In situ detection of transrenal gene mutations without DNA isolation and amplification using Array Piezoelectric Plate Sensor (PEPS).

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Dedications

Dedicated to:

Elçin Elgün Kırımlı
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Abstract
In situ detection of transrenal gene mutations without DNA isolation and amplification using Array Piezoelectric Plate Sensor (PEPS).
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Low molecular weight (LMW) DNA fragments from distant organs can pass through kidneys and exist in urine as ”transrenal DNA”. Transrenal DNA provides a noninvasive means to access cell-free DNAs from distant organs such as liver, pancreas, colon that are otherwise difficult to obtain. Shih and Shih’s laboratory at Drexel has developed a highly sensitive piezoelectric plate sensor (PEPS) based on 8-μm thick highly piezoelectric lead magnesium niobate-lead titanate (PMN-PT) freestanding layer. For detection, receptor specific to a target analyte is immobilized on the PEPS. Binding of the target analyte to the receptor on the PEPS surface decreases its resonance frequency. In-situ, label-free detection of the target analyte is achieved by monitoring the PEPS resonance frequency shift. The detection resonance frequency shift of PEPS is enhanced more than 1000 times due to the PMN-PT layer’s capability to change its crystalline orientation upon analyte binding. As a result, PEPS has exhibited unprecedented attoM (aM) sensitivity in label-free detection of single-stranded DNA.

The purpose of this study was to further develop PEPS as a tool for rapid (in < 40 min), label-free, multiplexed and highly sensitive detection of transrenal gene mutations of distant organs in urine. 1762T/1764A double mutation of hepatitis B virus (HBV), a risk factor associated with hepatocellular carcinoma (HCC) and Kras point mutation at codon 12, a biomarker present in 50% of colorectal carcinomas (CRC) will be the model transrenal gene mutations in this study. To achieve the goal, a signal processing algorithm was devel-
oped to reduce noise in the resonance spectrum to increase the resolution of the detection resonance frequency shift. A flow system was implemented with temperature-controlled compartments to effectively dehybridize the naturally double-stranded target DNA so that the target DNA can be detected without the need for DNA isolation, concentration, and amplification. The use of temperature, flow speed and locked nucleic acid (LNA) were exploited to enhance the detection specificity of the mutated DNA against the wild type and an array of PEPS is used to detect multiple target DNAs simultaneously. Results indicate that PEPS could directly detect Kras point mutations in urine with 0.1 aM (60 copies/ml) sensitivity and 1:1000 mutant to wild type specificity without labeling, DNA isolation and amplification.
1. Introduction

1.1 Structure of DNA

A deoxyribonucleic acid (DNA) molecule is composed of two polynucleotide chains each of which contains four different types of nucleotides. Each of these chains is also called a "strand" of DNA. Each nucleotide contains base portions through which two strands are attached one another by hydrogen bonding. Four bases contained in nucleotide of a single DNA strand may be either Adenine (A), Cytosine (C), Guanine (G) or Thymine (T) (Figure 1.1).
Figure 1.1: Complementary base pairs making up the double helix are shown. Adenine hybridizes with Thymine and Guanine with Cytosine. GC pairs hybridize with 3 hydrogen bonds whereas AT pairs hybridizes with two and antiparallel backbones of complementary strands are also shown. (Image adopted from [1]).

The nucleotides in a DNA strand are covalently attached to each other through the sugars and phosphates which creates the "backbone" of a DNA strand. Backbone of a DNA strand makes the structure negatively charged due to abundance of negatively charged and
perfectly aligned phosphate groups (Figure 1.2).

Figure 1.2: DNA and its constituent substructures are shown on the top left. They covalently bond with each other in a sequence to form the single stranded DNA shown on top right. In the bottom left diagram the DNA is shown straightened out for ease of understanding however the real DNA structure which is shown on the right is twisted to form the double helix. (Image adopted from [1]).
A DNA strand is also polarized in the sense that one end of a DNA chain ends with a 5’ phosphate and the other end terminates with a 3’ hydroxyl group as each nucleotide in the sequences of the strand is covalently attached to the next from these groups. This polarity is indicated by abbreviating the ends of a DNA strand as ”5’end” and ”3’end”. The three dimensional structure of a double stranded DNA is called ”the double helix” (Figure 1.3). The helical structure of the double stranded DNA arises from the structural features of its polynucleotide chains held together by hydrogen bonding. The underlying mechanism can best be described by the hydrogen bonding taking place between the bases. The bases of the nucleotides are always on the inside of the helical structure whereas the sugar-phosphate back bone faces outwards. Moreover, in any hydrogen bonding event, a two-ring base (also called ”Purines”) is paired with a single-ring base (also called ”Pyrimidines”) as shown in Figure 1.3.
Figure 1.3: (A) A space-filling model of helical double stranded DNA is shown on the left. This 1.5 turn (10.4 base pairs per turn) model shows the major and minor grooves in the structure. (B) A short section of double stranded DNA is also illustrated on the right to show the real bond patterns between nucleotides. 5’ and 3’ ends can also be shown together with the phosphate backbone. (Image adopted from [1]).

In order to fold into a structure which is energetically favorable, the length of each hydrogen bond is minimized by winding nucleotides around each other to form a helical structure which makes a complete turn in every ten base pairs. In order for the hydrogen bonding to take place each strand should also antiparallel. Thus if one strand is in a direction of 5’ to 3’ ends the complementary should be in 3’ to 5’ direction. The alignment
of two perfectly complementary strands and formation of hydrogen bonding in between to generate a helical double stranded DNA (dsDNA) is also called a "hybridization" event, and the reverse process is often called "de-hybridization" or "denaturation".

1.1.1 Hybridization of Single Stranded and Denaturation of Double Stranded DNA Fragments

In cells, during replication or other processes such as transcription, two strands of the double helix is separated from each other, if not all, in a local region. This can only be accomplished by disrupting the hydrogen bonds between base pairs. This process can be done in the laboratory by heating a solution of DNA or by adding acid or alkali to ionize its bases.

When heat treated, the dissociation of one strand from the other occurs precipitously at a certain temperature range as shown in Figure 1.4. The melting temperature ($T_m$) of DNA is defined as the temperature at which half the helical structure is lost. The melting temperature of nucleic acids is usually monitored by measuring their absorption of light at 260 nm because stacked bases absorb less ultraviolet light than unstacked ones (hypochromism). When the temperature is lower than $T_m$, complementary sequences start forming the double helix by a process called renaturation or reannealing. Other than temperature, sequence of the strands (G-C content) and the sequence length is the major factors determining how fast this process would occur.
Figure 1.4: Hypochromism investigated by spectroscopy. (A) Single-stranded DNA absorbs more light with respect to double stranded helical DNA. (B) The absorbance increases as the double stranded DNA denatures to form single stranded DNA which can be monitored in real time to determine the melting temperature $T_m$ of any DNA sequence. (Image adopted from [2]).

1.2 Cell-free Nucleic Acids

The existence of cell-free nucleic acids (cf-NA) is first discovered in 1948 by Metais and Mandel [6]. However, unlike what one would expect, the importance of this discovery is understood rather lately, as most of the reviews on the subjects are published in the last 3 years. The spotlight could only be turned on this phenomena by 1994, with the discovery of Ras gene fragments([7, 8]) in the blood of cancer patients. Moreover not only DNA
but also mRNA and miRNA fragments are shown to be in high concentrations in blood of cancer patients. Different genetic and epigenetic signatures then shown to be associated with cfNAs.

The formation of cfNA in blood can be stemmed from different pathways. Apoptosis (a normal, genetically regulated process leading to the death of cells and triggered by the presence or absence of certain stimuli, as DNA damage.) and necrosis (the death of one or more cells in the body, usually within a localized area, as from an interruption of the blood supply to that part) can cause the disruption of the cell membrane, hence release of genetic material into the blood stream. Both of these occurrences are common in all all types of cancer. Secretion is another way cfNA forms. Phagocytosis of the necrotic and apoptotic cells by macrophages or other scavenger cells have been shown in in vitro cell cultures and secretion of digested DNA from macrophages is studied before [9]. However it has been shown that there are not enough circulating cells to justify the amount of DNA found in the plasma [10] by lysis alone. It would have to be assumed that 10,000 tumor cells per mL are circulating in the blood to account for the DNA found in breast cancer patients which is far more than has ever been found in any patient [11]. It has been proven afterwards that cellular nuclear acid fragments can also be actively released or secreted [12], as shown in Figure 1.5.
Figure 1.5: Not only mutations but also methylations, DNA integrity, microsatellite alterations and viral DNA has been shown to be associated with cf-DNA in blood. Both single stranded and double stranded DNA can be released into the bloodstream through different ways as discussed. Moreover individual tumor type can release more than one form of cf-DNA. (Image adopted from [3]).

Although the size and quantity of the total DNA fragments in blood of cancer patients varies a lot. It has been estimated that, for a tumour that weighs 100 g, which corresponds to $3 \times 10^{10}$ tumour cells, up to 3.3% of tumour DNA may enter the blood every day [13]. On average, the size of this DNA varies between small fragments of 70 to 200 base pairs and large fragments of approximately 21 kilobases.
The composition of cfNA not only consists of DNA derived from cancer cells but also from healthy cells as well. The ratio of DNA derived from cancer and normal cells depends on the cancer type and mostly on the stage of the cancer and size of the tumor as well [3]. Moreover nucleic acids also have a half-life. They are generally cleared from the bloodstream in 15 minutes to several hours [14]. The structure of the DNA is also a very important factor affecting the half-life of certain fragments. For instance double stranded DNA has a significantly higher half-life with respect to single stranded one, as shown by Bendich et. al. [15]. Moreover, circular DNA released into the bloodstream by viruses may also have a longer half-life than the linear one as postulated in the same study.

1.2.1 Concentration of Cell Free Nucleic Acids

It has been shown by various studies that amount of cf-DNA in cancer patients is higher than normal subjects, however, the amount of cf-DNA in blood or plasma from both groups varies considerably. Although cancer patients have higher cfDNA levels than healthy control donors, the concentrations of overall cfDNA vary considerably in plasma or serum samples in both groups [16–18]. For example, the average cf-DNA in blood of cancer patients is 180 ng per ml, however this average is calculated from a range from 0 to over 1000 ng per ml. [19–22] Average concentration of cf-DNA in blood of normal subjects is approximately 30 ng per ml, an average of a population with cf-DNA concentration ranging from 0-100 ng per ml. [14]. However these results are inconclusive due to the fact that DNA is isolated using different methods in different studies and sizes of the populations used in these studies are usually not suitable to perform any powerful statistical tests. [3]
cf-DNA not only consists of genomic DNA, but also of mitochondrial DNA, and the level at which these two types of DNA is affected by the existence of a cancer varies as shown by diverging results [3].

### 1.3 Transrenal DNA

Transrenal DNA (Tr-DNA) was first discovered in 2000 by Botezatu et. al. [23]. Labeled DNA has been injected into the peritoneal cavity of mice and the distribution of radioactivity was analyzed. Fragments of 150-160 bp. were detected in urine. This is also confirmed in humans as well. For example, DNA from Y chromosome was detected in urine of women pregnant with male fetuses. [24–26]. This discovery lead the way for possibility of prenatal diagnosis from transrenal DNA.

Another study demonstrated that male derived DNA fragments were present in the urine of female subjects transfused with male blood [27]. Association with cancer on the other hand was shown when mutant kRas gene fragments were detected in urine of colorectal and pancreatic cancer patients who had tumors with the same exact kind of mutations [23]. This finding was later confirmed and further investigated and confirmed in precancerous tissue by Su et. al. [28].

As expected (judging by the studies on cf-DNA) Tr-DNA also includes DNA not only from cancerous tissue but also from infections caused by pathogens. For example Epstein-Barr virus can be excreted into urine in nasopharyngeal carcinoma patients and the fraction
of excretion is negatively associated with the size of the DNA molecules [29]. Moreover Tuberculosis can be diagnosed by detecting mycobacterial DNA in urine by nucleic acid amplification methods [4]. Figure 1.6 illustrates how the cf-DNA in blood is filtered through the kidney and form Tr-DNA which can then be tested for diagnosis.
Figure 1.6: Transrenal DNA production in a patient with pulmonary tuberculosis is shown. M tuberculosis bacilli from infective foci in the lungs are destroyed by the immune response releasing cell-free nucleic acids in plasma. Filtered cf-DNA forms Tr-DNA which can then be detected. (Image adopted from [4]).
DNA fragments derived from tumors of different cancer types were also found in urine. A study on kRas gene fragments associated with colorectal and pancreatic cancer has already been discussed above. A mutation, a G:C to T:A transversion at codon 249 (249T) of p53 gene found to be associated with Hepatocellular Carcinoma (HCC), is detected in urine of patients with HCC. [30] Another important region leading to the same cancer type is induced by Hepatitis B Virus (HBV). 1762T/1764A double mutation of HBV is found in the urine of patients with HBV induced HCC [31]. Not only mutations, but also other epigenetic factors associated with cancer is studied in Tr-DNA. For example, hypermethylated vimentin gene promoter fragments were detected in urine of patients with CRC [32].

To summarize, DNA fragments of dying cells can be released into the blood stream by several mechanisms such as apoptosis, necrosis or secretion by active transport, and some portion (especially low molecular weight (150-250 bp.)) of this cf-DNA can pass through kidney barrier to form Tr-DNA.

1.4 DNA Biosensing

Although there are many different biosensor platforms used to detect DNA, most important and study extensively by others are Polymerase Chain Reaction (PCR) dependent, Quartz Crystal Microbalance (QCM), Surface Plasmon Resonance (SPR), nanostructure associated (such as carbon nanotubes), Piezoelectric microcantilever (PEMS), Atomic Force Microscopy (AFM), and Electrochemical biosensors. Because PCR is the ”Golden Standard” of any detection scheme involving DNA markers, PCR will be discussed in detail and examples from other platforms will be given including their advantages and disadvantages.
1.4.1 Polymerase Chain Reaction

PCR can be described in one sentence as in vitro DNA polymerization of a target DNA fragment which is usually called "amplicon". As the name implies ampicon is the sequence which is "amplified" through making copies of it, in "cycle" of polymerizations. One cycle of PCR includes 3 main stages. "Denaturation","Primer Annealing“ and "Polymerization/Elongation/Primer Extension". These stages separated from each other by a sudden change in the temperature, which is required to increase the rate at which each reaction occurs.

In the denaturation part of the cycle, first double stranded amplicon is denatured by heat treatment into its constituent complementary single stranded chains as shown in Figure 1.7

![Figure 1.7: Illustration of Denaturation part of a cycle of PCR reaction. (Image adopted from www.btci.org).](image)

Then in the primer annealing part of the cycle, two primers required for the enzyme Taq. Polymerase (or subunits of this enzyme in different types of PCR) anneals on each strand. Primers are designed to be complementary to the denatured sister strands from their
3’ ends, because Taq polymerase has only a 5’ to 3’ polymerization capability as shown in Figure 1.8.

Figure 1.8: Illustration of annealing part of a cycle of PCR reaction. Forward and reverse primers anneal on complementary strands of the double stranded amplicon. (Image adopted from www.btc.org).

In the extension or elongation part of the cycle, Taq polymerase enzyme binds on the -primer annealed- double stranded portion of each strand, and elongates or extends the primers with the deoxyribonucleotidetriphosphates (dNTPs) dissolved in the solution as you can see in Figure 1.9.
Figure 1.9: Illustration of extension part of a cycle of PCR reaction. Forward and reverse primers anneal on complementary strands of the double stranded amplicon. (Image adopted from www.btci.org).

Cycles of PCR follow each other until a predetermined number of cycles is reached for amplification. As number of cycles increases the number of amplicon copied increases exponentially. Thus a chain of cycles can potentially produce millions of copies of the region intended for amplification as illustrated in Figure 1.10.
The reason why PCR is considered the standard technique for DNA detection is its unprecedented sensitivity. PCR has been shown previously to be capable of amplifying single DNA molecules in a reaction chamber [33–37]. All of the studies described above involving cf-DNA and Tr-DNA, involved PCR in one form or another. However PCR also suffers from some issues which is still being addressed by researchers.
One of the main disadvantages of PCR is its requirement for DNA isolation. Like most other reactions involving proteins and enzymes, contamination inhibits the reaction catalyzed by the enzyme, in this case Taq polymerase. Moreover, in the case of isolation of Tr-DNA from urine for PCR, things are even more complicated. It has been shown by electrophoresis that, DNA isolated from urine reveals two major fractions: High-molecular-weight DNA and Low-molecular-weight DNA. High molecular weight DNA is majorly DNA from intact cells including infectious disease agents as well. On the other hand low molecular weight DNA is usually 150-200 bp in length and constitutes the Tr-DNA [23]. This finding is further supported by a study on mutant kRas DNA fragments of approximately the same length, in urines of patients with colorectal tumors [28]. Most of the current isolation kits however are not designed to isolate such low molecular weight DNA, they are designed to isolate nuclear DNA from intact cells with high molecular weights (>350 bp.), and there is a need for a simple, cost effective method for isolation of small DNA fragments from body fluids [38].

Another disadvantage when working with PCR is inhibition by co-isolated factors. Not only nucleases but also other proteins may interfere with the reaction catalyzed by PCR. Moreover amplicon size also limits the use of PCR on urine. As it is described before, the amplicon by definition should be smaller than the DNA fragments to be analyzed. However in reality the fragments in urine, usually rarely randomly fragmented, meaning a target sequence usually resides in fragments cleaved differently. For example if there are 160 copies of 160 bp DNA fragments including a target sequence of interest, only 70 of them will be amplified with a 90 bp amplicons or only 40 of them will be amplified with a 120 bp amplicons. The rest of the copies will not have full amplicons inside [39]. This can be
generalized to deduce that as the amplicon size increases the sensitivity of detection from Tr-DNA by PCR drops significantly. It has been claimed by the studies of Umansky et. al. that the sensitivity of the PCR reaction for detecting the presence of Tr-DNA in urine specimens falls drastically as the amplicon size exceeds 150 bp. and in all experiments, the sensitivity was reduced to zero with amplicons of more than 300 bp. [38].

