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ARTICLE *in* BIOCONJUGATE CHEMISTRY · SEPTEMBER 2009

Impact Factor: 4.51 · DOI: 10.1021/bc900162u · Source: PubMed

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Construction of Oligonucleotide Microarrays (Biochips) via Thioether Linkage for the Detection of Bacterial Meningitis

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Oligonucleotide-based arrays are increasingly becoming useful tools for the analysis of gene expression and single-nucleotide polymorphism. Here, we report a method that allows the direct immobilization of thiolated oligonucleotides onto an epoxy-activated glass surface via a stable thioether linkage under microwaves. The described chemistry efficiently immobilizes the probes via terminal thiol groups with uniform spot morphology. The thioether linkage could endure repeated PCR-like heat cycling with only 2.5% loss after 20 cycles, indicating that the chemistry can be used in integrated PCR/microarray devices. The highlighting feature of the proposed method is that the detection limit for the probe concentration can be reduced to 0.25 μM with 20-mer oligonucleotides. The efficiency of the projected method (~33%) indicates its advantage over the existing standard methods, viz., NTMTA (~9.8%), epoxide-amine (~9.8%) and disulfide (~1.7%). The constructed microarrays were validated through the detection of base mismatches and bacterial meningitis. These features make the projected strategy ideal for manufacturing oligonucleotide arrays and detection of mismatches and bacterial diseases.

INTRODUCTION

Oligonucleotide microarrays have lately emerged into the academic and commercial arena owing to their usefulness in high-throughput, parallel gene expression, SNP analysis, and comparative genome hybridization (*1*−*4*).

DNA microarrays can be produced essentially in two different ways: (i) by direct chemical synthesis on the solid surface using photolithography technique (*5, 6*); (ii) by immobilization of presynthesized oligonucleotides on selected surfaces (*7*−*10*). An example of the first approach is the Affymetrix method, which involves stepwise synthesis of oligonucleotides using photocleavable protecting groups. It is advantageous in generating high-density (10^6 sequences/cm^2) microarrays but lacks flexibility and economy. There are also some practical limits to the length of oligonucleotides to be synthesized. The immobilization method allows the tethering of long as well as modified base-incorporated oligonucleotide sequences and flexibility of a variety of ligands to be fixed on a surface of choice. That is why patterning of presynthesized oligonucleotides is preferred in research laboratories and commercial applications, where low to medium density arrays are required. In addition to the chemistry involved in fixing oligonucleotides to the polymer surface, the choice of polymer matrix, particularly glass, plays an important role in influencing the quality of the constructed microarrays.

Since oligonucleotides bind poorly to virgin glass, a number of methods for noncovalent and covalent immobilization of oligonucleotides on modified glass have been reported (*11*−*23*). The simplest is electrostatic adsorption of probes on polylysine and aminopropyl-coated microslides (*10*), but it results in low hybridization efficiency, as ionic interactions encourage the probes to lay flat on the surface. Therefore, a single terminal covalent attachment is preferred for short oligonucleotides, as it allows the entire oligonucleotide to be available for hybridization. Some common combinations of surface/oligonucleotide modifications that have been demonstrated include the following: carboxyl−amine, thiol−disulfide, amine−aldehyde, aldehyde−oxyamine, biotin−streptavidin, gold−thiol, zirconylated-surface−phosphate, epoxide−amine, and so forth. In addition, there are strategies employing heterobifunctional reagents, such as *N*-(2-trifluoroethanesulfonatoethyl)-*N*-(methyl)-triethoxysilylpropyl-3-amine (NTMTA) and *N*-(3-trifluoroethanesulfonyloxypropyl)anthraquinone-2-carboxamide (NTPAC), previously reported from the authors' laboratory (*7*), for construction of oligonucleotide microarrays. These methods make use of single terminal attachment of oligonucleotides on modified glass surfaces. In some cases, immobilization was also realized by photochemical methods (single or multipoint contact). All of the above-mentioned approaches have their own limitations with respect to factors like involvement of multisteps for the introduction of functional groups on either the oligomer or glass surface, time-consuming reactions to realize immobilization with low to moderate immobilization efficiency, low thermal and chemical stability, and so forth.

Recently, Bocking et al. (*9*) have reported the immobilization of thiolated oligonucleotides on epoxylated silicon (111) surface. The method involves a one-step DNA immobilization; however, the functionalization of silicon (111) to generate epoxy groups is quite tedious and requires a multistep reaction scheme. Similarly, Preininger et al. (*16*) have generated high-capacity epoxy surfaces by coating 1% epoxy resin using different methods for achieving signal enhancement and immobilized amine- and thiol-modified oligonucleotides. However, reproducibility of the coating process and enhanced background signals limit the viability of the protocol. In search of a simple and efficient immobilization method, which would overcome the limitations of the existing ones, we explored the thioether linkage for microarray fabrication.

