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Immobilization of self-quenched DNA hairpin probe with a heterobifunctional reagent on a glass surface for sensitive detection of oligonucleotides

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ABSTRACT

A new sensitive method for the detection of nucleic acids on a glass surface has been described. The self-quenched DNA hairpin probe is immobilized on a glass surface utilizing heterobifunctional reagent, *N*-(3-triethoxysilylpropyl)-4-(isothiocyanatomethyl)-cyclohexane-1-carboxamide (TPICC). In the closed state fluorescence intensity was quenched due to the presence of guanosine residues in close vicinity of fluorophore while on hybridization with perfectly matched complementary target strand fluorescence was restored. Efficiency and specificity of immobilization as well as thermal stability at variable temperature and pH conditions have been discussed in detail. The method employed has potential for the detection of single nucleotide variations and other diagnostic studies.

1. Introduction

Nucleic acids based detection and quantification methods play an important role in the field of DNA-diagnostics and drug discovery. The labeled oligonucleotides with organic fluorophores instead of radioisotopes are routinely being used to recognize the DNA sequences in homogeneous as well as in heterogeneous hybridization assays.[1,2] Oligonucleotides having hairpin like structure—hairpin probes, are labeled with a fluorophore at one end and a quencher molecule (conventional/natural) at the other terminal. Recently, such kind of single labeled hairpin probe has emerged as an effective diagnostic tool in molecular biology studies similar to the conventional molecular beacons.[3,4] The molecular beacons comprise stem–loop like structure (ideally, 4–7 nucleobase pairs in stem region and 15–25 nucleobase pairs in loop region) and are usually dual labeled with a fluorophore (donor dye) at 5′-end and with an acceptor (as a quencher), which may or may not be fluorescent, at the 3′-end. In the absence of perfectly matched complementary target strand fluorophore and quencher molecule remain in proximity and as a result of dipole–dipole interaction between them, the fluorescence intensity of the probe quenches efficiently by the phenomenon of fluorescence resonance energy transfer (FRET).[5]

In the case of molecular beacons, the strategy requires labeling at both ends of a single stranded oligonucleotide with a specific fluorescent dye which generally limits the yield of synthesized oligonucleotide probe and is also an expensive approach. To circumvent the limitations possessed by the molecular beacons, hairpin probes have been designed and developed involving a single fluorescent molecule at one end and an acceptor molecule, like nucleobase or amino acid, on the other end, to quench the fluorescence intensity of the fluorophore in the closed state conformation. For this purpose, a number of unique non-fluorescent quenchers instead of traditional ones, ranging from DNA nucleotides (guanosine, deazaguanosine) to gold nanoparticles have already been introduced successfully.[6–10] Additionally, instead of utilizing interactions between the two extrinsic probes, interaction of fluorophores with DNA nucleobases or with amino acids has potentially used for the specific detection of DNA or RNA sequences and antibodies at the single molecular level.[11–13]

The fluorescence quenching by mono-labeled hairpin probe is based on the phenomenon of photoinduced electron transfer (PET), in between the first excited singlet state of fluorophore and ground state of a quenching molecule when present in proximity, in which electron donating moiety like guanosine oxidized to form radical ion pair ($G^{+\cdot}$) and return to ground state via radiationless charge recombination.[14,15] Both the tools are successfully applied in diagnostics to detect the presence of the specific target nucleic acid sequences, after hybridization with matched complementary target strands, by strong increase in the fluorescence intensity signal. Therefore, makes the homogeneous assay more reliable, sensitive and versatile.

For high-throughput screening of biomolecules microarray technology (biochip), in last decades, has become a potent tool with diversified applications in genetic analysis, molecular diagnostics, and drug discovery.[16–26] Of two well-established method-

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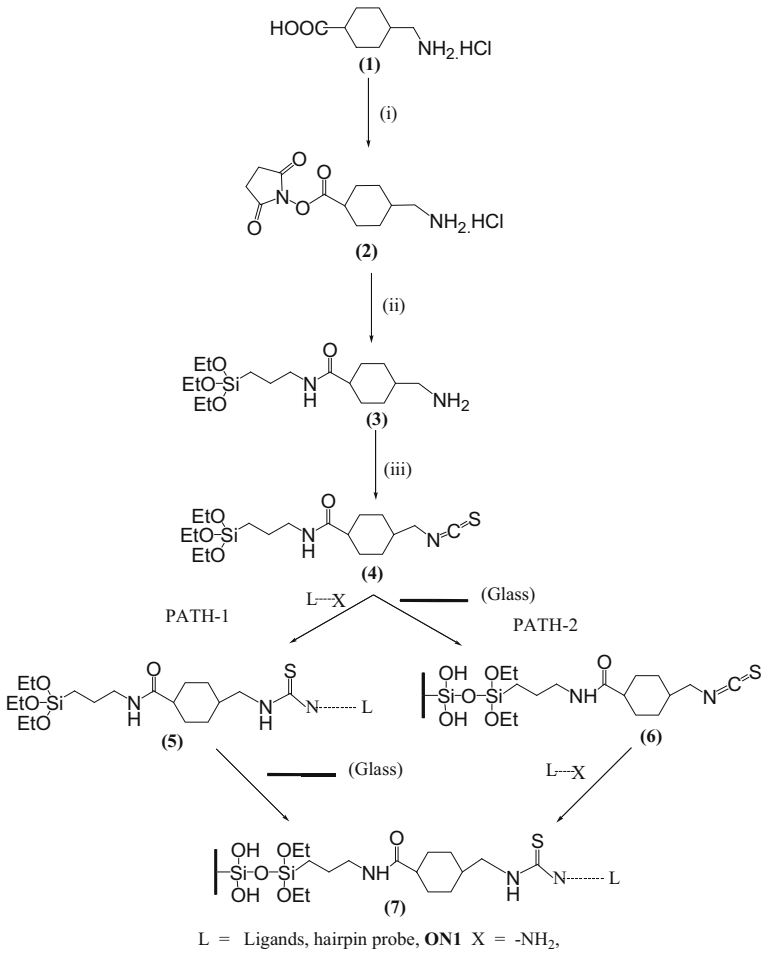
ologies for the construction of microarrays—in situ synthesis[27,28] and deposition method,[29] the latter has become more popular to construct low to medium density microarrays with great affordability and flexibility. This approach is also useful because modified/unmodified biomolecules synthesized chemically or enzymatically can be characterized and purified prior to immobilization process on a solid or polymeric surface. The immobilization of biomolecules on a surface like glass microslide by the latter approach, can be achieved by suitable chemical reactions and then by interactions between surface-bound reactive groups and functionalized biomolecules.[30,31]

