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N-(3-Triethoxysilylpropyl)-4-(*N*′-maleimidylmethyl)-cyclohexanamide (TPMC): A heterobifunctional reagent for immobilization of oligonucleotides on glass surface

Arvind Misra*

Department of Chemistry, Faculty of Science, Banaras Hindu University, Varanasi-221 005, India

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Abstract—A new heterobifunctional reagent, namely, *N*-(3-triethoxysilylpropyl)-4-(*N*′-maleimidylmethyl)cyclohexanamide (TPMC) was developed and its potentiality for fixing of thiol (-SH) modified oligonucleotides were tested. The covalent attachment of oligonucleotides with the reagent was achieved through its maleimide functionality at one end via stable thioether linkage while the other end bearing triethoxysilyl functionality has been utilized for coupling with the virgin glass surface with simplified methodologies. Immobilization of oligonucleotides was achieved by two alternating ways. The PATH-1 involves formation of conjugate of reagent and SH-modified oligonucleotides through thioether linkage and was subsequently immobilized on unmodified glass surface through triethoxysilyl group and alternatively, PATH-2 involves reaction of reagent first with unmodified glass surface to get maleimide functionality on the surface and then the SH-modified oligonucleotides were immobilized via thioether linkage. The specificity of immobilization was tested by hybridization study with complementary fluorescein labeled oligonucleotide strand.

Nucleic acid based detection and quantification methods play an important role in the medical diagnostics and drug discovery. The development of reliable, fast and inexpensive detection methods is important. The measurement of nucleic acid hybridization under heterogeneous condition provides a variety of advantages such as convenience, real time and accurate quantification for diverse applications. To determine the presence of a specific DNA sequence it is necessary to do hybridization with a fluorescently labeled oligonucleotide by immobilizing the biomolecules on the solid surface. Recently, microarray technology (biochip) has emerged as a promising tool for gene discovery,[1] genome analysis,[2] medical diagnostics for genetic diseases,[3] detection of single nucleotide polymorphism,[4] nucleic acid–ligand interaction,[5] DNA sequencing by hybridization[6] and DNA computing.[7]

Photolithographic technique[8] and deposition methods[9] are two very well-established methodologies for the immobilization of biomolecules on different solid surfaces like polypropylene, polyethylene, nylon, poly (methyl methylacrylate), glass, silicon, etc. The quality of microarray which is synthesized by deposition method, in which a pre-fabricated nucleic acid is either covalently[10–13] or noncovalently[14] immobilized with great flexibility, depends upon the nature of solid surface selected and chemistry used for fixing biomolecules. In general, the solid surface is first subjected to chemical modifications with the help of an appropriate reagent to generate reactive functionality on it and then pre-modified oligonucleotides are immobilized on the activated surface using suitable reagents and activators. Thus, the process of immobilization involves multisteps and expensive reagents. Out of several polymeric supports used for the construction of microarrays, virgin glass is a preferred one because of ease of modification by silane chemistry, low cost, low intrinsic fluorescence, relatively homogeneous surface, resistance to heat and also its favourable optical properties for highly sensitive fluorescence imaging.

Several heterobifunctional and/or cross linking reagents are in practice for immobilization of oligonucleotides on pre-functionalised polymeric surfaces.[15–21] However,

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* Tel.: +91 0542 2307321x104; fax: +91 0542 2368127/2368175; e-mail: arvindmisra2003@yahoo.com

