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N-(3-Triethoxysilylpropyl)-6-(*N*-maleimido)-hexanamide: An efficient heterobifunctional reagent for the construction of oligonucleotide microarrays

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Abstract

Synthesis of a new heterobifunctional reagent, *N*-(3-triethoxysilylpropyl)-6-(*N*-maleimido)-hexanamide (TPMH), for the preparation of oligonucleotide microarrays is described. Its triethoxysilyl function is specific toward virgin glass surface and maleimide function undergoes conjugate addition to 3′- or 5′-thiol-modified oligonucleotides. The construction of microarrays using TPMH has been realized via two routes. In Route A, TPMH was reacted first with 3′- or 5′-thiol-modified oligonucleotide under microwaves, thereby producing oligonucleotide–triethoxysilyl conjugate in 15 min, which was then brought in contact with virgin glass microslide, resulting in immobilization of an oligonucleotide sequence. In Route B, immobilization involves generation of maleimide functions on virgin glass surface by the reaction with TPMH, followed by coupling with thiol-modified oligonucleotides under microwaves in 15 min to produce surface-bound oligonucleotides. The microarrays constructed using both routes were analyzed by hybridizing with tetrachlorofluorescein-labeled complementary oligonucleotide. Subsequently, these microarrays were successfully used in the discrimination of single and double nucleotide mismatches based on fluorescence intensity.
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Bifunctional cross-linking reagents have been used extensively for a variety of purposes including preparation of affinity matrices, modification and stabilization of diverse structures, identification of ligand and receptor binding sites, structural studies, etc. [1]. Because of some obvious reasons, heterobifunctional reagents, which contain two different functional groups, have proven to be more advantageous as cross-linking can be controlled both selectively and sequentially. By taking advantage of the differential reactivity of the two reactive groups, these reagents are finding extensive use in DNA microarray technology, one of the hottest areas in current biological research. The technology, in a very short span of time, has become an indispensable tool for fundamental and applied research, particularly, in biochemistry and biomedical sciences. In the past 2–3 years, it has provided high-throughput analysis to aid gene discovery, disease diagnosis, drug screening, polymorphism analysis, and other applications [2–12].

Two main technology platforms for manufacturing microarrays have emerged. The first platform, the deposition method, uses the immobilization of pre-fabricated oligonucleotides by spotting on functionalized polymer surfaces [13–24]. The second platform uses direct *in situ* synthesis of oligonucleotides (~20 to 70 mers) on polymer surfaces [25–28]. The later platform produces high-density arrays; however, it needs sophisticated equipments, which are quite expensive for production and utilization of simple customized oligonucleotide microarrays. Therefore, the deposition method has become a method of choice, which offers excellent flexibility in that a wide variety of other ligands can also be immobilized. This method is most

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widely used for creating low-to-medium-density microarrays, which are quite often used by research laboratories for routine applications. Quality of microarrays fabricated by the later platform is influenced by two important factors, viz., (a) the nature of the solid surface used, and (b) the chemistry employed for fixing the oligonucleotides. Of the various polymer supports tried so far, glass is preferred due to its easy modification by silane chemistry, resistance to high temperature, low cost, low intrinsic fluorescence, and favorable optical properties that allow highly sensitive laser-induced fluorescence imaging. Usually, the solid surface is first subjected to modification using an appropriate reagent to generate reactive functional groups and then pre-modified oligonucleotides are immobilized on the activated surface using suitable reagents or activators. Thus the immobilization process requires several steps and expensive reagents to immobilize oligonucleotides on a solid surface. Though a large number of chemical methods for immobilization of oligonucleotides on pre-functionalized polymer surfaces, have been described in the literature, there are only a few available reagents, viz., 3-mercaptopropyltrimethoxysilane (MPTS)[1] [29], (3-glycidyloxypropyl) triethoxysilane, (GOPTS) [30] and *N*-(2-trifluoroethane sulfonatoethyl)-*N*-(methyl)-triethoxysilylpropyl-3-amine (NTMTA) [31], that can be used directly for fixing oligonucleotides on virgin glass surface. The GOPTS method requires a longer reaction time (~8 h), the MPTS method results in immobilization of oligonucleotides via disulfide linkage, which is a labile linkage, and the NTMTA method involves a multistep synthesis strategy. Therefore, a glass-specific reagent, capable of fixing oligonucleotides rapidly on virgin glass surface and bypassing the limitations of the earlier reagents, is still elusive.

Recently, microwaves have offered an excellent alternative to conventional energy sources for carrying out organic reactions under mild conditions with higher yields and specificities in relatively short spans of time [32,33]. Therefore, to expedite the immobilization process, we decided to incorporate the advantages of microwaves to immobilize the premodified oligonucleotides on glass surface.