In this Communication, we propose the covalent attachment of thiol-modified probes to epoxy-activated glass slides under microwaves, where it takes only 12 min for immobilization with

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10.1021/bc900162u CCC: $40.75
Published on Web 08/13/2009

Table 1. List of Oligonucleotide Sequences Synthesized with Their Deprotection Conditions and Yields

no.	oligonucleotide sequence	deprotection conditions	yield (A_{260})
1.	TET-d (TCT GTA TGT CTA TTC TCG TT)-$OPO_3(CH_2)_6$-SH	Aq. NH_4OH (30%) containing 0.1 M DTT, 16 h, 55 °C.	24.3
2.	TET-d (TCT GTA TGT CTA TTC TCG TT)	Aq. NH_4OH (30%), 16 h, 55 °C.	27.1
3.	TET-d (AAC GAT CGA TTT GCT TAC GT)	Aq. NH_4OH (30%), 16 h, 55 °C.	25.1
4.	TET-d (TAC GAC CGT CTA TGC CGA)	Aq. NH_4OH (30%), 16 h, 55 °C.	23.1
5.	HS-$(CH_2)_6$-OPO_3-d (ACG TAA GCA AAT CGA TCG TT)	Aq. NH_4OH (30%) containing 0.1 M DTT, 16 h, 55 °C.	24.3
6.	HS-$(CH_2)_6$-OPO_3-d (ACG TAA GCA AAT CGA TCG GT)	Aq. NH_4OH (30%) containing 0.1 M DTT, 16 h, 55 °C.	24.6
7.	HS-$(CH_2)_6$-OPO_3-d (ACG TAA GCA AAT CGA TCA GT)	Aq. NH_4OH (30%) containing 0.1 M DTT, 16 h, 55 °C.	22.1
8.	HS-$(CH_2)_6$-OPO_3-d (TTT TTT TTT TTT TTT TTT TT)	Aq. NH_4OH (30%) containing 0.1 M DTT, 16 h, 55 °C.	23.2
9.	HS-$(CH_2)_6$-OPO_3-d (TCG ACG AAC TGA TCA TCC)	Aq. NH_4OH (30%) containing 0.1 M DTT, 16 h, 55 °C.	23.6
10.	HS-$(CH_2)_6$-OPO_3-d (CGT CGA GTT GTT CAT CTC GCA).	Aq. NH_4OH (30%) containing 0.1 M DTT, 16 h, 55 °C.	24.5
11.	H_2N-$(CH_2)_5$-OPO_3-d (ACG TAA GCA AAT CGA TCG TT)	Aq. NH_4OH (30%), 16 h, 55 °C	25.3
12.	TET-d(TCG ACG AAC TGA TCA TCC) Forward primer	Aq. NH_4OH (30%), 16 h, 55 °C	23.1
13.	TET-d(TCG GCA TAG ACG GTC GTA) Reverse primer	Aq. NH_4OH (30%), 16 h, 55 °C	22.5

~24% immobilization efficiency. A major advantage of the projected method is that it requires neither a coupling reagent nor chemical manipulations for the immobilization of prefabricated oligonucleotides. The proposed strategy is demonstrated with respect to pH and thermal stabilities, immobilization efficiency, functional availability of the probes for hybridization, and detection of mismatches. Also, it has been compared with the standard methods, viz., NTMTA, epoxide−amine, and disulfide approaches, to highlight its advantages over the existing ones. Subsequently, the oligonucleotide-based biochip was validated for detection of bacterial meningitis in human samples.

EXPERIMENTAL PROCEDURES

Materials. Virgin glass microslides (75 × 22 mm^2), *N*-methylimidazole, and 3-glycidyloxypropyltrimethoxy silane (GOPTS) were procured from Sigma-Aldrich Chemical Co., St. Louis, MO. Tetrachlorocarboxyfluorescein (TET) phosphoramidite was obtained from Applied Biosystems Inc., Foster City, CA. Other reagents and chemicals were of analytical grade and used as received from commercial sources, unless indicated.

Buffers. Reaction buffer: 0.1 M *N*-methylimidazole containing DMSO (10%), pH 8.0. Hybridization buffer: 125 mM sodium citrate containing 750 mM NaCl, pH 7.0. Capping buffer: 0.1 M Tris containing 50 mM ethanolamine, pH 9.0.

Glass Silanization. *Step 1: Glass Cleaning.* Glass microslides were cleaned by immersing them in NaOH (1.0 M) for 2 h at 35 °C followed by rinsing with Milli Q water (2 × 100 mL). The slides were then subjected to treatment with a solution of hydrochloric acid (2.0 M) for 30 min, followed by rinsing with Milli Q water (3 × 100 mL), and finally submerged in ~95% ethanol for 1 h. The slides were dried under vacuum and stored in an inert atmosphere.

Step 2: Silanization of the Slides. The precleaned slides were immersed in a solution of GOPTS (2%, v/v) in toluene (75 mL) for 4−5 h at 50 °C, followed by washings with toluene (2 × 75 mL) and ethanol (2 × 75 mL), and dried under vacuum. Finally, these were baked for 1 h at 100 °C and stored in an inert atmosphere.

Oligonucleotide Synthesis and Purification. Oligonucleotides and their thiol-modified analogues (*23*) were synthesized at 0.2 μmol scale on a Pharmacia Gene Assembler Plus using the standard phosphoramidite approach following the manufacturer's protocol (Gene Assembler Plus Manual, Uppsala, Sweden, 1988). The oligonucleotide sequences were assembled, and their deprotection conditions and yields are given in Table 1. The ammoniacal solution of oligomers was concentrated in a speed vac concentrator; the residue obtained was redissolved in water (200 μL) and applied onto a desalting column (reverse-phase ODS-silica gel column). The oligomers were eluted with 30% acetonitrile in water, concentrated in a speed vac, purified on RP-HPLC, and stored at 4 °C.

