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N-(3-Triethoxysilylpropyl)-4-(isothiocyanatomethyl)-cyclohexane-1-carboxamide (TPICC): A heterobifunctional reagent for immobilization of biomolecules on glass surface

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ABSTRACT

An efficient heterobifunctional reagent, *N*-(3-triethoxysilylpropyl)-4-(isothiocyanatomethyl)cyclohexane-1-carboxamide (TPICC) has been developed by a simple 'two step reaction' for the preparation of bioconjugates and immobilization of biomolecules such as oligonucleotides, peptides and proteins on the glass surface. The isothiocyanate functionality at one end of the reagent, TPICC was found specific for the ligands having either aminoalkyl (RNH_2) or me]rcaptoalkyl (R-SH) functionality. The synthesis of bioconjugates with the reagent was achieved through its isothiocyanate functionality at one end via the formation of stable thiourea linkage with aminoalkyl and dithiocarbamate linkage with mercaptoalkyl derivatives. The triethoxysilyl functionality of the reagent has been utilized for specific coupling with the virgin glass surface by a very simple methodology.

Nucleic acids 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 convenience, real time and accurate quantification for diverse applications. Recently, microarray technology (biochip) due to its high flexibility and versatility has emerged as an indispensable 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]

Two well established methodologies are available for construction of microarrays. The first methodology[8] involves the direct on surface synthesis (in situ) whereas the second[9] one, popularly known as 'deposition method' involves the immobilization of prefabricated modified biomolecules with either covalent[10–13] or noncovalent[14] bond formation on the surface. The later method utilizes 'spotting technique' and is more popular to construct low to medium density microarrays on account of added advantages such as affordability and flexibility. The microarrays constructed through deposition method is also useful in the fact that modified/unmodified biomolecules prepared chemically or enzymatically can be fully purified and characterized prior to immobilization by spotting method.

The construction of quality microarrays requires a suitable chemical method for functionalization of the surface to facilitate an efficient and unperturbed immobilization of biomolecules with a well defined stable and specific architecture. Heterobifunctional reagents which contain two different reactive functional groups at either ends have been extensively used as a cross-linking reagents and have also gained importance for surface immobilization and pattering of biomolecules on various kind of solid supports.[15–23] Recently, glass/silicon surfaces especially, glass microslide has been used extensively in microarray technology for construction of oligonucleotide, peptide, protein and other biomolecules microarrays. The application of microslides is attributable to ease of modification by silane chemistry, low intrinsic fluorescence, homogeneous surface, resistance to heat, favorable optical properties for sensitive fluorescence imaging and cost effectiveness.

The morphology of the constructed quality microarrays basically depends upon the nature of surfaces selected as well as on employed chemical methods. Generally, solid surface is first, activated through a chemical method using suitable reagents/activators, to make surface functional/reactive corresponding to the respective modified biomolecules. The functional groups on the surface generated can be of either electrophilic nature such as aldehyde, epoxy, isocyanate, isothiocyanate, *N*-hydroxysuccinimide ester (NHS) or of nucleophilic nature such as semicarbazide and amino.[22–24] Electrophilic nature of surfaces are found highly reactive to amino, thiol and hydroxyl reactive functional groups and can be used potentially for efficient covalent immobilization of different classes of compounds such as oligonucleotides, peptides and proteins under milder and ambient reaction

