7.1 Introduction

Over the past few years, oligonucleotide based arrays have emerged as a promising and convenient tool for high throughput experimentation in disease diagnosis, mapping and gene expression analysis, as it offers advantages of using very small amount of biological probes (picomoles), along with capability of screening thousands of analytes in parallel.\textsuperscript{1-7}

A number of solid surfaces have been proposed for the fabrication of biochips.\textsuperscript{7-21} Of these, glass has been found to be most suitable as it is an inexpensive material with low intrinsic fluorescence and a relatively homogeneous chemical surface, however, low functional group density limits its applications for sensitive recognition of biomolecular interactions. Particularly, to prepare biochips for disease diagnosis, highly functional chip matrices are required, as it allows high immobilization capacity and good accessibility of target molecules.

In order to produce high functional group density chips, several strategies employing polymeric supports or polymeric coatings have been reported in the literature. In 2000, Waddell et al.\textsuperscript{9} described a method for testing the immobilization and hybridization of oligonucleotides on polymethymethylacrylate (PMMA) substrate. Following the similar idea, Fixe et al.\textsuperscript{16,17} developed a method for array fabrication of biomolecules using modified PMMA surfaces. In another elegant approach, a method has been described that employs chitosan-coated glass slides for the construction of oligonucleotide microarrays.\textsuperscript{13} In 2006, Marie et al.\textsuperscript{21} described a method for the direct immobilization of amino-functionalized DNA onto SU-8 coated microslides. Of all the employed reagents, the epoxy-based photoresist, SU-8 has been employed for a large variety of lab-on-a-chip applications, as it possesses a number of properties that makes it attractive as a structural material in micro-fabricated devices. SU-8 is biocompatible, rigid, inexpensive, thermally and chemically stable, and transparent to light above 360 nm.\textsuperscript{14, 15, 21-23}

In this chapter, a process of biochip fabrication by directly immobilizing modified oligonucleotides (aminoalkyl-, thiophosphoryl-, and phosphorylated oligomers) onto a SU-8 coated microchip has been described. The proposed strategy not only resulted in high signal reproducibility but also showed
superior spot morphology and homogeneity. The study undertaken herein includes investigation of optimal thickness of SU-8 layer required for immobilization followed by determination of the threshold concentration of oligomer required for visualization. The thermal and pH stability of constructed microarrays were evaluated. The constructed microarrays were then used for discrimination of base mismatches and detection of bacterial meningitis and typhoid.

7.2 Experimental

7.2.1 Cleaning, Silanization and SU-8 Coating of Glass Slides

Silanization of glass slides was carried out, as described in Chapter II. Coatings of SU-8 of different thicknesses (2, 2.5 and 3 μm) on the glass microslides was carried out by spin-coating, according to manufacturer’s protocol. Subsequently, the microslides were subjected to soft bake 1 min at 65°C, to get rid of excess solvent, and 2 min at 95°C, for curing process. The coated microslides were stored under inert atmosphere.

7.2.2 Determination of Optimal Thickness Required for Immobilization

A labeled oligomer, TET-d(CTT CTT TTT CCT GTT ACC GT)-OPO₃²⁻, was dissolved in the reaction buffer (used in Chapter V) and spotted (0.5 μl) onto SU-8 coated glass slide (2 μm thickness) at a concentration of 0.1 μM using a pipettmen followed by incubation at 45°C for 2 h. After the reaction, the microslide was washed with 1 x SSC buffer (2 x 50 ml) followed by MilliQ water (2 x 50 ml), and dried under vacuum. Similarly, this oligomer was immobilized on the microslides of 2.5 and 3 μm thicknesses. Subsequently, the spots on the microslides were visualized under a laser scanner and quantified.

