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Preparation of oligonucleotide microarrays on modified glass using a photoreactive heterobifunctional reagent, 1-*N*-(maleimidohexanoyl)-6-*N*-(anthraquinon-2-oyl) hexanediamine (MHAHD)

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ABSTRACT

A novel approach for the construction of oligonucleotide microarrays under the influence of light and microwaves is described. For that purpose, a new heterobifunctional crosslinking reagent, 1-*N*-(maleimidohexanoyl)-6-*N*-(anthraquinon-2-oyl) hexanediamine (MHAHD), possessing a photoactive anthraquinone moiety on one terminus and an electrophilic maleimide group on the other one, was developed. The immobilization of oligonucleotides using the reagent, MHAHD, was realized via two routes (A and B). In order to speed up the immobilization procedure, the reaction between 3′- or 5′-mercaptoalkylated oligonucleotides and maleimide moiety of MHAHD was carried out under microwaves in just 15 min. The oligonucleotide arrays produced by both the routes were analyzed by hybridization experiments (hybridization efficiency 30.13%) and subsequently used for the discrimination of base mismatches. The constructed microarrays were found to possess good thermal stability (only ~4.5% loss of fluorescence intensity observed after ten cycles).

1. Introduction

Chemical crosslinking reagents find a wide variety of applications such as preparation of affinity matrices, modification and stabilization of diverse structures, identification of ligands and receptor binding sites and structural studies and so forth [1]. The ability to link two proteins or molecules having different binding specificities or catalytic activities has opened the potential for creating a new universe of unique and powerful reagent systems for assay and targeting applications. Chemical crosslinking reagents, an outgrowth of protein modification chemistry, are designed to have specific reactivity for functional groups contained in each reactant. A large number of homo- and heterobifunctional reagents are known [2–8] in the literature. Since heterobifunctional reagents possess two selectively reactive groups that allow coupling to be carried out in a stepwise manner, better control of the conjugation chemistry is attainable. These reagents find a variety of applications in modern molecular biology and serve as molecular tools for attachment of biomolecules such as proteins, peptides, nucleic acids and enzymes on a variety of surfaces. Recently, these covalently immobilized biomolecules have yielded bio-surfaces for molecular analysis, separation, targeting, synthesis and diagnosis. Most commonly used reagents are based on the thermochemical reactive groups [1–8]. Light-dependent immobilization procedures have been introduced [3] to circumvent drawbacks imposed by thermochemical reactions, such as requirement of the 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 on polymer surfaces for the construction of microarrays. Most of them utilize functionalized polymer surfaces for this purpose [9].

Recently, oligonucleotide arrays have emerged as a powerful tool for genetic analysis with higher throughput and reproducibility than the traditional gel-based methods. Arrays of DNA molecules, either as double stranded segments or short single-stranded oligonucleotides have been utilized for different molecular biology applications including mutation detection [10,11], gene expression monitoring [12,13], DNA sequencing [14], genome analysis [15,16], immunology [17] and medical diagnostic of genetic diseases [18]. Two general methods have established themselves for producing DNA microarrays, viz., (a) direct on-surface synthesis of oligonucleotides [19–22], and (b) the immobilization of presynthesized oligonucleotides, i.e. deposition method [23–30]. Concerning the direct synthesis of oligonucleotides on the surface, the best known method is photolithographic oligonucleotide

Abbreviations: MHAHD, 1-*N*-(maleimidohexanoyl)-6-*N*-(anthraquinon-2-oyl) hexanediamine; THF, tetrahydrofuran; NHS, *N*-hydroxysuccinimide; DCC, dicyclohexylcarbodiimide; TLC, thin layer chromatography; NMI, *N*-methylimidazole; DMSO, dimethylsulfoxide

