Chapter 4: To identify miRNA levels of RB serum on a novel microfluidics

The act of miniaturization has led to increased usage of lab-on-a-chip concept in various clinical applications due to their competitive advantages such as reduced sample and reagent consumption, analysis time and increased automation (Figeys and Pinto 2000). An important consequences of miniaturizing are the increased surface to volume ratio, increased interaction between analyte and substrate (Maheshwari, Kottantharayil et al. 2010, Séguin, McLachlan et al. 2010). The devices are usually fabricated using silicon, glass, and polymers. Traditional device micro fabrication includes materials which are etched through photolithography and sealed using thermal or anodic bonding. Glass is a promising option with well-defined surface chemistries, and superior optical transparency. But due to its cost and fabrication time, the glass is not commonly preferred. Some of the various polymers used are poly-dimethylsiloxane (PDMS), polystyrene, polymethylmethacrylate (PMMA), polyvinylchloride (PVC), and polycarbonate (PC) (Makamba, Kim et al. 2003).

Among these material, PDMS is the most widely used polymer for lab on chip devices in both laboratory and commercial sector. The flow of liquids inside the microfluidic channel is achieved primarily by capillary force. The use of PDMS as a substrate material in microfluidics research has increased tremendously in the last decade. The modification of the hydrophobic PDMS to hydrophilic opens an additional wide range of applications in future prospects of modified PDMS surfaces such as molecular separations, cell
culture in micro channels, lab-on-chip proteomic analysis, bio molecular detection via immunoassays, microelectronics, and protective coating for medical devices (Almutairi, Ren et al. 2012).

Various surface coating methods have been applied to change the hydrophobic nature of PDMS surfaces. The majority of coating protocols include an initial process of exposing PDMS to high energy like O$_2$ plasma (Ren, Bachman et al. 2001, Vickers, Caulum et al. 2006) or UV/ozone treatment (Tan, Fukuda et al. 2007) or chemical treatment. The functional groups on PDMS is oxidized by oxygen plasma or UV/ treatment before surface coatings and covalent modifications. The commonly used chemical treatments such as APTES (3-Aminopropyl) triethoxysilane) and PAV (polymeric amine layer) method showed variation in the efficiency of probe immobilization on the PDMS. Several studies had showed that PDMS can be used for protein based analyte detections such as in immunoassays (Luo, Fu et al. 2005, Pla-Roca and Juncker 2011), studying the cell dynamics (Zhang, Choi et al. 2013), and also detection of small oligonucleic acid molecules called microRNAs.

MicroRNAs (miRNAs) are few nucleotides in size, highly conserved like mRNAs, ribosomal RNAs, and non-protein-coding with single-stranded RNAs. MicroRNAs not only present in the cells but also present as free circulating body fluids in extremely low levels. The expression profiles of circulating miRNAs can differentiate or classify stages of cancers in the human body. Therefore, sensitive miRNA detection will be a challenge and opened a new field of cancer diagnosis. Though the existence of various techniques
such as quantitative polymerase chain reaction (qPCR), northern blotting, advanced deep sequencing, oligo microarrays, and several new sensitive methods are emerging for easy detection of miRNA. All these techniques allow detection and quantity measurements of miRNAs despite of the obstacles such as small size, high similarity and having different melting temperatures. Among them only the technique which discovers novel miRNA and homologous miRNAs with high similarity is the deep sequencing method. In economic scale, high-throughput detection of miRNAs is performed on microarray platforms but with low reliability and sometimes bias with the artefact signals. In a regular and minimal miRNA number analysis, qPCR is the most reliable tool.

The circulating serum miRNAs have been used as molecular signatures for identifying breast cancer (Kumar, Keerthana et al. 2013), retinoblastoma (Beta, Venkatesan et al. 2013), and colorectal cancers (Li, Yang et al. 2013). The accurate assessment of miRNAs related to the disease condition in the serum samples would help in early diagnosis (Kroh, Parkin et al. 2010, Ichikawa and Akiyama 2013). The existing standard methods for quantifying miRNAs are Northern blotting and qRT-PCR methods that require high amount of input RNA template and lengthy processing time which is not suitable for routine clinical diagnostics on serum samples. Albeit the above advantages, there are other parameters besides high sensitivity, such as a short detection time and small sample volume. For point of care diagnosis, the detection process should be completed on a miniature portable device which doesn’t require processing steps. So, to identify miRNAs on microfluidics overcomes the problems which may all the current techniques couldn’t meet. We have chosen widely used PDMS and PMMA materials as a
choice of developing microRNA detections form serum samples. Both the materials are good for POC diagnosis; the PDMS is more economic compared to PMMA materials and both needs hydrophilic surface treatment before immobilizing biomolecules. In addition to that PMMA is validated as a microfluidic device for tissue extracted nucleic acids and microRNAs. PMMA can be used as PDMS for protein absorption studies. Likewise, on PDMS, F.Fixe et al., immobilized DNA (Fixe, Dufva et al. 2004) and Wen et al., immobilized antibody by multilayer approach on PMMA surfaces (Wen, He et al. 2009). In a study by N.Wongkaew et al., detected RNA from Cryptosporidium parvum by increasing the binding efficiency on PMMA by micro mixing channels (Fixe, Dufva et al. 2004). Micro-mixing strategy of PMMA was used for detection of free bilirubin (Sun, Nie et al. 2010). The PMMA microfluidic device (Hitachi) was used for the miR-222 detection in PC12 cells (Kalani, Mohan et al. 2013).

