New Protocol for Oligonucleotide Microarray Fabrication using SU-8-Coated Glass Microslides

D. Sethi,[§] A. Kumar,[§] R. P. Gandhi,[†] P. Kumar,[§] and K. C. Gupta*[,‡,§]

Indian Institute of Toxicology Research (CSIR), Mahatma Gandhi Marg, Lucknow-226001, India, and Institute of Genomics and Integrative Biology (CSIR), Mall Road, Delhi University Campus, Delhi-110007, India. Received June 9, 2010; Revised Manuscript Received July 19, 2010

Microarray technology has become an important tool for detection and analysis of nucleic acid targets. Immobilization of modified and unmodified oligonucleotides on epoxy-functionalized glass surfaces is often used in microarray fabrication. Here, we demonstrate a protocol that employs coating of SU-8 (glycidyl ether of bisphenol A) onto glass microslides to obtain high density of epoxy functions for efficient immobilization of aminoalkyl-, thiophosphoryl-, and phosphorylated oligonucleotides with uniform spot morphology. The resulting microarrays exhibited high immobilization (∼65%) and hybridization efficiency (30−36%) and were sufficiently stable over a range of temperature and pH conditions. The prominent feature of the protocol is that spots can be visualized distinctly at 0.05 μM probe (a 20-mer oligonucleotide) concentration. The constructed microarrays were subsequently used for detection of base mismatches and bacterial diseases (meningitis and typhoid).

INTRODUCTION

Over the past few decades, oligonucleotide-based arrays have emerged as a promising and convenient tool for high-throughput experimentation in disease diagnosis, mapping, and gene expression analysis, as these offer distinct advantages in terms of requirement of a very small amount of biological probes (nanomoles and picomoles) (*1*−*6*).

Direct syntheses (in situ) of oligonucleotides on the chip surface following conventional or photolithographic methods and immobilization of presynthesized oligonucleotides on selected substrates (the deposition method) are two predominant methods for fabrication of oligonucleotide microarrays (*6*−*21*). The in situ synthesis protocol, which allows the preparation of high-density oligonucleotide microarrays, suffers from certain drawbacks (*6*−*8, 17*). The deposition method, on the other hand, offers flexibility, in the sense that a variety of biomolecules can be immobilized on a surface of choice but is limited to generation of low-to-medium-density arrays.

The choice of substrate and the chemistry employed for attachment of oligonucleotides are key factors that govern the quality and efficacy of constructed microarray significantly, especially in cases of low probe and target concentrations. A number of solid surfaces have been proposed for the fabrication of biochips (*20*−*34*). Of these, glass and PMMA appear attractive because these supports can easily be derivatized generating reactive functional groups such as aminoalkyl, mercaptoalkyl, carboxyl, aldehyde, and so forth on the surface. However, glass, an inexpensive material, proffers some advantages, as it reveals low intrinsic fluorescence and has a relatively homogeneous chemical surface. Recently, in order to realize the full potential of biochip technology for disease diagnosis, limitation of sensitivity has been a major concern. A general drawback of glass slides is the low functional group density due to its nonporous nature, which limits the number of reactive groups that can participate in the binding of oligonucleotides, resulting in poor surface coverage with the probes. Therefore, to develop such biochips, highly functionalized matrices having high immobilization capacity with good accessibility to target molecules are required. For this purpose, a vast variety of reagents and polymers have been used for coating to obtain highly functionalized glass surfaces (*30*−*34*). In one of the approaches, Preininger et al. (*31*) reported high-capacity ARChip epoxy as the reactive chip obtained after coating the glass microslide with 1% epoxy resin for immobilization of modified oligonucleotides with higher immobilization and hybridization efficiency; however, poor reproducibility of the coating process and enhanced background signals limited its commercial feasibility. Likewise, other methodologies, which were developed, required multistep processing (*32*−*34*). Recently, the epoxy-based photoresist, SU-8, a biocompatible, rigid, inexpensive, thermally, and chemically stable and transparent-to-light above 360 nm (*25, 26, 35*−*37*), has been employed as a structural material in microfabricated devices such as probes for scanning probe microscopy, waveguides, optical-based devices, and a large variety of lab-on-a-chip applications. Besides, an attempt has also been made to develop SU-8 surfaces for immobilization of DNA and proteins (*36, 37*).

Here, we demonstrate a process of biochip fabrication by directly immobilizing modified oligonucleotides (aminoalkyl-, thiophosphoryl-, and phosphorylated oligomers) onto SU-8 coated high-density glass microslides. This protocol provides higher signal reproducibility with superior spot morphology and homogeneity. The optimal thickness of the SU-8 layer required for immobilization and threshold concentration of oligomer required for visualization and thermal and pH stability of constructed microarrays were evaluated in a detailed study before validating these biochips for detection of base mismatches and of bacterial diseases such as meningitis and typhoid.

EXPERIMENTAL PROCEDURES

General. Virgin glass microslides (75 × 22 mm^2), *N*-methylimidazole (NMI), and 3-glycidyloxypropyltrimethoxysilane (GOPTS) were obtained from Sigma-Aldrich Chemical Co., St Louis, MO. SU-8 2002 was purchased from MicroChem

* E-mail: kcgupta@iitr.res.in; kcgupta@igib.res.in; Tel.: +91 522 2621856.

[†] INSA Honorary Scientist.

[‡] Indian Institute of Toxicology Research.

[§] Institute of Genomics and Integrative Biology.