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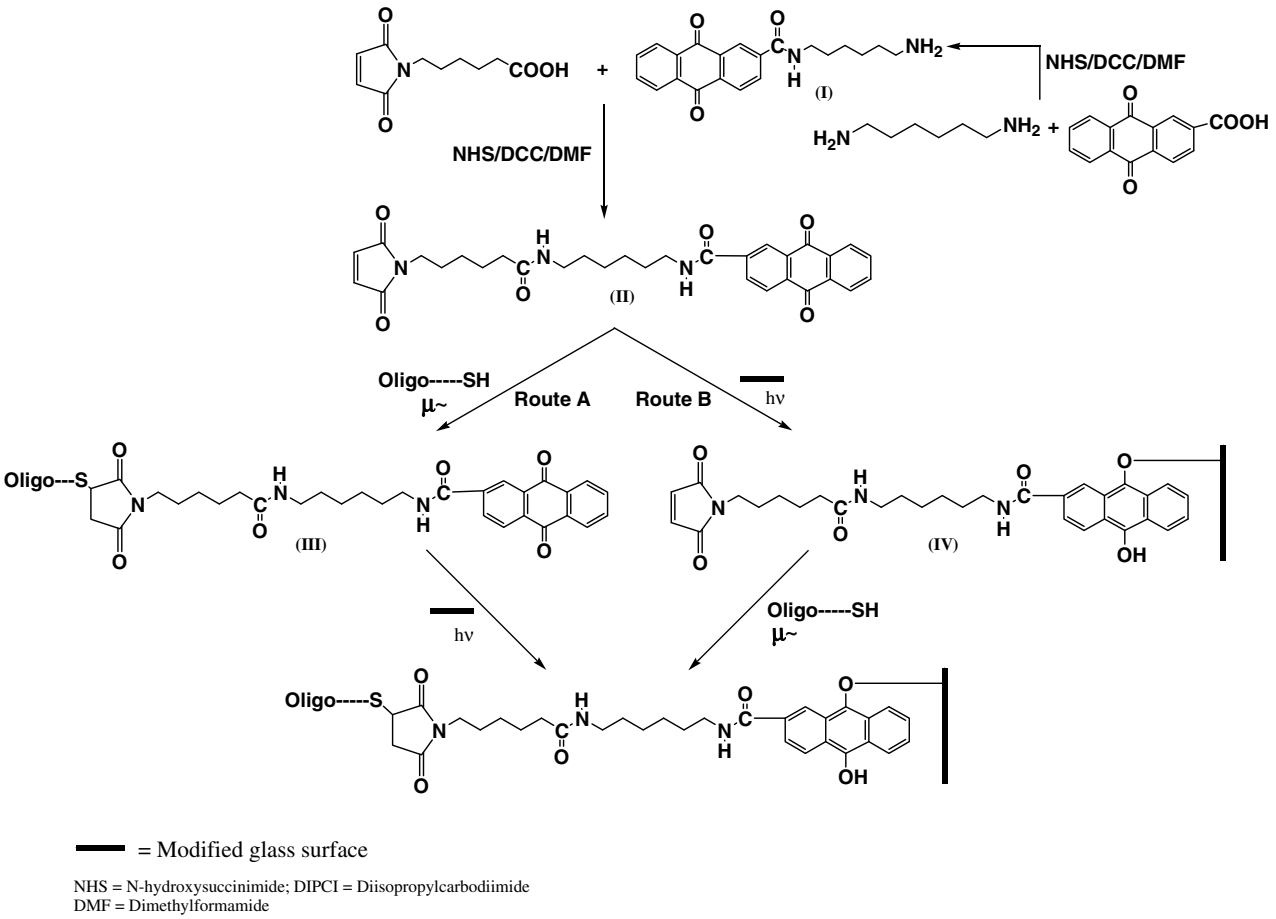

Scheme 1. Synthesis of the reagent, 1-*N*-(maleimidohexanoyl)-6-*N*-(anthraquinon-2-oyl) hexanediamine (MHAHD), and immobilization of oligonucleotides.

