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ARTICLE *in* BIOCONJUGATE CHEMISTRY · NOVEMBER 2008

Impact Factor: 4.51 · DOI: 10.1021/bc800241k · Source: PubMed

Article

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Bioconjugate Chem., **2008**, 19 (11), 2136-2143 • Publication Date (Web): 22 October 2008

A Facile Method for the Construction of Oligonucleotide Microarrays

Dalip Sethi, A. Kumar, K. C. Gupta,* and P. Kumar*

Institute of Genomics and Integrative Biology (CSIR), Delhi University Campus, Mall Road, Delhi-110007, India. Received June 14, 2008; Revised Manuscript Received September 17, 2008

In recent years, the oligonucleotide-based microarray technique has emerged as a powerful and promising tool for various molecular biological studies. Here, a facile protocol for the construction of an oligonucleotide microarray is demonstrated that involves immobilization of oligonucleotide−trimethoxysilyl conjugates onto virgin glass microslides. The projected immobilization strategy reflects high immobilization efficiency (∼36−40%) and signal-to-noise ratio (∼98), and hybridization efficiency (∼32−35%). Using the proposed protocol, aminoalkyl, mercaptoalkyl, and phosphorylated oligonucleotides were immobilized onto virgin glass microslides. Briefly, modified oligonucleotides were reacted first with 3-glycidyloxypropyltriethoxysilane (GOPTS), and subsequently, the resultant conjugates were directly immobilized onto the virgin glass surface by making use of silanization chemistry. The constructed microarrays were then used for discrimination of base mismatches. On subjecting to different pH and thermal conditions, the microarray showed sufficient stability. Application of this chemistry to manufacture oligonucleotide probe-based microarrays for detection of bacterial meningitis is demonstrated. Single-step reaction for the formation of conjugates with the commercially available reagent (GOPTS), omission of capping step and surface modification, and efficient immobilization of oligonucleotides onto the virgin glass surface are the key features of the proposed strategy.

INTRODUCTION

Oligonucleotide arrays have emerged as a powerful and convenient tool for parallel and high-throughput experimentation in molecular biological research (*1*−*6*). Array technology has considerably simplified the process involved in disease diagnosis and gene mapping, as it offers advantages of using a very small amount of biological probes (nanomoles and picomoles), along with the capability of analyzing thousands of analytes in parallel (*7*).

Direct syntheses (in situ) of oligonucleotides on the chip surface following conventional or photolithographic methods and immobilization of presynthesized oligonucleotides on the selected substrates are the two predominant methods for construction of oligonucleotide microarrays. The in situ synthesis protocol allows the preparation of high-density oligonucleotide microarrays; however, it suffers from certain drawbacks (*8*−*12*). The deposition method, on the other hand, offers flexibility in the sense that a variety of biomolecules can be immobilized on the surface of choice but limited to generation of low to medium density arrays (*14*−*19*). Therefore, the deposition method has become a method of choice for routine applications in research and commercial laboratories. The choice of surface and the chemistry employed for attachment of oligonucleotides are the key factors that govern the quality of constructed microarray. Out of various surfaces used, glass is a substrate of choice owing to its widespread availability, low cost, well-characterized silane chemistry for functionalization, and favorable optical properties, which permits highly sensitive laser-induced fluorescence imaging.

* Correspondence address. Dr. P. Kumar, Institute of Genomics and Integrative Biology, Delhi University Campus, Mall Road, Delhi-110007, India. Tel: +91-11-27666156, Fax: +91-11-27667471, E-mail: pkumar@igib.res.in. Dr. K.C. Gupta, Institute of Genomics and Integrative Biology, Delhi University Campus, Mall Road, Delhi-110007, India. Tel: +91-11-27662491, Fax: +91-11-27667471, E-mail: kcgupta@igib.res.in.

For the attachment of oligonucleotides, various covalent and noncovalent oligonucleotide immobilization techniques have been documented. The simplest of them is the immobilization of probes via electrostatic adsorption onto a charged priming layer like aminopropyl or polylysine-coated glass slides; however, it results in poor hybridization efficiency (*13*). Covalent attachment of probes onto the solid support is a preferred approach, as it results in higher stability and hybridization efficiency as compared to noncovalent immobilization. Some commonly used attachment chemistries (surface-probe) are semicarbazide-aldehyde (*14*), epoxide-amine (*15*), aldehyde-amine (*16*), gold-thiol (*17, 18*), and thiol-thiol (*19*) interactions. Various hetero- and homobifunctional reagents have also been reported for tethering oligonucleotides to polymeric surfaces (*20*−*24*). In addition to the covalent chemistries employed for the fabrication of arrays, some "organic−inorganic" interactions have also been utilized to realize immobilization of oligonucleotides. Attachment of thiol-modified probes to metallic gold via sulfur−gold linkage and phosphorylated oligonucleotides on zirconylated surface are the representative examples of this approach (*18, 25*).

Here, we describe a simple two-step method that involves attachment of modified oligonucleotides (aminoalkyl, mercaptoalkyl, and phosphorylated oligomers) onto a virgin glass surface. The first step involves the reaction between a modified probe and a commercially available reagent, 3-glycidyloxypropyltriethoxysilane (GOPTS), to form a silylated conjugate, which, in turn, reacts with silanol functionalities of the glass slide to result in surface-bound oligonucleotides. The proposed strategy not only resulted in signal reproducibility but also showed superior spot morphology and homogeneity. The study undertaken herein includes investigation of optimal time required for the formation of oligomer−trimethoxysilyl conjugate followed by their immobilization on a virgin glass surface and determination of the threshold concentration of conjugate required for visualization. The thermal and pH stability of constructed microarrays was evaluated. The constructed microarrays were compared with the standard method to clearly

10.1021/bc800241k CCC: $40.75
Published on Web 10/22/2008

highlight the advantages of the projected strategy in terms of immobilization and hybridization efficiency.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. Virgin glass microslides (75 × 22 mm^2), *N*-methylimidazole (NMI), and 3-glycidyloxypropyltrimethoxysilane (GOPTS) were obtained from Sigma-Aldrich Chemical Co., St. Louis, MO. Absolute ethanol was procured from Tedia Company Inc., Fairfield, U.S. 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 the commercial source, unless specified.

Instrumentation. Scan Array Lite Scanner (GSI Lumonics), Speed Vac concentrator (Hetovac VR-1), Lambda Bio 20 UV−vis spectrophotometer (Perkin-Elmer, USA), and *Quant Array* software for quantification of fluorescence intensity of the spots (Packard Biosciences, USA) were used.

