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# Construction of Oligonucleotide Microarrays (Biochip) Using Heterobifunctional Reagents

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**Summary**

A number of hetero- and homobifunctional reagents have been reported to immobilize biomolecules on a variety of supports. However, efforts are on to search for a method, which is relatively simple, involving minimum of steps, cost effective, easy to reproduce, and that produces stable oligonucleotide arrays. Two new reagents, viz., [N-(2-trifluoroethanesulfonatoethyl)-N-(methyl)-triethoxysilylpropyl-3-amine], and [N-(3-trifluoroethanesulfonyloxypropyl)anthraquinone-2-carboxamide] have been designed considering the above points. These reagents contain different functional groups at their two ends. In [N-(2-trifluoroethanesulfonatoethyl)-N-(methyl)-triethoxysilylpropyl-3-amine], one end (triethoxysilyl) is capable of binding to the virgin glass surface and the other one consists of trifluoroethanesulfonate (tresyl) function specific toward aminoalkyl and mercaptoalkyl functionalities, which are easy to introduce at the 3′- or 5′-end of oligonucleotides. Likewise, in [N-(3-trifluoroethanesulfonyloxypropyl)anthraquinone-2-carboxamide], one end consists of photoactivatable moiety (anthraquinone) capable of reacting to a C–H containing surface and the tresyl function at the other end reacts specifically with aminoalkyl and mercaptoalkyl functionalities in modified oligonucleotides. These reagents have successfully been utilized to construct a number of oligonucleotide arrays and subsequently used for the detection of mismatches.

**Key Words:** Glass surface; heterobifunctional reagents; immobilization; microarray; NTMTA; NTPAC; oligonucleotides.

## 1. Introduction

Detection, analysis and characterization of oligonucleotide/DNA sequences in the traditional way, i.e., one gene-one experiment, are quite laborious and time consuming. Recently developed microarray technology has proved to be a

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boon in this direction. It is a powerful tool that allows simultaneous detection of different target molecules present in a sample.

Microarrays are flat surfaces on which different molecules of oligonucleotides/DNAs have been affixed at discrete locations in an ordered manner ***(1,2)***. The simultaneous measurement of expression patterns of thousands of genes ***(3–5)*** is made possible by microarrays thus making it an effective research tool. The majority of applications are in the area of large-scale screening of mutations, studies of gene polymorphism, differential-gene expression analysis, disease diagnosis, identification and characterization of pathogens, and so on. For the preparation of oligonucleotide arrays, several surface materials have been tried, viz., nylon ***(6)***, glass ***(7–9)***, polyacrylamide ***(10)***, polypropylene ***(11)***, polyesters ***(12)***, poly(methyl methacrylate) (PMMA) ***(13)***, silicon ***(14,15)***, optical fibers ***(16)***, gold ***(17,18)***, polyethylene glycol grafted on silica surfaces ***(19)*** and so on. The choice of surfaces for microarray preparation depends on their homogeneity, stability upon storage and reactivity toward biomolecules. Based on these criteria, glass and polypropylene could be opted as they can easily be modified to generate functional groups on them. However, glass is most commonly used as it is easily available, inexpensive and can be used in laser scanners in which it offers low background.

Basically, two methods are followed for preparing an oligonucleotide array: (1) *in situ* ***(20–22)*** or on chip synthesis of oligonucleotides and (2) arraying of prefabricated oligonucleotides. The former one is based on photolithographic methods, wherein the oligonucleotides are synthesized at the predetermined sites on the solid or polymeric surface, using photolabile protecting groups. Using this technique, a density of $10^6$ sequences/$cm^2$ has been achieved on a biochip ***(21)***.

In the second method, oligonucleotides are first prepared and then immobilized on suitable solid surfaces (inorganic and organic polymeric materials). A plethora of reports on the attachment of readily prepared oligonucleotides to a surface have appeared. Noncovalent ***(23)*** and covalent attachments to surfaces have been analyzed and the latter was found to be superior. In the covalent attachment of immobilization, generally, electrophilic-glass surfaces ***(24)*** are reacted with nucleophilic oligonucleotides. Many modifications to the surface as well as to the oligonucleotides have been carried out for improving the quality of the microarrays.

To minimize the number of steps in the preparation of arrays, homo- and heterobifunctional reagents (linkers) have been used to couple oligonucleotides directly to virgin/functionalized glass surface ***(25–27)***. These linkers have either two similar or different functional groups, one capable of attaching to the surface and the other that can react with a suitably modified oligonucleotide. Heterobifunctional crosslinkers, viz., succinimidyl 4-(maleimidophenyl)butyrate,

m-maleimidobenzoyl-N-hydroxysuccinimide ester, succinimidyl-4-(N-male imidomethyl)cyclohexane-1-carboxylate (*SMCC*), N-(γ-maleimidobutyryloxy) succinimide ester, maleimidopropionic acid N-hydroxysuccinimide ester and N-succinimidyl(4-iodoacetyl)aminobenzoate bearing both thiol and amino reactive moieties have been developed for covalent attachment of thiol-modified oligomers to aminosilane monolayer films ***(28)***. In case of succinimidyl 4-(maleimidophenyl)butyrate, a surface density of about 20 pmol of bound oligonucleotide/cm$^2$ was obtained. This method appeared to be superior to other methods of attachment both in terms of density of immobilized oligonucleotides and also the reduced concentration of oligomers required to achieve this density.

The use of heterobifunctional crosslinkers has proved to be attractive but the hunt is on for a surface which could confer reproducibility to the array preparation. It must also be stable to conditions of hybridization and show no nonspecific binding. Recently, Sulfo-SMCC has been used to attach oligonucleotides on Si(111) and Si(001) surfaces ***(14,29)***. However, the surface had to be modified first by reaction with an 11-undecylenic acid methyl or trifluoroethylester under photolight (254 nm), then hydrolyzed to yield a carboxylic acid modified surface that was then reacted with polylysine and Sulfo-SMCC to get a maleimide-activated surface. Fixing of oligonucleotides to virgin glass surface has been made possible by two reagents, viz., 3-mercaptopropyltriethoxysilane and 3-glycidyloxypropyltrimethoxysilane ***(25)***. 3-Mercaptopropyltriethoxysilane results in immobilization of oligonucleotides through disulfide linkage, which has proved to be a labile linkage and 3-glycidyloxypropyltrimethoxysilane requires longer reaction time (~8 h).

Recently, a novel heterobifunctional crosslinker, [N-(2-trifluoroethane-sulfonatoethyl)-N-(methyl)-triethoxysilylpropyl-3-amine] (NTMTA) has been developed, which contains a tresyl function on one side and a triethoxysilyl function on the other side ***(30,31)***. The tresyl function can couple to a 5′-aminoalkyl or mercaptoalkyl-functionalized oligonucleotide/biomolecules, whereas, the triethoxysilyl function can bind to the virgin glass surface. The method is suitable for the construction of oligonucleotide microarrays on a glass surface without employing any additional coupling reagent. The density of immobilized DNA was found to be comparable or better than the existing glass-specific methods and far superior to these methods in terms of reaction time for construction of microarrays and signal-to-noise ratio (>98). Moreover, the covalently immobilized oligonucleotides were stable to varying heating conditions (encountered during PCR). The thermal stability studies indicated that the microarrays obtained by this method are quite stable and can be exploited successfully in biological research.

Photoreactive groups have also been used for immobilization of biomolecules including oligonucleotides. Recently, a new photolabile heterobifunctional

reagent was developed, N-(3-trifluoroethanesulfonyloxypropyl)anthraquinone-2-carboxamide (NTPAC). The idea was to have a reactive functional group (tresyl) at one end, which can be specific toward aminoalkyl- or mercaptoalkyl moiety commonly found in biomolecules and a photoreactive group (anthraquinone) at the other end, which can react with a variety of C–H containing polymer matrices, such as polypropylene, polyethylene, polystyrene, and modified glass surfaces, and so on ***(32,33)***. Reagents, based on photoactivable azides and protected carbenes ***(34–36)***, have also been initially considered for this purpose but dropped because the generated reactive species (i.e., nitrenes and carbenes, respectively) produce a number of side products leading to lower reaction yields on photoirradiation.

The immobilization techniques are elucidated using the above two important reagents, namely, NTMTA and NTPAC.

## 2. Materials

1. Acetonitrile, dichloromethane (DCM), ethylene dichloride (EDC), and toluene are dried by refluxing over calcium hydride, distilled and stored over 4 Å molecular sieves.
2. Methanol, petroleum ether, and diethyl ether are dried over sodium metal, distilled, and stored in dry amber-glass bottles.
3. N,N-dimethylformamide (DMF) is purified by azeotropic distillation with benzene and stored over 4 Å molecular sieves.
4. 1H-tetrazole is purified by sublimation.
5. Pharmacia. LKB Gene Assembler Plus (Uppsala, Sweden).
6. FAM amidite (Applied Biosystems, Foster City, CA).
7. Fluorescein at C-5 position in thymidine-phosphoramidite (Glen Research Inc., USA).
8. Other reagents and chemicals are purchased from reputed manufacturers (e.g., Sigma-Aldrich (St. Louis, MO), Fluka (Chemie AG, Buchs, Switzerland), Merck ltd. (Mumbai, India) and so on). The reagents required in this work are: thioacetic acid, thiobenzoic acid, 6-bromohexanol, 18-crown-6, N,N,N′,N′-tetraisopropyl-2-cyanoethyl-phosphoramidite, N,N′-dicyclohexylcarbodiimide (DCC), controlled pore glass (CPG), dithiothreitol (DTT), 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride), anthraquinone-2-carboxylic acid, 3-aminopropanol, 5-aminopentanol, N,N-diisopropylethylamine, N-methylaminoethanol, 3-aminopropyltriethoxysilane, octyltrimethoxysilane, 3-chlorotrimethoxysilane, 1-*O*-(4,4′-dimethoxytrityl)-5-aminopentanol ***(37)***, 1-*O*-(4,4′,-dimethoxytrityl)-6-mercaptohexanol ***(38)*** chloro-(2-cyanoethyl)-N,N′,-diispropylaminophosphine, N-hydroxysuccinimide (NHS).
9. Buffers mentioned in this work are as follows:
   a. Buffer 1: 0.1 *M* triethylammonium acetate, pH 7.1.
   b. Blocking buffer 2: 0.1 *M* Tris-HCl, pH 8.5.
   c. Reaction buffer 3: 0.1 *M* sodium phosphate containing 0.5 *M* NaCl, pH 8.0.
   d. Washing buffer 4: 0.1 *M* sodium phosphate containing 0.5 *M* NaCl, pH 7.1.
   e. Hybridization buffer 5: 0.1 *M* sodium phosphate containing 1 *M* NaCl, pH 7.1.
   f. Buffer 6: 0.1 *M* sodium citrate containing 0.5 *M* NaCl, pH 7.1.