In this paper, we describe a microwaves-aided immobilization of oligonucleotides on virgin glass surface using a novel heterobifunctional reagent. The reagent, *N*-(3-triethoxysilylpropyl)-6-(*N*-maleimido)-hexanamide (TPMH) **II** (Scheme 1), carries maleimide function on one end to react specifically with thiol-modified oligonucleotides to give a stable thioether linkage. The other terminal bears a triethoxysilyl function destined to be coupled to the virgin glass surface. The immobilization of oligonucleotide using the proposed reagent was realized via two routes. In the first route (Route A), the maleimide–ligand conjugate was formed by allowing the reaction of TPMH with thiol-modified oligonucleotide under microwaves and the resulting conjugate was then brought in contact with virgin glass microslide to obtain immobilized oligomer. In the second route (Route B), the reagent was allowed to react first with virgin glass microslide through its triethoxysilyl functionality to generate maleimide functions on the surface, which in subsequent step reacted with thiol-modified oligonucleotides under microwaves to produce surface-bound oligonucleotides. Both routes work satisfactorily and the constructed microarrays were tested by hybridization assay and successfully used in detection of nucleotide mismatches. The thermal stability of the immobilization chemistry was evaluated by subjecting the spotted glass slide to PCR-like conditions.

Materials and methods

Materials

3-Aminopropyltriethoxysilane, dicyclohexylcarbodiimide, 1,6-hexanediamine, *N*-hydroxysuccinimide, and virgin glass microslides were procured from Sigma Chemical Co. (St. Louis, MO). TET-phosphoramidite was obtained from Applied Biosystems Inc. (Foster City, CA). Other chemicals and reagents were procured from local companies and used as received.

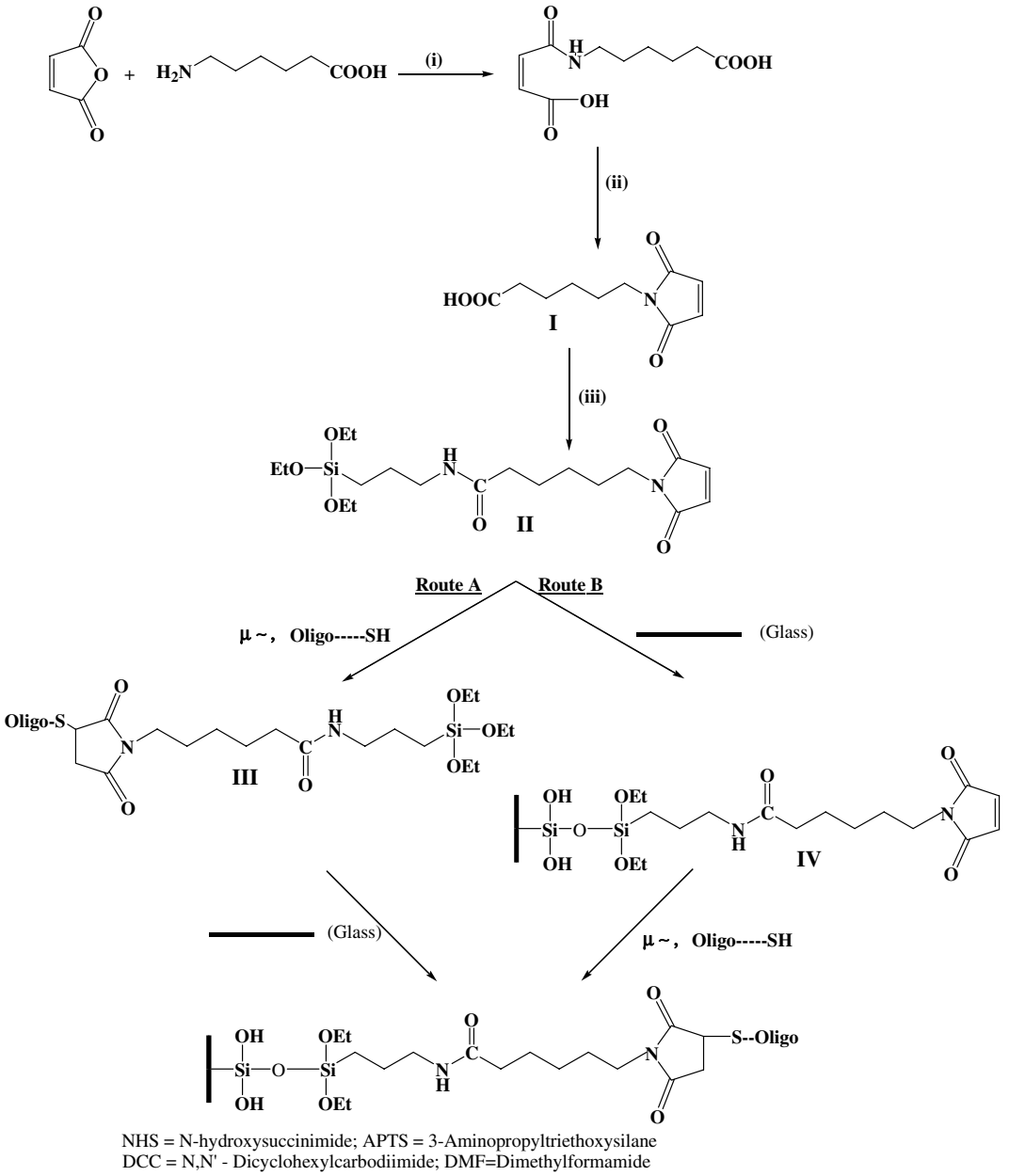
Instrumentation

Microwaves-assisted reactions were performed in a microwave oven (BPL-Sanyo, India), operating at 800 W (2750 Hz). Microslides were scanned under a laser scanner at 570 nm using Scan Array Lite, MicroArray Analysis System, GSI Lumonics. Fluorescence intensities of the immobilized oligonucleotides were determined by Quant Array software.