Cleaning of Glass Slides. The glass slides were treated with 1 M NaOH for 1 h followed by washing with MilliQ water (3 × 150 mL). Then, the slides were treated with 1.5 M HCl for 1 h followed by extensive washings with MilliQ water (3 × 150 mL). Finally, the slides were immersed in absolute ethanol (2 × 150 mL, for 15 min each), dried, and stored under inert atmosphere.

Reagents and Buffers. (i) GOPTS solution (0.0136 M): Prepared by dissolving 30 μL GOPTS in 10 mL of dimethylformamide (DMF)/tetrahydofuran (THF)/acetonitrile (ACN). (ii) Reaction buffer (0.1 M NMI): Prepared by dissolving 80 μL in 10 mL of MilliQ water. (iii) Washing buffer: 125 mM SSC buffer containing 750 mM sodium chloride, pH 7.0. (iv) Hybridization buffer: 125 mM SSC buffer containing 1 M sodium chloride, pH 7.0.

Oligonucleotide Synthesis and Purification. Oligonucleotides, used in this study, were synthesized on 0.2 μmol scale on a Pharmacia LKB Gene Assembler Plus using the standard phosphoramidite approach following the manufacturer's protocol (*26*). Labeled oligonucleotides were prepared by performing the last coupling with TET-phosphoramidite in an analogous manner to that of normal nucleoside−phosphoramidites, except that an extended coupling time (5 min) was employed. A list of oligonucleotide sequences assembled along with their deprotection conditions and yields are given in Table 1. The ammoniacal solutions in all the cases were concentrated in a speed vac; the residue obtained was dissolved in water (200 μL) and applied onto a desalting column (reverse-phase ODS-silica gel column). The oligonucleotides were eluted with 30% acetonitrile in water, concentrated, purified, and stored at 4 °C.

Determination of Optimal Conditions Required for Immobilization of Modified Oligonucleotides on a Virgin Glass Microslide. *Preparation of Oligonucleotide−Trimethoxysilyl Conjugate.* Oligonucleotide sequence, TET-d(CTT CTT TTT CCT GTT ACC GT)-OPO_3^{2-} (0.6 nmol), dissolved in the reaction buffer (100 μL), was allowed to react with a GOPTS solution (100 μL) prepared in DMF for 8 h on an Eppendorf mixer. The reaction mixture was then concentrated, and the viscous material obtained was suspended in MilliQ water (40 μL) and centrifuged. The supernatant was collected and concentrated. The residue was finally dissolved in 10% DMSO in water to fix the concentration of the probe solution to 5 μM and then spotted (0.5 μL) manually using a 2 μL pipetteman onto a virgin glass microslide. After spotting, the microslide was kept at 45 °C for 35 min. The slide was washed with 1× SSC buffer (2 × 50 mL for 15 min each), followed by MilliQ water (2 × 50 mL), and dried under vacuum. Subsequently, the spots on the microslide were visualized under a laser scanner and quantified.

Likewise, the experiment was performed in other solvent systems, viz., THF/water (1:1, v/v) and ACN/water (1:1, v/v).

Time Kinetics for the Synthesis of Conjugate. To determine the optimum time required for the formation of conjugates, a kinetic study was performed. Twelve Eppendorfs were equally charged with GOPTS solution (100 μL) in DMF and TET-labeled oligonucleotide, TET-d(CTT CTT TTT CCT GTT ACC GT)-OPO_3^{2-} (0.6 nmol in 100 μL reaction buffer). The Eppendorfs were kept at 45 °C and withdrawn at different time intervals. Subsequently, the reaction mixture was worked up and microarray constructed, as described above. After usual washings and drying, the spots on the microslides were scanned under a laser scanner and quantified. The extent of reaction was evaluated by plotting fluorescence intensity of the spots against time. A similar study was carried out with aminoalkyl and mercaptoalkylated oligonucleotides.

Threshold Concentration of the Conjugate Required for Visualization. In order to determine the threshold concentration of conjugate required for visualization under a laser scanner, oligomer−trimethoxysilyl conjugate, TET-d(CTT CTT TTT CCT GTT ACC GT)-OPO_3^--$CH_2CH(OH)CH_2O(CH_2)_3$-Si-$(OMe)_3$, was dissolved in 10% DMSO in MilliQ water and spotted manually in duplicate in different concentrations (0.5, 1, 2.5, 5, 10, and 20 μM) on a glass microslide. The spotted slide was kept in a humidity chamber at 45 °C for 35 min, followed by washings with the washing buffer (2 × 50 mL for 15 min each) and MilliQ water (2 × 50 mL). The slide was dried under vacuum and scanned under a laser scanner.

Likewise, the conjugates of aminoalkyl and mercaptoalkylated probes were studied.

Evaluation of Glass Surface for Immobilization. In order to evaluate the homogeneity of glass surface, an experiment was performed. An oligomer−trimethoxysilyl conjugate, TET-d(CTT CTT TTT CCT GTT ACC GT)-OPO_3^--$CH_2CH(OH)CH_2O(CH_2)_3$-Si$(OMe)_3$ (5 μM), was spotted manually on virgin glass microslides at different locations and kept at 45 °C for 35 min. After washing and drying, as described above, the slide was scanned.

Hybridization Studies. Oligonucleotide sequence, d-(ACG GTA ACA GGA AAA AGA AG)-OPO_3^- (0.6 nmol), dissolved in 100 μL of reaction buffer, was mixed with 100 μL solution of GOPTS in DMF. The reaction mixture was agitated for 270 min at 45 °C. Then, the solution was concentrated under vacuum to obtain a syrupy residue of oligonucleotide−trimethoxysilyl conjugate, which was resuspended in MilliQ water (40 μL). The mixture was centrifuged to get rid of excess GOPTS as undissolved material. The supernatant was concentrated under vacuum to obtain a residue, which was reconstituted in MilliQ water containing 10% DMSO (v/v) to make an oligonucleotide conjugate concentration to 5 μM. The conjugate was then spotted manually in duplicate on a glass slide. The spotted microslide was incubated at 45 °C in a humidity chamber for 35 min, followed by washings with washing buffer (3 × 50 mL for 15 min each) and then subjected to hybridization experiment. The microslide was kept in a hybridization chamber and treated with a solution of the complementary fluorescent oligonucleotide, TET-d(CTT CTT TTT CCT GTT ACC GT) (40 μL, 40 μM), prepared in hybridization buffer, under a coverslip at 45 °C for 1 h and then at 25 °C for 12 h. After removal of coverslip, the glass microslide was then washed with hybridization buffer (3 × 50 mL for 15 min each) to get rid of excess complementary oligomer. Subsequently, the microslide was dried, examined under a laser scanner, and quantified.

Similarly, the aminoalkyl and mercaptoalkylated oligonucleotide conjugates were immobilized on a microslide and hybridization assay was performed.