10. Glass microslides are precleaned by immersing in 25% ammonium hydroxide solution overnight and then rinsed with double-distilled (dd) water (3 × 50 mL). Finally, these are submerged in anhydrous ethanol (~95%) for 30 min, dried under vacuum in a desiccator and stored in a dust-free container.

## 3. Methods

For the immobilization of oligonucleotides using NTMTA and NTPAC reagents, 5′-modified oligonucleotides are required, whereas for quantification, 3′-modified oligonucleotides are required. The following **Subheadings 3.1.** and **3.2.**, give the procedure for the preparation of 5′- and 3′-modified oligonucleotides. The oligonucleotide sequences synthesized in this work along with their deprotection conditions are presented in the **Table 1**.

### *3.1. Preparation of 5′-Modified Oligonucleotides*

A reactive functional group (aminoalkyl or mercaptoalkyl) can be introduced in the protected form with the help of a suitable linker as last coupling step, which during deprotection step generates oligomers with free nucleophilic reactive groups ***(39–41)***.

#### *3.1.1. Synthesis of 5′-Mercaptoalkylated Oligonucleotides*

**Scheme 1** depicts the steps involved in the preparation of reagent, S-acyl-6-mercaptohexyl-2-cyanoethyl-N,N-diisopropylphosphoramidite **3a**, **3b**. This reagent is further used in the last coupling step to obtain 5′-mercaptoalkylated oligonucleotides using Gene Assembler Plus synthesizer.

##### 3.1.1.1. Preparation of Potassium Thioacetate **1a** or Thiobenzoate **1b**

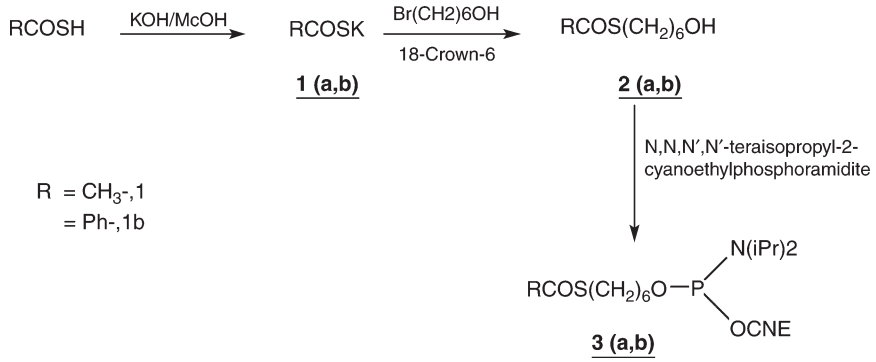
1. To a cold solution of thioacetic or thiobenzoic acid (10 mmol) in methanol, add a methanolic solution of potassium hydroxide (10 mmol) dropwise over a period of 5 min with constant stirring.
2. After complete addition, stir the solution at room temperature for 15 min, filter it to remove any insoluble material, and concentrate on a rotary evaporator to reduce the volume to about 10 mL.
3. Triturate the above solution with diethyl ether (200 mL) to precipitate the potassium salt of thioacetic or thiobenzoic acid.
4. Filter on a sintered-disc glass funnel, and dry under vacuum in a desiccator.

##### 3.1.1.2. Preparation of S-Acyl-6-Mercaptohexanol **2a**, **2b**

1. Mix potassium thioacetate **1a** (2.4 mmol), 6-bromohexanol (2.0 mmol), and 18-crown-6 (0.2 mmol) in toluene (20 mL) in a round-bottom flask and reflux for 15 min.
2. Filter off the insoluble material and dilute the filtrate with toluene (30 mL).
3. Wash the previously described filtrate successively with saturated aqueous sodium hydrogen carbonate (2 × 20 mL), sodium chloride (1 × 20 mL), and water (1 × 20 mL).

**Table 1**
**Oligonucleotides Synthesized With Their Deprotection Conditions**

| No. | Oligomer sequence | Deprotection conditions |
|---|---|---|
| 1. | $H_2N$-$(CH_2)_5$-$OPO_3$-d(CAG AGG TTC TTT GAG TCC TT) | Aq $NH_4OH$, 16 h, 60°C |
| 2. | HS-$(CH_2)_6$-$OPO_3$-d(CAG AGG TTC TTT GAG TCC TT) | Aq $NH_4OH$ + 50 m*M* DTT, 16 h, 60°C |
| 3. | HS-$(CH_2)_6$-$OPO_3$-d(CTC CTG AGG AGA AGG TCT GC) | Aq $NH_4OH$ + 50 m*M* DTT, 16 h, 60°C |
| 4. | $H_2N$-$(CH_2)_5$-$OPO_3$-d(CTC CTG AGG AGA AGG TCT GC) | Aq $NH_4OH$, 16 h, 60°C |
| 5. | $H_2N$-$(CH_2)_5$-$OPO_3$-d(CTC CTG AGG CGA AGG TCT GC) | Aq $NH_4OH$, 16 h, 60°C |
| 6. | FAM-d(AAG GAC TCA AAG AAC CTC TG) | Aq $NH_4OH$, 16 h, 60°C |
| 7. | FAM-d(GCA GAC CTT CTC CTC AGG AG) | Aq $NH_4OH$, 16 h, 60°C |
| 8. | d($T^F$TT TTT TTT TTT TTT TTT TT)-$OPO_3$-$(CH_2)_6SH$ | Aq $NH_4OH$ + 50 m*M* DTT, 16 h, 60°C |
| 9. | $H_2N$-$(CH_2)_5$-$OPO_3$-d(CTC CTG CGG AGA ACG TCT GC) | Aq $NH_4OH$, 16 h, 60°C |
| 10. | $H_2N$-$(CH_2)_5$-$OPO_3$-d(CTC CTG CGG CGA ACG TCT GC) | Aq $NH_4OH$, 16 h, 60°C |
| 11. | $H_2N$-$(CH_2)_5$-$OPO_3$-d(TTT TTT TTT TTT TTT TTT TT) | Aq $NH_4OH$, 16 h, 60°C |
| 12. | $H_2N$-$(CH_2)_5$-$OPO_3$-d(AAT CGT TAC TTT TTA TTA TCC) | Aq $NH_4OH$, 16 h, 60°C |
| 13. | HS-$(CH_2)_6$-$OPO_3$-d(AAT CGT TAC TTT TTA TTA TCC) | Aq $NH_4OH$ + 50 m*M* DTT, 16 h, 60°C |
| 14. | HS-$(CH_2)_6$-$OPO_3$-d(TTG GGT CCG CCA CTC CTT CCC) | Aq $NH_4OH$ + 50 m*M* DTT, 16 h, 60°C |
| 15. | HS-$(CH_2)_6$-$OPO_3$-d(TTG GGT CCG CTA CTC CTT CCC) | Aq $NH_4OH$ + 50 m*M* DTT, 16 h, 60°C |
| 16. | Fluorescein-d(GGA TAA TAA AAA GTA ACG ATT) | Aq $NH_4OH$, 16 h, 60°C |
| 17. | Fluorescein-d(GGG AAG GAG TGG CGG ACC CAA) | Aq $NH_4OH$, 16 h, 60°C |
| 18. | Fluorescein-d(TTT TTT TTT TTT TTT TTT TT)$OPO_3$-$(CH_2)_6SH$ | Aq $NH_4OH$ + 50 m*M* DTT, 16 h, 60°C |




Scheme 1. Preparation of reagents for 5′-mercaptoalkylation of oligonucleotides.

4. Separate the organic layer and dry it over anhydrous sodium sulfate, concentrate and purify by silica gel column chromatography using petroleum ether: ethyl acetate (8:2 v/v) as an eluent.
5. Collect the fractions containing the desired material and concentrate to obtain a yellowish oily material (~92% yield). $R_f$: 0.45 (hexane: ethyl acetate, 6:4).
6. Characterize the compound S-acetyl-6-mercaptohexanol **2a** by nuclear magnetic resonance (NMR) spectrum: $^1$H NMR ($CDCl_3$) δ: 1.6 (m, 8H), 2.4 (s, 3H), 2.95 (t, 2H), and 3.8 (m, 2H).

S-Benzoyl-6-mercaptohexanol **2b** can be synthesized in an analogous manner. $R_f$: 0.52 (hexane: ethylacetate, 6:4). $^1$H NMR ($CDCl_3$) δ: 1.54 (m, 8H), 3.04 (t, 2H), 3.7 (m, 2H), and 7.6 (m, 5H).

### 3.1.1.3. Preparation of S-Acyl-6-Mercaptohexyl-2-Cyanoethyl-N,N-Diisopropylphosphoramidite **3a**, **3b**

1. Dissolve the compound **2a** (1 mmol) in anhydrous acetonitrile (10 mL) and add to it N,N,N′,N′-tetraisopropyl-2-cyanoethylphosphoramidite (1.5 mmol).
2. To the previously described mixture, add tetrazole (0.75 mmol) dissolved in acetonitrile (1.5 mL) dropwise at room temperature.
3. Stir the resulting mixture for 2 h and monitor the progress of the reaction on thin layer chromatography (TLC) and after the reaction is complete, quench it by adding dry methanol (0.2 mL).
4. Concentrate the reaction mixture under reduced pressure and take the resulting syrupy mass in ethyl acetate (20 mL).
5. Wash the previously described mixture with sodium chloride solution (2 × 10 mL). Separate the organic phase and dry over anhydrous sodium sulfate.
6. After filtration, remove the solvent by evaporation and purify the resulting material using silica gel column.

7. Elute the desired compound with hexane: ethylacetate: triethylamine (TEA) (8:1:1, v/v/v), pool together the fractions containing the pure material and concentrate on a rotary evaporator to obtain the title compound **3a** in about 85% yield.

Similarly, prepare the phosphoramidite of compound **2b** and purify to obtain compound **3b** in about 85% yield.