Although the material of choice plays a key role in microfluidic device development but also a sensitive sensor probe assay is required for to make a successful device and further can be used for clinical or biological molecules detection. An earlier reports on development of miRNA sensor probe assays for microRNA detection used a two-step method for rapid and sensitive detection by a molecular beacon structured probe which includes an additional step of removing non hybridized probe in the assay (Baker, Bao et al. 2012). The assay uses locked nucleic acid (LNA) modified molecular beacon probes for to overcome the technical difficulties of sensitive detection and stable hybridizations. Here we have taken well-studied miR-18a in the human serum samples (Garrison and Biggs 1990, Morimura, Komatsu et al. 2011, Beta, Venkatesan et al. 2013) for
microRNA hybridization studies. To the best of our knowledge, this application has not been reported for direct serum microRNA hybridizations. Moreover, in this method a minimum volume of 0.1 μL human serum is used to determine the miRNA copy numbers by fluorescence spectroscopy and microscopy methods. The assay then validated on PDMS static method (miRNA sensor probe immobilized and target miRNA as a passing solution on it) and PMMA fluidic model method (miRNA sensor probe and target miRNA are in fluidic state and mixes with each other).

In the static PDMS models, we have used two surface chemistry methods to immobilize the miRNA sensor probe on hydrophobic PDMS surface and interpreted the results of two methods i.e PAV (Thorslund, Sanchez et al. 2005) and APTES coating (Belder, Deege et al. 2001, Wang, Meng et al. 2006, Yu, Li et al. 2007) (Figure-4.1). In this objective, both the static PDMS and fluidic PMMA model prototypes were thoroughly evaluated for plausible translational application.

**Figure 4.1.** *PDMS general device model:* 3D image of PDMS slab having two sample inlets and a reservoir or outlet for sample collection is shown in the figure. MicroRNA pairing is specific in the channel i.e. hoogsteen base pairing takes place.
4.1. MicroRNA sensor probe assay development

4.1.1. Materials and Methods

4.1.1.1. Serum Sample collection

A volume of 1.0 ml blood sample was collected directly into vacutainer from healthy and Retinoblastoma (RB) individuals. The whole blood was allowed to stand for 30min at room temperature (RT) and centrifuged at 1800Xg for 20 minutes at RT. The resultant serum was aliquot into sterile diethylpyrocarbonate (DEPC)-treated, RNAase free 1.5 ml tubes and stored at -80°C freezer. A total of six non-retinoblastoma age matched healthy and six retinoblastoma samples were used for this study. Commercial fetal bovine serum was used for in vitro studies. Our institution ethics review board approved for the collection of serum samples to study serum miRNA profile in retinoblastoma (Ethics code: 49B-2011-P).

4.1.1.2. MiR-18a Beacon Probe design

The design of miR-18a probe was 5′/6-FAM/CCGAGCTATCTGCAC TAGATGCACC TTAC/iBiodT/CGG/3′Dab/. Briefly, microRNA probe designed for mature miR-18a (MIMAT0000072) or hairpin loop region of precursor miR-18a contained total number of 33 nucleotides in length. It comprises the middle ‘23’ nucleotides complementary to the miR-18a seed sequence; (highlighted), a 6-carboxyfluorescein (FAM) fluorphore at the 5′end, a Biotin (iBio) moiety, and 4-Dimethylamino-Azobenzene 4 carboxylic acid (Dab or Dabsyl) quencher at the 3′ end. The seed sequence was flanked at both end by 5 residues; CCGAG/CTCGG to form stem structure of the hair pin loop (Figure-4.1). Approximate number of Locked nucleic acid (LNA) modifications for the seed sequence were calculated based on given DNA/RNA Tm of the synthesized probe and found
equivalent to be 10 numbers (Based on Exiqon Oligo Tm prediction tool, www.exiqon.com/RNA-tm; Underlined in the probe sequence). The synthesized probes were HPLC purified and re-constituted in nuclease free water, stored at -20°C freezer.

**Figure 4.2. Schematic representation of LNA hybridization with miRNA**: LNA probe binds to target miRNA, and the hybridization results in opening of beacon loop and quenching of FAM dye.