10.1021/bc100262n
Published on Web 08/16/2010

Table 1. Synthesis of Oligonucleotides, Deprotection Conditions, and Yields

Sample number	Oligomer sequence	Deprotection conditions[a]	Yields O.D. at A_{260}
1	TET-d(CTT CTT TTT CCT GTT ACC GT)-O- (PO_3^{2-}/ PSO_2^{2-}/ PO_2^{-}-$O(CH_2)_2$-O-$(CH_2)_2$-NH_2)	Aq. NH_4OH, 55 °C, 16 h	20.5−22.4
2	d(CTT CTT TTT CCT GTT ACC GT)-O- (PO_3^{2-}/PSO_2^{2-}/ PO_2^{-}-$O(CH_2)_2$-O-$(CH_2)_2$-NH_2)	-Do-	22.1−24.5
3	TET-d(ACG GTA ACA GGA AAA AGA AG)	-Do-	22.1
4	d(CTT CTT TTT ACT GTT ACC GT)-O- (PO_3^{2-}/PSO_2^{2-}/ PO_2^{-}-$O(CH_2)_2$-O-$(CH_2)_2$-NH_2)	-Do-	24.1−24.8
5	d(TTT TTT TTT TTT TTT TTT TT)-OPO_3^{2-}	-Do-	23.7
6	TET-d(ACC GGT GGA TGT GGC TTC CTT G)	-Do-	21.2
7	TET-d(TGG TCT GCA GCA CCT TTT GAA C)	-Do-	20.5
8	d(ACC GGT GGA TGT GGC TTC CTT GTT TT)-OPO_3^{2-}	-Do-	24.2
9	TET-d(GCT GCG GTA GGT GGT TCA A)	-Do-	21.2
10	TET-d(TTG TCG CGG ATT TGC AAC TA)	-Do-	20.6
11	d(ACG TGT CAG CTG CAC ATT CGT TTT T)-OPO_3^{2-}	-Do-	23.8

[a] In the case of thiophosphorylated oligomers, aq. NH_4OH containing 50 mM DTT was used.

Corp., Newton, MA. Absolute ethanol was procured from Tedia Company Inc., Fairfield. 4,5,6,7-Tetrachlorofluorescein (TET) phosphoramidite was obtained from Applied Biosystems Inc., Foster City, CA. All other reagents used were of analytical grade and used as received from commercial source, unless specified.

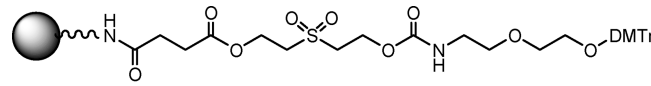
Instrumentation. ScanArray Gx Plus Microarray Scanner (Perkin Elmer), Speed vac concentrator (Hetovac VR-1), and Lambda Bio 20 spectrophotometer (Perkin-Elmer) were used. Spin-coating of microslides with SU-8 was carried out on Deltaspin (*38*)(Delta Scientific, Delhi, India).

Reagents and Buffers. Reaction buffer: 100 mM *N*-methylimidazole (NMI) was prepared by dissolving 80 μL in 10 mL of Milli-Q water (containing 10% DMSO). Capping buffer: 0.1 M Tris buffer containing 50 mM ethanolamine, pH 8.0. Washing buffer: 125 mM SSC buffer containing 750 mM sodium chloride, pH 7.0. Hybridization buffer: 125 mM SSC buffer containing 1 M sodium chloride, pH 7.0.

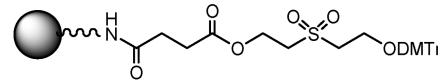
Cleaning, Silanization, and SU-8 Coating of Glass Slides. The glass slides were cleaned and silanized following the reported protocol (*15*). After washing and drying, the slides were stored under inert atmosphere. SU-8 2000 photoresist was coated with thickness of 2, 2.5, and 3 μm by spin-coating onto a glass microchip according to manufacturer's protocol (*38*). After spinning, the excess solvent was evaporated in a soft bake at 65 °C for 1 min and at 95 °C for 2 min. The microslides were stored under inert atmosphere.

Oligonucleotide Synthesis and Purification. In the present study, oligonucleotide sequences (Table 1) were assembled on Pharmacia Gene Assembler Plus at 0.2 μmol scale following the standard phosphoramidite chemistry. 3′-Modified oligonucleotides were synthesized on the engineered polymer supports (**I** and **II**). For 5′-labeled oligonucleotides, synthesis was carried out following the conventional protocol and the last coupling was performed with the TET-phosphoramidite reagent in a manner analogous to the normal nucleoside phosphoramidite coupling. Post-synthesis work-up and purification were carried out following the reported protocol (*14*).

Determination of Optimal Thickness Required for Immobilization. In order to arrive at optimal thickness of coated SU-8 photoresist (2, 2.5, 3 μm) onto glass microslides required for efficient immobilization of oligomers, attachment of probe was carried out following the reported protocol (*15*). Briefly, a labeled oligomer, TET-d(CTT CTT TTT CCT GTT ACC GT)-OPO_3^{2-}, was dissolved in the reaction buffer and spotted (0.5 μL) onto SU-8 coated glass slides at a concentration of 0.1 μM using a pipettmen followed by incubation at 45 °C for 2 h. Then, the microslide was washed with 1 × SSC buffer (2 × 50 mL) followed by Milli-Q water (2 × 50 mL), and dried under vacuum. Subsequently, the spots on the microslide were visualized under a laser scanner and quantified.



I : Support for synthesis of 3'-aminoalkylated oligonucleotides



II : Support for synthesis of 3'-phosphoryl- and thiophosphorylated oligonucleotides

Evaluation of Chip Surface for Immobilization. To evaluate the homogeneity of the SU-8 coated microslide surface, the following experiment was performed. An oligomer sequence, TET-d(CTT CTT TTT CCT GTT ACC GT)-OPO_3^{2-} (0.1 μM), dissolved in the reaction buffer, was spotted manually on a coated glass microslide at different locations all over the slide and kept at 45 °C for 2 h. The slide was processed and visualized, as described above.