Thus, in conjunction to our previous work with hairpin probe for the detection of target strands in solution phase[32] and development of heterobifunctional reagents for the immobilization of biomolecules on a glass surface,[33–36] in present communication we wish to report an approach, for an appropriate heterogeneous surface-based assay, to immobilize a modified 3′-aminoalkyl mono-labeled hairpin probe on a glass surface utilizing our previously developed new heterobifunctional reagent viz. *N*-(3-triethoxysilylpropyl)-4-(isothiocyanatomethyl)-cyclohexane-1-carboxamide (TPICC),[33] through a urea linkage. The trans arrangements of both aminomethyl and carboxylic functionalities present at the stable chair conformation of cyclohexane moiety of molecule have been explored for a specific patterning of hairpin probe on a glass surface as well as for maintaining an appropriate distance between the probe and solid surface. The specificity of the current methodology was achieved by the hybridization studies with different target strands, complementary to the loop region of hairpin probe. The observed enhanced fluorescence signal with a perfectly matched target strand confirms the presence of hairpin probe in two states—closed and open, and also the diminished fluorescence signal in the case of non-complementary and complementary strands having mismatches shows the specificity of the approach.

2. Results and discussion

The proposed reagent, TPICC was synthesized (Scheme 1) as reported previously[33] keeping in mind an objective for a versatile heterobifunctional reagent specifically for amino/mercapto-alkyl ligands and biomolecules through a straightforward simple, cost effective and short chemical method. The stable isothiocyanate functionality of the reagent is expected to form a stable thiourea/dithiocarbamate type of linkages with amino/thiol derivatives in organic and/or aqueous medium in a suitable pH range 6.5–8.5. The presence of triethoxysilane group at another terminus of reagent has liking for silanol functionality, present on the solid surface, thus making the reagent specific for glass/silicon surfaces through the formation of stable silyl-etheral (–Si–O–Si–) linkage which is frequently in use for the immobilization of biomolecules and construction of biochips.

First loading on the glass support (i.e., the amount of functional groups, μmol/g of solid support or μmol/m^2 on a microslide) has been estimated kinetically as established previously[33] through a



Scheme 1. Synthesis of heterobifunctional reagent, TPICC. (i) *N*-Hydroxysuccinimide (NHS) (ii) 3-aminopropyltriethoxysilane (APTS)/*N*,*N*′-dicyclohexylcarbodiimide (DCC)/DMF; (iii) CS_2/TEA/tosyl chloride/0 °C/THF.

more favorable and feasible route, path-2, and optimum time, 2 h, was further utilized to immobilize hairpin probe. In brief, control pore glass beads (CPG-500 Å) were reacted with reagent **4** (Scheme 1) in *N*,*N*′-dimethlyformamide (DMF), containing 1.5% triethylamine (TEA) at 45 °C for 45–60 min. After proper washing and drying, TPICC-activated glass beads, **6**, were charged with a solution of model ligand, *O*-(4,4′-dimethoxytrityl)-6-aminohexan-1-ol (0.01 M in DMF containing 1.5% TEA), to get **7**. The reaction vials were withdrawn at regular time intervals and were washed sequentially with DMF and diethylether containing TEA and dried. Kinetics was done to know optimum time period required for maximum extent of immobilization of ligand/oligonucleotide.

2.1. Optimum pH condition for immobilization of hairpin probe on a glass surface

The optimal pH requirement for effective covalent attachment of hairpin probe on a TPICC-activated glass surface was determined indirectly by reacting, model ligand, DMTr–O–$(CH_2)_6$–NH_2 and glass beads under different pH conditions, 6.5, 7.5, 8.5, 9.5, 10.5, and 11.5. The reaction was allowed for optimum time and then was treated with 3% trichloroacetic acid (TCA). The released dimethoxytrityl cation was estimated spectroscopically at 505 nm. A graph between loading on glass beads support and pH values illustrates (Fig. 1) maximum loading (~32–33 μmol/g) in pH range 8.5–9.5 and hence, the efficiency of immobilization in alkaline condition. Additionally, the extent of immobilization of model ligand on TPICC-activated glass support increases by increasing the alkalinity of the reaction medium and on average was found 31 μmol/g in pH range 6.5–9.5. However, in more alkaline condition (at pH 11.5), the loading on support surprisingly decreased (~25%) to a minimum. This relative decrease may be attributed to either hydrolysis of amide linkage or competitive nucleophilic addition reactions between hydroxyl and amino functionality. Thus, optimal pH 8.5 was further utilized to determine the extent of immobilization of hairpin probe on a glass surface.