Oligonucleotide synthesis and purification

The oligonucleotides were synthesized at 0.2 μmol scale on a Pharmacia Gene Assembler Plus following the standard phosphoramidite approach. To incorporate reactive thiol (-SH) group at 5′ end of the oligonucleotides, the last coupling was performed with the reagent $BzS(CH_2)_6P[N(iPr)_2]O(CH_2)_2CN$ [34]. The coupling was performed in a manner analogous 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. Furthermore, labeled oligonucleotide having thiol modification at 3′ end was synthesized on a thiol polymer support [35] by carrying out the last coupling with TET-

[1] *Abbreviations used:* MPTS, 3-mercaptopropyltrimethoxysilane; GOPTS, (3-glycidyloxypropyl)triethoxysilane; NTMTA, *N*-(2-trifluoroethanesulfonatoethyl)-*N*-(methyl)-triethoxysilylpropyl-3-amine; TPMH, *N*-(3-triethoxysilylpropyl)-6-(*N*-maleimido)-hexanamide; TET, 2′,4,7,7′,-tetrachlorofluorescein; THF, tetrahydrofuran; NHS, *N*-hydroxysuccinimide; DCC, *N,N′*-dicyclohexylcarbodiimide; FTIR, Fourier transform infrared; CPG, controlled pore glass; DIPEA, diisopropylethylamine; DMSO, dimethyl sulfoxide; DMF, dimethylformamide; TCA, trichloroacetic acid; EDC, dichloroethane.



Scheme 1. Synthesis of the reagent *N*-(3-triethoxysilylpropyl)-6-(*N*-maleimido)-hexanamide and immobilization of oligonucleotide. Reagents: (i) glacial acetic acid, (ii) acetic anhydride, sodium acetate, 90 °C, 2 h, (iii) APTS, NHS, DCC, DMF.

phosphoramidite. After usual work-up and purification, the oligomers were concentrated and stored at 4 °C.

Buffers

Reaction buffer was 0.1 M sodium phosphate containing 0.1 M NaCl (pH 7.0). Capping buffer was 10 mM phosphate-buffered saline containing 2% bovine serum albumin. Hybridization buffer was 0.1 M sodium phosphate containing 1.0 M NaCl (pH 7.5).

Synthesis of N-maleimidocaproic acid

N-Maleimidocaproic acid **I** (Scheme 1) was prepared according to the published procedure [36].

Synthesis of N-(3-triethoxysilylpropyl)-6-(N-maleimido)-hexanamide

To a solution of *N*-maleimidocaproic acid **I** (1 mmol) in dry tetrahydrofuran (THF) (15 ml), *N*-hydroxysuccinimide (NHS) (1.5 mmol) and dicyclohexylcarbodiimide (DCC) (1.2 mmol) were added. The reaction was allowed to stir for 3 h at room temperature. After completion of the reaction (monitored on TLC), dicyclohexylurea was filtered off and to the filtrate was added 3-aminopropyltriethoxysilane (1.2 mmol) and triethylamine (1.2 mmol). The reaction was allowed to stir for a further 6 h at room temperature. The reaction mixture was concentrated under vacuum to a syrupy mass, redissolved in anhydrous benzene, and filtered to get rid of NHS. The filtrate was concentrated to obtain

N-(3-triethoxysilylpropyl)-6-(*N*-maleimido)-hexanamide, (Scheme 1) in 78% yield, which was characterized by FTIR and ^{1}H-NMR.

^{1}H-NMR, $CDCl_3$, δ: 1.25 (s, 9 H, 3 × –CH_3), 1.31–1.65 (m, 6 H,3 × –CH_2-), 2.25 (t, 2 H,-$COCH_2$-), 3.2–3.3 (m, 4 H, 2 × -NCH_2-), 3.73 (m, 6 H, 3 × -OCH_2-), 6.75 (br d, 2H, -CH = CH-). FTIR: ν (cm^{-1}): 2968, 2929, 1702, 1618, 1576, 1248, 1168.

Cleaning of glass microslides

The glass microslides were cleaned by immersing in 1 M NaOH (100 ml) for 2 h followed by washing with distilled water. Slides were then submitted to a solution of 1 M HCl (100 ml) for 2 h followed by washing with distilled water and finally rinsed with ethanol. The glass slides were dried under vacuum and stored under inert atmosphere.

Time optimization for immobilization of oligonucleotide on glass microslide

To optimize the time required to immobilize 5′-thiol-modified oligonucleotides on virgin glass surface using reagent **II** (Scheme 1), a model experiment was designed to study kinetics of the reaction between *O*-(4,4′-dimethoxytrityl)-6-mercaptohexan-1-ol{$DMTrO(CH_2)_6SH$} and controlled pore glass (CPG)-500 Å support via two routes.