7.2.3 Determination of Threshold Concentration Required for Visualization

In an attempt to arrive at the threshold concentration of oligonucleotides required for fluorescence detection, a serially diluted oligomer solution (1, 0.5, 0.1, 0.05, 0.025 and 0.01 μM) was spotted onto a SU-8 coated glass microslide (2.5 μm thickness) and processed as described in Chapter IV.
7.2.4 Thermal and pH Stability

The thermal and pH stability of the constructed microarray on SU-8 coated microslides (2.5 μm thickness) were carried out as described in Chapter IV.

7.2.5 Hybridization Studies

In order to study the accessibility of attached probe sequence to target sequence, an oligomer sequence, d(CTT CTT TTT CCT GTT ACC GT)-OPO₃²⁻, dissolved in the reaction buffer, was spotted onto SU-8 coated glass microslide at 0.5 μM and processed as described in Chapter IV.

7.2.6 Base-mismatch Detection

The specificity of the system was demonstrated by immobilizing modified oligomers on SU-8 glass microslides. Three oligonucleotides, viz., d(CTT CTT TTT CCT GTT ACC GT)-OPO₃²⁻, d(CTT CTT TTT ACT GTT ACC GT)-OPO₃²⁻ and d(TTT TTT TTT TTTTTT TTT TT)-OPO₃²⁻, perfect match, single mismatch and non-complementary probe, respectively, were spotted onto a glass microslide at 0.5 μM and kept at 45°C for 2 h. Subsequently, the microslide was washed, dried and was hybridized with a complementary labeled TET-d(ACG GTA ACA GGA AAA AGA AG) as described in Chapter IV. Similar studies were carried out using aminoalkyl- and thiophosphorylated oligomers.

7.2.6 Signal Evaluation and Quantification

For quantification of immobilized oligonucleotides, a TET-labeled oligonucleotide sequence was diluted from 0.5 to 0.005 μM concentrations and spotted on a SU-8 coated glass 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).

7.3 Results and Discussion

Since the advent of microarray technology, glass has remained a substrate of choice as it offers the advantages of being non-porous, durable and stable at high temperatures often required for hybridization assays. However, due to low silanol functional group density of glass, glass microslides coated with a functional polymer has gained attention as substrate for the fabrication of microarrays. Of all the polymers employed for coating of slides, SU-8 has garnered attention as a possible material for a variety of biomedical
SU-8 is a biocompatible, chemically amplified, epoxy-based negative photoresist and very commonly used as a structural component in fabrication of bioanalytical microdevices, microarrays, sensors, bioassays, and drug delivery vehicles, because of its chemical and thermal resistance, as well as its ability to produce layers of a wide range of thicknesses. Its high epoxy group density, chemical stability with light transparency above 360 nm make SU-8 an ideal polymer coating for microarray fabrication. Nucleic acids can be immobilized on to SU-8 coated surfaces by covalent linkages or by non-covalent hydrophobic interactions.\textsuperscript{15, 21-23} Covalent attachment of probes is a preferred approach, as it results in higher stability of the constructed microarrays. The presence of epoxy functionalities on the surfaces coated with SU-8 photoresist, allows attachment of oligonucleotides with different nucleophilic functionalities. By taking advantage of above mentioned points, a rapid and clean protocol has been developed for direct immobilization of oligonucleotides bearing different functional groups onto SU-8 coated glass microslides.

Homogeneity of the epoxy-functionalities on the surface of SU-8 coated microslides was evaluated and found that the fluorescence intensity of spots were in the range of $\sim$18,000-18,500 A.U., which showed that the density of epoxy-functionalities on the coated glass surface was almost uniform throughout.

The results of the optimal thickness study revealed that the highest immobilization efficiency was obtained on glass microslides coated SU-8 with 2.5 $\mu$m thickness. Figure 1 shows the comparison of oligonucleotides immobilized on different glass microslides coated with SU-8 at different thicknesses.