#### 3.1.1.4. Synthesis and Deprotection of 5′-Mercaptoalkylated Oligonucleotides

1. Assemble an oligonucleotide chain at 0.2 µmol scale on a Gene Assembler Plus following manufacturer's recommendations using standard nucleoside containing polymer support.
2. Perform the last coupling step with one of the phosphoramidite reagents **3a** or **3b** in an analogous manner to the normal nucleoside phosphoramidites.
3. Deprotect the mercaptoalkylated oligonucleotides from the support and cleave the protecting groups by treatment with 30% aq ammonia containing 50 m*M* DTT at 60°C for 16 h (*see* **Note 1**).
4. Concentrate the ammoniacal solution in a speed vac and subject to desalting on a C-18 silica gel column.
5. Elute the 5′-mercaptoalkylated oligonucleotide with a solution of 30% aqueous acetonitrile solution and concentrate in a speed vac (*see* **Note 2**).

#### 3.1.1.5. Purification of 5′-Mercaptoalkylated Oligonucleotides by Affinity Chromatography

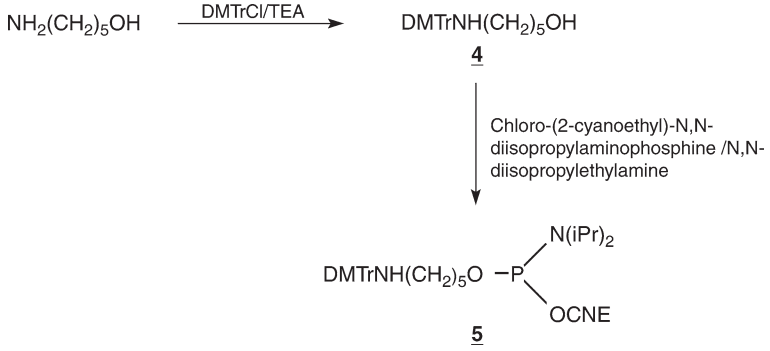
1. Pack a small chromatography column with 5-nitro-2-thiopyridyl-activated 3-mercaptopropyl-fractosil support and equilibrate with buffer 2 (10 mL).
2. Load the column with the crude mixture of 5′-mercaptoalkylated oligonucleotide dissolved in buffer 2 containing 20% acetonitrile.
3. Allow the solution to react with the column matrix for 1 h and then wash with the buffer 2 containing 20% acetonitrile (10 mL).
4. After all the truncated sequences are removed, elute the desired sequence with buffer 2 containing 50 m*M* DTT and 20% acetonitrile.
5. Concentrate and desalt the oligomer using Sephadex G-50 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) column using buffer 1.

### *3.1.2. Synthesis of 5′-Aminoalkylated Oligonucleotides*

5′-Aminoalkylated oligonucleotide can be prepared by performing the last coupling in a Gene Assembler Plus, using the reagent N-(4,4′-dimethoxytrityl)-5-aminopentyl-2-cyanoethyl-N,N-diisopropylphosphoramidite **5**. **Scheme 2** depicts the synthesis of this reagent.

#### 3.1.2.1. Preparation of N-(4,4′-Dimethoxytrityl)-5-Aminopentan-1-ol **4**

1. To a cold solution of 5-aminopentanol (10 mmol) in dry DCM (50 mL) and TEA (11 mmol), add a solution of 4,4′-dimethoxytrityl chloride (10 mmol), dissolved in DCM (25 mL), dropwise over a period of 30 min with constant stirring.
2. After complete addition, stir the solution at room temperature for 2 h.




Scheme 2. Preparation of reagent for 5′-aminoalkylation of oligonucleotides.

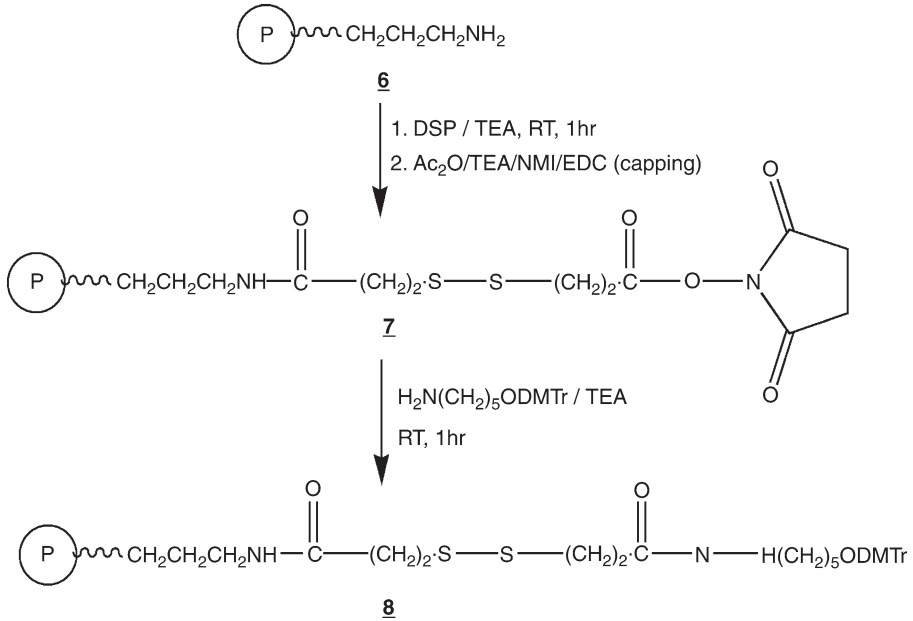
3. Monitor the reaction on TLC and if it is complete, wash the organic phase sequentially with cold 2.5% aq citric acid (2 × 25 mL), 5% aq sodium bicarbonate (2 × 25 mL), and saturated sodium chloride solutions (2 × 25 mL).
4. Collect the organic phase, dry over anhydrous sodium sulfate, filter, and concentrate on a rotary evaporator to get the desired compound as a syrupy material in about 88% yield.
5. Characterize the desired compound by NMR. $^1$H NMR ($CDCl_3$) δ: 1.6 (m, 6H), 2.9 (t, 2H), 3.3 (t, 2H), 3.8 (s, 6H), and 6.8–7.5 (m, 13H).

#### 3.1.2.2. Preparation of N-(4,4′-Dimethoxytrityl)-5-Aminopentyl-2-Cyanoethyl-N,N-Diisopropylphosphoramidite **5**

1. Dissolve the compound **4** (1 mmol) in anhydrous EDC (10 mL) and add N,N-diisopropylethylamine (4 mmol) to it.
2. Cool the previously described mixture in an ice-bath and add chloro-(2-cyanoethyl)-N,N-diisopropylaminophosphine (1.5 mmol) dropwise.
3. Stir the resulting mixture for 1 h at room temperature, monitor the advancement of the reaction on TLC and after the reaction is complete, quench it by adding dry methanol (0.2 mL).
4. Dilute the reaction mixture with EDC (50 mL) and wash sequentially with 5% aq sodium carbonate (2 × 25 mL) and saturated sodium chloride (2 × 25 mL) solutions.
5. Separate the organic phase and dry over anhydrous sodium sulfate.
6. After filtration, remove the solvent by evaporation on a rotary evaporator and purify the resulting material using silica gel column.
7. Elute the desired compound with hexane: ethylacetate: TEA (8:1:1, v/v/v), pool together the fractions containing the pure material and concentrate to obtain the title compound in about 85% yield.

#### 3.1.2.3. Synthesis and Deprotection of 5′-Aminoalkylated Oligonucleotides

1. Synthesize an oligonucleotide chain at 0.2 μmol scale on a Gene Assembler Plus following manufacturer's recommendations using standard nucleoside containing polymer support.




Scheme 3. Preparation of polymer support for 3′-mercaptoalkylated oligonucleotides.

2. Perform the last coupling step with phosphoramidite reagent **5** in an analogous manner to the normal nucleoside phosphoramidites except with an extended coupling time (5 min).
3. Deprotect 5′-aminoalkyl-oligonucleotide from the support and cleave the protecting groups by treatment with 30% aq ammonia at 60°C for 16 h.
4. Concentrate the ammoniacal solution in a speed vac and subject to desalting on a C-18 silica gel column.
5. Elute the 5′-aminoalkylated oligonucleotide with a solution of 30% aqueous acetonitrile solution and concentrate in a speed vac.

## *3.2. Preparation of 3′-Modified Oligonucleotides*

### *3.2.1. Synthesis of 3′-Mercaptoalkylated Oligonucleotides*

Unlike 5′-end modifications, 3′-modifications are not that straightforward. Because 3′- hydroxyl group of the leader nucleoside is not available for manipulation during solid phase synthesis as it is attached to the support through its hydroxyl group, modified supports have been designed to incorporate modifications at the 3′ end of oligonucleotides ***(42–46)***.

Out of a number of polymer supports that have been designed for this purpose, universal polymer support ***(38)*** is of considerable importance. The method used is described in **Scheme 3**. The synthesis can be carried out using a Gene Assembler Plus in the usual way. A coupling efficiency of >98% has been found using these supports.

#### 3.2.1.1. Preparation of Derivatized Support

1. To a suspension of 3-aminopropylated controlled pore glass **6** (3-AP-CPG) (1 g, 100 μmol amino groups) in dry EDC (5 mL) containing TEA (100 μL), add 10-fold molar excess of 3, 3′-diothio-bis(propionic acid N-hydroxysuccinimide ester) (DSP) (1 mmol).
2. Shake the reaction mixture at room temperature for 1 h.
3. Filter the polymer support on a sintered disc glass funnel and remove the excess reagent by washing sequentially with EDC (3 × 20 mL) and diethyl ether (2 × 10 mL).
4. Cap the residual amino groups on support with a solution of $Ac_2O$:TEA: N-methylimidazole:EDC (1:1:0.4:6, v/v) for 30 min at room temperature followed by washing of the support on the glass funnel with EDC (2 × 20 mL) and diethyl ether (2 × 10 mL).
5. Take a weighed amount of polymer support **7** (500 mg) in dry EDC (5 mL) and add 1-(*O*-4,4′-dimethoxytrityl)-5-aminopentanol (0.5 mmol), dissolved in anhydrous EDC (2 mL) containing TEA (50 μL).
6. Keep the reaction suspension at room temperature for 1 h with occasional shaking.
7. Transfer the support on to a sintered disc glass funnel and wash it with EDC and diethyl ether (2 × 10 mL of each).
8. Dry the fully functionalized support **8** under vacuum and determine its loading.

