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UV light-aided immobilization of oligonucleotides on glass surface using *N*-(3-trifluoroethanesulfonyloxypropyl)anthraquinone-2-carboxamide (NTPAC) and detection of single nucleotide mismatches†

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A novel heterobifunctional reagent, *N*-(3-trifluoroethanesulfonyloxypropyl)anthraquinone-2-carboxamide (NTPAC) has been developed for construction of oligonucleotide microarrays. Immobilization of oligonucleotides on a glass surface has been realized via two routes. In the first one, (A), mercapto- or aminoalkyl-oligonucleotides react with NTPAC to form oligonucleotide-anthraquinone conjugate which, in a subsequent reaction with modified glass surface under UV light (365 nm), results in surface bound oligonucleotides. In the second route, (B), the reagent, NTPAC, is allowed to react first with modified glass surface under UV light whereby it generates trifluoroethanesulfonate ester functions on it, which in a subsequent step react with mercapto- or aminoalkyl oligonucleotides to generate polymer bound oligonucleotides. The constructed microarrays are successfully used in single nucleotide mismatch detection by hybridizing these with fluorescein labeled complementary oligonucleotides. The difference in fluorescence signal is used as an indicator of mismatch.

Heterobifunctional and homobifunctional reagents[1-7] have been, of late, of considerable interest in the area of genomics and proteomics. Generally, these reagents are organic molecules which consist of two reactive functional groups, either identical (homobifunctional reagents) or different (heterobifunctional reagents). The functional groups in the reagents react with nucleophilic groups (aminoalkyl-, mercaptoalkyl-, hydroxyl) of ligands (biological or abiological), either intramolecularly or intermolecularly. These reagents serve as molecular tools for attachment of biomolecules such as proteins, peptides, nucleic acids and enzymes on various surfaces. The immobilized biomolecules are finding increasing use in studies relating to antigen-antibody interactions, structure of multi-enzyme complexes, arrangement of proteins in membranes and other biological systems[8]. In particular, the heterobifunctional reagents offer a unique and distinct advantage in having a differential reactivity profile for the two functional groups and permit cross-linking to be controlled both selectively and sequentially.

Again, microarrays of biomolecules such as oligonucleotides constitute one of the most recent tools, which is finding wide applications in molecular biology and genomics[9-12]. The methodology for construction of microarrays requires covalent fixing of oligonucleotides (small fragments of DNA), cDNA, PCR fragments, etc. on polymer surfaces (organic or inorganic). The DNA chips can then be used in studies like mutation detection, SNP analysis, disease diagnosis, differential gene expression analysis, etc. Also, arrays of short synthetic oligonucleotides, peptides, proteins etc. have been used in drug development, DNA sequencing, medical diagnostics, nucleic acid-ligand binding studies, DNA computing etc.[10-12]. A number of methods have been reported for the preparation of oligonucleotide arrays[13-21].

