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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**POINT OF CARE DIAGNOSTICS FOR
CARBONYL/OXIDATIVE STRESS BIOMARKERS**

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Interdisciplinary Graduate School

NTU Institute for Health Technologies

2018

**POINT OF CARE DIAGNOSTICS FOR
CARBONYL/OXIDATIVE STRESS BIOMARKERS**

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**Interdisciplinary Graduate School
NTU Institute for Health Technologies**

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2018

STATEMENT OF ORIGINALITY

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

21-AUG-2018

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Date

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Ammanath Gopal

SUPERVISOR DECLARATION STATEMENT

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

21-AUG-2018

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Prof. Bo Liedberg

AUTHORSHIP ATTRIBUTION STATEMENT

This thesis contains material from 4 papers published in the following peer-reviewed journals where I was the first and/or corresponding author.

A part of Chapter 3 is published as Rajwar, D., Ammanath, G., Cheema, J. A., Palaniappan, A., Yildiz, U. H., & Liedberg, B. Tailoring Conformation-Induced Chromism of Polythiophene Copolymers for Nucleic Acid Assay at Resource Limited Settings, ACS applied materials & interfaces 8.13 (2016): 8349-8357. DOI: 10.1021/acsami.5b12171.

The contributions of the co-authors are as follows:

- Dr. Palaniappan and Dr. Yildiz provided initial project direction and revised the manuscript drafts.
- I wrote the drafts of the manuscript along with Dr. Rajwar and Mr. Cheema and edited by Prof. Liedberg.
- Dr. Rajwar synthesized the polythiophenes and conducted NMR spectra.
- I co-designed the nucleic acid sensing platform along with Dr. Palaniappan and Dr. Yildiz. I performed the experimental work at Centre for Biomimetic Sensor Science (CBSS) along with Mr. Cheema and analysed/discussed the data along with Prof. Liedberg, Dr. Palaniappan and Dr. Yildiz.

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The contributions of the co-authors are as follows:

- Prof. Liedberg and Dr. Palaniappan initiated the project and edited the manuscript.
- I wrote the drafts of the manuscript and Dr. Yildiz revised the manuscript.
- I chose the biomarker and co-designed the sensing platform along with Dr. Palaniappan and performed all the laboratory experiments at CBSS and evaluated the data.

- Prof. Liedberg, Dr. Palaniappan and Dr. Yildiz provided guidance in the interpretation of the results.

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The contributions of the co-authors are as follows:

- Dr. Palaniappan initiated the project and wrote the manuscript. Prof. Liedberg edited the manuscript.
- Dr. Rajwar synthesized the polythiophene and conducted the NMR spectra.
- Mr. Cheema and I performed the experiments and analyzed the data with Prof. Liedberg, Dr. Palaniappan and Dr Yildiz.
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- Dr. Rajwar and Dr. Palaniappan initiated the project. Prof. Liedberg edited the manuscript.
- Dr. Rajwar synthesized the polythiophenes and conducted the NMR spectra.
- Dr. Tu and I wrote the manuscript and performed the experiments. Dr. Palaniappan and Dr. Yildiz revised the manuscript.
- Dr. Tu and I analyzed the data and discussed/interpret the results with the guidance from Prof. Liedberg, Dr. Palaniappan and Dr. Yildiz.

21-AUG-2018

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Ammanath Gopal

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LIST OF ABBREVIATIONS

Notations	Descriptions
T2D	Type 2 diabetes
WHO	World Health Organization
RCS	Reactive Carbonyl Species
ROS	Reactive Oxygen Species
UV	Ultraviolet
AGEs	Advanced glycated end products
RNA	Ribonucleic acid
miRNA	microRNA
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
HPLC	High-performance liquid chromatography
MS	Mass spectrometry
PCR	Polymerase Chain Reaction
POC	Point of care
LFA	Lateral flow assay
VFA	Vertical flow assay
μ-PAD	Microfluidic paper-based analytical device
HRP	Horseradish peroxidase
CP	Conjugated polymer
PT	Cationic polythiophene
mir21	MicroRNA-21
8-OHdG	8-hydroxy-2'-deoxyguanosine
Gly-AGEs	Glyceraldehyde derived advanced glycation end products
PVDF	Polyvinylidene fluoride
PES	Polyether sulfone
CA	Cellulose Acetate
CPEs	Conjugated polyelectrolytes
QCM	Quartz Crystal Microbalance
VOCs	Volatile Organic Compounds

ABSTRACT

Multiple triggers like exogenous and endogenous factors could affect the equilibrium levels of free radicals and reactive oxygen species in humans. For instance, elevated concentration levels of reactive carbonyl species (carbonyl stress) or reactive oxygen species (oxidative stress) damages a myriad of important biological molecules such as proteins, lipids, carbohydrates and nucleic acids. Damage to these molecules causes cell death and tissue injury, thus contributing to various disorders like type 2 diabetes mellitus (T2D), cancer, and cardiovascular diseases. Therefore, assays for stress related biomarkers could assist in the early diagnosis and could be predictive for disease prognosis. The conventional assay methodologies such as chromatographic and electrophoretic techniques are cumbersome, skill intensive and require expensive instrumentation. Thus, there is sufficient scope to develop a point of care (POC) assay for these biomarkers such that advantages of low cost, fast response time and high sensitivity as well as selectivity can be realized.

Carbonyl/Oxidative stress plays a major role in the onset and progression of T2D, and a simple, low cost way of measurement of carbonyl/oxidative stress biomarkers can be beneficial in screening patients for early diagnosis of T2D and its complications thereby assisting current clinical practice. The focus of this thesis was to develop colorimetric assays for monitoring carbonyl/oxidative stress biomarkers associated with T2D. Cationic polythiophene (PT); a water-soluble conjugated polymer was used as a luminescent reporter for the optical detection of these biomarkers. The developed assay consists of two modules; a sample fractioning unit; a filter paper (FUSION 5) for removal of large molecules from clinical samples without requiring external stimuli, and a transduction unit utilizing polyvinylidene fluoride (PVDF) membrane impregnated with (PT) for colorimetric sensing in a flow-through format.

The sensing strategy is based on monitoring the changes in optical properties of PT with its associated conformational changes when interacting with a peptide nucleic acid (PNA)/aptamer in the presence and in the absence of target biomarkers. As a proof of concept, the developed assay methodology utilizing PT and PNA/aptamer was validated using different carbonyl/oxidative stress biomarkers such as microRNA (mir21), DNA damage molecule; 8-hydroxy-2'-deoxyguanosine (8-OHdG) and Glyceraldehyde derived Advanced Glycation End Products (Gly-AGEs) without requiring tedious sample pre-treatment and clean up protocols. Colorimetric

responses for these biomarkers were obtained at clinically relevant concentrations and could therefore find applications in relation to the management of metabolic diseases such as T2D in a clinical setting as well as in the patient's home, thus providing attractive and affordable healthcare solutions for field diagnostics.

CHAPTER 1: INTRODUCTION

1.1 Background and Significance

Metabolic diseases are the disorders that cause energy and redox imbalance in metabolic pathways. Metabolic disorders are mainly characterized by the risk factors such as glucose intolerance, defective insulin secretion, dyslipidemia, hypertension, obesity and chronic inflammation [1, 2]. These risk factors are collectively known as metabolic syndrome, which in turn is associated with the development of type 2 diabetes (T2D) and cardiovascular diseases [3, 4]. T2D is one of the major chronic metabolic diseases characterized by progressive loss of glucose homeostasis. Around 9% of the world's population is affected by diabetes, according to the data published by World Health Organization (WHO), in 2014, and has been increasing at an alarming rate [5]. The percentage of residents in Singapore with diabetes aged between 18 and 69 years old has increased from 8.2% in year 2004 to 11.3% in year 2010 [6]. It is projected that by 2050, there will be 1 million diabetics in Singapore and this creates a major health problem with unmet needs. The pathogenesis of T2D is primarily driven by insulin resistance in target organs (i.e., liver, skeletal muscles, and adipose tissues) with subsequent dysfunction and/or destruction of the insulin-producing beta cells in the islets of Langerhans of the endocrine pancreas [7, 8]. Recent studies indicate that carbonyl or oxidative stress (a condition where there is imbalance between generation and inactivation of reactive carbonyl species (RCS) and reactive oxygen species (ROS)) has a direct role in initiation and pathogenesis of T2D [5, 9].

ROS/RCSs are chemically reactive molecules containing either oxygen or carbon that are endogenously produced as by-products of various physiological, metabolic, and biochemical processes. While low levels of ROS/RCS are required for cell signal transduction and immune response, an increase in their steady-state levels contributes to physiological stress [10]. Thus, heightened concentrations of ROS and RCS, above physiological levels cause oxidative and carbonyl stress, respectively [10, 11]. The carbonyl and oxidative stress are interrelated; recent studies have shown that the increase in RCS levels emanate from oxidative stress and vice versa, thereby forming a cyclic reaction [10]. The increase in ROS can either be endogenously or exogenously triggered. The endogenous sources come from the byproducts of metabolic reactions such as energy generation in mitochondria, whereas the exogenous sources include factors such as ultraviolet (UV) radiation, smoking, alcohol

consumption and long-term intoxication of aluminum chloride in drinking water. Subsequent imbalance in oxidants and anti-oxidants in the normal redox state of the cells produces peroxides and free radicals that damage proteins, lipids, carbohydrates and nucleic acids [12, 13]. The damage in these molecules causes cell death (e.g. insulin-producing cell death) and tissue injury and progresses to various metabolic diseases like T2D and cardiovascular complications [14, 15]. There is no remission for T2D. Although lifestyle intervention and bariatric surgery mitigates the severity of T2D, it does not eliminate T2D completely. A major factor for the non-remission is the loss of insulin-producing cells (low proliferating) in the pancreas. The elevated oxidative or carbonyl stress occurs before the onset of T2D and can, therefore be considered, a precursor state of T2D, otherwise also known as pre-diabetes in humans [14]. The pre-diabetes state represents a therapeutic phase in which early detection can prevent the onset of non-remission, full-blown diabetes, thus saving the patient from getting T2D. Hence, the detection of measurable carbonyl/oxidative stress related biomarkers could assist in the early diagnosis as well as in monitoring of T2D pathogenesis.

Carbonyl/Oxidative stress leads to damaged biomolecules such as lipids, nucleic acids, antioxidants or proteins, which can be used as stress related biomarkers for T2D for diagnosis [14]. The presence/concentration of these molecules can be used to evaluate a physiological state or condition. Carbonyl damage in proteins “carbonyl proteins” is one of the most occurring damage caused by oxidative stress [12, 16]. Advanced glycated end products (AGEs) formed by glycation (non-enzymatical addition of carbohydrate to protein) has been established as a biomarker for carbonyl stress [12, 14, 17]. Recent studies also suggest that microRNAs (miRNAs) are potential biomarkers for oxidative stress. Here, the specific expression pattern (upregulation/downregulation) of the miRNA is linked to T2D, vascular disorders and inflammation [9, 18, 19]. Henceforth, it is of importance to leverage on these known ‘circulating markers’ (i.e. biomarkers) of T2D and to develop assays to measure and evaluate these biomarkers for early diagnosis of T2D.

Carbonyl/oxidative stress biomarkers such as carbonyl proteins, DNA damage, miRNA and AGE have been measured by (i) enzyme-linked immunosorbent assays (ELISA) using monoclonal or polyclonal antibodies, (ii) fluorescence spectroscopy, (iii) high-performance liquid chromatography (HPLC), (iv) mass spectrometry (MS) and (v) polymerase chain reaction (PCR) [20]. A combination of these approaches

have been evaluated for the biomarkers assay, for instance, liquid chromatography (LC) with triple quadrupole MS detection for the quantitative measurement of protein glycation, oxidation and nitration adducts [21, 22]. Recent studies also show that matrix-assisted laser desorption ionization-mass spectrometry with time-of-flight detection (MALDI-TOF/MS) seems to be a promising tool for biomarkers assay [23, 24]. Although several assay platforms have been reported for biomarkers detection, most of these methodologies require sophisticated instrumentation and are time consuming. Furthermore, these methodologies require trained personnel to conduct the analysis. Even though MS provides sensitivity and specificity, current antibody-based methods remain the clinical standard, which require laborious tissue analysis procedures. Moreover, most of the commercially available kits lack specificity and their significance for clinical research has not fully been substantiated. Thus, it is highly necessary to develop a point of care (POC) assay for these biomarkers.

Point of care tests (POCTs) are rapid detection assays that are low cost, require minimal sample amounts and limited resources [25-27]. Modern day POCTs show good correlation to laboratory experiments and are largely reliable. Different materials such as glass, silicon, PDMS, polymer (plastics) and paper have been explored for POC device fabrication. Among these POCTs, paper-based assays are facile, cost-effective, easy to use, enabling diagnosis in resource-limited settings. Paper-based POCT can be classified into different types such as dipstick assay, lateral flow assay (LFA), vertical flow assay (VFA) and microfluidic paper-based analytical device (μ -PAD). The mechanism of detection and the detection limits depend on the target analytes, fabrication protocols and the detection labels used. They can be quantitative, semi-quantitative and qualitative in nature, depending on the assay techniques. The sensitivity of paper-based POCTs is limited by interferences from complex clinical samples such as blood, urine and saliva thereby requiring extraction or sample clean-up prior to analysis. Since rapid and less expensive assays are desirable, state-of-the-art extraction processes involving tedious protocols may not be applicable and therefore alternative sample fractioning unit utilizing filter membranes need to be developed. The sensitivity of paper-based POCTs are also influenced by the properties of materials they are made of, such as the transducers (electrodes, nanoparticles, dyes) and recognition molecules (antibodies, peptides, aptamer, etc.) [28].

Among the various detection methodologies/transducers, colorimetric assays have drawn most attention due to their quick visual response without requiring

sophisticated instrumentation [29-31]. Various optical reporters such as horseradish peroxidase (HRP), gold nanoparticles, magnetic nanoparticles, carbon nanotubes, dyes, quantum dots and conjugated polymers (CPs) have been explored for colorimetric assaying [32]. CPs have attracted significant interest in biosensing because of delocalized π -electrons along their conjugated backbone that yield unique optical and electronic properties. The optical properties of CPs could be varied with external stimuli such as temperature, solvent or pH change, electric field, and interaction with other molecules that may induce chirality in the supramolecular structure, subsequently changing the conjugation length of CPs. Among the biocompatible CPs, cationic polythiophenes (PT) have been mainly explored for biosensing due to their superior optical and electronic properties. The optical transitions of PT are due to the conformational alternations in backbone that increase or reduce the effective conjugation length leading to red or blue shift, respectively, of UV-vis absorption maximum. The extent of conformational alternations in backbone is mainly controlled by energy required for interrupting twisting [33].

Another important aspect of the paper-based POC is the bio receptor or recognition element. Currently, POC assays for T2D mainly rely either on antigen-antibody interaction or with analyte specific enzymes for specificity. The advances in nucleic acid engineering helped in the development of bio recognition elements such as aptamers. Aptamers are single-stranded oligonucleotides that can form 3-dimensional conformations to bind specifically to a target analyte. Aptamers have been found to be highly stable and selective in binding to its targets. The synthesis of aptamers is a facile process compared to antibody production, thereby decreasing the overall costs. Since, its synthesis process is chemical in nature, it is easier to manipulate and thus manufacturing aptamers against a large variety of target molecules is feasible. Aptamer-based assays can also eliminate the need for washing step and thus reducing the assay time [34]. Recently, aptamers have been designed and developed for various T2D biomarkers such as apolipoprotein E, glucagon, 8-hydroxy-2'-deoxyguanosine (8-OHdG) and AGEs [35-38]. For example, a gold nanoparticle based assay with good sensitivity for apolipoprotein E using a DNA aptamer as bio receptor is developed [39]. Another example is the noncompetitive affinity assay of glucagon using an aptamer rivalling the specificity of antibodies [40].

So, based on the combined advantages of both cationic polythiophene and aptamers, this thesis will focus on the development of colorimetric paper-based assays

utilizing cationic polythiophene for rapid and facile detection of carbonyl/oxidative stress biomarkers associated with T2D. The simplicity of this colorimetric paper-based assay together with the use of robust molecular components such as aptamers offer an attractive route for low-cost assays without requiring expensive instrumentation. The detection of these biomarkers could help in early detection of T2D than through standard measurements and will have an earlier window to intervene from getting diabetic complications through lifestyle changes.

1.2 Hypotheses

1. A paper-based vertical/flow-through POC assay utilizing cationic polythiophene as the optical reporter can be developed for the visual detection of carbonyl/oxidative stress biomarkers at clinically relevant concentrations, without using tedious standard procedures and instrumentation.

Among the POC tests available, paper-based tests, both lateral and vertical flow assays, are the easiest to handle and are cost effective. Gold-standard/laboratory techniques utilize multistep processes for sample preparation and analysis are not suitable in low resource settings [41]. Cationic polythiophene is predominantly studied in solution-based assays as both colorimetric and fluorometric probe. The utilization of cationic polythiophene as optical reporter instead of commonly used detection labels like enzymes (HRP), nanoparticles (colloidal gold and carbon), latex beads or fluorescent tags (Europium and quantum dots) on paper-based assay could be used in the low resource settings for the high-performance assay of carbonyl/oxidative stress biomarkers with minimal instrumentation.

2. Aptamers can be utilized for selective detection of biomarkers of carbonyl/oxidative stress at clinically relevant concentrations.

Aptamers are single stranded nucleic acid sequences that can fold into secondary and tertiary structures to bind to their ligands with high affinity and specificity. Compared to the conventional antibodies, they are small sized and can be easily modified [38]. They are also easy to handle and significantly cheaper than antibodies. Literature shows that aptamers potentially could be used in paper-based assays [42, 43]. The utilization of aptamers instead of antibodies could help in improving the quality of paper-based assay for the detection of carbonyl/oxidative stress biomarkers on site due to their stability (extended shelf life), low cost, specificity (especially for small molecule targets) and ease of modification [44].

1.3 Objective

People suffering from hyperglycemia (high levels of circulating glucose) generates reactive oxygen species (ROS) and reactive carbonyl species (RCS), within the circulatory system, that in turn either cause damage to the cells that secrete insulin and/or induce insulin resistance in peripheral tissues [5, 9]. The main aim is to develop a simple, easy to use diagnostic assay or technological tool aimed at measuring levels of carbonyl/oxidative stress biomarkers with high sensitivity and specificity for the early detection of T2D. The overarching aim is to develop a diagnostic assay that could assist in clinical decision-making and thereby guide clinical practice of managing T2D patients. The specific aims are to develop assays for the following carbonyl/oxidative stress biomarkers associated with T2D utilizing cationic polythiophene and aptamers.

1. mir21, a small non-coding RNA that modulates the level of ROS and associated with T2D. It is a circulating biomarker (present in blood) that elevates under oxidative stress.
2. 8-hydroxy-2'-deoxyguanosine (8-OHdG), a urinary biomarker for oxidative stress, in case of DNA damage. Studies have shown that 8-OHdG is a biomarker for various diseases such as T2D and cardiovascular complications.
3. Glyceraldehyde derived advanced glycation end products (Gly-AGEs) are elevated under oxidative stress in plasma/serum and associated with insulin resistance, endothelial dysfunction and vascular inflammation.