### 4.1.1.3. Fluorescence spectra of LNA probe hybridization with synthetic miRNA

Initial experiments were conducted in PBS spiked with synthetic miR-18a and miR-129 to study the specificity and sensitivity of the custom miR-18a LNA probe hybridization. The molecular beacon probe designed with sequence complimentary to miR-18a, was labelled with 5’FAM and 3’Dabcyl reporter dye molecules (Figure-4.2). Different concentration of synthetic miR-18a and miR-129a (non-specific) starting from 500nM, 1000nM, 1500nM, 2000nM, to 2500nM was hybridized with 500nM of custom LNA
The miR-18a probe at room temperature for 30mins. The fluorescence of hybridized probe was detected at 525nm (excitation 485nm) using fluorescence spectrophotometer.

4.1.1.4. The miRNA copy number/concentration (nM) determination in serum samples by spectroscopic method

The experiments were done with healthy human serum with different volume ranging from 0.1 to 5µl. Copy number/ concentration (nM) was determined from above synthetic miRNA standard graph. Biological variation in the miRNA copy number and linearity in hybridization between healthy and RB serum (n=15) was also determined for the serum volumes ranging from 0.1 to 5µL.

4.1.1.5. Fluorescent microscopy imaging & quantification of hybridized miRNA

Florescence images were taken for all the hybridized samples at 4X and 10X magnification fields using microscopy with FITC filter and were captured through an attached CCD camera. Experiments were carried out in 384 well plates; each well was incubated with 500nM of custom LNA probe, standard miR-18a template ranging from 500nM to 2500nM and unknown’s concentration of 0.1 µL (LOD) human serum in a final volume of 20 µL each. The emission intensity was measured and used to calculate miRNA copy number from the standard graph. The standard graph was obtained by calculating the area under the curve by measuring the pixel of the image using Image J software. The difference in the fluorescence intensity between healthy and RB serum has validated in (n=10) samples.
4.2 Design and validation of microRNA Bio Assay on PDMS platform (model-1)

4.2.1. Materials:

P-type wafer, (4’,100mm), PDMS (Sylgard 184) material was purchased from Dow and Corning’s company, PAV (polymeric amine) from, Glutaraldehyde was from, heparin sodium salt (Sigma-Aldrich, H3393-100KU), ATR-FTIR from M/s. Brukeroptics, Ellipsometry (JA woollam) and streptavidin from sigma Aldrich, Zeiss Axio observer Z1-Inverted Fluorescent Microscope, Biotin, Tye665, dabsyl labeled LNA probe from Exiqon (500150) (Denmark).

4.2.2. Methods:

4.2.2.1. Micro channel fabrication and PDMS preparation

The desired micro channels were produced using SU-8 resist structured silicon wafers by rapid prototyping and photo lithography techniques at MEMs facility, IIT Chennai. The micro channels were designed with dimension of 100 microns wide, 200-micron depth. PDMS silicone elastomer was mixed with curing agent in a ratio of 10:1 (w/w) and poured onto a structured silicon wafer, degassed under vacuum condition for 30 mins. PDMS was further cured at 80°C for 1hr (Figure-4.3). The PDMS device was peeled off from the wafers, and further exposed to UV radiation for immobilization procedure.
Figure 4.3. *PDMS mask:* Image A shows the blue print of PDMS mask and image B shows the casted PDMS device on master mold.

4.2.2.2. Layer by Layer method (Poly amine)-heparin and glutaraldehyde (PAV)

PDMS channels were coated with polyamine layer (PAV), glutaraldehyde (GA), Heparin and Streptavidin followed by LNA (locked nucleic acid) probe (Luo, Fu et al. 2005, Thorslund, Sanchez et al. 2005). The channels were rinsed thoroughly with deionized water. The PDMS channel was incubated with PAV (0.2 mg/mL in borate buffer-pH 9.0) for the duration of 5 min, glutaraldehyde (1%) for 15 min, heparin (0.1 mg/mL in 0.15 M NaCl (pH 7.0) for 15 min and streptavidin (0.1 mg/mL, in water) for 10min. The experiments were all performed at room temperature. After each coating step, the PDMS surface was carefully rinsed in 0.15 M NaCl. Further, biotin labeled 10μM LNA miR-18a probe was immobilized on to the PDMS surface as depicted in the Figure-4.4.
4.2.2.3. APTES method

APTES solution was prepared in 5% absolute ethanol (v/v) and applied to PDMS surface. It was then incubated for 30min at room temperature followed by washing with 96% ethanol and air dried. The prepared PDMS was stored in a clean, air tight containers. The layer coating was depicted in the Fig 4.5. The glutaraldehyde (1%) layer was further coated on PDMS as second layer. Then LNA modified molecular beacon probe was immobilized on top of the surface.
4.2.2.4. Surface coating Analysis by Infra-Red Spectra spectroscopy

The layers coated on native PDMS layers were subjected to Infra-Red spectroscopy analysis. The samples were prepared as PDMS film with different layers coated on top of it. The spectra were recorded in transmittance mode in Bruker Tenzor 27 series. The deuterated triglycine Sulfate (dTGS) detector and ZnSe beam splitter was used in the instrument for recording the spectra.