2.2. Synthesis and properties of oligonucleotide

The sequences of oligonucleotides were designed with the aid of OligoAnalyser (IDT, USA) and caution has been taken to minimize the formation of unwanted hairpins and/or secondary structures due to cross hybridization and the formation of self-dimers. The oligonucleotides were custom synthesized. The custom synthesis of hairpin probe was carried out at 0.2 μmol/g on a 3′-amino modifier, viz. (2-dimethoxytrityloxymethyl-6-fluorenylmethoxycarbonylamino-hexane-1-succinoyl)-long chain alkylamino-CPG.

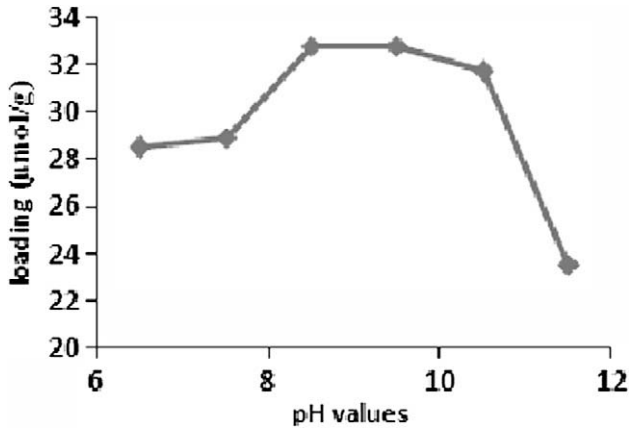


Figure 1. Kinetics with model aminoalkyl ligand shows optimum pH required to immobilize hairpin probe on a glass surface via path-2.

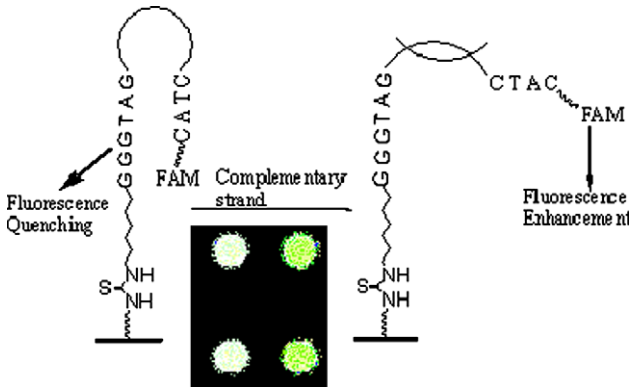


Figure 2. Systematic representation of hairpin probe and change in fluorescence signal after hybridization with matched target strand.

The last coupling was done with FAM–phosphoramidite to obtain 5′-labeled hairpin probe (**ON1**). The hairpin probe includes—a loop region of homogeneous deoxyadenosine (dA) nucleotide sequence and stem portion of four different complementary nucleotides (CATC·GATG). Two extra 'dG' units were introduced at dangling arm to ensure efficient fluorescence quenching.

2.3. Immobilization of hairpin on a glass support

The hydrated unmodified/virgin glass slides were first treated with reagent **4** to get TPICC-activated microslides. After proper washing and drying, as mentioned above, hairpin probe, **ON1** (10 μM, 0.5 μl) dissolved in a reaction buffer was spotted. The spotted slide was kept in a humid chamber for 2 h and then subjected to washings with reaction buffer and dried in vacuum. Prior to hybridization, residual isothiocyanate groups on activated glass microslide were blocked by treating with a capping buffer solution for 1 h, followed by washing steps, dried and visualized under a laser scanner. In the absence of complementary target strand (Fig. 2), hairpin probe remains in the closed state and guanosine residues, present at the dangling arm of hairpin probe and xanthene-based dye, fluorescein, are in proximity as a result of which on photoirradiation, due to low oxidation potential of guanosine and photoinduced electron transfer phenomenon[10–12] between fluorophore and guanosine moieties, diminished fluorescence was observed. However, on charging the second duplicate spot of immobilized hairpin probe with a perfectly matched strand (**ON2**), fluorescence signal was restored. The relative enhancement in fluorescence signal is attributed to the separation of fluorophore and dangling 'dG' residues far apart from each other. The driving force for conformational reorganization of hairpin probe is due to the formation of stable duplex on hybridization and as a consequence the involvement of more number of H-bondings in the loop region of hairpin probe than in the stem region.

2.4. Determination of threshold concentration of oligonucleotide required for fluorescence detection and hybridization study

In order to estimate threshold concentration required to construct a good quality of microarray of mono-labeled hairpin probe on a glass surface with clear illustration of fluorescence spot, steady state kinetics has been performed. Modified aminoalkyl hairpin probe, **ON1** (0.20 A_{260} unit) (100 μl) dissolved in a reaction buffer was serially diluted (15, 10, 7.5, 5, 2.5 and 1.5 μM concentration) and spotted (0.5 μl in all cases) in duplicates on a TPICC-activated glass slide. Microslides were incubated for 2 h in a humid chamber at 25 °C and were washed sequentially with reaction as well as with

