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An efficient and versatile approach for the construction of oligonucleotide microarrays

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Abstract—A new immobilization chemistry for covalent attachment of phosphorylated oligonucleotides on epoxy-activated glass surface via opening of oxirane ring is described. The proposed strategy results in excellent immobilization efficiency, spot homogeneity, and morphology. The constructed microarray was successfully demonstrated for discrimination of nucleotide mismatches.

During the last decade, the DNA-microarray technology has emerged as a powerful tool for gene discovery, detection of mutations, and mapping.[1,2] Two major strategies for the preparation of oligonucleotide microarrays have been developed. The first one is based on in situ synthesis (on-chip synthesis)[3] and the second one involves the immobilization of pre-synthesized DNA on the selected substrate.[4,5] The on-chip synthesis method represents the most efficient way of preparing high-density arrays, but it lacks in flexibility and requires expensive robotic instruments. The second method, that is, immobilization procedure, allows the immobilization of base modified oligonucleotides as well as other biomolecules after purification, on a surface of choice, thus offering flexibility in terms of ligands and surfaces used. Altogether these features make the deposition or immobilization method attractive to research laboratories dealing with low- to medium-density microarrays. Besides the chemistry involved in fixing biomolecules on polymer surface, the polymer matrix plays an important role in influencing the quality of constructed microarray. Glass has been the most preferred surface owing to its inherent advantages like low intrinsic fluorescence and superior optical characteristics required for fluorescent scanning. Since oligonucleotides bind poorly to glass, surface derivatization is often required.

Nowadays, a myriad of methods for covalent as well as non-covalent attachment of oligonucleotides on glass surface has been reported.[6–16] The simplest of them being the immobilization of probes via electrostatic adsorption on charged priming layer like aminopropyl or polylysine coated glass slides,[6] but unfortunately, it results in poor hybridization efficiency. Other attachment methods involve the generation of active functional groups on the surface, which, in turn, react covalently with the specific groups available on probe molecules. For example, 5′-amino modified oligonucleotides have been anchored on aldehyde-functionalized or epoxide-activated glass surface[7–9] and 5′-thiol modified probes are immobilized on glass modified with thiol groups via disulfide bond formation.[10] Heterobifunctional reagents have also been employed to realize immobilization of thiol or amino modified oligonucleotides on virgin and modified glass slides using NTMTA and NTPAC reagents,[11–13] already reported from authors' laboratory. In line with the use of covalent linkage formed between organic moieties, 'organic–inorganic' interactions have also been utilized to realize immobilization of oligonucleotides, attachment of thiol modified probes to metallic gold via sulfur–gold linkage[14,15] and phosphorylated oligonucleotides on zirconylated-surface.[16]

Looking to the limitations of the existing methods with respect to stability of immobilized probes, time-consuming reaction for effective immobilization, low

Abbreviations: NTMTA, *N*-(2-trifluoroethanesulfonatoethyl)-*N*-(methyl)-triethoxysilylpropyl-3-amine; NTPAC, *N*-(3-trifluoroethanesulfonyloxypropyl)anthraquinone-2-carboxamide; SSC, saline sodium citrate.

Keywords: Phosphorylated oligonucleotides; Microarray; Immobilization; Epoxylated glass microslide; Fluorescence; Hybridization.

