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A new heterobifunctional reagent for immobilization of biomolecules on glass surface

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Abstract—Synthesis of a new heterobifunctional reagent, [*N*-(2-trifluoroethanesulfonatoethyl)-*N*-(methyl)-triethoxysilylpropyl-3-amine] (NTMTA) is described for the immobilization of a variety of biomolecules on glass surface. Its triethoxysilyl group reacts with glass surface and trifluoroethanesulfonate ester structure reacts selectively with aminoalkyl/mercaptoalkyl function in biomolecules. The immobilization can be achieved by two ways involving two steps. The first route involves the reaction of NTMTA with glass beads followed by attachment of aminoalkyl- or mercaptoalkylated biomolecules. The second one involves the reaction of biomolecules, viz., oligonucleotides, proteins, etc., with NTMTA via their aminoalkyl or mercaptoalkyl functions to form a biomolecule conjugate, which is then reacted with glass beads (unmodified) to complete immobilization process. This has been demonstrated by successful immobilization of 5′-mercaptoalkyl- or aminoalkylated oligonucleotides and some commonly used enzymes on glass beads using NTMTA reagent.

A large number of reagents[1–7] (homobifunctional and heterobifunctional) have been reported for introducing modifications in proteins and other biomolecules. These have also been used in cross-linking and forming bridges involving intermolecular and intramolecular reactions. Some of the reagents[8–10] including photoactivatable and photocleavable properties have been used to biotinylate biomolecules, making conjugates, etc., for biological studies. Intramolecular cross-linking has been introduced in proteins and enzymes to provide better conformational stability to their structures. Immobilization of biomolecules on different matrices has resulted in an easy access to a tool to design affinity systems for the purification of precious biomolecules.

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 (biochips).[11–14] For this purpose, glass surface is first modified with the help of suitable reagents to generate reactive functional groups, which in turn are used to fix biomolecules using suitable reagents. The immobilization process involves several steps as well as reagents. In order to simplify this, we wish to report here a new heterobifunctional reagent, [*N*-(2-trifluoroethanesulfonatoethyl)-*N*-(methyl)-triethoxysilylpropyl-3-amine] (NTMTA), which can be used to immobilize biomolecules, viz., modified oligonucleotides, peptides, proteins, etc., directly on unmodified glass beads or microslides. The usefulness of the reagent NTMTA has been demonstrated successfully by immobilizing a number of biomolecules, several enzymes and 5′-mercaptoalkyl-/5′-aminoalkylated oligonucleotides on glass beads.

Preparation of the reagent, NTMTA, was performed by a two-step procedure (Scheme 1). First, 3-chloropropyltriethoxysilane (1 equiv) was reacted with *N*-methyl-2-aminoethanol (1 equiv) in the presence of a tertiary amine (1.1 equiv) under anhydrous condition and inert gas atmosphere. The resultant compound, *N*-methyl,*N*-(2-hydroxyethyl)-3-aminopropyltriethoxysilane **3** was isolated in 65% yield after vacuum distillation under argon gas. The compound **3** (1 equiv) was then converted to the desired compound, *N*-(2-trifluoroethanesulfonatoethyl)-*N*-(methyl)-triethoxysilylpropyl-3-amine] (NTMTA) **4** by its reaction with trifluoroethanesulfonyl chloride (tresyl chloride) (1 equiv) in the presence of triethyl-

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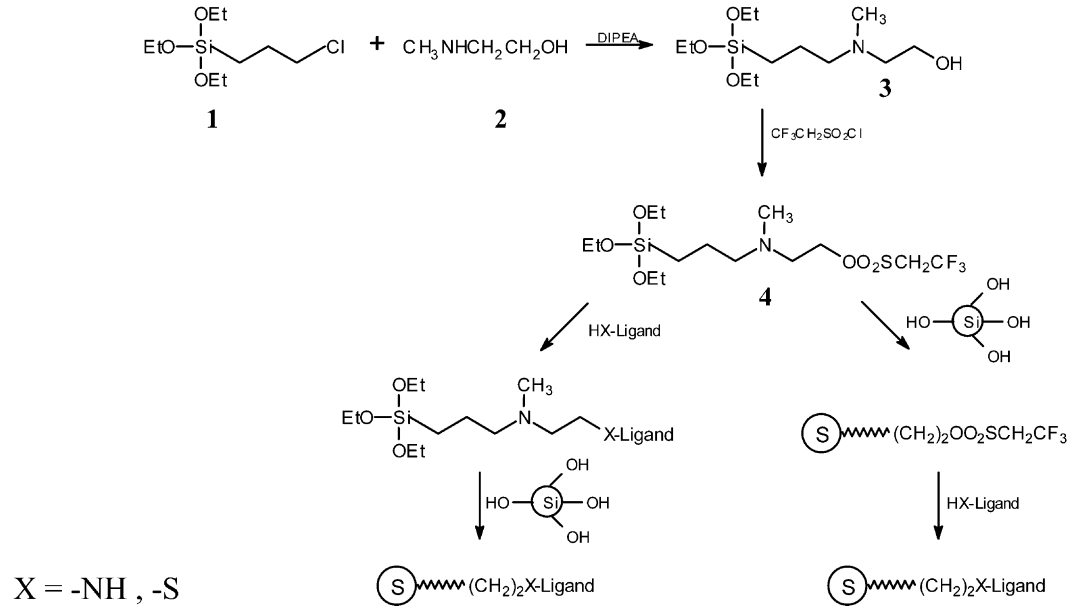