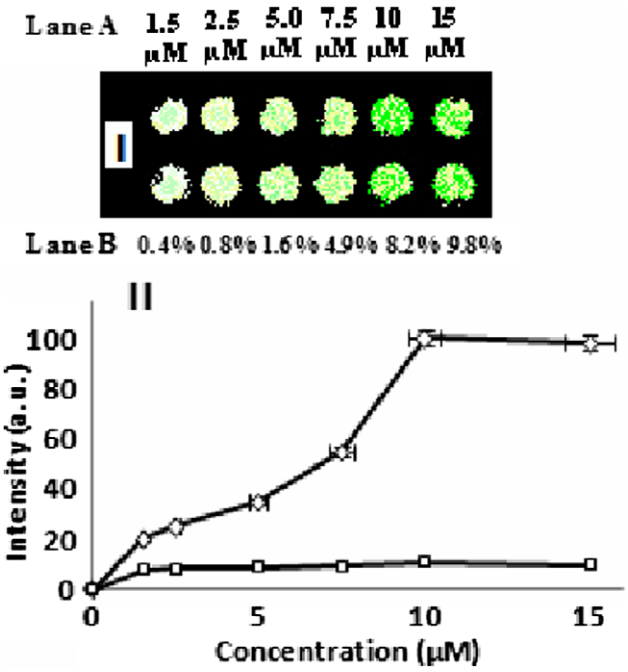


Figure 3. Scanned fluorescence images. (I) Optimum concentration for hairpin probe (**ON1**) on hybridization with a perfectly matched target strand (**ON2**). Graph (II) shows a relative increase in fluorescence signal for **ON1**. Lane A: concentration of spots. Lane B: Immobilization efficiency (including ±3% error and 1% SD).

washing buffers, dried and scanned. Diminished fluorescence signal for almost all spotted concentrations was observed, showing the presence of hairpin probe in closed state (Fig. 3II (square markers)). To minimize non-specific immobilization reaction, the remaining isothiocyanate active sites were capped as mentioned above.

For hybridization study **ON2,** complementary to the loop region of hairpin probe was dissolved in hybridization buffer in excess and charged at pre-spotted areas (20 μl, 0.40 A_{260} unit) on a glass surface. To ensure complete hybridization between complementary strands slides were kept at 55 °C for 2 h in a humid chamber (relative humidity ~80–89%) and then left overnight at room temperature. The slides were washed sequentially with hybridization and washing buffer to remove unhybridized complementary strand and dried before scanning under fluorescent scanner (Fig. 3I and II). Microslide shows relatively enhanced fluorescence signals for all the concentration tested however, the intensity observed for spot concentrations 7.5, 10 and 15 μM was found intense compared to other concentration levels. The DNA duplex formed for 10 μM concentration has shown a maximum enhancement in fluorescence intensity with clear visibility of spot. Since (Fig. 3I) intensity signal reaches a plateau at 10 μM concentration, it was considered as an optimum concentration to construct quality of arrays for hairpin probe. The results also demonstrate that the attachment efficiency of modified hairpin probe is almost proportional to the concentration of oligonucleotide and thus, clearly indicated that at 10 μM concentration complementary strands met the optimum stoichiometry to form stable duplex on a glass surface. However, higher concentration (15 μM) does not influence the fluorescence signal. The considerable variation observed in fluorescence intensity corresponding to the different concentrations of immobilized hairpin probe is in fact governed by the number of probes immobilized on the surface as well as on the availability of quantums of fluorophoric unit.

The relative fluorescence signal generated before hybridization corresponding to different concentrations of immobilized probe (Fig. 3II) was utilized to estimate the percentage of background fluorescence. The maximum enhancement in fluorescence signal observed for 10 μM concentration of immobilized hairpin probe was ~33–37%, including ~11–13% background fluorescence, compared to lower concentration levels and hence concomitant maximum density of immobilization on the glass surface. Further, at 15 μM concentration we observed a small decrease in fluorescence intensity, which may be attributed to over-crowding and/or lower accessibility of the surface-bound probe. In addition, the efficiency of hybridization and enhancement in fluorescence signal are guided by other important factors such as availability of active sites to interact, attachment of oligonucleotides on surface and extent of hybridization.

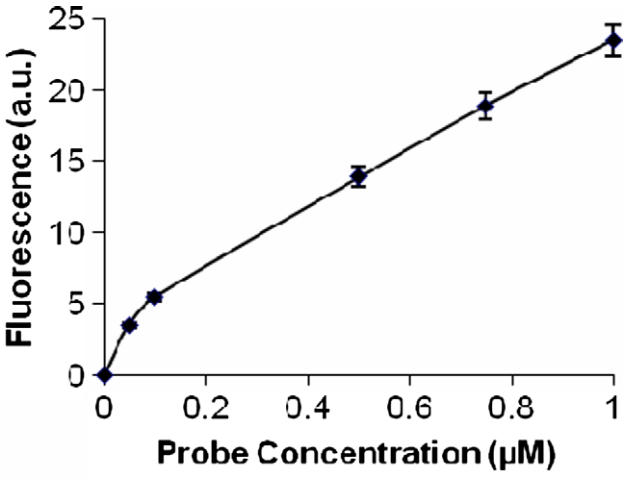


Figure 4. Calibration curve shows a linear correlation in between fluorescence intensity and concentration of spotted oligonucleotide **ON1** on a virgin glass surface (spotted in 0.05–1.0 μM).

2.5. Efficiency of immobilization of hairpin probe on a glass surface

In an attempt to determine and quantify the efficiency of immobilization for hairpin probe on a glass surface, **ON1**, was serially diluted 0.05, 0.1, 0.5, 0.75, 1.0 μM and was spotted on a virgin/unmodified glass surface as reported previously.[34,40] The microslide was dried and directly scanned to obtain a calibration curve by plotting the fluorescence intensity as a function of oligonucleotide concentrations (Fig. 4). An approximately straight line indicated a linear correlation between the fluorescence intensity and concentrations of spotted oligonucleotide. The immobilization efficiency on the basis of calibration curve was estimated and found to be 9.8%, 8.2%, 4.9%, 1.6%, 0.8%, and 0.4% against each concentration, respectively.