Table 1. Oligonucleotide Sequences Synthesized, Their Deprotection Conditions, and Yields

no	oligonucleotiode sequence	deprotection conditions	yield (A_{254} nm, OD)
1	TET-d (CTT CTT TTT CCT GTT ACC GT)OPO_3^{2-}	aq NH_4OH, 16 h, 60 °C followed by aq AcOH (80%), 4 h	24.3
2	d (AGC GTA ACA GGA AGA AGC TG)OPO_3^{2-}	same conditions	25.6
3	d (AGC GTA ACA GGA AGA TGC TG)OPO_3^{2-}	same conditions	23.4
4	d (AGC GTA CCA GGA AGA TGC TG)OPO_3^{2-}	same conditions	28.6
5	d (TTT TTT TTT TTT TTT TTT TT)OPO_3^{2-}	same conditions	24.2
6	d (AGC GTA ACA GGA AGA AGC TG)OPO_2-$(CH_2)_6NH_2$	aq NH_4OH, 16 h, 60 °C	20.2
7	d (AGC GTA ACA GGA AGA TGC TG)OPO_2- $(CH_2)_6NH_2$	same conditions	21.5
8	d (AGC GTA CCA GGA AGA TGC TG)- OPO_2- $(CH_2)_6NH_2$	same conditions	20.6
9	d(TTT TTT TTT TTT TTT TTT TT)- OPO_2- $(CH_2)_6NH_2$	same conditions	24.4
10	d (AGC GTA ACA GGA AGA AGC TG)OPO_2-$(CH_2)_6SH$	aq NH_4OH, 16 h, 60 °C containing 0.1 M DTT	21.1
11	d (AGC GTA ACA GGA AGA TGC TG)OPO_2- $(CH_2)_6SH$	same conditions	25.2
12	d (AGC GTA CCA GGA AGA TGC TG)- OPO_2- $(CH_2)_6SH$	same conditions	20.3
13	d(TTT TTT TTT TTT TTT TTT TT)- OPO_2- $(CH_2)_6SH$	same conditions	24.3
14	TET-d (CAG CTT CTT CCT GTT ACG CT)	aq NH_4OH, 16 h, 60 °C	24.1
15	TET-d(GCT GCG GTA GGT GGT TCA A) F. primer	same conditions	22.7
16	TET-d(TTG TCG CGG ATT TGC AAC TA) R. primer	same conditions	23.1
17	d(ACG TGT CAG CTG CAC ATT CGT)-OPO_3^{2-} (probe sequence)	aq NH_4OH, 16 h, 60 °C Followed by aq AcOH (80%), 4 h	23.5
18	d(CGT CGA GTC GGG CAT CTT TTT)-OPO_3^{2-} (control)	same conditions	24.5

Detection of Base Mismatches. The specificity of the system was demonstrated by immobilizing oligomer−trimethoxysilyl conjugates on virgin glass microslides. Four oligonucleotide conjugates, viz., d(ACG GTA ACA GGA AAA AGA AG)-OPO_3^--$CH_2CH(OH)CH_2O(CH_2)_3$-$Si(OMe)_3$, d(ACG GTA ACA GGA AAA AGC AG)-OPO_3^--$CH_2CH(OH)CH_2O(CH_2)_3$-$Si(OMe)_3$, d(ACG GTA ACA CGA AAA AGC AG)-OPO_3^--$CH_2CH(OH)CH_2O(CH_2)_3$-$Si(OMe)_3$, and d(TTT TTT TTT TTT TTT TTT TT)-OPO_3^--$CH_2CH(OH)CH_2O(CH_2)_3$-$Si(OMe)_3$ (5 μM), having zero, one, and two mismatches and noncomplementary probe, were spotted on a virgin glass slide and processed as described above. Subsequently, the spots on the microslide were hybridized with a complementary labeled TET-d(CTT CTT TTT CCT GTT ACC GT) and kept at 45 °C for 1 h and then at room temperature for 12 h. After washings with hybridization buffer (3 × 50 mL for 15 min each), the microslide was subjected to laser scanning followed by quantification. Under identical conditions, conjugates of aminoalkyl and mercaptoalkylated oligomers were immobilized, hybridized, and quantified.

In order to demonstrate that the epoxy group of GOPTS specifically reacts with terminal functional groups of end-modified oligonucleotides, a comparative study was performed. Conjugates of two oligonucleotide sequences, viz., TET-d(CTT CTT TTT CCT GTT ACC GT) and TET-d(CTT CTT TTT CCT GTT ACC GT)-OPO_3^{2-}, were prepared, as described above and after usual workup, the conjugates (5 μM) were spotted manually on virgin glass slide. After usual washings, the spots were visualized under a laser scanner and quantified.

Quantification of Immobilized Oligonucleotides. To quantify the density of immobilized oligonucleotides, an oligomer sequence, TET-d(CTT CTT TTT CCT GTT ACC GT)-OPO_3^{2-}, diluted to different concentrations (0.05, 0.10, 0.25, 0.50, 0.75, 1.0, 2.0 μM), was spotted on a glass microslide in duplicates. After drying the spots under vacuum, the microslide was visualized under a laser scanner and the fluorescence signal intensity corresponding to each spot was measured. Using these values, a standard curve was plotted between fluorescence intensity (A.U.) and concentration (μM) of the spot.

Thermal and pH Stability. To evaluate the thermal stability of probe immobilization, a microarray was constructed by spotting oligonucleotide−trimethoxysilyl conjugates at a concentration of 5 μM. The fabricated microarrays were treated under PCR-like conditions for 0, 5, and 10 cycles in 1× SSC buffer (pH 7). 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 washed, dried, and visualized under a laser scanner.

The pH stability was evaluated by preparing microarrays in a similar way and then subjecting them to washings with 1× SSC buffer of different pH (7, 8, and 9) (3 × 50 mL for 15 min each). The microslides, after usual washings and drying, were visualized under a laser scanner.

Detection of Bacterial Meningitis. *PCR-Amplification.* The PCR reaction was performed in 25 μL PCR Eppendorf containing 0.25 mM dNTPs (of each dATP, dCTP, dGTP, and dTTP), forward primer TET-d(GCT GCG GTA GGT GGT TCA A), and reverse primer TET-d(TTG TCG CGG ATT TGC AAC TA) (0.4 μM of each), genomic DNA (50 ng/reaction), Taq-polymerase (0.75 units), and MilliQ 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 50 s at 95 °C (denaturation step), 40 s at 58 °C (annealing step), 1 min at 72 °C (extension step); (3) 7 min at 72 °C (final extension step); hold temperature: 4 °C. The labeled PCR-amplifications, 111bp fragment of *ctrA* gene (*Neisseria meningitidis*; serogroup A), were analyzed by agarose gel electrophoresis, and detection was performed by ethidium bromide (10 μg/mL) in standard TAE buffer, pH 8.