1.4.2 Other Platforms

For disease screening and personalized medicine, the ability to rapidly detect multiple genetic markers and examine the genetic profile of the disease at low cost is greatly needed. Current genetic detection technologies under development rely on fluorescence [40], quartz crystal microbalance (QCM) [41, 42] electrochemical [43] binding to nano-metal particles [44], surface plasmon resonance (SPR) [45], silicon-based microcantilever sensor as well as piezoelectric microcantilever sensor [46, 47]. For DNA detection, QCM exhibited a concentration sensitivity of 0.1 fM [41, 48, 49]. Direct conductivity measurement of metal nanoparticles exhibited a concentration sensitivity of 500 fM [43]. The SPR exhibits concentration sensitivity of ∼ 1 fM [45, 50]. The electrochemical methods also exhibit concentration sensitivity on the order of 1 aM [42, 51] Nanowires [52] and nanotubes [53–56]; exhibit concentration sensitivity ranging from 100 fM to 0.2 fM. Microcantilevers coupled with nano-metal particles exhibited 0.01 nM concentration sensitivity [57]. Although many of these methods such as QCM, SPR, silicon based microcantilever sensor as well as lead zirconate titanate (PZT) piezoelectric microcantilever sensor [46, 47] are label-free, the sensitivity is still many orders of magnitude away from the attomolar requirement. Similarly, the $10^{16}$ M sensitivity achieved by magnetic beads isolation coupled with electro-
chemical enhancement was not sufficient [58]. Although nano-scale mechanical imaging by atomic force microscopy (AFM) can differentiate unhybridized single-stranded DNAs (ssDNAs) from hybridized double-stranded DNAs (dsDNAs) at attomolar level it requires sophisticated instrument such as AFM [59]. Although several recent studies showed attomolar sensitivity detection, they were not label-free. For example, carbon nanotubes exhibited attomolar sensitivity using streptavidin horseradish peroxidase labeling for signal amplification [60]. Electrochemical biosensor involving magnetic beads exhibited attomolar sensitivity using target DNA biotinylation [61] or electrocatalytic amplification [62]. GaN nanowire based extended-gate field-effect-transistor exhibited attomolar realtime sensitivity [63] requiring the dilution of the buffer more than 100 times, indicating that the sensitivity before dilution was only 0.1 fM. Table 1.1 at the end of the chapter compares advantages and disadvantages of these methods and indicates the limit of detection of each method.
Table 1.1: Comparison of other DNA biosensing platforms

<table>
<thead>
<tr>
<th>Method</th>
<th>Sensitivity</th>
<th>Disadvantages</th>
<th>Advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quartz Crystal Microbalance</td>
<td>0.1 fM [48,49]</td>
<td>Low sensitivity</td>
<td>Low Cost [64]</td>
</tr>
<tr>
<td></td>
<td>Time Consuming</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface Plasmon Resonance (SPR)</td>
<td>1 fM [50]</td>
<td>Expensive</td>
<td>Multiplexed [65]</td>
</tr>
<tr>
<td></td>
<td>Low Sensitivity [65]</td>
<td></td>
<td>9.5min-1.5 hrs. [66]</td>
</tr>
<tr>
<td>Carbon NanoTubes (CNTs)</td>
<td>35 fM [56]</td>
<td>Low</td>
<td>Low cost [67]</td>
</tr>
<tr>
<td></td>
<td>Low Sensitivity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Piezoelectric Microcantilevers</td>
<td>10 pM [57]</td>
<td>Low</td>
<td>Low cost</td>
</tr>
<tr>
<td></td>
<td>Low Sensitivity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atomic Force Microscopy (AFM)</td>
<td>Attomolar [59]</td>
<td>Expensive</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>Expensive Equipment</td>
<td></td>
<td>High Sensitivity</td>
</tr>
<tr>
<td>Electrochemical</td>
<td>Attomolar [51]</td>
<td>Time Consuming</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>(4 Hrs.) [66]</td>
<td>High Sensitivity</td>
<td></td>
</tr>
</tbody>
</table>

### 1.5 Piezoelectric Microcantilever (PEMS) and Piezoelectric Plate Sensor

This section and its subsections are written by the approval of the inventors (Wan Y. Shih, Wei-Heng Shih, Mehmet C. Soylu, and Wei Wu) of a yet unpublished patent dis-
closure entitled "Piezoelectric Plate Sensor (PEPS)". PEPS is a sensor that consists solely of a thin piezoelectric layer such as lead magnesium niobate-lead titanate solid solution, \((\text{PbMg}_{1/3}\text{Nb}_{2/3}\text{O}_3)_{0.63}(\text{PbTiO}_3)_{0.37}\) (PMN-PT) or lead zirconate titanate (PZT) less than 33 \(\mu\text{m}\) in thickness thinly coated with an electrode <150 nm in thickness on either side of the plate. A PEPS may be square, rectangular, circular in shape, or hollow in the center of the plate, and 100-2000 \(\mu\text{m}\) in its lateral dimension, and the edge of a PEPS may be completely or partially fixed on a substrate for handling. A schematic of the cross-section view of a PEPS is shown in Figure 1.11.
Figure 1.11: A schematic of (a) a PEPS which consists of solely a piezoelectric layer thinly coated with two electrodes on the opposite sides and (b) a PEPS electrically insulated with MPS-W9 coating for in-liquid detection.

For detection in air, receptors specific to the target analyte will be coated on a PEPS surface and excited electrically at its length extension mode (LEM), width extension mode (WEM), or any other planar extension mode (PEM) resonance frequency, f, typically in the range of 0.4 to 50 MHz. A schematic of first mode LEM, WEM, and PEM vibrations are shown in Figures 1.12a-c. Binding of the target analyte to the specific receptor on the PEPS surface shifted the LEM, WEM, or PEM resonance frequency. Detection of the target
analyte is achieved by monitoring a PEPS LEM, WEM, or PEM resonance frequency shift.

Figure 1.12: (a) A schematic top view of the first LEM, (b) WEM, and (c) PEM vibration where the shaded bars illustrated the initial position of the PEMS and the dash-dotted shapes illustrate the bent or extended positions.

For detection in a biological fluid or water, a PEPS must be electrically insulated. The detection sensitivity as defined by the relative resonance frequency shift, Δf/f, is governed by (1) the piezoelectric property of the piezoelectric layer, (2) the thickness of the piezo-
electric layer, and (3) the quality of the electrical insulation.

One important reason why PEPS is chosen to detect Tr-DNA in this thesis is its high sensitivity as a result of piezoelectric layers Youngs modulus change due to binding [68]. It has been shown in this study that flexural resonance frequency shift was more than 300 times larger than could be accounted for by mass loading. Moreover PEPS is proven as a solid platform which can detect $1.6 \times 10^{-18}$ M target DNA sequences by monitoring the first longitudinal extension mode (LEM) resonance frequency shift. One major advantage of PEPS over PCR is it does not require 2 primers which has to be specific. It may be very difficult in some cases to design probes that would specifically hybridize to the region of interest with no cross hybridization. Moreover the probe DNA sequence could be shorter than PCR primers just because they only need to be specific to one position with respect to forward and reverse primers both of which has to be specific. Detection also depends solely on hybridization, and amplicon size would not affect the sensitivity in the sense that occurs in PCR.

Detection using PEPS can also multiplexed as shown before. Array PEPS are used for in situ, real-time, all-electrical detection of Bacillus anthracis (BA) spores in an aqueous suspension using the first longitudinal extension mode of resonance. [69] All-electrical detection scheme allows for detection using multiple PEPS with relays. Moreover detections using PEPS is label-free. For example label-free detection of complex body fluids such as serum is shown before. Highly sensitive detection of HER2 extracellular domain in the serum of breast cancer patients by piezoelectric microcantilevers is done. [70]
1.5.1 Piezoelectric Property Dependence

The most unique characteristic of a PEPS is that the detection sensitivity as defined by the relative resonance frequency shift, $\Delta f/f$, can be controlled by the improvement in the piezoelectric property of the piezoelectric layer. In PEPS geometry, the electric field is applied in the thickness direction to excite mechanical vibrations in the length and width directions. Under such conditions, the electromechanical coupling coefficient, $k_{31}$, or the piezoelectric coefficient, $d_{31}$, are the most relevant properties. The electromechanical coupling coefficient, $k_{31}$, measures the efficiency of conversion of the electric energy associated with applying an electric field in the thickness direction to mechanical energy associated with extensional vibration energy in the length or width direction. The piezoelectric coefficient, $d_{31}$, correlates the elastic strain in the lateral direction, $\varepsilon_{11}$ to the applied electric field in the thickness direction, $E_3$, as $\varepsilon_{11} = d_{31} E_3$. The $k_{31}$ constant can be obtained by a resonance method using the LEM or WEM resonance peak. In Figure 1.13, the phase angle, (blue) and the impedance, $z$, (red) resonance spectra are shown around the WEM resonance peak of a PEPS.
Figure 1.13: Phase angle (blue) and impedance (red) versus frequency of a PEPS with a $k_{31} = 0.34$ around the WEM resonance peak at 3.17 MHz as indicated by the peak in the phase angle plot and the characteristic frequencies, $f_a$ and $f_p$, are the frequencies at which the minimum and maximum of the impedance occur, respectively.

As can be seen, the $\Theta$ (electrical impedance) versus $f$ plot exhibited the WEM peak at 3.17 MHz. The $z$ versus $f$ plot exhibits the characteristic frequencies, $f_a$, which exhibits a minimum in $z$ and $f_p$, which exhibits a maximum in $z$. The electromechanical coupling constant $k_{31}$ can be obtained by using $f_p$ and $f_a$ to determine $k_{eff}^2$ as; [71–75].
Once $k_{eff}$ is determined, $k_{31}$ can be related to $k_{eff}$ numerically [71].

1.5.2 Insulation Quality Dependence

3-mercaptopropyltrimethoxysilane (MPS) coating was investigated at various pH and water contents. It was found that PEPS electrically insulated with a new MPS insulation procedure MPS-W9 yielded much better insulation results than the earlier MPS-5 insulation. The old MPS-5 insulation entails soaking a PEPS in a 1% MPS solution in ethanol at pH = 4.5-5.5 for 12 hr three times. PEPS with MPS-5 insulation typically exhibited a maximum current density larger than 600 $\mu$A/cm$^2$ as measured by cyclic voltammetry (CV) [76]. Such high current densities created a large noise background rendering the PEPS not reliable enough for in-liquid detection. The large background noise level made it hard to track the peak frequency reliably, thus leaving the sensor not responsive to binding of target analyte to the sensor surface.

The new MPS-W9 insulation procedure on the other hand called for soaking a PEPS in a 0.1% MPS solution in ethanol at pH 9 with 0.5% of water for 12 hr three times, which allows the condensation of the silanol groups of the MPS to occur more effectively. As a result, a denser MPS coating $> 100$ nm in thickness could be achieved that reduced the maximum current density of a PEPS in a CV test to less than 2 $\mu$A/cm$^2$ [76]. As a result, the noise level as judged by the baseline difference between the spectrum of a PEPS in a phosphate buffer saline (PBS) solution and that of the same PEPS in air was much reduced.
A schematic of a PEPS electrically insulated with MPS-W9 is shown in Figure 1.11b.

As an example, the in-air and in-PBS resonance spectra of two similar PEPSs -one electrically insulated with MPS-5 and the other with MPS-W9 are shown in Figures 1.14 a and b, respectively. As can be seen, both PEPS exhibit the first LEM peak around 500-600 kHz and the first WEM peak at around 3 MHz. Note that there is little damping effect on the LEM and WEM peak frequencies of a PEPS due to the much smaller vibration amplitude compared to those of bending modes. As a result, both PEPSs in Figs. a and b exhibited LEM and WEM peaks that showed minimal change in the peak height intensity and negligible change in peak frequencies when in PBS as compared to in air. However, the PEPS coated with MPS-5, the in-PBS baseline was about 13 degrees higher than the in-air baseline at the base of the 1st LEM resonance peak (Figure 1.14 a). In contrast, PEPS coated with MPS-W9, the in-PBS baseline was only 2 degrees higher than the in-air baseline at the base of the 1st LEM resonance peak (Figure 1.14 b). The higher in-PBS baseline of the PEPS coated with MPS-5 insulation layer indicates a much higher noise level, which would reduce the sensitivity of the PEPS.
Figure 1.14: The in-air and in PBS resonance frequency spectra of a PEPS (a) with MPS-5 (old) insulation and with MPS-W9 insulation.

1.6 Objective and Specific Aims

Sensitivity and specificity are the major factors determining the performance of a biosensor. In this thesis different aspects of the development of a PEPS is studied to increase the overall sensitivity and specificity. Clinical requirements for diagnosis of cancer from transrenal DNA are very stringent.

The objective of this thesis is, to develop a method that can be applied to detection of transrenal DNA Mutations satisfying the following to be practical;

- Have at least attomolar ($10^{-18}$ M) detection sensitivity.([77])
- Be specific enough to detect mutant DNA in a background with abundant Wild Type DNA (ratio of Wild Type to Mutant can exceed 240 [28])
- Be multiplexed, as most clinical conditions require detections from multiple loci for di-
agnosis

- Be robust (sample-to-answer in less than 40 min.)

For this purpose the following specific aims are achieved:

- Development of a signal processing algorithm to increase the sensitivity by reducing noise in the resonance spectrum to achieve $10^{-19}$ M limit of detection (chapter 2)
- Initial optimization of flow speed and temperature for real time mutation detection using glass slides with fluorescent microspheres (chapter 3)
- Development of a validation methodology of specific mutation detection using fluorescent reporter microspheres (chapter 4)
- In situ mutation detection with optimal temperature and flow speed with at least 1:250 MT/WT specificity in detecting both single mutation and double mutation (chapter 4)
- Development of in situ double-stranded target DNA mutation. (chapter 5, target DNA can be detected without the need for DNA isolation, concentration, and amplification)
2. Detection of DNA hybridization in buffer and Urine

2.1 Introduction

Cell free DNA is first discovered by Mandel and Mtais in 1948 [6], a phenomena importance of which fathomed only after its association with cancer, as mutant Ras gene fragments were discovered in the blood of patients [7, 8]. Till that time circulating DNA has been studied extensively for its diagnostic and even prognostic association with different cancer types including but not limited to, bladder [78–80], breast [81–91], cervical [92–94], colorectal [28, 32, 95–108], hepatocellular carcinoma [30, 31, 109–116], lung [117–127], non-Hodgkin’s lymphoma [128–132], melanoma [133–141], ovarian [142–146], pancreatic [100, 147–149], and prostate cancer [150–159]. These studies involve detection of cancer not only from mutations in chromosomal DNA but also from mitochondrial DNA [159, 160] and viral DNA, epigenetic factors, such as methylations [79, 84, 85, 92, 93, 102, 108, 111, 121, 122, 125, 141, 142, 147, 148, 150, 151, 153, 161] and microsatellite alterations [80, 126, 136–138, 162–164]. The passage of circulating DNA through the kidney barrier has been neglected for many years due to the selective behavior of the nephron. As a result of this incorrect assumption, DNA fragments observed in urine mostly attributed to have originated from organs and tissues of urogenital tract. However it was later found out that low molecular weight DNA fragments can actually pass through kidneys [23] as glomerular filtrate, moreover, kidneys play a major role in preparation of clinical specimens of cell-free DNA [38]. The standard method for detecting DNA fragments in body fluids is Polymerase Chain Reaction (PCR). Although PCR has the ability to amplify one
molecule in a reaction chamber (reference), it has limitations such as amplicon size [39] and potential inhibition by co-isolated factors. Moreover PCR depends on DNA isolation techniques specialized for transrenal DNA as low molecular weight DNA is abundant in urine. Currently, many methods of DNA isolation do not preserve the short fragments, since they are normally intended for isolation of nuclear DNA from intact cells [38]. Other genetic detection technologies under development rely on fluorescence [40], quartz crystal microbalance (QCM) [41, 42], electrochemical [43] binding to nano-metal particles [44], surface plasmon resonance (SPR) [45], silicon-based microcantilever sensor as well as piezoelectric microcantilever sensor. For DNA detection, nanoparticle amplified QCM exhibited a concentration sensitivity of 1 pM [165]. Nanoparticle enhanced SPR exhibits concentration sensitivity of 10-100 aM [166]. The electrochemical methods involving nanofibers and nanotubes also exhibit concentration sensitivity on the order of 30 fM [167]. Nanowires [52, 168–171] and nanotubes [53, 54] exhibit concentration sensitivity ranging from fM to 1 fM. Microcantilevers coupled with nano-metal particles exhibited 0.01 nM concentration sensitivity [57]. Although many of these methods such as QCM, SPR, silicon-based microcantilever sensor as well as lead zirconate titanate (PZT) piezoelectric microcantiliver sensor (PEMS) [46, 47] are label-free, the sensitivity is still many orders of magnitude away from the attomolar requirement. Similarly, the $10^{-16}$ M sensitivity achieved by magnetic beads isolation coupled with electrochemical enhancement was still not sufficient [58]. Nano-scale mechanical imaging by atomic force microscopy (AFM) can differentiate unhybridized single-stranded DNAs (ssDNAs) from hybridized double-stranded DNAs (dsDNAs) at attomolar sensitivity it requires sophisticated instrument such as AFM [59]. There are also other biosensor platforms involving very sensitive
detection of DNA. A GaN nanowire based extended-gate field-effect-transistor is capable of detecting attomolar concentrations of target DNA in situ [63]. Streptavidin horseradish peroxidase functionalized carbon nanotubes are used for indirect amperometric detection of target DNA (tDNA) in attomolar concentrations [60]. In another study label free carbon nanotube impedance biosensors are used to detect 100 aM of target DNA which was again lower than required levels of clinical applications [55]. Another electrochemical biosensor based on an integrated chip is shown to reach attomolar sensitivity however that detection was also not in real time [62]. Recently a disposable electrochemical biosensor based on magnetic bead amplification and target DNA modification was able to reach attomolar sensitivity. Although very sensitive and disposable, this biosensor depended on amplification and labeling [61].

PMN-PT PEPS is a new type of piezoelectric sensor consisting of a PMN-PT freestanding film 8 mm in thickness [172] thinly coated with gold electrodes on the two major surfaces and encapsulated with a thin electrical insulation as schematically shown in 2.1. Receptor specific to a biomarker is immobilized on the surface of the electrical insulation layer. Binding of the target biomarker to the receptor on the PEPS surface shifts the PEPS length-extension-mode (LEM) (Fig.2.1b) or width-extension mode (WEM) (Fig.2.1c) resonance peak frequency, f. Detection of a target protein or DNA marker is achieved by directly immersing a PEPS in the biological fluid and monitoring the LEM or WEM resonance frequency shift, Df in real time. Detection of tDNA fragments using LEM of PEPS is studied before and a limit of detection of 1 aM is accomplished. [173] Since WEM resonance frequency was higher than the LEM (due to the geometry of PEPS as discussed in
chapter 1), limit of detection in theory should be possible as absolute frequency shifts in resonance peak due to stress generated by hybridization would be much larger. However the noise in the WEM resonance frequency peak was also increased preventing this. In order to do sensitive detections in higher frequencies noise levels in the background should be lowered.
Figure 2.1: A schematic of (a) a piezoelectric plate sensor (PEPS), (b) the first length extension mode (LEM), (c) width extension mode (WEM) vibration of a PEPS where the shaded bars illustrated the initial position of the PEPS and the dash-dotted shapes illustrate the extended positions, (d) a top-view optical micrograph. The gold color in (d) and the thin layers lining the top and the bottom of the PMN-PT are 110 nm Cr/Au electrodes.

In this study, we examine the detection sensitivity of lead magnesium niobate-lead titanate (PbMg$_{1/3}$Nb$_{2/3}$O$_3$)$_{0.65}$(PbTiO$_3$)$_{0.35}$ (PMN-PT) piezoelectric plate sensor(PEPS) in real-time, label-free, in situ MT tDNA hybridization detection in full urine and 1x PBS
buffer solution without isolation and amplification. In order to accomplish this a peak determination method is developed that decreases the noise of a WEM resonance frequency peak that resulted in a detection limit of 100 zM in buffer and an even lower 50 zM in urine. HBV-DM tDNA is used as a model, since conditions on hybridization of this model is studied before in chapter 3 and is submitted for publication as of time this thesis is written, and more importantly this model is used published results on LEM resonance peak detections [173].