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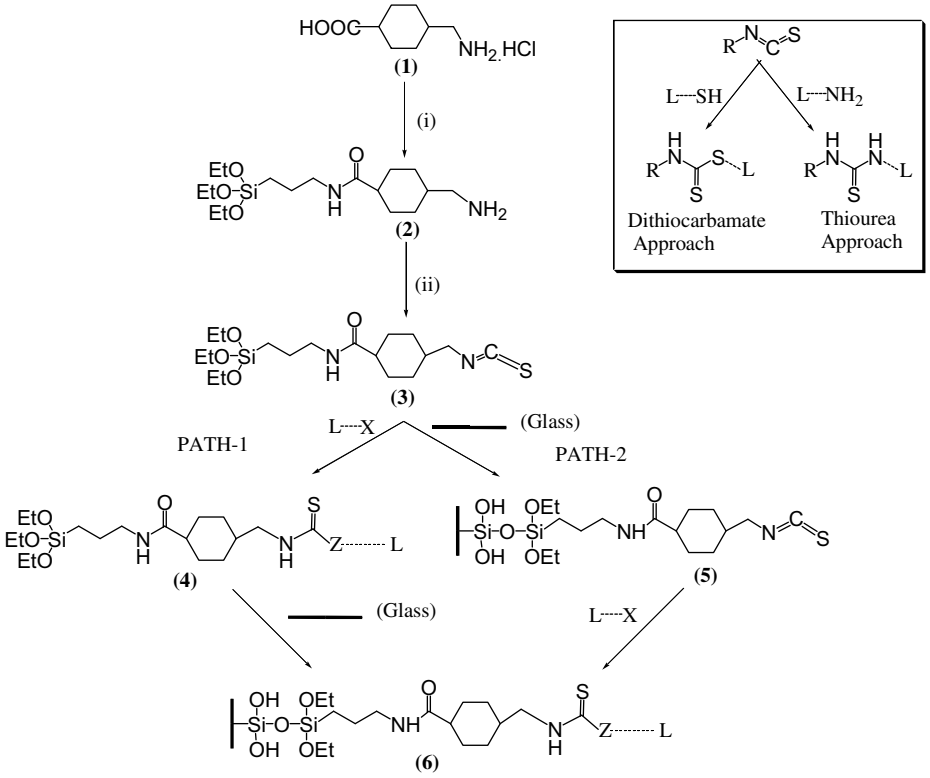
conditions. A large number of combinations, including different functional group containing surfaces and biomolecules have been in practice and most of them popularly include maleimide-thiol,[25,26] alkyne-azide,[27] hydrazide-aldehyde,[28] aminooxy-aldehyde,[20] epoxy-glycol,[23,29] gold-thiol, isothiocyanate-amino,[22] etc. Recently, a trifluoroethanesulphonate ester approach, using heterobifunctional reagent *N*-(2-trifluoroethanesulfonatoethyl)-*N*-triethoxysilylpropyl-3-amine (NTMTA) has been reported[30] to immobilize and construct microarrays of amino and mercaptoalkyl derivative of biomolecules (e.g., oligonucleotides). Consequently, the design and development of an alternative chemical approach for immobilization of aminoalkyl/mercaptoalkyl modified biomolecules on the solid surface, such as glass/silicon is significantly important and still elusive in the area of microarray technology.

Here, we describe the design and synthesis of a new heterobifunctional reagent *N*-(3-triethoxysilylpropyl)-4-(isothiocyanatomethyl)cyclohexane-1-carboxamide (TPICC) with some modification in our previously reported thiol specific reagent[31] to generate isothiocyanate functionality. The applicability of the reagent on the glass beads and microslides has been tested for the immobilization of aminoalkyl/mercaptoalkyl functional group containing ligands and biomolecules such as oligonucleotides and smaller peptides.

The reagent was synthesized at room temperature by stirring suspension of *trans*-4-(aminomethyl)cyclohexane carboxylic acid hydrochloride, **1** (1.5 mmol) in THF (15 ml), *N*-hydroxysuccinimide (NHS) (2.0 mmol) and dicylohexylcarbodiimide (DCC) (1.2 mmol) for 2 h under argon atmosphere (Scheme 1). After the completion of reaction (monitored on TLC), the reaction mixture was kept at 0 °C for 1 h to precipitate out dicyclohexylurea as a byproduct and concentrated in vacuum to leave a solid which was redissolved, filtered (three times to remove further dicyclohexylurea) and dried to get *N*HS ester of *trans*-4-(aminomethyl)cyclohexane carboxylic acid hydrochloride in 71% yield. Finally, the *N*HS ester of *trans*-4-(aminomethyl)cyclohexane carboxylic acid hydrochloride was taken in anhydrous DMF (10 ml) and kept in an ice-bath, filtered and to the filtrate, 3-aminopropyltriethoxy silane (APTS) (1.6 mmol) and triethylamine (TEA) (1.1 mmol) were added dropwise with continuous stirring for 1 h. The reaction mixture was allowed to come at room temperature and left for overnight stirring. After the completion of reaction, (monitored on TLC) solvents were evaporated in vacuum. The crude product was taken in hexane/petroleum ether and washed comprehensively (3 times) with 3% $NaHCO_3$ solution (25 ml), containing triethylamine (TEA). The organic layer was pooled and evaporated to dryness to afford *N*-(3-triethoxysilylpropyl)-4-(aminomethyl) cyclohexane-1-carboxamide, **2** in 75% yield. The hydrochloride salt of unreacted amino acid, *N*-hydroxysuccinimide and triethylamine hydrochloride were removed in washing step and subsequently amino functionality become free for further reaction.