4.2.2.5. Surface coating analysis by Ellipsometry

The layers coated on PDMS film were measured for its thickness using ellipsometry (JA Woollam & Co). The spectroscopic ellipsometry data was recorded as wavelength versus psi angle for thickness measurement. Individual layer coating thickness was measured.

4.2.2.6. Immobilization of LNA microRNA probes

LNA Capture probe (complementary to miRNA sequence) was prepared at 10μM concentration in RNA dilution buffer (kit from Origene). The LNA probe specific to
target miRNA was coated with micro tips on micro channels and incubated for 1hr at room temperature under dark environment. The PDMS micro-channels were then washed with phosphate buffered saline for 3 times for 5min each. The surface modified PDMS was then air dried and used for hybridization experiments.

4.2.2.7. Hybridization of microRNA

The coated and immobilized channels were tested for hybridization with target miRNA. 10 μL to 20 μL of commercial serum spiked with miRNA-18a (10μM) was added to the channels, allowed to hybridize at room temperature. Hybridization signal was captured using fluorescent microscope and images were quantified.

4.2.2.8. Fluorescence Imaging

Fluorescence imaging was performed using Inverted Fluorescent Microscope (on a Zeiss Axio observer Z1) with a 10 X objective lens. The samples were washed thoroughly with 0.15M NaCl and dried. The samples were kept in a plastic tray and placed on the stage of the microscope and captured using Alex 488 filter. The images were taken in fluorescence, phase contrast mode. The images were analyzed using the Image J open software.

4.3 Results and Discussion (4.1. microRNA-18a sensor probe assay development)

A gradual increase in the fluorescence was observed by fluorescent microscopy for 500nM custom LNA probe hybridization with different concentration of synthetic miR-18a ranging from 500nM, 1000nM, 1500nM, 2000nM to 2500nM (Figure-4.6A) in buffer solution with image intensity pixel values using Image J software (R^2= 0.94) (Figure-4.6B). An increased fluorescence was observed for increased volume of healthy
serum such as 0.1µl, 0.2µl, 0.5µl, 1µl, 5µl with 500nM when hybridized with custom LNA-18a probe (Figure-4.6C). The difference in fluorescence intensity between healthy serum and RB serum (n=10) was found to significant (Figure-4.6D).

Figure 4.6. MicroRNA bioassay with sensor miR-18a probe:
A). Microscopic imaging of hybridization experiments of custom LNA-miR-18a probe with spiked miR-18a with known concentrations and an unknown concentration of human serum for hybridization. The known concentrations of custom LNA-miR-18a probe to spiked miR-18a was in the ratio of 500:500nM (Figure-A), 500:1000nM (Figure-B), 500:1500nM (Figure-C), 500:2000nM (Figure-D), 500:2500nM (Figure-E) and whereas 0.1µl healthy serum was incubated with 500nM of custom LNA-miR-18a probe (Figure-F), water (Figure-G), 500nM LNA probe alone (Figure-H) and FBS alone (Figure-I) was used as controls.
B). Standard graph obtained using Image J software for Figure-4.6A.
C). Microscopic images of human serum hybridized with custom LNA miR-18a probe (volume from 0.1 to 5µl both healthy and RB (Test) serum.
D). Microscopic Imaging values derived using ImageJ software was plotted as Bar Diagram, values were shown in Mean ± SD. Bar diagram shows the mean fluorescence units with standard deviation. Student unpaired t-test was applied to derive P-values. Significant difference was observed between healthy and RB samples (p<0.01).
Similarly, gradual increase in hybridization was observed with known standard concentrations of synthetic miR-18a (500nM, 1000nM, 1500nM, 2000nM and 2500nM) in buffer solution ($R^2 = 0.97$) (Figure-4.6A). There was no gradual increase in the fluorescence of LNA beacon miR-18a probe with non-target miR-129 indicating the specificity of the probe to the target miR-18a. The copy numbers of miRNA in serum samples were calculated by using the above standard graph. The spectral analysis of varying volume (0.1 to 0.5μL) of healthy serum had shown the difference in the fluorescence intensity (Figure-4.6B). Further, it was validated in fifteen healthy samples (n=15) for copy number/concentration variation (Figure-4.6C).

