays makes comparative data analysis between groups problematic. Although some work has been recently published that addresses several of these issues, (2–7,9–13) there is still little consensus about which design features and end user protocols are optimum for highest quality microarray data. In a recent attempt to develop microarray standards, the authors of the MIAME (minimum information about a microarray experiment) protocol have introduced guidelines for establishing standards concerning the information requirements for a more effective comparative analysis of microarray data between groups (10). The emphasis on these guidelines is however on documentation and not on engineering guidance. This paper aims at providing engineering guidance in the fabrication of cDNA and oligonucleotide microarrays.

The glass surfaces of DNA microarrays have been modified in various ways to immobilize DNA (oligonucleotides and/or cDNA) (5–9). Common surface modifications for printing and affixing DNA onto glass slides are: poly-L-lysine (PLL) (14), 3-aminopropyltrimethoxysilane (APS) (3,5,9), 3-glycidoxy-propyltrimethoxysilane (GPS) (7,9) and aldehyde or carboxylic acid (5). DNA has also been directly printed onto unmodified glass (9). Amine-terminated cDNA and amine-terminated oligonucleotides may be covalently coupled to epoxide, isothiocyanate and aldehyde activated glass surfaces (7). Non-terminated DNA has also been spotted onto amine-functionalized surfaces such as PLL, APS and surfaces that

To whom correspondence should be addressed at Center for Bioelectronics, Biosensors and Biochips (C3B), Virginia Commonwealth University, PO Box 843038, 601 West Main Street, Richmond, VA 23284-3038, USA. Tel: +1 804 827 7016; Fax: +1 804 827 5002; Email: ...

were functionalized and derivatized with polyamidoamine dendrimer (PAMAM) (6).

One possible advantage of GPS, APS and PAMAM over PLL is that the former are covalently immobilized to the silicon bearing hydroxide functional groups on the surface of glass, while PLL is immobilized by adsorption, the result of acid–base interactions and hydrogen bonding with the amphoteric glass surface (15). Moreover, it has been reported that aminosilanes and PAMAM surfaces offer a more consistent surface than PLL, with lower background and higher overall fluorescent signal intensities (6). Given that there are ~5.0 silanol groups/nm^2 on a fully hydroxylated silica surface that is supplemented by a few layers of surface bound water, and given that the APS molecule could pack to a limit of ~5 molecules/nm^2 (perfect hydrocarbon chain packing, e.g. c-axis of polyethelene crystals packs at ~5.2–5.4), then it is likely that a well-packed APS layer would typically present in the range 3.5–4.0 amine groups/nm^2 (16,17), while PAMAM derivatized surfaces present ~66 amines/nm^2 (18). In addition, PLL surfaces generally require an induction period of ~2 weeks before they can produce consistent microarray results (3). PLL, APS and PAMAM all present amine functional groups suitable for interaction with DNA via hydrogen bonding and, potentially, via electrostatic interactions (9) under the appropriate pH conditions. DNA is commonly 'cross-linked' on these surfaces by exposure to UV light, however this process is poorly understood but is believed to involve the creation of radicals that induce interchain cross-linking. GPS, in contrast, allows amine-terminated DNA to be covalently immobilized to the surface (19) via an amine-initiated nucleophilic ring opening reaction that leads to covalent bond formation between the GPS and the amine-terminated DNA.

Blocking reactions are typically employed to prevent labeled reverse transcription product from adsorbing to the surface of the printed microarray during the hybridization reaction. Blocking methods provide the added advantage of washing away unbound DNA from the surface that would otherwise compete with the labeled species (3). Two of the most common blocking methods to address non-specific adsorption on amine-modified microarrays involve blocking with succinic anhydride (SA) (3,14) or bovine serum albumin (BSA) (3). Both are intended to block the unreacted functional groups of the printed microarray with chemistries that have low affinity for DNA.