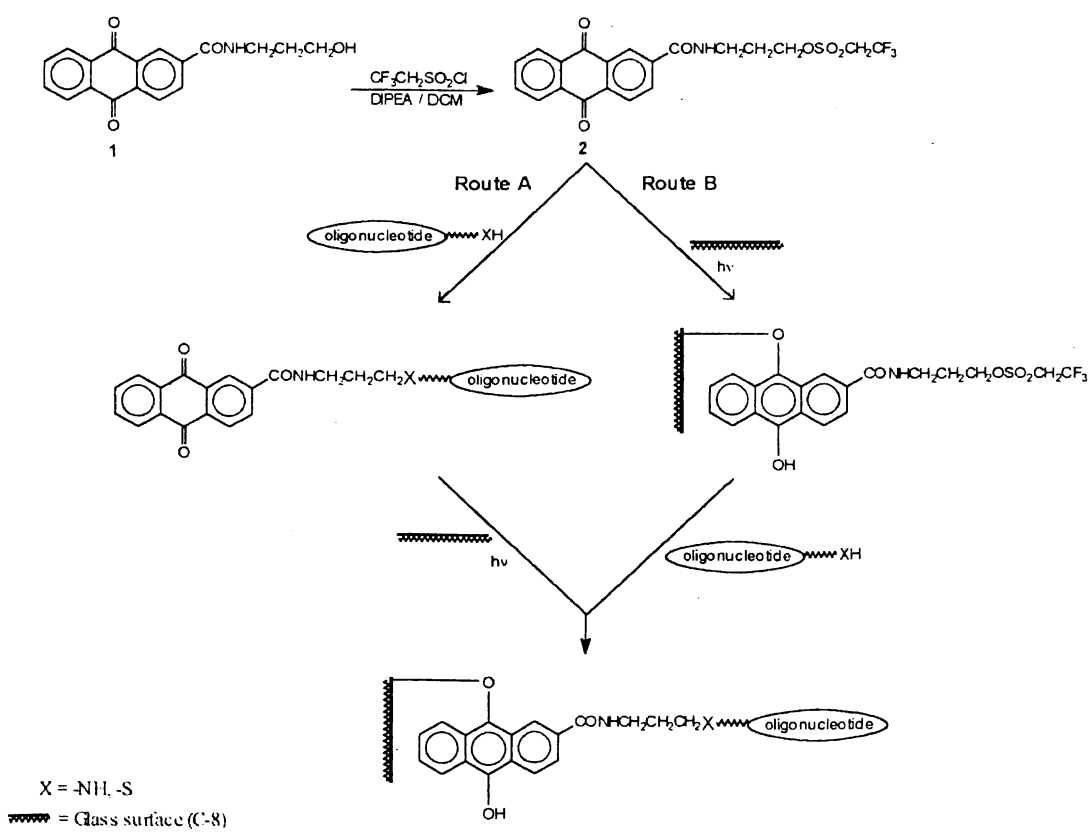
Besides the chemistry involved in fixing biomolecules on polymer surface, the polymer matrix plays an important role in influencing the quality of constructed microarrays. A number of polymer matrices such as nylon, nitrocellulose, polypropylene, polystyrene, silicon, glass, teflon, etc. have been examined for this purpose. Of these, glass and polypropylene appear attractive because these materials can easily

†Dedicated to Professor S. M. Mukherji, a scholar, an outstanding teacher and researcher, and a fine human being.

be derivatized generating functional groups such as aminoalkyl, carboxyl, aldehyde, mercaptoalkyl, etc. on the surface. Glass, in particular, offers an additional advantage in so far as this can be subjected to laser scanners in order to visualize the fluorescent spots on its surface.

In general, two approaches are commonly used for construction of oligonucleotide arrays. In one protocol, oligonucleotides are directly synthesized on the polymer surface at pre-selected positions following conventional photolithography[13,14]. The alternate method is based on fixation of pre-fabricated oligonucleotides, peptides/proteins on the solid surface[15–21]. This method offers flexibility in so far as a variety of ligands can be fixed after purification. In the latter approach, oligonucleotides are first modified to have reactive functionalities, viz., mercaptoalkyl- or aminoalkyl- moieties on its 5′- or 3′-termini, and are subsequently immobilized on modified polymer surfaces in the presence of suitable coupling reagents[15–19]. This approach, however, involves a multi-step support modification procedure and the use of expensive coupling reagents. In a recent study, oligonucleotides-anthraquinone conjugates have been immobilized on a few polymer surfaces under UV light (365 nm). However, the oligonucleotide-anthraquinone conjugates involve the preparation of much time-consuming phosphoramidite derivatives[22] and some of these derivatives are not very stable.

We describe here a novel general method involving UV light for immobilization of pre-synthesized modified oligonucleotides on glass surfaces through intervention of a new heterobifunctional reagent, *N*-(3-trifluoroethanesulfonyloxypropyl)anthraquinone-2-carboxamide (NTPAC). In designing the new heterobifunctional reagent, NTPAC (Scheme 1), we were guided by the following considerations: (a) one end of the reagent should be specific towards aminoalkyl- or mercaptoalkyl- moiety, commonly found in biomolecules, and which are easy to introduce in synthetic oligonucleotides, and (b) the other functional group should be photoreactive (capable of n-π^* excitation) in order for



Scheme 1. Preparation of NTPAC and immobilization of oligonucleotides using this reagent.