2.6. Optimum pH condition for hybridization study

To arrive at optimum pH required for hybridization on a glass surface, **ON2** was dissolved in hybridization buffer and treated with immobilized hairpin probe at 6.5, 8.5, and 10.5 pHs. After washing and drying steps as mentioned above, microslides were scanned. Fig 5B shows maximum fluorescence signal at pH 8.5 while less intensity was observed in acidic and strong alkaline conditions. Thus, confirming the applicability of the present methodology for further biological applications.

2.7. Thermal stability of constructed array

Thermal stability of the constructed arrays was evaluated by repeating annealing process and by monitoring the respective change in the fluorescence intensity. In the beginning (0 cycles) diminished fluorescence intensity was observed (Fig. 5A) which

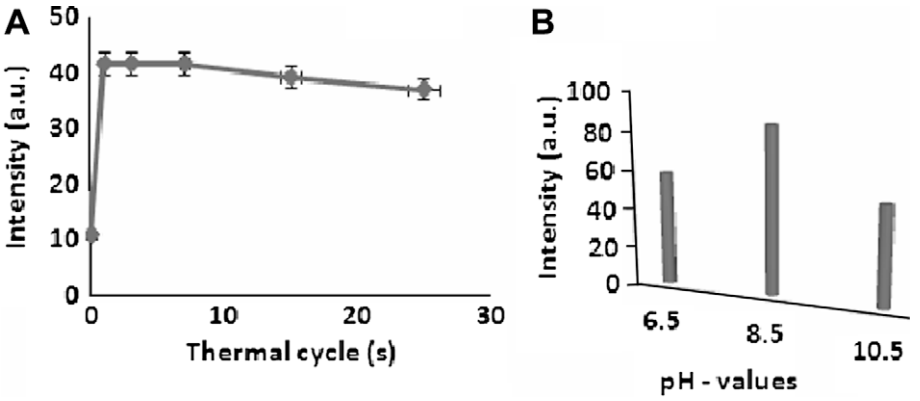


Figure 5. (A) Illustrate thermal stability of hairpin probe on a glass surface and change in fluorescence intensity after repeating number of thermal cycles (including ±3% error and 1% SD). (B) Bar diagram on the basis of fluorescence signal obtained on the hybridization of hairpin probe with a perfectly matched target strand at different pH conditions.

confirms the presence of fluorophore and quencher in proximity—closed state of hairpin probe. When the probe was subjected to first thermal cycle enhanced fluorescent signal was observed that persist for seven repeated thermal cycles. However, on extending the number of thermal cycles (15–25) we observed 6–11% decrease in the average fluorescence signal. In the absence of complementary target sequence, enhancement in intensity clearly indicates the breaking of H-bonds of stem part of hairpin probe to obtain a disrupted random coil conformation on a glass surface, as was previously observed in aqueous medium,[32] therefore, separating the fluorophore and quencher far apart from each other. Thus, results clearly demonstrated the quenching efficiency of guanosine residue as well as the thermal stability of the hairpin probe on solid surface.

2.8. Comparison with a previous known standard method

The efficiency of the proposed method was compared with a known standard epoxide method. An equal concentration of the probe **ON1** (10 μM) was used in both the methods and employed for different reaction times as reported elsewhere.[40] Immobilization efficiency was estimated after hybridization with the complementary target strand under identical condition followed by washing and drying steps. Microslides were scanned and a histogram (Fig. 6) was plotted corresponding to the observed fluorescence intensity. It can be seen that the immobilization efficiency of the proposed method (8.2%) although less but comparable to the epoxide method (9.7%). However, the proposed method (2 h) is far superior to the epoxide method (8 h) in terms of immobilization time and reaction conditions.

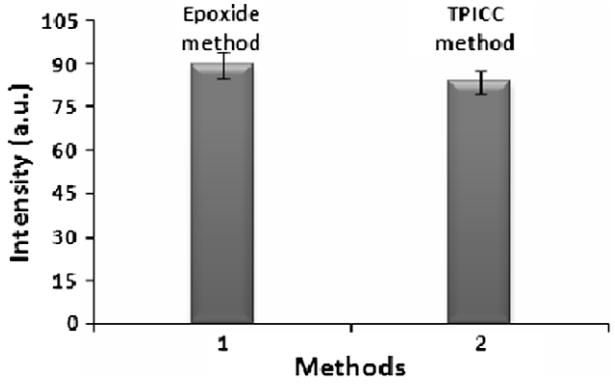


Figure 6. Comparison of immobilization efficiency; (1) epoxide method. (2) TPICC method.

2.9. Specificity of immobilization chemistry and detection of mismatches

To validate the present methodology, specificity for hybridization and application in sensitive diagnostics studies to detect mismatches, hybridization study was performed with different complementary target strands. The microslide having immobilized hairpin probe was subjected to hybridization process as discussed above with the following target complementary strands; **ON2**, perfectly matched (PM); **ON3**, non-complementary (NC); **ON4** having one mismatch (1MM) and **ON5** having two mismatches (2MM). Microslide was washed, dried and analyzed. Fig. 7 shows an utmost enhancement in fluorescence signal only for perfectly matched DNA duplex (Fig. 7E). However, the diminished fluorescence signal was obtained in the case of duplex having non-complementary