2.2 Experimental

2.2.1 PEPS fabrication

Two PEPS (PEPS A and PEPS B) used in this study were 2.5 mm long and 0.4 mm wide. They were fabricated from \((\text{PbMg}_{1/3}\text{Nb}_{2/3}\text{O}_3)_{0.65}(\text{PbTiO}_3)_{0.35}\) (PMN-PT) freestanding films 8 m thickness (ref) that was coated with 110 nm thick Cr/Au electrodes by thermal evaporation (Thermionics VE 90) and cut into 2.5 mm by 0.4 mm strips by a wire saw (Princeton Scientific Precision, Princeton, NJ). Gold wires 10 m in diameter were glued to the top and bottom electrodes of each strip using conductive glue (XCE 3104XL, Emerson and Cuming Company, Billerica, MA). The rear end of the strip was fixed on a glass substrate by a nonconductive glue (Loctite 1C Hysol Epoxy Adhesive) to form the PEPS geometry. It was then poled at 15KV/cm at 90°C for 60 min in an incubator (Digital Control Steel Door Incubator 10-180E, Quincy Lab). The dielectric constant of the PEPS was measured using an electrical impedance analyzer (Agilent 4294A) to be about 1800 with a loss factor of 2.5-3.7% at 1 kHz.
2.2.2 Electrical Insulation

The PEPS were electrically insulated to stabilize the resonance peaks for in-liquid detection by a new 3-mercaptopropyltrimethoxysilane (MPS) (Sigma-Aldrich Co. LLC.) solution coating scheme involving enhanced MPS cross-linking at pH=9.0 and with the addition of water.(Ref. mehmet’s paper) First, the PEPS was cleaned in a Piranha solution (two parts of 98% sulfuric acid (Fisher) with one part of 30% hydrogen peroxide (Fisher)) for 1 min, followed by rinsing in water and ethanol. Before coating the PEPS with MPS at pH=9.0, we dipped the PEPS in 50 ml of a 0.01 mM MPS solution in ethanol (Fisher) with 0.5% of de-ionized (DI) water for 30 min to promote hydrolysis followed by rinsing with water and ethanol. It was then subject to 5 12-hr of MPS coating in 50 ml of a 0.1% MPS solution with 0.5% of DI water in ethanol at pH = 9.0 (adjusted by adding KOH (Fisher)). For each 12-hr of MPS coating, the PEPS was always rinsed with water and ethanol first before being immersed in a fresh 0.1% MPS solution at pH=9.0 with 0.5% water. At the end of the 5\textsuperscript{th} round of MPS coating, the PEPS was rinsed with DI water and ethanol before further coating with receptors for detection. A schematic of the cross-section of a PEPS is shown in Fig. 2.1. After insulation, the resonance spectra of the PEPS were measured using an impedance analyzer (AIM 4170 C, Array Solutions). The phase-angle-versus-frequency resonance spectra of the PEPS in air (black) and in phosphate buffer saline (PBS) solution (red) are shown in Fig. 2.2.
Figure 2.2: (a) In-air (black) and in-PBS (red) phase angle-versus-frequency resonance spectra, and (b) relative resonance frequency shift, $\Delta f/f$, of the PMN-PT PEPS during the various steps of probe cDNA immobilization and target DNA detection. The insert in (b) shows a schematic of the molecules involved in the immobilization and detection.


2.2.3 Target DNA, probe DNA, and reporter DNAs

The target DNA (tDNA) used in this study was a 200-nucleotide (nt) long single-stranded DNA (Integrated DNA Technologies) containing the nucleotide sequence of the Hepatitis B virus genome (GeneBank Accession #X04615) centered around the 1762T/1764A double mutations. Part of the sequence of the tDNA containing the double mutations is shown in Table 2.1 where the two mutation sites were underlined. The probe DNA (pDNA) was a 16-nt long synthetic single-stranded DNA (Sigma) complementary to the 16-nt sequence of the tDNA centered around the double mutation sites as shown in Table 2.1. The pDNA had a biotin with a 12-polyethyleneglycol (PEG) spacer at the 5’ end. The melting temperature of the pDNA with the tDNA was 47°C as estimated under the experimental conditions Refs and listed in Table 2.1.

Table 2.1: The sequences and $T_m$ of the pDNA, tDNA, urDNA and drDNA

<table>
<thead>
<tr>
<th>Type of DNA</th>
<th>Sequence (5’ to 3’)</th>
<th>$T_m$ with tDNA($^\circ$C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tDNA</td>
<td>5’-...GGTTAATGATCTTTGT...-3’</td>
<td>–</td>
</tr>
<tr>
<td>pDNA</td>
<td>5’-...ACAAAGATCATTAACC...-3’</td>
<td>47</td>
</tr>
<tr>
<td>pDNA</td>
<td>Biotin-5’-ACAAAGATCATTAACC-3’</td>
<td>–</td>
</tr>
<tr>
<td>urDNA</td>
<td>Amine-5’-ACAGACCAATTTATGCCT</td>
<td>76.3</td>
</tr>
<tr>
<td></td>
<td>ACAGCCTCCTAG-3’</td>
<td></td>
</tr>
<tr>
<td>drDNA</td>
<td>Amine-5’-AATCTCCTCCCTTCCCTCCCAACTCCTCCCATCTTTT-3’</td>
<td>77.4</td>
</tr>
</tbody>
</table>

To immobilize the biotin-activated pDNA on the PEPS surface, the MPS-coated PEPS was first immersed in 200 µl of 5 mg/ml of maleimide activated biotin (Maleimide-PEG11-Biotin) (Pierce) in PBS for 30 minutes. The maleimide reacted with the thiol group on the MPS surface to immobilize the biotin on the PEPS surface. It was then followed by
immersing the PEPS in 200 µl of 1 µM of streptavidin in PBS to bind streptavidin to the biotin on the PEPS surface. Afterwards, the PEPS was immersed in 200 µl of a 10 µM solution of the probe DNA in PBS for an hour to allow the biotin at the 5’ end of the pDNA to bind to the streptavidin on the PEPS surface. Steps of immobilization is illustrated in inset in Fig.2.2b. The details of the chemical reaction of the immobilization steps are contained in Fig.2.3 [173].
Figure 2.3: cDNA immobilization Steps on PEPS surface: (1) In the first step, the maleimide of the maleimide-PEG-biotin linker reacted with the sulphydryl of 3-(trimethoxysilyl)-propyl-methacrylate (MPS) on the MPS coating surface to form a thiolester bond that covalently linked the maleimide-PEG-biotin on the MPS surface; (2) In the second step, the biotin of the immobilized maleimide-PEG-biotin reacted with streptavidin to immobilize streptavidin on the PEPS surface; (3) In the third step, the biotin at the 5’end of the cDNA reacted with the streptavidin bound on the biotin of the immobilized maleimide-PEG-biotin to finally immobilize cDNA on the PEPS surface.
There were two 30-nt long reporter DNAs (rDNAs) (Sigma). One was complementary to the sequence upstream of what was complementary to the pDNA and the other was complementary to the sequence downstream of what was complementary to the tDNA. The upstream rDNA was amine-activated with a 12-PEG spacer at the 5’ end while the downstream rDNA was amine-activated with a 7-PEG spacer at the 3’ end. The sequence of the upstream rDNA and that of the downstream rDNA are also shown in Table 2.1. In real DNA fragments, the mutation sites may be located anywhere in the fragments and in some cases the mutated sites may be too close to the edge for strong enough rDNA binding. Under such conditions, an rDNAs in the opposite stream would permit the binding of the rDNA to captured tDNA on the sensor surface. For this reason, we included both upstream and downstream rDNA in the study even though in the present synthetic tDNA the mutation sites were centrally located. The melting temperature for the binding the upstream rDNA (urDNA) to the tDNA was 76.3 °C and that of the downstream rDNA (drDNA) to the tDNA was 77.4 °C, which are also listed in Table 2.1. Fig. 2.4 is a schematic illustrating the relations between the tDNA, the pDNA, the urDNA, and the drDNA.
Figure 2.4: A schematic of the immobilization steps and nucleotide sequences of target DNA and its hybridization on pDNA, upstream rDNA and downstream rDNA.

2.2.4 Urine Sample Preparation

Urine samples were collected in a 50ml centrifuge in a first morning sample collection fashion after emptying the bladder the previous evening. Samples are kept in $4^\circ$C refrigerator for detection. Blocking of non-specific binding is accomplished by dissolving 3% BSA in urine equilibrated to room temperature.
2.2.5 Flow Setup

All the tDNA detections were carried out in a flow. A schematic of the flow system consisting of a polycarbonate detection chamber 18.5 mm long 3.5 mm wide and 5.5 mm deep (volume = 356 µl), three reservoirs, and a peristaltic pump (Cole-Parmer 77120-62) interconnected with 0.8-mm wide tubing as illustrated in Fig.3.3b and c. The PEPS was vertically placed in the center of the flow in the detection chamber with its major faces parallel to the flow. In each detection event, only one reservoir was connected to the detection chamber. The total volume of the liquid was 50 ml including the liquid in the reservoir, the detection chamber and the connecting tubing. In what follows, all detections were carried out with a flow rate of 1 ml/min corresponding to an average flow velocity of 1.4 mm/s at the PEPS surface. Furthermore, in this setup, the detection could transition from one detection experiment involving the sample in one reservoir to another detection experiment involving the sample of another reservoir by turning the valves. Typically, a 20-second period for valves turning without data recording was sufficient for a smooth transition from one detection experiment to another.

2.2.6 Blocking of Non-specific Binding in Urine

In order to block binding of non-specific binding on PEPS in urine, PEPS surface is treated with Bovine Serum Albumin (BSA) following pDNA immobilization. This is done by dipping the PEPS in a 100 µl of 3% BSA dissolved in 1 x PBS.
2.2.7 Data acquisition

A detection experiment involves recording of impedance spectrum of a resonance frequency with time continuously until the recording of data is stopped by the user. Fig.2.5 illustrates the screen of the laptop in a real detection experiment. Recording of impedance spectrum of a resonance frequency is achieved by scanning a given frequency window (W) using the impedance analyzer (AIM 4170 C, Array Solutions). As can be seen in figure 2.5, there is always an inherent noise in an impedance spectrum due to the noise in the impedance analyzing device. Since the analyzer itself can be simulated with a RLC circuit, as any RLC circuit it generates a noise. The difficulty is then to reduce this noise and find out the real peak position of the resonance frequency peak of PEPS. In order to achieve this goal, first, each impedance scan is time labeled and recorded in a text file. A peak finding algorithm determines the peak position of the resonance peak and adjusts the start and stop frequencies for the next frequency range to be scanned without changing the window size (frequency difference between a start and stop frequency) such that the next peak position remains closer in the window scanned. This ”Moving Window” algorithm is developed to keep pace with the moving resonance peak during a detection experiment, due to binding on the PEPS surface. Simultaneously, a time versus peak position graph is plotted and recorded in another text file for each resonance peak monitored every time a scan is finished so that user can monitor the frequency shift in real time. The designed algorithm also enables use of a relay for multiplexed detection setups involving monitoring resonance peaks of multiple PEPS simultaneously. The order of resonance frequency scans is given by the user as an input before the detection software is run so that after each scan the software automatically adjusts the relay and for the next PEPS. The software includ-
ing the peak finding and peak monitoring algorithms are completely automated until the user stops it, requiring no input from the user during detection. To summarize the inputs for the software developed for multiplexed resonance frequency monitoring are the number of PEPS used in the detection, the sequence indicating the order for impedance scans PEPS used (if more than one PEPS is used), the initial start and stop frequencies for each resonance peak to be monitored, and the file name and position for recording the raw data.

Figure 2.5: Three resonance frequency peaks are monitored in real time. Each resonance peaks raw impedance spectrum is plotted on top and their corresponding time versus peak position graph is plotted in the bottom. Peak positions are determined using the peak determination method discussed below.
2.2.8 Resonance peak frequency determination

The control of the resonance spectrum measurement, the determination of the resonance peak frequency, and the output of the resonance frequency with time were programmed in MatLab and were completely automated. To start detection, an initial start frequency and an initial stop frequency are input to define the initial frequency window in which the resonance spectrum is measured. To determine the resonance frequency from the measured spectrum, it is important to define an origin around which data points will be chosen to fit second order polynomials. A simple way to define origin is to use the peak of the raw impedance spectrum as illustrated in Fig.2.6a. This can be followed by fitting parabolas of different neighborhood sizes around the origin. A typical resonance spectrum includes 1000 data points and maximum number of data points around the origin is typically 480 points from either side of the origin. Parabola fitting is carried out over a range of data points around origin with multiples of ten up to the maximum available. To illustrate, 20 points are chosen around the origin to fit a parabola as shown in Fig.2.6a and 390 points is chosen in Fig.2.6b on the same spectrum. Parabolas fitted are shown in pink.
Figure 2.6: Phase angle versus frequency of WEM resonance spectrum of a PEPS. black full circles represent the raw data. The blue data points represent a neighborhood of 10 (a) and maximum neighborhood available (b) for parabola fitting (blue, in insets), raw peak value is indicated by an arrow on graphs, , polynomials fitted on these neighborhoods (pink, in insets) and peak positions of the fitted polynomials (red circles).

After outlier removal by an outlier removal method (American Society for Testing and Materials E178, k=1.5), the resonance frequency of the measured spectrum is finally obtained by averaging all the peak values of the fitted parabolas (excluding the outliers) as illustrated in Fig.2.7d with black squares. A typical number of peak values used for averaging is close to $35\pm4$. In order to maximize the predictive power of this method it is obvious that one needs a resonance peak spectrum which has a peak in the middle of the scanning frequency window which will maximize the available data points for parabola fitting. It is important here to note that data points chosen for parabola fitting is always defined by equal number of data points around a pre-determined origin. Thus, the distance
of the real peak frequency of a resonance peak from the midpoint of the frequency scan window is inversely proportional to the number of parabolas that are fitted on available data points around the origin. The raw peak frequency may be further from the real peak position as can be seen in Fig.2.6a. Thus instead of choosing this point as the origin one may first smooth the raw impedance spectrum by local regression using a weighted linear least square fit to a second-degree polynomial model with a span of 0.1 (2.7a). Parabola fitting can be done exactly as explained before on this smoothed spectrum instead of the raw spectrum as illustrated in Fig.2.7b and c (fitting on 20 and 430 data points respectively). As can be seen in Fig.2.7d this will not only increase the number of parabolas that can be fitted on the same spectrum with respect to an origin defined as the maximum raw spectrum but also decrease the variation in peaks of the parabolas fitted, thus decreasing the noise in determination of peak frequency from a given impedance spectrum.
Figure 2.7: a. Maximum of raw and smoothed data is shown in blue and pink respectively. 
b. Polynomial fitting is done on a neighborhood of 41 points on smoothed spectrum. c. 
Polynomial fitting is done on maximum available neighborhood of on smoothed spectrum. 
d. Neighborhood size versus peak of polynomials fitted on smoothed (black) and raw (red) 
data is plotted.

Another problem during a real detection experiment is to keep the origin determined 
after smoothing the raw frequency in the middle of the frequency scan window. As analytes
bind or hybridize on the PEPS surface the resonance frequency shifts to higher or lower frequencies depending on the stress generated. In order to overcome this situation the following formula is used to determine the start and stop frequencies of the next frequency scan window;

\[ f^{i+1}_{\text{start}} = \frac{2f_{\text{peak}}^i + f_{\text{start}}^i - f_{\text{stop}}^i}{2} \]

\[ f^{i+1}_{\text{stop}} = \frac{2f_{\text{peak}}^i - f_{\text{start}}^i + f_{\text{stop}}^i}{2} \]

where \( f^{i+1}_{\text{start}} \) and \( f^{i+1}_{\text{stop}} \) are the start and stop frequencies of the next frequency scan window respectively as determined from start and stop frequencies of the current frequency scan window \( f_{\text{start}}^i \) and \( f_{\text{stop}}^i \), respectively. This formula measures how far away the peak position of a resonance peak from the middle of the frequency scan window and moves the scanning window in the next scan such that the current resonance frequency peak will be in the middle in the next scan. This algorithm makes sure as long as the shift of the resonance peak stops at the steady state of the hybridization/binding, the resonance frequency peak will be kept in the middle of the scanning window. This generated a lag in algorithm to estimate the resonance frequency peaks position. As a future work this lag can be minimized using improved algorithm taking invaluable information from historical data on the time versus frequency shifts of the current and past detection experiments into account. Fig.2.8a below shows the resonance frequencies’ raw peak positions versus time during hybridization on PEPS surface of 10 μM tDNA dissolved in 1x PBS. This hybridization experiment is monitored using a fixed scanning window regime and the moving scanning window regime discussed above. Impedance spectra recorded using fixed window and
moving window are plotted before hybridization (t=0 min) and after hybridization (t =30 min) as illustrated in Fig.2.8b.

Figure 2.8: (a) Detection of 10 M of tDNA in 1x PBS is plotted using raw peak positions of phase angle versus frequency plots of WEM resonance peak. (b) Impedance spectra of resonance frequency peaks (red stars in (a)) at time 0 (filled symbols) and 30 (open symbols) min. are plotted for the fixed (red circles) and moving (black squares) window impedance scanning methods

2.2.9 Re-plotting Detection Experiments

Detection experiments can be re-plotted using a MatLab routine developed enabling access to raw impedance spectra and time dependent behavior of peak positions of different experiments and different peaks simultaneously. Frequency shifts are plotted in kHz versus time as can be seen in Fig. 2.9. Moreover user can plot experiments done in an order (such as different steps of immobilization and/or tDNA or FRM hybridization steps) in one frequency shift versus time plot. Each experiment is plotted and the frequency shift
in that experiment is written on the curve together with the file name assigned to record that experiment’s raw impedance spectra data. As can be seen in Fig.2.9, background signal checking in 1xPBS, covalent binding of maleimide activated biotin, conjugation of streptavidin and biotinylated pDNA and hybridization of tDNA followed by a second background signal check in 1x PBS is plotted for the length and width extension modes of the same PEPS. User can also click on any of the data points on time versus frequency shift graphs of any peak and the raw impedance spectra at that point is automatically drawn on the right of the graph as illustrated in Fig.2.9.
Figure 2.9: Detection experiment involving simultaneous monitoring of 7 peaks of the same PEPS is done. Experiment consisted of the following steps; background signal checking in 1xPBS, maleimide activated biotin binding, streptavidin binding, biotinylated pDNA binding, tDNA hybridization and second background signal checking in 1xPBS. Two peaks (LEM and WEM) is drawn in this re-plotting. A sample raw impedance spectra of each peak is drawn on the right of time versus frequency shift graphs for peaks.

