Bioorganic & Medicinal Chemistry 17 (2009) 5442–5450

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

# Polymer supported synthesis of aminooxyalkylated oligonucleotides, and some applications in the fabrication of microarrays

D. Sethi [a], S. Patnaik [b], A. Kumar [a], R. P. Gandhi [c], K. C. Gupta [a,d,*], P. Kumar [a,*]

[a] Institute of Genomics and Integrative Biology, Mall Road, Delhi University Campus, Delhi 110 007, India
[b] Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, Fogarty Hall, University of Rhode Island, Kingston, RI 02881, USA
[c] Dr. B.R. Ambedkar Centre for Biomedical Research, University of Delhi, Delhi 110 007, India
[d] Indian Institute of Toxicology Research, M.G. Marg, Lucknow 226 001, U.P., India

ARTICLE INFO

*Article history:*
Received 23 April 2009
Revised 18 June 2009
Accepted 19 June 2009
Available online 23 June 2009

*Keywords:*
Aminooxyalkyl-oligonucleotides
Solid phase synthesis
Microarray
Hybridization
Base mismatch
Disease diagnosis

ABSTRACT

A new protocol has been described for solid phase preparation of 3′- and 5′-aminooxylalkylated oligonucleotides using commercially available reagents. This involves attachment of linker **4** either with an LCAA-CPG support via succinoylation followed by synthesis (3′-aminooxyalkylated oligomers) or formation of its phosphoramidite **6** followed by coupling with desired oligomer (for generating 5′-aminooxyalkylated oligomers). Both the routes produced modified oligonucleotides in sufficiently high yields and purity (on HPLC) via conventional oligonucleotide synthesis on an automated synthesizer and deprotection step using aqueous ammonia (16 h, 60 °C). Aminooxyalkylated oligonucleotides were used to construct microarrays on glass surface (biochips). The performance of the biochips was evaluated by immobilizing modified oligonucleotides on epoxylated glass microslides under different sets of conditions with respect to pH, temperature and time. Further, the constructed microarrays were successfully used for detection of nucleotide mismatches and bacterial typhoid.



## 1. Introduction

During the past decades, solid phase synthesis methodology has remained a proficient and preferred tool for preparation of unmodified oligonucleotides and their modified analogues.[1–5] Synthesis of oligonucleotides containing unnatural 3′-terminal groups has become increasingly important for various biomedical applications, since modifications at the 5′-terminal preclude $^{32}$P-labeling of oligonucleotides through 5′-phosphorylation. In addition, in order to incorporate modifications at the 5′-terminal via solid phase phosphoramidite chemistry, the preparation of linker phosphoramidites is required, which is rather tedious and necessitates strictly anhydrous and inert conditions. Modifications at the 3′-terminal can be easily carried out by using engineered solid supports.[5,6] Therefore, there is an increasing interest in synthesis of oligonucleotides with functionalities such as aminooxy, carboxyl, or phosphate groups attached at the 3′-terminal.

One important application of end-modified oligonucleotides is their use in the fabrication of oligonucleotide microarrays. Recently, microarrays have widely been used as research tools for various applications such as gene expression analysis, DNA sequencing, mutation detection and medical research. The technology allows highly parallel throughput processing and screening of different biological samples (e.g., mRNA, cDNA and proteins).[7–15] DNA microarrays are mostly based on hybridization of targets with double-stranded DNA or single-stranded oligonucleotides (probes) immobilized on a polymer support (usually glass), followed by detection by a scanning process. Of the two established methods, viz., on-chip synthesis and deposition method, the latter is the preferred route for the preparation of oligonucleotide microarrays for routine applications, as it offers flexibility in respect of different chemistries for attachment of probes on a surface of choice. Different strategies of microarray fabrication involve tethering of different functional groups containing oligonucleotides and their analogues directly through use of a linker to the support bound functionalities.[16–32] Aminoalkylated oligonucleotides are generally used for post-synthetic attachment of oligonucleotides to the solid substrate owing to high nucleophilicity of the primary amino groups under basic conditions. However, the aminooxy group is a better nucleophile than the primary amino group. The enhanced nucleophilicity of the aminooxy groups is perhaps due to α-effect,[33] which refers to the increased nucleophilicity of a functional group due to the presence of an adjacent atom with a lone pair of electrons. The presence of oxygen atom adjacent to amino group enhances the nucleophilicity of the aminooxy group. Additionally, p$K_a$ of aminooxy groups is in the range of 5–6, as compared to amino groups (p$K_a \sim 9$), which keeps aminooxy groups unprotonated

* Corresponding authors. Tel.: +91 11 27662491; fax: +91 11 27667471.
*E-mail addresses:* kcgupta@igib.res.in (K.C. Gupta), pkumar@igib.res.in (P. Kumar).

0968-0896/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2009.06.038

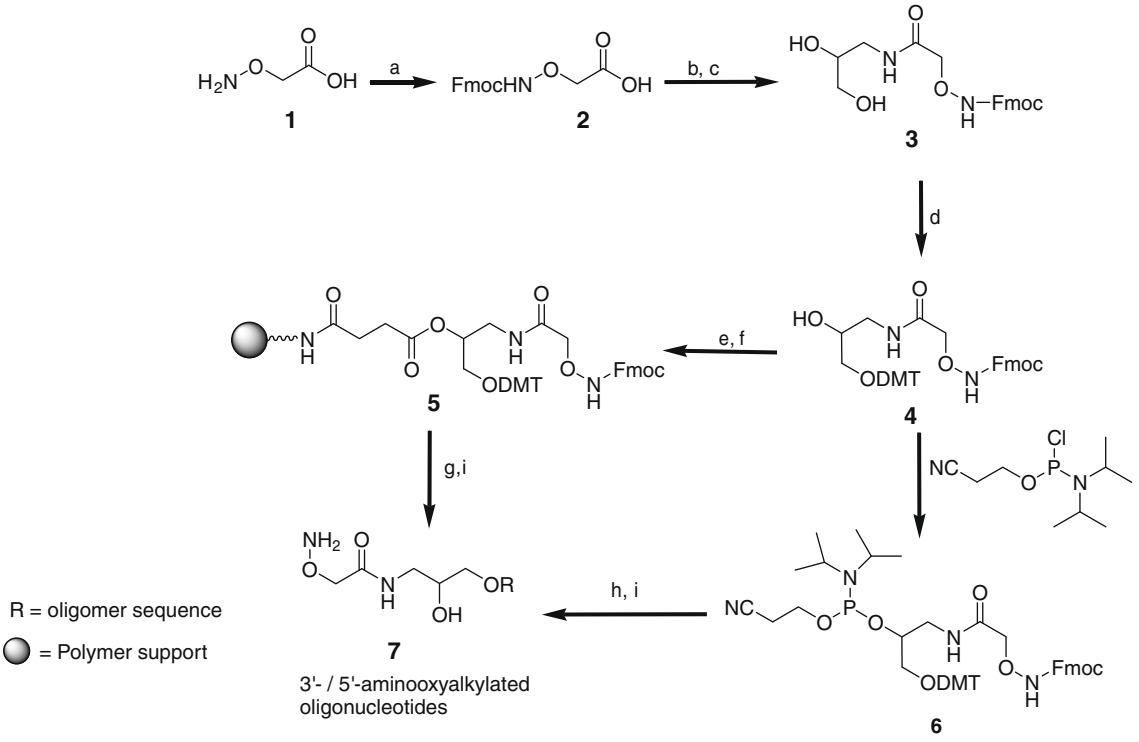
even at a low pH. This has already been reported that in acidic pH, the aminooxy-modified oligomers react more rapidly than their amino counterparts with electrophilic substrates.[34,35] Salo et al.[36] have reported a synthesis of aminooxyalkylated oligonucleotides. However, the method is quite tedious and requires an additional deprotection step, which may also result in the modification of nucleobases.