PCR Amplicon Capture. Two oligonucleotide−trimethoxysilyl conjugates, d(ACG TGT CAG CTG CAC ATT CGT)-OPO_3^--$CH_2CH(OH)CH_2O(CH_2)_3$-$Si(OMe)_3$ (complementary) and d(CGT CGA GTC GGG CAT CTT TTT)-OPO_3^--$CH_2CH(OH)CH_2O(CH_2)_3$-$Si(OMe)_3$ (control, noncomplementary) 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 the hybridization assay. After usual washings, the slide was visualized under a laser scanner.

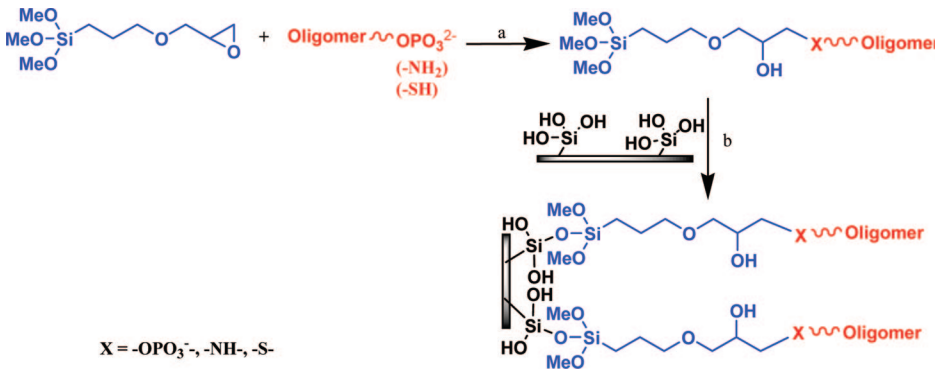
Signal Evaluation and Quantification. Microarrays were scanned on a Scan Array Lite Scanner (GSI Lumonics) fitted with a Cy3 optical filter with 30 μm resolution; laser power and PMT were set to 85% and 80%, respectively. Spot intensities were quantified using *Quant Array* software (Packard Bioscience) and graphs were plotted in Microsoft *Excel*.

For each experimental condition tested on the microarrays, the experiments were repeated 2−3 times. The immobilization and hybridization data presented are the average of these repetitions, and the error bars represent the percentage error (∼3−5%) observed on this average.

RESULTS AND DISCUSSION

Homo- and heterobifunctional cross-linkers have been finding extensive use for various applications, such as preparation of affinity matrices, modification and stabilization of diverse structures, identification of ligand and receptor binding sites,

Scheme 1. Immobilization of Oligonucleotides on Virgin Glass via Oligonucleotide−Trimethoxysilyl Conjugates[a]



[a] Conditions: (a) 45 °C, 4.5 h (phosphoryl-oligomer); 45 °C, 3 h (aminoalkyl-oligomer); 45 °C, 2 h (mercaptoalkyl-oligomer); and (b) 45 °C, 35 min.

structural studies, and so forth. Of these, heterobifunctional reagents, which contain two different functional groups, have proven to be more advantageous, as cross-linking can be controlled both selectively and sequentially. By taking advantage of the differential reactivity of the two reactive groups, these reagents are finding extensive use in DNA microarray technology for tethering oligonucleotides to solid surfaces (*20*−*24*). Recently, immobilization of biomolecules on glass/silicon wafers has gained importance, as these are being used in constructing microarrays of biomolecules (e.g., oligonucleotide, peptide, protein, polysaccharides, and so forth). The primary aim of the present investigation was to design a facile and economical protocol for immobilization of aminoalkyl, mercaptoalkyl, and phosphorylated oligonucleotides, as they can easily be synthesized using commercially available reagents and chemicals, onto the virgin glass surface. While designing such a protocol, the following points were kept in mind: (i) the methodology should not require modified surfaces, (ii) the reagents should be commercially available and inexpensive, and (iii) the protocol should not involve complex chemistry and stringent reaction conditions. Guided by these considerations, we could find out three reagents, viz., 3-glycidyloxypropyltrimethoxy silane (GOPTS), 3-isocyanatopropyltriethoxysilane (IPTS), and 3-mercaptopropyltriethoxysilane (MPTS), for this purpose. Among these reagents, IPTS can be used for immobilization of amino- and mercaptoalkylated oligonucleotide, however, due to highly moisture-sensitive nature, the results are not reproducible. MPTS results in immobilization of mercaptoalkylated oligomers via disulfide linkage, which is quite a labile linkage. Only GOPTS, which fulfills all the requirements, was considered to be the reagent of choice for immobilization of modified oligonucleotides (-NH_2, -SH, and -OPO_3^{2-}). The epoxy function present at one of the ends can react efficiently with a vast variety of nucleophiles as well as electrophiles under mild conditions, and the trimethoxysilyl functions at the other end of the reagent can specifically react with silanol functionalities on the virgin glass surface following silanization chemistry (Scheme 1). By taking advantage of these points, a rapid and clean protocol has been developed for immobilization of oligonucleotides bearing different functional groups.

Optimization of Reaction Conditions. In order to find an appropriate solvent system for the preparation of oligonucleotide−trimethoxysilyl conjugates, we surveyed the literature and observed that trialkoxysilane groups get hydrolyzed in aqueous solution in the presence of either strong ionizable acids or bases (*27*−*29*). However, the rate of hydrolysis is very dependent on the presence of an organic solvent in the reaction mixture (*30, 31*). Among the various solvent systems reported, viz., acetone−water, methanol−water, dioxane−water, ethanol−water, and DMF−water, the slowest rate of hydrolysis of

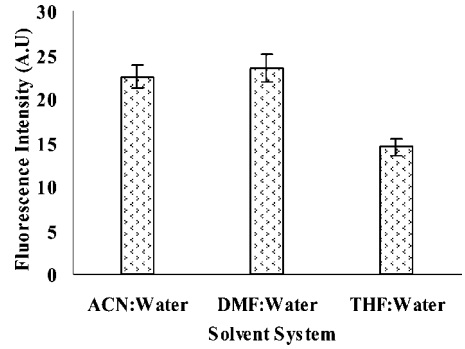


Figure 1. Effect of solvent systems on the reaction of end-modified oligonucleotides with GOPTS.