The copy number of miRNA in healthy serum was calculated for both microscopy and spectroscopy methods using standard curves [Figure-4.6B and Figure-4.7B]. The copy number of healthy serum found to be 349808.4±3375 copies (1399.23±13.5nM) for microscopic method (Figure-4.6C) (n=10) whereas 356743.3±18300 copies (1426.92±73nM) were obtained for spectroscopic method (n=15) (Figure-4.7B) in 0.1μl. Similarly, for RB samples microscopic method (n=10) showed RB 528815.3±9975 copies (2115.2±39.9nM) and spectroscopic method (n=15) showed 595862.3±24970 copies (2383.22±99nM) copies. Significant differences were obtained in both methods (p<0.01) (Figure-4.6D and Figure-4.7C)
Figure 4.7. MicroRNA bioassay with sensor miR-18a probe-Spectroscopic method:
A). Figure shows the graphical representation of custom LNA miR-18a hybridization in serum spiked with miR-18a. The ratio of custom LNA miR-18a to spiked serum miR-18a was in the range of 500:500nM, 500:1000nM, 500:1500nM, 500:2000nM, 500:2500nM. 0.1µl of healthy, blank, and commercial serum (FBS) were also incubated with 500nM custom LNA miR-18a probe (Standard graph shown in sub figure).

B). Fluorescent spectra of healthy serum and RB serum samples hybridized with 500nM of Custom LNA miR-18a probe. The coloured lines and a peak at 525nm indicate increase in hybridization and it was observed in the ascending order from 0.1 to 5µl volume (0.1, 0.2, 0.5, 1, 5µl).

C). Validation of LNA probe hybridization in healthy (Control=15) and RB serum (n=15) using spectroscopy method. Bar diagram shows the mean fluorescence units with standard deviation. Student unpaired t-test was applied to derive p-values. Significant difference was observed between healthy and RB samples (p<0.01).
4.4. FTIR spectroscopy (PAV Method) peak analysis showed coatings on PDMS

It was evident from the peak analysis of PAV method that the coated layers had expected functional groups present in each coating on PDMS surface, which clearly indicates that the surface was modified as per the earlier protocol (S. Thorslund, et al., 2005) (Figure-4.8). Qualitative information of respective functional groups of each layer spectrum was tabulated (Table-4.1). It was observed that the properties of the native PDMS was being reduced because of masking after each coated layer, (Table-4.1). The native PDMS had Si-O-Si backbone and functional –CH3 side chains. The peaks representing different CH3 vibrational modes of PDMS were decreased gradually on addition of different layers on PDMS in PAV method (Figure-4.8) representing adsorption process over PDMS. The peaks corresponding to CH3 was observed at 3380, 1253(cm⁻¹). Also, other peaks at 2962 cm⁻¹ (CH stretching), 1413 cm⁻¹ (CH3 asymmetric), 1257 cm⁻¹ (Si-CH3), 1010 cm⁻¹ represents (Si-O-Si stretching), 790 cm⁻¹ (Si-C stretching and CH3 rocking) vibrations were present in the backbone of PDMS.
Figure 4.8. Schematic representation of different layers coated on PDMS: PDMS coated with poly vinyl amine followed by bi functional cross linker glutaraldehyde, heparin, avidin and miRNA-18a probe.

Primary layer of polymeric amine coating revealed a clear NH peak at 3400 cm$^{-1}$ in the spectrum which was absent in native PDMS (Figure-4.9). This shows that the PDMS layer was modified by the addition of PAV solution. The other changes in the spectrum for heparin and streptavidin layers was noticed majorly at two regions; NH peaks at 3400 cm$^{-1}$ and amide I (C=O) peaks at 1642 cm$^{-1}$ (Figure-4.10). The increase in amide (NH) vibration was more in coated layers with a sharp peak. Finally, the increase in the amount of NH and CO bond observed after streptavidin coating increases the wettability of the PDMS surface. The resultant PDMS was used for probe immobilization. The results on amount of adsorption by different layers were given in the Table-4.1.
<table>
<thead>
<tr>
<th>Expected Vibration (cm⁻¹)</th>
<th>PAV Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-H stretching at 3380</td>
<td>Yes</td>
</tr>
<tr>
<td>C-H Sym. stretching at 2962</td>
<td>Yes</td>
</tr>
<tr>
<td>C=Н Stretching at 2155</td>
<td>Yes</td>
</tr>
<tr>
<td>C=O, C=N Stretching at 1640</td>
<td>Yes</td>
</tr>
<tr>
<td>CH3 Sym. bending at 1257</td>
<td>Yes</td>
</tr>
<tr>
<td>RNA ribose C-O stretching at 1013</td>
<td>Yes</td>
</tr>
<tr>
<td>Purine &amp; Pyrimidine residue at 788</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 4.1. *PAV Method*: FTIR analysis for each layer coated on PDMS surface in PAV method
Figure 4.9. FTIR analysis of PAV method: FTIR analysis peaks are shown for each layer in different colors. The major peak vibrations are highlighted with pointed arrows. The color codes for LNA probe, avidin, heparin (Hep), glutaraldehyde (GA) and polymeric amines (PAV) are given below.