2.2.10 Simulations

To determine the performance of the resonance peak frequency determination explained above, it is tested on a simulated detection experiment. The known resonance frequency shift versus time plot was is generated using a sigmoidal function (Fig.2.10a). Each data point on this plot represented the frequency of a resonance peak at a given time, and each
of these peaks is modeled using a second, or and third order polynomial and or a Gaussian function (Figures 2.10b,c,d). Gaussian function is adjusted so that peak position and inflection points intersect the second order polynomial (Fig. 2.10b). For each resonance frequency peak model, average increment in phase angle in used defined as 1 a unit of ”noise” parameter. Normally distributed random noise is generated using multiples of this unit as the standard deviation. The noise is added on the modeled peaks and peak finding algorithm is used to determine the peak positions. Window size is another parameter, effect of which is studied by simulations. A frequency window is defined as the difference in frequency between start and stop frequencies of each impedance scan. Third order polynomials are used to generate asymmetrical peaks. The ratio of distances of start and stop frequencies to the peak position of the modeled third order function is used as the parameter, ’R’, representing extend a measure of asymmetry of a generated model function. (Please observe that, if \( P_n(x) \) is defined as set of all polynomials of degree n, than \( \forall P(x)|P(x) \in P_3(x); 1 < R < 2, \text{Fig.2.10d} \)
Figure 2.10: a. Simulated model of a detection experiment representing graph of time versus resonance frequency peak position. b. Gaussian and second order simulated peaks without noise. c. A third order polynomial, with "R" representing the extend of asymmetry of polynomial. d. Simulated model of third order polynomials with different "R" values.
2.3 Results and Discussion

2.3.1 Analysis on Simulation Parameters

Width extension mode resonance frequency peaks are almost always asymmetrical due to the increase in baseline phase angle at high frequencies due to the inductive effect. Any circuit can be modeled as a serial RLC circuit, phase angle of which is can be calculated from;

\[ \theta = \frac{j\omega L - \frac{1}{j\omega C}}{R} \]

where \( \theta \) is phase angle, \( \omega \) is angular frequency which is \( 2\pi f \), where \( f \) is frequency, \( L \) is inductance, \( R \) is resistance and \( C \) is capacitance. As the frequency increases while effect of capacitance gets smaller, effect of inductance on the phase angle increases. This, asymmetry is modeled using a third degree polynomial with a degree of asymmetry measured by the parameter ”R”. The higher the R, value the more asymmetric the simulated resonance peak model is. Two types of error are measured in each simulation, relative error and error in frequency shift (\( \Delta f \)). For any simulation, frequency shift is calculated from the difference in frequencies of the peak positions with highest and lowest frequency peak positions (\( E_{\Delta f} = \Delta f_{\text{real}} - \Delta f_{\text{calculated}} \), where \( E_{\Delta f} \) is error in frequency shift). Relative error is calculated by averaging the differences in peak positions in the simulated and calculated resonance frequency peak positions for every time values. As can be seen in Figures 2.11 and b, after 10 simulations although the relative error increased with increasing R, standard deviations did not change significantly, whereas error in frequency shifts are not affected on average, however increased in standard deviation. This shows the peak monitoring algorithm
is capable of measuring the frequency shifts correctly although the calculated resonance frequency peaks are not close to the real values. This is due to the fact that the resonance frequency peaks retains their shape and asymmetry is not changing in an experiment due to binding, and even though there is an error due the asymmetry, because almost the same amount of error is retained in each peak position calculation, the net movement in the peak position is also retained. Figures 2.11c and d shows that, as the noise added on each modeled resonance frequency peak is increased both relative error and error in frequency shift increases. Window size on the other hand showed an increased effect in relative error in the asymmetrical model, with respect to the symmetrical models. However this effect is not seen in error in frequency shift as can be seen from Figures 2.11e and f.
Figure 2.11: Error analysis of the peak monitoring algorithm by simulations is shown. a. Effect of parameter, "R", on relative and frequency shift errors are plotted (a and b). Effect of "Noise" added on simulated resonance peaks on relative and frequency shift errors are plotted (c and d). Effect of "Window Size" on relative and frequency shift errors are plotted (e and f).
2.3.2 BSA blocking

In order to determine the amount of BSA required to block the PEPS surface for detections done in urine, resonance peak frequency of PEPS treated with different amounts of BSA is monitored for non-specific binding in urine using a flow rate of 1ml/min. This is followed by washing with 1x PBS using a 6ml/min flow rate. It is important to determine the least amount of BSA required for completely blocking non-specific binding because as the concentration of BSA used to block the PEPS surface is increased the sensitivity of PEPS also decreases as pDNA immobilized on the surface is hindered by the bound BSA. In Fig. 2.12 relative resonance peak frequency shift in non-specific binding and washing steps of a PEPS treated with no BSA and 1, 2, and 3 % is plotted time. Relative resonance peak frequency shift refers to the ratio of the resonance peak frequency shift to the position of the resonance peak frequency. As can be seen 3% BSA totally blocked the PEPS surface, enabling detection in urine.
Figure 2.12: Relative frequency shifts of PEPS B in urine treated after 1, 2 and 3 percent BSA and no BSA followed by a washing step in 6ml/min flow rate.

2.3.3 Dose response in 1 x PBS and urine and visual confirmation

Detection of tDNA concentrations from 100zM to 10 nM and from 50 zM to 10 nM are done in 1x PBS and in urine. tDNA hybridizations are followed by hybridizations of FRMs. Fig.2.13a and b show the relative resonance peak frequency shifts during hybridization of different concentrations of tDNA. Average relative frequency shifts in the last five
minutes of each hybridization reaction (25\textsuperscript{th}-30\textsuperscript{th} min. and 55\textsuperscript{th}-60\textsuperscript{th} min for hybridizations of tDNA and FRMs respectively) are plotted versus tDNA concentration for in urine and 1 x PBS detections in Fig.2.13. Relative frequency shifts after FRM hybridizations are also plotted versus number of FRMs hybridized on PEPS surface as can be seen in Fig.2.13d. This indicated a direct proportion between relative frequency shifts in FRM hybridizations and number of FRMs hybridized on PEPS surface.

As can be seen in Fig.2.13c, although in semi-log plot of concentration versus relative frequency shift graph there is a linear behavior between concentrations of 100 zM and 100 pM in detections in both urine and 1 x PBS, there is a steep decrease in the relative frequency shift in 50zM concentration with respect to 100 zM. This change in relative frequency shift can be best described by a threshold concentration mechanism, that when exceeded, generates detectable surface stress on PEPS surface. Moreover hybridization of FRMs on tDNA on PEPS surface generated almost an equal 1.2 times higher frequency shifts which occurred in tDNA hybridizations.
Figure 2.13: Standard curve of HBV DM detection in urine (a) and in 1xPBS (b) with PEPS A and PEPS B respectively at room temperature with 2ml/min flow rate. (c) Relative frequency shifts at 30 min (after tDNA hybridization) and 60 min (after FRM hybridization) are plotted versus concentration of tDNA. (d) FRM micrographs after tDNA and FRM hybridizations in urine for control, and concentrations; 50zM, 100zM, 1aM, 10aM and 100aM of tDNA.

Micrographs of PEPS surface after detection of some of the lowest concentrations in
urine are shown in Fig.2.14. As can be seen from the images FRMs are not aggregated and are evenly distributed on PEPS surface at high concentrations of tDNA. Number of FRMs on PEPS surface at these concentrations are plotted in Fig.2.13d.

Figure 2.14: FRM micrographs after tDNA and FRM hybridizations in urine for control, and concentrations; 50zM, 100 zM, 1 aM, 10 aM and 100 aM of tDNA. Width of PEPSB is 420 m and is shown by dashed parallel lines in micrographs.
2.3.4 Simulation Results

Random noise added on the simulated resonance peak frequencies are generated using normally distributed pseudorandom numbers as described before. By comparing the standard deviation of raw peak positions of simulated resonance peak frequencies generated by adding pseudorandom numbers with different standard deviations on a fixed second order polynomial, with standard deviation of a WEM resonance peak frequency monitored in 1 x PBS where there is no hybridization on PEPS surface. In 1 x PBS any frequency shift in resonance peak is considered as background noise. Standard deviation in raw peak positions is chosen as the criteria to compare the simulations with real noise in the background to eliminate any effect from signal processing. Fig.2.15 illustrates the noise versus standard deviation of raw peak positions of such simulated peaks and standard deviation in raw peak positions of WEM resonance peak in 1 x PBS is shown with a horizontal dashed line.
Figure 2.15: (a) Noise parameter of simulated detection experiments with zero frequency shifts are plotted versus corresponding standard deviation of the raw peak positions for each simulated experiment. Standard deviation in raw peak position of WEM resonance peak of a PEPS in 1xPBS is shown with dashed line. (b) Experimental data in (a) and simulated experiment with noise parameter "27" is plotted. This value is used for further simulations

In order to assess the effect of resonance peak frequency determination method developed a ”signal to noise ratio(SNR)” is defined as the ratio of relative frequency shift in the last 5 minutes of any hybridization detection experiment to the standard deviation of resonance peak position in 1 x PBS. WEM of PEPS A is monitored for tDNA detections of different concentrations in 1 x PBS. Same WEM resonance peak is monitored using a fixed window scanning method and the resonance frequency peak determination method discussed in this study simultaneously. In Fig.2.16a, three different methods are applied to determine the peak frequency, and SNR is plotted versus tDNA concentrations from 100 zM to 10 nM. In method one, raw peak positions are determined from fixed window scan-
ning method. In the second method peak positions determined by fixed window scanning and fitting 100 data points around the raw peak position of each scan. In third and final method peak determination methods discussed in this study is applied. As can be seen resonance frequency peak determination method results in an order of increase in SNR with respect to method one and 4-5 times increase with respect to method two.

In order to estimate the error done in relative frequency shifts simulations with the noise level determined in Fig.2.15a and b is used to generate simulations of detections of different tDNA concentrations. Percentage error of frequency shifts in the last five minutes of each simulation is calculated using the following formula:

\[
\frac{|\Delta f_{\text{simulation}} - \Delta f_{\text{actual}}|}{\Delta f_{\text{actual}}}
\]

where \(\Delta f_{\text{simulation}}\) is the average of relative frequency shift in last five minutes (25th min and 30th min) in each simulated tDNA detection
Figure 2.16: (a) Dose response experiment in 1x PBS is done recording peak resonance frequencies applying the peak determination algorithm and a fixed window scheme simultaneously. SNR of fixed window resonance frequency monitoring is plotted versus concentration of tDNA with single parabola fitting and raw peak positions and compared with that of peak resonance frequency determination method (b) Percentage of error in relative frequency shifts between 25th and 30th minutes are plotted using simulated resonance peaks with the same noise in experimental peaks.

2.4 Summary of Resonance Frequency Peak Determination

We have investigated real-time, in situ DNA hybridization detection using piezoelectric plate sensors (PEPSs) consisting of a highly piezoelectric lead magnesium niobate-lead titanate (PMN PT) layer 8 mm in thickness thinly coated with Cr/Au electrodes and electrically insulated with 3-mercaptopropyltrimethoxysilane (MPS) encapsulation. With probe complementary DNA (cDNA) immobilized on the PEPS surface and by monitoring the first
width extension mode (WEM) resonance frequency shift of the PEPS we showed that we could detect hybridization of the target DNA (tDNA) to the probe DNA on the PEPS surface at 50zM in urine and 100 zM in 1 x PBS with a signal to noise ratio of 14 and without isolation and amplification, which was validated in situ by the detection of fluorescently labeled microspheres coated with reporter DNAs complementary to the tDNA but different from the probe DNA following the detection of the tDNA. Simulations are done to measure the effectiveness of the developed method and a 5 fold increase in SNR is shown with respect to single parabola fitting and less than 12% error in final frequency shift in observed at the limit of detection. It has also been shown by simulations that, asymmetrical nature of WEM is not affecting the final frequency shift after each experiment.
3. Flow-Enhanced Detection Specificity of Mutated DNA against Wild Type with Reporter Microspheres

Cancer is a genetic disease and gene mutation is an important form of genetic defects that play an important role in cancer pathways. Detecting gene mutation is essential for cancer diagnosis, cancer therapy decision, as well as therapy efficacy monitoring. Many genetic cancer markers are known to circulate in body fluids such as serum and urine. Detecting circulating genetic markers in serum or urine is minimally invasive or non-invasive, which can be an integral part of the therapy monitoring when the primary tumor is removed or hard to get to. One challenge of detecting cancer genetic markers in serum or urine is that the wild type shed by the normal cells may be far more abundant than the defected gene shed by the cancer. For example, the wild type (WT) kRas gene is known to outnumber the mutant (MT) kRas gene by a factor of 240. [39] Gene mutation is among the most challenging to detect as one must be able to detect and differentiate the MT from the WT given that the WT outnumbers the MT and the genetic difference between the MT and WT is often one nucleotide. Currently WT can be discriminated using methods such as denaturing gradient gel electrophoresis, [174] temperature gradient gel electrophoresis, [175] single strand conformation polymorphisms, [176–178] heteroduplex analysis, [179] chemical cleavage method, [180, 181] protein truncation tests, [182, 183] DNA chips [184, 185] and high resolution melting temperature analysis. [186] All of the above methods are based on the fact that the hybridization kinetics between the WT and the probe DNA (pDNA) is different from that between the MT and the pDNA. For a pDNA that is perfectly com-
plementary to MT, the melting temperature of the MT, the de-hybridization temperature between the pDNA and the MT, $T_{MT}$, is higher than the melting temperature of the WT the de-hybridization temperature between the pDNA and the MT, $T_{WT}$. This melting temperature difference is important in differentiating the MT from the WT. Typically MT is detected at a temperature between $T_{MT}$ and $T_{WT}$. [186–188] While raising the temperature within the $T_{MT} - T_{WT}$ window may minimize the binding of the WT to improve specificity; it can also reduce the binding of the MT, thus reducing the sensitivity. Some single-nucleotide mutations may have a $T_{MT} - T_{WT}$ window as small as less than 1 C, [189] making it difficult to differentiate MT from WT by the temperature means alone. Recent development of locked nucleic acid (LNA) pDNA that can help widen the window between $T_{MT}$ and $T_{WT}$ is one way to help better differentiate MT from WT. [5]

Fluorescent polystyrene microspheres have been established as a means of detecting DNA hybridization and conjugation of DNA stabilizes the surface charge of microspheres. [190] In a systematic study involving shear flow, microspheres were coated with single stranded DNA to hybridize with pDNA on a surface under the influence of various levels of shear stress. It was shown that shear stress played an important role similar to that of temperature in that DNA became increasingly de-hybridized with an increasing shear stress and that there existed a critical shear stress above which microspheres became detached from the surface and that DNAs with a single mismatch exhibited a somewhat lower number of attached microspheres per unit area. [191] In addition, flow had also been shown to minimize cross-binding of closely related species of bacillus anthrax (BA) such as B. thuringiensis (BT), B. cereus (BC), and B. subtilis (BS) to anti-BA antibody immobilized on a sensor situated at the center of a laminar flow. [69] Fluid flow in a narrow channel
has also been shown to help enhance detection sensitivity by reducing nonspecific binding. [192–194] Therefore, it seems possible that fluid flow may be an auxiliary tool to further improve specificity of mutation detection.

The purpose of this study was to investigate how flow can affect the specificity of mutation detection in addition to temperature with the help of fluorescent reporter microspheres (FRMs). The model mutant (MT) gene was the 1762T/1764A HBV double mutation which is present in 50-85\% of hepatocellular carcinomas (HCC). [195–199] The HBV-DM MT gene has two mutated sites that are close to each other as shown in the schematic in Fig. 3.1a and in Table 3.1. A gold-coated glass (GCG) with pDNA complementary to the MT covalently immobilized on its surface was vertically immersed in the center of a laminar flow of the MT or WT solution for the MT or WT to hybridize to the pDNA on GCG. Separately, FRMs are covalently coated with reporter DNA (rDNA) that is complementary to MT and WT but different from pDNA (see the schematic in Fig. 3.1 and Table 3.1). After MT or WT binding, the GCG was immersed in a flow of FRMs for the FRMs to hybridize to the captured MT or WT on the GCG. By varying the flow rate at various temperatures between $T_{MT}$ and $T_{WT}$ we will be able to determine if flow helps improve the specificity of mutation detection.
3.1 Experimental

3.1.1 Target DNAs, probe DNA, and reporter DNAs

The MT used in this study was a 200-nucleotide (nt) long single-stranded DNA (Integrated DNA Technologies) containing the nucleotide sequence of the Hepatitis B virus genome (GenBank Accession #X04615) centered around the 1762T/1764A double mutations. [31] Part of the sequence of the MT around the double mutations is shown in Table 3.1 where the two mutation sites were denoted by the underline. Partial sequence of the 200-nt long WT is also shown in Table 3.1. 16-nt long synthetic single-stranded pDNA and 30-nt reporter DNA (rDNA) were purchased from Sigma. The pDNA was complementary to MT targeting the 16-nt sequence focused around the double mutation sites of the MT. The sequence of the pDNA is also shown in Table 3.1 The pDNA was amine-activated and
had a 12-polyethylene glycol (PEG) spacer at the 5’ end. The melting temperature of the MT with pDNA was 47°C and that of WT with pDNA was 23°C as estimated using salt adjustment for phosphate buffered saline (1x PBS) [200, 201]. These two melting temperatures are also listed in Table 3.1.

Table 3.1: The sequences and corresponding melting temperatures adjusted with salt concentration in 1xPBS

<table>
<thead>
<tr>
<th>Type of DNA</th>
<th>Sequence (5’ to 3’)</th>
<th>( T_m ) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( MT^a )</td>
<td>5’…GGTTAATGATCTTTGT…-3’</td>
<td>47</td>
</tr>
<tr>
<td>WT</td>
<td>5’…GGTTAAAAGGTCTTTGT…-3’</td>
<td>23</td>
</tr>
<tr>
<td>pDNA</td>
<td>Biotin-5’-ACAAAGATCATTACC-3’</td>
<td>—</td>
</tr>
<tr>
<td>UpstrDNA(^b)</td>
<td>Amine-5’-ACAGACCAATTTATGCCTACAGCCTCCTAG-3’</td>
<td>76.3</td>
</tr>
<tr>
<td>DwstrDNA(^c)</td>
<td>Amine-5’-AATCTCCTCCCCCAACTCCTCCAGTCTTT-3’</td>
<td>77.4</td>
</tr>
</tbody>
</table>

\(^a\) Mutation sites indicated by underlines, \(^b\) Upstream rDNA, \(^c\) Downstream rDNA

The melting temperatures of the two rDNAs with the MT and those of the rDNA to the
WT are also listed in Table 3.1. Fig 3.1a and 1b are schematics illustrating the relationship between MT, pDNA, and upstream and downstream rDNAs and that between WT, pDNA, and upstream and downstream rDNAs, respectively. The upstream and downstream rDNAs were designed to have much stronger binding to the target MT or WT than the pDNA to the target MT or WT. Therefore, when unbinding due to the flow-induced impingement force occurred it would occur at the binding sites between the pDNA and the MT or WT but not at that between the rDNA and the MT or WT. Because the binding of the pDNA to the WT was much weaker than that of pDNA to the MT, theoretically, the flow-induced impingement force could more easily overcome the weaker binding between the pDNA and the WT than that between the pDNA and the MT to allow us selectively detect MT but not WT.

### 3.1.2 Substrate Preparation and pDNA Immobilization

Glass microscope coverslips (22 mm 22 mm) were deposited with 100 nm thick gold using thermal evaporation. The coverslips were then cut into small rectangular pieces of approximately 3mm3mm. In the following we will refer to these 3mm3mm Gold-Coated Glass coverslips as GCGs. The surface of these GCGs were cleaned by immersing them into 100 times diluted piranha solution (1:1 Sulfuric Acid: Hydrogen Peroxide by volume) for 2 minutes and then washed by deionized water and anhydrous ethanol. The GCGs were then immersed in 50 ml of 0.01 mM 3-mercaptopropyl trimethoxysilane (MPS) (Sigma) solution in ethanol to coat the MPS on the gold surface of the GCGs. The pDNA was immobilized on the MPS surface using Sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) (Pierce) as the bi-function. The maleimide end of the SMCC
reacted with the thiol of the MPS on the GCG surface and the NHS ester end of the SMCC reacted with the amine at the 5’ end of the pDNA, thereby, covalently immobilized the pDNA on the GCG surface (Fig. 3.2a). The 12-PEG spacer at the 5’ end of the pDNA would allow the pDNA to be at a distance from the GCG surface for easier hybridization to the tDNA. After MPS coating, the MPS-coated GCGs were immersed in a 1M pDNA solution with 5 mM sulfo-SMCC for 1 hour to immobilize the pDNA. The GCG was then rinsed with DI water and phosphate buffer saline (PBS) solution and ready for MT or WT binding.