Concurrent detection of mir21, 8-OHdG and Gly-AGEs could yield vital information on the pathogenesis and progression of chronic complications and to estimate the prevalence of undiagnosed T2D and pre-diabetes. Table 1.1 shows the clinically relevant concentrations of the target biomarkers with its gold standard techniques and drawbacks.

Table 1.1: Clinically relevant concentrations of target biomarkers and its gold standard techniques.

Target	Clinically relevant concentrations range	Gold standard, Analysis time	Drawbacks	Refs
mir21	~ 1-10 ng/ml	Quantitative reverse transcription polymerase chain reaction (RT-qPCR) (> 3 hours)	Low to medium throughput and expensive	[45-48]
8-OHdG	~ 80 ng/ml	High Performance Liquid chromatography tandem mass spectroscopy (HPLC-MS) (> 2 hours)	Cumbersome (Elaborate extraction steps), high cost, low throughput, required trained personnel	[49-51]
Gly-AGEs	~ 10 µg/ml	Lack gold standard; Liquid chromatography tandem mass spectrometry (LC-MS/MS) in multiple reaction monitoring (MRM) using stable isotope dilution approach is most preferred (> 4 hours)	Time consuming, Multiple step (several injections per sample)	[52-54]

1.4 Scope

The scope of this project is to develop a colorimetric assay device for monitoring carbonyl/oxidative stress biomarkers associated with T2D such as mir21, 8-OHdG and Gly-AGEs. The proposed assay consists of a sample fractioning unit; a filter paper for removal of large molecules from clinical samples without requiring external stimuli, and a transduction unit cationic polythiophene for detection of biomarkers. The proposed system could be ideal for point-of-care applications and for rapid detection of carbonyl/oxidative stress biomarkers in clinical samples without requiring expensive instrumentation. Carbonyl/Oxidative stress plays a major role in

the T2D, and a simple, low cost way of measurement of carbonyl/oxidative stress biomarkers can be beneficial in screening patients for early diagnosis of T2D and its complications.

1.5 Overview

This thesis consists of 7 chapters. In Chapter 1, a brief introduction is presented to describe the background and significance of this thesis. It also includes the hypotheses, motivations and objectives of the research with its scope. Chapter 2 reviews about carbonyl/oxidative stress biomarkers and their conventional assay methodologies. This chapter also reviews about point of care diagnostics, conjugated polymers and current aptamer-based paper assays. Chapter 3 describes the sensing strategies for the visual detection of nucleic acids in both solution and paper-based platform utilizing cationic polythiophenes. This chapter also discusses about the development of flow-through device and logic gate system. Chapter 4 presents the optical detection of DNA damage molecule such as 8-OHdG utilizing the flow-through assay format. Chapter 5 describes the development of solution based colorimetric assay for advanced glycation end products. Chapter 6 discusses about collaborative works utilizing polythiophene such as detection of miRNA using Quartz Crystal Microbalance (QCM), colorimetric detection of aluminum ions and volatile organic compounds (VOCs). Chapter 7 outlines the summary and the future outlook of this dissertation.

CHAPTER 2: LITERATURE REVIEW

2.1 Carbonyl/Oxidative Stress and Metabolic Syndrome

Oxidative stress is the accumulation of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radicals due to the reduction in antioxidants and/or increase in oxidative mechanisms [55, 56]. The oxidative stress can arise from environmental or/and cellular sources causing overwhelming of antioxidant defense system creating ROS. The increase in ROS causes damage in tissues through oxidation of proteins, carbohydrates, nucleic acids and lipids and thus forming carbonyl stress as shown in Figure 2.1 [57]. Then, carbonyl stress induces protein damage by non-enzymatic modifications that changes the function of protein. This damage in the mitochondrial proteins could further drive the production of ROS endogenously. These stress are involved in the pathogenesis of various human diseases, and mainly in the onset and the progression of metabolic diseases such as diabetes, cardiovascular disorders, cancer and neurodegenerative diseases [58].

Figure 2.1: Oxidative stress from environmental and cellular sources causing tissue damage and thus resulting in carbonyl stress formation [57].

Recent studies show that carbonyl/oxidative stress is linked to metabolic syndrome that is a cluster of risk factors consisting of glucose intolerance, defective insulin secretion, dyslipidemia, hypertension, obesity and chronic inflammation [58]. As an example, obesity causes the increase in production of oxidation of lipids (lipid peroxidation) [59]. Also, the patients with metabolic syndrome showed lower

antioxidant defense, and people with type 2 diabetes (T2D) displayed an increase in carbonyl/oxidative stress biomarkers. The increase in ROS produces reactive carbonyl species (toxic aldehydes/RCS) causing the activation of stress-signalling pathways such as nuclear factor- κ B pathway and cell damage triggering the onset of T2D [60, 61]. These toxic aldehydes play a crucial role in the damage of insulin function on tissues and carbonylation of insulin peptide, and thus inducing insulin resistance causing metabolic syndrome [62]. These RCS can also react with proteins or lipids to form advanced glycation or lipoxidation end products that also plays a major role in the onset of diabetes. The role of carbonyl/oxidative stress on the pathogenesis of T2D is shown in Figure 2.2 [61].

Figure 2.2: The role of carbonyl/oxidative stress on the onset and progression of diabetes [61].

2.1.1 Carbonyl/Oxidative Stress Biomarkers

An excess of ROS/RCS causes modification or damage of biomolecules like lipids, proteins and DNA. Due to this damage, there is a bigger risk of several chronic disorders like diabetes, cancer, cardiovascular and neurodegenerative diseases [63,

64]. These damaged biomolecules can be used as biomarker for carbonyl/oxidative stress related disease progression. Different types of Carbonyl/Oxidative stress biomarkers are described in the following sections.

2.1.1.1 Lipid Peroxidation

The oxidation of poly unsaturated fatty acids (PUFA) or cell membrane phospholipids by free radicals that attack the carbon-carbon double bond is termed as lipid peroxidation [65]. It can occur through different processes like enzymatic oxidation, ROS-independent non-enzymatic oxidation, and ROS-mediated non-enzymatic oxidation. Due to these reactions, it proceeds via formation of primary and secondary lipid peroxidation products causing oxidative stress and oxidative damage and apoptosis [10, 66]. The most commonly formed RCS from oxidation of PUFA are malondialdehyde (MDA), hexanal and 4-hydroxy-trans-2-nonenal (HNE). The reactive carbonyls and other end products can react with nucleophilic groups of biomolecules to form adducts and crosslinks known as advanced lipoxidation end products (ALE) which causes ROS formation and in turn causing oxidative damage [10, 14].

2.1.1.2 Protein Carbonylation

Protein carbonylation is a form of protein oxidation where the native amino acids side chains are modified into carbonyl (aldehyde or ketone) [67]. It is an irreversible oxidative process or a non-enzymatic post-translational modification. This modification can be induced directly by ROS or indirectly by the secondary by-products of oxidative stress. Protein carbonyls are produced by direct oxidation, oxidative cleavage of proteins, from lipid peroxidation and by RCS produced by reducing sugars. Among the amino acids, cysteine and methionine are the most prone to the attack [12, 68]. Protein carbonyls are more stable biomarker as compared to those formed by lipid peroxidation and they circulate for longer periods of time in blood [69]. Thus, they can be used as diagnostic biomarkers of the human diseases that are linked to carbonyl/oxidative stress [70].

2.1.1.3 MicroRNA

MicroRNAs (miRNAs) are short (18-24 bases) noncoding molecule of single stranded RNA which regulates post transcriptional gene expression [71]. Some of the miRNAs are involved in promoting oxidative stress by targeting relevant genes as they

modulate the redox imbalance. Different miRNAs are linked with mitochondrial metabolism and they modulate the ROS production [72]. Various physiological and pathological conditions are critically regulated by miRNA including glucose homeostasis and inflammatory response modulation. A change in normal physiological levels of miRNA can cause disease development due to oxidative damage. The circulating miRNAs are very stable biomarkers for monitoring the disease progression [46]. Among different miRNAs, mir21 is a very important miRNA, which is upregulated in many diseases and regulates the formation of ROS. The major ROS species are Superoxide (O_2^-) and hydrogen peroxide (H_2O_2). The family of superoxide dismutases (antioxidant enzymes) such as SOD1, SOD2 and SOD3 helps in metabolizing ROS and neutralize or reduce the ROS produced by forming oxygen and hydrogen peroxide. So, when mir21 is upregulated, it decreases the function of superoxide dismutase-2 (SOD-2) causing the increase in ROS formation [18, 73, 74]. mir21 is involved in pathogenesis of diabetes and its complications such as diabetic nephropathy. In T2D, mir21 regulates secretion of insulin and contribute β -cell dysfunction [46].

2.1.1.4 DNA Oxidation

The oxidative damage of DNA caused by ROS is called DNA oxidation leading to mutations diseases and ageing process [75]. The damage can be initiated by UV light, ionizing radiation or normal metabolic process which results in the electron removal [76]. The single or double stranded breaks, covalent crosslinks and modification in bases cause to form so many different type of DNA damage products [77]. Nuclear and the mitochondrial DNA in tissue are the mostly affected area by oxidative damage. The hydroxyl radical ($HO\bullet$), an oxygen-free radical cause main damage to DNA. It can be mainly produced by various mechanisms such as Fenton reaction of hydrogen peroxide [78]. The mitochondrial DNA strands attacked by the $HO\bullet$ leads the formation of oxidation products by the addition of radicals to DNA bases [79].

ROS induced oxidation results in formation of numerous DNA fragments. Among them, 8-hydroxy-2-deoxyguanosine (8-OHdG) is a repaired product of oxidative guanine lesions and an important biomarker of oxidative DNA damage. 8-OHdG is formed by the interaction of $HO\bullet$ with the guanine nucleobases of the DNA strand by the generation of adducts followed by an electron subtraction [79, 80]. The

hydroxyl group is added at the 8th position of guanine. Guanine will react more with HO• compared to other nucleobases due to its low oxidation potential [81]. The chemical structure of 8-OHdG is shown in Figure 2.3. It can be used as a risk factor for various diseases like diabetes, cancer and neurodegenerative disorders [63].

Figure 2.3: Chemical structure of (A) guanine base (B) oxidized guanine (C) 8-OHdG derived from RNA (D) 8-OHdG derived from DNA [80].

2.1.1.5 Advanced Glycation End Products (AGEs)

Advanced Glycation End Products (AGEs) are heterogeneous group of bioactive molecules that are formed via glycation, sugar auto oxidation and sugar metabolic pathways. AGEs are formed predominantly through Maillard reaction or glycation that occurs in three steps. The first phase is the non-enzymatic reaction between reducing sugars and amino groups of lysine residues, guanidino group of arginine residues, or N-terminal α -amino group of proteins to form a Schiff base [82, 83]. Then the reversible Schiff base undergoes the chemical rearrangement to create a more stable Amadori products or early glycation end products. The subsequent oxidation and dehydration of these products create AGEs. The other pathways to form AGEs are auto oxidation of sugar and lipid peroxidation due to an increase in oxidative stress as well as through the metabolic pathways such as glycolysis and polyol [84, 85]. The formation of AGEs is shown in Figure 2.4. In addition to the endogenous formation of AGEs, these molecules are absorbed exogenously by high

AGEs content food and smoking. Due to these different pathways, there are several chemically distinct classes of AGEs derived from glucose, fructose, dicarbonyl compounds and α -hydroxyaldehydes. The products of these highly reactive intermediate carbonyl groups like α -dicarbonyls accumulate during the Amadori reorganization causing carbonyl stress [86].

Figure 2.4: Formation of advanced glycation end products (AGEs) [84].

The formation and accumulation of AGEs occur at a normal rate during aging, but in an accelerated rate under carbonyl/oxidative stress or hyperglycemic conditions. The elevated levels of AGEs are pathogenic and promote oxidative stress and inflammation and play a key role in the development of micro- and macro vascular diabetic complications, ageing, cardiovascular diseases, atherosclerosis, sarcopenia, and neurodegenerative disorders such as Alzheimer's and Parkinson's disease [87, 88]. The interactions between the AGEs and the receptor for AGEs (RAGE) plays a key role in vascular complications. Among various reducing sugar molecules, Glyceraldehyde (triose monosaccharide) reacts rapidly with free amino group of proteins to form glyceraldehyde derived AGEs (Gly-AGEs), and also has the strongest binding affinity to RAGE, among the various AGEs. These AGEs are elevated under

oxidative stress and associated with insulin resistance, endothelial dysfunction, vascular inflammation and cause accelerated atherosclerosis in diabetes [89, 90].

2.2 Current Status of Analytical Techniques

Various classic analytical methods for the detection of carbonyl/oxidative stress have been developed like spectrophotometry, chromatography, immunoassay and electrophoresis. A detailed review of these techniques is described below.

2.2.1 Spectrophotometric Assay

This is one of the classical approaches for the detection and assessment of carbonyl proteins. Here the carbonyl groups are derivatized with 2,4-dinitrophenylhydrazine (DNPH) to form 2,4-dinitrophenylhydrazones in various biological samples like plasma, tissue homogenates or isolated proteins as shown in the Figure 2.5 [91, 92]. This yellow product that has maximum absorption at 370 nm is quantified spectrophotometrically. For expressing the carbonyl content, UV analysis of total protein at 280 nm is needed.

Figure 2.5: Carbonyl group derivatization using DNPH [20].

Some of the disadvantages of this method are potential interference of compounds that absorb in the same region (360-380 nm). Moreover, it is necessary to remove nucleic acids as they also react with DNPH. This method also requires high amounts of protein as the protein loss during the washing steps is high [20].

2.2.2 Chromatography

Chromatography is an analytical technique where the separation of molecules (due to the difference in composition, structure, charge etc.) occurs by moving the sample over a stationary phase. The various stationary phases can be liquid, volatile gases, paper and glass plates. To enhance selectivity and sensitivity, derivatization can be used [93]. Various types of chromatography techniques like high-performance

liquid chromatography with electrochemical detection (HPLC-ECD) or with diode array detector; HPLC tandem mass spectrometry (HPLC-MS); gas chromatography-mass spectrometry (GC-MS) have been used to analyze carbonyl proteins and DNA oxidation [63, 94]. HPLC is used to separate and quantify the sample that is dissolved in the solution. The separation of protein fractions can be done using gel-filtration. HPLC-MS has both the separation and mass analysis capabilities and can be selectively applied to analyze the carbonyl compounds. Some of the disadvantages of these methods relates to solubilization of proteins, co-elution of proteins, high pH required for the derivatization and expensive instrumentation [20].

2.2.3 Electrophoresis

Electrophoresis is an analytical technique used in laboratories to separate macromolecules like proteins based on size by applying negative charge. There are different types of electrophoresis techniques like capillary electrophoresis, gel electrophoresis, isotachopheresis and so on. In polyacrylamide gel electrophoresis, the separation is based on size, density and purity of the sample. For example, the carbonyl groups are reduced with sodium tritiated borohydride and then followed by gel electrophoresis. Here the tritiated proteins were separated using SDS-PAGE and then quantified [95]. The disadvantage of this technique is the low selectivity because tritium binds to the existing Schiff bases [20]. Another technique uses capillary electrophoresis with laser induced fluorescence detection (CSE-LIF) by labeling the carbonyl group with Alexa 488 hydrazide for quantification of carbonyl proteins [96].

2.2.4 Immunoassays

An analytical assay which measures or determine quantitatively the concentration of analyte using an antibody or immunoglobulin. The principle of these assays is the capability of an antibody to selectively bind the target molecule [97]. These so-called immunoblotting assays are mainly done using the commercially available kits. Different blotting techniques like dot blot and slot blot are used in the assay. For example, by slot blotting the protein samples on a PVDF membrane and then subsequently DNPH derivatization and staining with anti-dinitrophenyl antibody followed by the interaction with a HRP conjugated secondary antibody [98]. Another technique is a sensitive enzyme-linked immunosorbent assay (ELISA) based on the formation of hydrazone through the DNPH derivatization and subsequently the

adsorption to the ELISA plate using biotinylated DNP antibody with the interaction of streptavidin linked HRP [99]. The disadvantages of these methods are the interference from the other molecules due to the cross reactions and batch to batch variability of antibodies [20].

Even though the above analytical techniques are able to detect and measure the oxidative damage to DNA or proteins, they are laboratory-based techniques that require expensive instrumentation, are time consuming and require trained personnel to conduct the analysis.

2.3 Introduction to Point of Care Diagnostics

Point of Care (POC) diagnostics can play a key role in healthcare for assessing a person's condition. In developing countries, the laboratory set up is not well equipped as in developed countries. The major factors affecting the diagnosis in a low resource setting are lack of essential equipment, laboratory consumables, limited trained staff, water supply, power supply and its quality. POC can be used as an alternative to laboratory procedure where doctors can make the initial assessment and also patients can monitor their health remotely [100]. POC diagnostic devices are normally fast, cheap, portable and require small sample volumes. POC diagnostics has proven useful in detecting disease markers, monitor therapies, detect biological and chemical hazards and many more [101]. Even though POC diagnostics are most popular in limited resource settings, it can be also be beneficial for the emergency response, at home and in military settings [100]. They can also be considered as personalized medicine as patients can involve in their own disease management [102].

According to the World Health Organization (WHO), an ideal POC diagnostic device should follow “ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Deliverable to the end-user)” [103]. An ideal POC device is shown in Figure 2.6 where all the functions are fully integrated [104]. The turnaround time is less and reduced manual interference helps in quick management of the diseases.

The emergence of new point of care technologies such as microfluidic devices and nanoparticles could help in delivering effective and cost-efficient healthcare in developing countries. The advancement in these technologies helps in improving assay sensitivity, miniaturization reduced costs and multiplex detection. The point of care

Figure 2.6: An ideal POC device [104].

diagnostics also could strengthen the management of chronic diseases in low resource settings to deliver appropriate, consistent and integrated care [105, 106]. Different materials for POC device can be glass, silicon, PDMS, polymer (plastics) and paper. The comparison of a chip based and a paper-based device is shown in Table 2.1 [107]. Among these POCs, paper-based POC assay are robust, low-cost and easy to use without requiring an external driving force.

Table 2.1: Comparison of a chip based and a paper-based device [107].

Comparison	Chip Based Device	Paper-Based Device
Material	Glass, Silicon, Polymer	Paper and Membrane
Manufacture	Channel Fabrication and Surface Modification	Hydrophilic channels and Hydrophobic barriers
Driving Force	Pump	Capillary force and Evaporation
Result Analysis	Reader	Reader or Visual Detection

2.4 Paper-based Analytical Devices

Paper is typically produced from cellulose by pressing moist fibers and drying them into flexible sheets. Paper has been used extensively in analytical and clinical chemistry due to its low cost, abundance, biodegradability, biocompatibility,

hydrophilicity so that it wicks fluid, versatile functionality and the inertness to the chemical reactions. Due to these unique properties paper is one of the best material for making ASSURED diagnostic devices [108-110].

Paper membranes with different materials, properties and structures are needed for different reagents in several applications. Morphological characteristics such as pore size, symmetry and porosity affect the binding/capture of the various analytes such as proteins and nucleic acids onto the membrane [111]. They are bound onto the membrane by different mechanisms such as electrostatic, van der Waals (dipole induced), hydrogen bonding or hydrophobic interactions. Usually, a membrane with small pore size and high porosity is necessary for high protein immobilization due to the large surface area. This also create slower wicking rate and thus providing more time for the reagent to interact with the membrane. Also, the wicking time depends upon the pore size, porosity and properties of the materials used during the fabrication of membrane [112].