Compound **2** (1 equiv) and TEA (4 equiv) were taken in THF (10 ml), cooled to 0 °C and CS_2 (1.5 equiv) was added dropwise (2 h) with continuous stirring. After complete addition, reaction mixture was allowed to come at room temperature and tosyl chloride (1.5 equiv) was added and stirred the reaction mixture further for 20–30 min to generate isothiocyanate functionality. The solvents were evaporated in vacuum and to the crude product acidic water (20 ml) was added. The desired product was extracted with diethylether (3× 20 ml). The combined organic layer was dried over anhydrous sodium sulphate, filtered and concentrated in vacuum. The desired reagent TPICC **3** was eluted with hexane by passing over short silica column as a semisolid mass in 82% yield.[32]

The proposed reagent was synthesized keeping in mind the objective of a versatile heterobifunctional reagent, specific for ami-



Scheme 1. Synthesis of heterobifunctional reagent TPICC. Reagents and conditions: (i) *N*-hydroxysuccinimide (NHS)/3-Aminopropyltriethoxysilane (APTS)/*N*,*N*′-dicyclohexylcarbodiimide (DCC)/dimethylformamide (DMF); (ii) CS_2/TEA/Tosyl chloride/0 °C/tetrahydrofuron (THF).

noalkyl/mercaptoalkyl ligands and biomolecules through a straightforward simple, cost effective and short chemical methodology. The stable isothiocyanate functionality of the reagent, TPICC is expected to form stable thiourea/dithiocarbamate type of linkages with amino/thiol derivatives in aqueous and organic medium at a suitable pH range 6.5–8.5. While on the other hand the presence of triethoxysilane group further, makes the reagent specific for glass/silicon surfaces through the formation of stable silyl-eteral (–Si-O-Si–) linkages.

The time kinetics for the formation of conjugates in between heterobifunctional reagent, TPICC and aminoalkyl/mercaptoalkyl ligands was carried out alternatively as reported previously.[31] In first alternative (PATH-1), reagent TPICC was treated with glass beads CPG 500 Å through its triethoxysilyl functionality and then covalently conjugated separately with ligands, viz., DMTr-$(CH_2)_6$-NH_2 and DMTr-$(CH_2)_6$-SH in dimethylformamide (DMF) and 1.5% TEA. In the second alternative (PATH-2), first reagent TPICC was conjugated with both the ligands through their amino and thiol functionality to form reagent–ligand conjugates or bioconjugates with the formation of stable thiourea and dithiocarbamate linkages, respectively. The reagent–ligand conjugates so formed were subsequently attached on to unmodified glass beads, CPG 500 Å surface through its triethoxysilyl function. The progress of the reaction was monitored by withdrawing the reaction vials after a regular time intervals and after proper washing and drying steps, weighed amount of support (1–2 mg) was treated with 3% trichloroacetic acid (TCA) in 1,2-dichloroethane for 5 min.[33] The loading on support (Table 1) was estimated by spectrophotometric determination of DMTr cation released at 505 nm wavelength. A graph was plotted between time (min) and loading (μmol/g) on the glass beads support to estimate the extent of reaction and optimum time requirement for complete chemical reaction of immobilization. For the covalent bond formation between support and reagent TPICC, or in between glass support and reagent–ligand conjugates the reaction was allowed to proceed at 45 °C in an Eppendorf Thermomixer for 45–60 min.