In this paper, we report an evaluation of spotting concentration, surface chemistries and blocking strategies for their combined role in the performance of oligonucleotide and cDNA microarrays. Our goal was to establish optimum protocols for manufacturing, spotting, hybridization and scanning of microarrays. cDNA and oligonucleotide microarrays were therefore spotted on six different surfaces. These surfaces evaluated were: APS, GPS, DAB-AM-16-poly(propyleminime hexadecaamine) (DAB), and PLL. DAB is a generation 3 dendrimer that was linked to the glass surface via covalent coupling following surface modification with GPS. In addition, two unmodified blank slides: (i) RCA-cleaned, but not surface modified (RCA); and (ii) cleaned and immersed in Tris–EDTA buffer (TEB) were also evaluated. Microarrays were blocked with either SA (SA-blocked), BSA (BSA-

range of available surface chemistries. The GPS presents the reactive glycidoxy functional group to which amine-terminated oligonucleotides and cDNA, derived from amine-terminated primers, could be covalently affixed. The APS, PLL and DAB surfaces present varying densities of amine functionalities for hydrogen-bonding interactions with DNA. The RCA-cleaned glass slides served as a reference surface while the TEB immersion deliberately introduced surface contamination to otherwise cleaned glass slide surfaces. The non-blocked surface served as the control for blocking. These surfaces and blocking strategies were evaluated by fabricating microarrays of cDNA and 30mer oligonuclotides prepared using the human GAPDH gene sequence. The oligonucleotides and cDNA were spotted at five different concentrations and hybridized to Alexaflour 555-labeled GAPDH PCR product. Wettability of the surfaces was determined by contact angle measurements with hexadecane and ultrapure water. Surface morphology was characterized by atomic force microscopy (AFM).

MATERIALS AND METHODS

Cleaning, preparation and surface modification of microarray slides

In a class 1000 clean room, 50 VWR brand glass microscope slides (VWR 48300-025) were solvent cleaned by immersion for 1 min in boiling acetone followed by 1 min in boiling isopropanol. The slides were then washed in ultrapure H$_2$O (18 MOhm) for 1 min and dried with filtered nitrogen. Next, the slides were UV/ozonated for 15 min on one side using a Boekel UV Clean Model 135500 followed by ultrasonication in a Branson 1510 ultrasonicator in isopropanol for 5 min. The slides were then washed in diH$_2$O and dried using filtered nitrogen. Finally, the slides were activated by immersion in a (5:1:1) solution of diH$_2$O:hydrogen peroxide:ammonium hydroxide (RCA) at 60°C for 1 min, followed by diH$_2$O wash, placed in glass slide carriers and dried in a convection oven for 30 min at 80°C. After this step, RCA-cleaned slides were stored for subsequent spotting.

The cleaned slides were then partitioned into six groups. One group of nine slides was modified by immersion in a solution of γ-APS 0.1% v/v in anhydrous toluene for 30 min at 40°C, washed three times in anhydrous toluene, placed in a glass staining dish and cured in a convection oven for 20 min at 110°C. The slides were then stored until needed for printing. Twenty-four slides were chemically modified by immersion in a solution of GPS 0.1% v/v in anhydrous toluene for 30 min at 40°C, washed three times in anhydrous toluene, placed in a glass staining dish and cured in a convection oven for 20 min at 110°C. Nine of these slides were stored for printing, and the remaining slides were subsequently modified by immersion in a solution of DAB 1.0% v/v in absolute ethanol overnight at room temperature. After the overnight incubation, the slides were washed three times in ethanol, placed in a glass staining dish and cured in a convection oven for 20 min at 110°C. The nine remaining slides were immersed in TEB (1.0 M Tris, 0.1 M EDTA) for 30 min at room temperature, washed in diH$_2$O, dried in a convection oven and stored. Nine slides were modified with PLL. The slides were immersed in a solution of

560 ml of diH$_2$O, then incubated with gentle shaking for 1 h at room temperature. The slides were then washed five times in diH$_2$O, dried with filtered nitrogen and placed in a 55°C vacuum oven for 10 min. All slides were stored in a plastic microscope box wrapped in aluminum foil then placed in a desiccator cabinet until needed for spotting. The PLL-modified slides were stored for 1 week prior to microarray spotting.