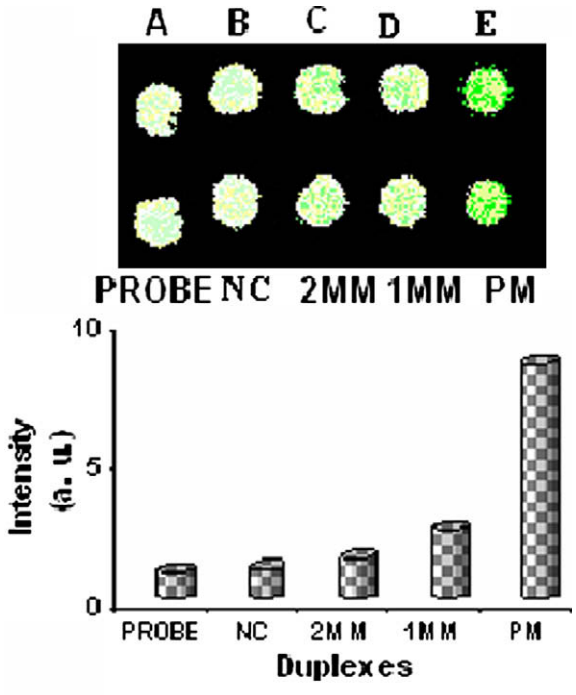


Figure 7. Fluorescence images showing specificity of arrayed hairpin probe (spotted in duplicate, 0.5 μl, 10.0 μM concentration) after hybridization with complementary targets (40 μl, 0.25 A_{260} unit). Spot A, **ON1** (hairpin probe); Spot B, **ON3** (NC); Spot C, **ON5** (2MM); Spot D, **ON4** (1MM); Spot E; **ON2** (PM). Bar diagram shows the relative fluorescence intensities of respective duplexes.

(**NC**) target strand (Fig. 7B) and has been found close to the contributing fluorescence signal, equivalent to background, of unhybridized hairpin probe. Similarly, the relative fluorescence signals generated after the hybridization of arrayed hairpin probe with targets containing two mismatches (Fig. 7C) and one (Fig. 7D) mismatch (A/C), respectively, were found 5–3 times less than the perfectly matched one. Thus, the results clearly demonstrated that the architected hairpin probe undergoes complete conformational reorganization with enhanced fluorescence signal in the presence of perfectly matched complementary target strand obviously, due to more number of H-bond interactions between complementary nucleobase pairs of loop region than of stem region. The non-complementary strand and those having mismatches were not able to form a complete stable duplex on a glass surface thereby resulting diminished or relatively low fluorescence signals, respectively.

3. Conclusion

In summary, a wide array of mono-labeled hairpin probe, utilizing a novel *N*-(3-triethoxysilylpropyl)-4-(isothiocyanatomethyl)-cyclohexane-1-carboxamide (TPICC) heterobifunctional reagent was synthesized for a specific recognition of target biomolecules (e.g., oligonucleotide) on a glass microslide. Although the immobilized hairpin probe attached through a linker arm with a fluorophore contributes little background fluorescence for some extent because of involvement of guanosine as a quencher but the approach has a significant interest to demonstrate the specificity of hybridization and further potential application in diagnostic studies and quenching ability of guanosine residues.

4. Experimental

4.1. Chemistry

4.1.1. General

All chemicals and solvents were of reagent grade and were purified or dried before use. Reactions were monitored by thin-layer chromatography (TLC) on pre-coated Silica Gel G plates at 254/365 nm under a UV lamp. Column chromatography was performed with silica gel (200–300 mesh). Spotting on glass plates (Sigma, USA) was done with the help of micropipettes (Thermo Fisher, USA). IR spectra in KBr were recorded on a Varian-3100 FTIR spectrophotometer. ^{1}H NMR spectra (chemical shifts in δ ppm) were recorded on JEOL AL 300 FT NMR (300 MHz) spectrophotometer using TMS as an internal standard. Fluorescence detection was done on a scanner (Scan-Array Lite, GSI, Lumonics) utilizing photomultiplier tube (PMT) gain and laser power at 60 arbitrary units for all measurements. Mean spot intensities were determined by QuantArray (Packard Biosciences) imaging software. The UV–vis spectra were recorded on a Shimadzu 1700 spectrophotometer using quartz cuvette of 1-cm path length. Melting points were recorded by open glass capillary method and are uncorrected.

4.1.2. Synthesis of reagent 4

4.1.2.1. *trans*-4-(Aminomethyl)cyclohexanecarboxylic acid hydrochloride (1). *trans*-4-(Aminomethyl)cyclohexanecarboxylic acid (mp; >300 °C) was dissolved in hydrochloric acid by continuous stirring at 55 °C for 4 h and then concentrated at 100 °C to obtain white crystal of *trans*-4-(aminomethyl)cyclohexanecarboxylic acid hydrochloride. The hydrochloride salt was suspended in acetone by continuous stirring for 2 h, filtered and washed. The residue was dried for 24 h over P_2O_5. The recorded melting point 248–253 °C was found near to the reported one.[37] R_f = 0.6 (MeOH). IR (KBr, cm^{-1}) 3151, 3043 (N–H str.), 2939 (NH_3^+), 2627, 1714, 1611, 1514.

4.1.2.2. *trans*-4-(Aminomethyl)-*N*-(3-triethoxysilylpropyl)cyclohexane-1-carboxamide (3). A suspension of compound, **1** (1.5 mmol) in THF (15 ml), *N*-hydroxysuccinimide (NHS) (2.0 mmol) and dicyclohexylcarbodiimide (DCC) (1.2 mmol) was stirred for 2 h at room temperature under argon atmosphere. After the reaction was complete (as monitored by TLC), the reaction mixture was kept at 0 °C for 1 h to precipitate out dicyclohexylurea and concentrated in vacuum to leave a solid which was re-dissolved in dichloromethane, filtered (three times to remove traces of dicyclohexylurea) and dried to get solid *N*HS ester of *trans*-4-(aminomethyl)cyclohexanecarboxylic acid hydrochloride, **2** in 80% yield. R_f = 0.9 (MeOH), ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.49 (br, 2H, NH_2), 2.95 (t, 4H, J = 6.6 Hz, succinimide), 2.82 (d, 2H, J = 6.3 Hz, –NCH_2), 2.47 (t, 1H, J = 12 Hz, cyclohexane ring), 1.90 (m, 4H, cyclohexane ring), 1.60 (m, 1H, cyclohexane ring), 1.30 (m, 4H, cyclohexane ring).