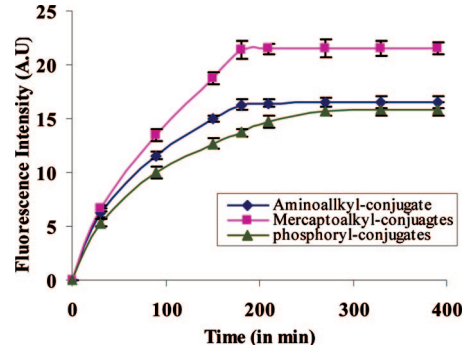


Figure 2. Kinetic study of the reaction between fluorescent-labeled end-modified oligonucleotides with GOPTS.

trialkoxysilanes was observed in DMF−water system, where DMF is believed to inhibit the hydrolysis of alkoxysilanes. Keeping these observations in mind, we carried out the conjugate formation (reaction between modified oligonucleotide and GOPTS) in DMF−water (1:1, v/v) using 0.1 M NMI, a nonionizable base, as reaction buffer. Even if, under such conditions, some of the trialkoxysilyl groups may get hydrolyzed, the use of a large excess of the conjugate for the immobilization reaction would hardly affect the efficacy of the system. Therefore, in order to evaluate the formation of oligonucleotide−trimethoxysilyl conjugates, three solvent systems, viz., ACN/water (1:1, v/v); DMF/water (1:1, v/v), and THF/water (1:1, v/v), were assessed. After carrying out the reaction for 8 h, the conjugate was spotted manually on a virgin glass microslide and incubated at 45 °C for 30 min. The results revealed that maximum fluorescence intensity (A.U.) of the spots was observed in case of DMF/water (1:1, v/v) containing 0.1 M NMI, indicating that the conjugation reaction proceeded efficiently in this solvent system (Figure 1). Therefore, in the

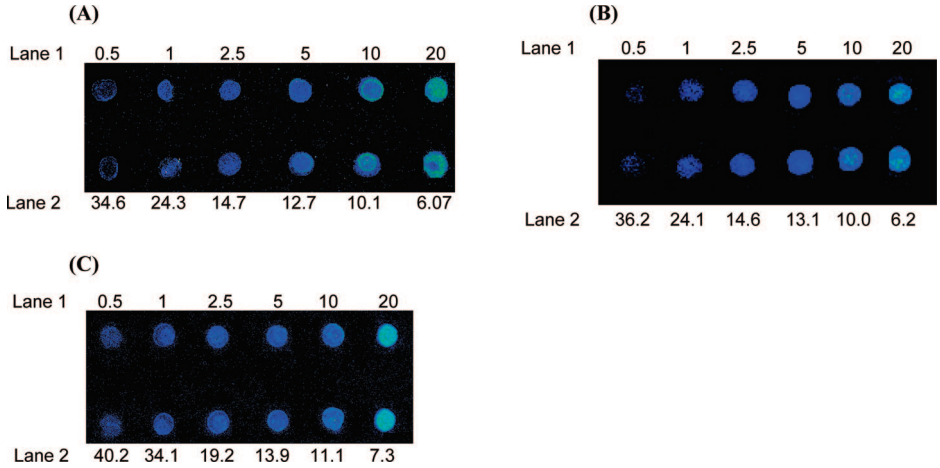


Figure 3. Pictorial representation of immobilization efficiency (%, lane 2) vs concentration (μM, lane 1) of (A) phosphoryl−trimethoxysilyl conjugate, (B) aminoalkyl−trimethoxysilyl conjugate, and (C) mercaptoalkyl−trimethoxysilyl conjugate.

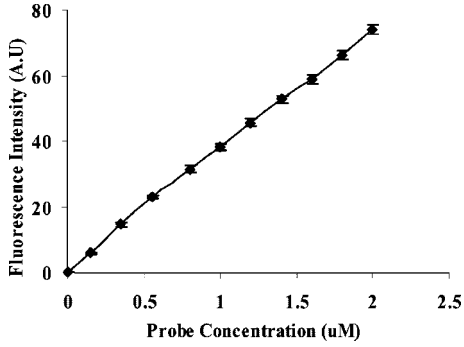


Figure 4. Correlation between concentrations of spotted labeled probe and fluorescence intensity. Fluorescent oligonucleotide was spotted in 0.05−2.0 μM concentrations.

rest of the experiments, oligonucleotide−trimethoxysilyl conjugates were prepared in DMF/water solvent system (1:1, v/v).

With the aim of determining the time required for the completion of reaction between aminoalkyl, mercaptoalkyl, and phosphorylated oligonucleotides and epoxy groups of GOPTS, kinetic studies were undertaken at 45 °C. Phosphorylated oligonucleotide, TET-d(CTT CTT TTT CCT GTT ACC GT)-OPO_3^{2-}, taken in twelve Eppendorf tubes, was allowed to react with GOPTS, and the tubes were withdrawn at different time intervals. After the usual workup, a microarray was constructed, as mentioned in the Experimental Section. The slide was visualized under a laser scanner, and fluorescence intensity (A.U.) of the spots was determined, which was subsequently plotted against time (min), as shown in Figure 2. The results indicate that the formation of the conjugate essentially completes in 180 min (3 h) in the case of aminoalkyl, 120 min (2 h) for mercaptoalkyl, and 270 min (4.5 h) for phosphorylated oligomers. In the subsequent experiments, a respective time period was employed for the preparation of conjugates between modified probes and GOPTS.

To determine the threshold concentration of the conjugate required for visualization under a laser scanner, an oligonucleotide−trimethoxysilyl conjugate, TET-d(CTT CTT TTT CCT GTT ACC GT)-OPO_3^--$CH_2CH(OH)CH_2O(CH_2)_3$-$Si(OMe)_3$, serially diluted from 20 μM to 0.5 μM, was spotted manually on a virgin glass slide and incubated at 45 °C. After the usual workup, the slide was scanned under a laser scanner and the spots were quantified. The pictorial view of the slide is depicted in Figure 3A. Similar experiments were carried out for aminoalkyl and mercaptoalkylated oligonucleotide conjugates (Figure 3B,C). The immobilization efficiency against each concentration was determined with the help of a standard curve (Figure 4). The results revealed that immobilization efficiency followed an inverse relation with concentration of spotted conjugates. The spots corresponding to 0.5−2.5 μM concentration were visible; however, to construct a good quality biochip, a concentration of 5 μM was selected and used for immobilization purposes.