4.5. FTIR analysis confirmed coating on PDMS using APTES Method

The APTES coating on PDMS was analyzed using Infra-Red spectroscopy (Figure-4.9). The functional groups of native PDMS such as CH3, Si-C-Si, Si-O-Si vibrations along with NH vibrations were observed (Figure-4.10.2). Primary amine (-NH), secondary
amine (-NH₂) were the predominant changes in different layer coating. The results revealed that the NH symmetric stretching had increased two-fold on APTES method when compared to PAV Method. Otherwise, other notable vibrations such as C-N stretching, C-H symmetry stretching, was less compared to PAV Method. The increased N-H stretching effect was reflected on LNA immobilization where C-O (RNA) stretching vibration had increased in two-fold. This was well corroborated by the two-fold increase in purine pyrimidine vibrations at 788cm⁻¹. The results were given in the Table-4.1.

**Figure 4.10.1.** Schematic representation of different layers coated on PDMS: PDMS coated with APTES followed by bi-functional cross linker glutaraldehyde, and miRNA-18a probe. Apart of image source taken from chuah YJ et al (Chuah, Kuddannaya et al. 2015)
Table 4.2. *APTES method*: FTIR analysis for each layer coated on PDMS surface in APTES method

<table>
<thead>
<tr>
<th>Expected Vibration (cm⁻¹)</th>
<th>APTES</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-H stretching at 3380</td>
<td>Yes</td>
</tr>
<tr>
<td>C-H Sym. stretching at 2962</td>
<td>Yes</td>
</tr>
<tr>
<td>C=N Stretcing at 2155</td>
<td>Yes</td>
</tr>
<tr>
<td>C=O, imine C=N Stretcing at 1640</td>
<td>Yes</td>
</tr>
<tr>
<td>–Si–CH₂–bending 1408</td>
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</tr>
<tr>
<td>CH₃ Sym. bending at 1257</td>
<td>Yes</td>
</tr>
<tr>
<td>RNA ribose C-O stretching at 1013</td>
<td>Yes</td>
</tr>
<tr>
<td>Purine &amp; Pyrimidine residues at 788</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Figure 4.10.2. FTIR analysis of APTES method: Peaks are shown for each layer and major peaks are highlighted with pointed arrow. The color codes for each layer APTES, glutaraldehyde and immobilized LNA probes are given.

4.6. Increase in thickness of coated layers on native PDMS

The thickness of various layers of PAV methods on PDMS were measured by ellipsometry. The spectroscopic measurement further confirmed coatings on PDMS and the thickness of different layers deposited on PDMS showed an overall thickness of 211 nm (PAV + GA+ Heparin+ Avidin) (Figure-4.10.3).
Figure 4.10.3. *PDMS coatings ellipsometry*: Image shows that increase in thickness of layers (PAV, GA, Heparin, and Streptavidin) was shown in the table. Units are represented in angstroms.

### Table

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>VALUE</th>
<th>ERROR BAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>k_{0}</td>
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<td></td>
</tr>
<tr>
<td>Roughness (\AA)</td>
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</tr>
<tr>
<td>Thickness # 1 (\AA)</td>
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<td></td>
</tr>
<tr>
<td>a</td>
<td>1.527±0.005</td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>-0.122±0.002</td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>0.015\pm0.0007</td>
<td></td>
</tr>
<tr>
<td>n of Cauchy film @ 632.8 nm</td>
<td>1.420±0.006</td>
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</tr>
<tr>
<td>k of Cauchy film @ 632.8 nm</td>
<td>0.00077085</td>
<td></td>
</tr>
</tbody>
</table>

4.7. **Fluorescence microscopy for PDMS surface immobilization**

The immobilized probes in two different methods on PDMS was evaluated for its hybridization with its specific target miRNA. Fluorescence intensity of hybridized miRNA was measured for both APTES method and PAV methods using fluorescent microscopy (Figure-4.11.1). The fluorescence was measured using FITC filter. The APTES method showed increase in fluorescence intensity than PAV Method. The intensity was measured using image J software and it revealed an increase in average fluorescence intensity from 88.4 (a.u) to 774.7 (a.u). This tenfold increase was observed (under 10X view) as an average of 3 different experiments.
**Figure 4.11.1. Hybridization on PDMS channels:** A) Uncoated PDMS slab with circuited channels. B) Native PDMS having little/no background in fluorescence imaging. C) Hybridization on APTES coated channels showing high green color. D) Hybridization of PDMS on PAV coated PDMS. Green color represents the fluorescence observed under microscopy.

**4.8. UV-Visible spectra of probe immobilization**

The probe immobilization on both PAV and APTES method was being compared using UV Visible spectra (Biospec Nano; Shimadzu, Japan). Before and after immobilization, the LNA probe concentration was measured using UV Visible spectroscopy. In APTES method, the initial stock concentration of 2.56 ng/μL solution was drastically reduced to 0.07 ng/μL after immobilization where as in PAV method, initial stock concentration of
1.79 ng/μL has reduced to 1.04 ng/μL only. The experiments were repeated for three times and results revealed that APTES method had absorbed more concentration of probe (3 fold) than PAV method (Table-4.2).