Route A. Five vials were charged with 1 ml solution of *O*-(4,4′-dimethoxytrityl)-6-mercaptohexan-1-ol [0.01 M in MeCN containing 1% diisopropylethylamine (DIPEA)] and 1 ml solution of reagent **II** (Scheme 1) (0.012 M in MeCN). The vials were placed in microwave oven and irradiated up to 25 min. After each exposure of 20 s, vials were cooled in an ice–water mixture to room temperature and withdrawn at regular time intervals (5, 10, 15, 20, and 25 min) from the oven. The resulting conjugate was then added to each of the five centrifuge tubes containing unmodified CPG-500 Å (~5 mg) and placed in an Eppendorf Thermomixer set at 45 °C for 40 min [31]. The polymer support, after having been washed with MeCN (5 × 1 ml) and diethylether (2 × 1 ml) containing 1% DIPEA, was subjected to drying under vacuum. The extent of reaction on support in each vial was determined by treating an accurately weighed amount of support (~1–2 mg) with 3% trichloroacetic acid (TCA) in dichloroethane (EDC) for 5 min. The released DMTr cation was measured spectrophotometrically at 505 nm to determine the loading of the support in μmol/g following the standard method [37]. A graph of the loadings on support (μmol/g) versus time of reaction (min) was plotted.

Route B. In an alternative route, CPG-500 Å (~30 mg) was suspended in a solution of reagent **II** (Scheme 1) (600 μl, 0.12 M in MeCN) and kept in an Eppendorf Thermomixer set at 45 °C. After 40 min, the support was recovered on a sintered glass funnel and subjected to washings with MeCN (5 × 1 ml) and diethylether (2 × 1 ml). After drying under vacuum, five glass vials were charged with TPMH-activated CPG (~5 mg) and a solution of *O*-(4,4′-dimethoxytrityl)-6-mercaptohexan-1-ol (1.0 ml, 0.01 M in MeCN containing 1% DIPEA) was added to each vial. The vials were kept in a microwave oven, exposed up to 25 min, and withdrawn at regular time intervals (5, 10, 15, 20, and 25 min). The content of each vial was washed sequentially with MeCN (5 × 1 ml) and diethylether (2 × 1 ml) containing 1% DIPEA followed by drying under vacuum. The loading on the support in each vial was determined as described above. A graph of the loadings on support (μmol/g) versus time of reaction (min) was plotted.

Optimization of concentration of oligonucleotide for fluorescence detection

To determine the optimal concentration required to visualize an oligomer on glass surface, an oligonucleotide sequence, TET-d(TTT TTT TTT TTT TTT TTT TT)-OPO_3-$(CH_2)_6SH$, was serially diluted in four concentrations (20, 10, 5, 2.5 μM) in a reaction buffer containing 10% dimethyl sulfoxide (DMSO) (v/v) and spotted on a TPMH-activated glass slide, followed by exposure under microwaves for 15 min (45 × 20 s). Then the glass microslide was washed with reaction buffer (3 × 15 ml), dried, and subjected to visualization under a laser scanner operating at 570 nm.

Oligonucleotide quantification

To quantify the density of immobilized oligonucleotides, an oligomer sequence, TET-d(TTT TTT TTT TTT TTT TTT TT)-OPO_3-$(CH_2)_6SH$, diluted to six concentrations, viz., 0.05, 0.1, 0.2, 0.5, 0.8, and 1.0 μM, was spotted on a virgin glass microslide in duplicates. After drying the spots, they were visualized under a laser scanner and the fluorescence intensity corresponding to each spot was measured. A calibration curve plotting the values of fluorescence intensity (A.U.) as a function of concentration of oligonucleotide (μM) was drawn.

Immobilization of 5′-thiol-modified oligonucleotides on glass surface under microwaves

Route A. An oligonucleotide sequence, HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG AGA AGG TCT GC) (0.15 A_{260} units) dissolved in reaction buffer (100 μl), was mixed with 100 μl of solution of reagent **II** (Scheme 1) [0.12 M in dimethylformamide (DMF)]. The reaction mixture was subjected to microwaves for 15 min (45 × 20 s). After completion of reaction, solvent was evaporated under vacuum to obtain dry residue, which was resuspended in distilled water. The mixture was centrifuged to get rid of excess reagent and supernatant was concentrated to obtain oligonucleotide–triethoxysilyl conjugate **III** (Scheme 1). The oligonucleotide conjugate was reconstituted in distilled water to make the concentration to 10 μM and spotted (0.5 μl) manually on a virgin glass microslide. The spotted microslide was

incubated at 45 °C in a humid chamber for 40 min, followed by washings with reaction buffer (3 × 15 ml). Subsequently, the glass microslide was hybridized with complementary oligomer, TET-d(GCA GAC CTT CTC CTC AGG AG), in hybridization buffer (40 µl) and kept at 45 °C for 1 h and then at room temperature overnight. After washings with hybridization buffer (3 × 15 ml), the slide was dried and subjected to a laser scanner for fluorescence detection.

Route B: In an alternative route, 1 ml solution of reagent **II** (Scheme 1) (0.012 M in DMF) was spread on a virgin glass microslide and kept at 45 °C in a humid chamber for 40 min. After thorough washings with DMF (5 × 15 ml) and diethyl ether (2 × 15 ml), the microslide was dried under vacuum. Subsequently, TPMH-activated glass microslide **IV** (Scheme 1) was spotted with an oligonucleotide sequence, HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG AGA AGG TCT GC) (10 µM, 0.5 µl) dissolved in a reaction buffer containing 10% DMSO (v/v). The spotted glass microslide was placed in a microwave oven and irradiated for 15 min (45 × 20 s). The glass microslide was then washed as described above and dried.