Here, we report a simple method for the synthesis of 3′- and 5′-aminooxyalkylated oligonucleotides and a methodology for the construction of biochips for detection of bacterial diseases. The synthetic route involves the use of inexpensive, commercially available chemicals and reagents for preparation of functionalized polymer support, and the deprotection step is carried out using aqueous 30% ammonia solution. The resulting aminooxyalkylated oligonucleotides are then used for fabrication of oligonucleotide microarrays on an epoxylated glass surface. The modified oligonucleotides were immobilized at pH 6 and 9 with high immobilization efficiency (~18% at pH 6 and ~22% at pH 9) and signal-to-noise ratio (~97). The hybridization efficiency was found to be ~32% at pH 6. The constructed microarrays were then used for discrimination of base mismatches. On being subjected to different pH and thermal conditions, the microarrays showed sufficient stability. Subsequently, oligonucleotide probe-based microarrays were prepared following the projected chemistry for detection of typhoid. The projected methodology was compared with the existing method (immobilization of aminoalkylated oligonucleotides on epoxylated glass surface) with respect to immobilization efficiency of oligonucleotides at pH 6 and 9.[33,34]

## 2. Results and discussion

In designing a simple route for synthesis of 3′-aminooxyalkyl-terminal oligonucleotides and in the construction of oligonucleotide microarrays for disease diagnosis, we kept the following points in view, viz., (a) the synthetic strategy should be simple and straight-forward, (b) it should involve commercially available reagents and chemicals, (c) synthesis and deprotection steps should not deviate from the standard procedures, (d) immobilization of modified oligonucleotides should not require any condensing/coupling reagent, (e) hybridization and immobilization efficiencies should be high enough to enable visualization of fluorescent spots at low concentration, and (f) the constructed microarrays should be able to discern base mismatches. We have thus employed the commercially available aminooxyacetic acid as a source of aminooxyalkyl moiety. The aminooxyalkyl group was protected by base-labile Fmoc protecting group to obtain *N*-(9-fluorenylmethyloxycarbonyl)-2-aminooxyacetic acid, **2**, in ~78% yield. This was coupled with 3-aminopropan-1,2-diol in the presence of *N,N*-diisopropylcarbodiimide, to obtain *N*-(2,3-dihydroxypropyl)acetamide-2-*N*-(9-fluorenylmethyloxycarbonyl) oxyamine, **3**, in ~88% yield. Subsequent reaction with 4,4′-dimethoxytrityl chloride resulted in the formation of compound **4**, which was succinoylated and coupled to long chain alkylamine-controlled pore glass (LCAA-CPG) to obtain the engineered polymer support, **5**, with aminooxy group loading of ~29 μmol/g of support (Scheme 1). All the intermediates were characterized by mass and $^1$H NMR spectra.

In order to demonstrate the utility of the polymer support **5** in the synthesis of modified oligonucleotides bearing aminooxy functionalities at 3′-end, oligonucleotide sequences (Table 1) were synthesized at 0.2 μmol scale following standard protocol on a Pharmacia Gene Assembler Plus. The oligomer, $NH_2OCH_2CONHCH_2CH(OH)CH_2\text{-}OPO_3^{2-}$-d(TTC GTC ATC AAG TAG TTC CT) was also assembled using the standard 5′-DMTr thymidine support with O-succinate linkage for comparison. The coupling efficiency per cycle based upon the released 4,4′-dimethoxytrityl cation exceeded 98.7% in both cases.




**Scheme 1.** Reagents and conditions: (a) FmocCl (1 equiv), $Na_2CO_3$ (1.25 equiv), dioxane; (b) NHS (1.2 equiv), DIPCI (1.2 equiv), THF; (c) 3-amino-1,2-propane diol (1.2 equiv); (d) DMTrCl (1.2 equiv), pyridine; (e) succinic anhydride (2 equiv), DMAP (0.5 equiv), TEA (2 equiv), EDC; (f) HBTU (1 equiv), LCAA-CPG (500); (g) oligo synthesis; (h) tetrazole, d(NNN)$_n$; (i) deprotection, cleavage.

**Table 1**
Oligonucleotide sequences synthesized, their deprotection conditions and yields

| No. | Oligonucleotiode sequence | Deprotection conditions | O.D. at $A_{254}$ nm |
|---|---|---|---|
| 1 | TET-d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ | Aq $NH_4OH$, 16 h, 60 °C | 20.6 |
| 2 | d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ | Aq $NH_4OH$, 16 h, 60 °C | 26.4 |
| 3 | d(TTC GTC *T*TC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ | Aq $NH_4OH$, 16 h, 60 °C | 21.1 |
| 4 | d(TTC GTC *T*TC AAG TAG *G*TC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ | Aq $NH_4OH$, 16 h, 60 °C | 23.2 |
| 5 | d(TTT TTT TTT TTTTTT TTT TT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ | Aq $NH_4OH$, 16 h, 60 °C | 20.3 |
| 6 | $NH_2OCH_2CONHCH_2CH(OH)CH_2$-$OPO_3^{2-}$-d(TTC GTC ATC AAG TAG TTC CT) | Aq $NH_4OH$, 16 h, 60 °C | 24.3 |
| 7 | TET-d(AGG AAC TAC TTG ATG ACG AA) | Aq $NH_4OH$, 16 h, 60 °C | 21.5 |
| 8 | TET-d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH_2CH_2CH_2CH_2CH_2NH_2$ | Aq $NH_4OH$, 16 h, 60 °C | 24.2 |
| 9 | TET-d(ACC GGT GGA TGT GGC TTC CTT G) F. primer | Aq $NH_4OH$, 16 h, 60 °C | 22.7 |
| 10 | TET-d(TGG TCT GCA GCA CCT TTT GAA C) R. primer | Aq $NH_4OH$, 16 h, 60 °C | 23.6 |
| 11 | d(ACC GGT GGA TGT GGC TTC CTT GTT TT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ (probe sequence) | Aq $NH_4OH$, 16 h, 60 °C | 20.5 |
| 12 | d(CGT CGA GTC GGG CAT CTT GGT ATT TT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ (control) | Aq $NH_4OH$, 16 h, 60 °C | 25.4 |

The deblocking of oligomers (modified as well as unmodified) was carried out by treatment with aq ammonia (30%) at 60 °C for 16 h. The one step process allows removal of all the protecting groups (β-cyanoethyl from phosphate and acyl from nucleic bases) together with the elimination of the Fmoc from aminooxy function. The ammoniacal solutions were concentrated and the oligomers desalted on RP-silica gel column. The crude oligomers thus obtained were analyzed on RP-HPLC. In one of the experiments, an oligomer, d(TTT TTT TTT TTT TTT TTT TT), was assembled on the standard d(T) support and modified polymer support **5.** After deprotection with aq ammonia and desalting, both the oligomers were analyzed on HPLC. A TET-labeled $T_{20}$ oligomer was also prepared on the modified polymer support. Figure 1A and B shows the HPLC profiles of crude oligomers, d(TTT TTT TTT TTT TTT TTT TT) prepared on the standard and modified polymer support **5**, respectively and Figure 1C shows the elution profile of a co-injection of both the oligomers. RP-HPLC elution of TET-labeled d($T_{20}$) is depicted in Figure 1D. Further, these oligomers were characterized by mass spectrometry.

In order to examine the efficacy of the projected strategy involving immobilization of aminooxy-oligonucleotides on to epoxylated glass surface to construct oligonucleotide microarrays (biochips) useful for disease diagnosis, several parameters were studied in detail. Epoxy functions on the glass surface were generated following the standard silanization procedure using 3-glycidyloxypropyltrimethoxysilane.[32]

To assess the quality of the epoxylated glass surface, a labeled oligomer, TET-d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$, was spotted randomly all over the surface of an epoxylated glass microslide. After usual incubation and washings, the slide was visualized under a laser scanner. The fluorescence intensity of individual spots on the slide was measured using QuantArray software. The average intensity of the spots after background subtraction was found to be in the range of ~7400–7700 A.U., indicating a uniform epoxylation of the glass surface.