Table 4.3. Comparison of absorbance readings of Probe solution on APTES, PAV coated method before and after coating layers

<table>
<thead>
<tr>
<th></th>
<th>APTES Coating (ng/μl)</th>
<th>PAV Coating (ng/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Probe</td>
<td>2.56 (1.79)</td>
<td>0.07 (1.01)</td>
</tr>
</tbody>
</table>

4.9. Discussion:

4.9.1. MicroRNA Bioassay

In this study, we have used LNA miR-18a probe directly to test the serum samples for microRNA-18a detection. Finally, we could detect miRNA directly from a minimum volume of 0.1μL serum despite serum complexities such as interference coagulation factors and other proteins. In contrast, the conventional methods require an usual volume of 200-500μL serum for extraction of microRNAs (Li and Kowdley 2012, Koberle, Kronenberger et al. 2013). Our method showed that miR-18a hybridization was more specific and there was no enhancement fluorescence signal observed in controls upon incubation of miR-18a with miR-129 and in the fetal bovine serum which may contains bovine originated non-specific microRNAs (Chen, Ba et al. 2008). The copy number
determination method can be performed in 384 well plates to enable this method to be as robust and economic for quantifying miRNA in serum samples. It was very evident from the results that the copy number detected by microscopic and spectroscopy methods was more in number indicating that it is more sensitive than qRT-PCR methods. The relative fold change difference between healthy and RB individuals was same in spectroscopy (n=15) and microscopy (n=10) methods which showed copy number ≥1-fold difference. Although the both methods showed uniform relative difference between healthy and RB, the spectroscopy method seems to be more sensitive in higher miRNA copy number determination compared to microscopic method. This suggest that the labelled LNA method is robust for quantification of miRNAs.

The usual lower yield of microRNA copy number from serum or plasma (Brase, Wuttig et al. 2010, Kroh, Parkin et al. 2010) by qRT-PCR is due to multiple steps involved in the process. This LNA molecular beacon direct hybridization approach can overcome the technical difficulties in improper copy number estimations (Blondal, Jensby Nielsen et al. 2013), which will be advantage for diagnostic purpose. Moreover, in clinical settings, it is important to measure intact microRNAs from biological samples to find out the actual number of microRNAs without any processing artefacts. Thus, LNA molecular beacon approach is very rapid and can detect microRNA in intact 0.1 µL healthy serum samples within 30 min. Thus, this approach may open new avenues for developing point of care device platforms.
4.9.2. Validation of miRNA-18a sensor probe assay on PDMS device

The PDMS is the most versatile material being used in fabrication of microfluidics based devices for biological assay. Several methods have tried to overcome the hydrophobic nature of PDMS for immobilization of Biomolecules. S. Thursland et al., 2005 (Thorslund, Sanchez et al. 2005) prepared a heamocompatible PDMS microchannels using multilayer PAV-GA-Heparin coating method which greatly increased the bio wettability of human plasma or blood samples. Mani K Naresh etal had used APTES for immobilizing concotamer DNA molecules on PDMS surface (Mani, Rudiuk et al. 2013). Almutairi et al had evaluated a process of grafting PDMS surface with poly ethylene glycol for immobilizing proteins on PDMS surface (Almutairi, Ren et al. 2012).

In this objective, we have compared existing PAV and APTES standard procedures for immobilizing microRNAs on PDMS. Both methods were used in microfluidics based devices for biological assays. There were earlier reports on chemical or physical means of immobilizing biological molecules on PDMS surface. The layers coated were confirmed using Ellipsometry studies. In the PAV method, the initial coating of poly amine layer makes the PDMS surface hydrophilic and leaves amine group for next layer crosslinking. The second layer, glutaraldehyde cross linked to amine group of heparin molecules and finally to avidin molecules by affinity binding. An affinity between biotin labelled LNA probe and coated avidin makes the probe layer for capturing target molecules in serum/plasma. Similarly, the amine group of APTES and ‘-CHO’ group biotin labelled
LNA modified beacon probe were cross-linked with homo bi functional glutaraldehyde layer.

Infra-Red spectra analysis of both methods revealed the presence of respective functional group. The vibrations of amino group after heparin and avidin coating in PAV method was compared to amino group exposure on APTES method. The APTES method showed more absorption in infra-red spectroscopy than PAV (Figure 4.11.1) method. The Ellipsometry analysis revealed that around 210 nm thickness of layers were coated on PDMS using PAV method. Both methods could capture serum miRNAs on our fabricated PDMS platform. Our method has an advantage over cost associated PEGylated method of protein immobilizations (Moustafa, Gadepalli et al. 2014).