Membranes with different materials such as nitrocellulose, nylon, polyvinylidene difluoride (PVDF), polyether sulfone (PES), cellulose acetate (CA) and glass fiber have different capillary properties and are used for diagnostics [113]. Among these membranes, nitrocellulose is most commonly used in paper-based assays due to its high binding capacity, pore connectivity and porosity. The nitrocellulose membrane bind to the protein due to the presence of nitrate group in membrane that interacts with peptide bonds of the protein rising electrostatic affinity [114]. But these membranes are brittle and has low resistance to highly acidic or alkaline environments. Nylon, CA, and PES membranes are mainly used in filtration. Nylon membrane is hydrophilic with excellent mechanical resistance and good pore size distribution [115]. PVDF can be both hydrophilic and hydrophobic membranes with high chemical and temperature resistance. This is due to the presence of highly electronegative fluorine atoms on the membrane [116]. Hydrophilic PVDF has moderate capillary flow rate with low protein binding capacity, whereas, hydrophobic PVDF has very less capillary flow with high protein binding capacity [115]. Glass fiber membrane filters is mainly used as a pre-filter for fluids containing many molecules and/or having high viscosity [117]. The above-mentioned membranes have smaller pore sizes ($\sim 0.1\text{-}5\text{ }\mu\text{m}$). Table 2.2 summarizes the characteristics of some of the commercially available paper membranes and their applications.

Table 2.2: Summary and characteristics of some commercially available membranes [111, 113].

Manufacturer	Model Number	Membrane	Characteristics	Applications
Merck	HF075	Nitrocellulose	Fast capillary flow	Analytical/Filtration
Merck	HF135	Nitrocellulose	Moderate capillary flow	Analytical/Filtration
Merck	HF180	Nitrocellulose	Slow capillary flow	Analytical/Filtration
Sterlitech	Nylon	Nylon	High protein binding, robust, low fluorescent background	Analytical/Filtration
Sterlitech	CA	CA	Low protein binding, high thermal stability	Analytical/Filtration
Sterlitech	PES	PES	Low protein binding, no surfactants	Analytical/Filtration
Merck	Durapore	PVDF	Low protein binding, moderate capillary flow, No fluorescent background	Analytical/Filtration
Whatman	FUSION 5	Glass fiber	Fast wicking rate, high tensile strength	Sample Pad, Analytical/Filtration
Whatman	470	Cellulose	High rate of absorption, bio-degradable	Wicking Pad

The above-mentioned membranes are used in litmus tests, dipstick assays, lateral flow assays (LFA), vertical flow assays (VFA) or flow-through assays and

paper microfluidic devices as the most commonly used paper-based diagnostic assays. Different types of paper-based analytical devices are shown in Figure 2.7.

Figure 2.7: Paper-based analytical devices (A) dipstick (B) LFA (C) μ PAD.

Litmus tests are form of pH indicator for obtaining acidity of the material. Here litmus (the extracted dye from lichens which is water soluble) are absorbed onto the filter paper by wicking and change color according to the acidity or alkalinity. In dipstick assays, the reagents are impregnated on the paper. This can be used for testing an analyte by dipping into the liquid sample like urine by changing the color based on the blotting [110, 118]. LFA is a sophisticated or highly engineered bioassay where specific binding reactions can be achieved with high sensitivity on a nitrocellulose paper. LFA also has reagents impregnated on the paper like dipstick and the flow paths for the sample are also integrated. It has mainly four parts consists of sample pad, conjugate pad, detection pad and the absorbent pad [119, 120]. The most commonly used LFA is the home pregnancy test [108]. Multiplexing in LFA is limited due to the eclipsing effect (depletion of sample) causing lower intensity signal for the test line. So, to obtain equal distribution of sample, flow-through or VFA are developed [121]. Flow-through assays contains plastic casing with the membranes such as microarray on top and absorbent pad at back whereas VFA are made up of syringe filter holder to place the membrane where analytes pass through vertically. VFA system has a controlled flow as sample is applied vertically using a syringe pump so that flow velocity can be adjusted. The comparison between LFA and VFA/flow-through assays is given on Table 2.3.

Table 2.3: Comparison between LFA and VFA/flow-through assays [121].

Lateral Flow Assay (LFA)	Vertical/Flow-through Assay (VFA)
Limited multiplexing (typically 1-4, including control line)	No limit in multiplexing from single sample
Possibility of non-specific binding as analyse in a sample are exposed to all reagents concurrently	For each test zone, unique reagents can be used
Moderate speed	Fast speed
Easy to use	Easy to use
Require co-formulation of reagents for storage	Can integrate assays easily from individual to multiplex
Mainly limited to immunoassays	Can perform different types of assays (immunoassay/enzymatic) on the same device
No wash steps usually	May/may not need wash steps
No need of syringe pump	Syringe pump may be required

Paper-based microfluidic devices (μ PADs) developed by Whitesides' group in 2007 by patterning the chromatography paper using photolithography (making membrane fully hydrophobic, followed by selective dehydrophobisation) [122]. Since then, different types of fabrication methodologies have been developed. These fabrication methods can be classified into two major groups as shaping/cutting of paper and patterning hydrophilic/hydrophobic channels on paper [122]. For the cutting section, the channeling of paper is generally done by cutting the paper using a cutter plotter or CO₂ laser cutting apparatus. These cut channels are usually then covered with a sticky tape as a backing material to provide strength and structure [123]. The major drawbacks of this method are requirement of a specialized equipment and wastage of raw material. However, for creating fluidic channel on paper with hydrophilic/hydrophobic patterns, there are various methods that can be further classified into subsections such as a) blocking of pores physically, b) by depositing hydrophobic materials on paper membrane, and c) modifying the paper membrane chemically. Different materials such as photoresist and polydimethylsiloxane (PDMS) are used for physical blocking, whereas, wax, polystyrene or ink are used for physical deposition. These materials help in changing the wetting characteristic of the paper membrane by adsorbing onto the pores of the membrane [122, 124]. In case of

Table 2.4: Comparison of μ PAD fabrication techniques [122].

Fabrication Technique	Equipment	Consumables/ Chemicals	Advantages	Drawbacks	Refs
Cutting	Computer, CO ₂ laser or X-Y plotter	None	Sharp defined features	Wastage of raw material, low stability	[125, 126]
Photolithography	Lithography equipment, hot plate or UV lamp printer	Photoresist, ultraviolet resin	Sharp barriers, High resolution	Complex, expensive equipment	[110, 127]
PDMS printing	Desktop plotter	PDMS	Flexible, cheap patterning	Inconsistent control in formation of hydrophobic barrier, low resolution	[128]
Ink-jet etching	Ink-jet printer modified	Polystyrene	Cheap, just require printer	Multiple printing steps, complex	[129]
Wax dipping	Heating unit, customized masks	Solid wax	Low cost and easy	Inconsistent control in formation of hydrophobic barriers between batches due to dipping	[130]
Wax printing	Wax printer, hot plate	Solid wax	Fast and simple	Low resolution	[131]
Stamping	PDMS stamp	Ink or paraffin	Simple, low cost	Inconsistent process and low resolution	[132]
Plasma treatment	Oven, vacuum plasma reactor, masks	Alkenyl ketene dimer (AKD), fluorocarbon	Low cost for consumable	High cost, need multiple masks or glass slides	[133]
Ink-jet printing	Ink-jet printer modified	AKD, UV curable acrylate	High resolution, only need printer	Different ink compositions for printing and extra heating step	[134]

chemical modification, the membranes are treated with cellulose reactive agents such as alkenyl ketene dimer (AKD) that reacts “OH” group in cellulose for creating hydrophobicity in the membrane [124]. The different fabrication techniques such as cutting, photolithography, PDMS printing, ink-jet etching, wax dipping, wax printing, stamping, plasma treatment and ink-jet printing are compared on the Table 2.4.

2.5 Modular Design of Diagnostics

The analytical procedure or clinical diagnostics contains several steps including sample transfer, sample preparation (processing and pretreatment), measurement, signal interpretation or evaluation, and presentation. The schematic of the analytical procedure is shown in Figure 2.8 [135].

Figure 2.8: Schematic of analytical procedure [135].

A paper-based diagnostic device is shown in Figure 2.9 [136] where the extraction, nanoparticle conjugation (for amplification purposes) and detection are all integrated. Sample preparation involves target isolation, sample pre-concentration and sample nanoparticle conjugation.

Figure 2.9: Ideal paper-based molecular diagnostic device [136].

2.5.1 Sample Pre-treatment and Separation

Sample pre-treatment or separation is mainly necessary when sample has interfering matrix components that might result in false positives or negatives, the analyte concentration is too low for detection, or the physical state of the sample is not suitable for the analytical technique [102]. For example, blood is one of the most important biological fluids used for disease diagnostics. The whole blood is too complex and might cause interferences in the assay due to the presence of blood cells. So, it is important to separate plasma from blood that contains several biomarkers like proteins, glucose, urea etc. for analysis. The conventional methods to separate blood plasma are centrifugation, filtration and flow cytometry which are time consuming and laborious [137].

Paper-based devices can be used for blood plasma separation with advantages like low cost, low volume and simple to use. The examples include RBC agglutination method by using blood plasma separation membranes. In RBC agglutination method, the chromatography paper is designed with a plasma separation zone and read out zones. Plasma separation zone is coated with anti A, B antibodies for agglutination of RBC. The schematic is shown in Figure 2.10(A) [138]. Another example is by utilizing blood plasma separation membrane in vertical and lateral flow format. Here blood separation membrane is attached or combined to a Whatman paper in vertical or lateral way as shown in Figure 2.10((B), (C)) [130, 139].

Figure 2.10: Schematic illustration (A) RBC agglutination (B) vertical flow (C) lateral flow [130, 138, 139].

2.5.2 Sample Pre-concentration

Sample pre-concentration is important because it enables detection of trace or low abundance species of the analyte. It is also necessary due to the micrometre dimensions of the fluidic channels resulting less sensitive optical detection compare to the conventional techniques. Pre-concentration helps in improving sensitivity and reliability of the sensing by significantly increasing signal-to-noise ratio. Sample pre-concentration can be done using surface binding, electro kinetic or nanoporous membrane techniques [140].

Figure 2.11: Simultaneous concentration and detection of the biomarker on paper [141].

For example, by combining an aqueous two-phase system (ATPS) with LFA where ATPS can help in concentrating the biomarker in one of the two phases. Here a 3-D paper design is integrated with LFA that enables the user to simultaneously concentrate and detect the biomarker with the addition of micellar ATPS. ATPS system can be comprised of either Triton X-114 surfactant or polyethylene glycol and potassium phosphate (PEG-salt) to concentrate the biomarker. It can increase the sensitivity by 10-fold. The schematic representation is shown in Figure 2.11 [141, 142].

2.6 Biosensor/Detection

A sensor is an analytical device that is designed to selectively detect a molecule or a class of molecules. When a biochemical/biological reaction or interaction (e.g. binding) is used to detect the molecule, it is called a biosensor.

Figure 2.12: Schematic of a biosensor [143].

The working principle of biosensor involves 3 major steps; recognition, transduction and signal processing/presentation. A biosensor is schematically shown in Figure 2.12 [143]. An ideal biosensor should possess high sensitivity, high selectivity, high specificity, as well as short response time and good working “dynamic” range. It should also be easy to operate.

A sensor also can be classified according to the transduction principle such as acoustic (mass), calorimetric (heat), electrical (conductivity), and optical (intensity/spectral) sensors. Among these, optical sensors have proven useful for the

detection of a biomolecules for easy use and will be further discussed in the following sections.

2.6.1 Colorimetric Sensor

Colorimetric analysis is based on the change in color due to the biochemical reactions between the target analyte (proteins, nucleic acids, organic solvent, and harmful gases) and the colorimetric probes or reporters. The concentration of the analyte can be determined with the aid of these probes [144]. There are many advantages with colorimetric sensor like cost effectiveness, ease of use and many of them do not require advanced instrumentation. In some case they are also designed to be interrogated with the naked eye. There are various colorimetric probes/reporters used for colorimetric sensing like enzymes, noble metals and luminescent polymers. These are further discussed in the following section.

2.6.1.1 Enzyme Based Detection

Enzymes are highly selective proteins with catalytic functions that can accelerate a chemical reaction by million times or more. The most commonly used analyte for enzyme-based detection is glucose using glucose oxidase (GOx) and horseradish peroxidase (HRP) that converts a chromogenic compound 3,3',5,5'-tetramethylbenzidine (TMB) [145] into a blue product in the presence of H₂O₂ (generated by GOx). Various other examples are for the detection of albumin, uric acid and ALP. For example, albumin was measured using tetrabromophenol blue as the colorimetric reagent while ALP was detected using nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolylphosphate [146]. The disadvantage of enzyme-based detection is the reduced long-term stability of the enzymes at room temperature causing reduction in activity resulting less sensitivity.

2.6.1.2 Nanoparticle Based Detection

In recent years nanoparticle-based detection strategies have become popular in bioanalysis. They exhibit high extinction coefficients and distinct electronic structures and properties, which offers improved sensitivity for the target analyte. Nanoparticles have been used to detect cancer biomarkers, bacteria, proteins and infectious diseases [146]. Various nanoparticles, like gold nanoparticles (AuNPs), silver nanoparticles (AgNPs) and ceria nanoparticles have been used for biosensing. AuNPs is most widely used due to its low toxicity, excellent biocompatibility and good stability.

For an example, AuNPs functionalized with peptide was utilized for the detection of cardiac troponin I (cTnI). Here, the size and concentration of AuNPs were varied to optimize the sensitivity and yielding a limit of detection of 0.2 ng/ml. In this, at the absence of cTnI, AuNPs display colloidal stability and remain in dispersed state, while the presence of cTnI causes the aggregation of AuNPs with color change based on cTnI concentration [147]. The assay schematic is shown in Figure 2.13.

Figure 2.13: Assay schematic of cTnI based on peptide functionalized AuNPs [147].

Another example is utilizing ceria nanoparticles and glucose oxidase co-immobilized on a filter paper for the detection of glucose. Here the physiochemical properties of the nanoparticles were utilized. When glucose is present, the filter paper color was changed from white to orange depending on the concentration of produced hydrogen peroxide with a detection limit of 0.5 mM. The schematic is shown in Figure 2.14 [148].

Figure 2.14: Detection of glucose using ceria nanoparticles [148].

The main disadvantage of the NP based sensors is related to the colloidal stability of the nanoparticle suspension. Subtle changes in the buffer composition (and/or contaminants), ionic strength and acidity might lead to uncontrolled aggregation of the NPs and subsequent colour change of the suspension [149].

2.6.1.3 Polymer Based Detection

Conjugated polyelectrolytes (CPEs) are a class of organic semiconducting polymers with hydrophobic π -conjugated backbones and hydrophilic ionic side groups [150]. According to different backbone structures, they can be classified into polyacetylene (PA), poly(para-phenylene) (PPP), polyfluorene (PF), polyaniline (PANI), polypyrrole (PPy) and polythiophene (PT) as shown in Figure 2.15 [150-152].

Figure 2.15: Chemical structures of various CPEs [153].

CPEs can be employed in various applications like light emitting diodes, solar cells, field effect transistors and biosensors by utilizing the delocalized π -electrons that give the polymer unique optical and electronic properties [154-157]. The properties of CPEs can be changed with temperature, pH, electric field, as well as upon binding to other molecules to form supramolecular structures [158-160]. The photophysical properties of the CPEs such as absorption and fluorescence emission can be tuned by changing the chemical nature of the substituents (R in Figure 2.15), effective conjugation length, intramolecular conformation and intermolecular packing. In addition, the charge density around the CPE backbone and its chain length have been used to tune the fluorescence emission wavelength [161]. With an electron donating side chain or by increasing the chain length, the emission of CPEs appeared red shifted. Similarly, the conformational change and the intermolecular packing of the CPE backbone have huge influence on emission properties as shown in Figure 2.16. During the sensing process, the optical changes in the CPEs are driven by electrostatic and hydrophobic interactions with other molecules due to the presence of the ionic (positive/negative) CPE side groups and the hydrophobic CPE aromatic/heterocyclic backbones.

- Chemical Nature

- Conjugation Length

- Conformation

- Interpolymer Packing

Figure 2.16: Factors influencing photophysical properties of CPEs [162].

Compared to small molecule-based sensors (organic dyes), CPEs are more advantageous as they can amplify the response from a binding or recognition event. In CPEs the binding event affects the conformation and/or packing characteristics of the entire polymer backbone resulting in amplification or quenching of fluorescence, whereas the binding on a small molecule-based sensors, the fluorescence is altered only on a single chromophore [163]. The small molecular organic dyes also exhibit poor photostability (limiting the long term use), photobleaching, blinking and presence of a high background signal in comparison with CPEs [164].

The various detection modes and mechanisms of CPEs are shown in Figure 2.17. They are fluorescence turn on (enhancing), turn off (quenching), fluorescence and visible color change.

Figure 2.17: Detection mechanisms and modes of optical detection for CPEs [163].

In case of turn-on mode, the binding event causes a conformational change or electron density change of the polymer backbone resulting in the onset of fluorescence. While in the turn-off mode, quenching of CPEs fluorescence occurs due to the intermolecular aggregation or non-radiative relaxation pathways of the polymer chain. The change in fluorescence is due to the alteration in electron density after target binding. This can be attributed to conformational change of CPEs, polymer aggregation and electron energy transfer. For all the fluorescent modes the signal can be amplified using fluorescence resonance energy transfer (FRET). The visible color mode occurs when a recognition event causes a change in the conjugation length of the polymer backbone resulting the change in absorption wavelength of the polymer. One significant advantage of CPEs is that, it can be used as both colorimetric and fluorometric probes. Moreover, they also can amplify the signal through collective effects so-called “collective system responses” [163].

Among the different CPEs, polythiophenes (PT) offer superior electronic and optical properties and are suitable for the bio sensing [165-167]. The optical transitions of PTs are due to the conformational change in the backbone of the polymer that in turn causes the blue or red color shift. Several PT biosensors have been developed for a broad spectrum of biological targets such as nucleic acids, proteins, various small biological molecules and ions. Some of these biosensors are discussed below.

The detection of single stranded DNA (ssDNA) using PT is shown in Figure 2.18. Initially the color of the solution was yellow that is typical for random coiled PT. When ssDNA was added to the PT solution, the conformation of the polymer changed from random coil to planar due to the electrostatic interactions that induced the formation of a duplex. Due to the conformational change, color of PT changed from yellow to red and fluorescence intensity was quenched. However, when a complementary ssDNA sequence was added to the solution, the nucleic acid sequences hybridized and formed a double stranded DNA (dsDNA). The PT wrapped around the dsDNA to form a triplex. Due to this fluorescence intensity was increased and the color revert back to yellow due to the reduction of conjugation length in PT. This two significantly different colors were utilized to detect the DNA hybridization [168].

Figure 2.18: (A) Schematic illustration of DNA hybridization event (B) Photographs of (a) PT (b) duplex formation (c) triplex formation [168].