#### 3.2.1.2. Synthesis of 3′-Mercaptoalkylated Oligonucleotide

Synthesize the oligonucleotides modified at their 3′-end using the above support **8** in a Gene Assembler Plus. After the synthesis of desired oligonucleotide chain, subject the support to deprotection conditions as given in **Subheading 3.2.1.4.**

#### 3.2.1.3. Synthesis of 5′-Labeled 3′-Mercaptoalkylated Oligonucleotide

After the synthesis of desired oligonucleotide chain in a Gene Assembler Plus, perform the last coupling with FAM amidite as discussed in **Subheading 3.1.1.4.**

#### 3.2.1.4. Deprotection

1. Treat the support bound oligonucleotide with aq ammonia (30%) containing 50 m*M* DTT in a sealed vial at 60°C for 16 h.
2. Concentrate the ammoniacal solution in a speed vac followed by desalting on C-18 silica gel.
3. Elute the oligomer sequence with 30% aq acetonitrile and again concentrate in a speed vac.

### *3.3. Preparation of Reagent, NTMTA*

#### *3.3.1. Preparation of N-(2-Hydroxyethyl)-N-(Methyl)-Triethoxysilylpropyl-3-Amine* **9**

1. Prepare a solution of N-methyl-aminoethanol (0.3 mol) and N,N-diisopropylethylamine (0.1 mol) in hot toluene (100 mL).
2. To this solution, add 3-chloropropyltriethoxysilane (0.1 mol) in one step.

3. Reflux the mixture for 10 h under inert (argon) atmosphere. Cool the mixture to 4°C and keep it for 16 h.
4. Filter the ammonium chloride that is formed in the reaction under an inert atmosphere through a sintered disc glass funnel and wash the residue with dry toluene (2 × 25 mL).
5. Collect the filtrate and the washings together in a round-bottomed flask and concentrate under vacuum.
6. Distill the syrupy crude material so obtained under vacuum. The title compound is obtained in about 65% yield.
7. Characterize the compound by NMR. $^1$H-NMR, $CDCl_3$ δ: 0.58 (t, 2H), 1.14 (m, 9H), 1.30 (m, 4H), 2.27 (s, N–$CH_3$), 2.3–2.6 (m, 7H, –$CH_2$), 3.58 (m, 6H, -O$CH_2$), and 3.66 (t, 2H).

### 3.3.2. Preparation of NTMTA **10**

1. Prepare a mixture of compound ***9*** **(**5 mmol) and TEA (6 mmol) in 25 mL of DCM.
2. Maintained at 0°C in an ice bath, add tresyl chloride (6 mmol) dropwise over a period of 5 min (*see* **Notes 3** and **4**).
3. After the addition is complete, take out the mixture from ice-bath and stir at room temperature.
4. Monitor the reaction by TLC and stir until complete conversion takes place (~2 h).
5. After the completion of the reaction, cool the reaction mixture to 0°C in an ice bath and unwanted amine hydrochloride is precipitated.
6. Remove the precipitate by filtration under argon. Concentrate the filtrate under vacuum to obtain a syrupy liquid of the compound ***10*** (*see* **Note 5**).
7. Characterize the product by NMR and MALDI-TOF. $^1$H NMR, $CDCl_3$ δ: 0.32 (m, 2H), 1.23 (m, 2H), 3.0–3.5 (m, 9H), 4.5 (br, t, 2H). MALDI-TOF: 384 $(M + H)^+$, matrix: 2,5-dihydroxybenzoic acid.

## 3.4. Preparation of Reagent NTPAC

### 3.4.1. Preparation of N-(3-Hydroxypropyl)Anthraquinone-2-Carboxamide **11**

1. To a solution of anthraquinone-2-carboxylic acid (2 mmol) and N-hydroxysuccinimide (2.5 mmol) in dry DMF (15 mL), add N,N′-dicyclohexylcarbodiimide (2.5 mmol).
2. Stir the reaction mixture at room temperature for 3 h.
3. After the reaction is complete, add 3-aminopropanol (3 mmol) and TEA (3 mmol) dropwise at room temperature.
4. Stir the reaction mixture at room temperature for 6 h.
5. Remove the precipitated N,N′-dicyclohexylurea by filtration and concentrate the filtrate to syrupy mass.
6. Dissolve the syrupy material in ethyl acetate (100 mL) and wash sequentially with 5% aq citric acid (2 × 25 mL), 5% aq sodium bicarbonate (2 × 25 mL), and saturated sodium chloride (1 × 25 mL) solutions.
7. Collect the organic phase, dry it over anhydrous sodium sulfate, filter, and concentrate under vacuum to obtain the title compound as solid material in 90% yield.
8. Characterize the compound by NMR. $^1$H NMR, $CDCl_3$ δ: 1.65 (m, 2H), 3.2–3.45 (m, 4H), 7.5–8.5 (m, 8H).

### *3.4.2. Preparation of NTPAC* **12**

1. Take N-(3-hydroxypropyl)anthraquinone-2-carboxamide ***11*** (1 mmol) in anhydrous DCM (10 mL) containing N,N-diisopropylethylamine (1.2 mmol).
2. Cool the reaction mixture in an ice bath and add tresyl chloride (1.2 mmol) dropwise over a period of 20 min (*see* **Notes 3** and **4**).
3. Stir the reaction mixture at room temperature for another 2 h for completion.
4. Cool the reaction mixture and filter the solution in an inert atmosphere to remove suspended ammonium chloride.
5. Concentrate the filtrate under vacuum and dry in a desiccator to obtain the title compound in quantitative yield as a syrupy material (*see* **Note 5**).
6. Characterize the reagent by MALDI-TOF (456, $[M + H]^+$; matrix: 2,5-dihydroxybenzoic acid) and UV absorptions at 325 and 255.8 nm.

## *3.5. Immobilization of Oligonucleotides*

Immobilization can be effected through two routes. In the first one, 5′-mercapto- or aminoalkylated oligonucleotides react with reagent (NTMTA or NTPAC) to form oligonucleotide-reagent conjugate, which in a subsequent reaction with glass surface (virgin or modified) (*see* **Note 6**) results in surface bound oligonucleotides. In the second route, the reagent (NTMTA or NTPAC) is allowed to react first with glass surface (virgin or modified) to generate tresyl functions on it, which, in a subsequent step, react with 5′-mercapto- or aminoalkylated oligonucleotides to generate surface bound oligonucleotides (*see* **Note 7**).

### *3.5.1. Immobilization of 5′-Mercapto- and Aminoalkylated Oligonucleotides Using NTMTA*

#### 3.5.1.1. Immobilization of 5′-Aminoalkylated Oligonucleotide Through Route A

1. Dissolve 0.15 $A_{260}$ units, 0.85 nmol of oligonucleotide sequence, $H_2N$-$(CH_2)_5$-$OPO_3$-d(CAG AGG TTC TTT GAG TCC TT), in 100 µL of reaction buffer 3.
2. Mix it with ethanolic solution of NTMTA (85 µL, 8.5 nmol).
3. Agitate the mixture on an Eppendorf mixer for 1 h at room temperature.
4. Concentrate the solution under vacuum to obtain a semidried residue of oligonucleotide-trimethoxysilyl conjugate.
5. Suspend this residue in 25 µL of dd water.
6. Centrifuge the mixture to get rid of excess of NTMTA as undissolved material.
7. Concentrate the supernatant under vacuum to obtain a residue that is reconstituted in dd water containing 10% DMSO (v/v) to make an oligonucleotide conjugate final concentration to 10 µ*M* (*see* **Note 8**).
8. Spot the oligonucleotide conjugate manually in multiplicates on a glass microslide.
9. Incubate the spotted microslide at 45°C in a humid chamber for 40 min.
10. After 40 min, wash the slide with washing buffer 4 (5 × 10 mL).
11. The slide is ready for hybridization (*see* **Note 7**).

#### 3.5.1.2. Immobilization of 5′-Mercaptoalkylated Oligonucleotide Through Route A

Immobilize the 5′-mercaptoalkylated oligonucleotide in a similar manner as previously described (*see* **Subheading 3.5.1.1.**) except that 30 min are required (**step 3**, **Subheading 3.5.1.1.**) to complete the reaction between mercaptoalkylated oligonucleotide and NTMTA.

#### 3.5.1.3. Immobilization of 5′-Aminoalkylated Oligonucleotide Through Route B

1. Treat the virgin glass microslide with ethanolic solution of NTMTA (85 µg, 0.2 µmol, and 2 mL) by evenly spreading over it.
2. Keep the microslide in a humid chamber maintained at 45°C for 40 min.
3. Wash the slide with ethanol (3 × 15 mL) and dry it under vacuum (*see* **Note 9**).
4. Spot the solution of 5′-aminoalkylated oligonucleotides (10 µ*M,* 0.5 µL) in reaction buffer 3 in triplicates.
5. Keep the spotted microslide in a humid chamber for 1 h at room temperature.
6. Remove the slide and keep it in blocking buffer 2 for 1 h followed by washing with washing buffer 4 (5 × 10 mL).
7. The slide is ready for hybridization (*see* **Note 7**).

#### 3.5.1.4. Immobilization of 5′-Mercaptoalkylated Oligonucleotide Through Route B

Immobilize the 5′-mercaptoalkylated oligonucleotide in a similar manner as previously described (*see* **Subheading 3.5.1.3.**) except that 30 min are required (**Subheading 3.5.1.3., step 5**) for completion of reaction between mercaptoalkylated oligonucleotide and tresyl functions on NTMTA-treated glass microslide.

### *3.5.2. Immobilization of 5′-Mercapto- and Aminoalkylated Oligonucleotides Using NTPAC*

#### 3.5.2.1. Immobilization of 5′-Aminoalkylated Oligonucleotide on C-8 Glass Microslide Through Route A

1. Take 1.5 $A_{260}$ units of the oligonucleotide sequence, $H_2N$–$(CH_2)_5$–$OPO_3$–d(AAT CGT TAC TTT TTA TTA TCC), dissolved in reaction buffer 3 (100 µL).
2. To the above solution, add NTPAC dissolved in DMF (9.1 mg, 0.2 *M*, 100 µL).
3. Agitate the reaction mixture at room temperature for 1 h.
4. After 1 h, concentrate the mixture under vacuum to remove the solvent.
5. Suspend the semisolid residue so obtained in 25 µL of dd water, centrifuge and discard the residue, which is nothing but the excess reagent (it is insoluble in water).
6. Dilute the decanted solution with 1 *M* LiCl to bring the final concentration of LiCl to 0.2 *M*.
7. Spot the anthraquinone-oligonucleotide conjugate so obtained on C-8 glass microslide (obtained after treatment of virgin glass microslide with a 5% solution

of octyltrimethoxysilane in toluene for 2 h at 50°C) in triplicates and allow the spots to dry in the air.
8. Keep the spotted slide in a UV chamber (operating at 365 nm, 150 W) for 30 min.
9. Then wash the slide with washing buffer 4 (3 × 20 mL) and followed by dd water (3 × 20 mL).
10. The slide is ready for hybridization (*see* **Note 7**).