Contact angle and AFM measurements

Contact angles of de-ionized water ($\gamma_L = \gamma_L{}^p + \gamma_L{}^d = 53 + 20 = 73$ mN m^{-1}) and anhydrous hexadecane ($\gamma_L \approx \gamma_L{}^d = 26$ mN m^{-1}) were measured at the cleaned or chemically modified microscope glass slides using an NRL Contact Angle Goniometer (Ramé-Hart Inc., Mountain Lakes, NJ). Octadecyltricholorsilane (OTS) was used as a reference surface and was prepared following solvent cleaning by immersion in 0.1% v/v OTS in anhydrous toluene at 40°C for 30 min. The slides were then rinsed three times with toluene and dried at 110°C for 20 min. In a contact angle measurement, a droplet (~15 μl) of probe solvent was placed on the cleaned or modified glass slide from a fixed height, and the contact angle was directly measured through the focusing lens of the goniometer. AFM was performed using a Digital Instruments Dimension 3100 Atomic Force Microscope. Scan rates were set between 5 and 8 Hz depending on the image quality, and the scan size was changed from 1 to 10 μm upon engagement of the cantilever. The instrument was operated in tapping mode to obtain the micrographs. The resulting height images were processed using Nanoscope III software. Images were flattened to remove scan lines, and the height scale was set to 75 nm. Feedback controls such as integral gain, proportional gain and amplitude set point were modulated in real time as the image was being generated. Integral and proportional gain were always set between 2 and 0.5.

Preparation of GAPDH cDNA for arraying

The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene fragment obtained from PCR was a source of cDNA for arraying onto the slides prepared in the previous step. Amine-modified PCR primers: forward: 5′ amine-C6-ccacccatgg-caaattccatggcaccgtca and reverse: 5′ amine-C6-ggtttttcta-gacggcaggtcaggtccacc, were diluted to a working concentration of 0.001 μg/μl and 10 μl was then mixed with 0.5 μl (5000 U/μl) of New England Biolabs (NEB) *Taq* polymerase (M0267S), 0.1 μl (200 mM) dNTPs (Invitrogen 10216-012, 014, 016, 018), 5 μl of 10× NEB PCR buffer, 0.5 μl of GAPDH template and 34 μl of diH$_2$O per 50 μl reaction for a total of 50 reactions. The reaction was initiated at 95°C for 30 s and cycled 29 times under the following conditions: melt at 95°C for 30 s, anneal at 50°C for 30 s and

extend at 72°C for 1 min using an MJ Research PTC-200 thermal cycler. After PCR, the reaction products were combined and distributed into three 1.7 μl centrifuge tubes. To each tube was added 750 μl of 100% ice-cold isopropanol and the tubes were centrifuged at 14 000 r.p.m. for 30 min in an Eppendorf Model 5804R centrifuge to pelletize the PCR product. The pellet was washed in 75% ethanol and re-pelleted by centrifugation at 14 000 r.p.m. for 30 min. After centrifugation the pellet was re-suspended in 20 μl diH$_2$O per tube and the contents of each tube were combined. The concentration of GAPDH in solution was quantified by UV spectroscopy with a Perkin Elmer Lambda 40 spectrometer. The GAPDH cDNA was diluted to the concentrations of 2.0, 1.0, 0.5, 0.2, 0.02 and 0.002 μg/μl. An equal volume of 2× spotting buffer (3 M Betaine, 6× SSC) was added to each of the dilutions to make the 1× spotting solution. The solutions were then distributed into separate 96 well V bottom micotiter plates using a Packard Biochip MultiProbeII Liquid Handling robot. The plates were stored at –20°C until needed for spotting.

Preparation of oligonucleotides for arraying

Oligonucleotide primers were designed using the GAPDH sequence (accession no. NM_002046) and synthesized by Integrated DNA Technologies. Table 1 lists the oligonucleotides, their 5′ modification and their position in the GAPDH sequence. The forward, interior and random primers were diluted to the 2× concentrations: 2.0, 1.0, 0.5, 0.2, 0.02 and 0.002 μg/μl in diH$_2$O and mixed with an equal volume of 2× spotting buffer (3 M betaine, 6× SSC). The forward, interior and random primers were arrayed on each type of chemically modified glass slide as well as onto the two groups of unmodified slides (RCA-cleaned and buffer immersed).