Real time monitoring of ssDNA cleavage was developed utilizing cationic polythiophene. The addition to ssDNA to the PT forms a duplex due to the electrostatic interaction, whereas, when ssDNA is digested by S1 nuclease or hydroxyl radical into small fragments, the duplex was not formed and the PT remained in the random coil formation and the polymer color didn't change as shown in Figure 2.19 [169].

Figure 2.19: (A) Schematic illustration of nuclease assay (B) Photograph of nuclease assay [169].

Label free detection of potassium ions using aptamer is shown in Figure 2.20. Here the aptamer which can specifically bind with potassium ions undergoes a conformational change from free form to G-quadruplex during binding. The two different conformations of the aptamer was detected using the PT. In case of the aptamer in free form, PT-aptamer formed a duplex due to the electrostatic interaction

and color of the PT change from yellow to red. While in case of G-quadruplex structure form of aptamer (due to the presence of potassium ions), the PT wrapped around this structure and formed an orange color as shown in Figure 2.20(B) [170].

Figure 2.20: (A) Schematic illustration of potassium ions assay (B) Photograph of (a) PT (b) LiCl (c) NaCl (d) KCl (e) RbCl [170].

Another example is of a cationic polythiophene derivative with anthracene side group (CPT) was developed for the dual mode (both colorimetric and fluorescent detection) of adenosine triphosphate (ATP). Here the sensing strategy was based on the self-promoting aggregation of CPT as shown in Figure 2.21. By adding ATP to the CPT, CPT and ATP interact due to the electrostatic interactions. This forced the CPT to adopt a planar formation, and the anthracene side groups in CPT assisted in polymer aggregation via interchain π - π stacking, providing sensitive detection of ATP [171].

Figure 2.21: (A) Schematic illustration of ATP assay (B) Change in color of CPT with other anions [171].

Polythiophenes were also used for water quality monitoring. As most of the microbes have an anionic surface (negatively charged), cationic polythiophene was used to detect the microbes. Here *E. coli* was used as a prototype for the detection of microbes and the sensor was based on fluorometric interrogation. When *E. coli* was added to the PT, the fluorescence of the polymer was quenched (decreased) without shift in wavelength due to change in conformation from random-coiled to planar as shown in Figure 2.22 [172].

Figure 2.22: Schematic illustration of the detection of microbial particles [172].

The detection of thrombin protein with an aptamer and PT on a solution and solid support was reported. In this, a new fluorescence signal amplification detection was proposed called fluorescence chain reaction (FCR). This occurs due to the combination of different interactions such as electrostatic, chromism and Förster resonance energy transfer (FRET) mechanisms that led to a novel fluorescence signal amplification detection. Here thrombin binding aptamer were labelled with cyanine dye (Cy3) at 3' end and an amine group at 5'-end. Cy3 was selected as its excitation spectrum was matched with PT's emission spectrum for efficient FRET. The PT was added to the aptamer and this solution was spotted onto surface-treated glass slide and formed covalent binding with the amine group as shown in Figure 2.23. When thrombin was added to the aptamer/PT complex, there was a significant increase in fluorescence. Bovine Serum Albumin (BSA) and immunoglobulin E (IgE) were also

added to the aptamer/PT complex to verify the specificity of the detection, and revealed no increase in fluorescence [173]. These studies were done in the buffer.

Figure 2.23: (A) Schematic illustration of thrombin assay on glass slide (B) Specific detection of thrombin protein [173].

Biosensing using PT is predominantly studied in the solution state. But there are very high non-specific interactions in solution state. Previously, our group developed a paper-based platform utilising PT for the detection of miRNA. In this, poly (vinylidene fluoride) (PVDF) paper was impregnated with cationic polythiophene

Figure 2.24: Schematic representation of miRNA detection using polythiophene [174].

and peptide nucleic acid (PNA), where PNA was used as a receptor. Here the non-complementary and single base mismatch sequences changed in the color (from orange to purple) due to the duplex formation, while the complementary miRNA sequence caused no color change due to the efficient hybridization with PNA thus forming a triplex. The schematic representation is shown in Figure 2.24. These studies were done in the buffer [174].

2.6.2 Aptamers

Another important aspect of a biosensor is the recognition element. The recognition element is used to specifically detect the target analyte. These recognition elements can be antibodies, peptides and aptamers. The term ‘aptamer’ is derived from the Latin word aptus- ‘to fit’. Aptamers are short, single-stranded oligonucleotides that can form 3-dimensional conformations upon specifically binding to a target analyte.

Figure 2.25: Selection of target aptamer through SELEX process [178].

The target can be proteins, nucleic acids, cells and even tissues [175]. The analyte-aptamer bond is relatively strong and is a result of hydrogen bonds and electrostatic interactions between the aptamer and analyte. A lot of other factors also come into

play such as complementary shape of target and aptamer, stacking interactions between aromatic compounds and the nucleobases of aptamers etc. [175, 176]. These short chain oligonucleotides can be both DNA and RNA chains and generally have 15-75 bases. The functionality of aptamers depends on length of nucleic acid molecule, primary chain sequence and their environmental conditions [175]. Aptamers are produced via chemical synthesis route, by a process called ‘SELEX’ or Systematic Evolution of Ligands by Exponential enrichment [177]. It involves oligonucleotide library selection, attaching binding molecules to the oligonucleotides, eluting the binding DNAs, amplifying them and finally repeating the entire process 7-8 times as shown in Figure 2.25 [34, 176, 178]. This process ensures that a homogenous batch of highly specific molecules with high binding affinity is obtained at the end of it. The process has very little or no batch to batch variation and allows incorporation of any chemical or functional modifications needed in the molecule during synthesis itself [176].

Aptamers are capable of folding into wide range of complex or three-dimensional structures enabling binding to target analytes. The structure of the aptamer is dependent upon the sequence of the nucleotides, characterized by hairpins (stem-loop), bulges, pseudoknots, triplexes or quadruplexes [179]. A hairpin loop forms when the regions are complementary to each other forming Watson-Crick base pairs, whereas, quadruplexes usually form in a guanine rich sequences, where four guanine bases are associated through hydrogen bonding. The structures are determined using either NMR spectroscopy or X-ray crystallography. Some of the aptamer secondary structures are shown in Figure 2.26 [180].

Figure 2.26: Schematic illustration of secondary structures of aptamers: (A) hairpin (B) pseudoknot (C) G-quadruplex [180].

Aptamers can be used as alternatives to antibodies, with numerous advantages over the latter as stated in Table 2.5. Aptamers are easy to handle, non-immunogenic, stable and cheaper.

Table 2.5: Advantages of aptamers over antibodies [34, 175, 176].

Aptamers	Antibodies
Chemically synthesized; In-vitro	Cell culture; In-vivo production
Process is less laborious and cheaper	Extremely laborious and expensive
Can be raised against targets, even toxic or non-immunogenic compounds	Difficult for toxic targets. Mainly for common proteins
Small size, easy to store and handle	Large size, difficult to store and handle
Batch to batch variation is less due to the chemical synthesis	Batch to batch variation is common
Long shelf life	Short shelf life
Fairly stable to temperature and pH and are easily refolded if denatured.	Sensitive to temperature and pH and undergo irreversible denaturation
Can be easily modified with other molecules	Difficult and expensive to modify

At present, colorimetric paper-based biosensors using aptamers are mainly achieved utilizing optical probes such as gold nanoparticles, quantum dots and dyes. Some of these biosensors are discussed below.

Aptamer was utilized for the detection of kanamycin- a bacterial antibiotic using gold nanoparticles (GNPs) on a paper chip as shown in Figure 2.27. Here, the citrate coated GNPs were incubated with the random coiled aptamer. This mixture was then added to the paper chip and dried. This paper was then used for the detection of kanamycin. Here in the absence of the target analyte, the aptamer adsorbed onto the GNPs protected from aggregation after the addition of NaCl, whereas, in the presence of the target analyte, the aptamer adsorbed was detached due to the conformational changes and GNPs were aggregated after addition of salt and resulting a change in color [181].

Figure 2.27: Schematic illustration of paper based detection of kanamycin [181].

Aptamers were also utilized in lateral flow assays (LFA). There are mainly two assay formats in aptamer based LFA; sandwich and competitive as shown in Figure 2.28. Aptamers can be modified easily to conjugate with different labels such as dyes and gold nanoparticles (GNP) that can give readable signals.

Figure 2.28: Aptamer based LFA: (A) Sandwich format (analyte- thrombin) [42] (B) Competitive format (analyte- ochratoxin) [183].

Sandwich assay is mainly utilized when there are two aptamers for the desired target. In this, one aptamer is conjugated with the label, while the other aptamer is immobilized at test line. Here, the target in the sample binds sequentially with labelled aptamer and the aptamer deposited at the test line respectively thus resulting a

Table 2.6: Summary of a few aptamer based optical paper biosensors.

Target	Optical Probes	Limit of Detection	Reference
Thrombin	Gold Nanoparticles	2.5 nM	[42]
Ramos cells	Gold Nanoparticles	4000 Ramos cells; 800 Ramos cells (with reader)	[43]
Adenosine	Gold Nanoparticles	20 μ M	[184]
Cocaine	Gold Nanoparticles	10 μ M	[184]
Ochratoxin A	Gold Nanoparticles	1 ng/ml; 0.18 ng/ml (with reader)	[183]
Vaspin	Gold Nanoparticles	0.105 nM	[185]
Ochratoxin A	Gold Nanoparticles	1 ng/ml	[186]
E. coli	Gold Nanoparticles	10 CFU/ml	[187]
Thrombin	Gold Nanoparticles	4.9 pM	[188]
HCV core antigen	Gold Nanoparticles	100 pg/ml; 10 pg/ml (with reader)	[189]
β -Conglutin	Gold Nanoparticles	9 fM	[190]
Aflatoxin B1	Cy5-labeled aptamer	0.1 ng/ml	[191]
Ochratoxin A	Cy5-labeled aptamer	0.4 ng/ml	[192]
E. coli	TMB/H ₂ O ₂	Not mentioned	[193]
Cancer cells	Quantum dots with graphene oxide	65 cells/ml	[194]
Adenosine	Iodine-starch	0.16 μ M	[195]
Pb ²⁺	Dye with hydrogel	200 nM	[196]

sandwich. At the control line, complementary oligonucleotides to labelled aptamer are immobilized [182]. The schematic of sandwich LFA is shown in Figure 2.28(A).

In case of competitive LFA format, the test line and control line were incubated with the complementary DNA sequence of aptamer which can form a duplex. Here, the conjugation pad of LFA contain detection labels such as aptamer modified GNPs. In this assay, when the sample does not contain the target, GNP-aptamer conjugates bind with complementary DNA in both test line and control line and forms the signal. But, in the presence of the target, GNP-aptamer prefers to bind with the target resulting the reduction in color signal in test line. The color intensity of the test line depend upon the concentration of the target [182]. The schematic of competitive LFA is shown in Figure 2.28(B).

A summary of a few aptamer-based biosensors for various targets are given below in Table 2.6.

Currently, paper-based biosensors mainly use gold nanoparticles and dyes as detection label. Also, most of the assays for carbonyl/oxidative stress biomarkers use antibodies as the recognition element. This thesis attempts to develop colorimetric sensors for the detection of carbonyl/oxidative stress biomarkers associated with T2D utilizing cationic polythiophene with high sensitivity and selectivity. As mentioned above, by taking advantages of both cationic polythiophene and aptamers, the assays for various carbonyl/oxidative biomarkers associated with T2D such as microRNA21 (mir21), 8-hydroxy-2-deoxyguanosine (8-OHdG) and glyceraldehyde derived advanced glycation end products (Gly-AGEs) were developed. The following chapters discuss about the assays developed for each of these biomarkers.

CHAPTER 3: VISUAL DETECTION OF NUCLEIC ACIDS

3.1 Introduction

Nucleic acids have been realized as potential markers for diagnosis of metabolic (e.g. T2D) and infectious diseases (e.g. tuberculosis, hepatitis) [197-200]. Polymerase chain reaction (PCR) and microarray methodologies are commonly adopted for nucleic acid assaying with good detection limits. These methodologies generally require instrumentation, trained personnel and are time-consuming, which prohibits the large-scale adoption [201-203]. Microfluidics and chip-based assay devices therefore have been explored in order to overcome these limitations; however, sophisticated fabrication protocols and external instrumentation may be required which increases the cost per test [204, 205]. On contrary, paper-based assays are facile, cost-effective, easy to use, enabling diagnosis on site and in resource-limited settings [206-208].

Efforts have been devoted to develop paper-based assays for rapid detection of nucleic acids [107, 209]. However, clinical samples (e.g., plasma, serum, urine) are usually complex and contain trace amounts of the target nucleic acids of interest, therefore requiring tedious additional procedures such as isolation and sample pre-treatment for minimizing interferences from sample matrices. Most of the nucleic acids assays reported involve either extraction or amplification protocols, for instance, using extraction kits for isolation of nucleic acids prior assaying [210, 211]. In the recent years, extraction and amplification processes have been integrated with paper-based assays [204, 212-214]. However, these approaches involve longer assay time, multiple processing steps (e.g. interference exclusion and cleaning step), and external functional units (e.g. heater, pressure supply) inhibiting point of care (POC) applications.

In this chapter, I take advantages of the optical changes of a cationic polythiophene as described in chapter 2 to develop solution and paper-based assays for nucleic acids. As described in chapter 2, optical transitions of PT are due to the conformational alternations in backbone that increase or reduce the effective conjugation length leading to red- or blueshift, respectively, of the UV-vis absorption maximum [215]. The extent of conformational alternations in backbone is mainly controlled by energy required for interring twisting, which is less than $2.5 \text{ kcal mol}^{-1}$ for disubstituted thiophene [215]. The dihedral angle between the conjugated rings

was calculated to be 67.8° for 3, 4-dimethyl substituted thiophene, while cyclopentene substitution on to polymer was reported to reduce the steric hindrance thereby lowering twisting angle ($55\text{--}56^\circ$). In this context, addition of pendant groups with varying chemical moieties to the conjugated polymer would further alter the twisting angle between rings and therefore introducing two different pendant groups in the same conjugated polymer backbone by statistical copolymerization of two monomers helps in tuning interring twisting of PT upon complexation with nucleic acids. The synthesized polythiophenes were first used in a solution-based assay and then translated to a paper-based platform. As a proof of concept, assay of two nucleic acid biomarkers, microRNA (mir21 associated with T2D [216]) and hepatitis B virus DNA (HBV-DNA) were performed. The validity of the developed flow-through device for the detection of nucleic acids was also demonstrated in plasma. A logic gate system was developed to utilize the colorimetric assay responses as inputs for discrimination of mir21 and HBV-DNA and subsequently to obtain a profile of nucleic acids in samples that exceed respective clinical threshold limits, thereby enabling rapid and point of care (POC) disease diagnosis.

3.2 Synthesis of Cationic Polythiophenes

Previous efforts at the Centre for Biomimetic Sensor Science (CBSS), were devoted to the synthesis of conjugated polyelectrolytes like cationic polythiophenes (PT) for sensor platform development. Homopolymers Poly (3-alkoxythiophene) polyelectrolytes (hPT1 and hPT2) with different functional groups (i.e. triethylamine and 1-methyl imidazole) at the terminal position of the side chain of thiophene and their random copolymers (cPT1, cPT2 and cPT3) in different monomer ratios (trimethylamine/1-methyl imidazole) of 1:1, 1:5, 5:1, respectively, have been designed and synthesized using oxidative polymerization. I acknowledge Dr. Deepa Rajwar for the help in synthesis of these polythiophenes. The detailed procedures for the synthesis of these polymers with their corresponding NMR spectra are given in Appendix I. The chemical structures of these polythiophenes are shown in Figure 3.1. The synthesized polythiophenes are water soluble with a stock concentration of 1 mg/10 mL ($\sim 300\text{ }\mu\text{M}$).

Figure 3.1: Chemical structures of homopolymers (hPT) and copolymers (cPT).

3.3 Solution based Sensing of Nucleic acids

3.3.1 Materials and Methods for Solution based Sensing

All the reagents and solvents were obtained from Sigma-Aldrich and used without further purification. MicroRNA sequences such as mir21 or target sequence (Tseq) 5' UAG CUU AUC AGA CUG AUG UUG A 3', Non-Complimentary Sequence/Control (NCseq) 5' AUG CAU GCA UGC AUG CAU GCA A 3' and single base mismatch sequence (SBseq) 5' UAG CUU AUC AGA GUG AUG UUG A 3' were obtained from IDT (Singapore). All sequences with stock solutions of 100 μ M were prepared with RNase free DI water and stored at -20 $^{\circ}$ C. The peptide nucleic acid (PNA) probe, N-TCA ACA TCA GTC TGA TAA GCT A-C, complementary to mir21 (Tseq) was purchased from Panagene (Daejon, Korea). Furthermore, steady state absorption and emission spectra were obtained by an Infinite M200Pro Tecan plate reader.

3.3.2 Experimental Findings in Solution based Sensing

Five different PTs, two hPTs (hPT1 and hPT2), and three cPTs (cPT1, cPT2 and cPT3) with varying pendant groups were systematically evaluated for miRNA assay. mir21, a miRNA sequence, is utilized in this study as the target sequence (Tseq) for evaluating colorimetric response of PT upon incorporation of a peptide nucleic acid sequence (PNA) complementary to mir21. PNA sequences are used as bio receptors instead of RNA/DNA as PNA binds to complementary RNA/DNA sequences with higher affinity than DNA/RNA due to the lack of electrostatic repulsion. This is because, PNA is DNA mimic where the negatively charged phosphate linkages in DNA are replaced with polyamide chains. Also, PNA is

resistant to nuclease degradation and have insensitivity to ionic strength and pH during hybridization [217]. PT exhibited a distinguishable colorimetric response upon formation of PT–PNA–Tseq triplexes compared to that of PT–PNA–NCseq/SBseq (noncomplementary sequences) mixtures or PT–Tseq/NCseq/SBseq duplexes as shown in Figure 3.2. The sensing strategy of the nucleic acid assay is based on monitoring color variations of PT upon interaction with Tseq/NCseq/SBseq in the presence and absence of PNA. In the absence of PNA, the resulting color would be due to PT–Tseq/NCseq/SBseq duplex formation (weakly fluorescent), whereas in the presence of PNA, the output color would be due to either the formation of duplex (PT–NCseq/SBseq), weakly fluorescent) or formation of triplex (PT–PNA–Tseq, fluorescent) thereby yielding different color signals.

Figure 3.2: Schematic illustration of PT–Tseq/NCseq duplex and PT–PNA–Tseq triplex formation using homo- and copolymers.

All the synthesized PTs were evaluated for their complexation capabilities with miRNA. The changes in the π – π^* transitions that arise from the conformational alternations in the PT backbone upon interaction with miRNA were monitored by absorption and fluorescence spectroscopy. Spectral characteristics recorded using 100 μ L of 150 μ M PT show that the absorbance (IPT) maxima of hPT1, hPT2, cPT1, cPT2, and cPT3 in DI appear at wavelengths (λ_1) 403, 459, 415, 436, and 406 nm, respectively, and are associated with random coil conformation of the PT derivatives [218]. Upon adding 1.4 μ M of Tseq, the absorbance maximum red-shifted with an observable color transition for all PTs as shown Figure 3.3. To facilitate comparison of colorimetric responses between PTs, the change (Δ) in absorbance ratio at λ_1 and λ_2 (wavelength corresponding to absorbance peak of PT–Tseq duplex) was evaluated (illustration for cPT3 is shown in Figure 3.3(f)) [219–221].