Determination of Threshold Concentration Required for Visualization. In an attempt to arrive at the threshold concentration of oligonucleotides required for fluorescence detection, a TET-labeled oligomer, TET-d(CTT CTT TTT CCT GTT ACC GT)-OPO_3^{2-} diluted (1, 0.5, 0.1, 0.05, 0.025, and 0.01 μM) in the reaction buffer, was spotted (0.5 μL) onto an SU-8 coated glass microslide and kept at 45 °C for 2 h. After the reaction, the slide was washed with 1 × SSC buffer (3 × 25 mL) and Milli-Q water (2 × 25 mL). The slide was dried and scanned under a laser scanner. The immobilization efficiencies were determined using the standard curve.

Immobilization of thiophosphoryl- and aminoalkylated oligonucleotides was also performed in a similar way. Subsequently, the slides were washed, dried, and visualized under a laser scanner.

Thermal and pH Stability. In order to evaluate the thermal stability of immobilization, a microarray was constructed by spotting a TET-labeled oligomer sequence, TET-d(CTT CTT TTT CCT GTT ACC GT)-OPO_2^{-}-O-$(CH_2)_2$-O-$(CH_2)_2$-NH_2 (0.1 μM), as mentioned above. After the usual washings, the constructed microarray was subjected to PCR-like conditions for 0 and 20 cycles in 1 × SSC buffer (pH 7). The microarray was first subjected to 94 °C for 30 s (denaturation step), followed by cooling to 54 °C for 30 s (annealing step), and then heating to 72 °C for 30 s (chain extension step). After thermocycling, the slide was washed, dried, and visualized under laser scanner. Likewise, thermal stability of immobilization of thiophosphoryl- and phosphorylated oligonucleotides was assessed.

The pH stability of the constructed microarray was evaluated by spotting the SU-8 coated microslides, as described above, and then subjecting them to washings with 1 × SSC buffer of different pH (7, 8, and 9). After usual washings and drying, the microslides were scanned under a laser scanner. The stability of the microarrays constructed using thiophosphoryl- and phosphorylated oligonucleotides was examined.

Hybridization Studies. In order to study the accessibility of attached probe sequence to target sequence, d(CTT CTT TTT CCT GTT ACC GT)-OPO_3^{2-}, dissolved in the reaction buffer, was spotted onto SU-8 coated glass microslide at 0.5 μM and kept at 45 °C for 2 h. Then, the spotted slide was washed with the capping buffer (3 × 50 mL) followed by Milli-Q water (2 × 50 mL) and dried. Subsequently, the spots on the microslide were hybridized with a complementary labeled oligomer. Briefly, in a glass Petri dish, the spotted glass slide was kept on a wet blotting paper, and the complementary oligomer, TET-d(ACG GTA ACA GGA AAA AGA AG) (40 μM dissolved in 40 μL of hybridization buffer), was spread on the spotted area. The area was covered with a coverslip and the slide kept at 60 °C for 5 min, and later at ambient temperature for 2 h and at 10 °C for another 2 h. The coverslip was removed, and the slide was washed with the washing buffer (3 × 50 mL). After washings with the hybridization buffer (3 × 50 mL), the microslide was dried and subjected to laser scanning followed by quantification.

Later, hybridization assays were performed on glass slides immobilized with aminoalkyl- and thiophosphoryl-oligomers.

Base-Mismatch Detection. The specificity of the system was demonstrated by immobilizing modified oligomers on SU-8 coated glass microslides. Three oligonucleotides, viz., d(CTT CTT TTT CCT GTT ACC GT)-OPO_3^{2-}, d(CTT CTT TTT A**C**T GTT ACC GT)-OPO_3^{2-}, and d(TTT TTT TTT TTTTTT TTT TT)-OPO_3^{2-} (perfect match, single mismatch, and noncomplementary probes, respectively), were spotted on a coated glass microslide at 0.5 μM and kept at 45 °C for 2 h. Subsequently, the microslide was washed, dried, and the spots hybridized with a complementary labeled TET-d(ACG GTA ACA GGA AAA AGA AG), as described above. Similar studies were carried out using aminoalkyl- and thiophosphorylated oligomer immobilized glass slides.

Detection of Bacterial Diseases. *Bacterial Typhoid. PCR-Amplification.* The PCR reaction was performed in 25 μL PCR Eppendorf tubes containing 0.25 mM dNTPs (of each dATP, dCTP, dGTP, and dTTP), forward primer [TET-d(ACC GGT GGA TGT GGC TTC CTT G)], and reverse primer [TET-d(TGG TCT GCA GCA CCT TTT GAA C)] (0.4 μM of each), genomic DNA (50 ng/reaction), Taq polymerase (0.75 units), and Milli-Q water. All PCR reactions were performed on an MJ Research thermocycler using the following profile: (1) 5 min at 95 °C (initial denaturation step); (2) 35 cycles of 60 s min at 95 °C (denaturation step), 40 s at 61.6 °C (annealing step), 2 min at 72 °C (extension step); (3) 7 min at 72 °C (final extension step); hold temperature 4 °C. The labeled PCR-amplifications, 503 bp fragment of *tyv* gene (*Salmonella typhi*), were analyzed by agarose gel electrophoresis, and detection was performed by ethidium bromide (10 μg/mL) in standard TAE buffer, pH 8.