Prior to hybridization, residual maleimido groups on activated glass microslide were blocked by treating with a capping buffer for 1 h, washed with reaction buffer, and dried. Subsequently, the immobilized oligonucleotide on the microslide was hybridized with complementary oligomer, TET-d(GCA GAC CTT CTC CTC AGG AG), as described above. The glass microslide, after washings and drying, was visualized under a laser scanner.

Immobilization of 5′-thiol-modified oligonucleotide on glass surface under thermal conditions

Route A. An oligonucleotide sequence, HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG AGA AGG TCT GC) (0.15 A_{260} units) dissolved in reaction buffer (100 µl), was combined with 100 µl of solution of reagent **II** (Scheme 1) (0.12 M in DMF). The reaction mixture was agitated for 4 h at room temperature [38,39]. Then the solution was concentrated under vacuum to obtain dry residue, which was resuspended in distilled water. The mixture was centrifuged to get rid of excess reagent and supernatant was concentrated to obtain oligonucleotide–triethoxysilyl conjugate **III** (Scheme 1). The oligonucleotide conjugate was reconstituted in distilled water to make the concentration to 10 µM and spotted on the virgin glass microslide. The spotted microslide was incubated at 45 °C in a humidified chamber for 40 min, washed with a reaction buffer, and dried. The glass microslide with immobilized oligonucleotide was hybridized with complementary oligomer, TET-d(GCA GAC CTT CTC CTC AGG AG), in a manner similar to that described above.

Route B: In an alternative route, virgin glass microslide was treated with 1 ml solution of the reagent **II** (Scheme 1) (0.012 M in DMF) at 45 °C in a humid chamber for 40 min. After thorough washings with DMF (5 × 15 ml) and diethylether (2 × 15 ml), the glass microslide **IV** (Scheme 1) was subjected to drying under vacuum and spotted with an oligonucleotide sequence, HS-$(CH_2)_6OPO_3$- d(CTC CTG AGG AGA AGG TCT GC) (10 µM, 0.5 µl) dissolved in a reaction buffer. The spotted slide was kept in a humidified chamber for 4 h and then subjected to washings with reaction buffer and dried. After blocking maleimido groups with a capping buffer, the hybridization was carried out as mentioned above.

Specificity of immobilization chemistry and detection of mismatches

Four oligonucleotide sequences, viz., HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG AGA AGG TCT GC), HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG TGA AGG TCT GC), HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG TCA AGG TCT GC), and HS-$(CH_2)_6OPO_3$-d(TTT TTT TTT TTT TTT TTT TT) dissolved in a reaction buffer, were spotted on a TPMH-activated glass microslide (Route B). The glass microslide was exposed to microwaves and after usual washings, the spots on microslides were hybridized with TET-labeled d(GCA GAC CTT CTC CTC AGG AG) in hybridization buffer. After washings with hybridization buffer, the microslide was dried and subjected to a laser scanner and fluorescence intensities were determined.

Thermal stability

The thermal stability of the immobilization chemistry was evaluated by subjecting the microslide, spotted with TET-d(TTT TTT TTT TTT TTT TTT TT)-OPO_3-$(CH_2)_6SH$, to three sets of temperature conditions. The microarray was first subjected to 94 °C for 30 s in a reaction buffer, followed by cooling to 54 °C for 30 s and then heating to 75 °C for 30 s. Likewise, microarray was submitted to 0, 5, 10, and 15 cycles. Subsequently, fluorescence intensities were determined by Quant Array software.

Results and discussion

The emergence of DNA microarrays has engendered a revolution in many aspects of biological research. Recently, immobilization of biomolecules on glass/silicon surfaces and particularly glass microslides has gained importance, as these are being used in constructing oligonucleotide, peptide, protein, and other biomolecule microarrays. The primary aim of the present work was to look for a versatile heterobifunctional reagent useful for immobilization of thiol-modified oligonucleotides on a virgin glass surface. While designing the proposed reagent, we were guided by a few considerations: (i) the preparation of the reagent should be simple and straightforward, (ii) the involvement of complex/tedious reactions should be avoided, (iii) one of the ends of the proposed reagent should be specific toward thiolated ligands, and (iv) the other end of the reagent should be specific toward virgin glass, thereby eliminating the extra step of surface modification. Of several thiol-

reactive groups reviewed for this purpose, maleimide was selected on the merits that it has sufficient stability in aqueous and organic environments and possesses specific reactivity toward sulfhydryl-group-containing ligands. The choice of the reactive group at the other end was limited as we wanted to immobilize ligands on virgin glass surface. Therefore, we selected triethoxysilyl group for this purpose, which is specific toward silanol functionalities of the virgin glass surface. Keeping these two reactive groups at different termini, a new heterobifunctional/cross-linking reagent, TPMH **II**, was synthesized, starting from 3-aminopropyltriethoxysilane and *N*-maleimidocaproic acid **I** (Scheme 1). *N*-Maleimidocaproic acid **I** was first converted to its *N*-hydroxysuccinimide-active ester by reacting with NHS in the presence of DCC for 3 h. The dicyclohexylurea was removed by filtration and the filtrate containing active ester of *N*-maleimidocaproic acid was subsequently reacted with 3-aminopropyltriethoxysilane in the presence of triethylamine at room temperature for 6 h. After completion of reaction (as monitored on TLC), solvent was evaporated to obtain the desired product as a residue, which was redissolved in benzene and filtered to remove residual NHS. The filtrate was evaporated to get the desired compound, **II**, in 78% yield. The reagent **II** was characterized by ^{1}H-NMR and IR. The peaks at δ 1.25 (-CH_3), 3.73 (-OCH_2-), and 6.75 (-CH═CH-) confirmed the synthesis of the reagent, TPMH. Subsequently, it was used for preparation of oligonucleotide microarrays.

