Robust and efficient synthetic method for forming DNA microarrays

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ABSTRACT

The field of DNA microarray technology has necessitated the cooperative efforts of interdisciplinary scientific teams to achieve its primary goal of rapidly measuring global gene expression patterns. A collaborative effort was established to produce a chemically reactive surface on glass slide substrates to which unmodified DNA will covalently bind for improvement of cDNA microarray technology. Using the p-aminophenyl trimethoxysilane (ATMS)/diazotization chemistry that was developed, microarrays were fabricated and analyzed. This immobilization method produced uniform spots containing equivalent or greater amounts of DNA than commercially available immobilization techniques. In addition, hybridization analyses of microarrays made with ATMS/diazotization chemistry showed very sensitive detection of the target sequence, two to three orders of magnitude more sensitive than the commercial chemistries. Repeated stripping and re-hybridization of these slides showed that DNA loss was minimal, allowing multiple rounds of hybridization. Thus, the ATMS/diazotization chemistry facilitated covalent binding of unmodified DNA, and the reusable microarrays that were produced showed enhanced levels of hybridization and very low background fluorescence.

INTRODUCTION

Accompanying the development of several high-throughput technologies that make it possible to sequence complex genomes is the challenge to not only identify genes, but to understand the function and expression of those genes. cDNA microarray technology, first developed by Patrick Brown and his colleagues at Stanford University (1), allows for the high-throughput measurement of expression patterns of thousands of genes simultaneously. Microarrays utilize a high-speed precision robot to affix thousands of DNA samples onto a solid support (a glass slide, chip or nylon membrane). This allows high spot densities to be obtained, thus increasing the number of samples that can be analyzed at once. The slides are simultaneously probed with fluorescently labeled cDNAs generated from mRNA isolated from cells or tissues in two different physiological, developmental or disease states (2–6). Each population of cDNAs is labeled with a different fluorescent dye, allowing direct comparisons on a single array. The relative intensities of the two fluorescent dyes within a spot correspond to the relative expression levels of the genes, reflected by the two RNA populations used to make the labeled cDNA. The use of microarrays, therefore, allows the expression of thousands of genes to be assayed in a single experiment.

While DNA microarray technology was initially developed to detect global patterns of gene expression, it has many other potential applications including identification of complex genetic diseases (7), mutation/polymorphism detection (8), and drug discovery and toxicology studies (9). Recently, tissue microarrays were used for the molecular profiling of tumor specimens (10).

The major steps of DNA microarray technology include the manufacture of microarrays, the fluorescent labeling of cDNA probes, hybridization of the probes to the immobilized target DNA and the subsequent analysis of the hybridization results. There are two predominant methods for producing DNA microarrays. The first was developed by Stephen Fodor and colleagues in the early 1990s (11), and it is often referred to as the Affymetrix method (Affymetrix Inc., Santa Clara, CA). In this process, DNA oligonucleotides are synthesized directly onto the microarray or DNA chip using photolabile protecting groups and masks to direct the selective addition of nucleotides.

In the second common method for producing microarrays, pre-existing DNA fragments are spotted onto a glass or membrane support using a precision robot (1). Both of these systems commonly permit only single usage of the DNA arrays. Reusability of microarrays would eliminate the variance between arrays, which are often presumed identical; the variance ultimately affects the experimental reliability of microarray-based analyses. The surface chemistry of the glass substrate is a major determinant of the stability of DNA attachment throughout the hybridization and washing steps.

The most popular substrates for spotting DNA are polylysine- and aminosilane-coated glass slides (12). These slides...
are available commercially, suitable for miniaturizing array dimensions and fluorescence detection, inexpensive and ready to use. The disadvantages of these slides include: (i) the spots are not uniform, limiting the accuracy of quantitative detection, and (ii) the high background commonly seen decreases the sensitivity of detection (13).

A glass substrate that allows covalent attachment of nucleic acids could solve the problem of reusability. The late 1970s saw the emergence of a procedure to covalently couple single-stranded nucleic acids (separated by gel electrophoresis) to Whatman paper that had been derivatized with diazobenzyloxymethyl groups, and the use of this paper for hybridization with DNA probes (14–18). This derivatization method has been extended in the current study to covalently immobilize DNA onto a glass substrate for subsequent hybridization with DNA probes.