In order to find out the optimal pH conditions required for efficient immobilization of aminooxyalkyl-modified oligonucleotides onto epoxylated glass microslides, the attachment of the labeled probe, TET-d(TTC GTC ATC AAG TAG TTC CT-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$, was carried out at pH ranging from 4 to 11, as described in Section 4. After washings and drying, the slide was subjected to laser scanning. The results showed that the spots corresponding to pH 6 and 9 exhibited the highest fluorescence intensity of ~7760 and ~9450 A.U., respectively (Fig. 2). This is because the aminooxy groups remain almost unprotonated at pH 6 but the epoxy oxygen gets protonated, which triggers an acid catalyzed opening of epoxide ring by the reaction with nucleophilic aminooxy groups. The enhanced nucleophilicity of aminooxy groups under basic conditions (pH 9) due to α-effect results in the maximum immobilization, i.e. spots with the highest fluorescence intensity. As the pH increased beyond 9, the fluorescence intensity decreased possibly because of partial hydrolysis of the epoxy functions at higher pH, whereas the lower attachment density at pH below 6 could be explained in terms of lower reactivity of the partially protonated aminooxyalkyl functions. Likewise, inadequate protonation of epoxide ring at pH 7 and 8 resulted in inefficient reaction of aminooxy groups with epoxylated glass surface. Therefore, the attachment of aminooxyalkyl-modified oligonucleotides was performed at pH values 6 and 9 in rest of the experiments.

Further, to arrive at the optimal time and temperature required for the immobilization reaction, an oligomer sequence, TET-d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ (1 μM), dissolved in the reaction buffers of pH 6.0 and 9.0 separately, was spotted onto epoxylated glass slides in duplicates and kept at 35 °C. Microslides were withdrawn at different time intervals (i.e., 60, 120, 180, 210, 240, 270, 300, 360 and 600 min) and subjected to usual washings and drying. Subsequently, the slides were scanned under a laser scanner and the spots were quantified. Similar study was carried out at 45 °C and 55 °C and the spots were quantified using QuantArray software. It was observed that, at pH 6.0, the reaction between 3′-aminooxyalkylated oligonucleotide and epoxy functions was completed in 270 min at 45 °C (having highest fluorescence intensity), while, at pH 9.0, the reaction took 240 min for completion at 45 °C (Fig. 3B). Deviating from 45 °C, the fluorescence intensity decreased at both the pH which might be due to either incomplete reaction (at 35 °C, Fig. 3A) or partial deactivation of the surface-bound epoxy functions (at 55 °C, Fig. 3C). Therefore, the subsequent experiments involving immobilization reaction were carried out at 45 °C.

Having optimized pH, time and temperature required for immobilization of aminooxyalkylated oligonucleotides onto epoxylated glass surface, threshold concentration of the probe required for visualization of spots under a laser scanner was determined by spotting manually a serially diluted (40, 20, 10, 5, 2.5, 1.0 and 0.5 μM) labeled oligomer, TET-d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ (dissolved in the reaction buffers of pH 6.0 and 9.0), for 270 and 240 min, respectively. After usual processing, the slide was visualized under a laser scanner (Fig. 4A and B represents the scanned images). It was inferred from the figures that the fluorescence intensity of the spots increased with increasing the concentration of the oligomer sequence, that is, density of the immobilized oligomer increases with the concentration reaching a plateau with oligomer concentration of 20 μM and higher.

The thermal stability of the constructed microarray was examined by subjecting the spotted microslide to 0, 10 and 20 cycles of PCR-like conditions. The results showed that oligonucleotides immobilized following the projected strategy were sufficiently stable (~10% and ~8.9% decrease in intensity after 20 cycles at pH 6

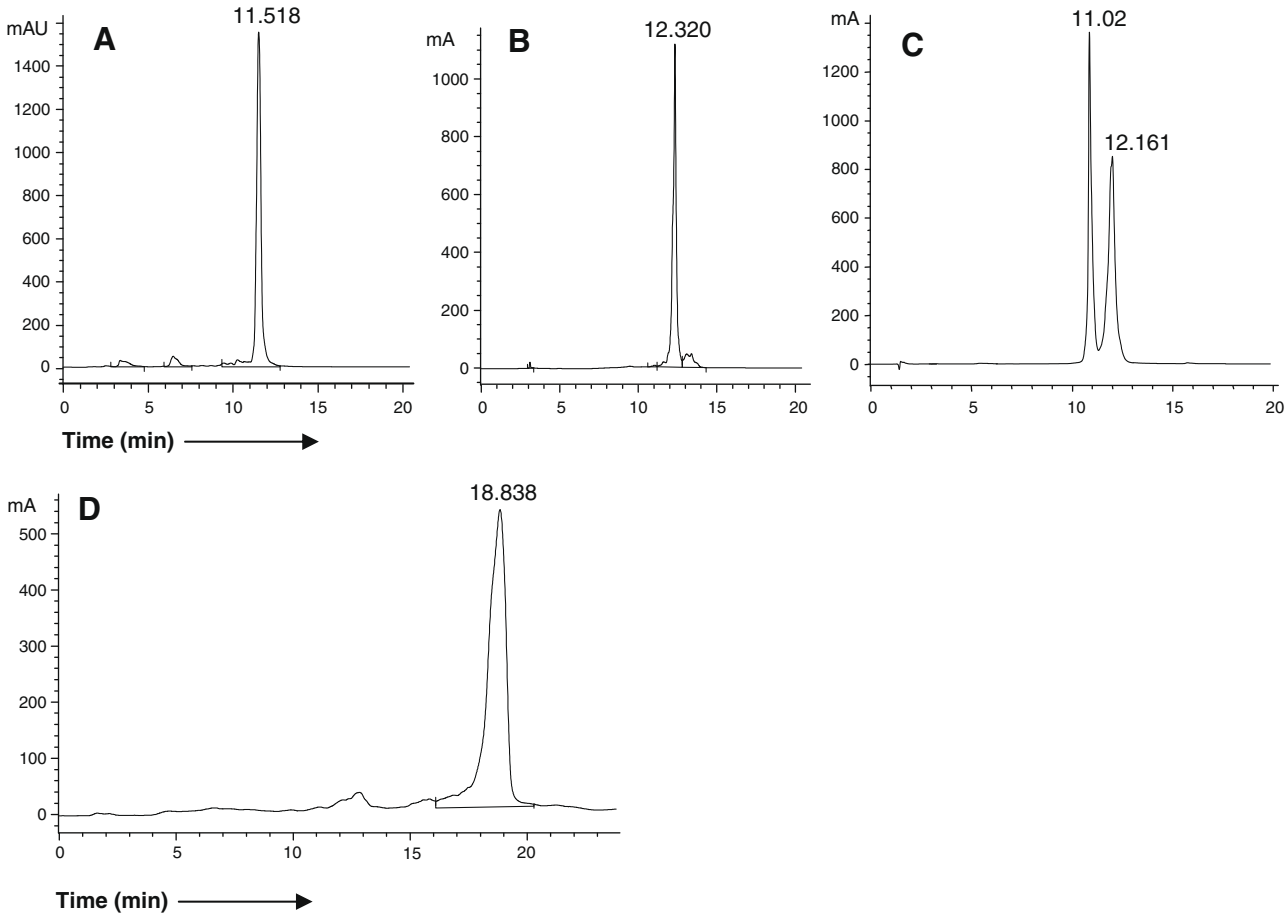



**Figure 1.** HPLC elution profiles of (A) Crude d(TTT TTT TTT TTT TTT TTT TT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ (*m*/*z*: 6246.8, $M^+$); (B) crude d(TTT TTT TTT TTT TTT TTT TT) (*m*/*z*: 6021.7, $M+H^+$); (C) co-injection of d(TTT TTT TTT TTT TTT TTT TT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ and d(TTT TTT TTT TTT TTT TTT TT), and (D) TET-d(TTT TTT TTT TTT TTT TTT TT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ (*m*/*z*: 6927, $M+Na^+$).