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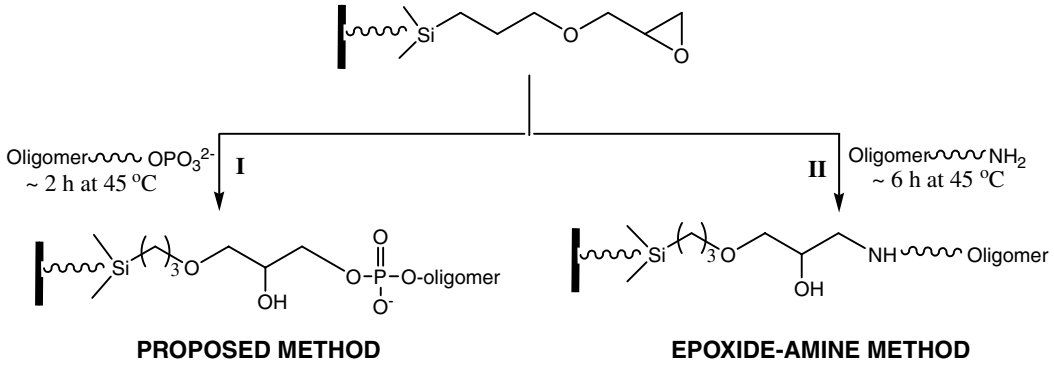
to moderate hybridization efficiency necessitates the need to develop alternative approaches for construction of oligonucleotide arrays that fulfill the changing requirements. For a new system to replace the existing methods to fulfill the changing requirements, methods have to be well characterized in relation to chemical and thermal stability, functional availability of probes for hybridization, and reproducibility of attachment chemistry. Keeping these points in mind, we report a simple, facile, and efficient method to synthesize oligonucleotide microarrays employing immobilization of 3′- or 5′-phosphorylated oligonucleotides on to epoxy-functionalized glass slides (Scheme 1). The highlighting feature of our strategy is that it does not involve the use of coupling reagents and other chemical manipulations to realize immobilization of phosphorylated oligomer on epoxylated-surface. The proposed strategy results in uniform spot morphology and excellent hybridization efficiency (defined as percent of covalently attached oligonucleotides that participated in duplex formation). The applicability of the new immobilization chemistry has further been demonstrated by utilizing it for discrimination of nucleotide mismatches.

To analyze the performance of the new approach for constructing arrays, epoxide function was generated on glass surface by silanization with commercially available 3-glycidyloxypropyltrimethoxy silane following published protocol.[17] Oligonucleotides and their modified analogs, viz., (**I**) = TET-d (TTT TTT TTT TTT TTT TT)-OPO_3^{2-}, (**II**) = TET-d (TTT TTT TTT TTT TTT TTT TT), (**III**) = d (ACC GTA ACA GAA TTT AGA AC)-OPO_3^{2-}, (**IV**) = d (ACC GTA ACA GAA TTT AGA AG)-OPO_3^{2-}, (**V**) = d (ACC GTA ACA GAA TTT AGC AG)-OPO_3^{2-}, (**VI**) = d (TTT TTT TTT TTT TTT TTT TT)-OPO_3^{2-}, (**VII**) = O_3^{2-} PO-$(CH_2)_6$-O-d (ACC GTA ACA GAA TTT AGA AC), (**VIII**) = NH_2-$(CH_2)_6$-O-d (ACC GTA ACA GAA TTT AGA AC), and (**IX**) = TET-d (GTT CTA AAT TCT GTT ACG GT), 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).

To evaluate the quality of epoxy-functionalized glass surface, 5′-labeled-3′-phosphorylated probe (**I**) was dissolved in a reaction buffer [*N*-methylimidazole (0.1 M) containing 10% dimethylsulfoxide (DMSO) (v/v), pH 10.0] and spotted manually at 20 μM concentration in 10 replica. The spotted slide was kept in a humid chamber at 45 °C for overnight, subjected to washings with 1× SSC buffer, pH 7.0 (4 × 50 ml), and Milli Q water (2 × 50 ml) each for 20 min followed by drying under vacuum. Subsequently, the microslide was visualized under a laser scanner (Scan Array Lite Scanner, GSI Lumonics, USA) fitted with a Cy3 optical filter at 30 μm resolution. The fluorescence of the spots on the slide was quantified using Quant Array software (Packard Bioscience, USA). The net intensity (after subtraction of the background noise) of the spots was found to be in the range of ~54.5–58.2 AU, implying a uniform concentration of epoxy groups present throughout the glass slide. In order to determine the immobilization and hybridization efficiencies, a calibration curve was constructed using a labeled oligomer as reported in the literature.[18] Briefly, an oligomer sequence, (**I**), was serially diluted in the reaction buffer to various concentrations viz., 1.0, 0.8, 0.4, 0.2, 0.1, 0.05, and 0.025 μM, and spotted on a virgin glass microslide. After drying, the slide was scanned under a laser scanner and the spots were quantified, a standard calibration curve was drawn by plotting fluorescence intensity (AU) versus concentration (μM), which was then used to quantify all the microarrays prepared following the present method (See Fig. 1, supporting information).