synthesis, which allows the generation of high-density oligonucleotide microarrays (up to 10^6 probes). The method is expensive and does not offer flexibility, which limits its widespread implementation. However, the patterning of pre-synthesized oligonucleotides is the preferred method for many research applications requiring low-to-moderate density arrays (up to few hundred probes). This method offers great flexibility and can accommodate different chemistry as well as surfaces of choice. However, for these microarrays to replace conventional diagnostic methods, substrates have to be well characterized with respect to thermal and chemical stability, absolute density of immobilized oligonucleotides, availability of oligonucleotide probes for hybridization and reproducibility of the attachment chemistry.

Primarily due to its transparency, low cost, non-porous nature and resistance to high temperature, glass is a popular material for oligonucleotide microarray technology. Glass possesses surface functionality, which renders it suitable for robust derivatization and oligonucleotide attachment chemistry. It also possesses very low intrinsic fluorescence, which improves the signal-to-noise ratio when high-intensity lasers are used for detection of labeled oligonucleotides. Central to the deposition technologies is the development of efficient chemistry for covalent attachment of oligonucleotides on glass. A number of attachment methods have been published, which vary widely in chemical mechanism, ease of use, probe density and stability. Most of the procedures reported for oligonucleotide immobilization are based on thermochemical reactions, i.e. covalent bond formation between ligands having amino, carboxyl, thiol or aldehyde functions and suitably modified solid supports. These methods often require derivatization of oligonucleotides, glass surface, or both. In addition, the thermochemical reaction may effect biological activity of the ligand and result in multiple-site attachment of individual molecules. Since photoactivatable reagents are topically addressable (i.e. regiospecific immobilization of oligonucleotide probes on a glass microslide in desired orientation), photoimmobilization of biomolecules have become a subject of intense research towards the development of biosensors and biomaterials.

Here, we report the synthesis of a new heterobifunctional reagent, 1-*N*-(maleimidohexanoyl)-6-*N*-(anthraquinon-2-oyl) hexanediamine (MHAHD) **II** (Scheme 1), for the preparation of oligonucleotide microarrays under the influence of light and microwaves. The reagent, MHAHD, carries sulfhydryl-reactive maleimide group on one of the termini, to react with 3′- or 5′-mercaptoalkylated oligonucleotides and an anthraquinone function on the other end, for light dependent covalent attachment to polymer surface. The immobilization of oligonucleotides using the proposed reagent was accomplished via two routes. In route A, an oligonucleotide-anthraquinone conjugate **III** (Scheme 1) was first formed by allowing the reagent, MHAHD, to react with mercaptoalkylated oligonucleotide under microwaves, an alternative energy source [31,32], which was subsequently immobilized on C–H containing polymer surface under UV irradiation (365 nm). In route B, MHAHD was brought in contact with polymer surface under UV light to generate maleimide functions on it **IV** (Scheme 1), which were subsequently coupled with 3′- or 5′-mercaptoalkylated oligonucleotides under microwaves in 15 min. Both the routes worked satisfactorily and the constructed microarrays were analyzed by hybridization assay. The versatility and applicability of

the proposed method were investigated by detection of nucleotide mismatches. The thermal stability of the immobilization chemistry was evaluated by subjecting the constructed microarray to PCR-like conditions.

2. Experimental part

2.1. Materials

Anthraquinone-2-carboxylic acid, dicyclohexylcarbodiimide, 1,6-hexanediamine, *N*-hydroxysuccinimide and Poly-Prep™ glass microslides were procured from Sigma Chemical Co. (St. Louis, MO). TET-phosphoramidite was obtained from Applied Biosystems, Inc. (Foster City, CA). All other chemicals were procured locally.

2.1.1. Photochemical reactions

Photochemical reactions involving anthraquinone moiety were carried out in an argon purged UV chamber (Gautam Enterprises, New Delhi, India) equipped with long wavelength tubes (365 nm) and a variable energy regulator.