Figure 3.2: A schematic of (a) pDNA immobilization on GCG surface using sulfo-SMCC, and (b) schematic of rDNA conjugation on carboxylated FRMs with sulfo-NHS and EDC.

3.1.3 rDNA conjugation to FRMs

Blue fluorescent polystyrene microspheres (FRMs) (Bright Blue, excitation: 360 nm, emission: 407nm) (Polysciences) 6 m in diameter were conjugated with two rDNAs. First, 0.1 ml of $2.1 \times 10^8$ particles/ml stock suspension of FRMs was diluted 10 times in PBS. After-
wards, the suspension went through the following washing steps three times: vortexing for 15 seconds, centrifuging at 3700 rpm (Centra, CL2, IEC, MA), discarding the supernatant, re-suspending the sediment in 10 ml PBS. For conjugation, FRMs suspensions at 2.1106 particles/ml were incubated with 3.3 M, 330 nM, 33 nM and 3.3 nM of mixed upstream and downstream rDNAs solutions at 1:1 ratio with 5 mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Sigma, MA) and 5 mg/ml sulfonated N-Hydroxysuccinimide (sulfo-NHS) (Pierce, IL) at pH=6 at room temperature for 1 hr (Fig 3.2b). The suspensions were then washed by centrifugation 3 times as described above. After the final washing, 10 ml of four different stock conjugated FRMs suspensions at 2.1 10^6 particles/ml were obtained from four different rDNA concentrations. In each experiment, 1 ml of a stock conjugated FRMs suspension was further diluted by 10 times to a volume of 10 ml and a concentration 2.1 10^5 particles/ml. In the following all results were obtained at 2.1 10^5 particles/ml. Fig. 3.3 shows a schematic of a FRM covalently coated with both the upstream and downstream rDNAs.
Figure 3.3: (a) A schematic of a FPM coated with upstream and downstream rDNA, (b) a schematic of flow cell where the GCG is placed in the center of laminar flow, and (c) a schematic of flow system.

3.1.4 tDNA Hybridization and Subsequent Capturing of FRMs

Hybridization of MT or WT to the probe DNA on a GCG and the subsequent hybridization of the FRMs by the bound MT or WT on the GCG were carried out in an open flow cell where a GCG was placed at the center of the detection chamber with the major faces of the GCG parallel to the direction of the flow as schematically shown in Fig. 3.3 at various temperatures controlled in an incubator (Digital Control Steel Door Incubator 10-180E, Quincy Lab). The custom-made flow cell was 18.5 mm long, 3.5 mm wide, and 5.5 mm deep (volume =356 l and cross-section area =19.25 mm$^2$) driven with a peristaltic pump (model 77120-62, Cole-Parmer’s Master Flex, Vernon Hills, IL). A schematic of the flow system is shown in Fig. 3.3c. An open container of deionized water was included in the incubator to control the humidity in the incubator to minimize evaporation from the open
flow cell.

To examine the effects of both the temperature and the flow rate, we carried out hybridization experiments at various temperatures and various flow rates. The temperatures examined were room temperature (RT), 30°C, and 35°C, which were between the melting temperature of the WT, 23°C, and that of the MT, 47°C. The flow rates examined were 0, 2, 4, and 6 ml/min. At the given temperature and flow rate, we first circulated the MT or WT solution through the flow cell for 30 min for the MT or WT to bind to the pDNA on the GCG as illustrated by the schematic in Fig. 3.4a. Afterwards, we circulate the FRMs suspension through the flow cell for 30 min to allow the FRMs to bind to the MT or WT captured on the GCG as schematically illustrated by Fig. 3.4b. PBS was then flown through the flow cell to wash off loosely bound FRMs at a flow rate of 2 ml/min for 30 in as schematically shown in Fig. 3.4c.
Figure 3.4: A schematic of a) tDNA sequence flown over pDNA immobilized on GCG, b) Upstream and downstream rDNA-conjugated FRMs flown over the GCG surface and FRMs hybridized on the flanking regions of the tDNA sequences, and c) unbound or loosely bound GCG washed.

3.1.5 FRMs counting

After washing, the GCG was air dried and examined in a fluorescent microscope (BX51, Olympus). The number of FRMs per unit area was determined with a custom made program turned into black and white using Otsu’s method. [202] As the brightness of each FRM was different in each image (due to different exposure times and FRMs fluorescence
decay), the number of white pixels per FRM could be different in each image after black and white conversion. To determine an average white pixel per FRM, clusters of white pixels were labeled and cluster size distribution was determined. The number of the FRMs per unit area in each image was determined from the pixel cluster size distribution as follows. First, clusters of white pixels less than 20 pixels were considered to be noise due to the gray-scale to black-white conversion and were not included in FRM counting. Second, in addition to well-separated FRMs, some FRMs were close together, they might appear as one large cluster. These large clusters of white pixels were separated from clusters of white pixels corresponding to single FRMs when determining the average number of white pixels per FRM. This was accomplished by applying an outlier removal algorithm (ASTM E178, k=1.5, ASTM=American Society for Testing and Materials) with the large outlying clusters representing groups of FRMs that were too close to be seen as separate FRMs. After the large outlying groups of white pixels were neglected, the average FRM size in terms of number of white pixels was determined by dividing the total number of white pixels in the remaining clusters by the total number of the remaining clusters. Once the average FRM size was determined, the total number of white pixels in all clusters including the large outliers was divided to this average number of white pixels per FRM to estimate the total number FRMs per unit area.
3.2 Results

3.2.1 FRM conjugation and initial room-temperature testing without flow

In order to determine the optimal condition for FRMs conjugation to the rDNAs, different concentrations (3.3, 33, 330, and 3,300 nM) were used to conjugate rDNAs to the FRMs as described above. To determine which concentration had the optimal conjugation conditions, we soaked GCGs with immobilized pDNAs in a 1-M solution of the 200-nt MT followed by rinsing with PBS. We then soaked the GCGs in suspensions of rDNA-conjugated FRMs obtained with different rDNA concentrations for 30 min followed by rinsing with PBS. Fig. 3.5a shows the number of FRMs/mm² on the GCG surface versus the rDNA concentration for rDNA conjugation to the FRMs. As can be seen from Fig. 3.5a, the FRMs conjugated in 330 nM rDNA solution exhibited the most number of FRMs/mm² captured on the GCG surface with identical pDNA immobilization and tDNA hybridization conditions, indicating that the optimal rDNA concentration for the rDNA conjugation on the FRMs was 330 nM. In what follows, all FRMs were conjugated with rDNAs at this concentration.
Figure 3.5: Number of FRMs per mm² captured on the GCG (a) followed by $10^{-10}$ M MT detection versus rDNA concentration and, (b) followed by MT detection versus MT concentration with pDNA optimally immobilized in 330 nM pDNA. Both (a) and (b) obtained at room temperature and without flow.

We then tested thus-obtained FRMs following MT hybridization to the GCG at various MT concentrations for 30 min followed by FRMs binding at $2.1 \times 10^5$ particles/ml and for 30 min where binding of both MT and FRM were carried out at room temperature without flow. The resultant number of FRMs/mm² versus MT concentration is plotted in Fig. 3.5b. As can be seen from Fig. 3.5b, at $10^{-16}$ M and $10^{-17}$ M of MT, the GCG showed about 450 and 300 FRMs/mm², respectively while at $10^{-18}$ M and $10^{-19}$ M of MT tDNA, the number of FRMs/mm² were comparable to that of the negative control at 0 M of MT. Sample fluorescent images of FRMs obtained at $10^{-18}$ M, $10^{-17}$ M, and $10^{-16}$ M MT are shown in inserts I, II, and III, respectively, indicating that the concentration sensitivity of this method without flow was about $10^{-17}$ M of MT.
3.2.2 Effect of Flow Rate and Temperature

Combinations of 3 different temperatures (room temperature, 30°C and 35°C) and 4 different flow rates (no flow, 2, 4 and 6 ml/min) were studied. The MT or WT was first flown for 30 min followed by the flow of FRMs for 30 min at the same temperature and flow rate, and finally washed with a flow of PBS at 2 ml/min at the same temperature as described above. The MT and the WT were kept at $10^{-10}$ M. The number of FRMs/mm$^2$ hybridized to MT on the GCG surface, that to the WT on the GCG surface and that to the negative control versus flow rate at room temperature, 30°C, and 35°C are plotted in Fig. 3.6a-3.6c. It is interesting to note from Fig. 3.6a that at RT, the flow increased the binding of both the MT and the WT in the 2-4 ml/min range but decreased the binding of both MT and the WT as the flow rate increased. Such increase of binding at a low flow rate and decrease in binding upon further increase of flow rate was also observed in the Anthraces bacillus detection. [69] The enhancement of binding by a flow has also been observed in antibody binding on antigen [203]. The enhanced binding at a lower flow rate was presumably due to that flow helped to bring more tDNAs to GCG surface for binding. Without flow, the DNAs would have relied solely on diffusion to get to the GCG surface. While flow can help bring more tDNAs to the GCG surface, it could also generate an impingement force on the FRMs [69] which could overcome the binding force between the tDNAs and pDNAs to unbind them on GCG, thus reducing the overall number of FRMs bound on the GCG surface as the flow rate was further increased. At 30°C and 35°C, though, the binding of both the MT and the WT decreased monotonically with an increasing flow rate, presumably due to the weakening of the binding of the pDNA to both the MT and the WT. Although the binding of the pDNA to both MT and WT decreased with an increasing flow rate at 30°C
and 35°C, what was of interest is that the binding of the pDNA to WT was suppressed to the levels similar to the negative controls at higher flow rates, suggesting that flow made the detection more selective at these two temperatures. In the following, we will refer to the number of FRMs/mm² captured on the GCG surface as the ”signal”, $S$ and $S_{MT}$, $S_{WT}$, and $SC$ refer to the numbers of FRMs/mm² captured on the GCG surface following flowing a $10^{-10}$ M MT solution, a $10^{-10}$ M WT solution, and a control blank PBS, respectively.
Figure 3.6: Number of FRMs per mm² captured by a GCG followed by detection in the control (no MT or WT, black), that followed by detection at $10^{-10}$ M WT (red), and that followed by detection (blue) at $10^{-10}$ M MT, (a) at room temperature, (b) at 30°C, and (c) at 35°C.
To examine if the flow helped increase the detection specificity, we plot the ratio, $S_{WT}/S_C$ versus flow rate in Fig. 3.7a with black for room temperature, red for 30°C and blue for 35°C. As can be seen, with flow, $S_{WT}/S_C$ was significantly reduced to close to unity not only at 35°C but also at 30°C. We further compared the $S_{WT}$ and $S_C$ using the Mann-Whitney U test. [204] The resultant p value versus flow rate is plotted, again, with black for room temperature, red for 30°C and blue for 35°C. As can be seen, at low flow rates of 0 and 2 ml/min $S_{WT}$ and $S_C$ were statistically different ($p<0.05$) at room temperature and at 30°C while at high flow rates of 4 and 6 ml/min $S_{WT}$ and $S_C$ were only statistically different at room temperature but not at 30°C. These results indicated that with the diminished significance of $S_{WT}$ against $S_C$ at a flow rate of 4-6 ml/min permitted specific MT detection at a lower temperature of 30°C as opposed to 35°C with a flow of 0-2 ml/min.
Figure 3.7: (a) $S_{WT}/S_C$ and (b) p value versus flow rate at room temperature (black), at 30°C (red), and at 35°C (blue) where $S_{WT}$ and $S_C$ are number of FRMs per mm$^2$ captured by a GCG followed by detection at 10$^{-10}$ WT and that captured by a GCG followed by detection in control (no WT or MT).

One measure of MT detection specificity with respect to WT is $S_{MT}/S_{WT}$. In Fig. 3.8, we plot $S_{MT}/S_{WT}$ versus flow rate for all three temperatures: room temperature (black), 30°C (red) and 30°C (blue). As can be seen, by increasing the temperature alone, $S_{MT}/S_{WT}$ increased only slightly from about 11 to about 12 at 35°C. With flow, $S_{MT}/S_{WT}$ increased dramatically. More importantly, at 30°C flow weakened the binding of WT more than that of MT to allow $S_{MT}/S_{WT}$ to reach around 24 at 4 ml/min, which was higher than the $S_{MT}/S_{WT}$ at any flow rate at 35°C, indicating that flow could indeed enhance the detection specificity at a low temperature to allow more sensitive detection. The above results indicated that with the present flow setup the optimal detection conditions for the current HBV-DM MT occurred at 30°C and a flow rate of 4 ml/min.
Figure 3.8: $S_{MT}/S_{WT}$ versus flow rate where $S_{MT}$ is number of FRMs per mm$^2$ captured by a GCG followed by detection at $10^{-10}$ MT and $S_{WT}$ is number of FRMs/mm$^2$ captured by a GCG followed by detection at $10^{10}$ WT at room temperature (black), at $30^\circ$C (red), and at $35^\circ$C (blue).

### 3.2.3 $S_{MT}/S_{WT}$ at $10$, $10^3$, $10^6$, and $10^7$ WT/MT concentration ratios

As can be seen from Fig. 3.5b, $10^{-17}$ M was the lowest MT concentration where MT could be detected without flow by the current detection scheme. To see how specific the
MT detection was at this lowest detectable concentration, we used the optimal conditions, i.e., 30°C flow rate of 4 ml/min and carried out MT detection at $10^{-17}$ M for 30 min followed by 30 min of FRMs hybridization. We then carried out WT detection at concentrations at $10^{-16}$, $10^{-14}$, $10^{-11}$, and $10^{-10}$ M (i.e., 10-fold, $10^3$-fold, $10^6$-fold, and $10^7$-fold that of the MT) followed with 30 min of FRMs hybridization. In Fig. 3.9a, we plot number of FRMs/mm$^2$ captured by hybridized WT at different concentrations, with and without 4ml/min flow rate (empty and pattern filled blue bars respectively), and number of FRMs/mm$^2$ captured by hybridized mixture of same concentrations of WT and $10^{-17}$ M of MT with and without 4ml/min flow rate (empty and pattern filled red bars respectively) versus WT concentration at 30°C. Also plotted in Fig. 3.9a are the horizontal bars indicating the number of FRMs/mm$^2$ captured by hybridized $10^{-17}$ M of MT only, at 30°C with and without 4ml/min flow rate (violet and green respectively) and that non-specifically bound of control (brown). In Fig. 3.9b, we plotted the $S_{MT}/S_{WT}$ and $S_{Mix}/S_{WT}$ ratios with and without 4ml/min flow rate deduced from Fig. 9a. As can be seen, without flow, $S_{WT}$ remained different from $S_C$. With a flow of 4 ml/min, $S_{WT}$ was not distinguishable from $S_C$. Moreover, mixture experiments showed that, at 30°C, even the addition of $10^7$ fold more amount of WT did not result in a significant increase in number of hybridized FRMs, when 4ml/min flow is applied. On the other hand, in the case of no flow, number of FRMs hybridized increased significantly. Moreover specificity increased significantly in flow conditions with respect to no-flow conditions as can be seen from Fig. 3.9b, and mixing significantly high concentrations of WT into a solution of $10^{-17}$ M MT did not change this ratio. This indicates that the presence of WT of any concentration at 30°C and 4ml/min had no discernible contribution to detection signal even at a MT concentration as
low as $10^{-17}$ M and that a flow of 4 ml/min made the detection as specific as $10^{-17}$ M MT/$10^{-10}$ M WT, or 1 MT to $10^7$ WT.

Figure 3.9: (a) Number of FRMs per mm$^2$ versus WT concentration and, (b) ratio of number of FRMs per mm$^2$ of MT/WT and Mixture/WT versus WT where mixture is $10^{-17}$ M MT mixed with different WT concentrations, and brown, blue and green horizontal bars represent number of FRMs per mm$^2$ for control, $10^{-17}$ M MT with flow and $10^{-17}$ M MT without flow conditions. Both (a) and (b) are done at 30°C, in 4ml/min flow rate or in no flow conditions.

### 3.3 Discussions

With a cross section area of the detection chamber being 19.25 mm$^2$, the average flow velocity $u$ was 1.7, 3.5, 5.2 mm/s for flow rates 2, 4, and 6 ml/min, respectively. The Reynolds number, $Re = \frac{\rho u w}{\eta}$ was just 6, 12, and 18 at flow rates 2, 4, and 6 ml/min, respectively where $\rho = 1010$ kg/m$^3$ was the density of the fluid, $\eta = 1.05$ cP the viscosity of the fluid and $w$ the width of the flow cell, well in the range of laminar flow. Furthermore
the entrance length, $I_e$, the length over which a fully developed velocity profile can be established once the flow entered the flow cell, can be calculated using:

$$I = 0.06d \, Re$$ \hspace{1cm} (3.1)

where $d$ was the width of the flow cell. According to Eq. (1) even at the highest flow rate which gave the largest $Re$, the entrance length was approximately 3.8 mm which was well below 7.7 mm, the distance from the inlet of the flow cell to GCG. Therefore, the flow in the detection chamber at the position of GCG, was laminar and the flow velocity profile in the width direction was parabolic as schematically shown in Fig. 3b: $u$ was zero at the cell wall and a maximum at the center of the flow. Thus, unlike other systems where the capture surface is part of a wall of the flow channel, [69] at which point the fluid velocity diminishes to zero, the present system situates the capture surface at the center of the flow where the flow velocity is at a maximum (as shown schematically in Fig. 3b). The concept of Goldman et al. [205] for a sphere parallel to a planar wall in a uniform flow could be applied to the FRMs bound on the GCG in the middle of a laminar flow where the flow velocity was uniform as discussed above. According to Goldman et al., in the middle of the flow cell where GCG is situated, one can obtain an analytical expression for the impinging force on a bound FRM as

$$F = (1.7)6\pi\eta(1.5u)$$ \hspace{1cm} (3.2)
where \( u \) was the average flow velocity and \( 1.5 u \) was the flow velocity at the centre of the flow, and \( a = 3 \text{ m} \) the FRM radius. The deduced impinging force on the FRMs by the flow was about 262, 524, and 787 pN with \( u = 1.7, 3.5, 5.2 \text{ mm/s} \) at 2, 4, and 6 ml/min, respectively. Although the exact binding forces between the pDNA and the WT and that between the pDNA and the MT were not known and it was unclear if there were more than one captured MT or WT bound to a FRM it suffices to say that the deduced force was consistent with 70-1500 pN found in the de-hybridization of a single double-stranded DNA [206, 207].