Time course of the reaction between TPMH and thiol-modified oligonucleotides (mercaptoalkylated ligand)

To determine the optimum time required for immobilization of thiol-modified oligonucleotides on virgin glass surface using reagent **II**, a kinetic study was performed using a model compound, *O*-(4,4′-dimethoxytrityl)-6-mercaptohexan-1-ol {DMTrO(CH_2)$_6$SH}, on CPG-500 Å via two routes. In Route A, five vials were charged with a solution of *O*-(4,4′-dimethoxytrityl)-6-mercaptohexan-1-ol and the reagent **II** dissolved in MeCN. Vials were subjected to microwave irradiation and withdrawn at different time intervals (5, 10, 15, 20, and 25 min). The conjugate so formed in each vial was reacted separately with CPG-500 Å at 45 °C for 40 min. The extent of reaction was determined by treating CPG support (after above sets of reaction) with 3% TCA in EDC. The loading values determined were plotted against time (min) as shown in Fig. 1a (Route A). In Route B, CPG support taken in an Eppendorf tube was treated with a solution of reagent **II** in MeCN and the reaction was allowed to proceed at 45 °C in an Eppendorf Thermomixer. After 40 min, support was recovered and, after usual washings, subjected to drying. In a subsequent step, five vials were charged with TPMH-activated CPG support and treated with *O*-(4,4′-dimethoxytrityl)-6-mercaptohexan-1-ol under microwaves for different time intervals (5, 10, 15, 20, and 25 min). The extent of reaction in each vial was determined as described for Route A. The loading values (μmol/g) determined were plotted against time (min) as shown in Fig. 1b (Route B). From these kinetics data, it can be inferred that immobilization of thiol-modified ligands on CPG-500 Å using reagent **II** essentially completes in 15 min under microwaves. Therefore, all experiments involving reaction of thiolated oligonucleotides and maleimide functions under microwaves were carried out for 15 min.

Quantification of immobilized oligonucleotides

Quantification of immobilized oligonucleotides was carried out by a graphical method. Here, an oligomer, TET-d(TTT TTT TTT TTT TTT TTT TT)-OPO_3-(CH_2)$_6$SH, diluted in six concentrations (0.05, 0.1, 0.2, 0.5, 0.8, and 1.0 μM), as detailed under Materials and methods, was spotted on a glass slide in duplicates and the fluorescence intensity of each spot was determined with the help of

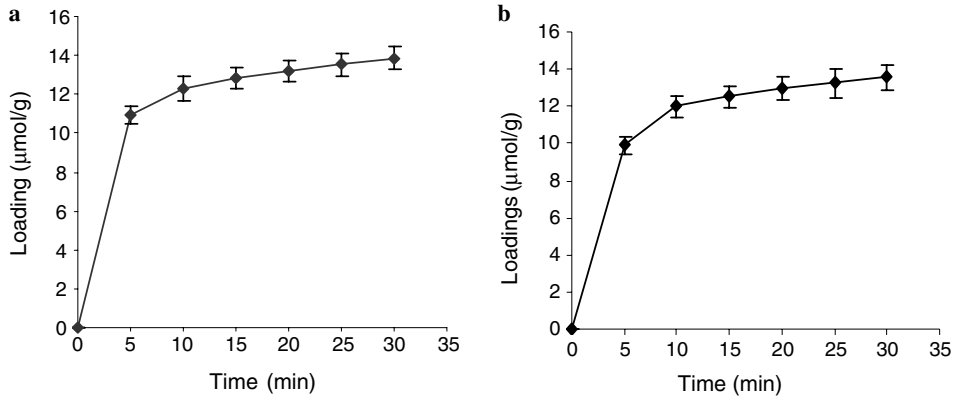


Fig. 1. Time kinetics of immobilization of *O*-(4,4′-dimethoxytrityl)-6-mercaptohexan-1-ol {DMTrO(CH_2)$_6$SH} on maleimido-activated controlled pore glass support (500 Å) via Route A (a) and Route B (b). In Route A, a solution of *O*-(4,4′-dimethoxytrityl)-6-mercaptohexan-1-ol was mixed with the reagent **II,** divided into five vials, and subjected to microwaves. The vials were withdrawn at different time intervals and the conjugate so formed in each vial was reacted separately with CPG-500 Å at 45 °C for 40 min. The extent of reaction was determined by treating CPG support with 3% TCA in EDC. In Route B, CPG support taken in an Eppendorf tube was treated with a solution of reagent II and the reaction was allowed to proceed at 45 °C for 40 min. After usual washings and drying, TPMH-activated support was divided in five vials and treated with *O*-(4,4′-dimethoxytrityl)-6-mercaptohexan-1-ol under microwaves for different time intervals. The extent of reaction in each vial was determined as described for Route A. $n = 3$.