this to react (abstract C–H hydrogen) from a variety of polymer matrices, viz., polypropylene, polyethylene, polystyrene, nylon, modified glass surfaces, etc. Reagents based on photoactivable azides and protected carbenes were considered for this purpose[23–25], but dropped as, upon photoirradiation, the generated reactive species, i.e. nitrenes and carbenes, respectively, produce a number of side products leading to lower reaction yields. Encouraged by a recent report from the Danish group[22] about the use of anthraquinone as photoactivable moiety for immobilization of biomolecules including oligonucleotides, we decided to use anthraquinone as photoexcitable moiety. Recently, anthraquinone-oligonucleotide conjugates have been implicated in studies on long range charge transfer in DNA and accompanying transient structural distortions in the DNA duplexes[26,27].

The immobilization methodology involves two alternate routes. In one protocol, an oligonucleotide-anthraquinone conjugate is formed by allowing the reagent, NTPAC, to react with an appropriately modified oligonucleotide followed by reaction with C–H containing polymers under UV irradiation (365 nm). In the alternate route, NTPAC is first brought in contact with glass surface and exposed to UV light (365 nm). Thereafter, the reactive trifluoroethanesulfonate ester functions on the polymer support react with the mercaptoalkyl or the aminoalkyl group-bearing oligonucleotides[18]. Based on both above protocols, we have immobilized a number of modified oligonucleotides on the glass surface. The constructed oligonucleotide microarrays were successfully used for detection of base mismatches.

The new reagent, *N*-(3-trifluoroethanesulfonyloxypropyl)anthraquinone-2-carboxamide (NTPAC, **2**), was made from reaction of *N*-(3-hydroxypropyl)anthraquinone-2-carboxamide, **1** with 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride) in dichloromethane for 2 h at room temperature. This was obtained in almost quantitative yield in a syrupy form and characterized by MALDI-TOF and UV spectroscopy.

Methodology for photo-immobilization and base mismatch detection

Determination of optimal concentration of oligonucleotides on the surface :

To arrive at optimal concentration required to visualize an oligomer immobilized on the glass surface, an oligomer, fluorescein-d (TTT TTT TTT TTT TTT TTT–SH), was allowed to react with the reagent, NTPAC, for 30 min at room temperature. The reaction mixture was dried under vacuum and the residue redissolved in water (100 μl). After thorough mixing, the suspension was centrifuged and the liquid decanted off. The resulting solution was then diluted with a solution of lithium chloride (1 *M*) to make three concentrations (5, 10 and 15 μM) in 0.1 *M* phosphate buffer, pH 8.5 containing lithium chloride (0.2 *M*). The oligomer-anthraquinone conjugate was spotted (0.5 μl) manually in triplicates with the help of a pipettman (2 μl). After irradiation for 30 min at room temperature with UV light (365 nm) in a photoreactor, the slides were washed with 0.1 *M* phosphate buffer containing 0.5 *M* sodium chloride, pH 7.5 and water, respectively. After drying the slides, the spots were visualized under laser scanner at 515 nm (Fig. 1). This revealed that 10 μM concentration of oligomers was sufficient to visualize spots under laser light. All other immobilization experiments were carried out with same concentration (10 μM).

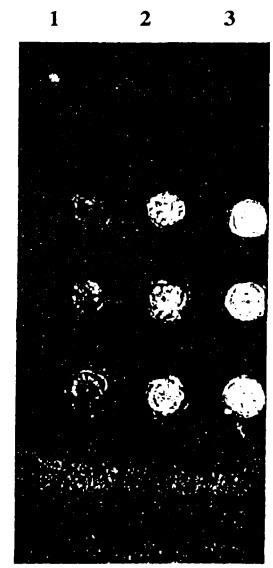


Fig. 1. Threshold concentration of oligonucleotide sequence required for observance of fluorescence under laser scanner. Lane 1 : 5 μM, Lane 2 : 10 μM and Lane 3 : 15 μM.