Determination of Optimal pH Required for Immobilization of Thiolated Oligomers on Epoxy-Activated Glass Surface. In order to study the effect of pH on immobilization of thiolated oligonucleotides on epoxylated polymer surface, the attachment of the probe was carried out at different pH values ranging 6−12. Briefly, a labeled oligomer, viz., TET-d (TCT GTA TGT CTA TTC TCG TT)-OPO_3-$(CH_2)_6$-SH, dissolved in the reaction buffer of different pH, was spotted onto a functionalized glass microslide and incubated in a humid chamber for 12 h, followed by capping of the residual epoxy functionalities with the capping buffer for 15 min at 50 °C. The slide was subjected to washings with 1× SSC buffer (4 × 50 mL), pH 7.0, for 20 min, and Milli Q water (2 × 50 mL), dried under vacuum and scanned under a laser scanner.

Determination of Optimal Time Required for Immobilization of Thiolated Oligomers on Epoxy-Activated Glass Surface. The optimal time required for immobilization of 3′-thiolated oligonucleotide onto epoxylated surface was evaluated by spotting an oligomer sequence, TET-d (TCT GTA TGT CTA TTC TCG TT)-OPO_3-$(CH_2)_6$-SH (40 μM), onto functionalized glass surface in duplicates using a 2 μL micropipet. The slides were then subjected to microwave irradiation up to 20 min (120 × 10 s) in a domestic microwave oven operating at 800 W (with no exposure longer than 10 s in a humid chamber, after each exposure, the slide was cooled in an ice−water mixture to room temperature). The microslides were removed at different time intervals (4, 8, 12, 16, and 20 min), followed by capping and washings as mentioned above. After drying, the spots were visualized under a laser scanner.

Similarly, to find out the optimal time required for immobilization under thermal conditions, the slides were spotted with a labeled oligomer as mentioned above and kept at 35 °C, followed by their withdrawal at different time intervals (2, 4, 6, 8, and 20 h) and subjected to capping, washings, and scanning, as described above.

Determination of Threshold Concentration of Oligonucleotides Required for Fluorescence Detection. In order to arrive at the optimal probe concentration required for visualization of spotted oligonucleotides under a laser scanner, a kinetic study was undertaken to immobilize a labeled oligomer at different concentrations under microwaves. Briefly, a labeled oligomer, viz., TET-d (TCT GTA TGT CTA TTC TCG TT)-OPO_3-$(CH_2)_6$-SH, was serially diluted to concentrations (20, 10, 5, 2.5, 1.0, 0.75, 0.5, 0.25 μM) and spotted (0.5 μL) onto the functionalized glass slide. The slide was then subjected to microwave irradiation for 12 min (72 × 10 s). After capping and usual washings, the slide was visualized under a laser scanner and the spots were quantified using *QuantArray* software (Packard Biosciences, USA).

The extent of involvement of exocyclic amino groups of heterocyclic base (guanosine) and backbone phosphodiesters in reaction with epoxy functions on glass surfaces was evaluated

by immobilizing 5′-labeled-3′-thiolated and 5′-labeled-3′-OH oligonucleotides at 5 μM concentration on an epoxy-coated slide under microwaves, followed by usual washings and drying. The slide was then subjected to a laser scanner followed by quantification.

Thermal and pH Stabilities. To evaluate the thermal stability of the immobilization chemistry, a microarray was prepared by tethering a TET-labeled oligomer at 0.5 μM to a functionalized glass slide under microwaves, as mentioned above. The slide was then subjected to PCR-like conditions and 0, 5, 10, 15, and 20 cycles were performed. PCR parameters were as follows: 94 °C for 30 s (denaturing step), 54 °C for 30 s (annealing step), and 72 °C for 30 s (extension step). After thermocycling, the slide was thoroughly washed and scanned under a laser scanner.

In an analogous way, vulnerability of the projected chemistry was evaluated at different pHs by subjecting the constructed microarrays to washings (4 × 15 min) with buffers of different pH values, ranging 7−9.

Hybridization Assay. To study the hybridization kinetics, two glass slides, each one spotted (0.5 μL) with 5 μM concentration of probe, viz., HS-$(CH_2)_6$-OPO_3-d (ACG TAA GCA AAT CGA TCG TT), were subjected to microwave irradiation for 12 min (72 × 10s) and 12 h at 35 °C, respectively. After the usual capping, washings, and drying, the microarrays were hybridized with 40 μL of complementary labeled oligonucleotide sequence, TET-d (AAC GAT CGA TTT GCT TAC GT) (40 μM), under a coverslip. The slides were then kept in a humidified chamber for 1 h at 45 °C and overnight at 35 °C. After hybridization, the coverslips were gently removed and the slides were successively washed with 2× SSC (2 × 15 min) and 1× SSC buffers (1 × 5 min), followed by drying under vacuum. The slides were scanned under a laser scanner and fluorescence intensity of the spots was quantified.