#### 3.5.2.2. Immobilization of 5′-Mercaptoalkylated Oligonucleotide on C-8 Glass Microslide Through Route A

The procedure for the mercaptoalkyl oligonucleotide is similar to the previously described (*see* **Subheading 3.5.2.1.**) except that the reaction time for oligonucleotide HS–$(CH_2)_6$–$OPO_3$-d(AAT CGT TAC TTT TTA TTA TCC) and NTPAC is 30 min at room temperature (*see* **Subheading 3.5.2.1., step 3**).

#### 3.5.2.3. Immobilization of 5′-Aminoalkylated Oligonucleotide on C-8 Glass Microslide Through Route B

1. Spread evenly a solution of the reagent NTPAC (91 mg; 2.0 mL, 0.1 *M*) in DCM on C-8-coated glass microslide.
2. Keep the slide in a fume-cupboard and allow the solvent to evaporate.
3. Place the dried microslide in a UV chamber (operating at 365 nm, 150 W) for 30 min.
4. Wash the slide several times with dry DCM (5 × 20 mL) and dry under vacuum.
5. Then spot a solution of 5′-aminoalkylated oligonucleotide sequence, $H_2N$-$(CH_2)_5$-$OPO_3$-d(AAT CGT TAC TTT TTA TTA TCC), (10 μ*M*, 0.5 μL) in reaction buffer 3, in triplicates.
6. Keep the spotted microslide in a humid chamber for 1 h.
7. Then treat the plate with blocking buffer 2 (10 mL) for 1 h.
8. Finally, wash the glass slide with washing buffer 4 (3 × 20 mL) followed by dd water (3 × 20 mL).
9. The slide is ready for hybridization (*see* **Note 7**).

#### 3.5.2.4. Immobilization of 5′-Mercaptoalkylated Oligonucleotide on C-8 Glass Microslide Through Route B

The 5′-mercaptoalkylated oligonucleotide, HS–$(CH_2)_6$–$OPO_3$-d(AAT CGT TAC TTT TTA TTA TCC), is immobilized on NTPAC-activated glass microslide in a similar fashion as described earlier (*see* **Subheading 3.5.2.3.**) except that the reaction time is 30 min instead of 1 h (**Subheading 3.5.2.3., step 6**).

### *3.6. General Method for Hybridization Assay*

#### *3.6.1. Hybridization of Immobilized Oligonucleotides With Complementary Oligonucleotides*

1. Dissolve the complementary oligomer (cf. glass microslide prepared in **Subheadings 3.5.1.** and **3.5.2.**), for example, fluorescein-d(GGA TAA TAA AAA GTA ACG ATT) in hybridization buffer 5 (500 μL).

2. Place a spotted microslide in a Petri dish and paste a hybri-slip (Sigma) on the spotted area of glass microslide.
3. Fill the chamber with complementary oligomer solution (500 µL).
4. Close the holes on the hybri-slip, and place the Petri dish along with microslide in heated chamber at 65°C for 10 min.
5. Allow it to cool slowly to room temperature and then to 10°C with gentle shaking and keep it at that temperature for additional 3–4 h.
6. Remove the complementary oligomer solution with the help of pipetman, remove the hybri-slip and wash the plates with the same buffer 5 and after drying, visualize the fluorescent spots under laser scanner at 570 nm.

### *3.7. Specificity of Immobilization Chemistry and Detection of Mismatches*

1. Dissolve oligonucleotide sequences, $H_2N$–$(CH_2)_5$–$OPO_3$-d(CTC CTG AGG AGA AGG TCT GC), $H_2N$–$(CH_2)_5$–$OPO_3$-d(CTC CTG AGG CGA AGG TCT GC), $H_2N$–$(CH_2)_5$–$OPO_3$-d(CTC CTG CGG AGA ACG TCT GC), $H_2N$–$(CH_2)_5$–$OPO_3$-d(CTC CTG CGG CGA ACG TCT GC) and $H_2N$–$(CH_2)_5$–$OPO_3$-d(TTT TTT TTT TTT TTT TTT TT), (10 µ*M* each) in reaction buffer 3.
2. Spot these oligomers on an NTMTA-activated glass microslide (Route B), and keep it in a humid chamber at room temperature for 1 h.
3. Place the microslide in blocking buffer 2.
4. Hybridize the spotted oligomers with a complementary FAM-d(GCA GAC CTT CTC CTC AGG AG) as discussed in **Subheading 3.6.**
5. Wash the microslide with buffer 5 and subject to visualization under laser scanner.

### *3.8. Determination of Optimal Concentration of Oligonucleotide Spots on Surfaces for Fluorescence Detection*

#### *3.8.1. Using NTMTA*

1. Prepare at least five different concentrations (15, 10, 7.5, 5, and 2.5 µ*M*) of 3′-mercaptoalkyl-oligonucleotide sequence d($T^F$TT TTT TTT TTT TTT TTT TT)-$OPO_3$–$(CH_2)_6$SH ($T^F$ is fluorescein attached at C-5 position in thymidine-phosphoramidite) by diluting the stock solution using the reaction buffer 3 containing 10% DMSO (v/v).
2. Spot the glass microslide activated with NTMTA (Route B) with the serially diluted fluorescent oligonucleotide solution (0.5 µL), in duplicate, using a micropipet.
3. Keep the microslide at room temperature for 30 min in a humid chamber.
4. Wash the microslide with washing buffer 4 (5 × 20 mL) and dry under vacuum.
5. Visualize the spots under laser scanner and calculate the optimum concentration.

#### *3.8.2. Using NTPAC*

1. Make three different concentrations of fluorescein-d(TTT TTT TTT TTT TTT TTT TT-S-AQ) (fluorescein-labeled anthraquinone-oligonucleotide conjugate) (5, 10, and 15 µ*M*) in 0.2 *M* LiCl.

2. Spot the oligomer (0.5 µL) manually in triplicates on C-8 glass microslide.
3. Irradiate the slide by placing it in a UV chamber (operating at 365 nm, 150 W) for 30 min.
4. After irradiation, wash the slide with buffer 4.
5. Dry the slide and visualize the spots under laser scanner at 570 nm.
6. Optimum concentration can be calculated by visualizing the bright spot with lowest concentration under laser scanner.

### *3.9. Quantification of Immobilized Oligonucleotides*

1. Prepare various concentrations of d($T^F$TT TTT TTT TTT TTT TTT TT)-$OPO_3$–$(CH_2)_6SH$ (0.025, 0.05, 0.10, 0.25, 0.50, 0.75, and 1 µ*M*) by dissolving in buffer 4.
2. Spot each of these solutions on a virgin glass microslide in duplicate.
3. Dry the spots on the microslide and visualize under a laser scanner; measure the fluorescence signal intensity corresponding to each spot.
4. Plot a graph of fluorescence intensity against concentration (*see* **Note 10**).

### *3.10. Determination of Optimal Time Required for Immobilization of Oligomers on Virgin Glass Surface*

In order to determine the optimum time required to immobilize aminoalkyl- and mercaptoalkylated oligonucleotides on the glass surfaces, it is advisable to carry out a model experiment to study the immobilization of 1-O-(4,4′-dimethoxytrityl)-5-aminopentanol or 1-O-(4,4′-dimethoxytrityl)-6-mercaptohexanol on virgin CPG support (500 Å) using NTMTA by two different routes.

#### *3.10.1. Attachment of O-(4,4′-Dimethoxytrityl)-5-Aminopentan-1-ol on to CPG Through Route A*

1. Dissolve *O*-(4,4′-dimethoxytrityl)-5-aminopentan-1-ol (40.5 mg, 100 µmol) in ethanol (1 mL).
2. Similarly, dissolve NTMTA (42.5 mg, 100 µmol) in 1 mL ethanol.
3. Mix the previously listed two solutions and allow the reaction mixture to swirl for 1 h at room temperature in a shaker.
4. After 1 h, add 200 µL of this solution of conjugate to each of the eight Eppendorf tubes containing approx 10 mg of virgin CPG.
5. Place all these Eppendorf tubes in thermo mixer set at 45°C.
6. Remove the tubes one by one at different time intervals (10, 20, 30, 40, 50, 60, 120, and 180 min).
7. Wash the support in each tube with EDC (5 × 1 mL) containing TEA (0.1%), dry it under vacuum in a desiccator and subject it to loading determination.

#### *3.10.2. Attachment of O-(4,4′-Dimethoxytrityl)-6-Mercaptohexan-1-ol on to CPG Through Route A*

Mix an ethanolic solution of *O*-(4,4′-dimethoxytrityl)-6-mercapto- hexan-1-ol (43.6 mg, 0.1 mmol, 1.0 mL) with ethanolic solution of NTMTA, allow it to stir

for 30 min instead of 1 h in case of *O*-(4,4′-dimethoxytrityl)-5-aminopentan-1-ol and follow rest of steps as discussed in **Subheading 3.10.1.**

### *3.10.3. Attachment of O-(4,4′-Dimethoxytrityl)-5-Aminopentan-1-ol on to CPG Through Route B*

1. Dissolve NTMTA (42.5 mg, 100 µmol) in 1 mL ethanol.
2. Weigh out approx 10 mg of CPG (500 Å) in eight different Eppendorf tubes and add the previously listed solution (100 µL) to each of them.
3. Keep the tubes in a thermo mixer set at 45°C.
4. Take out the tubes at different time intervals, i.e., 10, 20, 30, 40, 50, 60, 120, and 180 min.
5. Wash the support in each tube with ethanol (5 × 1 mL) followed by diethyl ether (2 × 1 mL).
6. After drying the support, add an ethanolic solution of *O*-(4,4′-dimethoxytrityl)-5-aminopentan-1-ol (4.05 mg, 10 µmol, 100 µL) to each of these tubes.
7. Keep the suspension on a mixer at room temperature for 1 h and then wash the contents of each tube with ethanol (5 × 1 mL) and diethyl ether (2 × 1 mL).
8. Dry the support in each tube under vacuum and subject it to loading determination.