Immobilization of aminoalkyl- and mercaptoalkyl-oligonucleotides on glass microslides :

Oligomers, viz. H_2N-$(CH_2)_5$-OPO_3-d(AAT CGT TAC TTT TTA TTA TCC) and HS-$(CH_2)_6$-OPO_3-d(AAT CGT TAC TTT TTA TTA TCC) were immobilized via two routes. First, the oligonucleotide-anthraquinone conjugate was formed by allowing the reagent, NTPAC, to react with H_2N-$(CH_2)_5$-OPO_3-d((AAT CGT TAC TTT TTA TTA TCC) (Table 1, Entry 1). After usual work up, the conjugate was spotted on C-8 glass slide followed by UV light irradiation (365 nm) for 30 min. In the second route, NTPAC was spread on the C-8 glass surface, dried and exposed to UV light (365 nm) for 30 min. Thereafter, the surface bound tresyl functions were reacted with H_2N-$(CH_2)_5$-OPO_3-d (AAT

Table 1. Oligonucleotide sequences with deprotection conditions and yields

Sl. no.	Oligonucleotide sequence	Deprotection conditions	Yield (O.D. at 260 nm)
1.	H_2N-$(CH_2)_5$-OPO_3-d (AAT CGT TAC TTT TTA TTA TCC)	Aq. NH_4OH (30%), 16 h, 60°	26.4
2.	HS-$(CH_2)_6$-OPO_3-d (AAT CGT TAC TTT TTA TTA TCC)	Aq. NH_4OH (30%), containing 0.1 *M* DTT, 16 h, 60°	23.5
3.	HS-$(CH_2)_6$-OPO_3-d (TTG GGT CCG CCA CTC CTT CCC)	Aq. NH_4OH (30%), containing 0.1 *M* DTT, 16 h, 60°	22.7
4.	HS-$(CH_2)_6$-OPO_3-d (TTG GGT CCG CTA CTC CTT CCC)	Aq. NH_4OH (30%) containing 0.1 *M* DTT, 16 h, 60°	24.3
5.	Fluorescein-d(GGA TAA TAA AAA GTA ACG ATT)	Aq. NH_4OH (30%), 16 h, 60°	27.7
6.	Fluorescein-d(GGG AAG GAG TGG CGG ACC CAA)	Aq. NH_4OH (30%), 16 h, 60°	28.4
7.	Fluorescein-d(TTT TTT TTT TTT TTT TTT TT)-OPO_3-$(CH_2)_6$SH	Aq. NH_4OH (30%) containing 0.1 *M* DTT, 16 h, 60°	21.9

CGT TAC TTT TTA TTA TCC). Rest of the sites were blocked by 0.1 *M* phosphate buffer containing 0.5 *M* tris base, pH 8.5. Similarly, 5′-mercaptoalkyl oligonucleotides were immobilized on the glass surface. Comparable results were obtained using both routes to immobilization.

Hybridization of immobilized oligonucleotides with complementary oligonucleotides :

The spotted plates were subjected to hybridization experiment with complementary oligomers. The plates were covered with a solution of complementary fluorescein labeled oligonucleotide (Table 1, Entry 5) in the hybridization chamber. After washing with phosphate buffer, the spots were visualized under laser scanner at 515 nm (Fig. 2).

Detection of single base mismatches :

In order to demonstrate the specificity of the labeled probes to their complementary targets, oligonucleotides, viz., HS-$(CH_2)_6$-OPO_3-d(TTG GGT CCG CCA CTC CTT CCC) and HS-$(CH_2)_6$-OPO_3-d (TTG GGT CCG CTA CTC CTT CCC) were immobilized in triplicates after reaction with the reagent, NTPAC, on the C-8 glass slides. The spots were hybridized with 5'-fluorescein-d(GGG AAG GAG TGG CGG ACC CAA) in a hybridization chamber. After thorough washings with phosphate buffer, the slides were scanned under laser scanner at 515 nm. The oligomer with