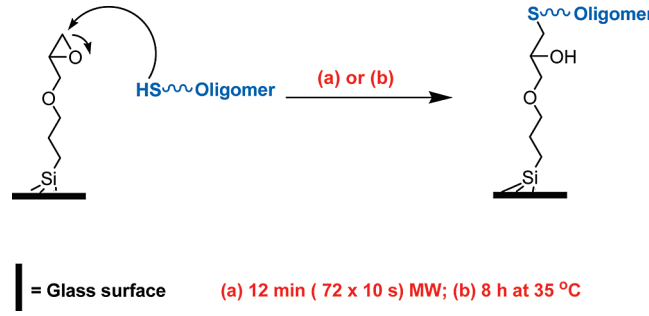
Detection of Mismatches. To investigate the sensitivity of the system, four oligonucleotides, viz., HS-$(CH_2)_6$-OPO_3-d (ACG TAA GCA AAT CGA TCG TT), HS-$(CH_2)_6$-OPO_3-d (ACG TAA GCA AAT CGA TCG GT), HS-$(CH_2)_6$-OPO_3-d (ACG TAA GCA TAT CGA TCG GT), and HS-$(CH_2)_6$-OPO_3-d (TTT TTT TTT TTT TTT TTT TT) having zero, one, and two base mismatches, and a noncomplementary sequence, respectively, were spotted at 5 μM onto an epoxy-coated glass slide followed by capping and usual washings. Subsequently, the microslide was subjected to hybridization experiment with a labeled oligomer, TET-d (AAC GAT CGA TTT GCT TAC GT), and washed with the hybridization buffer, as mentioned above. After drying under vacuum, the slides were subjected to a laser scanner.

Detection of Bacterial Meningitis. *PCR-Amplification.* Amplification of 430bp fragment of *Transposase* gene (*Neisseria meningitidis*; serogroup C) was performed by taking labeled forward primer TET-d (TCG ACG AAC TGA TCA TCC), labeled reverse primer TET-d (TCG GCA TAG ACG GTC GTA), genomic DNA, and Taq polymerase following a reported protocol (*22*). Analysis of the resulting PCR-amplicons was carried out on agarose gel electrophoresis.

Hybridization with PCR Amplicons. Two oligonucleotide sequences, HS-$(CH_2)_6$-OPO_3-d (TCG ACG AAC TGA TCA TCC) (probe) and HS-$(CH_2)_6$-OPO_3-d (CGT CGA GTT GTT CAT CTC GCA) (control) were immobilized on the glass slide, as described above. Amplicons were hot denatured (5 min, 95 °C), cooled on ice (10 min), and used for carrying out the hybridization assay. After usual washings and drying, the slide was subjected to laser scanning.

Signal Detection and Data Analysis. The arrays were imaged on a Scan Array Lite Scanner (GSI Lumonics) fitted with a Cy3 optical filter with 30 μm resolution. The laser power and PMT voltage were set accordingly to obtain optimum signal intensity. *QuantArray* software (Packard Bioscience) was used for analysis of intensity of the original images and the graphs were plotted in MS *Excel*. The fluorescence intensities in color images of slides were color coded, varying from blue (low) to green, yellow, and then white (saturation).

Scheme 1. Strategy for the Immobilization of Thiolated Oligonucleotides onto Epoxy-Activated Glass Microslides



DNA Quantification. In an attempt to quantify the density of immobilized oligonucleotides, varying amounts (0.0125, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 pmol) of an oligomer sequence, TET-d (TCT GTA TGT CTA TTC TCG TT)-OPO_3-$(CH_2)_6$-SH were spotted on a virgin glass microslide. After drying, the slide was scanned and the spots were quantified to obtain a standard calibration curve by plotting fluorescence intensity (A.U.) as a function of amount of the spotted probe (pmol) (Figure S1, Supporting Information).

For each experimental condition tested on the microarrays, oligomers were spotted in duplicates and the experiment was repeated at least three times. The immobilization and hybridization data presented are the averages of these repetitions, and the error bars represent the percentage error observed on this average.

RESULTS AND DISCUSSION

The use of oligonucleotide arrays is expanding beyond their high-profile role in genome sequencing to other fundamental applications like nucleic acid-ligand binding studies and medical diagnostics. The high-throughput screening technology enables researchers to rapidly profile the expression of thousands of genes simultaneously. Reports have shown that oligonucleotides immobilized through end-specific covalent linkage increase the availability of the immobilized probes for hybridization. Therefore, active groups are often introduced at either the 3′- or 5′-end (usually amino or thiol) of the oligonucleotides to allow end-specific immobilization onto the activated glass surface. For a new chemical method to be useful for the construction of microarrays, the functionalities on the surface should be compatible with the reactive groups on the probe molecules and the immobilization chemistry should be fast and reproducible and offer low background noise during detection. Holistic development of efficient chemistries for the manufacture of oligonucleotide arrays is essential to garner the potential of biochip technology. Microwave (MW) activation, as a nonconventional energy source, has become a very popular and useful technology in organic chemistry. A large number of articles describe significant acceleration in the reaction rates for a wide range of organic reactions, reducing the reaction time from hours to minutes with improved yields and reproducibility (*24, 25*). In contrast to thermal/photochemical reactions, the use of microwaves to construct oligonucleotide arrays has largely remained unexplored. We now present a method for covalent immobilization of 3′/5′-thiolated oligonucleotides onto an epoxy-activated glass surface under microwaves as illustrated in Scheme 1. The optimal pH for reaction, immobilization kinetics,

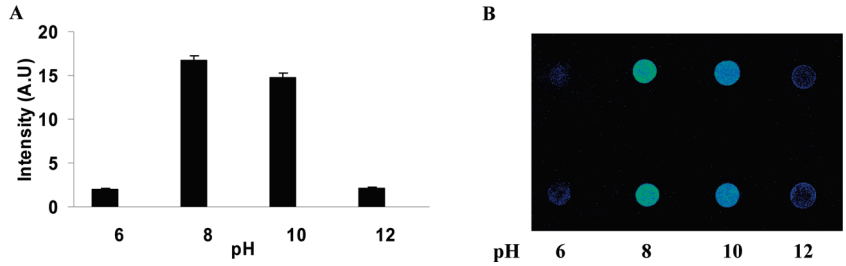


Figure 1. (a) A graph representing the influence of pH on the immobilization reaction of thiolated probes with epoxy-activated surface. (b) Fluorescence map obtained by immobilization of 5′-labeled, 3′-thiolated probe in the pH range 6−12.

