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Oligonucleotide microarrays with stem–loop probes: Enhancing the hybridization of nucleic acids for sensitive analysis

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ABSTRACT

We have demonstrated that the dynamics of nucleic acid hybridization in microarrays depend on the physical structure of immobilized probes. We have immobilized oligonucleotide-3′-phosphates with and without stem–loop structure on epoxylated glass surface, followed by hybridization under different conditions, viz., hybridization buffer, pH condition, temperature and ionic strength. In a comparative study, we have established that array constructed using probes with stem–loop structure displayed ~2.2 times higher hybridization signals than the probes without it. The stem–loop DNA array format is simple and flexible in design and thus potentially useful in various DNA diagnostic tests.

In the past decade, DNA arrays have been widely adopted in genomics because of their ability to simultaneously examine the expression level of thousands of genes. As a result, the scope of applications of microarrays has broadened rapidly, from drug discovery[1] to classification of cancers[2,3] and analysis of splice variants.[4] The principle of oligonucleotide arrays is based on the hybridization of target nucleic acids with DNA probes immobilized on a chip surface. Essentially, the fidelity of the hybridization relies on differences in the thermodynamic equilibrium between the bound and the free oligomers. Two approaches have established themselves for the fabrication of DNA arrays, viz., on-chip (in-situ) synthesis of nucleic acids, and the attachment of pre-synthesized oligonucleotides on the surface of choice.[5] Although each one has its own advantages, the deposition method involving the use of pre-synthesized ODNs is the most preferred and commonly used in DNA array fabrication.

A number of methods comprising the use of non-covalent, covalent and inorganic–organic interactions have been reported for immobilization of pre-synthesized probes, including molecular beacons (MB) on a substrate of choice, preferably glass.[6–11] The diversity of techniques that fall under the array umbrella as well as the diversity of uses for arrays complicates the understanding of the fundamental concepts that must be addressed before realizing the full potential of array technology. One approach to enhance the sensitivity in microarrays is based on the use of molecular beacons that resulted in fluorescence enhancement (ratio of net fluorescence intensity of the hybrid to that of MB) by a factor of ~2–5.5.[12] However, this small enhancement factor and the complicated purification techniques required to prepare pure MBs have impeded the exploration of full potential of molecular beacon based microarrays. Another approach relies on increasing the affinity of a probe (nucleic acid present on the array) for its target through various modifications such as chemistry of attachment, length and physical structure of the probe.[13–15] In an elegant approach to enhance the sensitivity of the constructed microarrays, Nonglaton et al.[16] have reported a new method to construct oligonucleotide arrays that utilizes binding of oligonucleotide probes bearing terminal phosphate groups to zirconium phosphonated glass microslides. Here, they observed that the use of a polyguanine spacer between the probe and the terminal phosphates significantly enhanced the fluorescence signal after hybridization with the fluorescent targets. Other research groups have also investigated the role of the spacer molecules and their effect on the hybridization behaviour of immobilized oligonucleotides. However, ideal systems with maximized sensitivity and data reproducibility are still elusive.

In the studies presented here, we have examined the dynamics of nucleic acid hybridization in microarray model system based on the physical structure of the immobilized species. We have constructed oligonucleotide arrays having stem–loop probes with

Abbreviations: HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; PBS, phosphate buffer saline; S.I., signal intensity; SSC, saline sodium citrate; TET, 4,5,6,7-tetrachlorofluorescein; TRIS, tris(hydroxymethyl)-aminomethane.