In order to determine the optimal time and temperature required for immobilization of phosphorylated oligonucleotide, briefly, oligomer (**I**) was diluted in reaction buffer to 20 μM concentration and spotted onto epoxy-functionalized glass microslides in quadruplicate and subjected to thermal condition at 35 °C. Slides were withdrawn at regular time intervals (120, 240, 360, 480, and 1200 min) and subjected to washings as described above. After drying under vacuum, the spots were visualized under a laser scanner and quantified. Similarly, immobilization reaction was also allowed to proceed at different temperatures viz., 45, 55, and 65 °C. The results (Fig. 1) indicate that the reaction between phos-



Scheme 1. Covalent attachment of modified oligonucleotides on epoxylated glass surface using (**I**) = phosphorylated oligonucleotides and (**II**) = aminoalkylated oligonucleotides.

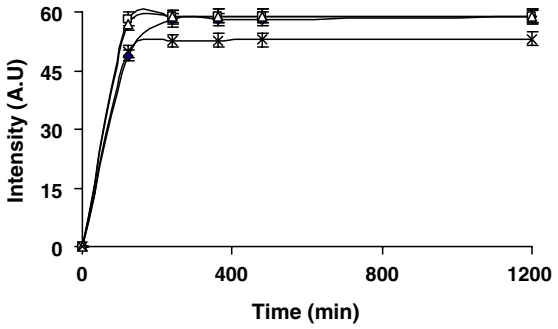


Figure 1. Graph representing time kinetics to determine optimal time required to immobilize phosphate ligands onto epoxy-functionalized glass microslides at different temperatures: (◊) 35 °C; (□) 45 °C; (△) 55 °C; (×) 65 °C.

phate and epoxy groups completes in ~120 min at 45 and 55 °C, whereas it takes ~240 min at 35 °C. The immobilization reaction completes in ~120 min at 65 °C but the value of net fluorescence intensity slightly decreases, this may be due to the partial deactivation of epoxy functionalities of the surface at higher temperature under basic condition. Therefore, the immobilization reaction was performed at 45 °C for ~120 min in rest of the experiments.

To optimize the probe concentration required for visualization of fluorescent oligonucleotide under a laser scanner, 5′-labeled-3′-phosphorylated probe (**I**) was dissolved in a reaction buffer and spotted manually in different concentrations on an epoxylated glass slide. The spotted slide was kept in a humid chamber at 45 °C for ~120 min, followed by capping of residual epoxy functionalities with a capping buffer (0.1 M Tris containing 50 mM ethanolamine, pH 9.0) for 15 min at 50 °C. The slide was then subjected to washings with 1× SSC buffer (4 × 50 ml), pH 7.0, and Milli Q water (2 × 50 ml) for 20 min each, dried under vacuum, and scanned under a laser scanner. Figure 2 shows a representative scanned image which clearly indicates that the detection limit for probe concentration before spotting can be reduced from 20 to 0.20 μM with 20mer oligonucleotides. Comparison of the fluorescence intensity of the spots confirms that the attachment density increases with increasing the concentration of the probe, reaching a plateau with probe concentration of 20 μM.

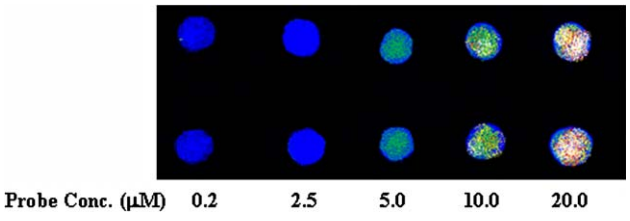


Figure 2. Fluorescence map of epoxylated glass surface spotted with TET-d (TTT TTT TTT TTT TTT TTT TT)-OPO_3^{2-} (**I**) in a concentration-dependent manner in duplicate. Slide was scanned at 75% of laser power and 70% of photomultiplier gain. The fluorescence intensities in color image of the slide are color coded, varying from blue to green, yellow, and then white (saturation).

The extent of binding of oligomer through terminal phosphate with the epoxylated-surface was evaluated by realizing the immobilization of a labeled probe containing 3′-phosphate and the same sequence without phosphate function on functionalized glass surface. Figure 3 clearly depicts the greater reactivity of terminal phosphate over the phosphodiester groups, as the net fluorescence intensity measured in case of phosphate-terminated-labeled probe was ~8.0-fold higher than non-phosphorylated one.