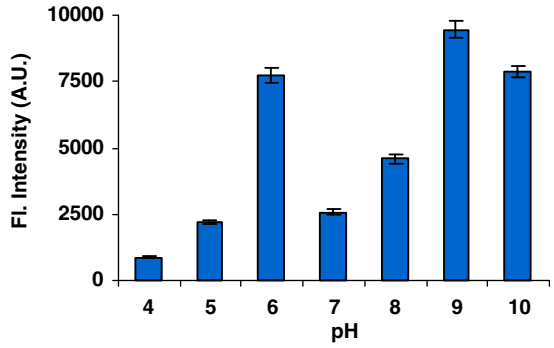



**Figure 2.** pH study of immobilization of TET-d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ onto epoxylated glass microslides.

and 9, respectively) and thus can be used in biological research. Figure 5 shows fluorescence intensity of untreated and heat-treated spots.

Likewise, stability to pH was evaluated by exposing the spotted microarrays to buffers of different pH (7, 8 and 9) for 30 min each. Figure 6 shows the results of this study, which indicated that fluorescence intensity decreases by ~3% at pH 8.0 whereas, at pH 9.0, it decreases by ~8%, as compared to fluorescence intensity observed at pH 7.0.

The practical applicability of the above methodology was assessed by hybridization experiments. In order to determine the extent of accessibility of the surface-immobilized oligomer sequence for efficient hybridization, oligomer sequences, d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ and $NH_2OCH_2CONHCH_2CH(OH)CH_2$-$OPO_3^{2-}$-d(TTC GTC ATC AAG TAG TTC CT), were immobilized onto epoxyalkylated glass microslides in duplicate at pH 6.0. After usual washings, the spots on the microslides were exposed to complementary labeled oligonucleotide sequence, TET-d(AGG AAC TAC TTG ATG ACG AA). The results (Fig. 7) showed the hybridization efficiency to be almost the same (~31.6%, 3′-end immobilization vs ~29.8%, 5′-end immobilization) irrespective of the nature of attachment of the probe sequences to the glass surface. Therefore, 3′-modified oligomers were employed in the study as these can easily be assembled on the engineered polymer support without affecting the standard synthesis and deprotection conditions.

The specificity and selectivity of the methodology was demonstrated by immobilizing aminoalkyl- and aminooxyalkylated oligonucleotides onto an epoxylated glass microslide at pH 6.0. The results of the experiment (Fig. 8) revealed that, under acidic environment, the aminooxyalkylated oligomer reacts efficiently with the epoxy function due to lower $pK_a$ of the aminooxy function and are immobilized with high fluorescence intensity, whereas, the aminoalkyl function, having $pK_a \sim 8$, may get protonated and thus result in a diminished reaction with surface-bound epoxy groups (which yield spots with lower fluorescence intensity). Therefore, at pH 6, the aminooxyalkylated ligands could be immobilized selectively on the epoxylated surface.

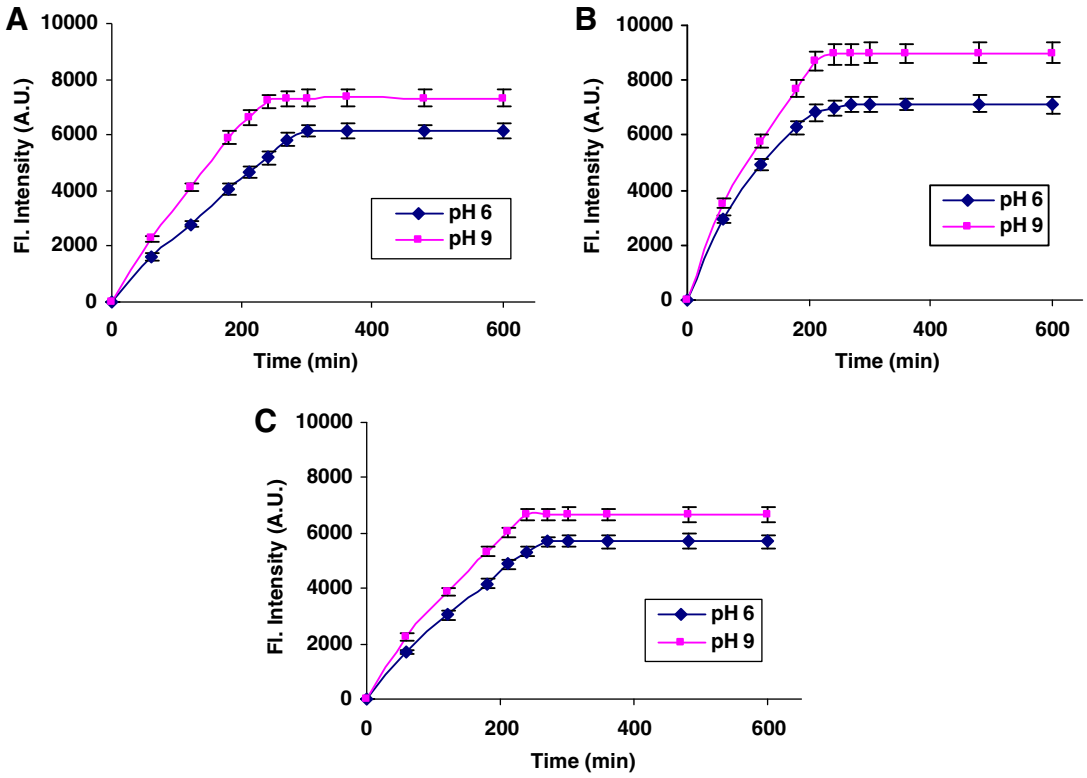



**Figure 3.** Graphs representing time kinetics to determine optimal time required for immobilization of aminooxyalkylated oligomers, in reaction buffers of pH 6 and 9, onto epoxy-functionalized glass microslides at different temperatures (A) 35 °C, (B) 45 °C, and (C) 55 °C.

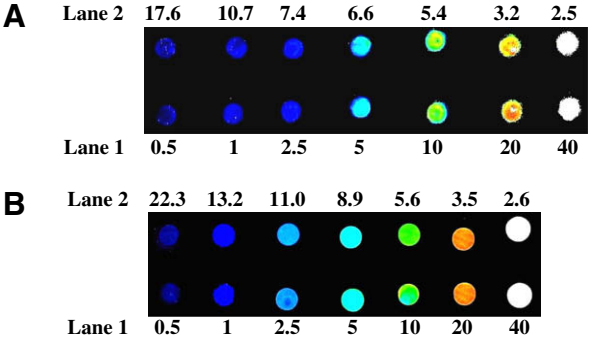



**Figure 4.** Threshold concentration of oligonucleotide sequence, TET-d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$, required for fluorescence visualization at (A) pH 6 and (B) pH 9. Lane 1, probe concentration (μM); lane 2, immobilization efficiency (%).

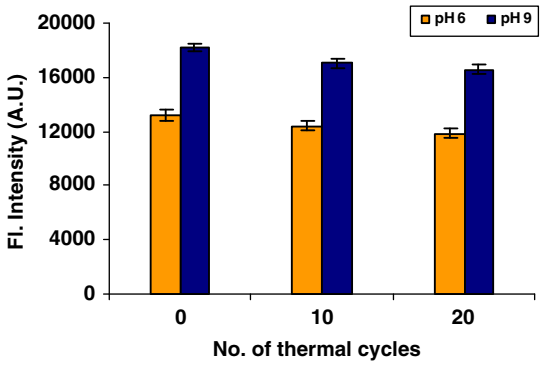



**Figure 5.** A graphical representation showing thermal stability of immobilized probe after 0, 10 and 20 cycles of PCR-like conditions ($n = 3$).