Bacterial Meningitis. PCR Amplification. In a similar fashion, PCR reaction was performed to obtain 111 bp fragment of *ctrA* gene (*Neisseria meningitides*, serogroup A) except by taking forward primer TET-d(GCT GCG GTA GGT GGT TCA A), and reverse primer TET-d(TTG TCG CGG ATT TGC AAC TA) using the following conditions: (1) 5 min at 95 °C (initial denaturation step); (2) 35 cycles of 50 s at 95 °C (denaturation step), 40 s at 58 °C (annealing step), 1 min at 72 °C (extension step); (3) 7 min 72 °C (final extension step); hold temperature 4 °C.

Immobilization of Probes and Detection of Diseases. Two oligonucleotide probes, d(ACC GGT GGA TGT GGC TTC CTT GTT TT)-OPO_3^{2-} (complementary to *tyv* gene) and d(ACG TGT CAG CTG CAC ATT CGT TTT T)-OPO_3^{2-} (complementary to *ctrA* gene) were immobilized on two SU-8 coated glass microslides. PCR amplicons of two diseases were hot-denatured (5 min, 95 °C), cooled on ice (10 min), and used for the hybridization assay. One of the microslides was hybridized with PCR amplicons corresponding to *ctrA* gene, and the other one was exposed to amplicons corresponding to *tyv* gene. After usual washings, the slides were visualized under a laser scanner.

Signal Evaluation and Quantification. Microarrays were scanned on a ScanArray Gx Plus Microarray Scanner fitted with a Cy3 optical filter at 20 μm resolution. PMT and laser power were set to 55% and 65%, respectively. Spot intensities were quantified and graphs were plotted in MS *Excel*.

For quantification of immobilized oligonucleotides, a TET-labeled oligonucleotide sequence, TET-d(ACG GTA ACA GGA AAA AGA AG), was diluted from 0.5 to 0.005 μM concentration and spotted on a virgin microslide. After drying, the slide was scanned and spots were quantified. A standard calibration curve was plotted between fluorescence intensity (A.U.) and concentration (μM).

For each experimental condition tested on the microarrays, the experiment was repeated 2−3 times. Immobilization and hybridization data presented are the average of these repetitions, and the error bars represent the percentage error (±2−4%) observed on this average.

RESULTS AND DISCUSSION

Since the advent of microarray technology, glass has remained a substrate of choice and several chemistries have been proposed for attachment of oligonucleotides onto it. However, low density of silanol groups often results in poor surface coverage with the probes, which ultimately affects the sensitivity of the constructed microarrays. In order to address this problem, attempts have been made to improve the sensitivity of an array by increasing the surface density of the functional groups (high-capacity biochips) to anchor oligomer probes. Recently, glass microslides coated with functional group-rich polymers (chitosan, polyethylenimine, acrylic acid-*co*-acrylamide, SU-8, etc.) have gained attention as substrates for the fabrication of microarrays. Of the polymers employed for coating of slides, SU-8 has garnered greater attention as a possible material for a variety of biomedical applications. Nucleic acids can be immobilized onto SU-8 coated surfaces by covalent linkages or by noncovalent hydrophobic interactions. Covalent attachment of probes is a preferred approach, as it results in higher stability of the constructed microarrays. The presence of epoxy functionalities on the surfaces, coated with SU-8 photoresist, allows attachment of oligonucleotides with different nucleophilic/electrophilic functionalities. By taking advantage of above-mentioned points, a rapid and clean protocol has been developed for direct immobilization of oligonucleotides bearing different functional groups onto SU-8 coated glass microslides.

Determination of Optimal Thickness Required for Immobilization. In order to study the effect of thickness of SU-8 photoresist on immobilization of oligomers, the attachment of a probe was carried out at different thickness (2, 2.5, and 3 μm). After usual washings and drying, the spots on the microslide were visualized under a laser scanner and quantified. The results obtained with a layer of thickness 2.5 μm (Figure 1) were found to be appropriate for immobilization, as it showed the highest immobilization efficiency. Therefore, in subsequent

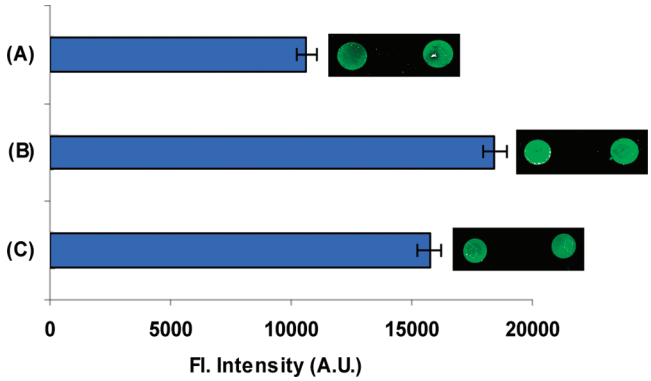


Figure 1. Fluorescence intensity data and pictorial representation of immobilization of TET-labeled oligonucleotide at (A) 2 μm, (B) 2.5 μm, and (C) 3 μm.

experiments, glass microslides coated with SU-8 (2.5 μm thickness) were employed.

Evaluation of Chip Surface for Immobilization. Homogeneity of the surface is one of the important factors for obtaining good-quality microarrays based on fluorescence detection. In the present study, the uniformity of epoxy functionalities on the surface of SU-8 coated microslides was evaluated by spotting an oligomer sequence at different locations all over the slide. After usual washings and drying, the slide was visualized under a laser scanner and the fluorescence intensity of spots was found to be in the range of ~18,000−18,500 A.U., which showed that the density of epoxy-functionalities on the coated glass surface was present almost uniformly throughout.