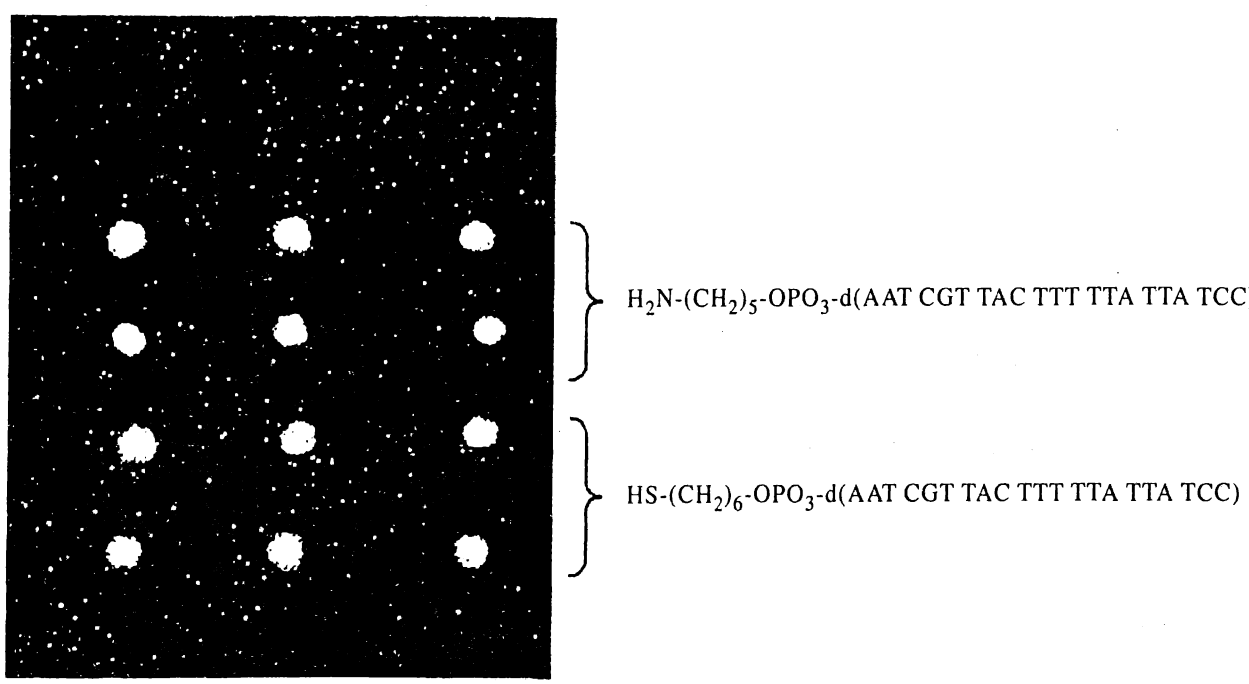


Fig. 2. Immobilization of oligonucleotides, H_2N-$(CH_2)_5$-OPO_3-d(AAT CGT TAC TTT TTA TTA TCC) and HS-$(CH_2)_6$-OPO_3-d(AAT CGT TAC TTT TTA TTA TCC), and their hybridization with fluorescein-d(GGA TAA TAA AAA GTA ACG ATT) and visualization under laser scanner.

perfect base match revealed intense bands, whereas the mismatched oligomer showed a weak band (lower fluorescence intensity) (Fig. 3).

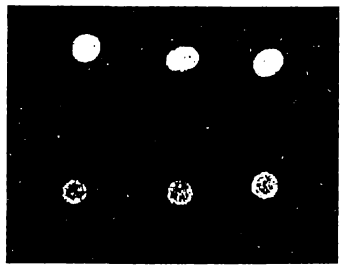


Fig. 3. Detection of single nucleotide mismatch via hybridization with fluorescein labeled complementary oligonucleotides. Lane 1 : HS-$(CH_2)_6$-OPO_3-d(TTG GGT CCG CCA CTC CTT CCC), Lane 2 : HS-$(CH_2)_6$-OPO_3-d(TTG GGT CCG CTA CTC CTT CCC). Hybridization was carried out using fluorescein-d(GGG AAG GAG TGG CGG ACC CAA).

Experimental

General : All reagents used in the present investigation were purified prior to their use. 2,2,2-Trifluoroethanesulfonyl chloride (tresyl chloride), anthraquinone-2-carboxylic acid, 3-aminopropan-1-ol and diisopropylcarbodiimide (DIPCI) were procured from Aldrich Chemical Co., St. Louis, MO. Fluorescein phosphoramidite was purchased from Applied Biosystems Inc., USA. All other reagents and chemicals were procured locally. All the reactions were carried out in an inert atmosphere unless stated otherwise.