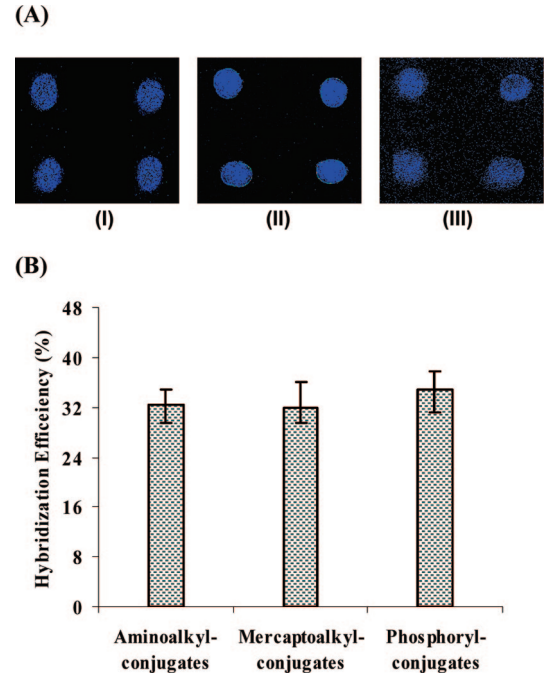


Figure 5. (A) Immobilization of (I) d(AGC GTA ACA GGA AGA AGC TG)OPO_3-$(CH_2)_6$NH- $CH_2CH(OH)CH_2O(CH_2)_3$-$Si(OMe)_3$, (II) d(AGC GTA ACA GGA AGA AGC TG)OPO_3-$(CH_2)_6$S-$CH_2CH(OH)CH_2O(CH_2)_3$-$Si(OMe)_3$, and (III) d(AGC GTA ACA GGA AGA AGC TG)OPO_3^--$CH_2CH(OH)CH_2O(CH_2)_3$-$Si(OMe)_3$ on a virgin glass surface followed by hybridization with labeled oligomer, TET-d(CAG CTT CTT CCT GTT ACG CT). (B) Quantitative hybridization efficiency.

Homogeneity of virgin glass microslides procured from Sigma Chemical Co., USA, was evaluated by manually spotting the TET-labeled conjugate at different positions on the glass microslides. The fluorescence intensity of spots was found to be in the range ~25.3−27.2 A.U., which showed that the density of silanol functionalities on the glass surface was present almost uniformly throughout the glass surface. The stability of the

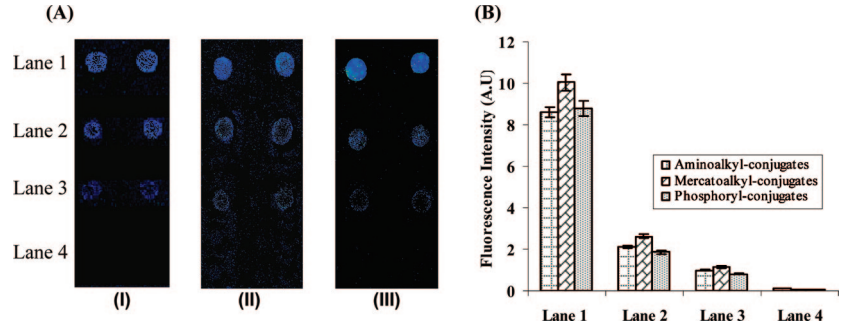


Figure 6. (A) Detection of base mismatches and specificity of immobilization after hybridization with TET-labeled complementary oligomer. Lane 1: zero mismatch. Lane 2: one mismatch. Lane 3: two mismatches. Lane 4: noncomplementary oligomer. Slide (I): Aminoalkyl−trimethoxysilyl conjugates. Slide (II): mercaptoalkyl−trimethoxysilyl conjugates. Slide (III): phosphoryl−trimethoxysilyl conjugates. (B) A histogram showing respective fluorescence intensity. Lane 1: Perfect match. Lane 2: One mismatch. Lane 3: Two mismatch. Lane 4: Non-complementary.

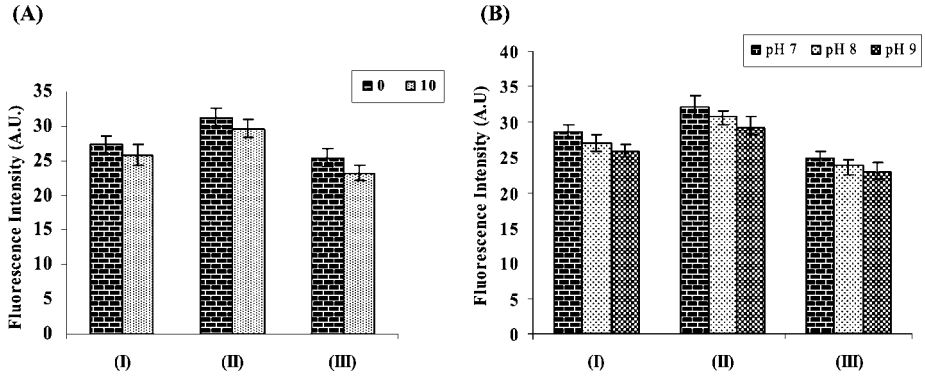


Figure 7. (A) Thermal stability of immobilized conjugates on glass slide and comparison of fluorescence signals obtained after 10 cycles of PCR-like conditions. (B) Stability of immobilized conjugates at pH 7−9.

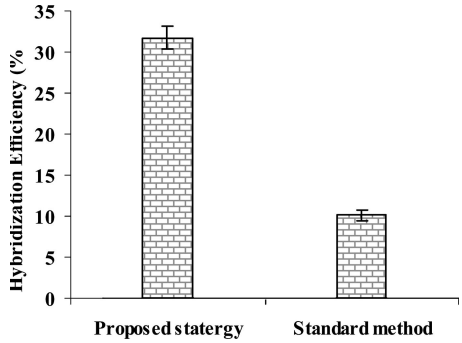


Figure 8. Comparison of hybridization efficiency of the proposed strategy with the standard one, viz., NTMTA.

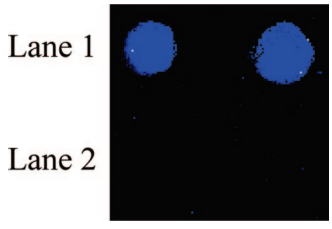


Figure 9. Detection of bacterial meningitis. Lane 1: Probe sequence. Lane 2: Control probe (non-complementary).

oligomer−trimethoxysilyl was also checked on storage at 4 °C for up to 30 days, and it was found to be stable.

Hybridization Assay. The applicability of the projected strategy was evaluated by performing hybridization assay of slides fabricated via immobilization of the trimethoxysilyl conjugates of aminoalkyl, mercaptoalkyl, and phosphorylated oligomers onto the glass slides at 5 μM concentration. The microarrays constructed were visualized by hybridizing them with the corresponding complementary TET-labeled oligonucleotides. After thorough washings and drying, the microslides were screened under a laser scanner and the fluorescent spots were visualized, as depicted in Figure 5A. The fluorescent spots on the microslides were quantified using *QuantArray* software. The quantification data is shown in Figure 5B. The proposed strategy resulted in high hybridization efficiency (∼32−35%). Furthermore, the spot morphology was also found to be almost identical.