Determination of Threshold Concentration Required for Visualization. In order to optimize the threshold concentration of oligonucleotides required for fluorescence detection, a labeled oligomer, diluted (1, 0.5, 0.1, 0.05, 0.025, and 0.01 μM) was spotted onto an SU-8 coated glass microslide and incubated at 45 °C for 2 h. After usual washings and drying, the slide was scanned under a laser scanner, and the pictorial view of the slide is depicted in Figure 2A. Immobilization of thiophosphoryl- and aminoalkylated oligonucleotides was performed in a similar way (Figure 2B,C). The immobilization efficiency against each concentration was determined with the help of a standard curve (Figure 3). From these results, it was observed that immobilization efficiency increased when increasing the concentration of spotted oligonucleotides, and the fluorescent intensity of spots reached saturation at a concentration corresponding to 1 μM. The threshold concentration to visualize spots was found to be 0.05 μM; however, to construct a good-quality biochip for hybridization assays, a concentration of 0.5 μM was selected, as the highest immobilization efficiency was attained at this concentration.

Thermal and pH Stability. The thermal stability of constructed microarrays was evaluated by immobilizing a TET-labeled oligomer sequence (0.1 μM), as mentioned above, followed by subjecting it to 0 and 20 cycles of PCR-like conditions in 1 × SSC buffer (pH 7). The percentage reduction in fluorescence intensity was observed to be ~6.2% for aminoalkyl-, ~8.5% for thiophosphoryl-, and ~7.9% for phosphorylated oligomers after 20 cycles (Figure 4A), indicating that constructed microarrays were sufficiently stable to be used in biological research. The stability of constructed microarrays was also analyzed at different pH values (7, 8, and 9). The results indicated that the constructed microarrays were sufficiently stable in SSC buffer of different pH. The decrease in fluorescence intensity was ~3.7% and ~5.6% for aminoalkyl-, ~7.0% and ~11.8% for thiophosphoryl-, and ~4.6% and ~8.3% for phosphorylated oligomers at pH 8 and 9, respectively, as compared to fluorescence intensity at pH 7 for the immobilized oligomers (Figure 4B).

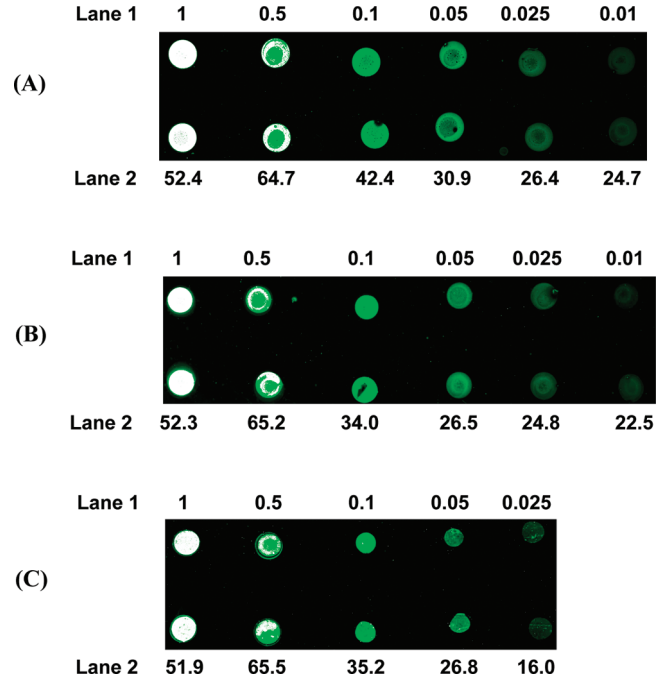


Figure 2. Threshold concentration of (A) phosphoryl-, (B) thiophosphoryl-, and (C) aminoalkylated oligonucleotides required for fluorescence visualization. Lane 1: Probe concentration (μM). Lane 2: Immobilization efficiency (%).

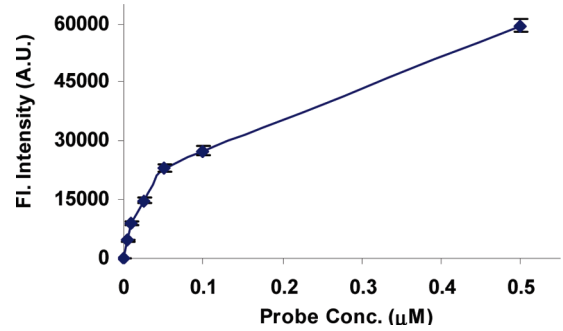


Figure 3. Correlation sketch between concentration of immobilized probe (μM), TET-d(ACG GTA ACA GGA AAA AGA AG), and fluorescence intensity (A.U).

Hybridization Studies. The applicability of the projected strategy was evaluated by performing hybridization assay of slides fabricated via immobilization of phosphoryl-, thiophosphoryl-, and aminoalkylated oligomers onto the SU-8 coated glass slides at 0.5 μM concentration. After incubation at 45 °C for 2 h, the slides were subjected to treatment with the capping buffer to block residual epoxy functions on the slide surface (*15*). Subsequently, the spots on the microslide were hybridized with complementary labeled oligomers. After washings with the hybridization buffer, the slides were scanned and fluorescent spots on the microslides were quantified. The projected strategy resulted in high hybridization efficiencies (~33.4% for aminoalkyl-, ~30.5% for thiophosphoryl-, and 35.7% for phosphorylated oligonucleotides).