From Figure 1 we observed that immobilization of mercaptoalkyl ligand on the support reached to saturation within 60 min with the formation of dithiocarbamate linkage while aminoalkyl ligand has taken much longer time (120 min), to complete the reaction through the formation of thiourea linkage. These optimum time periods were further utilized for the immobilization of other biomolecules on the glass surface. The immobilization process was found faster through dithiocarbamate approach however, the extent of loading on the support (Table 1) was found greater in case of thiourea approach. Further, as evident from kinetic data, although the alternative paths are showing promising results however, comparatively, first alternative (PATH-1) in which reagent TPICC was covalently attached with glass support, prior to immobilization of aminoalkyl/mercaptoalkyl ligand, has been found more favorable and feasible with greater extent of immobilization of biomolecules. Thus, the reagent TPICC developed was found

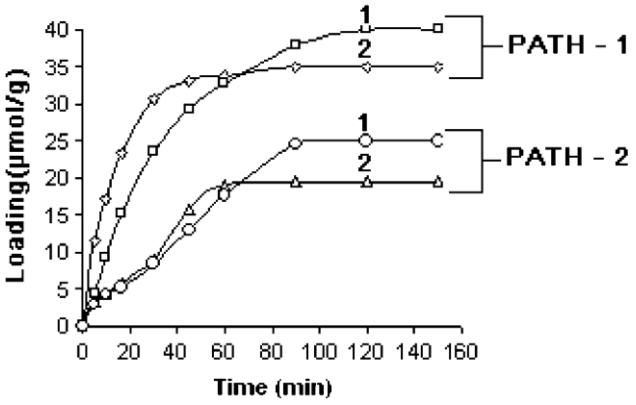


Figure 1. Time kinetics for aminoalkyl (**1**) and mercaptoalkyl (**2**) ligands, respectively, to know optimum time requirement for immobilize biomolecules on the glass surface (CPG 500Å), via both the alternating paths.

suitable for immobilization process on the glass support and can be potentially useful for the immobilization of biomolecules on the glass/silicon surfaces.

In order to test the potentiality of the newly developed heterobifunctional reagent TPICC, derivatives of tripeptides having free amino and thiol ends were prepared by coupling with the ligand *O*-4,4′-dimethoxytrityl-5-aminopentan-1-ol (DTAP) to bring dimethoxytrityl group at the other end.[34] Tritylated (DMTr) smaller peptides, dissolved in 1 M sodium borate buffer (pH 8.5) were subjected to reaction conditions of PATH-1 and also for same time period to complete the immobilization process on glass beads. After completion of reaction DMTr group was removed by treating glass beads with 3% TCA in 1,2-dichloroethane and as mentioned above loading was estimated in good yields (Table 1).

To validate further the current methodology of immobilization, unmodified microslides were charged with heterobifunctional reagent TPICC (0.3 M in DMF) at 45 °C in a humid chamber for 1 h with continues shaking. Washing of the microslides were carried out in DMF followed by diethyl ether (15 ml, 3 times) and dried under anhydrous condition to get isothiocyanate functionality on the glass surface. 5′-Aminoalkyl and mercaptoalkyl-oligonucleotides, viz., H_2N-$(CH_2)_6OPO_3$-d(AAA AAA AAA AAA AAA) and HS-$(CH_2)_6O$-PO_3-d(AAA AAA AAA AAA AAA) (0.20 A_{260} unit) (100 μL), dissolved in 0.1 M sodium phosphate buffer containing 1.5% TEA (pH 8.5), respectively, were spotted manually[26] (0.5 μL) with the help of pipette on the isothiocyanate activated microslides. The reaction was allowed to proceed for respective optimum time periods. After completion of reaction the microslides were washed and treated with capping buffer solution containing ethanolamine (1%), to neutralize the activated slides for further reaction. After proper washing and drying, immobilized microslide were hybridized with complementary fluorescent oligonucleotide probe strand **ODN1**,

Table 1
Immobilization of ligand and biomolecules using heterobifunctional reagent, TPICC

S.No.	Ligand	S-R + Ligand	R-L + Support
1.	SH-$(CH_2)_6$-ODMTr	33.9 μM/g of support	29.9 μM/g of support
2.	H_2N-$(CH_2)_6$-ODMTr	38.9 μM/g of support	32.9 μM/g of support
3.	H_2N-(Lys·Gly·βAla)-$(CH_2)_5$-ODMTr	24.5 μM/g of support	22.9 μM/g of support
4.	HS-(Cys·Gly·βAla)-$(CH_2)_5$-ODMTr	19.3 μM/g of support	15.4 μM/g of support
5.	H_2N-$(CH_2)_6OPO_3$-d(AAA AAAAAA AAA AAA)	Hybridization with Fluorescent probe **ODN1**	Hybridization with Fluorescent probe **ODN1**
6.	HS-$(CH_2)_6OPO_3$-d(AAA AAAAAA AAA AAA)	Hybridization with Fluorescent probe **ODN1**	Hybridization with Fluorescent probe **ODN1**