N-(3-Trifluoroethanesulfonyloxypropyl) anthraquinone-2-carboxamide (NTPAC) **2** :

N-(3-Hydroxypropyl)anthraquinone-2-carboxamide **1** (309 mg; 1.0 mmol)[22] was taken up in anhydrous dichloromethane (10 ml) containing diisopropylethylamine (155 mg; 1.2 mmol). The reaction mixture was cooled in an ice-bath and 2,2,2-trifluoroethanesulfonyl-chloride (tresyl chloride) (219 mg; 1.2 mmol) added drop-wise over a period of 20 min. The reaction continued for additional 2 h at room temperature. The solution was filtered in an inert atmosphere to remove suspended ammonium chloride and the filtrate concentrated under vacuum and further dried in a desiccator to obtain the title compound **2** in almost quantitative yield (472 mg) as a syrupy material. The reagent was characterized by MALDI-TOF (456, $M+H^+$; matrix : 2,5-dihydroxy-benzoic acid) (SEQ IV, Kratos, UK) and UV (λ_{max} 325 and 255.8 nm).

Oligonucleotides and their 5′-modified analogs : Oligonucleotides (Table 1) were synthesized on a Pharmacia. LKB Gene Assembler Plus at 0.2 μmol scale following standard protocol[29]. The 5′-mercaptoalkyl, aminoalkyl and 3′-mercaptoalkyl functionalities were introduced in the oligonucleotides according to published procedures[30–32] from this laboratory. The standard oligonucleotides and 5′-modified oligonucleotides were deprotected and desalted using standard protocol[29].

Complementary oligonucleotides labeled with fluorescein were synthesized by performing the last coupling reaction with fluorescein-phosphoramidite. After complete deprotection, the residue was dissolved in dd water (100 μl) and applied on Sephadex G-25 column. The labeled oligomer was eluted with 0.1 *M* triethylammonium acetate buffer, pH 7.5. The solution was concentrated to dryness and kept at 4° until use.

Immobilization of mercaptoalkyl- and aminoalkyl-oligonucleotides on C-8 glass surface :

Route A : Oligonucleotide with the sequence, H_2N-$(CH_2)_5$-OPO_3-d(AAT CGT TAC TTT TTA TTA TCC) (1.5 A_{260} units), dissolved in 0.1 *M* sodium phosphate buffer (100 μl), pH 8.5, was reacted with NTPAC **2** dissolved in DMF (9.1 mg; 0.2 *M*, 100 μl) for 1 h at room temperature and then concentrated under vacuum to remove the solvent (DMF) from the mixture. The dried residue was resuspended in dd water (25 μl) and the mixture was centrifuged to remove excess of reagent, **2**, which settles down at the bottom, being insoluble in water. The decanted solution was charged with lithium chloride (1 *M*) to bring the final concentration of LiCl to 0.2 *M*. The anthraquinone-oligonucleotide conjugate was spotted on C-8 glass plates in triplicates and the spots were allowed to dry in the air. The spotted plates were kept under UV light (365 nm) for 30 min. The plates were washed with 0.1 *M* sodium phosphate buffer containing 1 *M* sodium chloride, pH 7.5 (3 × 10 ml) and water (3 × 10 ml), respectively. The plates were kept in a moisturized chamber prior to hybridization experiments and stored at 4°.

Similarly, mercaptoalkyl oligonucleotide, HS-$(CH_2)_6$-OPO_3-d(AAT CGT TAC TTT TTA TTA TCC) was reacted with NTPAC **2**, for 30 min at room temperature followed by covalent attachment of the conjugate on C-8 glass plates under the influence of UV light (365 nm). Rest of the steps followed were as described above.

Route B : In an alternative route, a solution of the reagent, **2** (91 mg; 2.0 ml, 0.1 *M* in dichloromethane), was spread on C-8 coated glass microslide and the solvent allowed to evaporate in a fume cupboard. The dried plate was exposed to UV light (365 nm) for 30 min in Photoreactor (Gautam UV Cabinet, Delhi, India) equipped with 16 tubes (10 × 10 W and 6 × 8 W). The plate was thoroughly washed several times with dry dichloromethane (5 × 15 ml) and dried under