3.4 Summary of Temperature and Flow Enhanced Specificity using Fluorescent Reporter Microspheres

We have investigated the effect of a laminar flow on enhancing the specificity of MT detection at a lower temperature by immersing the detection GCG surface at the center of the flow of the target MT or WT at various flow rates and temperatures. pDNA complementary to the target MT DNA was immobilized on the GCG surface. 30 min of a flow of the MT or WT DNA solution was followed by a flow of \( 6 \times 10^5 \text{ FRMs/ml} \) for 30 min at the same flow rate and temperature. With HBV-DM as the model MT, we have shown that flow can increase the MT detection specificity by lowering the detection temperature to allow (1) a higher ratio of \( S_{MT}/S_{WT} \) and (2) a lower \( S_{WT} \) not distinguishable from \( S_C \). For the present system, a flow rate of 4-6 ml/min reduced the specific MT detection temperature from 35°C without flow to 30°C with a flow rate of 4-6 ml/min. Furthermore, optimal specific MT detection was shown to occur at a lower temperature with flow than the temperature without flow. For example, the detection specificity as measured by \( S_{MT}/S_{WT} \)
was 24 at 30°C and 4 ml/min as opposed to 15 at 35°C without flow. These results clearly indicate that flow can be utilized to help increase mutation detection specificity at a lower temperature.
4. Detection of mutations of single-stranded DNA

4.1 Introduction

Detection of mutations requires specificity that would differentiate one base differences in hybridization reactions. The melting temperature difference between MT and WT tDNAs can be less than 2 degrees Celsius as discussed before in chapter 3. As expected, as the number of mismatches increases the melting temperature difference between a MT and WT tDNA with a pDNA complementary to the MT tDNA would increase. Considering DNA denatures in an "unzipping" mechanism, the proximity of mismatches makes a big difference. Piezoelectric plate sensor (PEPS) demonstrates label-free detection of single stranded target DNA sequences in 1xPBS with a sensitivity level of 100 zM in width extension mode resonance peak without isolation and amplification as shown in chapter 2. In this study, we examine the detection sensitivity of lead magnesium niobate-lead titanate \((\text{PbMg}_{1/3}\text{Nb}_{2/3}\text{O}_3)_{0.65}(\text{PbTiO}_3)_{0.35}\) (PMN-PT) piezoelectric plate sensor(PEPS) in real-time, label-free, in situ MT tDNA mutation hybridization detection in full urine without isolation and amplification with high specificity that would allow detection in a background of excess amount of WT tDNA. A probe DNA (pDNA) sequence specific to HBV-DM used in chapters 3 and 2 and a pDNA specific to kRas codon 12 GGT \(\rightarrow\) GTT transversion are immobilized on the electrical insulation layer on PEPS surface. HBV-DM has been previously shown to be a risk factor for the development of Hepatocellular Carcinoma (HCC) [208]. A high percentage (\(>60\%\)) of HCC patients had HBV-DM in their sera [197, 198]. On the other hand, It has been reported that kRas gene mutations occur in up to 50% of colorectal
adenocarcinomas; these are mainly point mutations at codons 12 and 13, and less frequent at codon 61 [209]. Among the seven most common kRas mutations, codon 12 GGT → GTT transversion (Glycine → Valine) was associated with significantly higher colorectal cancer-specific mortality [210]. Probe DNA sequence used to detect kRas single mutation is composed of DNA and Locked Nucleic Acid (LNA) hybrid nucleic acids. Non-specific binding is prevented by treating the PEPS surface with 3% Bovine Serum Albumin (BSA). Hybridization detection of synthetic target DNA sequences in urine are accomplished at 30°C and 63°C using laminar flow. Further confirmation of detection is accomplished by Fluorescent Reporter Microspheres (FRM) as described in chapters 2. Detection limit of 100 zM is achieved. Furthermore an array of 6 PEPS is used to detect not only the GGT to GTT transversion but also 5 other possible mutations in codon 12 of kRas gene that would lead to colorectal carcinoma. Multiplexed detection setup is only done at 1 fM concentration, to measure cross hybridization between different mutations of the same codon and different pDNA immobilized on each PEPS. No cross hybridization at this concentration (which is probably more than what would be dissolved in any patients urine), is observed, although no dose response experiments are done.

4.1.1 Locked Nucleic Acids

Mismatch discrimination depends on differential hybridization between perfectly matched and mismatched strands hybridizing to form the double stranded duplex. A measure of efficiency of this discrimination is the differences in melting temperatures (Tm) between these species. The Δ Tm is usually small (0.53 °C) for a single mismatch. [211]

Hybrid probes including both DNA and LNA nucleotides were reported to enhance both
duplex stability and mismatch discrimination. [211–218] LNA monomers contain a modified ribose moiety. O-methyl group in LNA residues and bridges 2’ and 4’ carbons of the ribose ring. This covalent bridge locks the ribose in the N-type (3’-endo) conformation. This conformation enhances base stacking and phosphate backbone pre-organization [219] and results in improved affinity for complementary DNA or RNA sequences. LNA-DNA hybrid pDNAs increases T<sub>m</sub> of both perfectly matching and mismatching sequences, however increase in mismatching sequences is much less than that in perfectly matching sequences, widening the T<sub>m</sub> difference of a mismatching and perfectly matching sequence as shown in figure 4.1.
Figure 4.1: 1m indicates the melting temperature curve of a certain mismatching sequence (point mutation) with a pure DNA probe. 1p indicated the melting temperature curve of the same sequence with a perfectly matching pure DNA probe. 2m and 2p indicates the same melting curves with a LNA-DNA hybrid pDNA which contains 3 LNA bases centered around the mismatching base. Figure adopted from [5].

The use of LNA-DNA hybrid pDNAs were necessary to differentiate MT and WT tDNA with high specificity. The design in figure 4.1 is used to in this study to maximize the effect of LNA nucleotides as discussed in [5].
4.2 Materials and Methods

4.2.1 PEPS Fabrication

PEPS used in this study were fabricated from (PbMg$_{\frac{1}{3}}$Nb$_{\frac{2}{3}}$O$_3$)$_{0.63}$(PbTiO$_3$)$_{0.37}$ (PMN-PT) freestanding films 8 m thickness that was coated with 110 nm thick Cr/Au electrode by thermal deposition (Thermionics VE 90) cut into 2.5 mm by 0.7 mm strips by a wire saw (Princeton Scientific Precision, Princeton, NJ). Gold wires, 10 m in diameter, were glued to the top and bottom electrodes of each strip using conductive glue (XCE 3104XL, Emerson and Cuming Company, Billerica, MA). The rear end of the strip was fixed on a glass substrate by a nonconductive glue (Loctite 1C Hysol Epoxy Adhesive) to form the PEPS geometry as illustrated in Fig. 2.1d and poled at 15KV/cm at 90°C for 60 min. in an incubator (Digital Control Steel Door Incubator 10-180E, Quincy Lab). The dielectric constant of the PEPS was measured using an electrical impedance analyzer (Agilent 4294A) to be about 1800 with a loss factor of 2.5-3.7% at 1 kHz.

4.2.2 Electrical Insulation

The PEPS were electrically insulated to stabilize the resonance peaks for in-liquid detection by using a new 3-mercaptopropyltrimethoxysilane (MPS) (Sigma-Aldrich Co. LLC.) solutions coating scheme involving enhanced MPS cross-linking at pH=9.0 and with the addition of water.(135) First, the PEPS was cleaned in a Piranha solution (two parts of 98% sulfuric acid (Fisher) with one part of 30% hydrogen peroxide (Fisher)) for 1 min, followed by rinsing in water and ethanol. Before coating MPS at pH=9.0, we dipped the PEPS in 50 ml of a 0.01 mM MPS (98% v/v) solution in ethanol (Fisher) with 0.5% of de-
ionized water for 30 min to promote hydrolysis. It is then rinsed with water and ethanol and immersed in 50 ml of a 0.1% MPS solution in ethanol with 0.5% of DI water at pH=9.0–adjusted by adding KOH (Flakes/Technical, Fisher)–for 12 hr. The PEPS was then rinsed with water and ethanol and then subjected to four more rounds of 12-hr soaking in a fresh 0.1% MPS solution at pH=9.0 with 0.5% water with each soaking followed by rinsing in water and ethanol. It has been shown such a pH=9.0 MPS coating scheme resulted in a dense and smooth MPS coating at a rate of about 1.6 nm/hr and resultant current density of 2 A/cm², a more than 100 fold reduction than MPS coating obtained at pH=4.5 without water.(135) The in-air and in-phosphate buffer saline (PBS) solution phase angle versus frequency resonance spectra of a PEPS are shown in Figure 4.2a. As can be seen, the baseline and the length-extension-mode (LEM) resonance peak and the width-extension-mode resonance (WEM) peak of the in-liquid spectrum were close to that of the in-air spectrum, indicating the effectiveness of the new MPS coating at pH=9.0 with water.
Figure 4.2: (a) In-air (black) and in-PBS (red) phase angle-versus-frequency resonance spectra, and (b) relative resonance frequency shift, $\Delta f/f$, of the PMN-PT PEPS during the various steps of probe cDNA immobilization and target DNA detection. The insert in (b) shows a schematic of the molecules involved in the immobilization and detection.
4.2.3 pDNA Immobilization

4.2.3.1 Immobilization of pDNA for detections of kRas codon 12 GGT to GTT transversion and HBV-DM mutations

To immobilize the amine-activated pDNA on the PEPS surface, the MPS-coated PEPS was first immersed in a solution of 5mM of sulfo-SMCC dissolved in 200 µl 1xPBS (pH adjusted to 6.5) for 1 hour. The sensor is then washed three times with deionized water and then it is immersed in a solution of 10 µM of amine activated probe DNA dissolved in 200 µl of 1× PBS (pH 8.0) as shown in Figure 4.3. The relative resonance frequency shift, Δf/f, of the first WEM peak at various steps of the immobilization process is shown in Figure 4.2b. Also shown in the insert in Figure 4.2b is a schematic of the various steps involved in the immobilization process.
4.2.4 Immobilization of pDNA for 6 mutations on codon 12

The immobilization of biotinylated pDNAs designed for multiplexed detection are done using maleimide activated biotin as discussed in chapter 2.

4.2.5 Non-specific binding

Non-specific binding was prevented treating the PEPS with different percentages of Bovine Serum Albumin (Sigma) after pDNA immobilization. BSA is dissolved in 1x PBS.
and PEPS was immersed in this solution for 1 hour followed by washing 5 times with 1x PBS. WEM resonance peak of PEPS were monitored in urine in order to check for non-specific binding. This process was repeated systematically by increasing the BSA percentage used for blocking until no non-specific binding was observed as seen in Figure 2.12.

4.2.6 Target DNAs, probe DNA, reporter DNAs and FRMs

The probe DNAs (tDNA) used to detect HBV-DM was a 16-nucleotide (nt) long single-stranded DNA (Sigma) containing the nucleotide sequence of the Hepatitis B virus genome (GeneBank Accession #X04615) centered around the 1762T/1764A double mutations.(136) The probe DNA used to detect kRas codon 12 mutation was a 17 nucleotide DNA-LNA hybrid fragment (Exiqon, Inc) derived from the kRas gene sequence (Gene ID: 3845) centred around the point mutation. Three LNA bases are centred around the mutation site and the rest of the sequence consists of DNA bases (Figure 4.4a-b). This design is chosen to have the highest melting temperature between wild type and mutant tDNA hybridization reactions.(137) Target DNA sequences (Sigma) designed for both diseases are 50 nucleotides long and in both cases MT tDNA sequences consists of the region complementary to the pDNA and upstream region of the gene and WT tDNA sequences consists of the region complementary to the pDNA except the mutation sites and the downstream region of the gene (Figure 4.4a-b). Sequences and melting estimated temperatures are shown on Table 4.1. Both pDNA sequences had a biotin with a 12-polyethylene glycol (PEG) spacer at the 5’ end. The tDNA and pDNA sequences designed for multiplexed detections of kRas codon 12 mutations are shown on table 4.2. The tDNA sequences were 50 nucleotides long
(sequences not shown) as those in table 4.1, the only difference was in the mutation site where each tDNA were perfectly matching with the pDNA on that region.
Figure 4.4: Schematics of pDNA, Wild Type and Mutant tDNAs and upstream and downstream rDNAs designed for (a) HBV-DM and (b) kRas mutations. Mutation site(s) are shown with patterned boxes, dashed lines indicate the hybridization sites and mismatch(es) on Wild Type tDNAs is/are indicated. (c) Bright blue and yellow-green microspheres are conjugated with upstream and downstream microspheres to be used as Mt-FRM and Wt-FRM.
There were two different 30-nt long rDNAs (Sigma) complementary to the sequence of the MT tDNA upstream of the pDNA binding site and complementary to that of WT tDNA downstream of the pDNA binding site for both diseases. The upstream rDNAs were amine activated with a 12-PEG spacer at the 5’ end and the downstream rDNAs were also amine activated but with a 7-PEG spacer at the 3’ end. The sequence of the upstream rDNA and that of the downstream rDNA are also shown in Table 4.1. Figure 4.5 is a schematic illustrating the relationship between tDNA, pDNA, and upstream and downstream rDNAs. FRMs are prepared as explained previously in chapter 3.
Figure 4.5: Schematic of the detection setup. pDNA (Pink) is immobilized on SMCC conjugated PEPS surface via its primary amine attached on the 5’. WT tDNA (light green) and MT tDNA (dark blue) can hybridize on pDNA with mismatch(es) and no mismatches respectively. Bright blue FRMs (dark blue sphere) conjugated with MT rDNA (yellow) and Yellow Green FRMs (green sphere) hybridizes on MT tDNA and WT tDNA respectively.

4.2.7 Urine samples and Experimental Setup

Urine mixtures used for detection are prepared done by mixing different concentrations of tDNAs (as a mixture of WT and MT or MT alone or WT alone) in 50 ml of urine. PEPS is immersed in a flow cell as seen in Figure 3.3b. Experimental setup involving solutions of tDNA, FRM, PBS washings and Peristaltic Pump are prepared as explained previously. The whole setup was installed inside an incubator (Digital Control Steel Door Incubator 10-180E, Quincy Lab) for temperature control, including a 2 liter water bath for minimization
of evaporation during experimentation. Detection experiments involving HBV-DM were done using 4ml/min flow rate at 30°C as determined previously in chapter 3, and those involving kRas were done using 6ml/min flow rate at 63°C. This temperature is determined after experimentation with MT and WT at different temperatures. The array of 6 PEPS is shown in figure 4.6. Each PEPS is immobilized with one type of pDNA specific to one mutation shown in Table 4.2. Flow cell used for single PEPS detections is used. PEPS are connected to a relay which is controlled by the same MatLab routine discussed in chapter 2. Each time an impedance scan is performed by the analyzer, the relay is switched to the next PEPS (order of which is defined by the user) for the next impedance scan until the user stops the sequence.
Figure 4.6: Array of 6 PEPS is shown inside the flow cell (bottom), and a blow up of this array is shown on top. Each PEPS is shown in different colors indicating immobilization by different pDNAs, each specific to one type of mutation.
4.3 Results and Discussion

4.3.1 HBV-DM single stranded DNA detections

As previously discussed in Chapter 3, HBV-DM mutations can be detected specifically at 30°C with 4 ml/min flow rate 3.8. In Chapter 2 hybridization of HBV-DM MT tDNA is studied in room temperature and 2ml/min flow rate, 2.13a. However in this condition, hybridization of WT-tDNA is only studied in Chapter 3 on GCGs but not on PEPS.

4.3.1.1 Detection of HBV-DM MT and WT tDNA

Figure 4.7a below shows the detection of 10 aM and 100 zM of HBV-DM WT-tDNA at room temperature with 2 ml/min flow rate. Figure 4.7b shows the comparison of average relative frequency shifts between 25th-30th min.(signal of hybridization of tDNA) and 55th-60th min.(signal of hybridization of FRM) of MT tDNA and WT tDNA detections and these concentrations at this condition. As can be seen relative frequency shifts are very close at 10 aM. It is important to note here that in real patient samples WT tDNA is almost 10^3 fold higher in concentration. Thus it is obvious that this condition is not suitable for detection of mutations.
Figure 4.7: (a) Detection of HBV-DM Wt tDNA at room temperature and 2ml/min flow rate. 100zM and control experiments are shown in the inset. Detections were confirmed by FRM hybridizations and PBS background. (b) Average relative frequency shifts of tDNA and FRM hybridizations of 10 aM and 100 zM of MT-tDNA and WT-tDNA of HBV-DM are plotted. Experiments are done at room temperature and 2ml/min flow rate.

Detections of MT-tDNA and WT-tDNA of HBV-DM are also carried out in 30°C and and 4 ml/min flow rate separately. 10, 5 and 1 aM and 100 zM of MT-tDNA and 100,25 and 1 aM of WT-tDNA are detected as shown in Figure 4.8a and b respectively. Average relative frequency shifts between 25th-30th min. (\(S_{MT}\) and \(S_{WT}\)) and 55th-60th min.(\(S_{FRM}^{MT}\) and \(S_{FRM}^{WT}\)) of these detections are plotted in Figure 4.8c. The \(S_{MT}/S_{WT}\) and \(S_{FRM}^{MT}/S_{FRM}^{WT}\) ratio are plotted in Figure 4.8d.
Figure 4.8: Relative frequency shift versus time graph of detections of (a) 100zM, 100, 1.5 and 10 aM of MT tDNA followed by MT-FRM hybridization (b) 1 aM, 25 aM, 100 aM, 10 fM and 100 fM of WT tDNA followed by WT-FRM hybridization in urine at 30°C and 4ml/min flow rate (c) Relative frequency shifts in the last five minutes of tDNA and FRM hybridization parts (signal, "S") of experiments in (a) and (b) plotted versus concentration and (d) The ratios of $S_{MT}/S_{WT}$ are plotted for tDNA and FRM hybridization parts separately.

Figure 4.8b shows that at 25 aM of WT tDNA detection although there is detectable
hybridization of tDNA hybridization of FRMs was not detected. This is also confirmed by fluorescent microscopy as there was no WT-FRMs on the surface. This indicates that detection of tDNA by PEPS may not always be confirmed by FRM hybridization, especially at low concentrations or hybridizations of low affinity, which may occur in unfavorable conditions for a hybridization reaction (such as, high temperature, low salt concentration or pH). Figure 4.8c shows that as the concentration increased the number of hybridized tDNA is increased for both MT and WT tDNAs. The increase in relative frequency shift in MT is apparent even at 100zM. On the other hand, such an increase can only be observed at a 1000 fold higher concentration for WT. Moreover signal (S) of WT tDNA at 100aM is close to that of MT at a 100 fold lower concentration of 1aM. In figure 4.8d, although the increasing behavior of $S_{MT}/S_{WT}$ with MT tDNA concentration does not mean much since the $S_{WT}$ was already very close to control level in those low concentrations, the $S_{MT}/S_{MT}$ was at least 23. However it can still be deduced from figure 4.8c that $S_{MT}$ at 10 aM (long before $S_{MT}$ reaches plateau), is more than 6 times $S_{WT}$ at 100 fM (plateau is reached for $S_{WT}$).