The goals of this work were to produce a chemically reactive surface on glass slide substrates to which DNA will covalently bind and, subsequently, to hybridize to fluorescently labeled cDNA probes in a reproducible, consistent, uniform and stable manner. The covalent binding of DNA to solid substrates should increase the uniformity of attachment, and result in more uniform fluorescent signals and decreased DNA loss during the course of experiments. Covalently bound DNA should also permit the use of more stringent wash conditions, which would reduce background and thus allow for greater sensitivity. The binding chemistry described here provides increased reproducibility and consistency of results, and enhanced stability that allows the reuse of microarrays. In addition, this chemistry does not require prior synthetic modification of the DNA. It is predicted that this approach will also be applicable to the attachment of RNA, aptamers and ribozymes.

MATERIALS AND METHODS

Materials

p-Aminophenyl trimethoxysilane (ATMS) (90%; Gelest Inc., PA), sodium nitrate (Sigma, MO), sodium acetate (Sigma, MO), ethanol (absolute, 200 proof; Aaper Alcohols and Chemical Company, KY), glycine (Aldrich, WI), 20× SSC (0.3 M sodium citrate/3 M sodium chloride) and sodium dodecyl sulphate (SDS) were used. Polylysine-coated microscope slides were obtained from Sigma, silylated slides (aldehydeamine chemistry) from TeleChem International Inc. (CA) and Corning CMT-GAPSTM slides (aminosilane chemistry) from Corning Inc. (NY).

Preparation of DNA

Genomic DNA from the filamentous fungus Neurospora crassa was isolated as described (19) from the Neurospora crassa strain (FGSC strain 2490; Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City, KS). Neurospora crassa am DNA, corresponding to the glutamate dehydrogenase gene (20), was a 2 kb EcoRI–BamHI fragment from pBJ011 (21), gel purified with the QIAEX® II Gel Extraction System and QIAquick Gel Extraction Kit (Qiagen Inc., CA), or PCR-amplified using Takara Ex TaqSTM DNA Polymerase (PanVera, WI). These DNA samples were redissolved in 10 µl double-distilled (dd) H2O or 4× SSC and immobilized on diazotized glass surfaces at each of the following amounts per microliter: 100 ng ORSsa; 100 ng ORSsa plus 0.05 ng am; 100 ng ORSsa plus 0.5 ng am; 100 ng ORSsa plus 5.0 ng am and 100 ng am (where ORSsa refers to DNA from the entire genome of N.crassa). There is one gene equivalent of the am gene per one genome equivalent.

Preparation of labeled probes

The am DNA probes were fluorescently labeled with Cy3TM, dCTP or Cy5TM.dCTP (Amersham Pharmacia Biotech, NJ) using a Nick Translation Kit for radioactive and non-radioactive probe preparation (Amersham Pharmacia Biotech). After labeling, the probes were precipitated with 1 vol of 4 M ammonium acetate and 5 vol of 100% ethanol and redissolved in 7–9 µl of sterile ddH2O.

Silane layer formation on substrates

Glass slides were cleaned by immersion into piranha solution (70/30 v/v sulfuric acid and 30% hydrogen peroxide) for 30 min followed by washing in deionized water (22). Caution: piranha solution will react violently with most organic compounds and must be handled with extreme care. Cleaned substrates were coated with AMTS by immersing the substrates in 1 mM solution of AMTS in ethanol for 30 min. The glass substrates were then rinsed in ethanol and dried in a stream of N2. This procedure resulted in the formation of amine-terminated silane layer on the glass substrate. Formation of silane layers was confirmed by X-ray photoelectron spectroscopy (Y.Wu, P.L.Dolan, R.L.Metzenberg, M.A.Nelson and G.P.Lopez, manuscript in preparation). The thickness of the silane layers was estimated by ellipsometry of the silane layers formed on surface-oxidized Si wafers (22). The ellipsometric thickness of this silane layer was 4.9 ± 0.2 Å.

Diazotization of ATMS-treated surfaces

Just before reaction with nucleic acids, ATMS-reacted surfaces were converted to the diazobenzyl form by treatment with a solution containing 40 ml of water, 80 ml of 400 mM HCl and 3.2 ml of freshly prepared solution of NaNO2 (200 mM) for 30 min at 4°C (14). After 30 min, the ATMS-treated surfaces were washed three times, each for 3 min, with ice-cold sodium acetate buffer (50 mM, pH 4.7) followed by washing with ice-cold deionized water and then ethanol twice each (5 min washes). The diazotized surfaces were air dried and gently blotted with Kimwipes® (Kimberly-Clark, GA) while maintaining their temperature at 4°C.