Detection of Base Mismatches. The specificity of the system was demonstrated by immobilizing modified oligomers on SU-8 coated glass microslides. Three oligonucleotides, viz., d(CTT CTT TTT CCT GTT ACC GT)-OPO_2-$O(CH_2)_2O(CH_2)_2NH_2$, d(CTT CTT TTT ACT GTT ACC GT)-OPO_2-$O(CH_2)_2$-$O(CH_2)_2NH_2$, and d(TTT TTT TTT TTTTT TTT TT)-OPO_2-$O(CH_2)_2O(CH_2)_2NH_2$ (perfect match, single mismatch, and

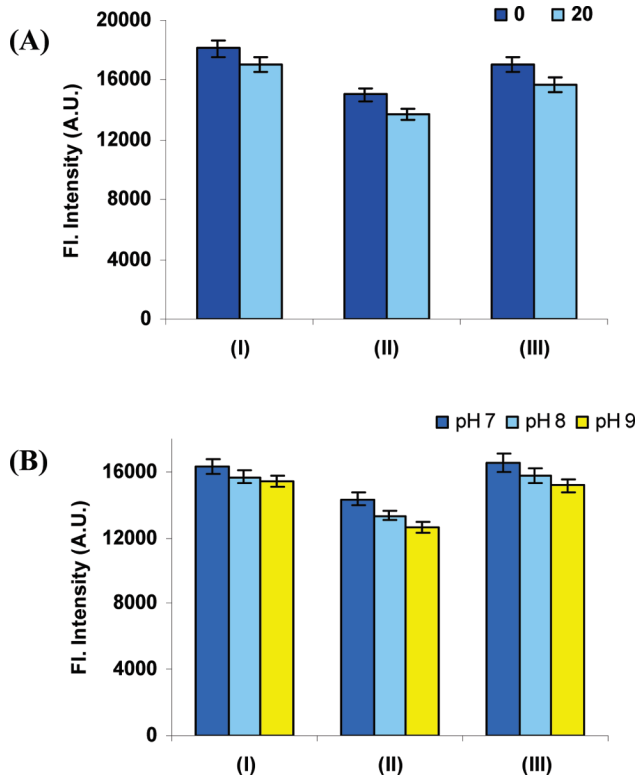


Figure 4. (A) Graphical representation showing thermal stability of immobilized (I) aminoalkyl-, (II) thiophosphoryl-, and (III) phosphorylated probes {TET-d(CTT CTT TTT CCT GTT ACC GT)—X} after 0 and 20 cycles of PCR-like conditions; (B) pH stability of the (I) aminoalkyl-, (II) thiophosphoryl-, and (III) phosphorylated probes immobilized onto SU-8 coated glass microslides ($n = 3$).

noncomplementary probes, respectively), were spotted on a glass microslide and incubated at 45 °C for 2 h. After treatment with the capping buffer followed by washings, the microslide was washed, dried, and hybridized with a complementary labeled oligomer, TET-d(ACG GTA ACA GGA AAA AGA AG), as described above. Figure 5A (I) clearly shows the base mismatches. The results revealed that the highest fluorescence intensity was obtained in the case of a perfect match and this decreased for single-mismatch, while the noncomplementary oligonucleotide did not exhibit significant signal intensity after hybridization, thus suggesting specificity of the hybridization in this case. Similar studies were carried out using thiophosphoryl- and phosphorylated oligomers, as shown in Figures 5A (II) and (III). The quantification data are depicted in Figure 5B.

Detection of Bacterial Diseases. In order to develop biochips for diagnosis of the bacterial infections, two microarrays were constructed on SU-8 coated glass microslides using a unique probe sequence of the *tyv* and *ctrA* genes, as described under Experimental Procedures. The spots on the first microslide were subjected to hybridization with the labeled PCR amplicons corresponding to the *ctrA* gene. After washings and drying, the slide was visualized under a laser scanner. Figure 6A shows the pictorial view of the slide. Spots of probe sequence specific to *ctrA* gene of bacteria (*N. meningitidis*) showed fluorescence, while the spots pertaining to *tyv* gene did not fluoresce at all. Spots on the second microslide were hybridized with PCR amplicons corresponding to *tyv* gene. Figure 6B depicts that spots of probe sequence specific to *tyv* gene exhibited fluorescence, while the spots pertaining to *ctrA* gene did not fluoresce at all. The experiment signifies the

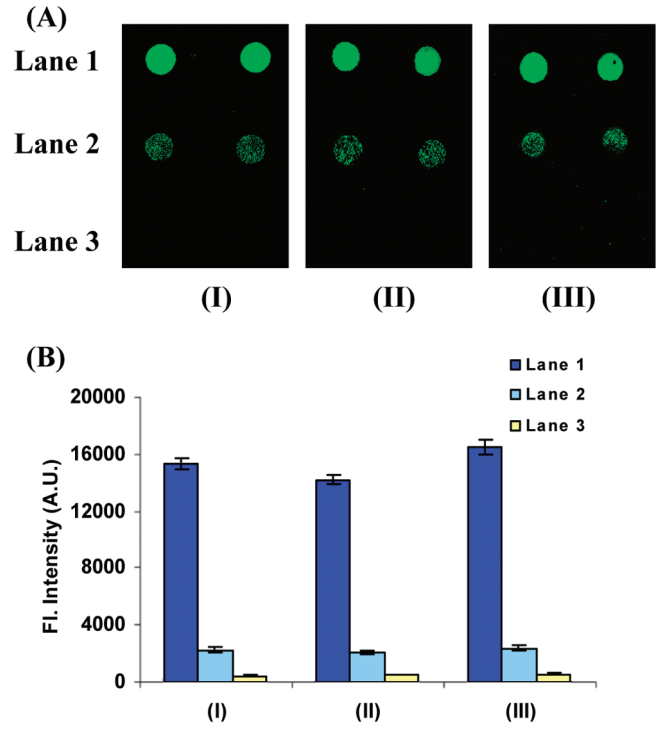


Figure 5. (A) Detection of base mismatches using (I) aminoalkyl-, (II) thiophosphoryl-, and (III) phosphorylated probes. (B) Quantitative data of the fluorescence intensity of the spots.