4.3.1.2 Detection of mixtures of HBV-DM MT and WT tDNA

As discussed in chapter 3 patients usually have extensive amount of WT tDNA with respect to the amount of MT tDNA. Thus it is not enough to detect low concentrations of MT tDNA most of the time. MT tDNA has to be detected in urine samples with a background of abundant WT tDNA. In order to test if the conditions optimized for this demanding specificity requirements a 1:250 ratio of MT to WT ratio is kept constant for mixture detections of HBV-DM.
For this purpose, 100zM, 1, 10 and 100 aM of MT tDNA is mixed with WT tDNA concentrations such that 1:250 MT to WT ratio is kept constant. Figure 4.9a shows the relative frequency shifts versus time graphs of hybridizations of MT and tDNA hybridizations which is followed by hybridizations of MT-FRM and WT-FRM. In order to check the interference of excess amounts of WT tDNA on hybridization of MT tDNA the signal (S) from hybridizations of MT and WT tDNA detections of the same concentrations done separately is added and compared with the mixture experiments as shown in figure 4.9b for both tDNA and FRM hybridization parts. As can be seen, as the concentration is increased from 100zM to 10aM the interference from WT tDNA becomes apparent as difference between the sum of signal from "WT tDNA-only"(red) and "MT tDNA-only"(black) experiments and mixture experiments (blue) increases. This was of course expected as WT tDNA interferes solely by its existence even though it does not hybridize with pDNA significantly. The same behavior is also observed in the FRM hybridization parts (patterned bars of the corresponding colors from tDNA experiments). Number of FRMs hybridized on PEPS surface are plotted versus concentration for these detections as shown in 4.9c and the micrographs are also shown on the lower right in the same figure. As can be seen although WT FRM hybridized on the surface increases as concentrations of tDNAs are increased, the rate of this increase is less than that of MT-FRM as can be deduced from the number of MT-FRMs on the surface.
Figure 4.9: (a) Relative frequency shift versus time graph of HBV-DM mixture detection experiments with 100zM, 1, 10 and 100 aM of MT tDNA mixed with 250 fold more amount of WT tDNA (Concentrations of MT tDNA in the mixture experiments are shown on the x axis). (b) Signal (S) of tDNA and FRM hybridization parts of mixture experiments are compared with sum of that of "MT-only" and "WT-only" experiments. (c) Number of MT-FRMs and WT-FRMs on PEPS surface was counted and plotted versus concentration of MT tDNA of mixture experiments.(d) Micrographs of PEPS after HBV-DM mixture detection experiments are shown. MT-FRMs are shown in blue and WT-FRMs are shown in green.
4.3.2 kRas single stranded tDNA detections

4.3.2.1 Determination of optimum temperature for detection of kRas mutations

In order to determine the optimum temperature at which the ratio of hybridizations of MT and WT tDNAs is maximized, detections of 10 fM of MT and WT tDNA are carried out at 55°C, 60°C and 63°C with 4ml/min flow rate (Figure 4.10a). These temperatures are chosen considering the $T_m$ of MT and WT tDNA are estimated at 70°C and 55°C respectively as shown in Table 4.1. As can be seen from figure 4.10b although the number of hybridized tDNAs on PEPS is inversely proportional with temperature for both MT (black) and WT (red) tDNAs (y axis on the left for relative frequency shifts), the ratio of signal from MT to WT (blue) was maximized at 63°C (y axis on the right for the ratio). Thus all the following detections of kRas mutations were carried out at 63°C at 4ml/min flow rate. 4.10
4.3.2.2 Detection of kRas MT and WT tDNA

kRas mutation detections represented even an harder specificity problem with respect to the HBV-DM detections. Not only because kRas is a point mutation instead of a double mutation, but also the mismatch sites were also close (one base apart) in HBV-DM detections as discussed in chapter 1. This is overcome with incorporation of LNA bases which required optimization of flow and temperature conditions once more. As shown previously in chapter 3, best specificity for the HBV-DM detections were achieved with a 4ml/min flow rate, thus mixture experiments of kRas mutation detections are carried out using this flow rate. Detections of 100 zM, 1, 10, and 100 aM of MT-tDNA (figure 4.11a) and 100 zM, 1 aM, 10 aM, 100 aM, 10 fM and 100 fM WT-tDNA (figure 4.11b) is done at this
condition. WT-tDNA did not show a significant signal until a threshold concentration of 10 aM is exceeded. Moreover 10 fM and 100 fM showed a very similar signal indicating the hybridization of WT reaches a plateau much sooner than MT-tDNA as can be seen in figure 4.11c. As expected the signal from the FRM hybridization was around 1.2 fold of that of tDNA hybridization for every concentration. The signal ratio of MT/WT tDNA are plotted in figure 4.11d. Contrary to the HBV-DM mutation detections shown in figure 4.8d, signal ratio of MT/WT is observed inversely proportional with concentration, although the ratio is higher for any given concentration with respect to HBV-DM mutation detections. This inverse proportionality is probably meaningless as because as can be seen in figure 4.11b these three concentrations (1 aM, 10 aM and 100 aM) are very close to control level. However as can be extrapolated from the figure 4.11c, $S_{MT}$ at 100 aM tDNA is more than 10 times higher than $S_{MT}$ at 1pM tDNA. Moreover $S_{MT}$ did not reach plateau at 100 aM whereas $S_{WT}$ is already in the plateau in 1pM.
Figure 4.11: Relative frequency shifts of detections of (a) 100 zM, 1, 10 and 100 aM of MT tDNA and (b) 100 zM, 1, 10, 100 aM, 10 fM and 100 fM of WT-tDNA are plotted versus time. (c) Signal(S) (average of relative frequency shifts between 25th-30th min. (tDNA hybridization) and 55th-60th min. (FRM hybridization) versus tDNA concentration of detections in (a) and (b). (d) $S_{MT}/S_{WT}$ plotted versus tDNA concentration.
4.3.2.3 Detection of mixtures of kRas MT and WT tDNA

At the optimum condition discussed above mixture of MT and WT kRas tDNA detections were done at a fixed ratio of MT:WT of 1:1000. 100zM, 1, 10 and 100 aM of MT tDNA is mixed with 1000 fold more amount of WT tDNA in urine samples. tDNA detections are followed by MT-FRM and WT-FRM hybridizations as shown in figure 4.12a. As expected, concentration and relative frequency shifts were directly proportional for both tDNA and FRM hybridization parts. Signal(S) of tDNA and FRM hybridization parts of kRas mixture experiments, HBV-DM mixture experiments and HBV-DM MT tDNA detection experiments (denoted by ”@RT” refering to ”room temperature”) done in chapter 2 are compared in Figure 4.12b. Highest signals were from HBV-DM MT tDNA detections which were carried out in room temperature. This was expected because room temperature and 2ml/min flow rate was a favorable condition for hybridization as there were no need of specificity against WT tDNA. However, kRas mixture experiments showed a higher signal for the tDNA hybridization with respect to that shown by HBV-DM mixture experiments. This was probably related to the high hybridization affinity of LNA containing pDNAs with tDNAs. However interestingly, FRM hybridization parts indicated the opposite. Although higher numbers of FRMs are hybridized on PEPS surface for the kRas mixture detections (as shown in Figure 4.12c), signal from the PEPS was higher in the HBV-DM mixture detections. This may be related to temperature dependence of stress generated on the PEPS surface by FRMs or to the extend that the stress is turned into resonance frequency shifts by PEPS at different temperatures. Micrographs of MT-FRMs and WT-FRMs are also shown in Figure 4.12d.
Figure 4.12: (a) Relative frequency shift versus time graph of kRas mixture detection experiments with 100zM, 1, 10 and 100 aM of MT tDNA mixed with 1000 fold more amount of WT tDNA (Concentrations of MT tDNA in the mixture experiments are shown on the x axis). (Signal of tDNA and FRM hybridization parts of kRas and HBV-DM mixture detection experiments compared with with that of HBV-DM MT tDNA detection experiments carried out in room temperature (denoted by "@RT") in chapter 2). (c) Number of MT-FRMs and WT-FRMs hybridized on PEPS surface after detections in (a). (d) Micrographs of PEPS surface after detections carried out in (a)
4.3.3 Multiplexed Detection of kRas codon 12 Mutations

The PEPS array composed of six sensors are illustrated in figure 4.6. Each PEPS is immobilized with one pDNA in table 4.2. In air impedance spectra of each PEPS used in the array is shown in figure 4.13 together with the $k_{31}$ calculated from the impedance using the formula in chapter 1.

![Impedance spectra of PEPS used in multiplexed detection.](image)

Figure 4.13: Impedance spectra of PEPS used in multiplexed detection.

In order to see if there is any cross hybridization of tDNAs with pDNAs on PEPS’ surfaces, 100 aM of all 6 tDNA's is dissolved in different urine samples. Each PEPS is immobilized with a different pDNA and 3% BSA blocking is done as explained before. Samples of urines containing different tDNAs are flown one by one, through the flow cell including the array of PEPS. Detections are carried out in the same condition as before (63°C and 4 ml/min flow rate). Relative frequency shifts of all PEPS are recorded simul-
taneously and plotted as seen in figure 4.14. As can be seen no cross hybridization is observed. This was expected as not only one but 2 bases were mismatching for half (15) of the cross hybridizations and the rest (15) were one mismatch cross hybridizations, which would be no different than separation of MT and WT tDNA as shown in previous results.
Figure 4.14: Relative frequency shifts of PEPS used in multiplexed detection of 6 mutations of kRas codon 12, known to be associated with colorectal carcinoma. 6 urine samples containing 6 different mutations are flown one by one over the PEPS array. Each sample was flown for 30 min.
4.4 Summary of Single Stranded DNA mutation detections

100 zM concentrations of HBV-DM and kRas MT tDNA can be detected in urine with a background of 250 and 1000 fold more WT tDNA respectively. Possibility of multiplexed tDNA detection was shown for 6 possible mutations of a single codon. Considering mutations/SNPs and other chromosomal abnormalities associated to a disease would occur in different regions rather than in a single codon, multiplexed detected can be easily accomplished using PEPS. LNA-DNA hybrid pDNAs showed an increase in specificity such that, even the \( \Delta T_m \) difference between MT and WT was much lower in the single mutation with respect to double mutation (2\(^{\circ}\)C and 20\(^{\circ}\)C respectively) if pDNA were pure DNA, MT tDNA could be detected in urine with a background of higher concentration of WT (1000 fold with respect to 250 fold in single mutation).
Table 4.1: The sequences and $T_m$ of the pDNA, tDNA, urDNA and drDNA for detection of mutations of kRas and HBV-DM

<table>
<thead>
<tr>
<th>Type of DNA</th>
<th>Sequence (5’ to 3’)</th>
<th>$T_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>drDNA (kRas)</td>
<td>Amine-C12-GCTGAAAATGACTGAATATA AACTTGTGGTAGT</td>
<td>73</td>
</tr>
<tr>
<td>urDNA (kRas)</td>
<td>GCAAGAGTGCCTTGACGATA CAGCTAATTCAGA-C7-Amine</td>
<td>78</td>
</tr>
<tr>
<td>pDNA (kRas)</td>
<td>Amine-C12-TGGAGCT+G+T+TGGCGTAG</td>
<td>—</td>
</tr>
<tr>
<td>MT-tDNA (kRas)</td>
<td>TCTGAATTAGCTGTATCGTAAGGCAC TCTTGCCCTACGCAAACAGCTCCA</td>
<td>70</td>
</tr>
<tr>
<td>WT-tDNA (kRas)</td>
<td>CTACGCCACCCAGCTCCAACCTACCAC AAGTTATATTCAGTCATTTTCAGC</td>
<td>55</td>
</tr>
<tr>
<td>drDNA (HBV-DM)</td>
<td>Amine-C12-ACAGACCAATTTATGC CTACAGCCTCTAG</td>
<td>76</td>
</tr>
<tr>
<td>urDNA (HBV-DM)</td>
<td>AATCTCCTCCCCCACAACCTCCTCC ACGTCTTT-C7-Amine</td>
<td>77</td>
</tr>
<tr>
<td>pDNA (HBV-DM)</td>
<td>Amine-C12-ACAAAGATCATTAACC</td>
<td>—</td>
</tr>
<tr>
<td>MT-tDNA (HBV-DM)</td>
<td>TTTAAAGACTGGGAGGAGTTGG GGGAGGAGGATTAAGTTAATGATCTTTGT</td>
<td>47</td>
</tr>
<tr>
<td>WT-tDNA (HBV-DM)</td>
<td>GGTTAAAAGGTCTTTGTACTAGG AGGCTGTAAGGCATAAAATTTGGCTGTTCA</td>
<td>23</td>
</tr>
</tbody>
</table>

Mutation sites indicated by underlines in MT-tDNAs and WT-tDNAs, and in bold letters in pDNAs

Fragments of tDNAs hybridizing with pDNA are shown in bold on pDNA sequences

LNA bases are indicated by a “+” sign preceding the nucleic acid

$T_m$’s are melting temperatures with tDNA(C°)
Table 4.2: The sequences of pDNAs and $T_m$’s of the pDNA with MT and WT tDNA, for detection of mutations of 6 kRas mutations

<table>
<thead>
<tr>
<th>PEPS’ name</th>
<th>Codon 12 Mutation</th>
<th>pDNA</th>
<th>$T_m^{MT}$</th>
<th>$T_m^{WT}$</th>
<th>$\Delta T_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEPS1</td>
<td>AGT</td>
<td>BioTEG-tggagc+t+A+gtgcgtag</td>
<td>68</td>
<td>52.7</td>
<td>15.3</td>
</tr>
<tr>
<td>PEPS2</td>
<td>CGT</td>
<td>BioTEG-tggagc+t+C+gtgcgtag</td>
<td>71</td>
<td>50.1</td>
<td>20.9</td>
</tr>
<tr>
<td>PEPS3</td>
<td>GAT</td>
<td>BioTEG-tggagct+g+A+gtgcgtag</td>
<td>70</td>
<td>54.7</td>
<td>15.3</td>
</tr>
<tr>
<td>PEPS4</td>
<td>GCT</td>
<td>BioTEG-tggagct+g+C+gtgcgtag</td>
<td>72</td>
<td>51.1</td>
<td>20.9</td>
</tr>
<tr>
<td>PEPS5</td>
<td>GTT</td>
<td>BioTEG-tggagct+g+T+gtgcgtag</td>
<td>70</td>
<td>54.3</td>
<td>15.7</td>
</tr>
<tr>
<td>PEPS6</td>
<td>TGT</td>
<td>BioTEG-tggagc+t+T+gtgcgtag</td>
<td>69</td>
<td>53.3</td>
<td>15.7</td>
</tr>
</tbody>
</table>

Codon 12 complementary region on pDNAs are indicated in bold letters
pDNA’s are 5’ biotinylated
Mutation sites are indicated by capital letters
LNA bases are indicated by a “+” sign preceding the nucleic acid
All sequences are from 5’ to 3’
Biotin-TEG is a biotin attached to a 15-atom mixed polarity triethylene glycol spacer
5. Detection of double stranded DNA

5.1 Introduction

Detection of any double stranded via hybridization may only be done by denaturation of the two complementary strands it composed of as described in chapter 1. Although hydrogen bonding can occur between triplets (RNA triplets [220], or DNA triplets [221]) and even quadruple helices in cells as discovered recently [222] in different occasions, none of these kind of hybridizations or recognitions would be sufficiently strong to detect minute amounts of transrenal DNA in urine.

Boiling a sample of urine would be sufficient in order to facilitate heat denaturation. However in order to prevent annealing or hybridization of heat denatured complementary strands in a sample, it has to be cooled down as fast as possible to the detection temperature. The denaturation or annealing of DNA can be monitored in real time using absorbance as it is discussed in chapter 1. In figure 5.1 [223], the effect of cooling rate is shown by measuring the absorption of light with 260nm wavelength. As can be seen, rapid cooling allows only the formation of local regions of dsDNA, formed by the base pairing or annealing of short regions of complementarity within or between DNA strands; the decrease in A260 is hence rather small. On the other hand, slow cooling allows time for the wholly complementary DNA strands to find each other, and the sample can become fully double-stranded, with the same absorbance as the original native sample.
In this study the effect of cooling rate is investigated and the fastest cooling method is developed for the detection of kRas codon 12 GGT to GTT transversion and HBV 1762T/1764A double mutation. In order to prevent annealing of denatured complementary DNA fragments further, capture DNA fragments, having exactly the same sequence as the target DNA, are immobilized on GCGs. Effect of this competitive inhibition on annealing is investigated. Detection of kRas and HBVDM double stranded DNA fragments in urine is achieved with 100 zM limit of detection in a background of 1000 and 250 fold more concentration of WT double stranded DNA fragments.
5.2 Materials and Methods

5.2.1 PEPS fabrication, electrical insulation, pDNA immobilization and BSA blocking

PEPS fabrication, electrical insulation and BSA blocking are done as discussed in chapters 4 and 2. pDNA immobilization is done using sulfo-SMCC as a linker between MPS covered PEPS surface and amine activated pDNA for detections of HBVDM double stranded and kRas codon 12 GGT to GTT transversion DNA mutations as discussed in chapters 3 and 4.

5.2.2 Target DNAs, probe DNA, reporter DNAs and FRMs

pDNA sequences used in this study was the same with those used in chapter 4 for detection of HBV-DM and kRas single stranded mutations (4.1). 90 bp. tDNAs on the other hand were double stranded and supplied from IDT (Integrated DNA Technologies) sequence of which are shown in table 5.1. urDNAs and drDNAs were the same as in table 4.1 in chapter 4. Only Bright Blue FRMs were used in this study since MT and WT tDNA both have the upstream and downstream regions thus it was not possible to hybridize different color FRMs on MT and WT tDNA when mixture experiments were being conducted.

Double stranded DNA is not only detected from synthetic sources, but also from SW480 cells cultured in cell medium. SW480 cell line is homozygous for kRas codon 12 GGT to GTT transversion. DNA is extracted from the cells using whole human genome DNA extraction kit and then sonicated to generate an average fragment size of 500 bps. The
Table 5.1: The sequences of tDNAs, used for detection of mutations of double stranded kRas codon 12 GGT to GTT point mutation and HBV-DM

<table>
<thead>
<tr>
<th>Type of DNA</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-tDNA (kRas)</td>
<td>GGTTCCTGAAATTAGCTGTACTCTGCAAGGCCTTTGCCTACGCCAACAGCTCCAACCTACCACAAGTTTTATATTCAAGTCATTTTCAGCACG</td>
</tr>
<tr>
<td>WT-tDNA (kRas)</td>
<td>GGTTCCTGAAATTAGCTGTACTCTGCAAGGCCTTTGCCTACGCCAACAGCTCCAACCTACCACAAGTTTTATATTCAAGTCATTTTCAGCACG</td>
</tr>
<tr>
<td>MT-tDNA HBV-DM</td>
<td>TTGGTTAAAGACTGGGAGGAGTTGGGGGGAGGTCTTTGAGTTAGGGAAGCTGTCTGTCA</td>
</tr>
<tr>
<td>WT-tDNA HBV-DM</td>
<td>TTGGTTAAAGACTGGGAGGAGTTGGGGGGAGGTCTTTGAGTTAGGGAAGCTGTCTGTCA</td>
</tr>
</tbody>
</table>

Mutation sites indicated by underlines in MT-tDNAs and WT-tDNAs. Fragments of tDNAs hybridizing with pDNA are shown in bold on pDNA sequences extracted cells are then dissolved in urine samples of required concentrations and detections were same as in kRas synthetic double stranded tDNA at 63°C and 4ml/min flow rate.