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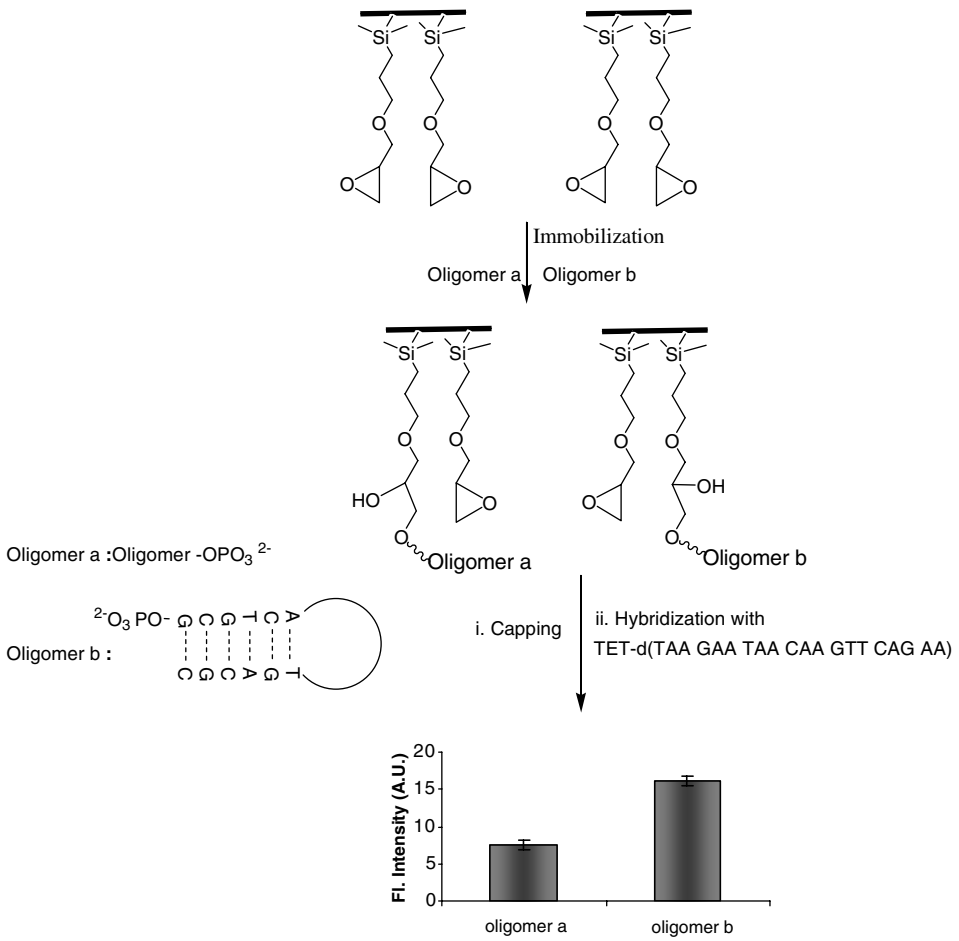
short single stranded overhangs (Scheme 1) bearing a phosphate group at 3′-terminus for immobilization on epoxylated glass surface. In a comparative study, we have demonstrated that array constructed by the use of probes with stem–loop structure displays ~2.2 times higher hybridization signal than the probes without it. The stem–loop DNA array format is simple and flexible in design and thus potentially useful in various DNA diagnostic tests.

To understand the dynamics of microarray hybridization with respect to the structure of immobilized probes, three sets of oligonucleotide probes, viz., **Set I**: (a) d(*CGC AGT* TTC TGA ACT TGT TAT TCT TA *TGA CGC*)-OPO_3^{2-}, (b) d(*CGC AGT* TTC TGA ACT TGT TAT TCT TA *ACT GCG*)-OPO_3^{2-}, **Target**: TET-d(TAA GAA TAA CAA GTT CAG AA); **Set II**: (a) d(*CGC AGT* ATG TGC TAT CTT TCT AGT CA *TGA CGC*)-OPO_3^{2-}, (b) d(*CGC AGT* ATG TGC TAT CTT TCT AGT CA *ACT GCG*)-OPO_3^{2-}, **Target**: TET-d(TGA CTA GAA AGA TAG CAC AT); **Set III**: (a) d(*CGC AGT* ATC TAT CGT TGC ATC CGT CT *TGA CGC*)-OPO_3^{2-}, (b) d(*CGC AGT* ATC TAT CGT TGC ATC CGT CT *ACT GCG*)-OPO_3^{2-}, **Target**: TET-d(AGA CGG ATG CAA CGA TGA AT), were synthesized at 0.2 μmol scale on an automated DNA synthesizer using the standard phosphoramidite approach following the manufacturer's protocol (Gene Assembler Plus Manual, Uppsala, Sweden, 1988). Phosphorylated oligonucleotide probes were synthesized on the commercially available long chain aminoalkyl-controlled pore glass (LCAA-CPG).[17] Immobilization of phosphorylated oligonucleotides on epoxylated microslide was carried out following the protocol already published from authors' laboratory.[7,8] Scan Array Lite Scanner, GSI Lumonics, USA fitted with a Cy3 optical filter at 30 μm resolution, operating at 80% of laser power and 80–85% of PMT voltage, was used for visualization of the constructed oligonucleotide arrays after hybridization. The fluorescence intensity of the spots on the slides was quantified using Quant Array software (Packard Bioscience, USA). Background as well as negative control spot intensity (array elements non-complementary to the target solution) was subtracted from all the values. All the experiments were repeated at least three times. The data presented here are the average of these repetitions and the hybridization efficiencies were calculated from a calibration curve, constructed using a labelled oligomer.[7]