Formation of DNA arrays onto diazotized surfaces

The experimental DNAs were printed onto the ice-cold diazotized surfaces (manually or using a micro spotting device). After air drying for 1–2 h at room temperature, the diazotized slides were either (i) immersed in 1% glycine solution (pH 7.2) twice, each time for 5 min, rinsed with ddH2O to remove the residue of glycine, and air dried, or (ii) UV-crosslinked at 90 mJ using a Stratalinker™ (Stratagene, CA) and baked at 80°C for 2 h. The printed slides were stored in the dark at room temperature until used for hybridization.

Hybridization of DNA array

The printed slides were placed into a Coplin jar and pre-hybridized for 45 min at 42°C in a buffer containing 50% formamide, 5× SSC, 0.1% SDS and 1% bovine serum albumin...
After pre-hybridization, the slides were washed in room temperature ddH₂O and isopropanol and then air dried. The Cy5-labeled probes were combined with equal volumes of 2× hybridization buffer (50% formamide, 10× SSC, 0.2% SDS) and denatured for 5 min in boiling water. The hybridization mixture was applied onto the array, and covered with a glass cover slip. The array was placed in a humid ArrayIt™ Hybridization Cassette (TeleChem International Inc.) and submerged in a 42°C water bath for 14–16 h. After hybridization, the array was washed at room temperature in 1× SSC, 0.1% SDS for 5 min, 0.1× SSC, 0.1% SDS for 5 min and 0.1× SSC for 5 min. The array was given a final, brief rinse in ddH₂O and air dried.

Scanning glass slides
In the initial stages of this project, individual, manually printed spots were visualized with a Nikon Diaplot laser-confocal microscope (Nikon Inc., NY) equipped with a Bio-Rad MRC-600 scanning head (Bio-Rad, CA). Recent arrays have been visualized with the confocal laser scanner, ScanArray 3000, produced by GSI Lumonics (CA).

Image processing
Both Cy3- and Cy5-labeled am DNA that had been affixed to the diazotized glass surfaces (with no hybridization) and the hybridized arrays were processed using Image Tool image processing program, Version 2.0 (University of Texas Health Science Center at San Antonio, TX), Bio-Rad COMOS (Bio-Rad) and Microsoft Excel (Microsoft Inc., WA). The steps included: (i) identifying each spot location, (ii) determining the spot intensity and the fluorescence background and (iii) calculating the background-subtracted intensities of each spot.

Stripping procedure
After hybridization with fluorescently labeled probes, the slides were immersed in stripping buffer (2.5 mM Na₂HPO₄, 0.1% SDS) at 95°C for 30 s, three to four times (13). They were then rinsed in ddH₂O, air dried, and stored in the dark at room temperature.

RESULTS
We have developed a chemical process for covalently linking DNA to an ordinary microscope slide in a manner that preserves the ability of the immobilized nucleic acid to hybridize to complementary sequences. A schematic of the overall process is shown in Figure 1. In brief, glass substrates were first cleaned with piranha solution and then functionalized with ATMS. Just before the reaction with nucleic acids (referred to herein as target DNA), ATMS-reacted surfaces were converted to the diazobenzyl form by treatment with a solution containing HCl and NaNO₂. Primary aromatic amines react with nitrous acid to yield diazonium salts. In this step, an electrophilic attack by +NO causes displacement of the H⁺ at the nitrogen (23):

\[
\text{ArNH}_2 + \text{NaNO}_2 + 2\text{HCl} \rightarrow \text{ArN}^\equiv \text{NCl}^- + \text{NaCl} + 2\text{H}_2\text{O}
\]

Because diazonium salts are unstable, this and all subsequent steps of this process, inclusive of nucleic acid spotting, were done at 4°C. After 30 min, the ATMS-treated substrates were washed successively with ice-cold sodium acetate buffer, ddH₂O and 100% ethanol. A gentle acidic buffer kept the diazonium salts active (23) on the glass surface. DNA was then spotted or microarrayed onto the diazotized glass surfaces, and air dried at room temperature for 1–2 h. The DNA reacts to form a covalent bond with the azo-terminus of the diazotized surface (23). In order to neutralize unreacted diazonium groups and to reduce non-specific binding of the probe to the slide, the surfaces were either immersed in 1% glycine solution or pre-hybridized in a buffer containing 50% formamide, 5× SSC, 0.1% SDS and 1% BSA as described above.
Diazonium salts can undergo a reaction referred to as ‘coupling’, in which certain aromatic compounds (such as certain bases in DNA) covalently bind to the positively charged nitrogen of the diazonium group (23). In the reaction between the diazonium salt on the glass surface and the DNA molecule, the aromatic rings of the DNA bases undergo attack by the diazonium ion. Because the diazonium ion is very weakly electrophilic, the aromatic ring must contain a powerful electron-releasing group, i.e. -OH, NR₂, -NHR or -NH₂. Covalent binding usually occurs para to the activating (electron-releasing) group (23). In the structure of the four bases of DNA, adenine, cytosine and guanine all contain an aromatic NH₂ group, which may undergo the coupling reaction with the diazonium ion. However, there is no strong electron-releasing group found in thymine. We thus assume that the covalent binding would occur between adenine, cytosine or guanine bases of the DNA and the diazonium salt. The thymine residue is covalently bound to the positively charged amine groups on the surface of the substrate by ultraviolet irradiation.