2.1.2. Microwave assisted reactions

Reactions involving maleimidoalkyl groups and mercaptoalkylated oligonucleotides were carried out in a domestic microwave oven (BPL-Sanyo, India), operating at 800 W (2750 Hz). Each exposure was of 20 s, followed by cooling in an ice-bath for 5 s to room temperature.

2.1.3. Laser scanning

Spotted glass microslides were visualized under a laser scanner operating at 570 nm (ScanArray Lite, MicroArray Analysis System, GSI Lumonics, Packard Biosciences, USA). Fluorescence intensity of the spots was determined by Quant Array software.

2.1.4. Buffers

(i) Reaction buffer, 0.1 M sodium phosphate containing 0.1 M NaCl (pH 7.0).
(ii) Capping buffer, 10 mM phosphate buffer saline (PBS) containing 2% bovine serum albumin (BSA).
(iii) Hybridization buffer, 0.1 M sodium phosphate containing 1.0 M NaCl (pH 7.5).

2.2. Synthesis of N-(6-aminohexyl)anthraquinone-2-carboxamide I

To a solution of anthraquinone-2-carboxylic acid (3.32 mmol) in anhydrous THF (20 ml), *N*-hydroxysuccinimide (NHS) (4.98 mmol) and dicyclohexylcarbodiimide (DCC) (3.984 mmol) were added. The reaction mixture was allowed to stir for 3 h at room temperature until complete conversion took place, as monitored by thin layer chromatography (TLC). The precipitated dicyclohexylurea was filtered off and the filtrate containing *N*-hydroxysuccinimide ester of anthraquinone-2-carboxylic acid was added dropwise to a solution of 1,6-hexanediamine (6.0 mmol) in THF (10 ml) in the presence of triethylamine (1.2 mmol) at 0 °C. The reaction mixture was further allowed to stir overnight at room temperature. After completion of reaction, the reaction mixture was evaporated to a syrupy mass. The residue was dissolved in dichloroethane and washed successively with 5% sodium bicarbonate (2 × 20 ml), 5% citric acid (2 × 20 ml) and saturated solution of sodium chloride (1 × 20 ml). The organic phase was collected, dried over anhydrous sodium sulfate and concentrated to obtain *N*-(6-aminohexyl)anthraquinone-2-carboxamide **I**, as a syrupy mass in ~60% yield. The compound was characterized by ^{1}H NMR and mass spectroscopy. ^{1}H NMR ($CDCl_3$) δ: 1.2–1.54 (m, 8H, 4 × – CH_2–), 2.92 (t, 2H, $-NCH_2$–), 3.18 (br t, 2H, $-NCH_2$–), 7.2–8.4 (m, 7H, Ar–H). MS (*m*/*z*): 351 ($M + H^+$).

2.3. Synthesis of 1-N-(maleimidohexanoyl)-6-N-(anthraquinon-2-oyl)hexanediamine (MHAHD) II

N-Maleimidocaproic acid required for the preparation of compound **II** was synthesized according to the published procedure [33]. To a solution of *N*-maleimidocaproic acid (1 mmol) in anhydrous THF (15 ml), NHS (1.5 mmol) and DCC (1.2 mmol) were added. The reaction was allowed to stir at room temperature for 3 h. After complete conversion of *N*-maleimidocaproic acid to its active-ester, (as monitored by TLC), dicyclohexylurea was filtered off and the filtrate was added to a solution of *N*-(6-aminohexyl)anthraquinone-2-carboxamide **I** (1 mmol) in THF (10 ml) and further allowed to stir for 6 h at room temperature. Then the reaction mixture was concentrated under vacuum, dissolved in dichloroethane (75 ml) and washed with aqueous 5% sodium bicarbonate (2 × 30 ml), 5% citric acid (2 × 30 ml) and saturated solution of sodium chloride (1 × 30 ml), respectively. The organic phase was collected, dried over sodium sulfate, and concentrated under vacuum to obtain the desired reagent, MHAHD **II**, as syrupy material. The crude reagent was purified by silica gel column chromatography using dichloroethane: methanol, 95:5 (v/v), as an eluent. The fractions containing the pure material were pooled together and concentrated on a rotary evaporator to obtain the compound **II** in ~82% yield. The reagent was characterized by ^{1}H NMR and mass spectroscopy. ^{1}H NMR ($CDCl_3$) δ: 1.23–1.57 (m, 14H, 7 × $-CH_2$–), 2.21 (t, 2H, $-CH_2CO$–), 3.25–3.52 (br m, 6H, $-NCH_2$–), 6.84 (dd, 2H, $-CH{=}CH$–), 7.34–8.58 (m, 7H, Ar–H); MS (*m*/*z*): 544 ($M + H^+$).