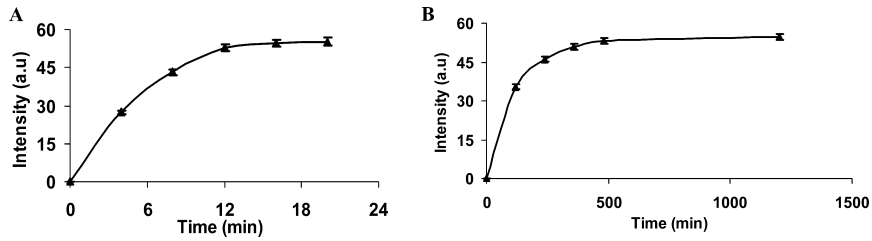


Figure 2. Time kinetics to determine the optimal time required for immobilization of thiolated probes under (a) microwave; (b) thermal condition (35 °C).

stability (thermal and pH), effect of probe, and target concentrations on hybridization efficiency have been optimized.

Immobilization of Thiolated Oligonucleotide onto Epoxy-Activated Glass Microslides. The optimal condition required for effective immobilization of thiol-modified oligonucleotides via thioether linkage was determined by spotting a labeled probe (at 1 μM) on epoxy-functionalized glass surface under different pH conditions, as mentioned in the Experimental Procedures. The results showed that maximum immobilization was achieved at pH 8.0 possibly through the availability of thiol groups in their anionic form (Figure 1A,B). As the pH was increased from 8 to 12, the fluorescence intensity decreased, which might have resulted due to partial oxidation of thiol functions at higher pH values, whereas the reason for lower attachment density attained at pH 6 could be due to the lower reactivity of thiol functionality at slightly acidic pH. On the basis of these findings, immobilization of thiol-modified probes was carried out at pH 8.0 for the rest of the experiments.

The kinetics of thioether linkage formation under both microwaves and thermal conditions were investigated by spotting a large excess of 3′-thiolated oligomer onto an epoxy-functionalized glass surface. The results show that the reaction between the thiolated probe and epoxy groups is essentially completed in 12 min under microwaves, while it takes ∼8 h at 35 °C under thermal conditions (Figure 2A,B). Therefore, the rest of the experiments were conducted for 12 min under microwaves for the immobilization of thiol-modified probes onto the functionalized glass surface. Similarly, to find the highest density of oligonucleotide attachment on the surface, a kinetics study of the probe immobilization at various amounts (0.125−10 pmol) was undertaken (Figure 3). It can be deduced from the results that the attachment density increases upon increasing the concentration of the probe, and the highest fluorescence intensity was obtained at 10 pmol in the spotting buffer (Figure 3). Subsequently, the immobilization efficiency was determined using a standard calibration curve (Figure 3, Lane 2) (Table S1, Supporting Information).

With the aim to determine the extent of involvement of terminal thiol groups of the probe with epoxy functions of glass surface, a microarray was prepared by spotting 5′-labeled oligonucleotides (5 μM) with and without thiol modification at 3′-terminus. After treatment with the capping buffer and usual

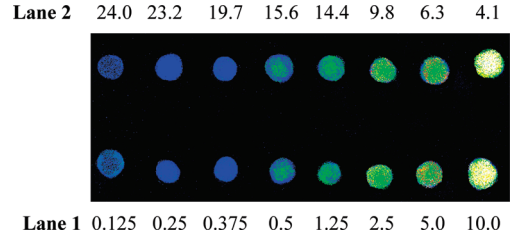


Figure 3. Threshold amount of probe, viz., TET-d (TCT GTA TGT CTA TTC TCG TT)-OPO_3-$(CH_2)_6$-SH, required for detection under a laser scanner. Lane 1: Amount of the spotted probe (pmol). Lane 2: Immobilization efficiency (%).

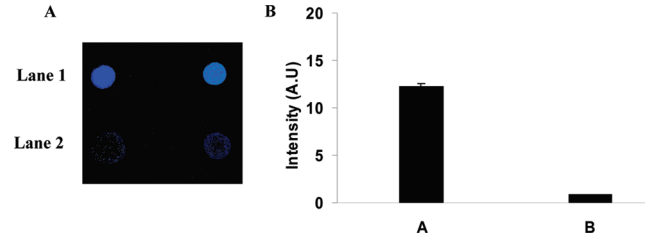


Figure 4. (a) Fluorescence map showing immobilization of TET-d (TCT GTA TGT CTA TTC TCG TT)-OPO_3-$(CH_2)_6$-SH (lane 1) and TET-d (TCT GTA TGT CTA TTC TCG TT) (lane 2) on epoxy-functionalized glass surface. (b) A graph representing the comparison of fluorescence intensity obtained by spotting thiol-modified (A) and unmodified oligomer (B) on epoxy-activated surface.