Attachment of Cy dye-labeled DNA to ATMS/diazotized glass slides

Preliminary results indicate that our immobilization method produces uniform spots that immobilize equal or greater amounts of DNA than current commercially available immobilization techniques (e.g. polylsine-based methods; 1). Figure 2 shows images that were captured from Cy3-labeled am DNA spotted onto ATMS/diazotized, polylsine-coated and TeleChem glass slides (Sigma, MO) and silylated slides (TeleChem International Inc.). Cy3-labeled DNA was manually spotted with a p10 micro Pipetman (Rainin Instrument Company, Emeryville, CA) onto diazotized, polylsine and TeleChem glass slides in dilutions of 1:10, 1:100 and 1:1000 using both H₂O and 4x SSC as diluents. The initial concentration of the Cy3-labeled DNA was 20–25 ng/µl. The protocol used for processing the TeleChem slides was that suggested by the manufacturer (http://arrayit.com/Products/Substrates/substrates.html). Processing of polylsine slides was as described previously (3). The spots on the diazotized slides were more homogeneous and uniform than those on the polylsine and TeleChem slides, and background fluorescence was significantly lower. In addition, the results with diazotized slides were quantifiable and reflected the serial dilutions used, in contrast to the spots on the polylsine and TeleChem slides (Fig. 3).

Also, in an attempt to evaluate and disrupt the covalent attachment, the ATMS/diazotized slides spotted with the Cy3-labeled am DNA were processed through a stripping procedure (as described in the Materials and Methods). The Cy5-labeled DNA remained affixed to the diazotized surface (data not shown).

Hybridization of DNA array

Unlabeled target DNA was immobilized on ATMS/diazotized, polylsine and Corning aminosilane-coated slides (CMT-GAPSTM, Corning Inc.). The protocols used for processing the polylsine and Corning slides were as described previously (3,24). Cy5-labeled am DNA was hybridized to increasing amounts of am-specific target DNA per microliter in the
spotted DNA solutions: 100 ng genomic (ORSa) DNA; 0.05 ng am DNA in 100 ng ORSa; 0.5 ng am in 100 ng ORSa; 5 ng am in 100 ng ORSa and 100 ng am DNA. Images of the Cy5-labeled am DNA hybridized to pure am DNA on the three types of slides are shown in Figure 5.

The spots on the ATMS/diazotized slide were very uniform and homogeneous, and the background was extremely low. Hybridization was detected with even the lowest amounts of am DNA (see below).

Whereas the spots on the Corning slide were also homogeneous, the background was very high, and ‘comet tails’ streaked from the detectable spots. Hybridization only with those spots corresponding to the two highest concentrations of target DNA (spotted at 100 ng am and 5 ng am in 100 ng ORSsa) was detectable; the three remaining spots had no hybridization signals (not shown).

The spots on the polylysine slide lacked clarity due to high background, low intensity, ‘comet tails’ and ‘shadows’. Only the spot corresponding to the highest amount of target DNA (pure am DNA) was detectable, and the intensity of this spot was much lower than that of the corresponding spots on ATMS/diazotized or Corning slides.

The ATMS/diazotized surface chemistry facilitated accurate detection of very low proportions of specific target DNA upon hybridization with a Cy5-labeled probe (Fig. 6); even the lowest amount of am DNA, corresponding to about one-thousandth of the DNA in the spot, was detectable. In contrast, measurable hybridization to target DNA on the Corning and polylysine slides was detected only with the highest (polylysine) or two highest (Corning) amounts of am DNA on their surfaces. The resolution of the spots on these slides was also reduced by high background signals.