Δ_{duplex} , $[I_{\text{duplex}}(\lambda_1)/I_{\text{duplex}}(\lambda_2) - I_{\text{PT}}(\lambda_1)/I_{\text{PT}}(\lambda_2)]$, and Δ_{triplex} , $[I_{\text{triplex}}(\lambda_1)/I_{\text{triplex}}(\lambda_2) - I_{\text{duplex}}(\lambda_1)/I_{\text{duplex}}(\lambda_2)]$ were observed in the following order; $\Delta_{\text{hPT1}} (3) > \Delta_{\text{cPT3}} (1.9) > \Delta_{\text{cPT1}} \sim \Delta_{\text{cPT2}} (0.9) > \Delta_{\text{hPT2}} (0.5)$ and $\Delta_{\text{cPT3}} \sim \Delta_{\text{hPT1}} (1) > \Delta_{\text{cPT2}} (0.6) > \Delta_{\text{hPT2}} (0.5) > \Delta_{\text{cPT1}} (0.3)$, respectively. Although hPT1 shows the largest interaction with Tseq, recovery upon PNA addition was relatively lower for hPT1 ($\Delta_{\text{triplex}}/\Delta_{\text{duplex}}$, $\sim 33\%$) when compared to cPT3 ($\sim 53\%$). Figure 3.3 illustrates that absorbance maxima of PT–Tseq duplex (red spectra) were centred at around 550 nm (λ_2). The

Figure 3.3: Absorption spectra of PTs in DI with various miRNA sequences in the presence and the absence of PNA for (a) hPT1 (b) hPT2 (c) cPT1 (d) cPT2 (e) cPT3 and (f) an illustration for calculating the change (Δ) in absorbance ratio at λ_1 and λ_2 .

deconvolution of these duplex spectra upon baseline correction reveal four minor components with absorbance maximum ranging from 424 to 587 nm. Absorbance maximum for all the spectra were observed at ~547 nm, which is attributed to the duplex conformation. The remaining minor components, for instance, for hPT1 at 424, 502, and 587 nm, correspond to twisted or planarized conformations [218]. The most significant spectral shift from 412 to 547 nm was observed for hPT1. The extent of shifts was observed to be in the following order: hPT1 [135 nm] > cPT3 [133 nm] > cPT1 [106 nm] > cPT2 [92 nm] > hPT2 [61 nm], in agreement with our analysis using absorbance intensity ratios at λ_1 and λ_2 (see above).

Experimental results therefore indicate the possibility of utilizing cPT, cPT3 for instance, that possess both favourable quenching and recovery capabilities among all synthesized PT for nucleic acid assaying. The main reason for the improvement of cPT behaviour is attributed to the introduction of hydrophobic pendant groups that weakens the electrostatic interaction of cPT with miRNA. The imidazolium group in cPT would still facilitate cPT-miRNA complexation via both electrostatic and hydrophobic interactions but to a lesser extent as compared to the amine group, thereby enabling better recovery compared to hPT1 [222, 223]. Experimental results suggest not only that “planarization of backbone” influences optical properties of PT but also that synergistic electrostatic and hydrophobic forces govern the PT-miRNA supramolecular complexation.

The emission spectra of hPT1, hPT2, cPT1, cPT2 and cPT3 in DI is a featureless band with the maximum peak at 536 nm, 591 nm, 566 nm, 581 nm, and 539 nm respectively as shown Figure 3.4(a)-(e). As expected the addition of miRNA quenches the all PTs, whereas the recovery of fluorescence with the addition of PNA is higher for PT-Tseq complex in comparison to the PT-NCseq/SBseq, which reveals a clear difference between Tseq and NCseq/SBseq. Among the 5 different PTs, cPT3 exhibited the best fluorescence recovery upon addition of PNA, in agreement with the absorption spectra.

The optical transitions of PT/CPs are due to the conformational alternations in backbone that increase or reduce the effective conjugation length leading to either red or blue shift of the fluorescence signal. cPT3 exhibited a distinguishable optical response upon formation of cPT3-Tseq-PNA triplexes compared to cPT3-Tseq/NCseq/SBseq duplexes or cPT3-PNA-non-complementary complexes tested as shown in Figure 3.4(f). The addition of miRNA sequences such as Tseq, NCseq and

SBseq changes the color of cPT3 from yellow to purple. While, in the presence of PNA, the color of PT-PNA-Tseq changes back to yellow, due to the hybridization of PNA and Tseq, but, in case of NCseq and SBseq, the PNA didn't hybridize with them and so the color remains purple due to their effective interactions with PT. The obtained colorimetric responses also concur with the spectral characterization as shown in Figures 3.3(e) and 3.4 (e).

Figure 3.4: Fluorescence spectra of PTs in DI with various miRNA sequences in the presence and the absence of PNA for (a) hPT1 (b) hPT2 (c) cPT1 (d) cPT2 (e) cPT3 and (f) Optical responses of cPT3 with miRNA under UV lamp where (1) cPT3 (2) cPT3+Tseq (3) cPT3+NCseq (4) cPT3+SBseq (5) cPT3+PNA+Tseq (6) cPT3+PNA+NCseq (7) cPT3+PNA+SBseq.

3.3.3 Summary of Solution based Assaying

There is a lack of discussion in the literature on how the sensing capacity of a CPEs may vary with structure of the pendant group. Here, by synthesizing PTs and taking advantage of different functional groups in a single polymer, it has been demonstrated that copolymer of PT yielded better optical responses for the miRNA detection in comparison to those of their homopolymers. Moreover, the proposed assay could be utilized for detection of other RNA sequences of interest upon introduction of a corresponding complementary PNA sequence. Due to the encouragement from the solution-based assay results, I then translated the solution-based platform to the paper-based which is described in the following sections.

3.4 Paper-based Sensing of Nucleic Acids

PTs are predominantly studied in the solution state. In the following section, the solution-based sensing platform is translated to a paper-based one for easy use.

3.4.1 Materials and Methods for Paper-based Sensing

All the chemicals for PT synthesis were purchased from Sigma–Aldrich and used without further purification. Deionized (DI) MilliQ water (resistivity of 18 MΩ cm) was used for buffer preparation and PT synthesis. The following ssDNA and PNA (complementary to HBV-DNA) sequences were purchased from IDT and Panagene, respectively. HBV-DNA was used for the optimization of PT coating on paper. The wild type HBV-DNA sequence was selected from YMDD gene (named after its amino acids motif tyrosine (Y), methionine (M), and aspartate (D)), which represents an active site of HBV reverse transcriptase [224]. PVDF filter centrifugal columns were purchased from Merck Millipore. A 13 Mega pixel Samsung galaxy note 3 camera was utilized for capturing digital images of the samples.

HBV-DNA: 5' TAT ATG GAT GAT GTG GTA TT 3'

PNA: N-AAT ACC ACA TCA TCC ATA TA-C

All the synthesized PTs as described above were used for the coating on paper. PTs were drop casted on different membranes such as cellulose, nitrocellulose and polyvinylidene fluoride (PVDF). Among them, PVDF membranes were chosen for the optimization of polymer as coating on PVDF membrane provided better homogeneity. PTs were coated onto PVDF centrifugal tubes by adding 200 μL of varying concentrations of PT. Tubes were centrifuged at 8000 rpm for 3 minutes. This process

was repeated several times to optimize the PT coating. Subsequently the deposited films were washed 2 times with DI water by centrifuging at 8000 rpm for 3 minutes each. The filter was then cut from the tubes. Samples were subsequently tested with HBV-DNA by adding 2 μ L of varying concentrations. Similarly, 2 μ L of pre-hybridized solution of 25 μ M PNA with varying concentrations of HBV-DNA was added to the PT coated membranes. These membranes were placed on UV illumination zone (portable UV lamp, at 365 nm, 8 W), and digital images were captured using a Samsung galaxy camera positioned at a fixed distance from the membranes. Subsequently, the captured digital images were imported to a computer in jpeg format for analysis using the ImageJ software. 75 x 75 pixels of each PT membranes were utilized to generate RGB values and delta E analysis.

3.4.2 Experimental Findings in Paper-based Sensing

All the 5 synthesized PTs were evaluated for coating on hydrophilic PVDF membranes and their complexation capabilities with ssDNA. Initial evaluation was carried out by coating 100 μ M of PTs with 7.5 μ M of HBV-DNA and 25 μ M PNA samples. The sensing strategy is based on observing the optical properties of PT impregnated in the PVDF membrane with HBV-DNA and HBV-DNA/PNA in analogy with the solution-based platform of miRNA. In brief, complexation of PT-HBV-DNA on the PVDF membrane produces a non-fluorescent duplex due to the electrostatic interactions between PT and the HBV-DNA, thereby leading to a fluorescence quenching and a color transition from orange to purple. On the other hand, the color transition of PT is not observed in the presence of PNA as triplex formation of PT-PNA-HBV-DNA gives fluorescent orange color. Hence, the orange color of PT-PNA-HBV-DNA and the significantly different purple color (PT-HBV-DNA) enables naked eye detection of HBV-DNA. Inspection of samples under UV illumination reveal that all PTs undergo noticeable quenching in the presence of HBV-DNA whereas no quenching was observed for samples containing mixtures of HBV-DNA-PNA complexes as shown in Figure 3.5. Among the 5 PTs the most distinguishable fluorescence quenching was observed for hPT1 followed by cPT1, cPT3, cPT2 and hPT2. The better performance of hPT1 could be attributed to its dominating trimethylamine group as compared to 1-methyl imidazole functional group in hPT2. Interactions with hydrophilic PVDF membrane are observed to aid

conformational changes in hPT1 allowing it to freely form duplexes as compared to hPT2 along with other copolymers.

Figure 3.5: Responses for 100 μM PTs coating (REF (only polymer)-without DNA or DNA/PNA) with 7.5 μM of HBV-DNA and HBV- DNA with complementary PNA.

Although naked eye responses are sufficient to ascertain superiority for hPT1 response, RGB analysis was further carried out to quantify the responses as shown in Figure 3.6 for all 5 polymers. Figure 3.6(a)-(e) shows that the change in RGB values for all the polymers after the addition of HBV-DNA (quenching), and after the addition of HBV-DNA-PNA (recovery). The changes in RGB colors are significant upon quenching, whereas, the corresponding changes for PTs and the PTs plus HBV-DNA-PNA are very small or insignificant. Among the 5 different polymers hPT1 showed the prominent response (most change in color) in RGB values. Furthermore, delta-E (ΔE) values, a metric (ranging from 0 to 100: $\Delta E > 2$ is regarded as the threshold value for visual differentiation of colors) for visual perception of color differences were then calculated from RGB values as shown in Figure 3.6(f) based on the International Commission of Illumination (CIE) 1976 algorithm [225] using the ΔE calculator provided by ColorMine.org. ColorMine library is an open-source MIT licensed .NET project. In this method, RGB values are converted to CIE $L^*a^*b^*$ color space where L^* , a^* and b^* are numerical values representing lightness, green–red and blue–yellow color components, respectively. Lab colors were designed to approximate human vision and align with how humans perceive color [226]. A high ΔE value implies that the change in color is more easily distinguishable. Thus, a suitable

Figure 3.6: RGB values for different polymers and with the addition of HBV-DNA and HBV-DNA-PNA (a) hPT1 (b) hPT2 (c) cPT1 (d) cPT2 (e) cPT3 (f) ΔE values of duplex and triplex formation with 5 different polymers.

polymer for nucleic acid assay should possess a high ΔE value upon duplex formation and a correspondingly low ΔE value upon triplex formation (indicative of good quenching and recovery, respectively). As shown in Figure 3.6(f) the ΔE value for duplex formation (quenching) was highest for the hPT1 polymer. For triplex formation, ΔE value should be as low as possible indicating PT and PT-HBV-DNA-

PNA have same color. The lowest ΔE value was seen for cPT3 with hPT1 as second lowest, indicating the efficient DNA-PNA hybridization and high recovery.

The preliminary results indicate the feasibility of all polymers to be utilized in the solid state. However, I chose to continue with hPT1 for further evaluation in assay development because of its superior performance. To maximize the assay performance, the optimization of hPT1 coating is important. I observed a close trade-off between coating uniformity and limit of detection. A too low hPT1 concentration would result in a poorly coated membrane making it difficult to discern quenched areas. A high hPT1 concentration provided excellent fluorescence and coating uniformity. However, when the positive charges of hPT1 outnumber the negative charges of HBV-DNA it becomes difficult to detect low concentrations of the target DNA. Based on these observations, flow-through device was then developed.

3.5 Development of Flow-through Device

3.5.1 Materials and Methods for Flow-through Device

The centrifugal filters containing PVDF membranes with 0.1 μm pore size were purchased from Merck Millipore. Fusion 5 membranes were obtained from Whatman. Human plasma was ordered from GeneTex, Taiwan. The following nucleic acid sequences (mir21, HBV-DNA and random sequences) and PNA sequences were purchased from IDT (Singapore) and Panagene (Daejeon, Korea), respectively.

mir21: 5' UAG CUU AUC AGA CUG AUG UUG A 3'

HBV-DNA: 5' TAT ATG GAT GAT GTG GTA TT 3'

PNA1: N-TCA ACA TCA GTC TGA TAA GCT A-C

PNA2: N-AAT ACC ACA TCA TCC ATA TA-C

Random sequences used for the: 5' TAT ATG GAT GAT GTG GTA TT 3'

validation of logic gate system: 5' AUG CAU GCA UGC AUG CAU GCA A 3'

3.5.2 Device Fabrication and Analysis

In the following sections, it has to be noted that hPT1 is referred to as PT. In brief, 200 μL of 1 μM PT was centrifuged using PVDF centrifugal tubes at 8000 rpm for 3 min. In order to obtain a homogenous coating of PT on PVDF membranes, the abovementioned step was repeated seven times, followed by washing with DI water for 2 times under same centrifugation conditions. These PT coated membranes are stored at room temperature overnight for drying. A vertical flow-through configuration

was adopted for its advantages over the lateral-flow format in terms of low sample volume and decreased assay time as described in chapter 2. Capillary or passive forces allow this system to operate without the requirement of an external pump [139]. The following steps were carried out for the development of flow-through device.

1. Upon drying, centrifugation tubes were cut by a clipper as shown in Figure 3.7(a)
2. After clipping off, a scalpel was used to peel out the PVDF membrane (Figure 3.7(b)) and this circular PT-coated PVDF membrane was placed on a petridish.
3. A 5-mm hole puncher was used to make a hole in the double-sided adhesive tape as shown in Figure 3.7(c)
4. Then a lamination sheet, which had been punched a hole too, was pasted on the sticky side of the tape (Figure 3.7(d)). This sheet acted as a ‘casing’ or a ‘barrier’ for the device. This lamination sheet also ensured proper handling of the device for later experiments.
5. Next, the Fusion 5 membrane (Figure 3.7(e) and the PT-coated PVDF membrane (Figure 3.7(f) were placed into the punched hole, using a tweezer.
6. Then, the red film of the double-sided tape was peeled off (Figure 3.7(g)) and another lamination sheet with a punched hole was stuck on the backside (Figure 3.7(h)).
7. The device made was then cut into proper dimensions as shown in Figure 3.7(i).

Figure 3.7: Development of flow-through device.

It has to be noted the above device developed is shown for just one PVDF membrane. For the multiplexed nucleic acid assay of both biomarkers such as mir21 and HBV-DNA, the device consists of an array of four PT impregnated PVDF membranes, three of them attached with Fusion 5 filter papers, stacked between two transparent lamination sheets. Double-sided adhesive tapes are utilized to secure the membranes to the laminated sheets as shown in Figure 3.8(a).

Figure 3.8: (a) Schematic illustration of the flow-through colorimetric assay with four individually addressable PVDF membranes (b) the strategy for sensing mir21 and HBV-DNA that yield colorimetric profiles (c) corresponding to the PT impregnated PVDF membranes (view from bottom) without PNA (membrane 1 and 2), with PNA1 complementary to mir21 (membrane 3), and PNA2 complementary to HBV-DNA (membrane 4).

PT impregnated PVDF membranes, numbered 1, 2, 3 and 4 as shown in Figure 3.8(c), are utilized as reference, for monitoring duplex response, for mir21 and HBV-DNA assaying, respectively. Membranes 3 and 4 were incubated with 25 μM complementary PNA (PNA1 and PNA2 for mir21 and HBV-DNA, respectively). For the assay, $\sim 15 \mu\text{L}$ of samples spiked with varying concentrations of mir21/HBV-DNA was added on top of the Fusion 5 membrane. Herein, plasma was used as the clinical matrix with known concentrations of mir21/HBV-DNA. Plasma spiked with mir21/HBV-DNA concentrations of [0 nM (control), 1 nM, 10 nM, 100 nM, 1 μM and

10 μ M] were diluted 10-fold in DI water before the assay and was added to flow-through devices. The sample passively flows through the Fusion 5 membrane, which filters out macromolecules from the sample, allowing nucleic acids to reach the PT membrane.

In order to observe the color changes of the PT impregnated PVDF membranes (membranes 2, 3 and 4, with respect to reference membrane 1) the flow-through membrane device was placed under a portable UV lamp (UVL-28, 365 nm, 8 W). Digital images were then recorded with a mobile phone camera (Samsung S7), perpendicularly located over the device at a fixed distance. Subsequently, the captured digital images were imported to a computer in jpeg format for analysis using the ImageJ software. 75 x 75 pixels of each PT membranes (average of three locations at the centroids of the images) were utilized to generate RGB values as shown in Figure 3.9 [227]. The fluorescence spectra were measured with Avantes spectrofluorometer using the spectroscopy software provided (AvaSoft 8.0).

Figure 3.9: Acquisition 75x75 pixels of the membrane followed by extraction of RGB values and calculation of ΔE values of the flow-through array samples shown in Figure 3.8(c) (lower right).

3.5.3 Experimental Findings in Flow-through Device

3.5.3.1 Interaction of PT with Nucleic Acids in Deionized Water and Plasma

Flow-through colorimetric assay was carried out by observing the changes in optical properties of PT impregnated PVDF membranes, upon complexation with the target nucleic acid in the presence/absence of complementary PNA sequence. Initially, all the four PVDF membranes are orange in color, which is the inherent colour of the PT, as neutral PNA (both PNA1 and PNA2) does not interact with positively charged PT. The sensing strategy, as illustrated in Figure 3.8(b), is based on monitoring the changes in optical properties of PT impregnated PVDF membrane, with and without peptide nucleic acid (PNA, complementary to corresponding target nucleic acid) upon

complexation with target nucleic acid (mir21/HBV-DNA). PT without PNA forms a duplex with nucleic acids (PT-mir21 or/and PT-HBV-DNA) through electrostatic interactions between PT and nucleic acids, leading to a color change from orange to purple and to a fluorescence quenching according to the concentration of the nucleic acids. In contrary, PT with complementary PNA (PNA1, complementary for mir21 or PNA2, complementary for HBV-DNA) forms a triplex (PT-PNA1-mir21 or PT-PNA2-HBV-DNA) upon interaction with target nucleic acid, weakening electrostatic interaction between PT and mir21/HBV-DNA, and therefore does not yield significant color and fluorescence changes. The orange color (PT-PNA-mir21/HBV-DNA) and the significantly different purple color (PT-mir21/HBV-DNA) indicates the presence or absence of mir21/HBV-DNA, respectively in the sample, enabling the visual detection of nucleic acids.