washings, the slide was visualized under a laser scanner (Figure 4A). The fluorescence intensity from spots of the unmodified oligonucleotide was found to be ∼6.5% of that measured from the probe with a 3′-thiol modification, implying specific binding of the terminal thiol groups to the epoxy-activated surface (Figure 4B). The low amount of fluorescent signal obtained with unmodified oligonucleotide could have arisen from weaker binding of internucleotidic phosphate groups and exocyclic amino groups of guanosine moieties in the probe with the epoxy-activated surface. Although nonspecific binding of the unmodified probe is small, a measurable fluorescent signal still resulted, indicating that the same interactions may result from the

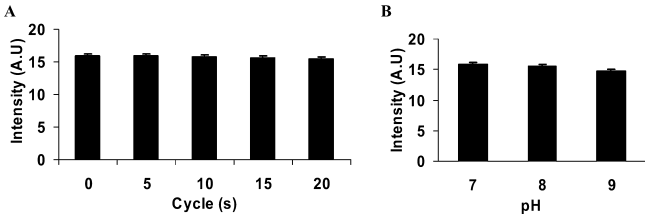


Figure 5. (a) Thermal stability of thioether linkage under PCR-like conditions and comparison of the fluorescent signal obtained after 0, 5, 10, 15, and 20 cycles; (b) stability of the constructed microarrays under a pH range of 7−9.

complementary targets during hybridization, leading to significant background and artifacts. This was eliminated by treating the spotted slides with a capping buffer to deactivate the residual epoxy functions.

Thermal and pH Stability. The heat stability of the constructed microarray was evaluated by submitting it to 0, 5, 10, 15, and 20 PCR-like heating cycles. The fluorescent signal of the heat-treated microarrays was found to be decreased by only ∼1.4% after 10 cycles and ∼2.5% after 20 cycles. Figure 5A shows a histogram obtained by comparing the fluorescent signal of heat-treated and pretreated microarray. This shows that the oligonucleotides immobilized on the activated glass surface following the projected strategy are sufficiently stable to be used in biological research.

In order to find the vulnerability of the constructed microarrays toward different pHs, the constructed microarrays were subjected to washings (4 × 15 min) with buffers of different pH, ranging 7−9. The quantification results reveal that the fluorescence intensity decreased by only 2.0% at pH 8.0 and 4.4% at pH 9.0 in comparison to that obtained at pH 7.0 (Figure 5B). Thus, the fabricated microarray can be used over a wide range of pH safely without significant change in its performance.

Hybridization Assay. The accessibility and specificity of the surface-bound probes during hybridization have been the major concern while preparing microarrays. In order to investigate these parameters, two microarrays were prepared by immobilizing oligonucleotide under microwaves (12 min) and thermal conditions (∼8 h) (Scheme 1). After the hybridization experiment, the spots were visualized under a laser scanner as shown in Figure 6A,B. Negligible fluorescence was obtained during hybridization with the noncomplementary oligonucleotide, demonstrating that nonspecific hybridization did not occur. The signal-to-noise ratio was on average 96, as calculated from signals obtained from complementary hybridized spots and nonhybridized spots.

To demonstrate the dynamics of microarray hybridization, the kinetics of hybridization reaction for different target oligonucleotide concentrations was followed. The results showed that, in the initial stage, the hybridization signal increases on increasing the target concentration, then reaches a steady state between 40 and 60 μM (Figure 7A). The results of kinetics of hybridization for different probe concentrations are shown in Figure 7B. The plot of hybridization efficiency (efficiency defined as percent of covalently attached oligonucleotides that participated in duplex formation) for different amounts of immobilized probes reveals that it reaches a maximum (∼33%) for an optimal immobilized probe concentration of 5 μM (Table S1, Supporting Information). Above this concentration, the efficiency did not increase, even if the concentration of the immobilized probe was increased, as the overloading of the surface with probe may cause a crowding effect that can lower the accessibility of the surface-bound probe molecules. In addition, the efficiency of hybridization depends on many factors like the number of epoxy groups on the surface accessible to

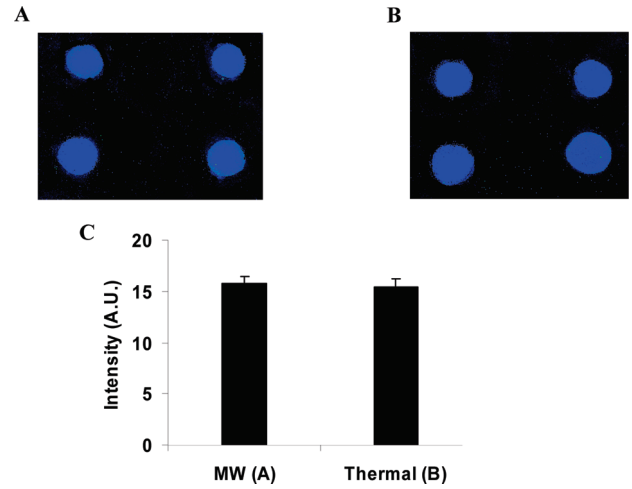


Figure 6. Fluorescence maps obtained after hybridization of HS-$(CH_2)_6$-OPO_3-d (ACG TAA GCA AAT CGA TCG TT) immobilized at 5 μM with target, TET-d (AAC GAT CGA TTT GCT TAC GT) (40 μM). Oligonucleotide is immobilized (A) under microwaves for 12 min (72 × 10s) at 800 W; (B) under thermal condition at 35 °C for ∼8 h. (C) Quantification representation of hybridization results of the two methods, A and B.

the 5′-thiolated oligonucleotides, attachment efficiency of oligonucleotides to the epoxy-activated surface, and availability of immobilized probes to the target oligomer.