2.4. Modification of Poly-lysine glass plates

Poly L-lysinylated microslides were treated with a capping solution (acetic anhydride: triethylamine: NMI: dichloroethane:: 1: 1: 0.4: 6, v/v, 50 ml) for 2 h at room temperature. Then the slides were washed extensively with dichloroethane (5 × 40 ml), diethylether (2 × 40 ml) and dried in a desiccator.

2.5. Oligonucleotide synthesis and purification

The oligonucleotides were synthesized at 0.2 μmol scale on an automated DNA synthesizer following the standard phosphoramidite approach. In order to incorporate reactive thiol (–SH) group at 5′-end of the oligonucleotides, the last coupling was performed with the reagent, *S*-benzoyl-6-mercaptohexyl-2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite [34]. The coupling was performed in an analogous manner to that of normal nucleoside phosphoramidite. Similarly, complementary labeled oligonucleotides were also synthesized in the same manner by performing the last coupling with TET-phosphoramidite. Labeled oligonucleotides having thiol modification at 3′-end were synthesized on the thiol polymer support [35] by carrying out the last coupling with TET-phosphoramidite. After usual work-up, desalting was carried out on ODS-silica gel column and elution was effected with 30% acetonitrile in water. Desalted oligomers were subjected to purification on RP–HPLC, followed by lyophilization.

2.6. Determination of optimal concentration of oligonucleotide for fluorescent detection

To arrive at the threshold concentration required to visualize an oligonucleotide on modified poly-lysinylated microslide, the modified slide was activated with MHAHD **II**. For this purpose, a solution of MHAHD **II** (1 ml, 0.01 M in DMF) was spread evenly using a spin coater on a modified microslide and the solvent was allowed

to evaporate in a desiccator under vacuum. The dried microslide was exposed to UV light (365 nm) in a photochemical reactor for 30 min [7]. After thorough washing with DMF (5 × 40 ml) and diethyl ether (2 × 40 ml), the microslide was dried under vacuum. An oligomer sequence, 5′ TET-d(TTT TTT TTT TTT TTT TTT TT)-OPO_3-$(CH_2)_6$SH 3′, was serially diluted in four different concentrations (2.5, 5, 10 and 20 μM) in the reaction buffer containing 10% DMSO (v/v) and spotted (0.5 μl) manually using a 2 μl pipetteman onto a MHAHD-activated microslide **IV** in duplicates. The spotted microslide was kept on a cold water soaked bloating paper in a covered petridish (~100% humidity) and exposed to microwaves for 15 min (45 × 20 s) [36], followed by washing with the reaction buffer (3 × 40 ml) each for 15 min. The microslide was dried and visualized under a laser scanner at 532 nm.