As observed from Figure 3.8(c), with addition of 1 μ M mir21 to the device (1 μ M mir21 added to membranes 2, 3 and 4), a color change from orange to purple was observed for membrane 2 (PT) and membrane 4 (PT-PNA2, non-complementary to mir21). The color changes could be attributed to the formation of 'PT-mir21' duplex due to the electrostatic interactions between the positively charged PT and the negatively charged mir21. The random coiled morphology of the PT changes to a planar π -stacking morphology, leading to a substantial quenching of the fluorescence signal and color transition from orange to purple. On the contrary, no significant color change (similar to the reference, membrane 1) was observed for the membrane 3 (PT-PNA1, complementary to mir21) due to the formation of a 'PT-PNA1-mir21' triplex structures. PT retains its random coiled morphology, owing to the hybridization between mir21 and PNA1, thereby retaining the fluorescence signal upon PT-PNA1-mir21 triplex formation. Similarly, with the addition of 1 μ M HBV-DNA to membranes 2, 3 and 4, a purple color representing the duplex formation was observed on membranes 2 and 3, whereas, orange color representing the triplex formation was observed on membrane 4 (PT-PNA2-HBV-DNA). Subsequently, responses were evaluated for a mixture of mir21 (1 μ M) and HBV-DNA (1 μ M) added to membrane 2, 3 and 4. A dark purple color was observed on membrane 2, indicating significant duplex formation, as both mir21 and HBV-DNA sequence interacts with PT. Figure 3.8(c) shows that a reddish-orange color for membranes 3 and 4 due to the formation of both duplex and triplex morphologies. Figure 3.8(c) further ascertains that the

obtained distinct color patterns of PT arrays could be utilized to estimate the presence of nucleic acids in samples.

Figure 3.10(a) and Figure 3.10(b) illustrate the color-coded digital array for mir21 assay in DI water and plasma, respectively, spiked with mir21 concentrations varying from 10 μ M to 1 nM (from left to right). A concentration-dependant color change was obtained in both cases (PT without PNA), indicating a direct correlation between degree/extent of PT planarization and the concentration of mir21 (duplex formation). On the contrary, for the assay in DI and plasma, (with PNA1) revealed no significant changes in color upon mir21 addition indicating efficient PNA1-mir21 hybridization that weakens PT-mir21 electrostatic interactions, at all mir21 concentrations tested. However, color changes were more distinct in DI, for example, the color of PT appeared as reddish-orange for the plasma samples without mir21, instead of orange (as observed for assay in DI), which could be attributed to the interactions of PT with the plasma components. The normalized fluorescence spectra of PT incorporated PVDF membranes after addition of various concentrations of mir21 in DI water and plasma are shown in Figure 3.10(c) and Figure 3.10(d), respectively. Upon excitation at 420 nm, an emission band centred at 568 nm and 578 nm are observed for PT in DI and plasma, respectively. The intensity of PT-mir21 gradually decreases with increasing concentration of mir21, indicating significant quenching due to the duplex formation. The normalized fluorescence spectra of PT-PNA1 membranes exposed to different concentrations of mir21 look almost identical to each other (therefore only the 1 μ M mir21 spectrum is shown here). Figure 3.10(d) reveals concentration dependent mir21 quenching in plasma, which concurs with the response in DI water. Further evidence of concentration dependent duplex formation was obtained from the RGB analysis of the color-coded digital array (Figure 3.10(e) and (f)) using ImageJ software. Significant differences in ΔE values (calculated from RGB values in (Figure 3.10(e) and (f)) between the duplex (ranging between ~ 49 and ~ 7 , for the entire test concentration range) and the triplex (ranging between ~ 10 and ~ 5) are also evident in Figure 3.10(g), indicating visual detection of mir21 in plasma is possible. ΔE analysis ascertains that the color difference between duplex (PT-mir21) and triplex (PT-PNA1-mir21) is visually distinguishable and that the proposed assay could be utilized for the detection of mir21 in plasma over a wide dynamic range (1 nM to 10 μ M) as shown in Figure 3.10(g). The correlation between the ΔE values and

Figure 3.10: Color-coded digital array of PT impregnated PVDF membranes (with/without PNA1) for various mir21 concentrations (10 μM to 1nM) in (a) DI water, (b) plasma, and (c), (d) their corresponding fluorescence spectra, respectively. mean RGB values and standard deviation for $n=3$ in (e) DI (f) plasma (g) ΔE values of duplex and triplex formation in both DI and plasma ($n = 3$). (h) Semi-logarithmic plot of the mir21 concentration versus ΔE in both DI and plasma ($n = 3$).

mir21 concentrations was evaluated and represented in a semi-logarithmic scale, as shown in Figure 3.10(h). The data points in Figure 3.10(h) is the average of ΔE values from three individual experiments ($n = 3$). The calibration curves were linear over 5 orders of magnitude in both DI ($Y = 7.60 \log_{10} x + 88.24, R^2 = 0.99$) and plasma ($Y = 6.44 \log_{10} x + 63.35, R^2 = 0.94$) and limits of detection (LODs) in DI water and plasma were ~ 0.6 nM and ~ 2 nM, respectively (for a signal that is 3 times larger than the background noise).

Similarly, colorimetric responses for HBV-DNA between 10 μ M to 1 nM concentration ranges spiked in both DI water and plasma are illustrated in Figure 3.11 (a, b). The fluorescence spectra, Figure 3.11(c) and 3.11(d), showed similar quenching behaviour as compared to that of mir21. Further confirmation of detection is evident from the ΔE values (calculated from the RGB values in Figure 3.11(e), (f)), which displays considerable difference between duplex and triplex formation is shown in Figure 3.11(g). LODs of ~ 0.6 nM and ~ 2 nM were achieved with calibration curves in DI water ($Y = 7.48 \log_{10} x + 86.53, R^2 = 0.99, n = 3$) and plasma ($Y = 5.16 \log_{10} x + 51.64, R^2 = 0.95, n = 3$), respectively (Figure 3.11 (h)) with an assay time of ~ 45 minutes (required for drying of the membranes).

LODs were also calculated from the fluorescence intensity. The correlation between the normalized fluorescence intensity and mir21/HBV-DNA concentrations was evaluated using: $(I_{PT} - I)/I_{PT}$, where I_{PT} and I represent the integrated fluorescence intensity of PT without and with mir21/HBV-DNA over 500 to 800 nm wavelength, respectively, and represented in semi-logarithmic scale, as shown in Figure 3.12. LODs for both mir21/HBV-DNA in DI and plasma was attained at ~ 0.5 nM and ~ 1 nM, respectively.

In both cases, the trend of the HBV-DNA detection remains similar to that of mir21, which indicates that the proposed assay can be applied for the detection of any nucleic acid sequence in plasma. It should be noted that 10-fold diluted plasma is used for evaluation of colorimetric responses of nucleic acids.

Figure 3.11: Color-coded digital array of PT impregnated PVDF membranes (with/without PNA2) for various HBV-DNA concentrations (10 μ M to 1nM) in (a) DI water, (b) plasma, and (c), (d) their corresponding fluorescence spectra, respectively. mean RGB values and standard deviation for $n=3$ in (e) DI (f) plasma (g) ΔE values of duplex and triplex formation in both DI and plasma ($n = 3$). (h) Semi-logarithmic plot of the HBV-DNA concentration versus ΔE in both DI and plasma ($n = 3$).

Figure 3.12: Semi logarithmic plot of the (a) mir21 and (b) HBV-DNA concentration versus integrated fluorescence intensity in both DI and plasma ($n = 3$).

3.5.3.2 Stability of the Flow-through Device

The shelf life of devices intended for POC application in sparsely equipped field laboratories is crucial to minimize the logistic burden on the supply chain. The influence of temperature and storage time on the stability of the flow-through device was then evaluated. The device was stored up to one month at varying temperatures (-20 °C, 4 °C, 10 °C, RT, and 30 °C). As PT is photosensitive, devices were stored in dark to prevent PT degradation (reduction in fluorescence intensity). Figures 3.13(a) and 3.13(b) represent the color-coded digital array and the RGB responses of the devices that are freshly prepared and stored for one month at different temperatures, respectively. The color-coded images illustrate that the devices stored at 10°C and RT were most stable after one month among the different temperature ranges tested, as no significant color differences of PT membranes were observed as shown in Figure 3.13(a). RGB analysis yield a least difference of ~0.09 in G/B ratio (G/B ratio was calculated for evaluating the colorimetric responses as significant variations in green and blue intensities were observed compared to red intensities) for the devices that stored at room temperature as compared to the devices stored at other temperatures for one month with respect to freshly prepared device. Color-coded digital array (Figure 3.13(c)) shows that mir21 assay could be carried out with devices that are stored at different temperatures even after one month. However, for the temperature ranges tested, mir21 assay responses (RGB values, Figure 3.13(d)) of the device stored at RT for one month shows a minimal deviation of ~0.05 in G/B ratio from the responses obtained using freshly prepared devices, indicating that devices should be stored

preferably at RT. Temperatures over 30 °C is not suitable for storage of the devices due to degradation in the color and fluorescence intensity of PT.

Figure 3.13: (a) The colorimetric responses of freshly prepared and membranes stored for one month at varying temperatures (-20°C, 4°C, 10°C, RT, 30°C) and corresponding (b) RGB analysis ($n = 2$). (c) The colorimetric responses of 1 μ M mir21 detection with freshly prepared and membranes stored for one month at varying temperatures and the corresponding (d) RGB analysis (without PNA1, $n = 2$), (New-Freshly Prepared, Old-After 1 month).

3.5.3.3 Logic Gate based Nucleic Acid Computing

A generalized logic gate computing system for obtaining a profile of nucleic acids in a sample is proposed. The colorimetric responses are utilized as inputs for the proposed logic gate system. As described in the Figure 3.8(c), membranes 1, 2, 3 and 4 were utilized as reference, for monitoring duplex response, for mir21 and HBV-DNA assaying, respectively. For generalization, extending the PT array yield membranes numbered 5, 6... n , which could be utilized for assaying other nucleic acid sequences of interest, upon incorporating the corresponding PNA sequences. Therefore,

colorimetric response of the membranes 2, 3, 4... n , upon addition of sample (except membrane 1), yielding ΔE values as ΔE_{Duplex} , $\Delta E_{Triplex-mir21}$, $\Delta E_{Triplex-HBV-DNA}$ $\Delta E_{Triplex-n}$, respectively, with membrane 1 as reference. The ΔE values are subsequently applied to the logic system for obtaining a profile of nucleic acids in the sample. The operational amplifiers (OA) are used as comparators that indicate the presence or absence of any specific nucleic acids at concentrations above the respective nucleic acid's clinical threshold limits. In order to realize this decision, the degree of the duplex/triplex formation as indicated by the ΔE values are compared with pre-set threshold values $\Delta E_{TH-Duplex}$ and $\Delta E_{TH-Triplex}$.

$\Delta E_{TH-Duplex}$ is the ΔE value obtained experimentally by adding the minimum concentration of a nucleic acid, among the n nucleic acids tested, that indicates a diseased condition. $\Delta E_{TH-Triplex}$ is evaluated from the response of a sample containing a mixture of minimum concentration of target nucleic acid at a diseased condition and a maximum possible concentration of total nucleic acid biomarker in samples (assuming 1 μ M). For OA_1 , if ΔE_{Duplex} is greater than or equal to $\Delta E_{TH-Duplex}$, then duplex formation is ascertained. For the subsequent OAs, if $\Delta E_{TH-Triplex}$ is greater or equal to the $\Delta E_{Triplex}$, then the presence of specific nucleic acids sequences is ascertained. The outputs of all these OAs subsequently serve as inputs for AND logic gates in order to execute logical conjunction with the output from OA_1 . The output of an AND gate is true only when both the duplex and triplex formations are true.

For illustration of the operation of the proposed logic gate system, OA_2 and OA_3 are assigned for assaying mir21 and HBV-DNA sequences, respectively. As illustrated in Figure 3.14(a), for samples containing only mir21, both OA_1 and OA_2 yield logic '1', when ΔE_{Duplex} is greater than $\Delta E_{TH-Duplex}$ and $\Delta E_{TH-Triplex}$ is greater than $\Delta E_{Triplex-mir21}$, respectively. If both inputs are true, AND_1 yields a final output as logic '1' ($D_1 = 1$) indicating the presence of mir21. On the other hand, due to the absence of HBV-DNA in the sample, OA_3 would yield a logic '0', as $\Delta E_{TH-Triplex} < \Delta E_{Triplex-HBV-DNA}$. In this case, one of the inputs of the AND_2 is false, yielding a final output as logic '0' ($D_2 = 0$, confirming absence of HBV-DNA). Figure 3.14(b) represents the truth table for dual biomarkers detection ($n = 2$) as illustrated in Figure 3.8(c).

Figure 3.14: (a) A generalized nucleic acid biomarkers logic gate-based system for specific detection of biomarkers from a mixture, illustrated using a truth table (b) for dual biomarkers ($n = 2$) detection.

In order to evaluate the feasibility of developing a generalized logic gate system for POC diagnosis, mir21 assay is demonstrated in the presence of varying concentrations of other random nucleic acid sequences. Figure 3.15(a) illustrates the logic gate system for mir21 assaying. As discussed earlier, for single nucleic acid assaying ($n = 1$), two operational amplifiers (OAs) and one AND logic gate are required. The logic gate yield outputs as logic '1' or logic '0', referring to presence or absence of mir21 at concentrations over the minimum concentration that could indicate the disease (for e.g.: 10 nM), respectively. The threshold values ($\Delta E_{TH-Duplex}$ and $\Delta E_{TH-Triplex}$) for OA_1 and OA_2 are set to equal ΔE values for 10 nM mir21 (minimum concentration of nucleic acid in the diseased state) and 10 nM mir21-PNA1+ 1 μ M random sequences (assuming total nucleic acid concentration in normal physiological conditions), respectively. The color coded digital array and ΔE values for threshold conditions are shown in Figure 3.15(b). $\Delta E_{TH-Duplex}$ and $\Delta E_{TH-Triplex}$ values are experimentally found as ~ 28 and ~ 21 , respectively. The colorimetric responses for different samples as well as their respective ΔE values are shown in

Figure 3.15(c) and (d), respectively. For sample (i) containing 1 μ M mir21 concentration, OA_1 and OA_2 yields logic '1', due to the formation of duplex with ΔE of ~ 45 (greater than $\Delta E_{TH-Duplex}$ of 28) and triplex with a ΔE of ~ 8 (lower than $\Delta E_{TH-Triplex}$ of 21). Since, both the inputs (A, B) of AND_1 logic gate are '1', the final output of the system is logic '1', indicating the presence of mir21. For samples (ii) to (v), with varying concentrations of mir21 (equal/ higher than 10 nM) in the presence of random nucleic acid sequences, the logic gate system yields a final output as logic '1', concurring with the colorimetric responses (Figure 3.15(c)). For sample (vi) containing 1 nM mir21 (non-diseased condition, below a threshold of 10 nM), the input A is zero as the ΔE_{Duplex} of ~ 21 is lower than the threshold value of ~ 28 yielding a final output of logic '0', indicating absence of mir21. For samples with

Figure 3.15: (a) A logic gate-based system based on the flow-through assay for mir21 assaying. (b) Color-coded digital array and ΔE values for threshold conditions. (c) The colorimetric inputs of the device and logic gate output for varying concentrations and mixtures of mir21 and random nucleic acid sequences (see section 3.5.1) and (d) their corresponding ΔE values. The ΔE threshold values taken from (b) are shown as dotted lines. The lower part of (d) shows the input values for AND_1 logic gate (also shown in (c)).

1 nM mir21 in a mixture of random nucleic acid sequences at a concentration of 1 μ M, (sample vii), OA_2 yields a logic '0' as $\Delta E_{TH-Triplex} < \Delta E_{Triplex-mir21}$, yielding a final output of logic '0'. In a similar manner, the proposed logic gate system could be utilized for obtaining a profile of nucleic acids that are over their respective threshold values. The proposed logic gate system outputs a reliable decision for obtaining a profile of nucleic acids in samples that are over their respective clinical threshold values, thereby enabling rapid POC diagnostics.

3.6 Comparison with Other Sensing Strategies

Conjugated polyelectrolytes (CPEs) based nucleic acid assays have been widely investigated as an alternative to conventional assays. For instance, CPE-based competitive detection methodology utilizing short DNA and miRNA sequences competing for hybridization with capture DNA probes have been reported to yield a limit of detection (LOD) of ~ 1 nM [228]. Other reports monitoring conformational and colorimetric transitions of a CPE upon interaction with miRNA have been reported to achieve nM level miRNA detection sensitivities [219]. Recently, polyfluorene-alt-phenylene type CPE were utilized to detect DNA in serum samples [229]. Another FRET-based strategy utilizing cationic poly(phenylenevinylene) and malachite green (as intercalating dye) was found to be sensitive between 10 and 1000 nM; however, the assay did not enable naked eye detection [230]. Naked eye detection of nucleic acids by using mixtures of CPEs and gold nanoparticles were successfully presented pending validation with clinical samples [231]. Electrochemical approaches on quantitative analysis of miRNA have claimed sub nanomolar detection sensitivities [232].

The PCR, electrochemical, optical, capillary-electrophoresis and nanostructured materials-based methodology also were used for nucleic acid biomarker detection with good detection sensitivity (pM to fM). Most of these methodologies require extensive resources such as labelled probes, specialized fabrication protocol and sophisticated instrumentation for the assay [202, 233, 234]. Approaches such as extraction kit, centrifugation, and dilution with buffers are typically adopted for isolation of nucleic acids from samples such as blood [235], serum [233], plasma [236, 237] and cells [234]. However, integration of sample pre-treatment modules with nucleic acid assay devices still remains challenging [197]. In this regard, the developed flow-through assay eliminates tedious sample pre-treatment protocols,

while offering detection of nucleic acids from plasma sample, upon dilution, with decent sensitivity (LOD of ~ 2 nM in plasma) and specificity in ~ 45 min. Furthermore, the fabrication of the flow-through device is simple, flexible, cost-effective, and reproducible, and it possesses a potential for multiplexed sensing upon extending the PT array and incorporating PNA that are complementary to the target nucleic acids. The assay shows good stability over time and temperature and therefore has a great potential to facilitate rapid POC diagnostics in resource-poor settings. Additionally, the logic gate system offers a reliable prediction of the disease for the samples containing target nucleic acid sequence in clinically relevant concentrations, and therefore, has a great potential to facilitate rapid POC diagnostics in resource-poor settings.

3.7 Conclusion

Homopolymers (hPT1 and hPT2) with different pendant groups (i.e. triethylamine and 1-methyl imidazole) at the terminal position of the side chain of thiophene and their random copolymers (cPT1, cPT2 and cPT3) in different monomer ratios of 1:1, 1:5, 5:1, respectively, have been designed and synthesized to understand how the sensing capacity of a PTs may vary with structure of the pendant group in nucleic acid assay. Even though, all the polymers gave good responses for the mi21 assay, copolymer (cPT3) gave better optical responses for the mir21 detection in comparison to other synthesized polymers in solution state suggesting tuning of pendant group could improve the sensitivity and selectivity.