Specificity of Immobilization Chemistry for Detection of Mismatches. In order to demonstrate the specificity of the system, four oligonucleotides were immobilized onto an epoxy-functionalized glass microslide at 5 μM concentration of each probe as mentioned in the Experimental Procedures. After treatment with the capping buffer, the microslide was subjected to a hybridization step with a labeled oligomer (complementary to zero mismatch sequence). Figure 8 clearly shows the base mismatches. The perfectly matched sequence gave the maximum intensity (lane 1), while the spots having one (lane 2) and two (lane 3) showed fluorescence intensity in decreasing order. No measurable nonspecific hybridization signal from the noncomplementary control was detected. This may be because the residual epoxy groups on the surface got masked during treatment with the capping buffer to exclude nonspecific interactions with the target oligonucleotides.

Quantification and Surface Coverage of the Immobilized Oligonucleotides. Quantification of the microarrays prepared following the proposed method was carried out by using a standard calibration curve plotted between fluorescence intensity (a.u.) and amount of the spotted probe (pmol), as mentioned in the Experimental Procedures. Surface coverage of the immobilized probe was calculated from the following formula (*9b*):

$$\text{surface coverage (molecules}/\mu\text{m}^2) = \frac{\text{amt. of immobilized probe (pmol)} \times 6.023 \times 10^{23}}{\text{area of spot } (\mu\text{m}^2)}$$

(spotting vol.: 0.5 μL)

Comparison of the Proposed Method with the Standard Methods. The projected method for microarray preparation was compared with the three existing standard methods, viz., NTMTA (*7*), epoxide-amine (*21*), and disulfide (*19*) approach (Scheme 2). For comparison purposes, an equal concentration of the appropriate oligonucleotide was used. However, different reaction times, as reported therein, were employed in all the three methods. After immobilization, the spots were hybridized with the complementary labeled oligonucleotide, and the mi-

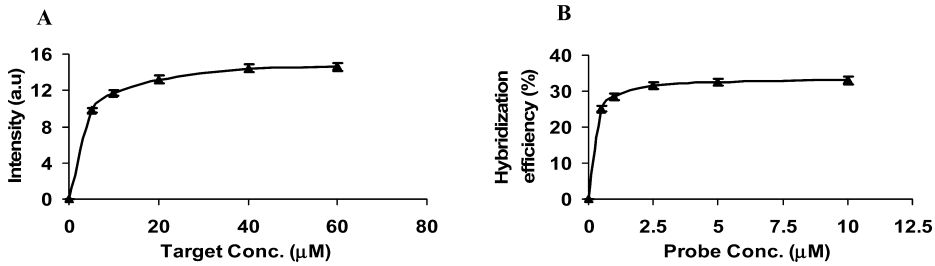


Figure 7. (a) Measurement of optimal target concentration for steady hybridization at 5 μM concentration of immobilized 5′-thiolated probe. (b) Hybridization efficiency obtained, by varying the amount of probes immobilized (0.5, 1, 2.5, 5, and 10 μM) onto epoxy-functionalized glass surfaces upon hybridization with a complementary strand (TET-labeled).

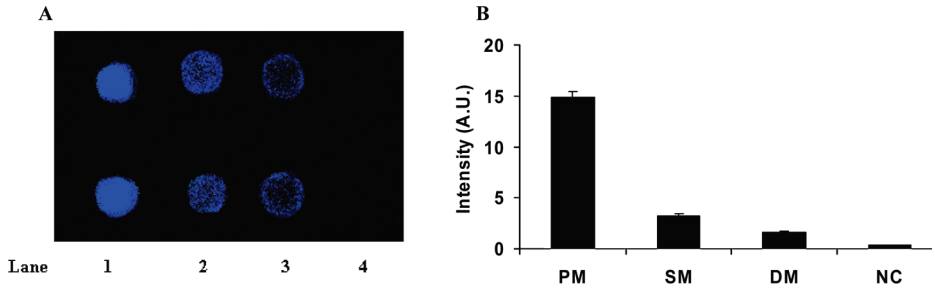
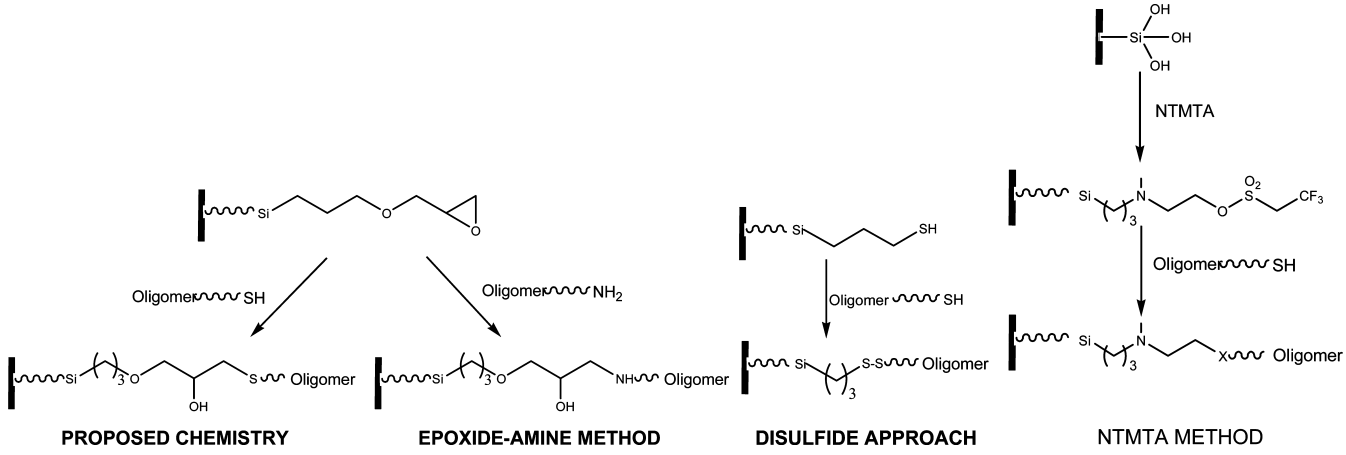