2.7. Specificity of immobilization chemistry and detection of mismatches

Four oligonucleotide sequences, viz., 5′ HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG AGA AGG TCT GC) 3′; 5′ HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG TGA AGG TCT GC) 3′; 5′ HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG TCA AGG TCT GC) 3′; 5′ HS-$(CH_2)_6OPO_3$-d(TTT TTT TTT TTT TTT TTT TT) 3′, dissolved in the reaction buffer containing 10% DMSO (v/v), were spotted on MHAHD-activated microslide **IV** (Route B) in triplicates, as describe above. The microslide was exposed to microwaves for 15 min (45 × 20 s) and after usual washings, the spotted microslide was subjected to hybridization with 5′ TET-d(GCA GAC CTT CTC CTC AGG AG) 3′, dissolved in the hybridization buffer (40 μl), first at 45 °C for 1 h and then overnight at room temperature followed by washing with the hybridization buffer. After drying, the microslide was subjected to scanning under a laser scanner.

2.8. Thermal stability

The thermal stability of oligonucleotides immobilized using MHAHD was evaluated by submitting the microslide spotted with 5′ TET-d(TTT TTT TTT TTT TTT TTT TT)-OPO_3-$(CH_2)_6$SH 3′ to PCR-like conditions. The microarray was first subjected to 94 °C for 30 s in 0.1 M reaction buffer, followed by cooling to 54 °C for 30 s and then heating to 72 °C for 30 s. Likewise, microarrays were submitted to 0, 5, 10 and 15 cycles. Subsequently, fluorescence intensity of the spots was measured by Quant Array software.

For each experimental condition tested on the microarrays, experiment was repeated three times. The immobilization and hybridization data presented are the average of these repetitions and the error bars represent the percentage error (±3–6%) observed on this average.

3. Results and discussion

Covalent ligand immobilization via photoreactive species offers the fascinating possibility of selective activation. Although psoralens, benzophenones, azides and carbene precursor are commonly employed in photochemical immobilization reactions, these photoreactive species suffer from several drawbacks. Therefore, a tricyclic quinone i.e. anthraquinone was employed as a photoreactive species due to its interesting properties for ligand immobilization [37]. The excited anthraquinone is highly reactive and reacts with almost all C–H containing substrates. The thiol reactive reagents have been widely used to modify peptides and proteins at specific sites because a thiol group is more nucleophilic than amino function under physiological conditions. After screening the number of thiol reactive groups, viz., maleimide, vinyl sulfones, haloacetyl derivatives, bromomethyl derivatives, and pyridyl disulfide, maleimide was preferred due to its highest selectivity towards the thiol functions. Keeping these two functional groups, i.e. anthraquinone and maleimide, at the two termini, a new heterobifunctional photocrosslinker reagent, MHAHD, was designed, which combines photoreactivity as well as selectivity towards thiol functions.

The synthesis of the reagent, MHAHD **II**, involves two-step process, as shown in Scheme 1. First, anthraquinone-2-carboxylic acid was converted to its *N*-hydroxysuccinimide ester using NHS in the presence of DCC. Succinimide ester of anthraquinone-2-carboxylic acid was coupled with 1,6-hexanediamine in the presence of triethylamine to afford *N*-(6-aminohexyl)anthraquinone-2-carboxamide **I** in ~60% yield. This reaction was performed carefully by taking 1,6-hexanediamine in excess and addition was done dropwise at 0 °C. The next step of the synthesis was the coupling of *N*-maleimidocaproic acid with *N*-(6-aminohexyl)anthraquinone-2-carboxamide **I**. For this step, *N*-maleimidocaproic acid was first converted to its *N*-hydroxysuccinimide ester derivative by usual method, followed by reaction with *N*-(6-aminohexyl)anthraquinone-2-carboxamide **I** in DMF at room temperature to obtain the crude compound, MHAHD **II**, which was purified by column chromatography to obtain the title compound in ~82% yield. All the compounds were characterized by ^{1}H-NMR and mass spectroscopy. Subsequently, it was used for the preparation of oligonucleotide microarrays.

In the present study, the oligonucleotide probes used for immobilization on the glass microslides were designed in such a way that there should not be any formation of secondary structure either due to self-complementarity, self-reaction or aggregation.