5.2.3 Urine samples and Experimental Setup

Urine samples were collected and prepared as it was done in chapters 2 and 4. Experimental setup involved a modified version of the setups used in those chapters. Two flow cells are used for detection as shown in figure 5.2.
Figure 5.2: Schematic of the experimental setup used to detect dsDNA. (a) Urine samples are heated to boiling temperature for 15 min. Hot sample (shown in red piping) is flown through a water bath that is inside an incubator which is equilibrated to detection temperature. (b) Schematic of the flow system is shown. Sample is flown through 2 flow cells including capture DNA immobilized GCG and pDNA immobilized PEPS in order. Detected sample is then discarded in the waste container. Dashed line illustrates the incubator in (a), everything inside the dashed line is in the incubator.

5’ amine activated capture DNA, having the same sequence as tDNA is immobilized
on 3mm$^2$ GCG as described in chapter 3. Capture DNA is used to hybridize to the already denatured double stranded DNA in urine. Because it has the same sequence as tDNA of interest, it competes with tDNA in hybridizing the complementary strand thus decreasing the probability of annealing. Moreover three different DNA cooling rates have been investigated. Three cooling regimes can be summarized as in the following.

1) **Slow cooling rate (22 min):** Boiled urine sample (50 ml) is passed through 1 m of plastic tubing (Tygon R3603) with an inner diameter of 1/16 inches. The tubing is immersed in iced water bath (4-10 °C). Collected sample was waited to be equilibrated with room temperature and detection is done afterwards. This method took approximately 22 min.

2) **Moderate cooling rate (4 min):** Boiled urine sample is passed through 2 m of same tubing. Tubing was in contact with ambient air. Total volume of the tubing was approximately 4 ml, which took 4 min. for the sample to pass through (flow rate was kept constant at 1 ml/min for all the detections) and the collected sample was at room temperature. Detection is directly done without no need of waiting for equilibration.

3) **Fast Cooling rate (1 min):** Boiled urine sample is passed through 50 cm. of tubing which is immersed in room temperature water bath. It took 1 min. for the sample to pass through the piping with 1 ml/min flow rate. Detection was made directly with no need for equilibration.
5.3 Results and Discussion

5.3.1 Comparison of Denaturation and tDNA capture methods

Effect of cooling rate and existence of capture DNA is investigated detecting 10 aM of 90 bp. HBV-DM double stranded DNA is done at room temperature after the above described cooling regimes. Figure 5.3a below is the relative frequency shift versus time graph for the experiments consisting of 6 possible combinations of 3 different cooling rates and 2 cases of existence of capture DNA. Relative frequency shift of 50 nts long ssDNA of same target done in room temperature (2.13b) is also plotted for comparison. As expected although the length of ssDNA was less than the dsDNA used in these detections the relative frequency shift was higher than any of the dsDNA detections. This is due to inevitable inefficiency of cooling. Most probably, some portion of denatured DNA is hybridized back with its complementary before sample hits the PEPS surface. The percentage of relative frequency shifts of different denaturation regimes are plotted in figure 5.3b. Black bars indicate the shifts in different cooling regimes without capture DNA. For each cooling regime difference in percentage relative frequency shifts of detections with capture and without capture DNA is plotted in red (In other words, for each cooling regime on the x axis, sum of black and red bars indicate the percentage relative frequency shift with capture DNA and black bars represents the detections with no capture DNA). As can be seen effect of capture DNA was least in the slowest cooling rate. This was expected since 22 min. was already enough time for renaturation of dsDNA. When the sample was run over the capture DNA it was already renatured. The effect of capture DNA in the fast cooling regime was less than the moderate cooling regime, most probably due to fast cooling already negated
the need of capturing any denatured complementary tDNA because the signal is already high when no capture is used in fast cooling regime.

Figure 5.3: (a) Relative frequency shifts of different cooling regimes are plotted for detection of 10 aM of HBV-DM dsMT tDNA in room temperature with 1ml/min flow rate. Relative frequency shift of same concentration of shorter ssDNA is plotted for comparison (b) Percentage of relative frequency shifts with respect to ssDNA detection of same concentration is plotted for different cooling regimes

5.3.2 HBV-DM double stranded mutation detections

Detection of 100 zM, 1 aM, 10 aM and 100 aM of HBV-DM dsMT tDNA (figure 5.4a) and 100 zM, 1 aM, 10 aM, 100 aM, 10 fM and 100 fM of HBV-DM dsWT tDNA (figure 5.4b) is done in urine, followed by FRM hybridizations. Average relative frequency shifts in the last five minutes of tDNA (25th-30th min) and FRM hybridizations (55th-60th min) are plotted for dsMT and dsWT tDNAs in figure 5.4c. As can be seen from these graphs dsWT tDNA reached plateau at 10 fM, and the signal was almost equal to that of 100 zM of
dsMT tDNA. Ratio of signal of dsMT tDNA to dsWT tDNA is also plotted in figure 5.4d. As can be seen, in the range between 100 zM to 100 aM, ratio was always higher than or equal to 15.

Figure 5.4: Relative frequency shifts of (a) 100 zM, 1 aM, 10 aM and 100 aM of HBV-DM dsMT tDNA and (b) 100 zM, 1 aM, 10 aM, 100 aM, 10 fM and 100 fM of HBV-DM dsWT tDNA is plotted versus time. Detections are followed by FRM hybridizations. (c) Signal (S) is plotted versus tDNA concentration for dsMT and dsWT tDNA and FRM hybridizations. (d) $S_{MT}/S_{WT}$ ratios are plotted for tDNA and FRM hybridizations versus concentration of tDNA.
5.3.3 HBV-DM double stranded mutation mixture detections

Detection of mixtures of HBV-DM dsMT and dsWT tDNA are done and confirmed by FRM hybridizations. However it is important to note that two color FRM scheme used in ssDNA detection in chapter 4 could not be used since both tDNAs have the upstream and downstream regions, thus FRMs were not specific to MT-tDNA or WT-tDNA, they could hybridize on both. Although a specific confirmation was not able to be performed a quantitative one was still possible. MT to WT tDNA ratio was kept at 250 as it was done in ssDNA detection of the same target. Figure 5.5a shows the relative frequency shift versus time graph for tDNA and FRM hybridization parts for mixture experiments involving dsMT tDNA of concentrations of 100 zM, 1 aM, 10 aM and 100 aM (250 fold more dsWT tDNA was mixed in each detection, concentration of dsMT was indicated on figure 5.5in a, b and c). The comparison of mixture experiments with “dsMT tDNA-only” and “dsWT tDNA-only” are also investigated. Average relative frequency shifts at the last five minutes of tDNA and FRM hybridization experiments are plotted versus concentration for mixture experiments and separately performed experiments of dsMT and dsWT tDNA (data from figure 5.4a and b respectively) in figure 5.5b. The sum of signal of separately performed dsMT and dsWT tDNA experiments were always higher than that of mixture experiments as expected because existence of dsWT tDNA interfered with the hybridization of dsMT tDNA as shown in the ssDNA case in chapter 4. Moreover the inhibitive nature of dsWT tDNA on dsMT tDNA was more aberrant in higher concentrations in terms of absolute relative frequency shift difference. Number of FRMs versus concentration graph was also plotted in figure 5.5c. As expected, a direct proportionality is observed between number of FRMs hybridized and concentration of tDNAs in the mixture. Micrographs of FRMs are
also shown on figure 5.5d. The width of each micrograph is approximately 400 nm.

Figure 5.5: (a) Relative frequency shift versus time graph of mixture of dsMT and dsWT tDNA followed by FRM hybridizations. Concentration of dsMT tDNA is indicated on figure. 250 fold more dsWT tDNA is mixed with dsMT tDNA in each experiment. (b) Plot of average relative frequency shifts in last five minutes of tDNA and FRM hybridization parts of mixture detections in (a) versus dsMT tDNA concentration and comparison with sum of average of relative frequency shifts of ”dsMT tDNA-only” and ”dsWT tDNA-only” detections in figure 5.4. (c)Plot of number of FRMs hybridized on PEPS surface after each mixture detection versus dsMT tDNA concentration. (d) Micrographs of FRMs on PEPS surface after mixture detection experiments in (a)
5.3.4 kRas double stranded mutation detections

Detection of 100 zM, 1 aM, 10 aM, and 100 aM of kRas dsMT tDNA (figure 5.6a) and 10 aM, 100 aM, 10 fM, 100 fM, and 1 pM of kRas dsWT tDNA (figure 5.6b) is done in urine, followed by FRM hybridizations. Average relative frequency shifts in the last five minutes of tDNA (25th-30th min) and FRM hybridizations (55th-60th min) are plotted for dsMT and dsWT tDNAs in figure 5.6c. As can be seen from these graphs dsWT tDNA reached plateau at 100 fM, and the signal was almost equal to that of 100 zM of dsMT tDNA. Ratio of signal of dsMT tDNA to dsWT tDNA is also plotted in figure 5.6d. As can be seen, for concentration of 10 aM and 100 aM, ratio was higher than or equal to 70, which is much larger than that observed in detection of HBV-DM double stranded tDNA (15 in the latter). As can be seen from figures 5.4b and 5.6b SMT of HBV-DM double stranded DNA at 100 aM was 0.65 and that of kRas was 0.85. There was already more signal in the kRas case. Moreover, the detections of kRas are done at a higher temperature (63°)C, in which the noise in the control was less with respect to noise in the control experiments of HBV-DM experiments which are done at 30°C. This decrease in the background noise can be attributed to the behavior of PEPS in high temperatures. The higher SMT can also be attributed to again the same behavior of PEPS in high temperature. The reason for this behavior of PEPS is that, high temperatures are closer to the Curie temperature of PEPS where domain switching in the crystal structure occurs.
Figure 5.6: Relative frequency shifts of (a) 100 nM, 1 aM, 10 aM and 100 aM of kRas dsMT tDNA and (b) 10 aM, 100 aM, 10 fM, 100 fM, and 1 pM of kRas dsWT tDNA is plotted versus time. Detections are followed by FRM hybridizations. (c) Signal (S) is plotted versus tDNA concentration for hybridizations of dsMT, dsWT tDNA and FRM. (d) $S_{MT}/S_{WT}$ ratios are plotted for tDNA and FRM hybridizations versus concentration of tDNA.
5.3.5 kRas double stranded mutations mixture detections

Detection of mixtures of kRas dsMT and dsWT tDNA are done and confirmed by FRM hybridizations. However again the two color FRM scheme used in ssDNA detection in chapter 4 could not be used since both tDNAs have the upstream and downstream regions, thus FRMs were not specific to MT-tDNA or WT-tDNA. MT to WT tDNA ratio was kept at 1000 in kRas case as it was done in ssDNA detection of the same target. Figure 5.7a shows the relative frequency shift versus time graph for tDNA and FRM hybridization parts for mixture experiments involving dsMT tDNA of concentrations of 100 zM, 1 aM, 10 aM and 100 aM (1000 fold more dsWT tDNA was mixed in each detection, concentration of dsMT was indicated on figure 5.7 in a, b and c). The comparison of mixture experiments with "dsMT tDNA-only" and "dsWT tDNA-only" are also investigated. Average relative frequency shifts at the last five minutes of tDNA and FRM hybridization experiments are plotted versus concentration for mixture experiments and separately performed experiments of dsMT and dsWT tDNA (data from figure 5.6a and b respectively) in figure 5.7b. The sum of signal of separately performed dsMT and dsWT tDNA experiments were always higher than that of mixture experiments as expected because existence of dsWT tDNA interfered with the hybridization of dsMT tDNA as shown in the ssDNA case in chapter 4. Moreover the inhibitive nature of dsWT tDNA on dsMT tDNA was more aberrant in higher concentrations in terms of absolute relative frequency shift difference but not as much as that occurred in HBV-DM double stranded tDNA detections. Number of FRMs versus concentration graph was also plotted in figure 5.7c. As expected, a direct proportionality is observed between number of FRMs hybridized and concentration of tDNAs in the mixture. Micrographs of FRMs are also shown on figure 5.7d. The width of each
Figure 5.7: (a) Relative frequency shift versus time graph of mixture of dsMT and dsWT tDNA followed by FRM hybridizations. Concentration of dsMT tDNA is indicated on figure. 1000 fold more dsWT tDNA is mixed with dsMT tDNA in each experiment. (b) Average relative frequency shifts in last five minutes of tDNA and FRM hybridization parts of mixture detections in (a) is plotted versus dsMT tDNA concentration and compared with sum of average of relative frequency shifts of ”dsMT tDNA-only” and ”dsWT tDNA-only” detections made in figure 5.6. (c) Number of FRMs hybridized on PEPS surface after each mixture detection is plotted versus dsMT tDNA concentration. (d) Micrographs of FRMs on PEPS surface after mixture detection experiments in (a) is shown.
5.3.6 SW480 Cell Line Extracted double stranded DNA detections

Detection of SW480 cell line extracted DNA is done for 100 zM, 1 aM, 10 aM and 100 aM of tDNA. Calculation of concentration is done considering the cells are homozygous for the mutation (each cell has 2 copies of mutation). Relative frequency shift versus time graph of SW480 tDNA and FRM hybridizations are plotted in figure 5.8a. The relative frequency shifts in the last five minutes of tDNA and FRM hybridization parts of ds-SW480 tDNA detections are compared with synthetic kRas dsMT tDNA (data from figure 5.6a).

The major difference in these detections are in ds-SW480 detections fragment size is not exact, however on average it is 500 bp. Thus hybridization of a 500 nts tDNA would be different than a 90 nts tDNA in dsMT tDNA detections. On the other hand, FRMs have the potential to hybridize on any tDNA captured by the pDNA on PEPS surface in synthetic tDNA detection case, since all the tDNAs possessed both the upstream and downstream regions certainly. On ds-SW480 tDNA case however, all tDNAs may not necessarily have upstream and/or downstream regions, or they may simply have portions of one and/or both. Thus, number of FRMs that could hybridize were expected to be less than that occurred in synthetic dsMT tDNA detection case. As shown in figure 5.8b, signals in both tDNA and FRM hybridization parts of ds-SW480 detections are slightly less than (if not equal to) those of dsMT tDNA. The uncertainty in tDNA sequence in ds-SW480 should not only affect the hybridization of FRMs but also hybridization of tDNA with pDNA on PEPS surface since, not all the fragments necessarily have the pDNA complementary region, some of them might have some portion of it. The length of pDNA on the hand was much less than the fragments size (16 to 500), decreasing the probability of such occurrences (probability that any fragments not including the whole pDNA sequence was 16/500 or
approximately 3%). The number of FRMs hybridized on PEPS surface after ds-SW480 tDNA detection are also plotted in figure 5.8c. As can be seen the direct proportionality of number of FRMs on surface with tDNA concentration was conserved as in all previous detections. However in the ds-SW480 tDNA detection case, there was a significant drop in number of FRMs hybridized on PEPS surface with respect to that occurred in the synthetic dsMT tDNA detection case (drop was observed in all concentrations at an approximately 60% rate). Micrographs of FRMs on PEPS surface after these detections are also shown in figure 5.8d.
Figure 5.8: (a) Relative frequency shift versus time graphs of ds-SW480 tDNA followed by FRM hybridizations. Concentration of ds-SW480 tDNA is indicated on figure. (b) Average relative frequency shifts in last five minutes of tDNA and FRM hybridization parts of ds-SW480 tDNA detections in (a) is plotted versus tDNA concentration and compared with average of relative frequency shifts of synthetic dsMT tDNA detections made in figure 5.6a. (c) Number of FRMs hybridized on PEPS surface after each ds-SW480 tDNA detection is plotted versus tDNA concentration. (d) Micrographs of FRMs on PEPS surface after mixture detection experiments in (a) is shown.
An important point that should be mentioned is how would PEPS behave given a real patient sample with an unknown ratio of MT and WT. In order to best answer this, the worst case where there was abundant WT tDNA should be considered in healthy subjects. As described in the introduction part, normal subjects have approximately 30 ng of cf-DNA per ml, an average of a population with cf-DNA concentration ranging from 0-100 ng per ml [14]. Thus in the worst case a normal subject would have 100 ng of cf-DNA in blood. Considering each cell have $3.2 \times 10^9$ bps and each bp has a molecular weight of 660 gr/mole on average, one cell would have approximately 3.5pg of DNA. Thus 100 ng of cf-DNA would mean approximately 29,000 cells per ml. Each cell would have 2 copies of the WT tDNA thus a normal subject would have at most approximately 100 aM of MT tDNA in blood. Of course not all of this DNA can pass through the kidney barrier but for simplicity we assume the concentration of DNA in urine and blood is same because DNA in urogenital tract can pass in to the urine.

As shown in figures 5.4 and 5.6, signal from hybridization of 100 aM of WT tDNA is approximately 4 and 5 times lower than that of 1 aM of MT tDNA in double mutation and point mutation cases respectively. Thus even in the worst case a positive detection of 1 aM of ds-tDNA is achievable by using PEPS.

5.4 Summary of Double Stranded DNA mutation detections

Detection of HBV-DM and kRas double stranded MT tDNA was accomplished with a 100 zM limit of detection on a background of 250 and 1000 fold more amount of WT
tDNA. Using a heat denaturation and rapid cooling (taking approximately 15 sec. with a 4ml/min flow rate) regime, although the signal strength (relative frequency shift) dropped to about 80% with respect to single stranded tDNA detection, limit of detection remained unchanged. Detection of SW480 cell line extracted double stranded tDNA fragments with an average length of 500 bp is accomplished with the same limit of detection with 100 zM. Moreover detections are confirmed with FRM hybridizations. These results indicate that PEPS is capable of detecting less than 1 aM of point mutations specifically, without need of amplification or isolation of DNA.
6. Conclusion

6.1 Optimization of Temperature and Flow Rate

- We have incorporated flow in gene mutation detection and used fluorescent microspheres to optimize the flow rate together with temperature and achieved better mutation detection specificity than by changing the temperature alone.

6.2 Resonance Frequency Peak Determination Method

- Peak determination method has increased the SNR and allowed detection of <60 copies/ml tDNA with an SNR of 10.
- Less than 12% error has been estimated as shown by the simulations

6.3 Detection of Single Stranded DNA fragments

- Single-stranded tDNA of HBV-DM and kRas codon 12 GGT → GTT point mutation have been detected in urine at the optimum temperature and flow rate.
- Detection of 100 zM single stranded tDNA has been accomplished in urine and unambiguous confirmation of MT and WT tDNA has been achieved using 2 color FRM hybridization
- MT tDNA was detected in urine and confirmed at the lowest concentration of 100 zM in a background of 250 fold and 1000 fold more WT tDNA in double mutation and point mutation cases respectively.
LNA-DNA hybrid probes used to detect point mutations showed increased specificity (WT/MT = 1000) yet at a higher temperature (63°C) with respect to detections of double mutation (30°C).

6.4 Multiplexed Detection

10^{-15} M of 6 different tDNAs (each perfectly matching with one pDNA immobilized on an array of 6 PEPS) have been run through the array PEPS one by one and hybridizations only detected on PEPS with pDNA perfectly matching with the tDNAs.

At the optimum temperature of 63°C and 4 ml/min flow rate no cross hybridization has been observed.

6.5 Detection of Double Stranded DNA

Sample at boiling temperature was cooled down to room temperature in 30 sec. for direct detection.

Using capture DNA a 87% single stranded coverage was achieved.

Without capture DNA the coverage dropped only 10% to yield 77% coverage

100 zM MT tDNA of HBV-DM and kRas codon 12 GGT \rightarrow GTT point mutation was detected in urine.

100 zM of SW480 cell line (homozygous for kRas codon 12 GGT \rightarrow GTT point mutation) extracted DNA was detected in urine and confirmed by FRM hybridization.
Bibliography