PTs are mainly studied in the solution state. However, solution-based assays are not easy to handle as compared to paper-based assays and often suffers from low selectivity in complex matrices. So, the idea was to translate the solution-based nucleic acid assay into a paper-based assay using the 5 synthesized polythiophenes. However, in case of paper-based platform, hPT1 showed better performance than cPT3 as in solution-based assay.

From the promising results from the paper-based platform, a flow-through colorimetric assay is then demonstrated for the naked-eye detection of nucleic acids in plasma sample at concentrations over five orders of magnitudes. The integration of filter papers with the optical reporter (PT) incorporated PVDF membranes enable sample pre-treatment and detection in the same platform, thus improving the device robustness and functionality. The assay yields rapid detection of nucleic acids with

good specificity, which can be employed for the early diagnosis of nucleic acid biomarker related diseases. Colorimetric responses evaluated with RGB/ ΔE analysis provide LOD in the low nM regime for both the mir21 and HBV-DNA with assay time of ~ 45 min. A generalized logic system is also described and evaluated for rapid decision making of colorimetric responses of mir21. The developed integrated colorimetric/logic gate methodology possesses a great potential for POC diagnostics.

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CHAPTER 4: LUMINESCENT DEVICE FOR THE DETECTION OF DNA DAMAGE

4.1 Introduction

ROS induced oxidation results in formation of numerous DNA damage products or fragments. Among them, 8-hydroxy-2'-deoxyguanosine (8-OHdG) is a repaired product of oxidative guanine lesions and an important biomarker for oxidative stress associated with T2D [79, 238] and provide knowledge about oxidative DNA damage. 8-OHdG has been quantified in various biological samples such as urine, saliva, blood and tissue [239-242]. Reports indicate that the average levels of 8-OHdG in urine are ~ 20 ng/ml (70 nM) and ~ 80 ng/ml (280 nM) in healthy and diseased subjects, respectively, thereby requiring sensitive methodologies for their assay [241, 243]. The most commonly used analytical techniques for the detection of 8-OHdG in urine are high performance liquid chromatography-electrochemical detection (HPLC-ECD) [244], HPLC tandem mass spectrometry (HPLC MS/MS) [245], capillary electrophoresis with electrochemical detection (CE-ECD) [242, 246], electrochemical methods including cyclic voltammetry (CV) [247-249], differential pulse voltammetry [250] and linear sweep voltammetry (LSV) [251]. Ultraviolet spectroscopy [252, 253], fluorescence spectroscopy [254, 255], resonance light scattering (RLS) [256] and enzyme-linked immunosorbent assay (ELISA) [80] have also been explored for the detection of 8-OHdG. However, most of them are laboratory techniques requiring sophisticated instrumentation and trained personnel to perform the assay. Thus, it is highly desirable to develop a point of care (POC) assay for 8-OHdG. Over the recent years, the concept of paper-based sensing has gained significant attention for fabrication of lab-on-a-chip devices enabling point of care analysis [257-261]. Although paper-based biosensors for 8-OHdG analysis have been reported, most of the approaches involve antibodies, which pose stability concerns [63, 262].

In this chapter, I take advantages of the optical changes of a cationic polythiophene as described in chapter 2 to develop a paper-based luminescent device for 8-OHdG assay utilizing aptamer. The validity of the developed device for the detection of 8-OHdG is also demonstrated in artificial urine.

4.2 Experimental Section

4.2.1 Materials and Chemicals

8-OHdG, artificial (synthetic) urine and all the reagents and solvents were obtained from Sigma-Aldrich and used without further purification. The 8-OHdG aptamer (sequence, 5'-GCG GGC GAT CGG CGG GGG GTG CGT GCG CTC TGT GCC AGG GGG TGG GAC AGA TCA TAT GGG GGT GCT-3') [37], was synthesized by IDT, Singapore followed by PAGE purification. PVDF centrifugation tubes of 0.1 μm pore size were purchased from Merck. Fusion 5 membranes made of glass fibers were purchased from Whatman and used for the pre-treatment of samples.

4.2.2 Synthesis, Sample Preparation and Analysis

Cationic polythiophene, with triethylamine pendant groups (PT) was synthesized using the protocol described in Appendix I. The flow-through device was prepared as described in chapter 3. In brief, 200 μL of 5 μM PT was added to a centrifugation tube. Tubes were centrifuged at 8000 rpm for 3 min. The above step was repeated for 3 more times. After impregnation of PT, the PVDF membranes were washed twice using DI water at 8000 rpm for 3 min and dried overnight. PVDF membranes deposited with PT was then peeled off from the centrifugation tube for device fabrication. The device consisted of 2 laminated sheets containing a FUSION 5 filter paper and a PVDF membrane impregnated with PT for colorimetric sensing. The lamination sheets containing FUSION 5 and PVDF paper were then attached using a double-sided adhesive tape as shown in Figure 4.1.

PVDF membrane impregnated with PT was incubated with 1 μM aptamer followed by addition of varying concentrations of 8-OHdG ($\sim 5 \mu\text{L}$). To ascertain the obtained color changes, the devices were placed on a UV illumination zone (portable UV lamp, at 365 nm, 8 W) and digital images were captured using a Sony Xperia Z5 premium mobile phone camera positioned at a fixed distance from the device. Subsequently, the digital images were transferred to a computer as jpeg files for image processing. The raw images were imported into "ImageJ" software and the centroids (75×75 pixels) of paper membranes were utilized to create a digital array. The RGB values were evaluated using ImageJ software in order to ascertain colorimetric responses. The process of creating color-coded digital array is shown in Figure 4.2. Fluorescence measurements were performed using Avantes spectrofluorometer.

Figure 4.1: Schematic representation of the luminescent paper device illustrating distinguishable colorimetric responses of poly(3-alkoxy-4-methylthiophene) (PT) for the G-quadruplex-secondary structure of aptamer in the presence of 8-OHdG and for the PT-aptamer duplex in the absence of 8-OHdG.

Figure 4.2: Acquisition 75x75 pixels of the membranes followed by calculation of RGB values of the flow-through array samples.

4.3 RESULTS AND DISCUSSION

4.3.1 Interaction of PT with Aptamer and 8-OHdG

Cationic polythiophene (PT), with triethylamine pendant groups, were impregnated in the PVDF membranes along with an aptamer as the 8-OHdG recognition element. A guanine-rich aptamer sequence, reported to exhibit a high affinity for 8-OHdG, was utilized in this study [37]. The aptamer utilized for affinity binding to 8-OHdG with K_d value of $0.1 \mu\text{mol L}^{-1}$ [37] was modified by removing the primer 1 and primer 2 sequences at the 3' and 5' in order to serve as an effective recognition element for the proposed assay. $5 \mu\text{M}$ PT and $1 \mu\text{M}$ aptamer (in DI water) were determined to be the optimum concentrations for monitoring 8-OHdG at clinically relevant levels (pM to nM). As illustrated in Figure 4.1, the sensing strategy for naked eye detection of 8-OHdG is based on monitoring the changes in optical properties of PT upon complexation with the aptamer in the presence and in the absence of 8-OHdG. Figure 4.3 illustrates that addition of $1 \mu\text{M}$ aptamer onto the PT-impregnated PVDF membrane yields a color change from orange (PT on the PVDF membrane) to purple (PT-aptamer complex on PVDF membrane), column 1 to column 2. The duplex formation is associated with backbone planarization and aggregation of the PT due to the electrostatic interactions between the positively charged PT (associated with the triethylamine groups) and the negatively charged aptamer. These electrostatic interactions cause a change from a random-coiled morphology of PT to a planar π -stacking morphology, leading to a substantial quenching of the fluorescence signal and a concomitant color transition. Saturated colorimetric response for 8-OHdG is expected to overlap with the color of PT, provided complete 8-OHdG-aptamer complex formation and consequent weakening of electrostatic interaction between PT and aptamer, whereas, assay concentrations would yield intermediate colorimetric responses between columns one and two depending on the strength of interaction between the PT and the aptamer. Therefore, for the assay, different concentrations of 8-OHdG from 50 pM to 500 nM were added to the PT impregnated PVDF membranes containing the aptamer. 8-OHdG induces a conformational change of the aptamer containing guanine-rich nucleic acid sequence to form G-quadruplex structures as shown in Figure 4.1. The formation of the rigid structures of G-quadruplex reduces the electrostatic interactions between PT and aptamer, thereby leading to fluorescence and color recovery of PT. Figure 4.3((a), (b)) illustrates that both color change and

fluorescence recovery occur with increasing concentration of 8-OHdG, attributed to the formation of a G-quadruplex structures. Decreasing 8-OHdG concentration changes the color from orange to purple as shown in Figure 4.3(a). The two significantly different optical signals (PT-aptamer and PT-aptamer-8-OHdG) were subsequently analyzed and correlated to the concentration of 8-OHdG. Figure 4.3(a) shows a 75x75 pixel color-coded digital array of various concentration of 8-OHdG (500 nM to 50 pM, left to right) for subsequent analysis.

In order to validate the assay, fluorescence spectra were recorded for the PT-aptamer incorporated PVDF membranes for various concentrations of 8-OHdG. Upon excitation at 420 nm, an emission maximum centered at 566 nm and a shoulder at around 625 nm appeared as shown in Figure 4.3(b). The spectrum of the PT impregnated paper is red shifted (about 25 nm) with respect to the solution spectrum likely because of partial self-aggregation/confinement of PT [170, 174, 263]. Even though aggregation causes loss in fluorescence intensity, the PT impregnated paper shows clearly visible orange color emission upon UV exposure (365 nm), as illustrated in Figure 4.3(a). Moreover, steady state fluorescence spectroscopy reveals a substantial quenching in emission and red shift (about 15 nm) upon addition of aptamer (black to red) indicating a duplex formation between PT and aptamer. As observed from Figure 4.3(b), the fluorescence intensity gradually increases with the increasing concentration of 8-OHdG due to the formation 8-OHdG-aptamer G-quadruplex complexes. Further evidence of complex formation is obtained from the RGB analysis of the color-coded digital array (Figure 4.3(c)) via ImageJ software. As shown in Figure 4.3c, there is a substantial change in the G (from 150.34 to 68.45) and B (from 76.9 to 129.13) values upon addition of aptamer to PT, whereas upon addition of 500 nM 8-OHdG with the aptamer, the G (118.26) and B (74.5) values reverts back to that of PT indicating almost complete recovery of color. It could be observed that the G (from 118.26 to 63.06) and B (from 74.5 to 119.76) values decreases and increases, respectively, with the decreasing concentrations of 8-OHdG. In order to ensure reproducibility, spectral and RGB analysis of the impregnated paper were conducted for three independent assays. Figure 4.3(c) shows that RGB analysis is in agreement with the naked eye observation (Figure 4.3(a)). The correlation between 8-OHdG concentration and normalized fluorescence intensity is evaluated using the ratio I/I_{PT} , where I and I_{PT} represents the integrated fluorescence intensity from 450 nm to 750 nm for the assay and PT, respectively. The linearity of the response was analyzed

Figure 4.3: (a) Color-coded digital array of PT with aptamer and various 8-OHdG concentrations. (b) Normalized fluorescence spectra upon addition of aptamer and various concentrations of 8-OHdG. (c) Mean RGB values and standard deviation for n=3. (d) Semilogarithmic plot of 8-OHdG concentration versus normalized fluorescence intensity and the corresponding linear fit. (e) Semilogarithmic plot of 8-OHdG concentration versus ratio of G/B values and the corresponding linear fit. (f) Mean Delta-E values and standard deviation for (n=3) of various 8-OHdG concentrations with respect to PT-aptamer.

using a semi logarithmic plot of normalized fluorescence intensity versus concentration of 8-OHdG. Figure 4.3(d) shows the linear regression equation ($Y = 0.076 \log X + 1.39$, $R^2 = 0.989$, $n=3$), yielding a limit of detection of ~ 300 pM ($S/N=3$). Similarly, the correlation between concentration of 8-OHdG and colorimetric response is evaluated using the G and B values from the Figure 4.3(c). The linearity of the response was ascertained by a semi logarithmic plot of G/B ratio versus 8-OHdG

concentration ($n=3$), as shown in Figure 4.3(e) with a linear regression equation of ($Y= 0.255 \log X+3.09$, $R^2 = 0.96$, $n=3$) yielding a limit of detection of ~ 350 pM ($S/N=3$). Furthermore, Delta-E values, ranging between 0-100, is a metric for human eye perception of color differences, is calculated using International Commission of Illumination (CIE) 1976 algorithm [225]. A delta-E value > 2 is regarded sufficient for our eyes to distinguish colors. Figure 4.3(f) illustrates a broad range of delta E ($\sim$ from 8 to 53) for PT-aptamer-8-OHdG and PT-aptamer in the assay concentration range of 8-OHdG (50 pM to 500 nM), indicating that the color difference between PT-aptamer-8-OHdG and PT-aptamer could be visually distinguished.

4.3.2 Selectivity Studies

The selectivity of the platform was evaluated using metal ions, potentially interfering compounds and other 8-OHdG analogues that may coexist in urine. As observed from Figure 4.4(a), the metal ions, analogues and the interfering compounds yield almost similar visual responses (varying shades of purple) as that of the aptamer, whereas, 50 nM 8-OHdG with and without interfering molecules show a recovery of color to orange from purple. Figure 4.4(b) illustrates the fluorescence recovery of 8-OHdG, metal ions and other interfering compounds. The fluorescence recovery was calculated as $(I-I_{\text{aptamer}})/(I_{\text{PT}}-I_{\text{aptamer}})$, where I_{aptamer} represents the integrated fluorescence intensity of PT-aptamer complex from 450 nm to 750 nm. A small to negligible fluorescence recovery is observed upon addition of 8-OHdG analogues and metal ions, even at 100 times higher concentration than that of 8-OHdG, as shown in Figure 4.4(b). The higher concentrations of ions such as potassium and 8-OHdG analogues interfere with the detection of 8-OHdG (maximum of $\sim 20\%$ for potassium) as observed from Figure 4.4(b) suggesting that these molecules also could induce a conformational change of the aptamer [252]. However, such ions could be eliminated from the sample matrices by dialysis using a dialysis bag with a molecular weight cut off (MWCO) of 200 Da. In the mixture of 50 nM 8-OHdG with 8-OHdG analogues and interfering compounds such as guanosine, guanine, uric acid, ascorbic acid and cytosine, a recovery around 64% was observed as against 67% for 50 nM 8-OHdG without interfering compounds, illustrating excellent selectivity of the proposed methodology.

Figure 4.4: (a) Color coded digital array of proposed platform with 8-OHdG and interfering molecules. (b) Fluorescence recovery upon addition of 8-OHdG, metal ions, 8-OHdG analogues and other potentially interfering compounds (Mixture: 8-OHdG (50 nM) with interfering compounds such as uric acid, cytosine, guanine, guanosine and ascorbic acid (5 μ M)).

4.3.3 Analysis in Artificial Urine

In order to validate the proposed methodology for clinical applications, different concentrations of 8-OHdG were spiked in the synthetic urine and tested. As shown in Figure 4.5(a), varying colorimetric responses were obtained upon addition of different concentrations of 8-OHdG in spiked urine. The orange PT color appears relatively darker upon addition of artificial urine samples without aptamer and there is a reduction of the fluorescence intensity, as compared to that of DI water. A possible explanation is that other anions in artificial urine might contribute to the formation of planar π -stacking morphology upon electrostatic interactions with PT and thereby lowering the PT fluorescence. However, as shown in column 2 in Figure 4.5(a), the color changes to purple upon addition of aptamer, which is in agreement with the responses in DI. A color change from orange to pink was observed upon decreasing the concentration of 8-OHdG from 500 nM to 500 pM (left to right in Figure 4.5(a)). As shown in Figure 4.5(b) there is also a substantial quenching of fluorescence upon addition of the aptamer (black to red) and the fluorescence intensity recovers gradually with increasing 8-OHdG concentration (from 500 pM to 500 nM). Further evidence of 8-OHdG aptamer complex formation was obtained from the RGB analysis, Figure. 4.5(c). Upon the addition of aptamer, there was a significant change of RGB values from PT to PT-aptamer (orange to purple), whereas upon addition of 500 nM 8-OHdG, the RGB values almost reverts back to PT, indicating the change in color from purple

to orange. Changes in RGB values were also observed with varying concentrations of 8-OHdG. Figure 4.5(c) shows that RGB analysis is in agreement with naked eye observation and the proposed methodology can be used for the detection of 8-OHdG at clinically relevant concentration ranges in urine samples. The estimated LOD for 8-OHdG in artificial urine is around 500 pM.

Figure 4.5: (a) Color coded digital array of PT with aptamer and various 8-OHdG concentrations in artificial urine. (b) Normalized fluorescence spectra upon addition of aptamer and various 8-OHdG concentrations. (c) Mean RGB values and standard deviation for $n=3$.

4.4 Comparison with Other Sensing Strategies

Electrochemical methodologies such as cyclic voltammetry (CV) and differential pulse voltammetry (DPV) for quantitative analysis of 8-OHdG have claimed subnanomolar detection sensitivities [247, 250]. Similarly, spectroscopic methods such as ultraviolet-visible spectroscopy (UV-Vis) and fluorescence spectroscopy also have been explored for the detection of 8-OHdG [252-254] with subnanomolar and picomolar sensitivities. However, most of the reported methodologies involve tedious and sophisticated assay protocols. Table 4.1 illustrates that the LOD of the proposed methodology is either better or comparable to previous reports, while exhibiting a wider linear dynamic range for 8-OHdG detection. Our findings indicate a linear response for both color and the fluorescence intensity for

clinically relevant concentration ranges of 8-OHdG (50 pM to 500 nM) [243] with a limit of detection of ~ 350 pM and 300 pM, respectively.

Table 4.1: Comparison of various sensing platforms for the detection of 8-OHdG.

Methodology	Detection Range	Limit of detection (LOD)	Ref.
LSV	0.7–70 μ M	100 nM	[251]
DPV	0.5–35 μ M	31.3 nM	[250]
CV	0.0563-16.4 μ M	18.8 nM	[249]
CV	5.6–1155 nM	0.875 nM	[247]
CE-ECD	0.01-1.5 μ M	2.6 nM	[246]
UV–vis	5.6–282 nM	1.7 nM	[252]
UV-vis	0.466-247 nM	141 pM	[253]
Colorimetric and Electrochemical	35.3-706.1 nM	7.3 nM	[63]
Fluorescence	3.96-211 nM	1.19 nM	[254]
Colorimetric and Fluorescence	0.5-500 nM	~ 350 pM Colorimetric and ~ 300 pM Fluorescence	This work

In addition, the overall detection processes are conducted at ambient conditions and the experimental procedure is facile. Furthermore, the developed methodology possesses significant advantages over conventional sensing platforms such as ease of use, ease of fabrication and low cost with an assay time of 30 min.