High background and the streaking of spots by ‘comet tails’, as seen with the Corning and polylysine slides, have limited the accuracy and reproducibility of microarray analyses. However, with the ATMS/diazotized surface chemistry, hybridization of a probe corresponding to a single target gene allowed reproducible detection of the target sequence within genomic DNA, even when the target gene represented ~1/2000 of the genome (and therefore a small fraction of the DNA immobilized on the slide).

**Stripping and reuse of arrayed diazotized slides**

After an initial hybridization with a fluorescently labeled probe, the arrayed diazotized slides were stripped and rehybridized with a Cy5-labeled am DNA probe four successive
times. Stripping resulted in the removal of essentially all signals (not shown). Images of the spots resulting from each hybridization (times 1–5) are shown in Figure 7A; quantitation of mean background-subtracted intensities yielded the results shown in Figure 7B. Whereas the intensity of signals decreased with each successive hybridization, the spots on the ATMS/diazotized slide clearly remained detectable and proportionately accurate, reflecting the amounts of specific target DNA immobilized. The reusability of ATMS/diazotized slides makes this chemistry a significant contribution to the field of DNA microarray technology.

DISCUSSION

We describe a chemical process for covalently linking DNA to a glass substrate in a manner that preserves the ability of the immobilized nucleic acid to hybridize to complementary sequences. This surface chemistry promises to advance DNA microarray technology and analysis by increasing sensitivity, reproducibility and consistency, as well as stability, which permits reuse of the microarrays.

Two major DNA microarray technologies currently in use are the cDNA microarray format (25) and the in situ synthesized oligonucleotide microarray format (11,26). Many other approaches are also in use and/or in development (27–31). However, regardless of the format, an integral and much-discussed component of the technology remains the surface chemistries for attaching DNA to the solid substrate. The types of substrates used include nitrocellulose and nylon membranes and glass. Glass-based arrays are usually made on microscope slides that are coated with polylysine (1), aminosilanes (7) or amino-reactive silanes (25).

The cDNA array format developed at Stanford University is widely used; this methodology relies on the ionic binding of negatively charged DNA molecules to a positively charged...
polylysine surface on glass substrates (1). cDNA array designs using aminosilane-coated slides immobilize nucleotides by ultraviolet irradiation to form covalent bonds between the thymine residues in the DNA and the positively charged amine groups on the silane slides (32). Amino-reacting silane microscope slides will covalently bind amino-modified DNA molecules (25).

The *in situ* synthesized microarrays have oligonucleotides covalently bound to impermeable substrates through one end of the nucleotide strand (33). Fodor *et al.* utilized solid-phase chemistry, photolabile protecting groups and photolithography to chemically synthesize oligonucleotides directly onto solid substrates for *in situ* fabrication of arrays (11). A covalent linker molecule that terminates with a photolabile protecting group is used to derivatize a solid substrate. Light-directed oligonucleotide synthesis then proceeds as light is directed through a mask to deprotect and activate selected sites. The protected nucleotides then couple to the activated sites (34).

Another method for *in situ* synthesis of DNA utilizes ink-jet delivery of nucleotide precursors to an impermeable solid surface (35). Beier and Hoheisel (13) recently developed a linker system to covalently immobilize modified nucleic acids onto both glass and polypropylene. This system also supports *in situ* modification of oligomers onto these substrates. In future work, we will compare these technologies with the ATMS/diazotization method described in this paper.

The field of microarray technology is in its infancy, and opportunities for expansion, growth and innovation appear nearly limitless. The method described here for covalently attaching unmodified DNA to a glass substrate is a potential new ‘spoke’ in the expanding and complex ‘wheel’ of cDNA microarray analysis. It offers a paradigm for tethering nucleic acid onto a solid substrate that allows for considerable improvements in hybridization of complementary sequences, stability of affixed DNA, and reusability of arrayed slides, as well as greatly reduced background. This technology ultimately has the potential to enhance the field of global-gene expression analysis, as well as the analysis of DNA variation on a genome-wide scale.

CONCLUSION

We have developed an efficient ATMS/diazotization method for forming DNA microarrays onto solid substrates. Experimental results show that the covalent binding of DNA to ATMS/diazotized surfaces considerably increased the consistency of the attachment, and reduced DNA loss during the hybridization process. Also, better hybridization results were generated compared with those obtained using common commercially available immobilization techniques, and background fluorescence was minimal. With this immobilization process, arrayed slides were reusable after stripping at least four times, with reproducible and quantitative results.

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