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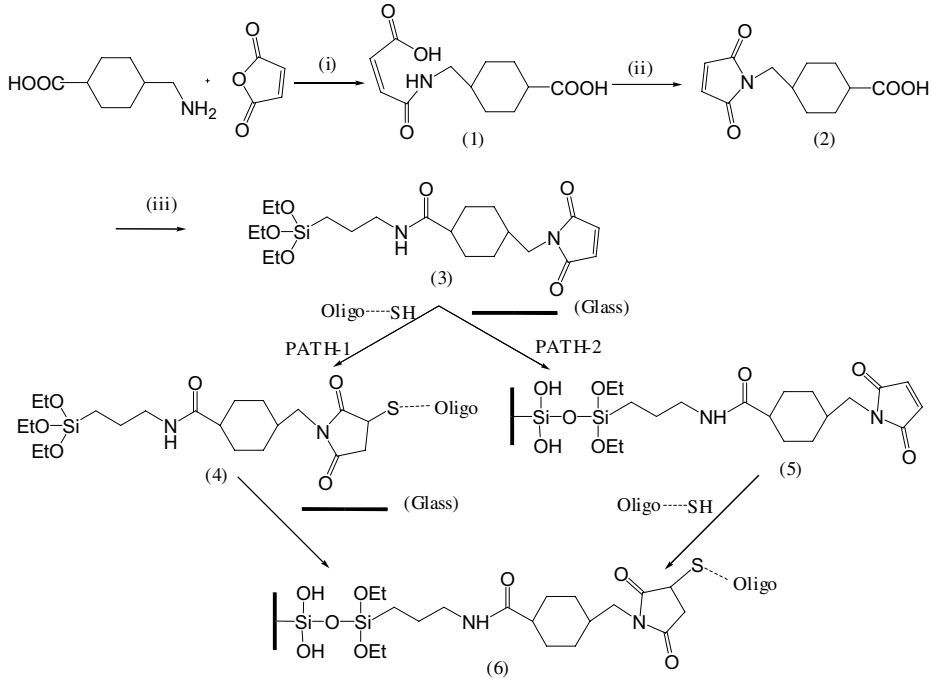
these reagents have their own limitations like 3-mercaptopropyltriethoxysilane[15] (MPTS) involves labile disulfide linkage, 3-glycidyloxypropyltriethoxysilane[16] (GOPTS) is a time-consuming process (~8 h), *N*-(2-trifluoroethanesulfonatoethyl)-*N*-(methyl)-triethoxysilylpropyl-3-amine[17,24] (NTMTA), *N*-(iodoacetyl)-*N*′-(anthroquinon-2-oyl)-ethylenediamine[18] (IAED), *N*-(3-trifluoroethane sulfonyloxypropyl)anthraquinone-2-carboxamide[19] (NTPAC) are multistep strategies for the immobilization of oligonucleotides on the glass surface. Therefore, encouraged with the utilities of heterobifunctional reagents for the immobilization of oligonucleotide on glass surface a new reagent, namely, *N*-(3-triethoxysilylpropyl)-4-(*N*′-maleimidylmethyl) cyclohexanamide (TPMC) was developed and its potentiality for fixing of thiol (-SH) modified oligonucleotides was tested. The covalent attachment of oligonucleotides with the reagent was achieved through its maleimide functionality at one end via stable thioether linkage, while the other end bearing triethoxysilyl functionality has been utilized for coupling with the virgin glass surface with simplified methodologies.

The objective of the present work was to develop a versatile heterobifunctional reagent which should be specific for the immobilization of thiol-modified oligonucleotide on a glass surface. The reagent was synthesized keeping in mind (i) simple, cost effective and straightforward methodology (ii) avoiding involvement of multisteps (iii) out of two functional ends, one end should be specific for thiolated ligands, while the other one for glass surface. The maleimide functionality was selected because it has greater degree of stability in the aqueous and organic medium and in the pH range of 6.5–7.5 it has greater specificity for thiol functionality, resulting in the formation of thioether linkage. The triethoxysilyl functionality on the other end of the reagent was selected because of immobilization process on the glass surface which has itself silanol functionality and both in turn have specificity for each other to develop silyl-etheral (–Si–O–Si–) linkages. Thus, by putting these reactive functional groups at different termini a new heterobifunctional reagent (TPMC) **3** was synthesized (Scheme 1) by treating *trans*-4-(aminomethyl) cyclohexane carboxylic acid with maleic anhydride in presence of glacial acetic acid at room temperature (rt) to generate N-substituted maleamic acid. Cyclisation of the intermediate was subsequently done by heating intermediate **1** in presence of acetic anhydride containing sodium acetate as a catalyst to get *N*-(carboxymethylcyclohexyl)-maleimide **2**. The desired reagent TPMC was obtained by stirring compound **2** (1 mM), dissolved in dry THF (15 mL), *N*-hydroxysuccinimide (NHS) (1.5 mM) and dicyclohexylcarbodiimide (DCC) (1.2 mM) for 3 h at room temperature. After completion of reaction (monitored on TLC), dicyclohexylurea was filtered off and to the filtrate 3-aminopropyltriethoxysilane (1.2 mM) and triethylamine (1.2 mM) were added and further stirred overnight at room temperature. The reaction mixture was concentrated in vacuum, redissolved in anhydrous benzene and filtered to remove particulate materials. The filtrate was concentrated in vacuum to obtain TPMC as a gummy mass in almost 70% yield, which was characterized by ^{1}HNMR and MALDI-TOFF.[22]