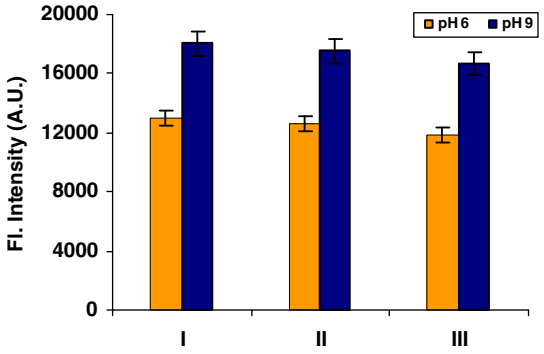



**Figure 6.** A graphical representation showing pH stability of the probes immobilized onto epoxylated glass slides at pH 6 (orange) and 9 (blue). Subsequently, the slides were subjected to washings with the washing buffer of pH 7, 8 and 9 (at ambient temperature) and fluorescence intensity of the spots was determined. I, II and III indicate stability at pH 7, 8 and 9, respectively.

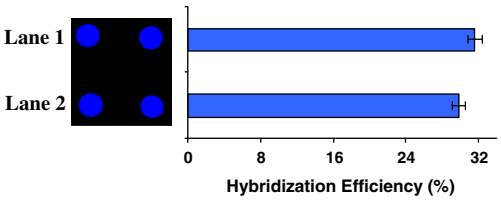



**Figure 7.** Fluorescence map after performing hybridization assay with labeled oligomer, TET-d(AGG AAC TAC TTG ATG ACG AA), of epoxylated glass slide spotted with, d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ (Lane 1) and $NH_2OCH_2CONHCH_2CH(OH)CH_2$-$OPO_3^{2-}$-d(TTC GTC ATC AAG TAG TTC CT) (Lane 2).

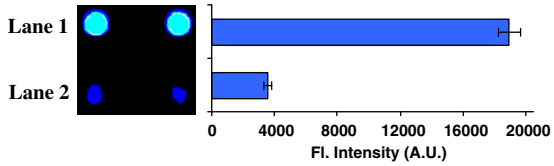



**Figure 8.** Fluorescence map of hybridization assay performed on epoxylated glass slide spotted with TET-d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)$-$CH_2NHCOCH_2ONH_2$ (Lane 1) and TET-d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH_2CH_2CH_2CH_2CH_2NH_2$ (Lane 2) at 5 μM concentration in reaction buffer of pH 6, with quantitative histogram of fluorescence intensity.

In order to demonstrate the capability of this methodology for detecting base mismatches, four oligomers, viz., d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$, d(TTC GTC *T*TC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$, d(TTC GTC *T*TC AAG TAG *G*TC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ and d(TTT TTT TTT TTT TTT TTT TT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ (2.5 μM) with zero, one, two mismatches and non-complementary with respect to target sequence, were immobilized. On hybridization, the perfect match gave the highest fluorescence followed by one and two mismatches with diminished fluorescence, while non-complementary sequences did not light up at all (Fig. 9). The signal-to-noise ratio was ~97 as calculated from the signals obtained from complementary hybridized spots and non-complementary spots. The quantitative data of the scanned image is depicted in Figure 9.

In order to construct a biochip for detection of bacterial typhoid, a microarray was constructed using a unique probe sequence of the gene and a non-complementary probe sequence as a control. The spots were subsequently hybridized with the labeled PCR amplicons. After usual washings and drying, the spots were visualized under a scanner. Figure 10 gives pictorial representation of the scanned image. While the spots of the probe sequence specific to *tyv* gene of bacteria (*Salmonella typhi*) showed fluorescence, the spots specific to non-complementary probe did not fluoresce at all, signifying the specificity of the system for preparing biochips for detection of bacterial typhoid in humans.

## 3. Conclusions

A simple and rapid method for the preparation of 3′- and 5′-aminooxyalkylated oligonucleotides has been developed. These oligomers were then used to construct biochips useful for the detection of nucleotide mismatches and bacterial typhoid. The methodology offers an added advantage in as much as the multiple

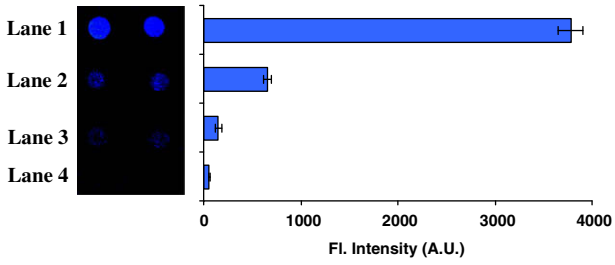



**Figure 9.** Fluorescence maps of nucleotide mismatch detection with quantitative analysis of fluorescence intensity. Four oligonucleotide sequences, d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ (Lane 1), d(TTC GTC *T*TC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ (Lane 2), d(TTC GTC *T*TC AAG TAG *G*TC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ (Lane 3), and d(TTT TTT TTT TTT TTT TTT TT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ (Lane 4), were immobilized on glass microslide. After usual washings, the spotted microslide was hybridized with TET-d(AGG AAC TAC TTG ATG ACG AA) and subjected to a laser scanner for visualization of fluorescent spots.

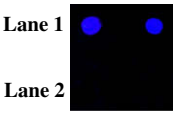



**Figure 10.** Detection of bacterial typhoid. Lane 1: probe sequence. Lane 2: control probe (non-complementary).

aminooxyalkyl groups can be incorporated in oligonucleotides and the aminooxyalkylated oligomers can be immobilized selectively with high immobilization efficiency as compared to aminoalkylated oligomer at pH 6.

## 4. Experimental

### 4.1. General

Reagents and chemicals used in the present investigation were purified prior to their use. The reactions requiring anhydrous conditions were carried out under a blanket of dry Argon gas. 3-Glycidyloxypropyltrimethoxysilane (GOPTS), *N*-methylimidazole (NMI) and virgin glass microslides were procured from Sigma–Aldrich Chemical Co., St. Louis, MO. Tetrachlorofluoresceinyl-phosphoramidite (TET-phosphoramidite) was purchased from Applied Biosystems Inc., Foster City, CA. Other ancillary reagents and chemicals were locally sourced. $^1$H NMR spectra were recorded on a Bruker Avance 300 spectrometer operating at 300 MHz and the chemical shifts ($\delta$) were reported in ppm relative to TMS. Mass spectra of the organic compounds were recorded on Waters KC455 TOF-MS and oligonucleotides on a SELDI-TOF (Surface enhanced laser desorption ionization-time of flight) PBS-II, Ciphergen Biosystems, Freemont, CA using an 8-well gold plated chip. 3-Hydroxypicolinic acid mixed with picolinic acid and diammonium citrate was used as matrix for oligomer analysis. Reversed-phase HPLC was performed on an Agilent 1100 series system fitted with a variable PDA detector set at (260 ± 8) nm, using Hypersil Gold C-18 column from Thermo Electron Inc., USA. Elutions were carried out with increasing gradient of MeCN. Solvent A: 0.1 M ammonium acetate, pH 7.0; solvent B: acetonitrile; gradient: 0–35% B in 25 min; flow rate: 1 ml/min.

### 4.2. Buffers used

(i) Reaction buffer: 0.1 M sodium acetate was prepared by dissolving 2.72 g in 200 ml of MilliQ water (containing 10% DMSO).

(ii) Capping buffer: 0.1 M Tris buffer containing 50 mM ethanolamine, pH 8.

(iii) Washing buffer (SSC buffer): 125 mM sodium citrate containing 750 mM sodium chloride, pH 7.