The accessibility and specificity of the surface-bound probe for hybridization have been the major concern while preparing the oligonucleotide microarrays. To ascertain the effect of placement of phosphate function at either of the two termini (3′ or 5′) in the probe on hybridization efficiency, an experiment was conducted by immobilization of 3′- and 5′-phosphorylated oligonucleotides (5 μM), followed by performing the hybridization assay with target (complementary oligonucleotide, 40 μM). The results revealed (see Fig. 2, supporting information) that there was not a significant change in the hybridization efficiency, therefore, in rest of the experiments, 3′-phosphorylated oligomers were employed as these can be synthesized economically using a universal support (aminoalkylated-CPG).

The specificity of the system is demonstrated by immobilizing four oligonucleotides, viz., (**III**), (**IV**), (**V**), and (**VI**) having zero, single, and double mismatches, and non-complementary on an epoxy-functionalized glass microslide each at 5 μM concentration. After treatment with capping buffer, the microslide was subjected to hybridization assay with a labeled oligomer (40 μM), (**IX**) (complementary to zero mismatch sequence). The microslide, after usual washings and drying, was subjected to laser scanning. Figure 4a shows the results of the hybridization experiment, the perfectly matched duplex gave the maximum intensity (lane 1), while spots having one (lane 2) and two (lane 3) base mismatches showed fluorescence intensity in decreasing order. No measurable non-specific hybridization signal from the non-complementary control (lane 4) was detected. The quantitative result of the scanned image is depicted in Figure 4b.

The microarray prepared following the present immobilization chemistry was compared with the standard method, viz., epoxide-amine.[8] For comparison purposes, an equal concentration of the appropriate oligonucleotide (each at 10 μM), (**VII**) and (**VIII**), dissolved in the corresponding reaction buffer, *N*-methylimidazole (0.1 M) containing 10% dimethylsulfoxide (DMSO) (v/v), pH 10.0, and sodium phosphate (150 mM), pH 8.5, was used for immobilization. However, different reaction time as obtained by immobilization kinetics viz., ~120 min for phosphorylated oligomer and ~360 min for amine modified probe (see Fig. 3, supporting information) was employed. After performing hybridization assay with target molecule (**IX**, 40 μM), the microslides were visualized followed by quantification of spots. Figure 5a and b show scanned images of both the methods, while Figure 5c depicts the quantitative data in the form

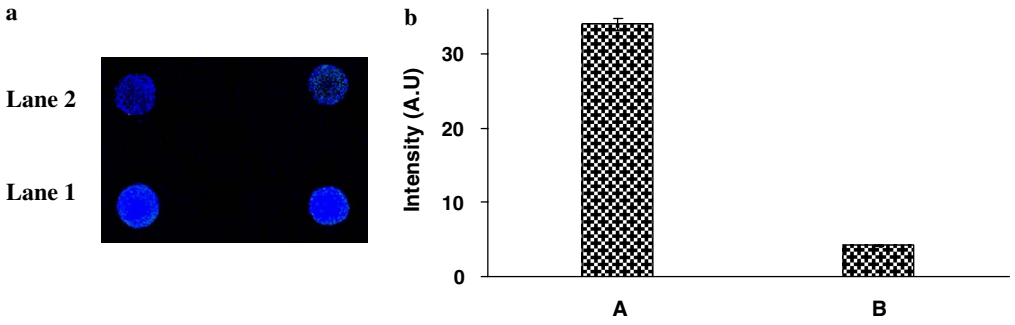


Figure 3. (a) Fluorescence map of epoxy-glass slide spotted with modified (**I**) (lane 1) and unmodified oligomer (**II**) (lane 2), each one spotted at 2.5 μM, slide was scanned at 75% of laser power and 70% of photomultiplier gain, (b) comparison of fluorescence intensity obtained by spotting, modified (**I**), (**A**), and unmodified oligomer (**II**), (**B**) on epoxylated glass slide each at 2.5 μM probe concentration.