The oligonucleotides were synthesized at 0.2 μM scale on an ABI 392 synthesizer following standard phosphoramidite approach. To incorporate the reactive thiol (-SH) group at the 5′-end the last coupling was performed with the reagent *S*-trityl-(6-mercaptohexyl)-(2-cyanoeth-



Scheme 1. Reagents and conditions: (i) glacial acetic acid; (ii) acetic anhydride, sodium acetate, 100 °C, 2 h; (iii) 3-aminopropyltriethoxysilane (APTS), *N*-hydroxysuccinimide (NHS), *N*,*N*′-dicyclohexylcarbodiimide (DCC), dimethylformamide (DMF).

yl)-*N*,*N*′-diisopropylphosphoramidite (Glen Res, Inc., Sterline, VA) and oxidation of the final cycle was done with 0.02 M iodine solution to minimize cleavage of the trityl-*S*-linkage. To get fluorescent oligonucleotide, postsynthetic labeling was done with *N*-succinimidyl ester of 6-carboxyfluorescein (6-FAM) as reported earlier on.[23] All the oligomers were treated with 30% aq. NH_4OH solution at 55 °C for 16 h. The ammonical solution was concentrated in a speed vac, redissolved in triple distilled water and desalted on reverse-phase C-18 silica gel column using 30% acetonitrile as eluant. 5′-end thiolated oligomer was obtained by suspending the product in 0.1 M triethylammoniumacetate (TEAA) buffer (pH 6.5) and vortexed with 1 M silver nitrate solution (200 μL) for 30 min at room temperature after that 1 M dithiothreitol (DTT) (250 μL) was added and vortexed further for 5–10 min at room temperature. Centrifugation was done to remove particulate materials, washed with 0.1 M TEAA buffer, pooled the supernatant, desalted, concentrated in a speed vac and stored at 4 °C. Finally, all the oligomers were purified on RP-HPLC using 0.1 M ammoniumacetate (pH 7.1) buffer and acetonitrile (0–50% B in 30 min).

The proposed reagent was synthesized keeping in mind the fast reactivity of the maleimide group for thiol group via stable thioether linkage while the presence of triethoxysilane group (has specificity for covalent bond formation with silanol function) at the other end makes the reagent specific for the glass/silicon surfaces, which is commonly being used for the preparation of biochips due to certain advantages. In order to demonstrate the applicability of the proposed reagent TPMC, kinetics has been studied to know the time intervals required and the extent of reactivity of the reagent towards glass surface. For that a model experiment was performed[24] with reagent DMTr-O-$(CH_2)_6$-SH on unmodified controlled pore glass (CPG, 500 Å) with reagent TPMC alternatively. First DMTr-O-$(CH_2)_6$-SH was treated with reagent TPMC expecting the formation of conjugate via thioether linkage between reagent and the ligand and then reacted with unmodified CPG, 500 Å in which triethoxysilane functionality of the conjugate was supposed to get covalently attached with the unmodified glass beads. Alternatively, reagent TPMC was first treated with unmodified glass beads in which triethoxysilane functionality of the reagent first formed covalent bond with the glass beads, hence generating maleimide functionality on the glass beads and then reacted with model ligand DMTr-O-$(CH_2)_6$-SH at room temperature in which the maleimide functionality of the reagent was expected to form thioether linkage with the ligand. Both the alternating ways were tried and reaction was monitored by withdrawing the reaction vials (6 each for both alternating paths) at regular time intervals and after proper washing and drying, loading on the support was determined by treating weighed amount of the support (~1–2 mg) with 3% trichloroacetic acid in dichloroethane for 5 min. The released DMTr cation was monitored on spectrophotometer at 505 nm wavelength. The loading was calculated in μM/g.[25] It was found that maximum amount of loading (38.4 μmol/g) and in turn the reactivity of the reagent towards the glass surface occurred approximately within 2 h (Fig. 1). This threshold time period was utilized further for the immobilization of the oligonucleotides on the glass surface following both alternating paths (Scheme 1).