(iv) Hybridization buffer: 125 mM sodium citrate containing 1 M sodium chloride, pH 7.

### 4.3. Preparation of polymer support 5 and phosphoramidite 6

#### 4.3.1. Synthesis of *N*-(9-fluorenylmethyloxycarbonyl)-2-aminooxyacetic acid, 2

To a pre-cooled aqueous solution (20 ml) of 2-aminooxyacetic acid hemihydrochloride **1** (10 mmol, 2.185 g) and sodium carbonate (25 mmol, 2.65 g) was added dropwise a solution of 9-fluorenylmethyl chloroformate (20 mmol, 5.174 g) in dry dioxane (20 ml) at 10 °C over a period of 15 min. After complete addition, the mixture was allowed to stir for 4 h at 35 °C. The completion of the reaction was monitored by TLC and after removal of the solvent, the residue was taken up in ethyl acetate (100 ml). To this was added 5% citric acid solution (50 ml). The organic layer was

collected and subsequently washed with saturated solution of NaCl (2 × 10 ml) followed by drying over anhydrous $Na_2SO_4$. After filtration, the solvent was removed on a rotavapor to give a white compound **2** (4.82 g, yield, ~78%). Mass calculated: 312; mass observed: 313.2 $(M+H)^+$.

#### 4.3.2. Synthesis of *N*-(2,3-dihydroxypropyl)acetamide-2-*N*-(9-fluorenylmethyloxycarbonyl) oxyamine, 3

To the compound **2** (5 mmol, 1.56 g), dissolved in dry THF, were added *N*-hydroxysuccinimide (6 mmol, 0.7 g), 4-dimethylaminopyridine (1 mmol, 122 mg) and *N,N*-diisopropylcarbodiimide (6 mmol). After stirring the mixture for 4 h at room temperature, 3-amino-1,2-propanediol (6 mmol, 546 mg) was added and the reaction was stirred further for 3 h at room temperature. The solvent was evaporated, the residue taken up in ethyl acetate (50 ml), washed with aqueous 10% citric acid solution (2 × 10 ml) and dried over anhydrous $Na_2SO_4$ to obtain a crude product, which was purified by column chromatography using 1,2-dichloroethane. The desired compound, **3**, was obtained in ~88% yields (~1.7 g) and characterized by its mass and $^1$H NMR. Mass calculated: 386; mass observed: 409.1 $(M+Na^+)$.

$^1$H NMR ($CDCl_3$) $\delta$: 2.6 (t, 2H, $-NCH_2$), 3.5–3.9 (m, 3H, –OCH–, $-OCH_2-$) 4.1–4.65 (m, 5H, $-CH_2OCO-$, $C_9H$, $-OCH_2CO-$) and 7.2–8.1 (m, 8H, Ar–H).

#### 4.3.3. Synthesis of *N*-{2-hydroxy-3-(4,4′-dimethoxytrityloxy)-propyl}acetamide-2-*N*-(9-fluorenylmethyloxycarbonyl)-oxyamine, 4

Compound **3** (770 mg, 2.0 mmol) was dried by co-evaporation with anhydrous pyridine (25 ml) and taken up in dry pyridine (25 ml). To this, DMAP (0.2 mmol, 24.4 mg) and DMTrCl (711 mg, 2.4 mmol) were added and the mixture was stirred overnight at room temperature. On completion of the reaction (as monitored on TLC), excess DMTrCl was hydrolyzed by the addition of methanol (0.5 ml). The reaction mixture was concentrated in vacuo and traces of pyridine were removed by co-evaporations with toluene (2 × 25 ml). The syrupy material, thus obtained, was dissolved in ethyl acetate (75 ml) and washed with saturated aqueous solutions of $NaHCO_3$ (2 × 25 ml) and NaCl (1 × 25 ml), respectively. The organic phase was dried over $Na_2SO_4$ and concentrated in vacuo to yield the crude product, which was purified by column chromatography using 1,2-dichloroethane containing triethylamine (1%), as an eluent. The desired compound, **4**, was obtained in ~85% yield (1.168 g) and characterized by its mass and $^1$H NMR. Mass calculated: 687; mass observed: 709 $(M+Na)^+$.

$^1$H NMR ($CDCl_3$) $\delta$: 2.7 (t, 2H, $-NCH_2$), 3.5–3.85 (m, 9H, 2 × $-OCH_3$, –OCH–, $-OCH_2-$) 4.2–4.6 (m, 5H, $-CH_2OCO-$, $C_9H$, $-OCH_2CO-$) and 7.15–8.2 (m, 21H, Ar–H).

#### 4.3.4. Preparation of engineered polymer support

**4.3.4.1. Succinoylation of 4.** A mixture of **4** (687 mg, 1.0 mmol), succinic anhydride (200 mg, 2.0 mmol), dry TEA (280 μl, 2.0 mmol) and DMAP (61 mg, 0.5 mmol) was dissolved in dry ethylene dichloride (5 ml) and stirred for 30 min at rt. The reaction was monitored on TLC and, after completion, it was quenched by the addition of MeOH (0.5 ml). The reaction mixture was further diluted with EDC (20 ml) and washed successively with cold 5% aqueous citric acid (2 × 15 ml) and saturated solution of NaCl (2 × 15 ml). The organic phase was collected, dried over anhydrous $Na_2SO_4$ and concentrated to obtain the title compound (745 mg; 95% yield), which was characterized by its mass. Mass ($m/z$) calculated: 785; observed: 785 $(M^+)$.

**4.3.4.2. Attachment of the above hemisuccinate to LCAA-CPG.** Succinoylated compound **4** (158 mg, 0.2 mmol) was dissolved in anhydrous DMF (2.0 ml). To this was added 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (0.2 mmol). The reaction mixture was swirled for 30 s and long chain alkylamine-CPG (500 mg) was added. The resulting suspension was stirred for 30 min at ambient temperature and then recovered on a sintered disc glass funnel and washed with DMF (3 × 10 ml) and diethyl ether (2 × 10 ml), respectively. The support was dried in a vacuum desiccator and subjected to capping (residual amino groups) with a solution consisting of $Ac_2O$:TEA:*N*-methylimidazole:DCM (1:1:0.4:6, v/v/v/v) for 30 min at rt. After washings with DCM (3 × 10 ml) containing triethylamine (1%, v/v), the functionalized polymer support, **5**, was dried in vacuo. The loading on the support was calculated following the standard protocol.

#### 4.3.5. Preparation of phosphoramidite reagent of 4

*N*-{2-Hydroxy-3-(4,4′-dimethoxytrityloxy)-propyl}acetamide-2-*N*-(9-fluorenylmethyloxy-carbonyl)-oxyamine, **4** (790 mg, 1.0 mmol) was dissolved in anhydrous acetonitrile (10 ml) and to this was added *N,N,N′N′*-tetraisopropyl-2-cyanoethylphosphoramidite (2 mmol). To this mixture, a solution of tetrazole (1 mmol, 70 mg dissolved in 2.5 ml of acetonitrile) was added dropwise at room temperature and the reaction was allowed to stir for 2 h. After completion of reaction (as monitored on TLC), methanol (1 ml) was added to quench the reaction. The solvent was evaporated on a rotary evaporator, the resulting syrupy residue taken up in ethyl acetate (25 ml) and washed with saturated sodium chloride solution (2 × 10 ml). The organic phase was collected, dried over anhydrous $Na_2SO_4$, filtered, and the solvent was removed. The material was purified over silica gel column and the desired material was eluted with hexane/ethyl acetate/triethylamine (5:2:1). The fractions containing compound **6** were pooled and concentrated on a rotary evaporator (~82% yield).