Probe immobilization

Array fabrication was performed using a Cartesian Technologies PixSys 5500SQ Pin Array Robot and Liquid Dispensing System. Forward, interior and the random oligonucleotide sequences were spotted in three sub-arrays on slides that were modified with GPS, APS, DAB, PLL and the unmodified slides (RCA-cleaned and buffer immersed). PCR amplified GAPDH cDNA was also spotted on these slides in three additional but separate sub-arrays. The DNA arrayed on these surfaces was spotted in graded concentrations using the betaine spotting solution. The final DNA microarray layout is shown in Figure 1. After spotting, the APS, DAB, PLL, RCA and buffer immersed arrays were cross-linked with 90 mJ/cm^2 in an Ultra-Violet Products CL-1000 UV cross-linker and baked at 80°C for 1.5 h. The GPS arrays were incubated at 42°C in 50% humidity for 8 h, rinsed with 0.2% SDS solution for 2 min by vigorous shaking, washed three

Table 1. Oligonucleotide sequence information

| Oligo name	Position	Modification	Sequence
Forward	228–258	Amine	ccacccatgg caaattccat ggcaccgtca
Reverse	802–811	Amine	ggtttttcta gacggcaggt caggtccacc
Interior	502–531	Amine	cagcctcaag atcatcagca atgcctcctg
Unlabeled competitor	Complement of interior	None	caggaggcat tgctgatgat cttgaggctg
Randomer	None	Amine	acctggacct gaatccgcca tatagcctac
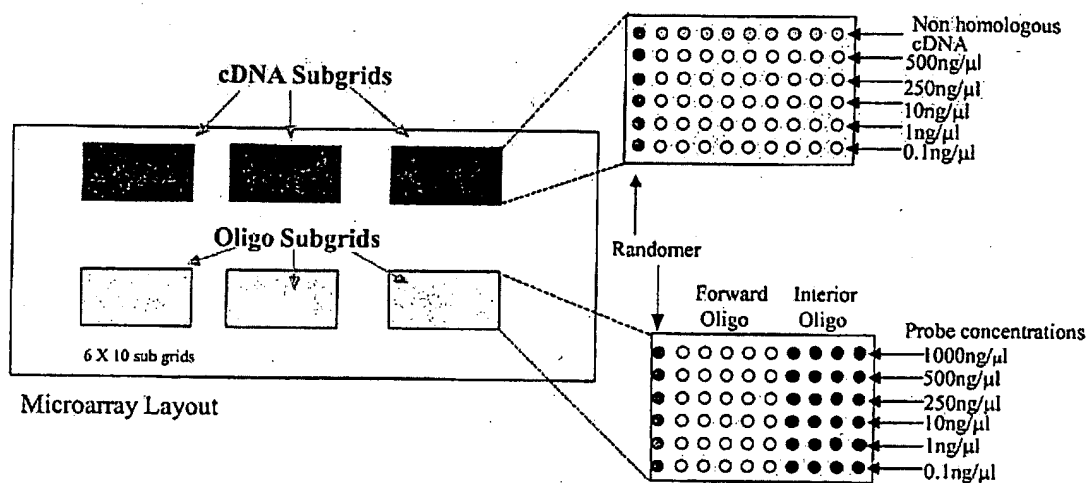
Figure 1. Microarray layout.

times in diH$_2$O, incubated in diH$_2$O at 50°C for 20 min then dried with filtered nitrogen. All arrays were then stored in foil-wrapped slide-boxes in a desiccator cabinet overnight prior to hybridization.

Labeling of GAPDH target

The forward and reverse oligonucleotide primers were used to amplify a 600 bp region of the GAPDH gene for fluorophore labeling. The previously described PCR protocol was used except that aminoallyl dUTP (Molecular Probes A-21664) was included in the reaction mixture at a ratio of 3:1 dUTP:TTP for a final concentration of 200 mM in each 80 µl reaction for a total of 60 reactions. The resulting PCR product was labeled using the ARES™ DNA labeling kit from Molecular Probes (A-21665) according to the supplied protocol.

Pre-hybridization blocking

Twelve slides were immersed in pre-hybridization buffer containing 5× SSC, 0.1% SDS and 1.0% BSA, incubated at 42°C for 45 min, washed 5× in diH$_2$O then dried using filtered nitrogen. Another 12 slides were immersed in SA pre-hybridization solution containing 15 ml sodium borate and 6 g SA in 350 ml 1-methyl-2-pyrrolidinone. The solution containing the slides was incubated on an orbital shaker for 20 min, quenched in boiling diH$_2$O, washed five times in 95% ethanol and dried using filtered nitrogen. Twelve slides were left unblocked. The remaining slides in the GPS and RCA groups were processed separately according to the same protocol.