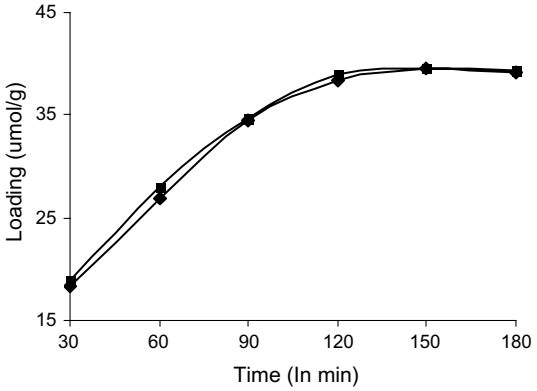


Figure 1. Time kinetics to determine the optimal time required to immobilize oligonucleotides on the unmodified surface via PATH-1 (♦) and PATH-2 (■).

Immobilization of the oligonucleotides has been done by adopting both the paths. PATH-1: 5′-thiolated oligonucleotide sequence [HS-$(CH_2)_6OPO_3$-d(AACCCAGCAC GACGTTTT)], (0.20 A_{260} unit) (100 μL) dissolved in 0.1 M sodium phosphate buffer containing 0.1 M NaCl (pH 7.0) was treated with reagent TPMC solution (100 μL, in 0.15 M DMF). The reaction mixture was vortexed for 2 h at room temperature. The mixture was centrifuged to remove particulate materials and the supernatant was concentrated and treated with capping buffer (10 mM phosphate-buffered saline containing 0.2% BSA) to neutralize the unreacted reagent. The reaction mixture was again centrifuged, washed with phosphate buffer and then concentrated in a speed vac. The oligonucleotide-triethoxysilane conjugate **4** so formed was reconstituted (10 μM) in triple distilled autoclaved water and was subsequently allowed to react with silanol function of (spotted manually, 0.5 μL) virgin glass microslide by incubation for 1 h in a humid chamber at 45 °C.

PATH-2: alternatively, the unmodified microslide was first treated with 1 mL solution of reagent TPMC (0.15 M in DMF) at 45 °C in a humid chamber for 1 h with continuous shaking. Washing was done with DMF (3 × 15 mL) and diethyl ether (3 × 15 mL) to get maleimide functionality on the glass surface and after drying in vacuum, 5′-thiolated oligonucleotide sequence [HS-$(CH_2)_6OPO_3$-d(AACCCAGCACGACGTTTT)], (0.20 A_{260} unit) was spotted manually under the same reaction condition as mentioned above. The spotted slide was washed with phosphate buffer and capping step was performed as above and dried in vacuum to get **5**.

After immobilization of oligonucleotides by both the alternating ways, both plates were hybridized with complementary fluorescent oligonucleotide probe strand (40 μL) FAM-$(CH_2)_3$-OPO_3-d(AAAACGTCGTGCTG

GGTT) (0.25 A_{260} unit) dissolved in 0.1M phosphate buffer containing 1.0 M NaCl (pH 7.5) by keeping for 1 h at 45 °C in a hybridization chamber and then at room temperature overnight. After thorough washing with phosphate buffer (3 × 15 mL) the slides were dried and visualized under fluorescence microscope (Fig. 2).

The minimum concentration of oligonucleotide required for the immobilization on the glass surface was determined by reacting a maleimide-activated glass microslide (PATH-2) with an FITC labeled oligomer d(AA AAAAAAAAAAAAAAA)-OPO_3-$(CH_2)_6$SH dissolved in phosphate buffer in four different concentrations (2.5, 5, 10 and 15 μM) and after washing with buffer and distilled water (3 × 15 mL) fluorescence intensity was measured. It was found that the spot of 10 μM concentration was sufficient for easy visualization (Fig. not given) and hence was selected for immobilization of oligonucleotides and its hybridization study.