Specificity of Chemistry and Base Mismatch Detection. To demonstrate the specificity of the proposed chemistry and the extent of involvement of internucleotidic phosphodiester groups in the formation of conjugates, a study was performed using TET-labeled phosphorylated oligomer and its nonphosphorylated labeled analogue, as described in the Materials and Methods section. Fluorescence intensity of spots of nonphosphorylated oligomer was only ∼10% as compared to that of phosphorylated oligonucleotides.

In order to demonstrate the specificity of the microarrays, four oligonucleotide−trimethoxysilyl conjugates having zero (d(AGC GTA ACA GGA AGA AGC TG)OPO_3^{2-}), one (d(AGC GTA ACA GGA AGA TGC TG)OPO_3^{2-}), and two (d(AGC GTA CCA GGA AGA TGC TG)OPO_3^{2-}) mismatches and a non-complementary sequence, d(TTT TTT TTT TTT TTT TT)OPO_3^{2-}, to the TET-d(CAG CTT CTT CCT GTT ACG CT) were immobilized onto a virgin glass slide. After the usual washings with SSC buffer and MilliQ water, the microarrays were subjected to a hybridization step with a labeled oligomer (complementary to zero mismatch sequence). Figure 6A(I) clearly shows the base mismatches. The results revealed that the fluorescence intensity of spots decreased for single-mismatch and was reduced to minimal for double-mismatch as compared to that of the perfect complementary oligomer.

Hybridization of the non-complementary oligonucleotide did not result in significant signal after hybridization, implying the specificity of the hybridization. Similarly, specificity of other microarrays constructed using amino- and mercaptoalkylated oligonucleotide−trimethoxysilyl conjugates was evaluated as depicted in Figure 6A(II and III). The quantification data are shown in Figure 6B.

Thermal and pH Stability. The thermal stability of constructed microarray was evaluated by subjecting it to 0, 5, and 10 cycles of PCR-like conditions. The percentage reduction in fluorescence intensity was observed to be ∼8.2% for phosphorylated oligomer conjugates, ∼5.8% for aminoalkylated oligomer, and ∼4.9% for mercaptoalkylated oligomer conjugates (Figure 7A), indicating that constructed microarrays are sufficiently stable to be used in biological research. The stability of constructed microarrays was also analyzed at different pHs (7, 8, and 9). The results indicated that the constructed microarrays are sufficiently stable in SSC buffers of different pH. The decrease in fluorescence intensity was ∼5% at pH 8 and ∼10% at pH 9 as compared to fluorescence intensity at pH 7 for the oligomer conjugates (Figure 7B).

Comparison of the Projected Strategy with Standard NTMTA Method. The efficacy of the proposed method was demonstrated by comparing with the NTMTA method (*20*). For comparison purposes, an equal concentration of an oligonucleotide (each at 5 μM) was used in all three methods. However, different reaction conditions as well as time were employed, as reported. After immobilization and hybridization with labeled complementary target, the slides were subjected to usual washings and drying. The slides were then subjected to scanning under a laser scanner for quantification, and the hybridization efficiency for each method was calculated. The hybridization efficiency of the proposed method (∼32−35%) was found to be significantly higher than the standard method, NTMTA (∼10.2%), as shown in Figure 8.

Detection of Bacterial Meningitis. In order to devise a biochip for the diagnosis of the bacterial infection, a microarray was constructed on a glass microslide using a unique probe sequence of the *CtrA* gene and a non-complementary probe sequence as control, as described in the Materials and Methods section. Subsequently, the spots on the glass surface were subjected to hybridization with the labeled PCR amplicons. After washings and drying, the slide was visualized under a laser scanner. Figure 9 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 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

An efficient and facile method for fabricating oligonucleotide microarrays has been developed employing conjugates of oligonucleotides. The method permits attachment of modified probes, which can be synthesized easily on the machine itself using commercially available reagents, onto virgin glass slides with high immobilization and hybridization efficiency and signal-to-noise ratio (∼98). The protocol offers an added advantage that it bypasses the capping step. The method involves commonly used reagents, chemicals, and simple chemistry; it is hoped that this will provide individuals, particularly the end users or nonchemists, an easy access to an oligonucleotide microarray for various applications including diagnosis of genetic as well as infectious diseases.

ACKNOWLEDGMENT

Financial support from the Department of Biotechnology, New Delhi, India, is gratefully acknowledged. One of the authors, D.S., acknowledges the University Grants Commission (UGC), New Delhi, India, for the award of Research Fellowship to carry out his work. Authors are thankful to Dr. Sunil Gupta and Dr. Shashi Khare, National Institute of Communicable Diseases (NICD), Delhi, India, for providing meningitis sample and facilities.

Supporting Information Available: Morphology, homogeneity and the reproducibility data in the projected method and comparison with the standard method, NTMTA. This material is available free of charge via the internet at http://pubs.acs.org.

LITERATURE CITED

(1) Mao, H., Lu, Z., Zhang, H., Liu, K., Zhao, J., Jin, G., Gu, S., and Yang, M. (2008) Colorimetric oligonucleotide array for genotyping of hepatitis C virus based on the 5′ non-coding region. *Clin. Chim. Acta 388*, 22–27.

(2) Sosnowski, R. G., Tu, E., Butler, W. F., O'Connell, J. P., and Heller, M. J. (1997) Rapid determination of single base mismatch mutations in DNA hybrids by direct electric field control. *Proc. Natl. Acad. Sci. U.S.A. 94*, 1119–1123.

(3) Zhu, T., Budworth, P., Han, B., Brown, D., Chang, H. S., Zou, G., and Wang, X. (2001) Toward elucidating the global gene expression patterns of developing Arabidopsis: Parallel analysis of 8300 genes by a high-density oligonucleotide probe array. *Plant Physiol. Biochem. 39*, 221–242.

(4) Drobyshev, A., Mologina, N., Shik, V., Pobedimskaya, D., Yershov, G., and Mirzabekov, A. (1997) Sequence analysis by hybridization with oligonucleotide microchip: identification of β-thalassemia mutations. *Gene 188*, 45–52.

(5) Boudreau, C. R., Yang, I., and Liau, L. M. (2005) Gliomas: advances in molecular analysis and characterization. *Surgical Neurology 64*, 286–294.

(6) Gallagher, J., Howlin, J., McCarthy, C., Murphy, E. P., Bresnihan, B., FitzGerald, O., Godson, C., Brady, H. R., and Martin, F. (2003) Identification of Naf1/ABIN-1 among TNF-α-induced expressed genes in human synoviocytes using oligonucleotide microarrays. *FEBS Lett. 551*, 8–12.