Scheme 1. Preparation of reagent NTMTA and immobilization of ligand on glass surface.

amine (1.1 equiv) under Ar atmosphere in anhydrous ethylene dichloride. The desired compound was fully characterized by its ^{1}H NMR and mass analysis on MALDI-TOF.[15]

The proposed reagent was prepared keeping in mind the fast reactivity of trifluoroethanesulfonate esters with aminoalkyl- or mercaptoalkyl- groups containing ligands/biomolecules. The presence of triethoxysilane group further makes the reagent specific for glass/silicon surfaces, which is being used commonly for the preparation of biochips due to certain advantages. In order to demonstrate the usefulness of the proposed reagent, **4**, we have selected organic compounds, enzymes and modified oligonucleotides with aminoalkyl- or mercaptoalkyl functionalities. The time kinetics of the reaction involving trifluoroethanesulfonate ester and aminoalkyl- or mercaptoalkyl functional groups containing compounds has already been reported.[16] Therefore, we have followed the same time period for completion of the reaction between them. The attachment of organic compounds, enzymes and modified oligonucleotides with aminoalkyl- or mercaptoalkyl functionalities was achieved in two ways, that is, the first one involves the reaction of NTMTA with the glass support through its triethoxysilyl function followed by the reaction of a specific ligand having aminoalkyl- or mercaptoalkyl functionalities. In second one, NTMTA reagent was allowed to react first with the ligand having aminoalkyl- or mercaptoalkyl functionalities through its sulfonate ester function resulting in a ligand conjugate, which is subsequently attached on to glass beads surface through its triethoxysilyl function. *O*-4,4′-Dimethoxytrityl-5-aminopentan-1-ol[17] and *O*-4,4′-dimethoxytrityl-6-mercaptohexan-1-ol[18] were used as model compounds to determine the extent of reactions of the reagent NTMTA with aminoalkyl- and mercaptoalkylated compounds. After reaction, the loading on the support was calculated on the basis of the released dimethoxytrityl cation after treatment with a detritylating reagent.[19] In case of immobilization of aminoalkyl- or mercaptoalkyl-oligonucleotides, the immobilized oligonucleotides, d(TTT TTT TTT TTT TTT TTT TT), were detected by hybridizing with FITC-labeled complementary oligonucleotides, d(AAA AAA AAA AAA AAA AAA AA) followed by visualizing under fluorescence microscope. Similarly, oligomers, $H_2N(CH_2)_6O$-(TTT TTT TTT TTT TTT TTT TT) and $HS(CH_2)_6O$-(TTT TTT TTT TTT TTT TTT TT), were immobilized on unmodified glass slide and hybridized with fluorescein-d(AAA AAA AAA AAA AAA AAA AA). After usual washings with buffer, the spots on the plate were visualized under laser scanner Figure 1 shows the fluorescent spots visualized under laser scanner. Activity of immobilized enzymes was determined by using their respective chromogenic substrates and by measuring the absorbance of the released moiety. The results are summarized in Table 1.

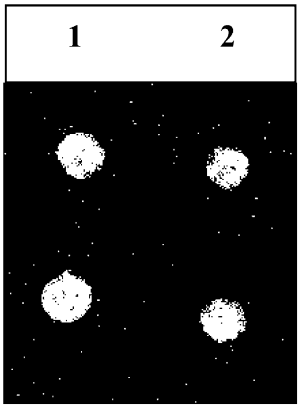

Figure 1. Immobilization of oligonucleotides, $H_2N(CH_2)_6O$-(TTT TTT TTT TTT TTT TTT TT) (Lane 1) and $HS(CH_2)_6O$-(TTT TTT TTT TTT TTT TTT TT) (Lane 2), in duplicates on unmodified glass plate, their hybridization with fluorescein-d(AAA AAA AAA AAA AAA AAA AA) and visualization under laser scanner.

Table 1. : Immobilization of ligands using NTMTA reagent

S.No.	Ligand	S-R + Ligand	R-Ligand + S
1.	$HS(CH_2)_6ODMTr$	37.4 μmol/g of support	32.2 μmol/g of support
2.	$H_2N(CH_2)_5ODMTr$	32.6 μmol/g of support	33.5 μmol/g of support
3.	Glucose oxidase (53 u/mg)	Activity: 137 u/g of support	Activity: 110 u/g of support
4.	Horseradish peroxidase (179 u/mg)	Activity: 87 u/g of support	Activity: 70 u/g of support
5.	Alkaline phosphatase (1.7 u/mg)	Activity: 12.99 u/g of support	Activity: 11.38 u/g of support
6.	$H_2N(CH_2)_6O$-(TTT TTT TTT TTT TTT TTT TT)	Hybridization with FITC labeled d(AAA AAA AAA AAA AAA AAA AA)	Hybridization with FITC labeled d(AAA AAA AAA AAA AAA AAA AA)
7.	$HS(CH_2)_6O$-(TTT TTT TTT TTT TTT TTT TT)	Hybridization with FITC labeled d(AAA AAA AAA AAA AAA AAA AA)	Hybridization with FITC labeled d(AAA AAA AAA AAA AAA AAA AA)

S = CPG (Polymer support); R = Reagent; the values given in the parenthesis against enzymes are activity of that enzyme in solution.

Encouraged by the efficiency of the reagent, NTMTA[20] in immobilization of a variety of biomolecules on glass surface, it has been thought to further explore the potential of this reagent in construction of microarrays of biomolecules (oligonucleotides, peptides, proteins, etc.) on glass microslides. The work is under progress and would be communicated shortly.

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