To demonstrate the specificity of the proposed chemical method for the immobilization of oligonucleotide strands (-SH), three oligonucleotide sequences, namely, 5′-HS-$(CH_2)_6OPO_3$-d(AACCCAGCACGACGTTTT); HS-$(CH_2)_6OPO_3$-d(CCAGGCAGTTCAAAATTT) and HS-$(CH_2)_6OPO_3$-d(CCACCGGGAATCTTTAAA)-3′ were immobilized on the microslide following PATH-2 in triplicate and hybridized with the complementary fluorescent oligonucleotide probe 5′-FAM-$(CH_2)_3$-OPO_3-d (AAAACGTCGTGCTGGGTT) as mentioned above. The fully matched duplex in microslide **1** (lane b) and microslide **2** (lane c) has shown the fluorescence (Fig. 3) while the unmatched complementary strands have not generated fluorescence at all. Thus, clearly confirmed the specificity of the immobilization chemistry and utility of the newly developed heterobifunctional reagent TPMC. Encouraged with the efficiency of the reagent and stability in the aqueous medium at pH range between 6.5 and 7.5, further work is under progress for the construction of microarray and immobilization of the biomolecules like peptides, proteins, etc. and would be communicated.

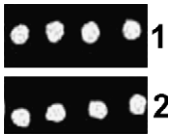


Figure 2. Immobilization of oligonucleotide sequence 5′-HS-$(CH_2)_6$O-PO_3-d(AACCCA GCACGACGTTTT) via PATH-1 (slide 1) and PATH-2 (slide 2).

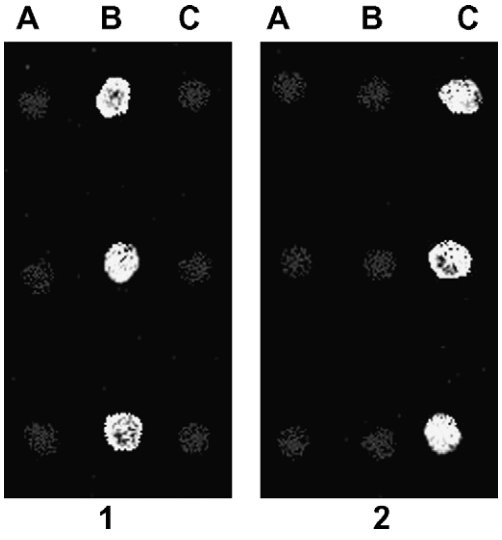


Figure 3. Specificity of immobilization. Three oligonucleotide sequences **in microslide 1**, viz., 5′-HS-$(CH_2)_6OPO_3$-d(CCAG GCAG TTCAAAATTT) (lane a); 5′-HS-$(CH_2)_6OPO_3$-d(AACCCAG CCGA CGTTTT) (lane b) and 5′-HS-$(CH_2)_6OPO_3$-d(CCACCGGGA ATC TTTAAA)-3′ (lane c). And **in microslide 2**, viz., 5′-HS-$(CH_2)_6OPO_3$-d (CCAGGCAGTTCAAAATTT) (lane a); 5′-HS-$(CH_2)_6OPO_3$-d(CC ACCGGGAATCTTTAAA)-3′ (lane b) and 5′-HS-$(CH_2)_6OPO_3$-d (AAC CCAGCACGACGTTTT) (lane c). Hybridization of both microslides with 5′-FAM-$(CH_2)_3$-OPO_3-d(AAAACGTCGTGCT GGGTT) fluorescent probe.