### 4.4. Synthesis and purification of modified oligonucleotides

A number of oligonucleotide sequences (Table 1) were assembled on the engineered polymer support **5** (for 3′-terminal modification). For 5′-end modifications, oligonucleotides were synthesized, and the last coupling was performed with the phosphoramidite reagent, **6**, in a manner analogous to the normal nucleoside phosphoramidite couplings. For comparison purposes, oligomers were also synthesized on the unmodified polymer supports. Synthesis was carried out on Pharmacia Gene Assembler Plus at 0.2 μmol scale following the standard phosphoramidite chemistry. After synthesis, the support bound oligonucleotides were subjected to 30% ammonia treatment for 16 h at 60 °C. Subsequently, the ammonical solutions were concentrated in a speed vac. Analysis and purification of oligonucleotides was carried out on Agilent 1100 series system.

### 4.5. Immobilization studies

#### 4.5.1. Glass silanization

The glass slides were treated with 1 M NaOH for 1 h followed by washing with MilliQ water (3 × 150 ml). The slides were treated with 1.5 M HCl for 1 h followed by washings with MilliQ water (3 × 150 ml). The slides were then dipped in absolute ethanol (2 × 15 min) and dried. The silanization was accomplished using GOPTS (2%, v/v) in toluene for 4–5 h at 50 °C. After washing and drying, the slides were stored under Ar atmosphere.

#### 4.5.2. Evaluation of glass surface for immobilization

To evaluate the homogeneity of the epoxylated glass surface, the following experiment was performed. An oligomer sequence, TET-d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHC$-$OCH_2ONH_2$ (1 μM), dissolved in reaction buffer, pH 6, was spotted

manually on an epoxylated glass microslide at different locations all over the slide and kept overnight at 45 °C. After the reaction, the microslide was treated with a capping buffer (0.1 M Tris containing 50 mM ethanolamine, pH 8.0) for 15 min at 50 °C and then washed with the washing buffer (2 × 50 ml) followed by MilliQ water (2 × 50 ml), and dried under vacuum. Subsequently, the spots on the microslide were visualized under a laser scanner and quantified.

#### 4.5.3. Determination of optimal pH required for immobilization

In order to study the effect of pH on immobilization of aminooxyalkylated oligomers on epoxylated glass slides, the attachment of probe was carried out at different pH (4–11). A labeled oligomer, TET-d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)$-$CH_2NHCOCH_2ONH_2$, was dissolved in reaction buffers having different pH and spotted (0.5 μl) onto epoxylated glass slides at a concentration of 1 μM using a pipettmen followed by overnight incubation at 45 °C. The slide was processed and visualized, as described above.

#### 4.5.4. Immobilization kinetics

The results of the above experiment was used to optimize the time and temperature required to fix the 3′-aminoxy modified oligonucleotides on the epoxylated glass surface, TET-labeled oligomer, TET-d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)$-$CH_2NHCOCH_2ONH_2$ (1 μM) was dissolved in the reaction buffer of pH 6. Microarrays were prepared by spotting the above solutions for different time intervals (60, 120, 180, 210, 240, 270, 300, 360, and 600 min) and were kept at 35 °C. After the reaction, the slides were washed with the washing buffer (2 × 50 ml), followed by MilliQ water (2 × 50 ml), and dried under vacuum. The microslides were subjected to scanning under laser. Similarly, the immobilization reaction was allowed to proceed at temperatures of 45 °C and 55 °C.

Likewise, the experiment was repeated at pH 9. The extent of reaction was evaluated by plotting fluorescence intensity (A.U.) with respect to time (min).

#### 4.5.5. Determination of threshold concentration of modified oligonucleotides required for visualization of fluorescence spots on glass microslides

In an attempt to arrive at the threshold concentration of aminooxyalkylated oligonucleotides required for fluorescence detection at pH 6 and 9, the reaction was carried out by spotting (0.5 μl) a serially diluted (40, 20, 10, 5, 2.5, 1.0 and 0.5 μM) TET-labeled oligomer, TET-d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)$-$CH_2NHCOCH_2ONH_2$, dissolved in reaction buffers of pH 6 and 9, onto epoxylated glass microslides and allowed to react at 45 °C for 270 min and 240 min, respectively. After the reaction, the glass slides were washed with the washing buffer and MilliQ water. The slides were dried and visualized under a laser scanner. The immobilization efficiencies were determined using the standard curve.

#### 4.5.6. Thermal and pH stability

In order to evaluate the thermal stability of immobilization, microarrays were constructed by spotting a TET-labeled oligomer sequence (2.5 μM), as mentioned above, in the reaction buffers of pH 6 and pH 9, respectively. After usual washings, the constructed microarrays were subjected to PCR-like conditions for 0, 10 and 20 cycles in SSC buffer (pH 7). PCR parameters were as follows; 94 °C for 30 s (denaturation step), 54 °C for 30 s (annealing step), 72 °C for 30 s (extension step). After thermocycling, the slides were dried and visualized under laser scanner.

The stability of the constructed microarrays was also evaluated by spotting the microslides in a similar way at pH 6 and 9, and then subjecting them to washings with the washing buffer of different pHs (7–9) at ambient temperature. The microslides were scanned, after usual washings and drying, under a laser scanner.

### 4.6. Hybridization studies, specificity of chemistry and base mismatch detection

#### 4.6.1. Comparison of hybridization of 3′-and 5′-aminooxy-oligonucleotides

In order to study the effect of attachment of probe sequence on the accessibility to target sequence, two oligomers, d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ and $NH_2OCH_2CONHCH_2CH(OH)CH_2$-$OPO_3^{2-}$-d(TTC GTC ATC AAG TAG TTC CT), dissolved in the reaction buffer of pH 6.0, were spotted onto epoxy-glass microslide and kept at 45 °C for 240 min. The residual epoxy functions were capped by treating the glass surface with a capping buffer, 0.1 M Tris containing 50 mM ethanolamine, pH 8, for 15 min at 50 °C. After usual washings with the washing buffer, the spots were subjected to hybridization with the labeled complementary target, TET-d(AGG AAC TAC TTG ATG ACG AA), as described above. After washings with the hybridization buffer (2 × 50 ml), the slide was dried and subjected to visualization of spots under a laser scanner and quantification.

#### 4.6.2. Specificity of chemistry

Two oligonucleotides sequences, viz., TET-d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ (labeled aminooxyalkylated) and TET-d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH_2CH_2CH_2CH_2CH_2NH_2$ (labeled aminoalkylated) (5 μM), were dissolved in reaction buffer of pH 6, spotted onto an epoxylated glass microslide in duplicates and kept overnight at 45 °C. After the reaction, the microslide was washed with the washing buffer (2 × 50 ml), followed by MilliQ water (2 × 50 ml), and dried under vacuum. Subsequently, the slide was visualized under a laser scanner.

#### 4.6.3. Detection of base mismatches

The specificity of the system was demonstrated by immobilizing modified oligomers on epoxylated glass microslides. Four oligonucleotides, viz., d(TTC GTC ATC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$, d(TTC GTC *T*TC AAG TAG TTC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$, d(TTC GTC *T*TC AAG TAG *G*TC CT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ and d(TTT TTT TTT TTT TTT TTT TT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ (2.5 μM), having zero, one, and two mismatches and non-complementary probe, were spotted on a glass microslide and processed as described above. Subsequently, the spots on the microslide were hybridized with a complementary labeled TET-d(AGG AAC TAC TTG ATG ACG AA) and kept at 45 °C for 1 h and then at room temperature for 12 h. After washings with the hybridization buffer (3 × 50 ml), the microslide was subjected to laser scanning followed by quantification.