vacuum. On the trifluoroethanesulfonylated (tresylated) plate was spotted a solution of 5′-mercaptoalkylated oligonucleotide, HS-$(CH_2)_6$-OPO_3-d(AAT CGT TAC TTT TTA TTA TCC) (10 μM, 0.5 μl) in 0.1 *M* phosphate buffer, pH 8.5, in triplicates, which was allowed to stand under inert atmosphere for 30 min. After the reaction, the plate was treated with 0.1*M* phosphate buffer containing 0.5 *M* tris base, pH 8.5, for 1 h (1 × 10 ml) followed by washing with 0.1*M* phosphate buffer, pH 7.5 (3 × 10 ml) and finally with dd water (3 × 10 ml). The plate was then dried at room temperature under vacuum and the spots were visualized by hybridization with fluorescein labeled complementary oligonucleotide sequence.

Similarly, 5′-aminoalkylated oligonucleotide, H_2N-$(CH_2)_5$-OPO_3-d(AAT CGT TAC TTT TTA TTA TCC), was immobilized on activated plates following the procedure described above except that the reaction was carried out for 1 h instead of 30 min.

Hybridization assay :

The oligonucleotides, viz. H_2N-$(CH_2)_5$-OPO_3-d(AAT CGT TAC TTT TTA TTA TCC) and HS-$(CH_2)_6$-OPO_3-d(AAT CGT TAC TTT TTA TTA TCC), immobilized on the glass plates, were hybridized with complementary oligomers labeled with fluorescein in a hybridization chamber. The complementary oligomer, fluorescein-d(GGA TAA TAA AAA GTA ACG ATT), was dissolved in 200 μl of 0.1 *M* phosphate buffer containing 1 *M* sodium chloride, pH 7.5, and spread over a glass plate. After covering the spots with microscope slide, the chamber was placed in a water bath at 65° and allowed to cool slowly to room temperature. It was further cooled to 10° and kept at that temperature for additional 2 h. The plates were washed with the same buffer system and fluorescent spots were visualized under laser scanner operating at 515 nm (Scan Array Lite Micro Array Analysis System, GSI Lumonics, USA).

Detection of mismatches based on fluorescence intensity :

In order to identify the mismatches in the oligonucleotide sequences based on fluorescence intensity, oligonucleotide sequences, viz. HS-$(CH_2)_6$-OPO_3-d(TTG GGT CCG CCA CTC CTT CCC) and HS-$(CH_2)_6$-OPO_3-d(TTG GGT CCG CTA CTC CTT CCC), were reacted with the reagent, **2**, and the conjugates spotted on the glass slide in triplicates as described above. The spots on the plate were hybridized with fluorescein labeled d(GGG AAG GAG TGG CGG ACC CAA) in 0.1 *M* phosphate buffer containing 0.5 *M* sodium chloride, pH 7.5. After usual washings, the spots on the plate were visualized under laser scanner.

Determination of optimal concentration of oligonucleotide spots on surfaces for fluorescence detection :

In order to arrive at optimal concentration required to visualize an oligomer immobilized on the glass surface, fluorescent oligomer-anthraquinone conjugate, fluorescein-d(TTT TTT TTT TTT TTT TTT TT–S–AQ), was diluted in three different concentrations (5, 10, 15 μM) in 0.2 *M* lithium chloride. The oligomer was spotted (0.5 μl) manually in triplicates on C-8 glass slide and irradiated under long wavelength UV light (365 nm) for 30 min. After irradiation, the slide was washed with 0.1 *M* phosphate buffer containing 0.5 *M* sodium chloride, pH 7.5. The slides were dried and the spots visualized under laser scanner at 515 nm.

Conclusion :

A novel heterobifunctional reagent, NTPAC, has been developed for construction of oligonucleotide arrays using end-modified oligonucleotides and a variety of inert surfaces. The new reagent provides flexibility in that this can be used in two ways. In one protocol, modified oligonucleotide is reacted with the reagent to furnish an oligonucleotide-anhraquinone conjugate, which subsequently reacts with a modified glass surface to generate surface bound oligomers. In the second pathway, NTPAC is first reacted with the modified glass surface followed by spotting of modified oligomers on the activated surface to obtain surface immobilized oligonucleotides. The constructed oligonucleotide microarrays, *interalia*, find use in detection of mismatches. The new reagent appears to hold potential for effective immobilization of oligonucleotides on varied polymer surfaces.

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