(7) Loy, A., and Bodrossy, L. (2006) Highly parallel microbial diagnostics using oligonucleotide microarrays. *Clin. Chim. Acta 363*, 106–119.

(8) Southern, E. M., Case-green, S. C., Elder, J. K., Johnson, M., Mu, K. U., Wang, J., and Williams, J. C. (1994) Arrays of complementary oligonucleotides for analysing the hybridization behaviour of nucleic acids. *Nucleic Acids Res. 22*, 1368–1373.

(9) Moorcroft, M. J., Meuleman, W. R. A., Latham, S. G., Nicholls, T. J., Egeland, R. D., and Southern, E. M. (2005) In situ oligonucleotide synthesis on poly(dimethylsiloxane): a flexible substrate for microarray fabrication. *Nucleic Acids Res. 33*, e75.

(10) Fodor, S. P., Read, J. L., Pirrung, M. C., Stryer, L., Lu, A. T., and Solas, D. (1991) Light directed spatially addressable parallel chemical synthesis. *Science 251*, 767–773.

(11) Singh-Gasson, S., Green, R. D., Yue, Y., Nelson, C., Blattner, F., Sussman, M. R., and Cerrina, F. (1999) Mask-less fabrication of light director oligonucleotide arrays using a digital micromirror array. *Nat. Biotechnol. 17*, 974–978.

(12) McGall, G. H., and Fidanza, J. A. (2001) Photolithographic synthesis of high density oligonucleotide arrays. *Methods Mol. Biol. 170*, 71–101.

(13) Burns, N. L., Van Alstine, J. M., and Harris, J. M. (1995) Poly(ethylene glycol) grafted to quartz: analysis in terms of a site-dissociation model of electro-osmotic fluid flow. *Langmuir 11*, 2768–2776.

(14) Podyminogin, M. A., Lukhtonaov, E. A., and Reed, M. W. (2001) Attachment of benzaldehyde-modified oligodeoxynucleotide probes to semicarbazide-coated glass. *Nucleic Acids Res. 29*, 5090–5098.

(15) Lamature, J. B., Beattie, K. L., Burke, B. E., Eggers, M. D., Ehrlich, D. J., Fowler, R., Hollis, M. A., Hollis, B. B., Kosicki,

B. B., Reich, R. K., and Smith, S. R. (1994) Direct detection of nucleic acid hybridization on the surface of a charge coupled device. *Nucleic Acids Res. 22*, 2121–2125.

(16) Defrancq, E., Hoang, A., Vinet, F., and Dumy, P. (2003) Oxime bond formation for the covalent attachment of oligonucleotides on glass support. *BioMed. Chem. Lett. 13*, 2683–2686.

(17) Brockman, J. M., Frutos, A. G., and Corn, R. M. (1999) A multistep chemical modification procedure to create DNA arrays on gold surfaces for the study of protein-DNA interactions with surface plasmon resonance imaging. *J. Am. Chem. Soc. 121*, 8044–8051.

(18) Herne, T. M., and Tarlov, M. J. (1997) Characterization of DNA probes immobilized on gold surfaces. *J. Am. Chem. Soc. 119*, 8916–8920.

(19) Rogers, Yu-H., Baucom, P. J., Huang, Z. J., Bogdanov, V., Anderson, S., and Boyce-Jacino, M. T. (1999) Immobilization of oligonucleotides onto a glass support via disulfide bonds: a method for preparation of DNA microarrays. *Anal. Biochem. 266*, 23–30.

(20) Kumar, P., Choithani, J., and Gupta, K. C. (2004) Construction of oligonucleotide arrays on a glass surface using a heterobifunctional reagent, N-(2-trifluoroethanesulfonatoethyl)-*N*-(methyl)-triethoxysilylpropyl-3-amine (NTMTA). *Nucleic Acids Res. 32*, e80.

(21) Kumar, P., Agarwal, S. K., and Gupta, K. C. (2004) N-(3-Tri-fluoroethanesulfonyloxypropyl)-anthraquinone-2-carboxamide: A new heterobifunctional reagent for immobilization of biomolecules on a variety of polymer surfaces. *Bioconjugate Chem. 15*, 7–11.

(22) Patnaik, S., Swami, A., Sethi, D., Pathak, A., Garg, B. S., Gupta, K. C., and Kumar, P. (2007) N-(Iodoacetyl)-N′-(anthraquinon-2-oyl)-ethylenediamine (IAED): A new heterobifunctional reagent for the preparation of biochips. *Bioconjugate Chem. 11*, 474–483.

(23) Hermanson, G. T. (1996) *Bioconjugate Techniques*, pp 239−242, Academic Press, San Diego.

(24) Chehade, K. A., and Spielmann, H. P. (2000) Facile and efficient synthesis of 4-azidotetrafluoroaniline: a new photoaffinity reagent. *J. Org. Chem. 65*, 4949–4953.

(25) Nonglaton, G., Benitz, I. O., Guisle, I., Piplier, M., Leger, J., Dubreuil, D., Tellier, C., Talham, D. R., and Bujoli, B. (2004) New approach to oligonucleotide microarrays using zirconium-phosphonate modified surfaces. *J. Am. Chem. Soc. 126*, 1497–1502.

(26) Pharmacia LKB. Gene assembler plus manual, Uppsala, Sweden, 1988.

(27) Torry, S. A., Campbell, A., Cunliffe, A. V., and Tod, D. A. (2006) Kinetic analysis of organosilane hydrolysis and condensation. *Int. J. Adhes. Adhes. 26*, 40–49.

(28) Shen, Y., Wu, T., Zhang, Y., and Li, J. (2005) Comparison of two typed (3-mercaptopropyl)trimethoxysilane-based networks on Au substrates. *Talanta 65*, 481–488.

(29) Li, H., Shih, W. Y., and Shih, W.-H. (2007) Stable aqueous ZnS quantum dots obtained using (3-mercaptopropyl)trimethoxysilane as a capping molecule. *IOP Nanotechnol. 18*, 495605.

(30) Jiang, H., Zheng, Z., and Wang, X. (2008) Kinetic study of methyltriethoxysilane (MTES) hydrolysis by FTIR spectroscopy under different temperatures and solvents. *Vib. Spectrosc. 46*, 1–7.

(31) Jiang, H., Zheng, Z., Li, Z., and Wang, X. (2006) Effects of temperature and solvents on the hydrolysis of alkoxysilane under alkaline conditions. *Ind. Eng. Chem. Res. 45*, 8617–8622.

BC800241K