![Figure 1: Fluorescence intensity data and pictorial representations of immobilization at (A) 2 $\mu$m, (B) 2.5 $\mu$m and (C) 3 $\mu$m](image)
The threshold concentration of oligonucleotides required for fluorescence detection was determined using a serially diluted TET-labeled oligomer, as described in the experimental section. The immobilization efficiency against each concentration (Figure 2) was determined with the help of a standard curve (Figure 3). The results revealed that immobilization efficiency followed an inverse relation with concentration of spotted oligonucleotides. The spots reached saturation at a concentration corresponding to 1 μM. The threshold concentration of visibility was 0.01 μM, however, to construct a good quality biochip for hybridization assays, a concentration of 0.5 μM was selected, as highest immobilization efficiency was attained at this concentration.

![Figure 2](image)

Figure 2: Threshold concentration of (A) aminoalkyl-, (B) thiophosphoryl- and (C) phosphorylated oligonucleotides required for fluorescence visualization. Lane 1: Probe concentration (μM); Lane 2: Immobilization efficiency (%).
The thermal stability constructed microarrays was evaluated by immobilizing a TET-labeled oligomer sequence (0.1 μM), as mentioned above. The percent reduction in fluorescence intensity was observed to be ~6.2% for aminoalkyl-, ~8.5% for thiophosphoryl- and ~7.9% for phosphorylated oligomers after 20 cycles (Figure 4), indicating that constructed microarrays are sufficiently stable at high temperature to be used in biological research.

Likewise, the stability of constructed microarrays was also analyzed at different pH (7, 8 and 9). The results indicated that the constructed microarrays were sufficiently stable in SSC buffer of different pH. The decrease in fluorescence intensity was ~3.7% and ~5.6% for aminoalkyl-, ~7.0% and ~11.8% for thiophosphoryl- and ~4.6% and ~8.3% for phosphorylated oligomers at pH 8 and 9 respectively, as compared to fluorescence intensity at pH 7 for the immobilized oligomers (Figure 5).
The applicability of the projected strategy was evaluated by performing hybridization assay of slides fabricated via immobilization of the phosphoryl-, thiophosphoryl- and aminoalkylated oligomers on to the SU-8 coated glass slides at 0.5 μM concentration. The proposed strategy resulted in higher hybridization efficiencies (33.4% for aminoalkyl-, 30.5% for thiophosphoryl- and 35.7% for phosphorylated oligonucleotides). The specificity of the system was also demonstrated by immobilizing modified oligomers on SU-8 glass microslides. Figure 6A shows the pictorial representation of the slides. The results of the study revealed that the fluorescence intensity of spots decreased for single-mismatch and that of the non-complementary oligonucleotide did not show any fluorescence after hybridization, implying the specificity of the hybridization. The quantification data is shown in Figure 6B.

In order to develop biochips for the diagnosis of the bacterial infections, two microarrays were constructed on SU-8 coated glass microslides using a unique probe sequence of the *ctrA* gene and *tyv* gene, as described in the experimental sections, Chapter IV and V. The spots on the first microslide were subjected to hybridization with the labeled PCR amplicons corresponding to the *ctrA* gene. After washings and drying, the slide was visualized under a laser scanner. Figure 7A shows the pictorial view of the slide. Spots of probe sequence specific to *ctrA* gene of bacteria (*N. Meningitidis*) showed fluorescence while the spots pertaining to *tyv* gene did not fluoresce at all. Spots on the second microslide were hybridized with PCR amplicons corresponding to *tyv* gene. Figure 7B shows that spots of probe sequence specific to *tyv* gene showed
fluorescence while the spots pertaining to \textit{ctrA} gene did not fluoresce at all. The experiment signifies the specificity of the system to prepare biochips for the detection of bacterial diseases in humans.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6}
\caption{(A) Fluorescence maps of base-mismatch detection using (I) aminoalkyl-, (II) thiophosphoryl- and (III) phosphorylated probes; (B) Quantitative data of the fluorescence intensity of the spots.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure7}
\caption{Detection of bacterial (A) meningitis and (B) typhoid.}
\end{figure}
7.4 References