Figure 8. Detection of nucleotide mismatches and specificity of immobilization via hybridization with labeled oligonucleotide, viz., TET-d (AAC GAT CGA TTT GCT TAC GT) (complementary to zero mismatch). (A) Lane 1, HS-$(CH_2)_6$-OPO_3-d (ACG TAA GCA AAT CGA TCG TT). Lane 2, HS-$(CH_2)_6$-OPO_3-d (ACG TAA GCA AAT CGA TCG GT). Lane 3, HS-$(CH_2)_6$-OPO_3-d (ACG TAA GCA AAT CGA TCA GT). Lane 4, HS-$(CH_2)_6$-OPO_3-d (TTT TTT TTT TTT TTT TTT TT). (B) Quantitative representation of the results of nucleotide mismatches. PM: Perfect match. SM: Single mismatch. DM: Double mismatch. NC: Noncomplemenatry.

Scheme 2. Comparison of the Proposed Strategy with the Standard Methods



croslides were visualized under a laser scanner for quantification of spots (Table S2, Supporting Information). The hybridization efficiency of the proposed method (~33%) is much higher than the method employing NTMTA reagent (~9.8%), epoxide-amine immobilization chemistry (~9.8%), and disulfide approach (1.7%), as shown in Figure 9.

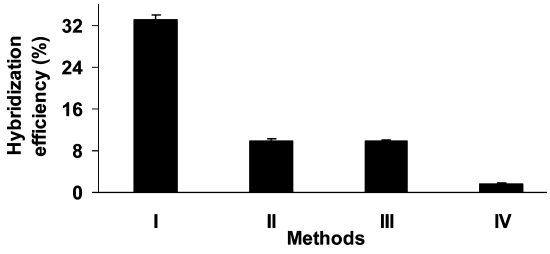


Figure 9. Comparison of hybridization efficiency of proposed method (I) with standard ones, viz., (II) = NTMTA, (III) = epoxide-amine, and (IV) = disulfide method.

Detection of Bacterial Meningitis. In order to demonstrate the ability of the constructed biochip to detect bacterial infection, a specific probe of the *Transposase* gene and a noncomplementary probe, as control, were immobilized onto an epoxylated glass slide, and after the usual washings, the spots on the glass surface were subjected to hybridization with the labeled PCR

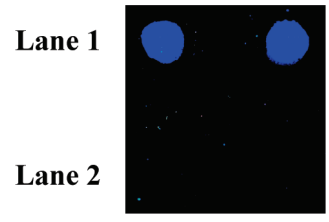


Figure 10. Detection of bacterial meningitis. Lane 1: Probe sequence, HS-$(CH_2)_6$-OPO_3-d (TCG ACG AAC TGA TCA TCC). Lane 2: Control sequence, HS-$(CH_2)_6$-OPO_3-d (CGT CGA GTT GTT CAT CTC GCA).

amplicons. After washings and drying, the slide was visualized under a laser scanner (Figure 10). Spots pertaining to probe sequence specific to *Transposase* gene of bacteria (*N. Meningitidis*, Serogroup C) showed fluorescence, while spots pertaining to the control did not fluoresce at all, signifying the specificity of the system to prepare biochips for the detection of bacterial meningitis in humans.

CONCLUSIONS

A simple and efficient method for preparation of oligonucleotide microarrays via a stable thioether linkage is established with rapid immobilization of thiolated oligomers onto epoxylated surface under microwaves with excellent immobilization efficiency (~24%), stable over a wide pH range and thermocyclic conditions, suggesting that the biochip could be integrated with PCR. The proposed chemistry enables the immobilized probes to be highly accessible to targets during hybridization with remarkable efficiency (~33%) and negligible background noise (signal-to-noise ratio 96). It also successfully discriminates the base mismatches and can detect bacterial diseases thus promising for other biomolecules and chemicals for diagnostic purposes.

ACKNOWLEDGMENT

This work is supported by the Deparment of Biotechnology, New Delhi and CSIR NWP013. D.S. thanks the University Grants Commission, New Delhi, India, for the award of a Senior Research Fellowship. Authors are also thankful to Drs. S. Gupta and S. Khare, NICD, Delhi, India for providing patient samples and facilities.

Supporting Information Available: Standard calibration curve for quantification, surface coverage data of immobilized probe and captured target and comparison data of the proposed method with the standard methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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BC900162U