To understand the reasons for the structure-dependent hybridization reaction, we examined the signal intensity for probes with and without stem–loop structure. Briefly, six arrays were constructed by immobilizing oligomer probes (a and b of **Set I**) in the concentrations, for example, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 μM on the epoxylated glass microslides under denaturing conditions (0.1 M *N*-methylimidazole in 10% DMSO, pH 10) for 2 h at 45 °C. After being arrayed, the slides were treated with a capping buffer (0.1 M Tris containing 50 mM ethanolamine, pH 9.0) for 15 min at 50 °C. The slides were then washed with Milli Q water (3 × 50 ml) for 15 min each and immersed in the hot 1× SSC buffer (pH 7.0, 55 °C) for 10 min and then at 25 °C for 2 h. Subsequently, the slides were dried under vacuum and subjected to incubation with 40 μl of target oligomer, TET-d(TAA GAA TAA CAA GTT CAG AA) (40 μM), in the hybridization buffer (1× SSC, pH 7.0) at 55 °C for 1 h and then at room temperature for 12 h.[8] The slides were then washed with 2× SSC buffer (pH 7.0, 3 × 15 min) at room temperature and dried under vacuum prior to visualization under a la-



Scheme 1. Outline of the fabricated oligonucleotide arrays using probes with and without stem–loop structure and their hybridization with target oligomer. **Set I**, (a) d(*CGC AGT* TTC TGA ACT TGT TAT TCT TA *TGA CGC*)-OPO_3^{2-}, (b) d(*CGC AGT* TTC TGA ACT TGT TAT TCT TA *ACT GCG*)-OPO_3^{2-}.

ser scanner (Fig. 1A). The intensity of the fluorescent spots was determined,[7,8] the signals were corrected for background intensity and then plotted as a function of the corresponding probe concentration (Fig. 1B). The results clearly showed that initially, with increase in probe concentration, the enhancement factor ($S.I._{\cdot stem-loop}/S.I._{\cdot without\ stem-loop}$) increased and reached a value of ~2.2, then it gradually decreased with further increase in the concentration of the spotting solution and reached a steady state at 5.0 μM with enhancement factor ~1.24. The observed effects might be due to high packing of the probes on the surface, which might hinder the probe to acquire stem–loop conformation. In principle, the probe sequence in the loop anneals to a complementary nucleic acid target sequence. The strong probe–target duplex overcomes the shorter hairpin stem, leading to a conformational reorganization. The stability of the probe–target helix forces the hairpin stem to unwind, however, with increase in probe concentration of the spotting solution, the repulsion between the strands of the immobilized probe and target sequence (due to negative phosphate backbone) becomes more predominant, resulting in the lowering of the enhancement factor. Therefore, in rest of the experiments, arrays were constructed using 1 μM concentration. Further, in order to investigate the effect of hybridization temperature on the enhancement factor, hybridization assay was repeated at different temperatures (60 °C, 70 °C and 80 °C for 1 h and then at room temperature for 12 h); however, no significant variation in the results was obtained.