Compound **2** was taken in anhydrous DMF (10 ml), cooled and filtered. To the filtrate, 3-aminopropyltriethoxysilane (APTS) (1.6 mmol) and triethylamine (TEA) (1.1 mmol) were added dropwise for 1 h with continuous stirring. The reaction mixture was allowed to reach room temperature and left for overnight stirring. After the reaction was completed, (monitored by TLC, almost negligible mobility in methanol) the solvent was evaporated in vacuum. The crude product was taken in hexane/petroleum ether and washed comprehensively (three times) with 3% aq $NaHCO_3$ 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, **3** in 65% yield. In the washing step, hydrochloride salt of unreacted amino acid, *N*-hydroxysuccinimide and triethylamine hydrochloride were removed, resulting in amino functionality free for further reaction.

IR (KBr, cm^{-1}) 3329 (NH_2), 2928 (aliphatic C–H str.), 2853 (aliphatic C–H str.), 1628, 1574, 1089 (Si–O–C); ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.78 (q, 6H, –OCH_2), 3.46 (br, 2H, NH_2), 3.21 (dd, 4H, J = 6.6 Hz, –NCH_2), 2.42 (m, 1H, cyclohexane ring), 1.90 (m, 4H, cyclohexane ring), 1.73 (m, 2H, –CH_2), 1.57 (m, 1H, cyclohexane ring), 1.30 (m, 13H, –CH_3 and cyclohexane ring), 0.60 (t, 2H, J = 7.8, 8.1 Hz, –$SiCH_2$).

4.1.2.3. *N*-(3-Triethoxysilylpropyl)-4-(isothiocyanatomethyl)-cyclohexane-1-carboxamide (TPICC) (4). CS_2 (1.5 equiv) was added slowly (2 h) to the well-stirred cold solution of compound **3** (1 equiv) and TEA (4 equiv) in THF (10 ml). After complete addition reaction mixture was allowed to proceed for 2 h at room temperature. The reaction mixture was again kept in an ice-bath and tosylchloride (1.5 equiv) was added. The reaction mixture was allowed to reach room temperature and stirred further for 30 min to generate isothiocyanate functionality which was further confirmed by the characteristic infrared band at 2121 cm^{-1} for –NCS group. The solvents were evaporated in vacuum. Acidic water (20 ml) was added and the product was extracted with diethylether (3 × 20 ml). The combined organic layer was kept over anhydrous sodium sulfate, filtered and concentrated in vacuum. The desired reagent TPICC, **4** obtained as a yellowish white solid was eluted with hexane (2% TEA) by passing over short silica column in 50–55% yield. IR (KBr, cm^{-1}) 3439, 2927, 2858, 2121 (–N=C=S), 1628, 1456, 1384, 1084 (Si–O–C). ^{29}Si NMR (59.6 MHz, $CDCl_3$, $(CH_3)_3SiCl$ as external reference,) δ 12.39 (s). ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 11.8, 21.8, 23.4, 24.8, 25.3, 25.5, 32.2, 33.8, 49.2, 168.4.

4.1.3. Synthesis of loading reagent, *O*-4,4′-dimethoxytrityl-6-aminohexane (DMTr–O–$(CH_2)_6$–NH)

The model ligands were prepared according to the reported procedure.[38,39] Yield 78% R_f = 0.34 (DCM:MeOH:9:1), IR: ν (cm^{-1}) = 750, 1039, 1251, 1444, 1633, 3626. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.8 (m, 8H, 4 –CH_2), 2.9 (t, 2H, –NCH_2), 3.3 (t, 2H, –OCH_2), 3.8 (s, 6H, –OCH_3), 6.8–7.5 (m, 13H, Ar–H).

4.2. Buffer solutions

Loading/reaction buffer: 0.1 M sodium phosphate buffer (5% DMSO, as a co-solvent) containing 1.5% TEA (pH 8.5); capping buffer (0.1 M Tris containing 50 mM ethanolamine); hybridization buffer, 10 mM Tris–HCl, 5.0 mM $MgCl_2$, 50 mM KCl (pH 8.3); washing buffer, 3× SSC buffer (pH 7.0).

4.3. Functionalization of the unmodified glass surface

The unmodified glass slides were first hydrated by dipping in 1 M aqueous ethanolic solution of NaOH (25 ml) for 2 h followed by washing with distilled water, aq 1 N HCl solution and again with distilled water. The glass slides were vacuum dried under nitrogen.

4.4. Oligonucleotide synthesis and purification

Purification and custom synthesis of oligonucleotides at 0.2 μmol/g were from Biochem Life Sciences (USA) and were used as such. The oligonucleotide sequences, **ON2**; perfectly matched (**PM**), 5′-d(TTT TTT TTT TTT TTT TTT TT)-3′, **ON3**; non-complementary (**NC**) 5′-d(AAA AAA CCC AAA CCC AAA AA)-3′, **ON4** having one mismatch (**1MM**) (5′-d(TTT TTT TTT T*C*T TTT TTT TT)-3′ and **ON5**, having two mismatches (**2MM**) 5′-d(TTT TTT TTT *CC*T TTT TTT TT)-3′ were designed with the aid of OligoAnalyser (IDT, USA) and caution has been taken to minimize the formation of undesired hairpins, self-dimers or any secondary structures due to cross hybridization. The custom synthesis of modified aminoalkyl hairpin probe [5′-FAM-$(CH_2)_6$-OPO_3-*catc*AAA AAA AAA AAA AAA AAA AA*gatggg*-OPO_3-$(CH_2)_6$-NH_2-3′ (**ON1**)] was carried out using 3′-aminomodifier, viz. (2-dimethoxytrityloxymethyl-6-fluorenylmethoxycarbonylaminohexane-1-succinoyl)-long chain alkylamino (LCAA)-CPG and last coupling was done with FAM–phosphoramidite.