S, glass support (CPG 500Å and microslides); R, heterobifunctional reagent (TPICC). Values reported are within range of ±2% percentage error.

viz., FAM-(CH_2)3-OPO3-d(TTT TTT TTT TTT TTT). **ODN1** (40 μl, 0.25 A_{260} unit), dissolved in 0.1 M phosphate buffer containing 1.0 M NaCl (pH 7.5) was charged on the spotted area and kept for 2 h at 45 °C in a hybridization chamber and then at room temperature overnight to facilitate complete hybridization between the complementary oligonucleotide strands. After thorough washing with phosphate buffer (15 ml, 3 times) the slides were dried and fluorescence intensity of the duplexes on the microslides were visualized under fluorescence microscopes (Fig. 2).

The minimum concentration of oligonucleotide required for the immobilization on the glass surface was determined by reacting a isothiocyanate-activated glass microslide (PATH-1) with a fluorescein labeled oligonucleotide strand d(AAA AAA AAA AAA AAA AAA)-OPO_3-$(CH_2)_6$SH dissolved in phosphate buffer, in four different concentration (2.5, 5, 10, and 15 μM) and after washing with buffer and distilled water (15 ml) fluorescence intensity was measured. It has been observed that the spot of 10 μM concentration was sufficient for easy visualization (figure not given) and hence was selected for immobilization of oligonucleotides and their hybridization study.

To demonstrate the specificity (Fig. 3) of the current methodology using newly developed heterobifunctional reagent, unmodified oligonucleotides strand d(AAA AAA AAA AAA AAA) and 5′-amino/mercaptoalkyl-oligonucleotides, viz., H_2N-$(CH_2)_6OPO_3$-d(AAA AAA AAA AAA AAA) and HS-$(CH_2)_6OPO_3$-d(AAA AAA AAA AAA AAA) (0.20 A_{260} unit) (100 μL), dissolved in 0.1 M sodium phosphate buffer containing 1.5% TEA (pH 8.5), respectively, were spotted on microslides as described above. After proper incubation, washing and capping steps microslides were dried and hybridized with fluorescent oligonucleotide **ODN1**. After thorough washing with phosphate buffer microslides were dried and visualized. No measurable amount of fluorescence signal was obtained in case of unmodified immobilized oligonucleotide duplexes (**1**, microslide C and D) thereby, confirming the specificity of current methodology for isothiocyanate and amino/mercaptoalkyl derivatives.

Conclusively, an efficient heterobifunctional reagent has been developed for the immobilization of aminoalkyl/mercaptoalkyl ligands and biomolecules on a glass/silicon surfaces through a stable thiourea/dithiocarbamate linkages. The further application of the reagent, its stability, suitability for construction of good quality of arrays of biomolecules and specificity of detection is under consideration and will be presented in further communications.

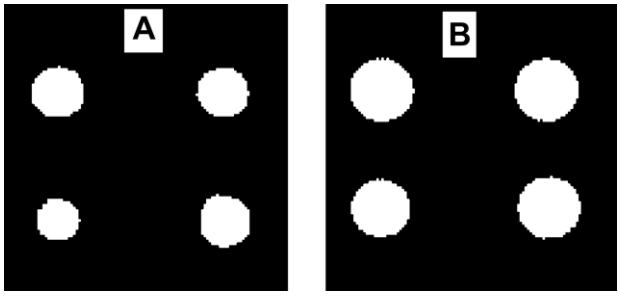


Figure 2. Fluorescence images of immobilized oligonucleotides, H_2N-$(CH_2)_6OPO_3$-d(AAA AAA AAA AAA AAA) (in microslide A) and HS-$(CH_2)_6OPO_3$-d(AAA AAA AAA AAA AAA) (in microslide B) (spotted in 0.5 μL, 10 μM) (PATH-1). Hybridization of both microslides with fluorescent oligonucleotide probe, **ODN1** (40 μL, 0.25 A_{260} unit).