In order to demonstrate the wider applicability of the concept, two more sets of oligonucleotide probes (**II** and **III**), varying in loop sequence, were immobilized under identical conditions and hybridized with their corresponding complementary fluorescent targets. The signal enhancement factor was found to be ~2.31 and ~2.25 with respect to probes in sets **II** and **III**, respectively (Fig. 1C), which implies that the enhancement factor in newly developed stem–loop probe arrays is independent of the loop sequence and can be used effectively for the detection of DNA targets.

To study the effect of various hybridization buffers on signal intensity, several buffers were screened, viz., saline sodium citrate (SSC), phosphate buffer saline (PBS), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and tris(hydroxymethyl)-aminomethane (Tris) (0.1 M containing 125 mM NaCl, pH 7.0) for performing hybridization assays on arrays constructed by spotting oligomers (a and b, **Set I**) on the epoxylated surface. Identical molar concentrations of each species were arrayed (1 μM) to ensure that the same number of binding sites would be available for the solution phase probe.

After hybridization reactions, the slides were subjected to washings and drying, followed by scanning under a laser scanner. The quantitative results clearly indicate that the enhancement factor increases in the order, HEPES < PBS < Tris < SSC (Fig. 2A) and the highest fluorescence signals are consistently obtained with 1× SSC buffer.

Further to optimize the concentration of the SSC buffer required to obtain maximum signal intensity, hybridization assays on the constructed arrays, as described above, were carried out in SSC buffer of different ionic strength (75, 100, 125, 150 and 175 mM). Subsequently, the microslides were subjected to usual washings with the respective buffers, dried under vacuum and then visualized under a laser scanner. As depicted in Figure 1B, the highest fluorescence was observed with the SSC buffer (125 mM, pH 7.0) (Fig. 2B). Deviation from this concentration resulted in decreased fluorescence intensity of the spots, which could be explained on the basis of the concentration of metal ions in hybridization buffer. By increasing the salt concentration, the metal ions stabilize the duplex structure by reducing the electrostatic repulsion between the negatively charged immobilized probe and the target. However, further increasing the salt concentration may again cause the repulsion due to excessive positive charge on the DNA duplex, which, in turn, may affect the hybridization.

Analogously, the effect of pH of the hybridization buffer, one of the key factors, which can affect the performance of the arrays, on signal intensity was also evaluated. To study this parameter, three arrays were constructed by immobilizing equimolar concentration (1 μM) of oligomers (a and b, **Set I**), followed by incubation with target oligomer in hybridization buffer (1× SSC) having different pHs, viz., 6, 7 and 8. After hybridization as-