Hybridization and imaging

Each group of slides was hybridized using a GenTac Hybridization Station (Genomic Solutions). 100 µl of hybridization buffer [4× SSC, 1× Denhardt's reagent, 5.0% SDS, 10% dextran sulfate, 40% formamide solution (50% v/v diH$_2$O)] containing 40 ng labeled GAPDH cDNA and, for some experiments, 24 ng unlabeled competitor, was added to each microarray hybridization solution. The hybridization was allowed to proceed for 16 h at 42°C. After hybridization, the arrays were sequentially washed with medium stringency buffer (2× SSC, 0.1% SDS) (Genomic Solutions 16004901),

high stringency buffer (0.1× SSC, 0.05% SDS) (Genomic Solutions 16004501), post wash buffer (0.1× SSC) (Genomic Solutions 16003501) and diH$_2$O. The arrays were then dried with filtered nitrogen. Each microarray was scanned at 5 µm resolution using a Perkin Elmer ScanArray 5000 microarray scanner using the 488 nm filter.

RESULTS

Surface chemistry and blocking strategy

Four chemically modified and two unmodified glass surfaces were studied for their characteristics relating to: (i) immobilization of cDNA and oligonucleotides, (ii) resulting slide background intensity after hybridization, (iii) signal intensity (spot intensity/slide background intensity) following hybridization and (iv) spotting uniformity. The surface chemistries evaluated were γ-APS, GPS, DAB (linked to the glass surface via GPS), PLL, a cleaned glass surface that had been immersed in TEB and a RCA-cleaned surface. These surfaces were selected because they are commonly used or otherwise cost effective/easy to implement in the microarray fabrication laboratory. While there are several alternative attachment chemistries (5,7), we limited this study to the most widely used and well-documented examples. Most cDNA microarray fabrication has been reported using PLL surfaces (2,3,14,15). However, Hegde *et al.* (3) and Liu *et al.* (20) have used APS surfaces for their cDNA microarray work and APS-modified glass surfaces are commercially available from Corning [CMT-GAPS slides (catalog no. 40004, Corning)] and Telechem [Super Amine slides (catalog no. SMM)] (web addresses for microarray substrates: Corning: http://www.corning.com/LifeSciences/pdf/gaps_ii_coated_slides_10_01_ss_cmt_gaps_002.pdf and Telechem: http://arrayit.com/Products/Substrates/substrates.html).