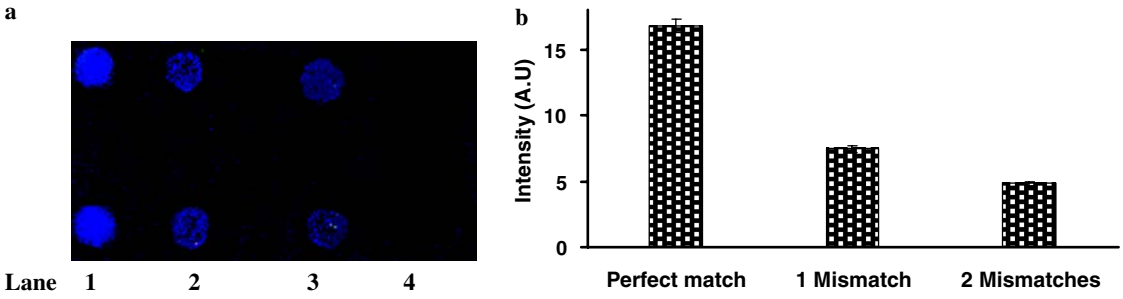


Figure 4. (a) Fluorescence map showing detection of mismatches and specificity of immobilization on hybridization with complementary labeled target, viz., TET-d (GTT CTA AAT TCT GTT ACG GT) (complementary to zero mismatch). Lane 1, d (ACC GTA ACA GAA TTT AGA AC)-OPO_3^{2-}; lane 2, d (ACC GTA ACA GAA TTT AGA AG)-OPO_3^{2-}; lane 3, d (ACC GTA ACA GAA TTT AGC AG)-OPO_3^{2-}; lane 4, d (TTT TTT TTT TTT TTT TTT TT)-OPO_3^{2-}, slide was scanned at 70% of laser power and 65% of photomultiplier gain, (b) quantitative data of hybridization experiment observed with perfect, single, and double mismatch in probe after hybridization with target (complementary to zero mismatch).

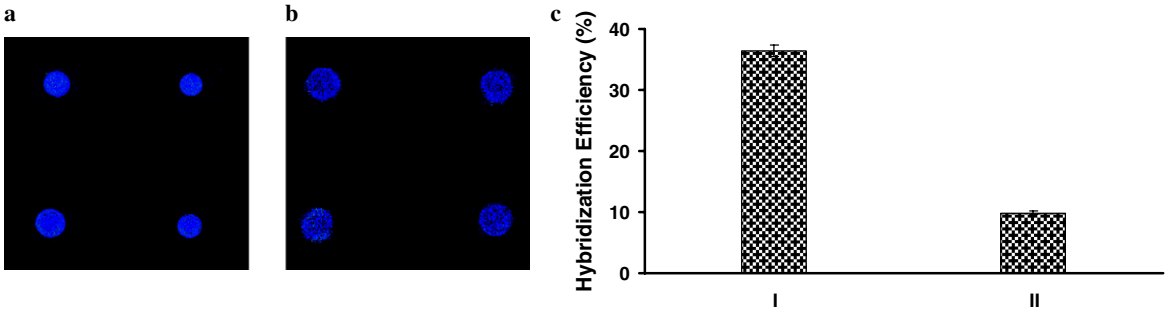


Figure 5. Fluorescence map after performing hybridization assay with labeled oligomer (**IX**), of epoxylated glass slide spotted with, (a) $^{2-}O_3PO$-$(CH_2)_6$-O-d (ACC GTA ACA GAA TTT AGA AC); (b)NH_2-$(CH_2)_6$-O-d (ACC GTA ACA GAA TTT AGA AC), slides were scanned at 75% of laser power and 70% of photomultiplier gain; (c) a histogram showing comparison of the present strategy (**I**) with epoxide-amine (**II**) immobilization chemistry.

of a histogram. It is clearly evident from these results that the present strategy is superior in terms of hybridization efficiency (~36.5%) as well as immobilization time (~120 min) relative to epoxide-amine approach (~9.86%, ~360 min).

In conclusion, we have demonstrated a new, efficient, and facile method for fabrication of oligonucleotide arrays. The technique relies on modification of oligonucleotide at the terminus with a free phosphate group, followed by reaction with epoxy-activated surface. The utility of the method is demonstrated by probing the performance and reproducibility of the constructed microarrays. The proposed strategy results in excellent immobilization as well as hybridization efficiencies.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.08.014.

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