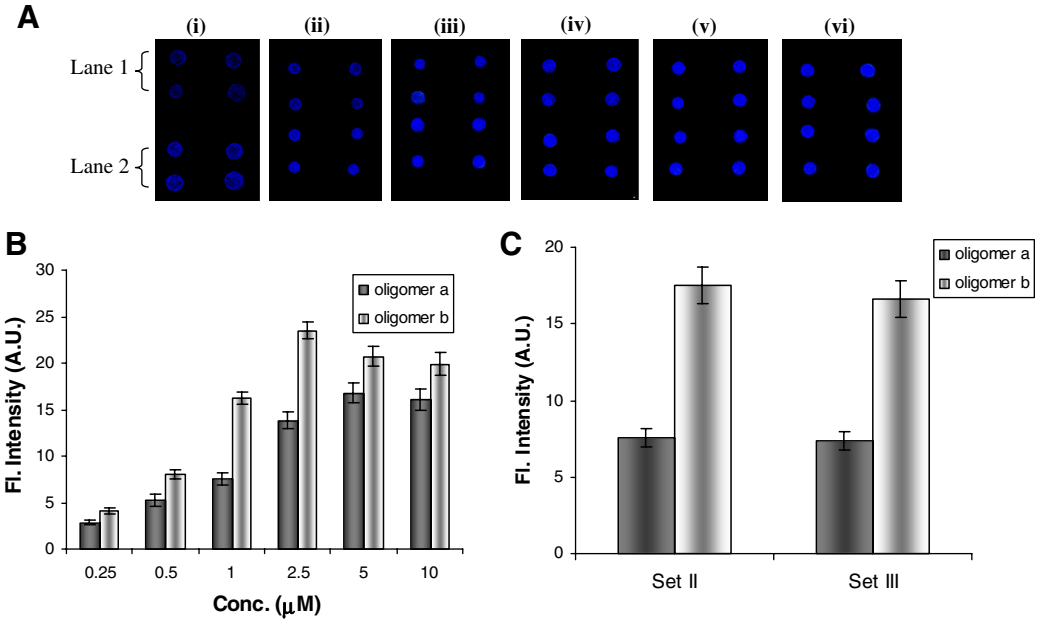


Figure 1. (A) Fluorescent maps obtained after hybridization assays of arrays constructed by immobilizing oligomers (a and b) of **Set I** on epoxylated glass microslides, (lane 1) probe (a) and (lane 2) probe (b) at different probe concentrations viz., (i), 0.25 μM; (ii), 0.5 μM; (iii), 1.0 μM; (iv), 2.5 μM; (v), 5.0 μM; (vi), 10.0 μM; (B) quantitative representation of the signal intensities obtained after hybridization of arrays, and (C) quantitative representation of data obtained with oligomers of sets **II** and **III** after hybridization with their corresponding complementary targets.

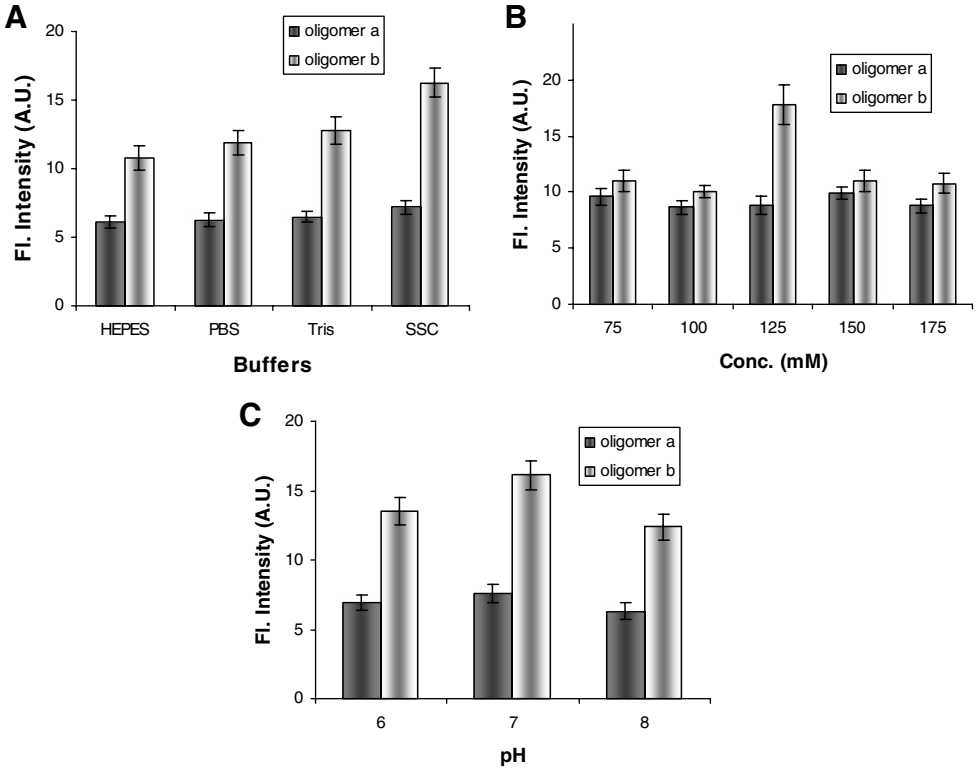


Figure 2. Histograms showing the comparison of signal intensities obtained after hybridization of arrays constructed by immobilizing oligomers of **Set I** (A) with complementary target dissolved in different buffers, viz., HEPES, PBS, TRIS, SSC, (B) with target dissolved in SSC buffer of different ionic strength, and (C) with target dissolved in SSC buffer (125 mM) of different pH (6–8).

say, the processed slides were scanned under a laser scanner and fluorescence intensity was determined. The quantitative results depicted that optimal hybridization was achieved with the buffer of pH 7.0 (Fig. 2C).