For determination of optimal concentration required for visualization of oligomers on the microslide, a maleimido-activated microslide was prepared using MHAHD **II** (Route B). An oligomer, 5′ TET-d(TTT TTT TTT TTT TTT TTT TT)-OPO_3-$(CH_2)_6$SH 3′, was spotted onto MHAHD-activated microslide **IV** in four different concentrations (2.5, 5, 10, 20 μM) and exposed to microwaves. After washings with the reaction buffer (3 × 40 ml) and drying, the spotted microslide was visualized under a laser scanner and the fluorescence intensity of each spot was measured using Quant Array software. The immobilization efficiency against each concentration was evaluated with the help of a standard curve (Fig. 1, see Supporting Information) and is depicted in Fig. 1. Though the spots corresponding to 5 μM can be visualized easily, a 10 μM concentration was preferred for the preparation of microarrays to have a better discrimination of base mismatches.

The applicability of the proposed strategy was demonstrated by immobilizing thiol-modified oligonucleotides under the influence of microwaves and light. For comparison purposes, arrays were

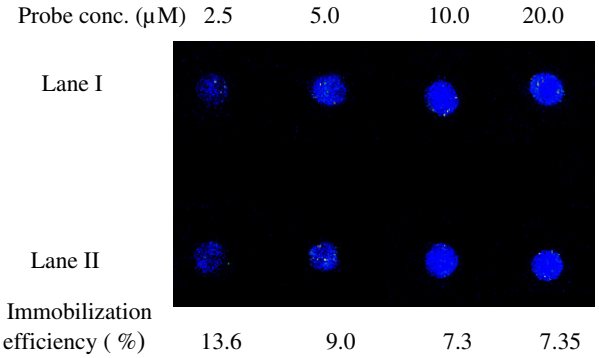

Fig. 1. Threshold concentration of oligonucleotide sequence, TET-d(TTT TTT TTT TTT TTT TTT TT)-OPO_3-$(CH_2)_6$SH, required for fluorescence visualization. Lane I, probe concentration; Lane II, immobilization efficiency. Slide was scanned at 85% of laser power and 80% of photomultiplier gain.

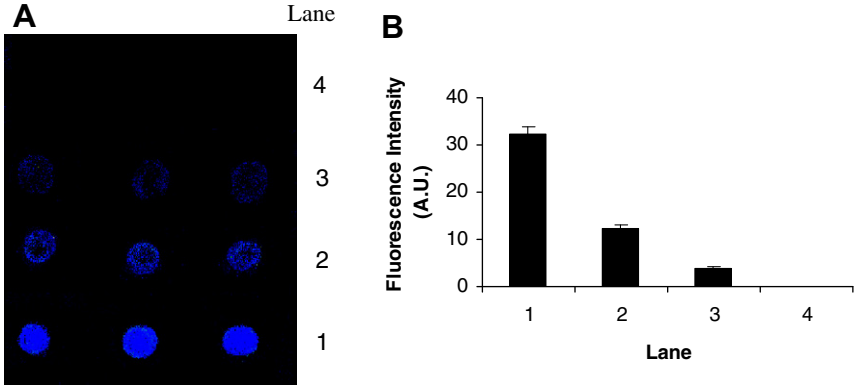

Fig. 2. (A) Detection of nucleotide mismatches and specificity of immobilization via hybridization with TET-d(GCA GAC CTT CTC CTC AGG AG). Lane 1, HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG AGA AGG TCT GC); Lane 2, HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG TGA AGG TCT GC); Lane 3, HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG TCA AGG TCT GC); Lane 4, HS-$(CH_2)_6OPO_3$-d(TTT TTT TTT TTT TTT TTT TT). (B) Quantitative data analysis of comparison of fluorescence intensity observed with mismatches in the probes after hybridization with target (complementary to zero mismatch); $n = 3$. Slide was scanned at 90% of laser power and 85% of photomultiplier gain.

constructed under thermal conditions too. Slides fabricated via both the routes (A and B) under microwaves as well as thermal conditions [38] were then subjected to hybridization (see Supporting Information).