Quant Array. The fluorescence intensity of the spots was plotted against its concentration (Fig. 2.) An almost linear correlation between the concentration of the spotted oligonucleotide and its fluorescence intensity was observed. The same curve was then used to quantify all microarrays prepared using the proposed method.

To determine the threshold concentration required for visualization of oligomers immobilized on the glass microslide, a maleimido-activated glass microslide was prepared using reagent **II** (Route B). An oligomer, TET-d(TTT TTT TTT TTT TTT TTT TT)-OPO_3-$(CH_2)_6$SH dissolved in reaction buffer in four concentrations (2.5, 5, 10, and 20 μM), was spotted onto TPMH-activated glass microslide **IV** (Scheme 1) and exposed to microwaves for 15 min. After washing the spotted microslide with copious amounts of reaction buffer and MilliQ water, 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 calibration curve (Fig. 2) and is illustrated in Fig. 3 (lane II). Though the spots corresponding to 2.5 and 5.0 μM concentration can be visualized easily, a 10 μM concentration was selected for construction

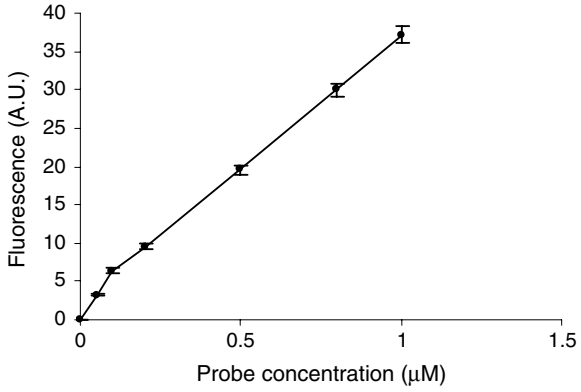


Fig. 2. Correlation between concentrations of immobilized probe and fluorescence intensity. Fluorescent oligonucleotide was spotted in 0.05–1.0 μM concentrations. The spotted microslide was scanned under a laser scanner. $n = 3$.

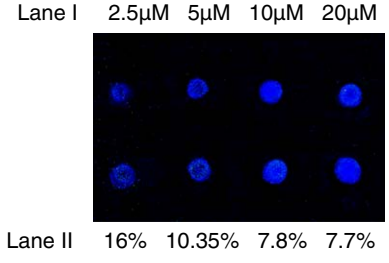


Fig. 3. Threshold concentration of oligonucleotide sequence TET-d(TTT TTT TTT TTT TTT TTT TT)-OPO_3-$(CH_2)_6$SH required for fluorescence visualization. Lane I, concentration of spots; Lane II, immobilization efficiency. Oligonucleotide dissolved in reaction buffer in four concentrations (2.5, 5, 10, and 20 μM) was spotted onto TPMH-activated glass microslide **IV** and exposed to microwaves for 15 min. After usual washings and drying, fluorescence intensity of each spot was measured using Quant Array software. $n = 3$.

of microarrays so that the constructed microarray could be used successfully for the discrimination of base mismatches.

Immobilization of thiol-modified oligonucleotides and hybridization assay

Having determined the optimal concentration and time required for immobilization of 5′-thiol-modified oligonucleotides on the glass surface after performing the above two experiments, immobilization of oligonucleotides was carried out using reagent **II** following both routes. As the design of reagent TPMH, a heterobifunctional reagent, includes two different reactive groups at either end, it was, therefore, considered necessary to explore whether this reagent could be used in two ways. In Route A, oligonucleotide sequence HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG AGA AGG TCT GC) was reacted with reagent **II** (Scheme 1) under microwaves to form oligonucleotide–triethoxysilyl conjugate **III** and the resulting conjugate was immobilized on a virgin glass microslide. The method is beneficial due to the stability of oligomer–triethoxysilyl conjugate over several months at 4 °C. In Route B, maleimido-functionalized glass slide was prepared by coating reagent **II** on a virgin glass microslide via its triethoxysilyl function. In the subsequent step, TPMH-activated glass microslide **IV** (Scheme 1) was spotted with an oligonucleotide sequence, HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG AGA AGG TCT GC), and exposed to microwaves for 15 min. Maleimido–glass slides are sufficiently stable and can be stored for longer durations at 4 °C. Oligonucleotide microarrays prepared following two routes (A and B) were hybridized with complementary-labeled oligomer, TET-d(GCA GAC CTT CTC CTC AGG AG), and after thorough washings with buffer, the slides were visualized under a laser scanner. For comparison purposes, thiol-modified oligonucleotide was immobilized on a virgin glass microslide using reagent **II** via both routes under thermal conditions (4 h, room temperature). The glass microslides with immobilized oligonucleotides were then analyzed by hybridization assay. Fig. 4 shows the fluorescent spots after the hybridization experiment by both routes under microwaves and thermal conditions. The results show that the fluorescence intensity of the spots immobilized under microwaves was comparable to that immobilized under thermal conditions. However, the time required for immobilization of oligomers under thermal conditions was much higher (4 h) than that under microwaves (15 min), which clearly indicates the advantage of the proposed methodology.