In conclusion, we have demonstrated that the physical structure of the immobilized probes is a key factor that determines the extent to which probe strands are able to capture target molecules in solution. The optimal probe concentration is 1 μM for maximum enhancement (~2.2) after hybridization under experimental conditions. However, it has also been observed that with both kinds of probes, the efficiency of duplex formation depends on the surface probe density and the enhancement factor reaches a value of ~1.24 at higher probe concentration. The presented array format has also been studied under different pHs and hybridization buffers. We expect that arrays of stem–loop oligonucleotides probes would be useful for various molecular biology applications.

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References and notes

1. Heller, R.; Schena, M.; Chai, A.; Shalon, D.; Bedilion, T.; Gilmore, J.; Woolley, D. E.; Davis, R. W. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2150.
2. Perou, C. M.; Sorlie, T.; Eisen, M. B.; van de Rijn, M.; Jeffrey, S. S.; Rees, C. A.; Pollack, J. R.; Ross, D. T.; Johnsen, H.; Akslen, L. A.; Fluge, O.; Pergamenschikov, A.; Williams, C.; Zhu, S. X.; Lonning, P. E.; Borresen-Dale, A.-L.; Brown, P. O.; Botstein, D. *Nature* **2000**, *406*, 747.
3. Bittner, M.; Meltzer, P.; Chen, Y.; Jiang, Y.; Seftor, E.; Hendrix, M.; Radmacher, M.; Simon, R.; Yakhini, Z.; Ben-Dor, A.; Sampas, N.; Dougherty, E.; Wang, E.; Marincola, F.; Gooden, C.; Lueders, J.; Glatfelter, A.; Pollock, P.; Carpten, J.; Gillanders, E.; Leja, D.; Dietrich, K.; Beaudry, C.; Berens, M.; Alberts, D.; Sondak, V.; Hayward, N.; Trent, J. *Nature* **2000**, *406*, 536.
4. Hu, G. K.; Madore, S. J.; Moldover, B.; Jatkoe, T.; Balaban, D.; Thomas, J.; Wang, Y. *Genome Res.* **2001**, *11*, 1237.
5. Seliger, H.; Hinz, M.; Happ, E. *Curr. Pharm. Biotechnol.* **2003**, *4*, 379.
6. Kumar, P.; Gupta, K. C. *Bioconjug. Chem.* **2003**, *14*, 507.
7. Mahajan, S.; Kumar, P.; Gupta, K. C. *Bioconjug. Chem.* **2006**, *17*, 1184.
8. Mahajan, S.; Kumar, P.; Gupta, K. C. *BioMed. Chem. Lett.* **2006**, *16*, 5654.
9. Du, H.; Disney, M. D.; Miller, B. L.; Krauss, T. D. *J. Am. Chem. Soc.* **2003**, *125*, 4012.
10. Shchepinov, M. S.; Case-Green, S. C.; Southern, E. M. *Nucleic Acids Res.* **1997**, *25*, 1155.
11. Broude, N. E.; Woodward, K.; Cavallo, R.; Cantor, C. R.; Englert, D. *Nucleic Acids Res.* **2001**, *29*, e92.
12. Yao, G.; Tan, W. *Anal. Biochem.* **2004**, *331*, 216.
13. Maskos, U.; Southern, E. M. *Nucleic Acids Res.* **1992**, *20*, 1675.
14. Maskos, U.; Southern, E. M. *Nucleic Acids Res.* **1992**, *20*, 1679.
15. Maskos, U.; Southern, E. M. *Nucleic Acids Res.* **1993**, *21*, 4663.
16. Nonglaton, G.; Benitez, I. O.; Guisle, I.; Pipelier, M.; Leger, J.; Dubreuil, D.; Tellier, C.; Talham, D. R.; Bujoli, B. *J. Am. Chem. Soc.* **2004**, *126*, 1497.
17. Letsinger, R. L.; Gryaznov, S. M. *Tetrahedron Lett.* **1992**, *33*, 4127.