### *3.10.4. Attachment of O-(4,4′-Dimethoxytrityl)-6-Mercaptohexan-1-ol on to CPG Through Route B*

For the attachment of O-(4,4′-dimethoxytrityl)-6-mercaptohexan-1-ol on to CPG, first follow **steps 1–5** as given in **Subheading 3.10.3.** Then add an ethanolic solution of *O*-(4,4′-dimethoxytrityl)-6-mercaptohexan-1-ol (4.36 mg, 10 µmol, 100 µL) to each of the eight tubes in **step 6** (*see* **Subheading 3.10.3.**) and allow it to react for 30 min. Follow rest of steps as discussed in **Subheading 3.10.3.**

### *3.10.5. Determination of Extent of Reaction on the Support*

As the dimethoxytrityl cation absorbs at 498 nm, the deoxyribonucleoside loading can be determined by spectrophotometric determination of the released dimethoxytrityl cation on acidic treatment of a sample of support ***(47)***.

1. Weigh an accurate amount of support (1–2 mg) and treat it with detritylating solution (a mixture of 51.4 mL of 70% perchloric acid and 46 mL of methanol) for 5 min.
2. Measure the released dimethoxytrityl cation at 498 nm.
3. Calculate the loading on the support in µmol/g using the formula:

$$\text{Loading (µmol/g of support)} = \frac{14.3\text{X } A_{498} \text{ X vol (mL) of detritylating solution}}{\text{wt. of support (mg)}}$$

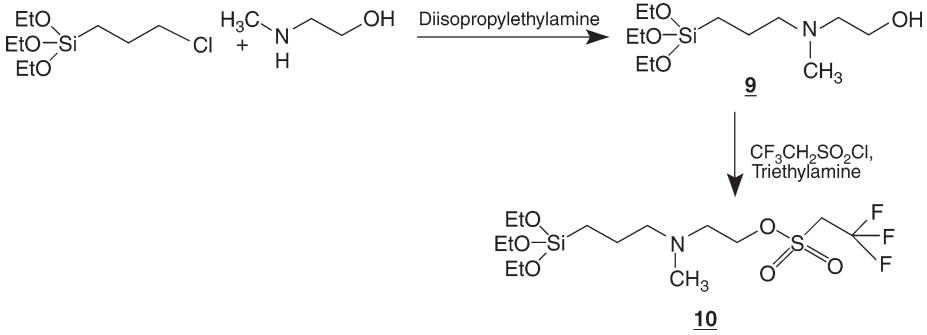
4. Plot a graph of loading on the support (µmol/g) vs time of reaction (min).

### *3.11. Discussion*

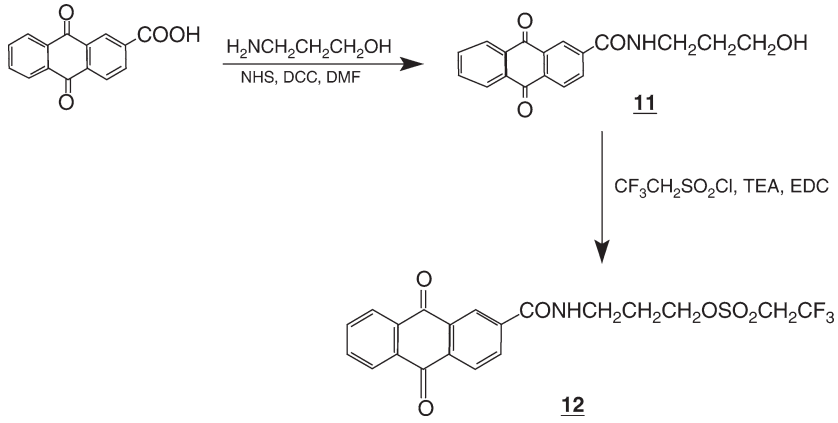
A number of hetero- and homobifunctional reagents have been reported to immobilize biomolecules on a variety of polymer supports. Most commonly used reagents are either based on thermochemical or photochemical reactive groups. Generally, the thermochemical reactive groups containing reagents require the presence of nucleophilic functions (–SH, $–NH_2$) in the ligand molecules, or on the polymer surface. A number of methods are currently available where modified oligonucleotides are immobilized covalently on polymer surfaces for the construction of microarrays. However, looking at the advantages of using heterobifunctional reagents for the construction of microarrays, the following points have been taken into consideration during designing of newer reagents (NTMTA and NTPAC): (1) the preparation of the reagents must be straightforward and involve commonly available reagents and chemicals; (2) these must be sufficiently stable during storage; and (3) one end of the reagents must contain an active chemical group specific toward aminoalkyl and mercaptoalkyl functionalities, easy to introduce at the 5′-end of oligonucleotides during synthesis in the machine itself, whereas, the other end of the reagents should possess either a thermochemical reactive group (triethoxysilyl in case of NTMTA, reactive toward glass surface) or a photochemical reactive group (anthraquinone in case of NTPAC, reactive toward any C–H containing surfaces).

During immobilization using these reagents and for finding the optimal concentration required for the visualization of the oligonucleotide, the oligonucleotide required modification at 3′- and 5′-end. The preparation of reagents and supports for modified oligonucleotides has been depicted in **Schemes 1–3**. These reagents have been used to incorporate modifications in the oligonucleotides in a regioselective manner. The preparation of new heterobifunctional reagents are shown in **Schemes 4** and **5** starting from commercially available chemicals. These are characterized by NMR and mass spectrometry. Subsequently, the utility of these reagents have been demonstrated by immobilizing a number of oligonucleotides on different surfaces following two routes (**Schemes 6** and **7**). The immobilized oligonucleotides can be seen under laser scanner after hybridizing the spotted oligomers with their complementary-labeled oligomers.

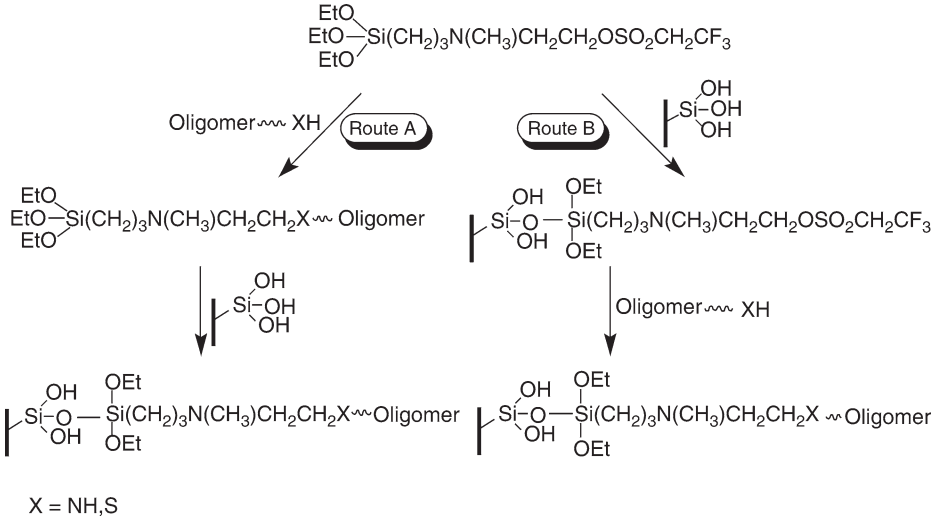
In order to arrive at the optimum time required to immobilize the 5′-modified (mercaptoalkyl or aminoalkyl) oligonucleotides on a glass surface using NTMTA, an indirect experiment has been designed using $DMTrO–(CH_2)_5-NH_2$ and $DMTrO(CH_2)_6-SH$ on CPG following both the routes. The results are shown in **Fig. 1A**,**B**. From these data, it can be concluded that the immobilization of mercaptoalkyl and aminoalkylated ligands on CPG using NTMTA essentially



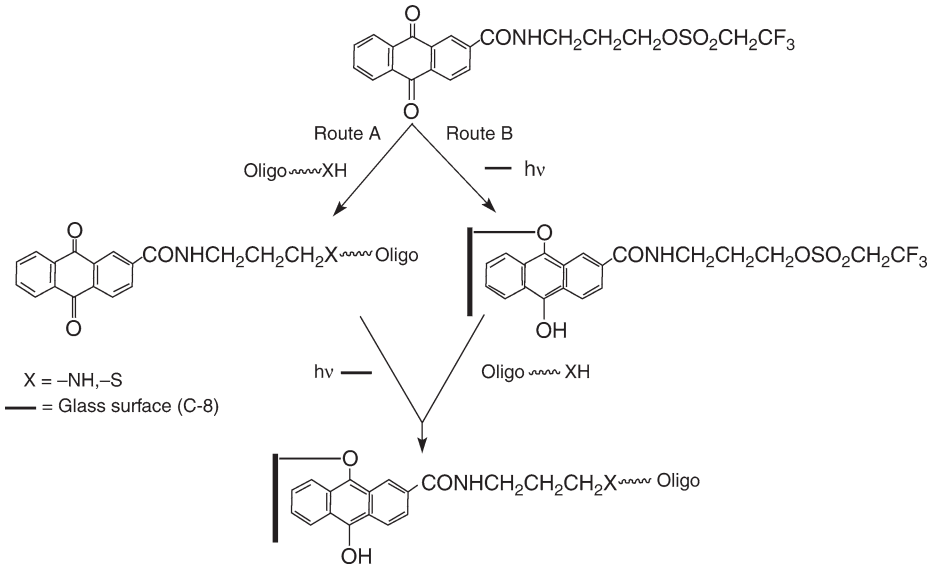

Scheme 4. Synthesis of NTMA reagent.




Scheme 5. Synthesis of NTPAC reagent.




Scheme 6. Immobilization of oligonucleotides using NTMA.




Scheme 7. Immobilization of oligonucleotide using NTPAC.

completes in 40 min at 45°C. Therefore, the same time period has been used for immobilization of oligonucleotides on glass surface through route A or B.

Similarly, to find out the optimal concentration required for visualization of an oligonucleotide on the glass slide, a virgin glass microslide was coated with NTMTA (route B) and spotted an oligomer, d($T^{F}$TT TTT TTT TTT TTT TTT TT)-$OPO_3$–$(CH_2)_6$–SH), in different concentrations (2.5, 5, 7.5, 10, and 15 μ*M*). After usual washings, the slide was scanned and the spots visualized under a laser scanner set at 570 nm (**Fig. 2**, **Lane 1**). The immobilization efficiency against each concentration can be determined with the help of the standard curve (**Fig. 3**) and is depicted in **Fig. 2** (**Lane 2**). Though the spots corresponding to 2.5–7.5 μ*M* concentration can be visualized easily, however, to have a good quality bright spots, a 10 μ*M* concentration was selected for construction of microarrays. The same experiment is repeated with NTPAC reagent and the results are shown in **Fig. 4**.