### 4.7. Detection of bacterial typhoid

*PCR-amplification*: The PCR reaction was performed in 25 μl PCR Eppendorf containing 0.25 mM dNTPs (of each dATP, dCTP, dGTP, and dTTP), forward primer TET-d(ACC GGT GGA TGT GGC TTC CTT G), and reverse primer TET-d(TGG TCT GCA GCA CCT TTT GAA C) (0.4 μM of each), genomic DNA (50 ng/reaction), Taq polymerase (0.75 units), and MilliQ water. All PCR reactions were performed on an MJ Research thermo-cycler using the following profile: (1) 5 min at 95 °C (initial denaturation step); (2) 35 cycles of 1 min at 95 °C (denaturation step), 40 s at 61.6 °C (annealing

step), 2 min at 72 °C (extension step); (3) 7 min at 72 °C (final extension step); hold temperature: 4 °C. The labeled PCR-amplicons, 503 bp fragment of *tyv* gene (*S. typhi*), were analyzed on agarose gel electrophoresis, and detected by ethidium bromide (10 μg/mL) in standard TAE buffer, pH 8.

*PCR amplicon capture*: Two oligonucleotides, d(ACC GGT GGA TGT GGC TTC CTT GTT TT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ (complementary) and d(CGT CGA GTC GGG CAT CTT GGT ATT TT)-$OPO_3$-$CH_2CH(OH)CH_2NHCOCH_2ONH_2$ (control, non-complementary) were immobilized on the glass slide and processed, as described above. Amplicons were hot denatured (5 min, 95 °C), cooled in ice (10 min), and used for the hybridization assay. After usual washings, the slide was visualized under a laser scanner.

### 4.8. Signal evaluation and quantification

Microarrays were scanned on a Scan Array Lite Scanner (GSI Lumonics) fitted with a Cy3 optical filter at 20 μm resolution, PMT and Laser power were set to 55% and 80%, respectively. Spot intensities were quantified using Quant Array software (Packard Bioscience) and graphs were plotted in MS Excel.

For quantification of immobilized oligonucleotides, a TET-labeled oligonucleotide sequence was diluted from 1 to 0.05 μM concentrations and spotted on a virgin micro slide. After drying, the slide was scanned and spots were quantified. A standard calibration curve was plotted between fluorescence intensity (A.U) and concentration (μM).

For each experimental condition tested on the microarrays, the experiment was repeated 2–3 times. The immobilization and hybridization data presented are the average of these repetitions, and the error bars represent the percentage error (±2–4%) on this average.

## Acknowledgements

Authors gratefully acknowledge the financial support from the Department of Biotechnology, New Delhi, India. One of the authors, DS, acknowledges the University Grants Commission (UGC), New Delhi, India, for the award of Senior Research Fellowship to carry out his work. Authors are also thankful to Drs. S. Gupta and S. Khare, National Institute of Communicable Diseases (NICD), Delhi, India, for providing typhoid sample and facilities.

## References and notes

1. Avino, A.; Guimil, G. R.; Albericio, F.; Mann, M.; Wilm, M.; Neubauer, G.; Eritja, R. *Bioorg. Med. Chem.* **1996**, *4*, 1649.
2. Beaucage, S. L. *Curr. Opin. Drug Discovery Dev.* **2008**, *11*, 203.
3. Carriero, S.; Damha, M. J. *Curr. Prot. Nucleic Acid Chem.* **2002**. Chapter 4: Unit 4.14.
4. Hirao, I.; Ishikawa, M.; Miura, K. *Nucleic Acids Symp. Ser.* **1985**, 173.
5. Iwase, R.; Toyama, T.; Nishimori, K. *Nucleosides Nucleotides Nucleic Acids* **2007**, *26*, 1451.
6. Pon, R. T. *Curr. Prot. Nucleic Acid Chem.* **2001**, Chapter 3: Unit 3.1.
7. Thaden, J.; Miller, P. S. *Bioconjugate Chem.* **1993**, *4*, 395.
8. Viazovkina, E.; Mangos, M. M.; Elzagheid, M. I.; Damha, M. J. *Curr. Prot. Nucleic Acid Chem.* **2002**. Chapter 4: Unit 4.15.
9. Gaynor, J. W.; Brazier, J.; Cosstick, R. *Nucleosides Nucleotides Nucleic Acids* **2007**, *26*, 709.
10. Markiewicz, W. T.; Wyrzykiewicz, T. K. *Nucleic Acids Res.* **1989**, *17*, 7149.
11. Beaucage, S. L. *Curr. Med. Chem.* **2001**, *8*, 1213.
12. Consolandi, C.; Severgnini, M.; Caredda, G.; De, B. G. *Anal. Biochem.* **2008**, *376*, 39.
13. Park, H. G.; Ham, H. O.; Kim, K. H.; Huh, N. *Biosens. Bioelectron.* **2005**, *21*, 637.
14. Ramsay, G. *Nat. Biotechnol.* **1998**, *16*, 40.
15. Cha, T. W.; Boiadjiev, V.; Lozano, L.; Yang, H.; Zhu, X. Y. *Anal. Biochem.* **2002**, *311*, 27.
16. Choithani, J.; Kumar, P.; Gupta, K. C. *Anal. Biochem.* **2006**, *357*, 240.
17. Chrisey, L. A.; Lee, G. U.; O'Ferrall, C. E. *Nucleic Acids Res.* **1996**, *24*, 3031.
18. Consolandi, C.; Severgnini, M.; Castiglioni, B.; Bordoni, R.; Frosini, A.; Battaglia, C.; Bernardi, L. R.; Bellis, G. D. *Bioconjugate Chem.* **2006**, *17*, 371.
19. Defrancq, E.; Lhomme, J. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 931.
20. Fixe, F.; Dufva, M.; Telleman, P.; Christensen, C. B. *Lab. Chip.* **2004**, *4*, 191.
21. Fontanel, M.; Bazin, H.; Teoule, R. *Bioconjugate Chem.* **1993**, *4*, 380.
22. Fuentes, M.; Mateo, C.; Garcia, L.; Tercero, J. C.; Guisan, J. M.; Fernandez-Lafuente, R. *Biomacromolecules* **2004**, *5*, 883.
23. Ghosh, S. S.; Musso, G. F. *Nucleic Acids Res.* **1987**, *15*, 5353.
24. Kimura, N.; Oda, R.; Inaki, Y.; Suzuki, O. *Nucleic Acids Res.* **2004**, *32*, e68.
25. Kremsky, J. N.; Wooters, J. L.; Dougherty, J. P.; Meyers, R. E.; Collins, M.; Brown, E. L. *Nucleic Acids Res.* **1987**, *15*, 2891.
26. Kumar, A.; Larsson, O.; Parodi, D.; Liang, Z. *Nucleic Acids Res.* **2000**, *28*, e71.
27. Kumar, P.; Gupta, K. C. *Bioconjugate Chem.* **2003**, *14*, 507.
28. Kumar, P.; Choithani, J.; Gupta, K. C. *Nucleic Acids Res.* **2004**, *32*, e80.
29. Kumar, P.; Agrawal, S. K.; Misra, A.; Gupta, K. C. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1097.
30. Kumar, P.; Agarwal, S. K.; Gupta, K. C. *Bioconjugate Chem.* **2004**, *15*, 7.
31. Lockett, M. R.; Phillips, M. F.; Jarecki, J. L.; Peelen, D.; Smith, L. M. *Langmuir* **2008**, *24*, 69.
32. Mahajan, S.; Kumar, P.; Gupta, K. C. *Bioconjugate Chem.* **2006**, *17*, 1184.
33. Isaacs, N. *Phys. Org. Chem.* **1995**, 276.
34. Misra, A.; Dwivedi, P. *Anal. Biochem.* **2007**, *369*, 248.
35. Podyminogin, M. A.; Lukhtanov, E. A.; Reed, M. W. *Nucleic Acids Res.* **2001**, *29*, 5090.
36. Salo, H.; Virta, P.; Hakala, H.; Prakash, T. P.; Kawasaki, A. M.; Manoharan, M.; Lonnberg, H. *Bioconjugate Chem.* **1999**, *10*, 815.