Specificity of immobilization chemistry and discrimination of mismatches

To demonstrate the specificity of the proposed chemistry for immobilization and to detect mismatches in the oligomer sequence, four oligonucleotide sequences, viz., HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG AGA AGG TCT GC), HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG TGA AGG TCT

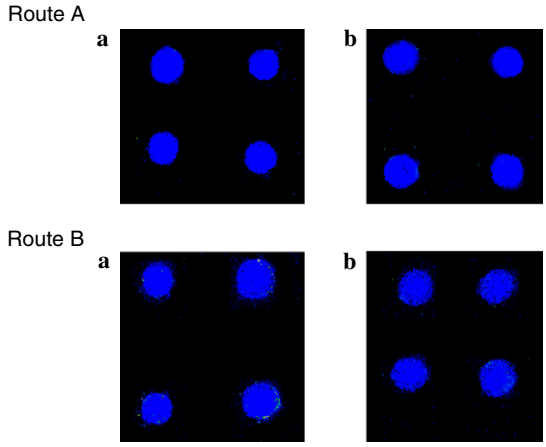


Fig. 4. Immobilization of oligonucleotide sequence HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG AGA AGG TCT GC) via Route A and Route B under (a) microwave irradiation and (b) thermal conditions, followed by hybridization with TET-d(GCA GAC CTT CTC CTC AGG AG) and visualization under a laser scanner.

GC), HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG TCA AGG TCT GC), and HS-$(CH_2)_6OPO_3$-d(TTT TTT TTT TTT TTT TT), were immobilized on glass microslide by Route B. After usual washings and drying, the slide was subjected to hybridization with TET-d(GCA GAC CTT CTC CTC AGG AG) in hybridization buffer followed by visualization under a laser scanner. The fully matched oligomer (lane 1) gave the maximum intensity (Fig. 5) while the oligomer in lane 2 with one mismatch showed diminished fluorescence. Similarly, the oligomer in lane 3 with two mismatches showed fluorescent intensity in decreasing order, whereas the oligomer in lane 4, the non-complementary one, showed no affinity for the TET-labeled oligomer. These results demonstrate that the immobilized oligonucleotides are specific toward complementary oligonucleotides and non-specific hybridization does not occur.

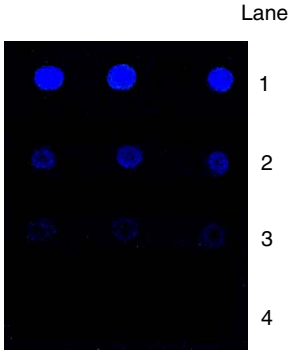


Fig. 5. Detection of nucleotide mismatches and specificity of immobilization. Four oligonucleotide sequences, HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG AGA AGG TCT GC) (lane 1), HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG TGA AGG TCT GC) (lane 2), and HS-$(CH_2)_6OPO_3$-d(CTC CTG AGG TCA AGG TCT GC) (lane 3), and HS-$(CH_2)_6OPO_3$-d(TTT TTT TTT TTT TTT TT) (lane 4), were immobilized on glass microslide by Route B. After usual washings, the spotted microslide was hybridized with TET-d(GCA GAC CTT CTC CTC AGG AG) and subjected to a laser scanner for visualization of fluorescent spots.

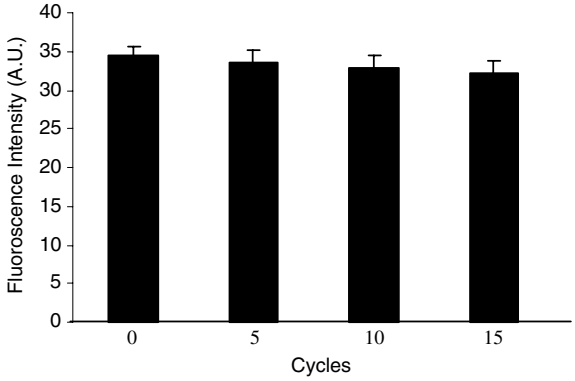


Fig. 6. Thermal stability of immobilized oligonucleotides (microarray). The microarray was first subjected to 94 °C for 30 s in 0.1 M reaction buffer, followed by cooling to 54 °C for 30 s and then heating to 75 °C for 30 s. The microarrays were compared on the basis of fluorescence signals obtained after hybridization of heat-treated and untreated arrays of oligonucleotides. $n = 3$.

Thermal stability

The thermal stability of the oligonucleotides immobilized onto maleimido-activated glass microslide was evaluated by submitting the oligonucleotide microarray to PCR-like conditions, i.e., 94 °C for 30 s, 54 °C for 30 s, and 74 °C for 30 s for 0, 5, 10, and 15 cycles (Fig. 6). After 10 cycles, the loss in fluorescence intensity was found to be ~4.5%. These results demonstrate that covalent linkage of thiol-modified oligonucleotide and maleimido-activated surface can be used for bioassays involving high temperature.

Conclusions

This study highlights the preparation of a novel heterobifunctional reagent, TPMH, and its use for immobilization of oligonucleotides on glass surface under microwaves. Immobilization has been realized via two different routes: Route A involves the reaction of thiol-modified oligomer with TPMH to generate triethoxysilyl–oligonucleotide conjugate under microwaves, which subsequently reacts with glass slide to obtain surface-bound oligomer, and Route B involves the reaction of TPMH with glass microslide to generate maleimido groups, which react with the thiolated oligomer to obtain surface-bound oligomer. The hybridization efficiency was found to be ~32% with signal-to-noise ratio (>98). Moreover, the covalently immobilized oligonucleotides were sufficiently stable under thermal conditions.

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