In an effort to identify a better microarray surface, one group has examined the amine presenting compound, PAMAM (6), and found it to have superior background and oligonucleotide capturing characteristics. We chose a closely related compound to that used by Benters *et al.* (6) for
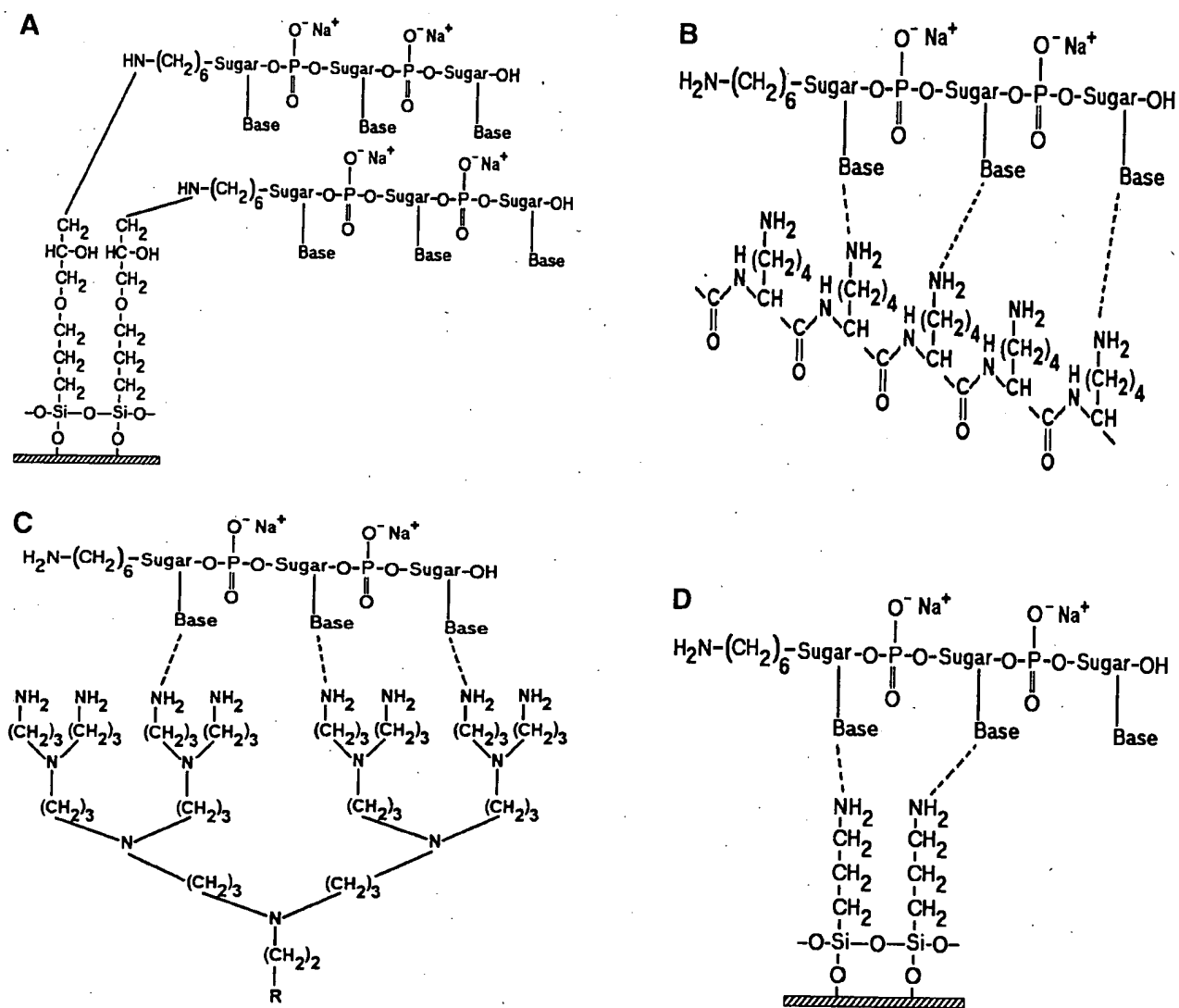
Figure 2. Schematic illustration of the various surface chemistries studied and the idealized interaction of DNA with functional groups on a glass surface. (A) GPS covalently bound to an amine-terminated oligonucleotide. (B) PLL hydrogen bonding with an oligonucleotide. (C) One-half of a DAB dendrimer hydrogen bonding with an oligonucleotide. (D) APS hydrogen bonding with an oligonucleotide.

covalent coupling, it has been reported that epoxy-silane (GPS) has been used for immobilizing amine-terminated oligonucleotides and cDNA (5,21). Figure 2 is a schematic illustration of the various surfaces studied.

The pre-hybridization blocking strategies studied were: no blocking, the adsorption of BSA and the reaction of SA. The ability of each of these three blocking strategies to reduce post-hybridization background intensity was investigated for each of the six surfaces. SA is commonly used as a blocking reagent in cDNA microarrays prepared on amine-functionalized surfaces (3,13). The anhydride readily reacts with the available amines forming the amide and thereby eliminating the amine from the surface with the intent of avoiding non-specific adsorption of DNA. Such an approach should be effective for both oligonucleotide and cDNA microarrays. A blocking solution containing BSA has been reported

by Hegde *et al.* (3) to result in lower background intensities when compared with SA. BSA is a neutral globular protein that readily adsorbs to surfaces and is commonly used in ELISAs.

There are two microarray platforms in wide usage: cDNA and oligonucleotide arrays. The oligonucleotide arrays vary in oligonucleotide length but are generally 25–70mers while printed cDNA typically ranges from 70 to 600 bp. Both types were evaluated in this study. The oligonucleotides selected were 30mers of the GAPDH gene and the cDNA was an ~600 bp PCR product amplified from GAPDH using amine-terminated primers. Both types of DNA were spotted over a broad range of concentration (0.001–0.5 µg/µl).

We measured spot quality as a function of spot and background intensities. All intensities were measured under

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.

fastcase
Smarter legal research.