Further, we have evaluated immobilized PDMS for hybridization efficiency with specific miRNA. Moreover, the stability of hybridized and immobilized probes was found to be stable for more than two weeks. Based on the above analysis, the APTES method was found to be more suitable for immobilizing capturing probe and hybridization efficiency. There was 8-fold high in fluorescence intensity. Moreover, the minimum number of steps involved APTES method reduces the technical problems associated with coating procedure and other washing steps.

4.10. Conclusion

Although the APTES and PAV methods are widely used and successful for biomolecule immobilizations, but in our study the APTES method gave promising result. So APTES
method is efficient in modifying PDMS surface as well as for capturing or immobilizing more nucleic acid probes in smaller surface areas of PDMS channels. Hence, APTES method can be more advantageous to immobilization-based microRNA assays in clinical application.

4.11. Design and fabrication of PMMA microfluidic platform (model-2)

Microfluidic design simulated in comsole software and final design dimensions were adjusted in autodcad by our bioengineering team. As shown in Figure-4.11.2, the design consists of three inlets ports and in which two inlets for blood serum injections of healthy and RB cancer individuals. A middle inlet was used for miRNA-18a sensor probe injection. The channels join at one point and continuous as a serpentine zigzag channel which can acts as a mixer and finally ends with a reservoir chamber. The overall dimension of the device was adjusted to 2.5cmX 3.6cm and total channel holding capacity of 12.5μL. The micro mixing fluidic device was prepared with PMMA material. Final fabrication of PMMA devices with the above-mentioned parameters were manufactured at Achira labs, Bangalore.
Figure 4.11.2. *PMMA design outlay:* Micro mixing model shows three inlet ports and two collection or outlet chambers. The middle Zigzag design is the mixer which enhances the mixing of fluid in the channel and increases the fluorescence. A) Design showing inlets, channels, and outlet ports. B) Design A was photo printed (mask) for fabrication of PMMA device.


The fabricated microfluidic prototype device was tested initially with synthetic microRNA-18a for the designed sensor probe. Before the miRNA-probe injections, the device mixing was tested using simple colored food dyes. The experiment performed several times to ensure that mixing should occur in the channel without any obstructions. Later, 500nM concentration of probe and 250nM of synthetic miRNA injected into
channels using micro syringe pumps and fluorescence increase was captured using microscope.

4.13. Results and Discussion:

4.13.1. Light microscopy observation for coloured dyes mixing experiment of PMMA device

PMMA Microfluidic device was connected to micro syringe pumps through peak tubing and graduated 1ml sample syringes under controlled flow rate (0.1μL/sec). Two different dyes passed through two inlets ports mixed and gave completely a different color at the end of channel. The gradual change in color was observed in a set of experiments conducted at IIT Chennai (Figure 4.11.3) and Achira Labs, Bangalore (Figure 4.11.4). These results indicated that device can be used for actual microRNA hybridization testing’s.

![Figure 4.11.3. Dye mixing experiment conducted at IIT Chennai: PMMA device connected to micro syringe pumps and microscope for direct monitoring.](image-url)
Figure 4.11.4. Dye mixing experiment conducted at Achira Labs, Bangalore. PMMA device connected to portable platforms which holds fine peak tubing and small volume sample reservoirs.

4.13.2. Fluorescence microscopy observation for PMMA device

Passing of 500nM of probe and 250nM of synthetic miRNA into the channels increased the fluorescence from starting region to ending region (Figure-4.11.4). Fluorescent intensity graph taken at starting region of channel mixer and ending region shows increase in fluorescence units in control serum and RB cancer serum. The increase in fluorescence is more for RB than control which indicates the microRNA levels are more in cancer that results in increase in hybridization and its fluorescence (Figure-4.11.5 and 4.11.6).
Figure 4.11.5. MicroRNA Hybridization in PMMA channels: miR-18a probe and serum passed into the PMMA channels using micro syringe pump. Gradual increase in fluorescence was observed from starting channel entry to the end of collection chamber. Green color shows the fluorescence captured under microscope.

Figure 4.11.6. MicroRNA Hybridization in PMMA channels - Microscopic spectral data-1: Fluorescent spectral graph taken for PMMA microfluidic mixing using fluorescent microscopy software. Initial fluorescence hybridization readings of Control and RB serum readings were marked on Y-axis.
4.14. Conclusion:

We have developed two cost effective models for fabricating and modifying PMMA and PDMS microfluidic devices. Although the both methods are inexpensive and highly efficient but there are certain limitations for each type. Both the methods are cheaper compared to glass made chips. Both the methods can integrate into portable and disposable point of care devices. The microRNA sensor probe is sensitive enough to discriminate cancer from non-cancer serum samples and further this basic platform technology can be applied to any other miRNA detection in any diseases or cancers. Here, the presented methods are meant for proof of concept, purely just prototypes. A further validation of these PDMS/PMMA devices with clinical samples on a portable handheld reader may help for developing a successful point of care devices and for commercial use.