Acknowledgment

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References and notes

1. Ramsay, G. *Nat. Biotechnol.* **1998**, *16*, 40.
2. Marshall, A.; Hodgson, J. *Nat. Biotechnol.* **1998**, *16*, 27.
3. Dorbyshev, A. N.; Mologina, N.; Shick, V.; Pobedimskaya, D.; Yershov, G.; Mirzabekov, A. D. *Gene* **1997**, *188*, 45.
4. Sosnowskii, R. E.; Tu, E.; Butler, W.; O'Connell, J.; Heller, M. *Natl. Acad. Sci. U.S.A.* **1997**, *94*, 1119.
5. Krylov, A. S.; Zasedateleva, O. A.; Prokopenko, D. V.; Rouviere-Yaniv, J.; Mirzabekov, A. D. *Nucleic Acids Res.* **2001**, *29*, 2654.
6. Pollock, J. R.; Perou, C. M.; Alizadeh, A. A.; Eisen, M. B.; Pergamenschikov, A.; Williams, C. F.; Jeffrey, S. S.; Botstein, D.; Brown, P. O. *Nat. Genet.* **1999**, *23*, 41.
7. Zhong, S.; Li, C.; Wong, W. H. *Nucleic Acids Res.* **2003**, *31*, 3483.
8. Pease, A. C.; Solas, D.; Sullivan, E. J.; Cronin, M. T.; Holmes, C. P.; Fodor, S. P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 5022.
9. Elder, J. K.; Johnson, M.; Milner, N.; Mir, K. U.; Sohail, M.; Southern, E. M. In *DNA Microarrays: A Practical Approach*; Schena, M., Ed.; Oxford Press: New York, 1999; p 77.
10. Seliger, H.; Hinz, M.; Happ, E. *Curr. Pharm. Biotechnol.* **2003**, *4*, 379.
11. Kumar, P.; Gupta, K. C. *Bioconjug. Chem.* **2003**, *14*, 507.
12. Defrancq, E.; Hoang, A.; Vinet, F.; Dumy, P. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2683.
13. Nonglaton, G.; Benitez, I. O.; Guisle, I.; Pipelier, M.; Leger, J.; Dubreuil, D.; Tellier, C.; Talham, D. R.; Bujoli, B. *J. Am. Chem. Soc.* **2004**, *126*, 1497.
14. Broude, N. E.; Woodward, K.; Cavallo, R.; Cantor, C. R.; Englert, D. *Nucleic Acids Res.* **2001**, *29*, e92.
15. Kumar, A.; Larsson, O.; Parodi, D.; Liang, Z. *Nucleic Acids Res.* **2000**, *28*, e71.
16. Moller, R.; Csaki, A.; Kohler, A. M.; Fritzsche, W. *Nucleic Acids Res.* **2000**, *28*, e91.
17. Kumar, P.; Choithani, J.; Gupta, K. C. *Nucleic Acids Res.* **2004**, *32*, e80.

18. Patnaik, S.; Swami, A.; Sethi, D.; Pathak, A.; Garg, B. S.; Gupta, K. C.; Kumar, P. *Bioconjug. Chem.* **2007**, *18*, 8.
19. Kumar, P.; Agarwal, S. K.; Gupta, K. C. *Bioconjug. Chem.* **2004**, *15*, 7.
20. Choithani, J.; Kumar, P.; Gupta, K. C. *Anal. Biochem.* **2006**, *357*, 240.
21. Beaucage, S. L. *Curr. Med. Chem.* **2001**, *8*, 1213.
22. ^{1}H NMR, $CDCl_3$ (δ ppm): 2.06–2.38 (br d, 2H, –CH–), 1.40–1.68 (m, 4H, 2 × $–CH_2–$), 8.0 (br d,–NH), 3.2–3.3 (m, 4H, 2 × $–NCH_2–$), 3.63 (m, 9H, 3 × $–OCH_2–$), 1.21–1.55 (m, 6H, 3 × $–CH_2–$), 6.73 (br, d, 2H, –CH═CH–). MALDI-TOF (2,5DHBA): 441 $(M+H)^+$.
23. Misra, A.; Kumar, P.; Gupta, K. C. *Anal. Biochem.* **2007**, *364*, 86.
24. Kumar, P.; Agrawal, S. K.; Misra, A.; Gupta, K. C. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1097.
25. Gait, M. J. *Oligonucleotide Synthesis: A Practical Approach*; IRL Press: Oxford, 1984.