4.4.1. Time kinetics to estimate loading on support

In an alternative route (path-2), CPG-500 Å (25 mg) was suspended in a solution of reagent **4** (Scheme 1) (0.12 M in DMF) containing 1.5% TEA and kept in an Eppendorf Thermomixer set at 45 °C. After 45–60 min, the support was recovered on a sintered glass funnel and subjected to washings with DMF (3 × 1 ml) and diethylether (3 × 1 ml). After drying under vacuum, TPICC-activated CPG^{*****} (≫5 mg) was charged with a solution of *O*-(4,4′-dimethoxytrityl)-6-mercaptohexan-1-ol (1.0 ml, 0.01 M in DMF containing 1.5% TEA). The reaction vials were withdrawn at regular time intervals and were washed sequentially with DMF (3 × 1 ml) and diethylether (3 × 1 ml) containing TEA and dried in vacuum.

4.4.2. Immobilization of hairpin on glass support

In an alternative route (path-2), virgin glass microslide was incubated in 5–10 ml solution of the reagent **4** (Scheme 1) (0.025 M in DMF) at 45 °C in a humid chamber for 1 h. After thorough washings with DMF (3 × 15 ml) and diethylether (3 × 15 ml), the glass microslide was dried in vacuum and hairpin probe dissolved in hybridization buffer was spotted (10 μM, 0.5 μl) with the help of pipetteman. The spotted slide was kept in a humid chamber at 25 °C for 2 h, then washed with reaction buffer and dried. The residual activated isothiocyanate groups on glass microslide were blocked by treating with capping buffer for 1 h, washed with reaction buffer and dried.

4.4.3. Determination of optimum concentration of oligonucleotides required for fluorescence detection and oligonucleotide quantification

First microslide was activated by silanization with reagent TPICC via path-2 and hairpin probe, **ON1** dissolved in reaction buffer was serially diluted (1.5, 2.5, 5, 7.5, 10, and 15 μM concentration) and spotted manually with the help of pipettman (0.5 μl in all cases). Microslide was incubated for 2 h in a humid chamber at 25 °C and after washing with reaction buffer followed by washing buffer (3 × 15 ml), dried and scanned on a fluorescent scanner with an excitation wavelength of 490 nm using an emission filter of 530 nm.

To determine and quantify the density of immobilization, **ON1** was diluted to the following known concentrations: 0.05, 0.1, 0.5, 0.75, and 1.0 μM, and was spotted on an unmodified glass surface. After drying the microslide was directly scanned and a calibration curve was drawn plotting the values of fluorescence intensity as a function of oligonucleotide concentration.

4.4.4. Determination of optimal pH required for immobilization of aminoalkyl modified hairpin probe on isothiocyanate-activated glass surface

A model experiment was performed by reacting ligand, DMTr–O–$(CH_2)_6$–NH_2 and glass beads (CPG-500 Å) charged with isothiocyanate functionality at pHs 6.5, 75, 8.5, 9.5, 10.5, and 11.5 at 45–55 °C in a Eppendorf Thermomixer for 1 h. The support was treated with TCA (3%) and released dimethoxytrityl cation was estimated spectroscopically at 505 nm.

Similarly, isothiocyanate-activated glass plate by reagent TPICC was reacted with hairpin probe, **ON1**. Hairpin probe was dissolved in reaction buffer (10 μM concentration) of different pHs in range as mentioned above and was spotted separately via path-2 (0.5 μl in each case). Microslides were incubated for 2 h in a humid chamber at 25 °C. After respective incubation time, microslides were washed with reaction buffer (3 × 15 ml) followed by washing buffer (3 × 15 ml), dried and scanned on a fluorescent scanner.

4.4.5. Thermal stability of constructed array

The slide was subjected to a manual; 3, 5, 9, and 17 thermal cycles on an Eppendorf Thermomixer. Each cycle consisted of heating to 90 °C for 1 min followed by a slow cooling step (in 30 min) to 5 °C for 1 min, and again heating to 70 °C for 2 min and then cooling step. Each time microslide was scanned at 25 °C and average of total fluorescence intensity signal was plotted against number of thermal cycles.

4.4.6. Hybridization

For hybridization assay spotted microslide with hairpin probe was kept on a wet blotting paper in a Petri dish and complementary oligonucleotide strands (40 μl, 0.25 A_{260} unit) dissolved in hybridization buffer were spread over pre-spotted area. Microslides were kept for 2 h at 55 °C in a hybridization chamber and then at room temperature overnight. After thorough washing with hybridization buffer (15 ml, four times) for 30 min followed by washing buffer (3 × 15 ml) the slides were dried under vacuum and analyzed under fluorescence scanner. Hybridization experiment for specificity of arrayed hairpin probe on a glass slide, **ON1** (0.5 μl, 10.0 μM concentration) was spotted in duplicates and target strands, **ON2–5** (40 μl, 0.25 A_{260} unit) were charged on pre-spotted areas, respectively.

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