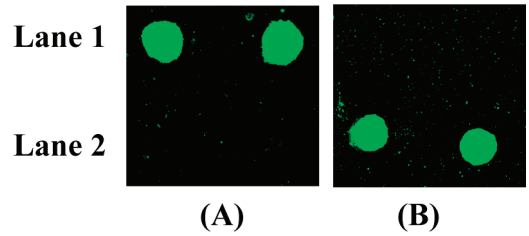


Figure 6. Detection of bacterial (A) meningitis and (B) typhoid. Lane 1: *ctrA* probe sequence {d(ACG TGT CAG CTG CAC ATT CGT TTT T)-OPO_3^{2-}}. Lane 2: *tyv* probe sequence {d(ACC GGT GGA TGT GGC TTC CTT GTT TT)-OPO_3^{2-}) were immobilized on SU-8 coated glass microslides.

specificity of the system to prepare biochips for the detection of bacterial meningitis and typhoid.

CONCLUSIONS

A versatile high-density epoxy-coated (SU-8) glass substrate has been developed for the construction of microarrays. Highlighting features of the projected strategy are as follows: covalent tethering of modified oligonucleotides with high immobilization and hybridization efficiency; stability of the immobilized probes toward a wide range of temperature and pH conditions; visualization of immobilized probes at the concentration of 0.05 μM (sensitivity almost 20—100 times); and ability to detect base mismatches and diagnose bacterial diseases. It is anticipated that the protocol will provide individuals, particularly end users, easy access to oligonucleotide microarrays for various routine biological studies.

ACKNOWLEDGMENT

Authors are thankful to Drs. S. Gupta and S. Khare, NICD, Delhi, India, for processing of patient samples. D.S. thanks the University Grants Commission, New Delhi, India, for the award of a Senior Research Fellowship.

LITERATURE CITED

(1) Grant, S. F., and Hakonarson, H. (2008) Microarray technology and applications in the area of genome-wide association. *Clin. Chem. 54*, 1116–1124.

(2) Miller, M. B., and Tang, Y. W. (2009) Basic concepts of microarrays and potential applications in clinical microbiology. *Clin. Microbiol. Rev. 22*, 611–633.

(3) Chen, X., Jorgenson, E., and Cheung, S. T. (2009) New tools for functional genome analysis. *Drug Discovery Today 14*, 754–760.

(4) Shen, Y., and Wu, B.-L. (2009) Microarray-based genomic DNA profiling technologies in clinical molecular diagnosis. *Clin. Chem. 55*, 659–669.

(5) Stangegaard, M., Wang, Z., Kutter, J. P., Dufva, M., and Wolff, A. (2006) Whole genome expression profiling using DNA microarray for determining biocompatibility of polymeric surfaces. *Mol. Biosyst. 2*, 421–428.

(6) Seliger, H., Hinz, M., and Happ, E. (2003) Arrays of immobilized oligonucleotides-Contributions to nucleic acids technology. *Curr. Pharm. Biotechnol. 4*, 379–395.

(7) Sethi, D., Gandhi, R. P., Kumar, P., and Gupta, K. C. (2009) Chemical strategies for immobilization of oligonucleotides. *Biotechnol. J. 4*, 1513–1529.

(8) Vaijayanthi, B., Kumar, P., Ghosh, P. K., and Gupta, K. C. (2003) Recent advances in oligonucleotide synthesis and their applications. *Ind. J. Biochem. Biophys. 40*, 377–391.

(9) Lane, S. M., Monot, J., Petit, M., Tellier, C., Bujoli, B., and Talham, D. R. (2008) Poly(dG) spacers lead to increased surface coverage of DNA probes: An XPS study of oligonucleotide binding to zirconium phosphonate modified surfaces. *Langmuir 24*, 7394–7299.

(10) Misra, A., and Shahid, M. (2009) Immobilization of self-quenched DNA hairpin probe with a heterobifunctional reagent on a glass surface for sensitive detection of oligonucleotides. *Bioorg. Med. Chem. 17*, 5826–5833.

(11) Wu, O., Ma, W., Zhu, L., Guo, Q., Zhang, B., Kulageri, S. M., and Zheng, W. (2008) Oligo-Microarray based on oligonucleotide immobilization on glass surface modified with activated acrylic acid-co-acrylamide copolymer. *J. Biomed. Mater. Res., Part B: Appl. Biomater. 87*, 67–72.

(12) Mahajan, S., Kumar, P., and Gupta, K. C. (2006) Oligonucleotide microarrays: Immobilization of phosphorylated oligonucleotides on epoxylated surface. *Bioconjugate Chem. 17*, 1184–1189.

(13) Lockett, M. R., Phillips, M. F., Jarecki, J. L., Peelen, D., and Smith, L. M. (2008) A tetrafluorophenylactivated ester self-assembled monolayer for the immobilization of amine-modified oligonucleotides. *Langmuir 24*, 69–75.

(14) Sethi, D., Patnaik, S., Kumar, A., Gandhi, R. P., Gupta, K. C., and Kumar, P. (2009) Polymer supported synthesis of aminooxy-alkylated oligonucleotides, and some applications in the fabrication of microarrays. *Bioorg. Med. Chem. 17*, 5442–5450.