In order to evaluate the specificity of the proposed chemistry for immobilization and to detect mismatches in the oligomer sequences, four oligonucleotide sequences, viz., 5′ HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG AGA AGG TCT GC) 3′; 5′ HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG TGA AGG TCT GC) 3′; 5′ HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG TCA AGG TCT GC) 3′; 5′ HS-$(CH_2)_6OPO_3$-d(TTT TTT TTT TTT TTT TT) 3′, were immobilized on glass microslide (Route B) in triplicates. After usual washings and drying, the slide was subjected to hybridization with 5′ TET-d(GAG GAC TCC TCT TCC AGA CG) 3′, followed by visualization under a laser scanner. The fully matched oligomer (lane 1) gave the maximum intensity (Fig. 2A), while the oligomer in lane 2 having one mismatch showed diminished fluorescence. Similarly, the oligomer in lane 3 having two mismatches showed fluorescent intensity in decreasing order, whereas the oligomer in lane 4, the non-complementary one, showed no affinity at all for the TET-labeled oligomer. The quantitative data of this experiment has been depicted in Fig. 2B. These results demonstrate the accessibility of the surface bound oligomers to specific hybridization and applicability of the method for the detection of nucleotide mismatches.

The thermal stability of the constructed arrays using the proposed reagent, MHAHD **II**, was demonstrated by subjecting the constructed microarray to three sets of temperature conditions, as used in PCR (Fig. 3). The spotted microarray was first subjected to heating at 94 °C for 30 s in the reaction buffer, followed by cooling at 54 °C for 30 s and then heating at 72 °C for 30 s. The loss in fluorescence intensity was found to be ~4.5% after ten cycles, inferring that the constructed microarray can withstand the high temperature conditions. The observed decrease in fluorescence intensity might be due to thermal degradation and photobleaching. Therefore, microarray prepared by the proposed reagent can be comfortably used in integrated PCR- machines, if required.

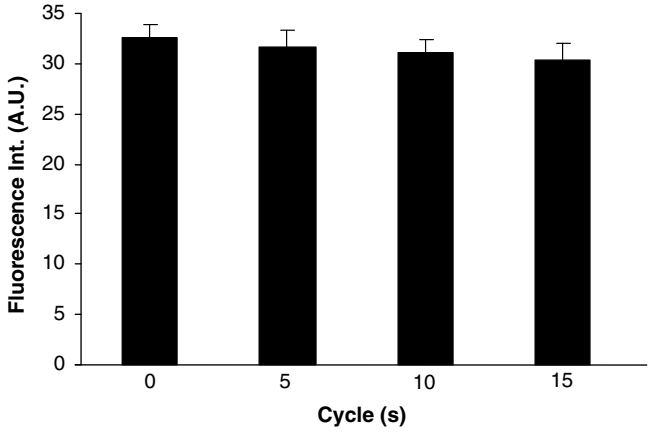

Fig. 3. A graphical representation showing thermal stability of immobilized probe after 0, 5, 10 and 15 cycles of PCR-like conditions $n = 3$.

4. Closing remarks

A new heterobifunctional reagent, MHAHD, has been proposed for the construction of oligonucleotide microarrays with high immobilization efficiency and in just 45 min. One of the termini carries anthraquinone moiety, which allows one to use it with a variety of polymer surfaces. The presence of two different functional groups offers a unique advantage to achieve immobilization via two routes under the influence of light and microwaves. The resultant microarrays were successfully used for discrimination of base mismatches. The immobilized oligonucleotides are sufficiently stable during PCR-like conditions, therefore, the strategy can be used for biological assays requiring higher temperatures. These data altogether demonstrate that the projected strategy may be useful for the preparation of new devices for DNA microarrays.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.susc.2008.05.020.

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