Using the earlier-mentioned parameters, i.e., optimal concentration and time required for immobilization of oligonucleotides through NTMTA, a number of 5′-amino- and mercaptoalkylated oligonucleotides have been spotted following both the routes and, visualized after hybridizing with complementary-labeled oligomers under laser scanner. The spots are shown in **Fig. 5A**,**B**. The fluorescent signals as well as morphology of the spots on microslides prepared through route A and route B are almost identical. Similarly, 5′-amino- and mercaptoalkylated oligonucleotides have been spotted using NTPAC reagent and

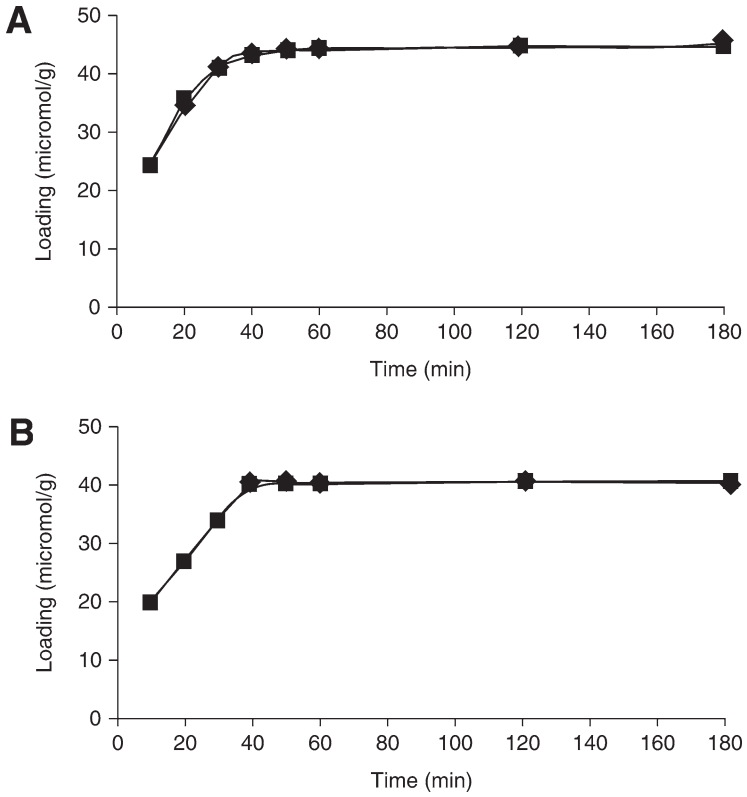



Fig. 1. Time kinetics to determine the optimal time required to immobilize oligonucleotides on unmodified glass surface through route A **(A)** and route B **(B)**. Squares, 5′-mercaptoalkylated ligand; diamonds, 5′-aminoalkylated ligand.

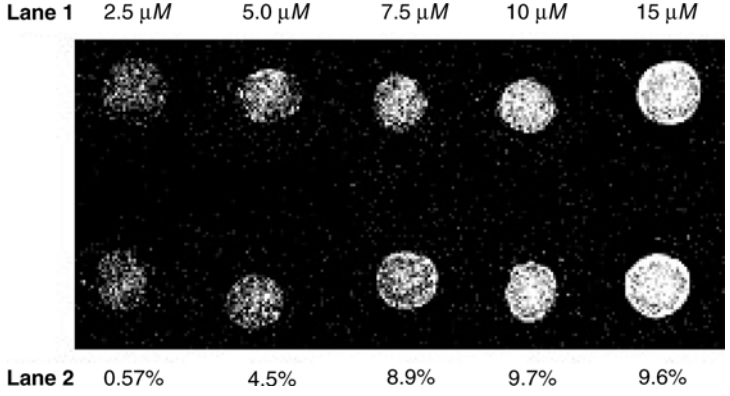



Fig. 2. Threshold concentration of oligonucleotide sequence, d($T^{F}$TT TTT TTT TTT TTT TTT TT)-$OPO_3$-$(CH_2)_6$SH (entry 8, **Table 1**), required for visualizing fluorescence under a laser scanner. Concentration of spots (2.5, 5, 7.5, 10, and 15 μ*M*) **(Lane 1)**; immobilization efficiency **(Lane 2)**. Reagent: NTMTA.

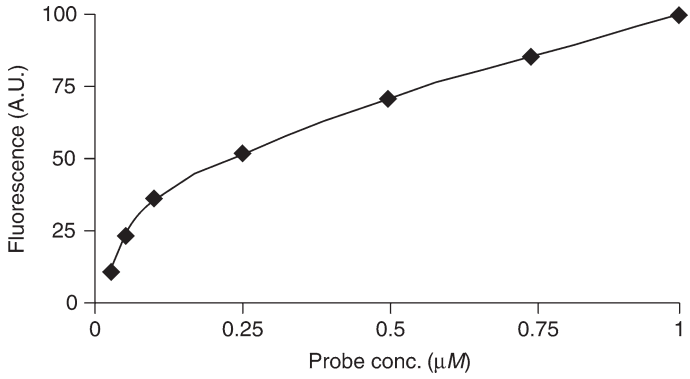



Fig. 3. A correlation sketch between concentration of immobilized probe and fluorescence intensity. Fluorescent oligonucleotide, d($T^F$TT TTT TTT TTT TTT TTT TT)-$OPO_3$-$(CH_2)_6$SH (entry 8, **Table 1**), was spotted in 0.025–1.0 μ*M* concentrations. The spotted microslide was scanned under a laser scanner. Reagent: NTMTA.

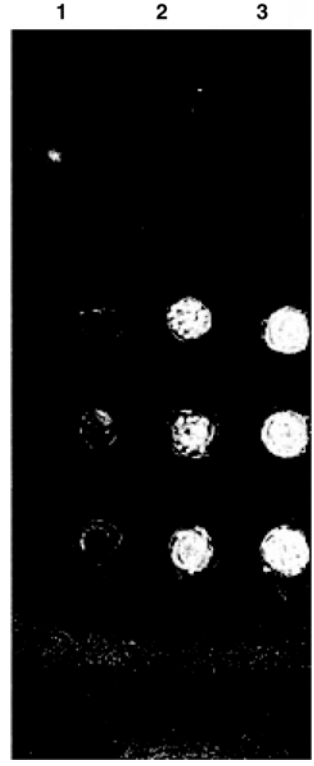



Fig. 4. Threshold concentration of oligonucleotide sequence, fluores-d(TTT TTT TTT TTT TTT TTT TT)$OPO_3$-$(CH_2)_6$S-AQ (entry 18, **Table 1**), required for observance of fluorescence under laser scanner. **Lane 1**, 5 μ*M*; **Lane 2**, 10 μ*M*; **Lane 3**, 15 μ*M*. Reagent: NTPAC.

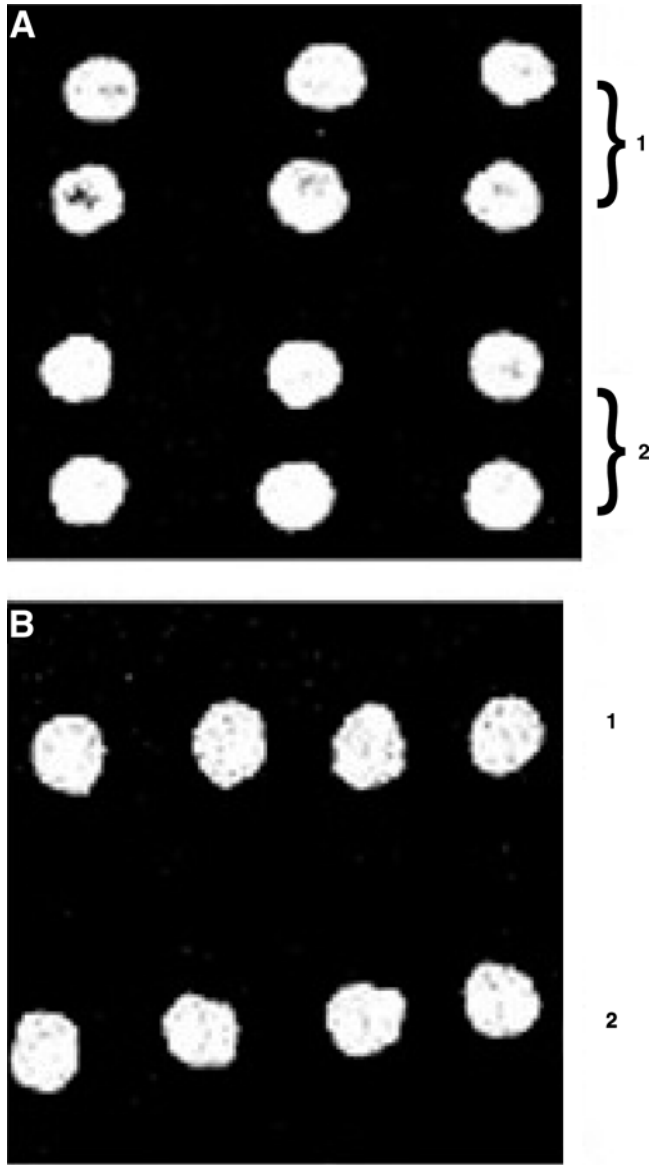



Fig. 5. **(A)** Immobilization of oligonucleotides using NTMTA through route A. (**Lane 1**) $H_2N$-$(CH_2)_5$-$OPO_3$-d(CAG AGG TTC TTT GAG TCC TT) (entry 1, **Table 1**) and (**Lane 2**) HS-$(CH_2)_6$-$OPO_3$-d(CAG AGG TTC TTT GAG TCC TT) (entry 2, **Table 1**) visualized after hybridization with FAM-d(AAG GAC TCA AAG AAC CTC TG) (entry 6, **Table 1**). **(B)** Immobilization of oligomers through route B. (**Lane 1**) $H_2N$-$(CH_2)_5$-$OPO_3$-d(CTC CTG AGG AGA AGG TCT GC) (entry 4, **Table 1**) and (**Lane 2**) HS-$(CH_2)_6$-$OPO_3$-d(CTC CTG AGG AGA AGG TCT GC) (entry 3, **Table 1**) visualized after hybridization with FAM-d(GCA GAC CTT CTC CTC AGG AG) (entry 7, **Table 1**).