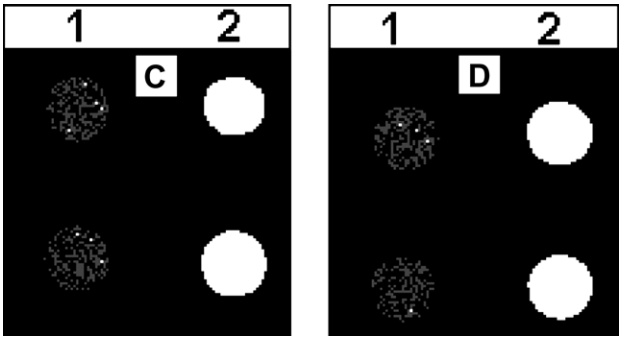


Figure 3. Fluorescence images showing the specificity of immobilization: **1,** unmodified oligonucleotide d(AAA AAA AAA AAA AAA) (in both microslide C and D); **2**, amino modified oligonucleotide H2N-(CH2)6OPO3-d(AAA AAA AAA AAA AAA) (in microslide C) and HS-(CH2)6OPO3-d(AAA AAA AAA AAA AAA) (in microslide D) (spotted in 0.5 μL, 10 μM) (PATH-1). Hybridization of both microslides with fluorescent oligonucleotide probe, **ODN1** (40 μL, 0.25 A260 unit).

Acknowledgments

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32. Characterization of **2**: ^{1}H NMR (DMSO-d_6) (δ ppm): 1.31–3.89 (m, 15H, $-CH_2CH_3$), 1.2 (t, 2H, $-CH_2$), 1.78 (m, 2H, $-CH_2$), 3.33 (m, 2H, $-CH_2NH-$), 8.1 (d, 1H, J = 6.9 Hz, –NHCO–), 1.3–1.62 (m, 8H, cyclohexane), 2.15 (m, 1H), 1.79 (m,1H), 2.65 (m, 2H, CH_2NH_2). MALDI-TOF: $[M+H]^+$ 360.54 (calc.), 362.1

(found). Characterization of **3**: ^{1}H NMR (DMSO-d_6) (δ ppm): 1.35–3.91 (m, 15H, $-CH_2CH_3$), 1.2 (t, 2H, $-CH_2$), 1.8 (m, 2H, $-CH_2$), 3.31 (m, 2H, $-CH_2NH-$), 8.21 (d, 1H, J = 6.7 Hz, –NHCO–), 1.35–1.61 (m, 8H, cyclohexane), 2.12 (m, 1H). MALDI-TOF: $[M+H]^+$ 402.45 (calc.), 403.4 (found).

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34. Characterization of ligand **3**: ^{1}H NMR (DMSO-d_6) (δ ppm): 1.19 (m, 4H, $-CH_2-$), 1.35 (m, 4H, $-NHCH_2$), 1.41 (m, 2H, $-CH_2$), 1.57 (m, 2H, $-CH_2$), 2.47 (t, 2H, J_1, J_2 = 7.12 Hz, $-CH_2CONH$), 2.85 (t, 2H, J = 6.7 Hz, $-CH_2NH_2$), 3.25 (t, 2H, J = 7.1 Hz, $-CH_2NH$), 3.57 (t, $-CH_2O-$), 3.38 (t, 2H, $-CH_2$), 3.66 (t, 1H, J = 8.15 Hz, $-CHNH_2$), 3.83 (s, 6H, $-OCH_3$), 3.97 (br, 4H, NH_2), 4.19 (s, 2H, $-CH_2$), 7.18 (m, 13H, DMTr), 7.67 (br, 3H, –NH). Characterization of ligand 4: ^{1}H NMR (DMSO-d_6) (δ ppm): 1.18 (m, 2H, $-CH_2$), 1.49 (m, 2H, $-CH_2$), 1.65 (m, 2H, $-CH_2$), 2.21 (s, 3H, $-COCH_3$), 2.64 (t, 2H, $-CH_2$), 3.26 (d, 2H, $-CH_2SH$), 3.1 (t, 2H, $-CH_2$), 3.47 (t, 2H, $-CH_2O-$), 3.41 (t, 2H, CH_2), 4.19 (s, 2H, CH_2), 3.63 (s, 6H, OCH_3), 4.89 (t, 1H, CHNH), 7.28 (m, 13H, DMTr), 7.57 (br, 4H, –NH).