(15) Mahajan, J., Sethi, D., Seth, S., Kumar, A., Kumar, P., and Gupta, K. C. Construction of oligonucleotide microarrays (biochips) via thioether linkage for the detection of bacterial meningitis. *Bioconjugate Chem. 20*, 1703–1710.

(16) Sethi, D., Kumar, A., Gupta, K. C., and Kumar, P. (2008) A facile method for the construction of oligonucleotide microarrays. *Bioconjugate Chem. 19*, 2136–2143.

(17) Beaucage, S. L. (2001) Strategies in the preparation of DNA oligonucleotide arrays for diagnostic applications. *Curr. Med. Chem. 8*, 1213–1244.

(18) Dufva, M. (2005) Fabrication of high quality microarrays. *Biomol. Eng. 22*, 173–184.

(19) Fuentes, M., Mateo, C., Garcia, L., Tercero, J. C., Guisan, J. M., and Fernandez-Lafuente, R. (2004) Directed covalent immobilization of aminated DNA probes on aminated plates. *Biomacromolecules 5*, 883–888.

(20) Afanassiev, V., Hanemann, V., and Wolfl, S. (2000) Preparation of DNA and protein micro arrays on glass slides coated with an agarose film. *Nucleic Acids Res. 28*, e66.

(21) Diaz-Quijada, G. A., and Wayner, D. D. M. (2004) A simple approach to micropatterning and surface modification of poly-(dimethylsiloxane). *Langmuir 20*, 9607–9611.

(22) Benters, R., Niemeyer, C. M., Drutschmann, D., Blohm, D., and Wöhrle, D. (2002) DNA microarrays with PAMAM dendritic linker systems. *Nucleic Acids Res. 30*, e10.

(23) Cha, T.-W., Boiadjiev, V., Lozano, J., Yang, H., and Zhu, X.-Y. *Anal. Biochem. 311*, 27–32.

(24) Consolandi, C., Severgnini, M., Castiglioni, B., Bordoni, R., Frosini, A., Battaglia, C., Bernardi, L. R., and De Bellis, G. (2006) A structured chitosan-based platform for biomolecule attachment to solid surfaces: Application to DNA microarray preparation. *Bioconjugate Chem. 17*, 371–377.

(25) Deepu, A., Sai, V. V., and Mukherji, S. (2009) Simple surface modification techniques for immobilization of biomolecules on SU-8. *J. Mater. Sci. Mater. Med. 20*, S25–S28.

(26) Erkan, Y., Czolkos, I., Jesorka, A., Wilhelmsson, L. M., and Orar, O. (2007) Direct immobilization of cholesteryl-TEG-modified oligonucleotides onto hydrophobic SU-8 surfaces. *Langmuir 23*, 5259–5263.

(27) Fontanel, M. L., Bazin, H., and Teoule, R. (1993) End attachment of phenol-oligonucleotide conjugates to diazotized cellulose. *Bioconjugate Chem. 4*, 380–385.

(28) Lie, L. H., Patole, S. N., Pike, A. R., Ryder, L. C., Connolly, B. A., Ward, A. D., Tuite, E. M., Houlton, A., and Horrocks, B. R. (2004) Immobilisation and synthesis of DNA on Si(111), nanocrystalline porous silicon and silicon nanoparticles. *Faraday Discuss. 125*, 235–249.

(29) Okutucu, B., and Telefoncu, A. (2004) Covalent attachment of oligonucleotides to cellulose acetate membranes. *Artif. Cells Blood Substit. Immobil. Biotechnol. 32*, 599–608.

(30) Fixe, F., Dufva, M., Telleman, P., and Christensen, C. B. V. (2004) One-step immobilization of aminated and thiolated DNA onto poly(methylmethacrylate) (PMMA) substrates. *Lab Chip 4*, 191–195.

(31) Preininger, C., Sauer, U., Dayteg, J., and Pichler, R. (2005) Optimizing processing parameters for signal enhancement of oligonucleotide and protein arrays on ARChip epoxy. *Bioelectrochemistry 67*, 155–162.

(32) Hong, B. J., Sunkara, V., and Park, J. W. (2005) DNA microarrays on nanoscale-controlled surface. *Nucleic Acids Res. 33*, e106.

(33) Lim, S. B., Kim, K.-W., Lee, C.-W., Kim, H.-S., Lee, C.-S., and Oh, M.-K. (2008) Improved DNA chip with poly(amidoamine) dendrimer peripherally modified with biotin and avidin. *Biotechnol. Bioprocess Eng. 13*, 683–689.

(34) Benters, R., Niemeyer, C. M., Drutschmann, D., Blohm, D., and Wohrle, D. (2002) DNA microarrays with PAMAM dendritic linker systems. *Nucleic Acids Res. 30*, e10.

(35) Abgrall, P., Conedera, V., Camon, H., Gue, A. M., and Nguyen, N. T. (2007) SU-8 as a structural material for labs-on-chips and microelectromechanical systems. *Electrophoresis 28*, 4539–4551.

(36) Joshi, M., Kal, N., Lal, R., Ramgopal, R. V., and Mukherji, S. (2007) A novel dry method for surface modification of SU-8 for immobilization of biomolecules in Bio-MEMS. *Biosens. Bioelectron. 22*, 2429–2435.

(37) Marie, R., Schmid, S., Johansson, A., Ejsing, L., Nordstrom, M., Hafliger, D., Christensen, C. B., Boisen, A., and Dufva, M. (2006) Immobilization of DNA to polymerized SU-8 photoresist. *Biosens. Bioelectron. 21*, 1327–1332.

(38) *Delta Scientific Equipments Pvt. Ltd. Manual*, Delhi, India (2007).

BC100262N