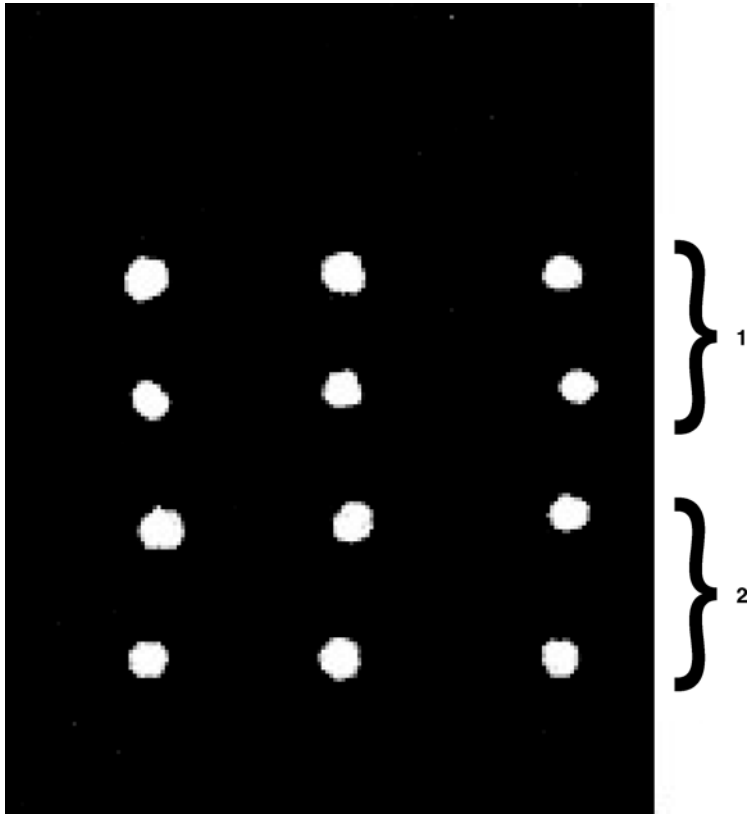



Fig. 6. Immobilization of oligonucleotides $H_2N$-$(CH_2)_5$-$OPO_3$-d(AAT CGT TAC TTT TTA TTA TCC) (entry 12**, Table 1**) (**Lane 1**) and HS-$(CH_2)_6$-$OPO_3$-d(AAT CGT TAC TTT TTA TTA TCC) (entry 13, **Table 1**) (**Lane 2**) in triplicates, using NTPAC and their hybridization with fluorescein–d(GGA TAA TAA AAA GTA ACG ATT) (entry 16, **Table 1**) and visualization under laser scanner at 570 nm.

visualized under laser scanner. **Figure 6** shows the immobilization pattern of oligonucleotides after hybridization with complementary-labeled oligomer.

The specificity of immobilization chemistry and detection of mismatches in case of oligonucleotides immobilized with the help of NTMTA have been depicted in **Fig. 7**. The perfectly matched duplex gives the maximum intensity (**Lane 1**) while the spots having one (**Lane 2**), two (**Lane 3**), and three mismatches (**Lane 4**) show fluorescence intensities in decreasing order and noncomplementary (**Lane 5**) did not emit any signal at all. It is quite evident from these results that the immobilized oligonucleotides are specific toward complementary oligonucleotides and nonspecific hybridization does not occur (**Lane 5**). The signal-to-noise ratio, in all the cases, is >98, as calculated from the signal obtained from complementary hybridized (**Lane 1**) and noncomplementary

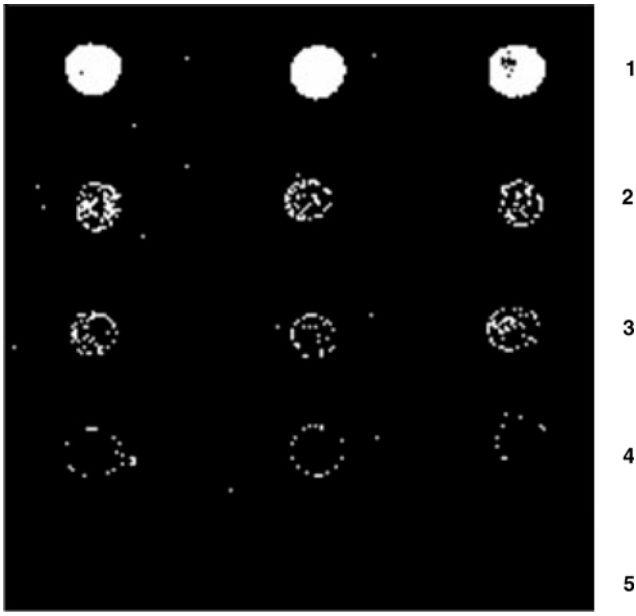



Fig. 7. Detection of nucleotide mismatches and specificity of immobilization through hybridization with FAM labeled complementary oligonucleotides. (**Lane 1**) $H_2N$-$(CH_2)_5$-$OPO_3$-d(CTC CTG AGG AGA AGG TCT GC) (entry 4, **Table 1**); (**Lane 2**) $H_2N$-$(CH_2)_5$-$OPO_3$-d(CTC CTG AGG CGA AGG TCT GC) (entry 5, **Table 1**); (**Lane 3**) $H_2N$-$(CH_2)_5$-$OPO_3$-d(CTC CTG CGG AGA ACG TCT GC) (entry 9, **Table 1**); (**Lane 4**) $H_2N$-$(CH_2)_5$-$OPO_3$-d(CTC CTG CGG CGA ACG TCT GC) (entry 10, **Table 1**); (**Lane 5**) $H_2N$-$(CH_2)_5$-$OPO_3$-d(TTT TTT TTT TTT TTT TTT TT) (entry 11, **Table 1**). Hybridization was carried out using FAM-d(GCA GAC CTT CTC CTC AGG AG) (entry 7, **Table 1**) followed by laser scanning. Reagent: NTMTA.

hybridized oligomers (**Lane 5**) at 10 μ*M* concentration. The low noise background in this system might be because of the fact that the surface is not aminated (positively charged) and therefore, not susceptible to nonspecific interactions with oligonucleotides (polyanions). A similar pattern has been obtained in case of oligonucleotides (matched and mismatched) immobilized with the help of NTPAC as shown in **Fig. 8**.

The thermal stability of the constructed arrays can be evaluated by exposing them five times to three sets of temperature conditions (encountered during polymerase chain reaction). These consist of 94°C for 30 s (denaturing step), 54°C for 30 s (annealing step), and then 75°C for 30 s (extension step) in buffer 6. **Figure 9** shows the comparison of intensities of fluorescence signals of heat-treated and untreated microarrays after hybridization with complementary-labeled oligomers. The signal intensity of heat-treated microarray decreases by only 1.6% as compared to pretreated microarray.

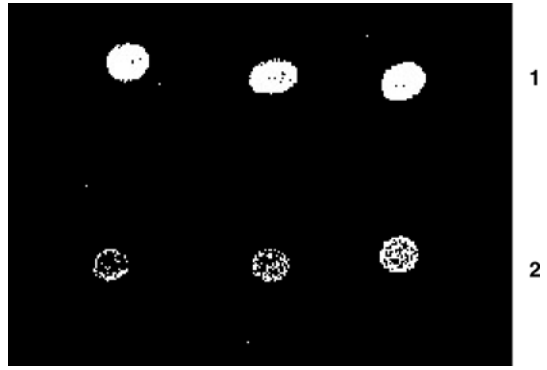



Fig. 8. Detection of single nucleotide mismatch through hybridization with fluorescein-labeled oligonucleotide. (**Lane 1**) HS-$(CH_2)_6$-$OPO_3$-d(TTG GGT CCG CCA CTC CTT CCC) (entry 14, **Table 1**), and (**Lane 2**) HS-$(CH_2)_6$-$OPO_3$-d(TTG GGT CCG CTA CTC CTT CCC) (entry 15, **Table 1**). Hybridization was carried out using fluorescein-d(GGG AAG GAG TGG CGG ACC CAA) (entry 17, **Table 1**). Reagent: NTPAC.

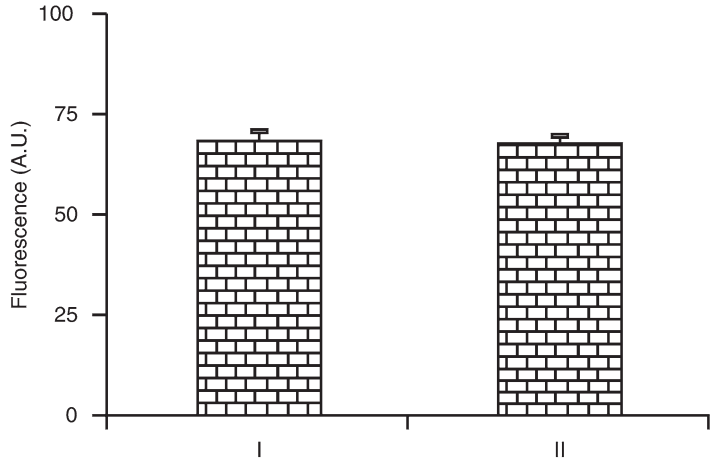



Fig. 9. Thermal stability of immobilized oligonucleotides (microarray). Comparison on the basis of fluorescence signals obtained after hybridization of heat-treated and untreated arrays of oligonucleotides. I, untreated microslide; II, heat-treated microslide.

## 4. Notes

1. Use freshly opened bottle of aq ammonia (30%) for clean deprotection of oligonucleotides.
2. It is always recommended to treat mercaptoalkylated oligonucleotides with 50 m*M* DTT solution before their use for immobilization reactions as they are susceptible to arial oxidation on storage.

3. Handle tresyl chloride container with great care as it might develop internal pressure. It is moisture sensitive and reacts violently with water.
4. All the apparatus must be baked in oven and flushed with argon. Because tresylation reaction is extremely moisture sensitive, carry out the reaction in a septum-sealed reaction-vessel fitted with a pressure equalizing syringe.
5. NTMTA and NTPAC should be stored in tightly closed containers under anhydrous conditions at 4°C.
6. Glass microslides must be thoroughly cleaned before their use to remove adsorbed or greasy materials.
7. Spotted microslides can be stored at 4°C and regenerated by placing them in a humid chamber for 1 h before hybridization assay.
8. This oligomer-triethoxysilyl conjugate is stable over several months at 4°C.
9. The tresylated microslides are stable and can be stored for several months at 4°C.
10. The same curve can be used to quantify all the microarrays prepared using the proposed method.

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