4.5 Conclusion

A disposable paper-based analytical device for the naked eye detection of oxidative stress biomarker was developed. The proposed methodology has been validated for 8-OHdG assay using luminescent reporter (PT) impregnated PVDF membranes. The naked-eye limit of detection for 8-OHdG in artificial urine is around 500 pM with an assay time of 30 min, which is below the clinical threshold levels in urine. Thus, the obtained colorimetric responses in artificial urine indicate the potential

for use in clinical applications for early diagnosis of various diseases such as T2D associated with reactive oxygen species. Besides, the proposed assay shows small to negligible interferences with the existence of metal ions and 8-OHdG analogues in high concentrations, indicating this methodology could be used in selective detection of 8-OHdG. Thus, the fabricated platform could be ideal for point-of-care applications and for detection of oxidative stress at locations where sophisticated instrumentation is sparse or even nonexistent.

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CHAPTER 5: COLORIMETRIC SCREENING ASSAY FOR ADVANCED GLYCATION END PRODUCTS

5.1 Introduction

Advanced Glycation End Products (AGEs) are heterogeneous group of bioactive molecules that are formed via glycation, sugar auto oxidation and sugar metabolic pathways as described in Chapter 2. AGEs play a key role in the development of micro and macro vascular diabetic complications, ageing, cardiovascular diseases, atherosclerosis, sarcopenia, and neurodegenerative disorders such as Alzheimer's and Parkinson's disease [82, 83]. The increase in AGEs causes the formations of ROS which in turn induce further AGEs formation resulting in mitochondrial dysfunction. The AGE measurement also has tremendous interest in both nutrition and metabolic syndrome fields. Maillard Reactions as described in chapter 2 are spontaneous and the Amadori products formed are known to be harmful. So, the end goal is to reduce the formation of AGEs and this is best done in a point of care management framework with a feedback loop of controlling food intake when levels start to rise. Hence it is very important to develop the assay of AGEs.

The most commonly used methodologies for the measurement of AGEs are immunochemical methods such as immunohistochemistry, enzyme-linked immunosorbent assay autoantibodies [264, 265], and bioanalytical methods such as Fluorescence Spectroscopy, UV–Visible spectroscopy, Surface Plasmon Resonance, Boronate Affinity Chromatography, High-Performance Liquid Chromatography (HPLC), Liquid Chromatography-Mass Spectroscopy (LC/MS), and Fluorescent Phenylboronate Gel Electrophoresis [266-269]. Biochemical techniques such as Nitroblue Tetrazolium Assay and Thiobarbituric Acid Colorimetric Method have also been explored for the detection of AGEs [270-272]. However, most of these methods are laboratory based that are cumbersome, require sophisticated instrumentation, and trained personnel to perform the analysis. Thus, it is highly significant to develop a point-of-care (POC) assay for AGEs.

In this chapter, I developed a novel solution-based bio sensing platform for both colorimetric and fluorometric detection of AGEs using cationic polythiophene and aptamer. To validate our approach, among various AGEs, Glyceraldehyde derived AGEs (Gly-AGEs), formed by glycation of sugar molecule glyceraldehyde (triose monosaccharide) that reacts rapidly with free amino group of Bovine Serum Albumin

(BSA) is selected. Such targets are cytotoxic and evoke oxidative stress and therefore could be used as a biomarker for vascular inflammation or atherosclerotic cardiovascular disease. A cytosine-rich aptamer (Apt) sequence, reported to exhibit a high affinity for Gly-AGEs, is used in this study as the recognition element [38, 273, 274]. The validity of the solution-based assay for the detection of Gly-AGEs is also demonstrated in plasma.

5.2 Experimental Section

5.2.1 Materials and Chemicals

AGEs and plasma were bought from MBL, Japan and GeneTex, Taiwan respectively. All the reagents and solvents were purchased from Sigma-Aldrich and used without further purification. The Gly-AGEs Apt (sequence, 5'-TGT AGC CCG AGT ATC ATT CTC CAT CGC CCC CAG ATA CAA G-3') was synthesized by IDT, Singapore, followed by PAGE purification. The stock solution of 100 μ M Apt was prepared with DNase free DI water and stored at -20 °C.

5.2.2 Synthesis, Sample Preparation, and Analysis

Cationic polythiophene, with triethylamine pendant groups (PT) was synthesized as described in Appendix I. For the Gly-AGEs assay, 15 μ L of various concentrations of Gly-AGEs in 10 mM PBS, pH 7.4 or in plasma was added to 5 μ L Apt and incubated for 20 minutes followed by the addition of 30 μ L of 116.67 μ M PT solution to these samples. To visualize the color change, the samples were placed on UV illumination zone (portable UV lamp, at 365 nm, 8 W), and digital images were captured using a Samsung mobile phone camera positioned at a fixed distance from the vials. Consequently, the digital images were transferred to the computer as jpeg files for processing and then the centroids of vials (75×75 pixel) were utilized to create a digital array. RGB analysis was further carried out using ImageJ image processing software in order to ascertain colorimetric responses. Steady state fluorescence and absorption spectra of the samples were measured using an Infinite M200Pro Tecan plate reader. Fluorescence spectra was obtained with excitation wavelength at 400 nm.

5.3 Results and Discussion

5.3.1 Interaction of PT with Aptamer and Gly-AGEs

The sensing strategy of Gly-AGEs is based on by observing the changes in optical properties of PT associated with its conformational changes upon interactions with the Apt in the presence and in the absence of Gly-AGEs, respectively, as shown in Figure 5.1. Apt was incubated with different concentrations of Gly-AGEs in 10 mM PBS, pH-7.4 for 20 min to form Apt/Gly-AGEs complex (i-motif complex). PT was added to Apt and Apt/Gly-AGEs complex with different concentrations to form PT-Apt duplex and PT-Apt/Gly-AGEs complex. In the absence of Gly-AGEs, a duplex is formed between PT and Apt due to the electrostatic interactions between the positively charged PT and the negatively charged Apt, causing a change from a random-coiled morphology of PT to a planar π -stacking morphology. This leads to a substantial fluorescence quenching and a concomitant color transition of the PT from yellow to blue. While, in the presence of Gly-AGEs, the single stranded cytosine rich Apt changes its conformation to form an intercalated motif (i-motif; a secondary structure of DNA that are formed in cytosine rich nucleic acid sequences) complex [274]. The formation of these rigid i-motif structures reduces the electrostatic interaction between PT and the Apt, thereby leading to less fluorescence quenching and a color transition

Figure 5.1: Schematic representation of the Gly-AGEs assay illustrating distinguishable colorimetric responses of poly(3-alkoxy-4-methylthiophene) (PT) for the i-motif structure of aptamer in the presence of Gly-AGEs and for the PT-Apt duplex in the absence of Gly-AGEs/presence of non-specific protein.

from yellow to pink. The assay would yield intermediate colorimetric responses between pink and blue depending upon the Gly-AGEs concentrations and amount of formation of i-motif complex. Thus, the pink color (PT-Apt-Gly-AGEs) and the significantly different blue color (PT-Apt) correspond to the presence and the absence of Gly-AGEs, respectively, thereby enabling the naked eye detection of Gly-AGEs.

Figure 5.2(a) illustrates the samples with different colors due to various concentrations of Gly-AGEs. The addition of Apt to the polymer changed the color from yellow to blue as shown in vial 1 and vial 2, respectively. The addition of Apt with Gly-AGEs as shown in vial 3 of Figure 5.2(a) showed a pink color. Decreasing Gly-AGEs concentration (from 300 $\mu\text{g/ml}$ (vial 3) to 1.5 $\mu\text{g/ml}$ (vial 8)) changes the color from pink to blue shown in Figure 5.2(a) due to the less formation of i-motif structures. The two significantly different optical signals (PT-Apt and PT-Apt-Gly-AGEs) are subsequently analyzed and correlated to the concentration of Gly-AGEs.

In order to validate the assay, fluorescence and absorption spectra were recorded for the samples shown in Figure 5.2(a). The emission spectra of PT, with the maximum peak at 556 nm, is shown in Figure 5.2(b). Steady state fluorescence spectroscopy reveals a substantial quenching in emission and red shift (about 36 nm) upon addition of Apt (black to red) indicating a duplex formation between PT and Apt. As observed from Figure 5.2(b), the fluorescence intensity gradually increases with the increasing concentration of Gly-AGEs due to the formation of Gly-AGEs-Apt i-motif complexes. Similarly, as shown in Figure 5.2(c), upon addition of Apt, the absorbance maximum is red-shifted (black to red) indicating the planar structure of PT and whereas, the addition of Gly-AGEs promotes the formation of i-motif complex, thus hindering the planarization of PT. Further evidence of complex formation is obtained from the RGB analysis of the vials (Figure 5.2(a)) via ImageJ software. Figure 5.2(d) shows the RGB analysis for the vials shown in Figure 5.2(a). As shown in Figure 5.2(d), there is a substantial change in the R (from 101.5 to 27.8) and G (from 106.5 to 51.3) values upon addition of aptamer to PT (no significant change in B channel), whereas upon addition of 300 $\mu\text{g/ml}$ Gly-AGEs with the Apt, the R (103.5) and G (112.3) values reverts back to that of PT indicating almost complete recovery of color. It could be observed that the R (from 103.5 to 28) and G (from 112.3 to 52.5) values decreases with the decreasing concentrations of Gly-AGEs. In order to ensure reproducibility, spectral and RGB analysis were conducted for three independent Gly-AGEs assays. Furthermore, Delta-E values, ranging between 0-100, is a metric for

human eye perception of color differences, is calculated using International Commission of Illumination (CIE) 1976 algorithm [225]. Figure 5.2(e) illustrates a

Figure 5.2: (a) Optical response of PT with various concentrations of Gly-AGEs under UV lamp where (1) PT (2) PT+Apt (3) 300 (4) 150 (5) 30 (6) 15 (7) 3 (8) 1.5 $\mu\text{g/ml}$. (b) Fluorescence spectra upon addition of Apt and various concentrations of Gly-AGEs to PT. (c) Absorption spectra upon addition of Apt and various concentrations of Gly-AGEs to PT. (d) Mean RGB values and standard deviation for $n=3$. (e) Mean delta E values and standard deviation for ($n=3$) of various Gly-AGEs concentrations with respect to PT-Apt. (f) Semilogarithmic plot of Gly-AGEs concentration versus delta E values and the corresponding linear fit (red line).

broad range of delta E from ~3 to 32 for PT-Apt-Gly-AGEs and PT-Apt in the assay concentration range of Gly-AGEs (1.5 µg/ml to 300 µg/ml), indicating that the color difference between PT-Apt-Gly-AGEs and PT-Apt could be visually distinguished. The correlation between the delta E values and Gly-AGEs concentrations was evaluated and represented in a semi-logarithmic scale, as shown in Figure 5.2(f). The data points in Figure 5.2(f) is the average of delta E values from three individual experiments ($n = 3$). The delta E data points are fitted over a linear function (red line as shown in Figure 5.2(f)) from 1.5 µg/ml to 300 µg/ml in PBS ($Y = 11.65 \log_{10} x - 0.101$, $R^2 = 0.91$) yielding a LOD of ~ 1.3 µg/ml.

5.3.2 Selectivity Studies

The selectivity of the solution-based assay was evaluated using other AGEs such as Glucose derived AGEs (Glu-AGEs), BSA, other proteins and sugar molecules that may coexist in plasma. As observed from Figure 5.3(a), 300 µg/ml of the proteins, sugar molecules yield almost similar visual responses as that of the Apt and a purple color in case of Glu-AGEs, whereas, 150 µg/ml of Gly-AGEs with and without interfering molecules show a color of pink instead of blue. Figure 5.3(b) summarizes the fluorescence recovery of Gly-AGEs, Glu-AGEs, BSA, other proteins and Apt at wavelength 592 nm, respectively. Small to negligible fluorescence recovery (less than 5%) is observed upon addition of proteins and sugar molecules, while the Glu-AGEs showed a 14% recovery implying Glu-AGEs could also induce the formation of i-motif at high concentrations (above 150 µg/ml).

Figure 5.3: (a) Color coded digital array of proposed platform with Gly-AGEs and interfering molecules. (b) Fluorescence recovery upon addition of Gly-AGEs, Glu-AGEs, proteins and sugar molecules (Mixture: Gly-AGEs (150 µg/ml) with BSA (300 µg/ml)).

5.3.3 Analysis in Plasma

In order to validate the proposed methodology for clinical applications, different concentrations of Gly-AGEs were spiked in plasma and tested. As shown in Figure 5.4(a), varying colorimetric responses were obtained upon addition of different concentrations of Gly-AGEs in spiked plasma. As shown in Figure 5.4(a), the color changes to purple from yellow upon addition of Apt to PT. Herein, vials 1 and 2 contain plasma without the spiked Gly-AGEs as control. The addition of PT to Apt and Gly-AGEs mixture observed a color change to pink as shown in vial 3 of Figure 5.4(a). A color change from pink to purple was observed upon decreasing the concentration of Gly-AGEs from 150 $\mu\text{g/ml}$ to 1.5 $\mu\text{g/ml}$ (vial 3 to vial 7 in Figure 5.4(a)). As shown in Figure 5.4(b) there is also a substantial quenching of fluorescence upon addition of the PT to Apt (black to red) and the fluorescence intensity recovers gradually with increasing Gly-AGEs concentration (from 1.5 $\mu\text{g/ml}$ (purple) to 150 $\mu\text{g/ml}$ (blue)). Similarly, the absorption spectra shown in Figure 5.4(c) also confirms the red shift in absorbance maximum upon addition of Apt. Further evidence of Gly-AGEs aptamer complex formation is obtained from the RGB analysis, Figure 5.4(d). Upon the addition of Apt, there is a significant change of RGB values from PT to PT-Apt (yellow to purple, refer to Figure 5.4(a)) whereas upon addition of 150 $\mu\text{g/ml}$ Gly-AGEs, the RGB values almost changes back to PT, indicating a change in color from purple to pink. Changes in RGB values were also observed with varying concentrations of Gly-AGEs. In order to ensure reproducibility, spectral and RGB analysis were conducted for three independent Gly-AGEs assays in plasma. Figure 5.4(e) illustrates the change in delta E values from ~ 3 to 22 between PT-Apt-Gly-AGEs and PT-Apt in the assay concentration range of Gly-AGEs (1.5 $\mu\text{g/ml}$ to 150 $\mu\text{g/ml}$), indicating that the color difference between PT-Apt-Gly-AGEs and PT-Apt could be visually distinguished in plasma. The correlation between the delta E values and Gly-AGEs concentrations was evaluated and represented in a semi-logarithmic scale, as shown in Figure 5.2(f). The calibration curves are linear over 1.5 $\mu\text{g/ml}$ to 150 $\mu\text{g/ml}$ in plasma ($Y = 8.81 \log_{10} x + 0.56, R^2 = 0.955$) yielding a LOD of ~ 1 $\mu\text{g/ml}$, implying the proposed methodology can be used for the detection of Gly-AGEs at clinically relevant concentration ranges in plasma samples.

Figure 5.4: (a) Optical response of PT with various concentrations of Gly-AGEs under UV lamp where (1) PT (2) PT+Apt (3) 150 (4) 30 (5) 15 (6) 3 (7) 1.5 $\mu\text{g/ml}$. (b) Fluorescence spectra upon addition of Apt and various concentrations of Gly-AGEs to PT. (c) Absorption spectra upon addition of Apt and various concentrations of Gly-AGEs to PT. (d) Mean RGB values and standard deviation for $n=3$. (e) Mean ΔE values and standard deviation for ($n=3$) of various Gly-AGEs concentrations with respect to PT-Apt. (f) Semilogarithmic plot of Gly-AGEs concentration versus ΔE values and the corresponding linear fit (red line).

5.4 Comparison with Other Sensing Strategies

Currently, there is no gold standard technique for the detection of AGEs. The main problem is that detection of the same AGEs shows inconsistency when measured by two different methods. Most commonly used method for the measurements of

AGEs are ELISA. But the quantitative measurements using ELISA are less reproducible and lack specificity [24]. HPLC combined with mass spectroscopy are accurate but very expensive and cumbersome. Immunohistochemistry is another method for the measurement of AGEs in tissues but have less specificity due to the lack of standardized antibodies. Recently, a non-invasive point of care methodology skin autofluorescence (SAF) has been developed to measure AGEs in skin using AGE Reader™ by company DiagnOptics Technologies. The drawback of this method is that is to measure AGEs level for people with dark skin and people using skin cream as the device uses light to detect AGEs [270]. In these regards, the developed methodology is simple to use and results are reproducible with good sensitivity. It has to be noted that further experiments are required to find out the specificity of this method with respect to other AGEs other than Gly-AGEs and Glu-AGEs.

5.5 Conclusion

A simple colorimetric and fluorescence assay based on luminescent reporter (PT) for the detection of carbonyl stress biomarker (Gly-AGEs) was successfully developed. The electrostatic assembly-disassembly between PT and cytosine rich aptamer sequence (Apt) provides evident color difference for naked eye observation. The limit of detection for naked eye observation by RGB/delta E analysis of Gly-AGEs is around 1 µg/ml in plasma with an assay time of 20 min, which is below the threshold clinical levels for inducing significant ROS generation. The obtained colorimetric responses in plasma indicate the potential for use in clinical applications for early diagnosis of various diseases such as T2D and atherosclerosis. Besides, the proposed assay shows negligible interferences with the existence of other high concentration sugar and protein molecules, indicating this methodology could be used in selective and sensitive detection for Gly-AGEs in analytically relevant concentration ranges without involving sophisticated instrumentation enabling point of care applications.

Manuscript is under preparation for this work.

CHAPTER 6: COLLABORATIVE PROJECTS USING POLYTHIOPHENES

This chapter will briefly introduce the projects done in collaboration with other researchers at CBSS using cationic polythiophenes. These works are already published or ready for submission.

6.1 Polythiophene Derivative on Quartz Resonators for miRNA Capture and Assay

6.1.1 Introduction

Assay formats based on Quartz Crystal Microbalance (QCM) have been reported, usually involving a recognition molecule immobilized on the QCM crystal surface that can specifically bind a target analyte [275-277]. As for the nucleic acid assay, PNA/DNA probes are usually immobilized on the QCM surface for detection of a particular DNA/RNA sequence of interest. Though sub-pM detection sensitivities have been reported based on QCM [276, 277] most of the reports possess the limitation of quantifying a specific sequence of RNA in the sample matrix. Unlike conventional nucleic acid probes that are immobilized on the QCM surface, cationic polythiophene (PT), enables capturing all miRNAs in a sample matrix, providing a unique possibility of detecting any miRNA sequence upon injection of a peptide nucleic acid (PNA) sequence complementary to the miRNA of interest. Avidin coated nanoparticles (ANPs) are subsequently utilized for signal amplification and to yield responses in clinically relevant concentration regimes. Avidin functionalized nanoparticles selectively bind to the biotin anchored to PNA. ANPs serve as mass enhancers amplifying the PNA hybridization signal. QCM frequency shift responses are then directly correlated with the miRNA concentration, thereby eliminating the need for PCR upon miRNA isolation. mir21, a miRNA sequence, is utilized in the study for validation of the proposed approach.

6.2.2 QCM Assay

The proposed assay as shown in Figure 6.1 involves sequential injection of the synthesized PT and BSA into the QCM chamber, followed by miRNA and a complementary sequence of biotinylated PNA (b-PNA) for hybridization with the target miRNA sequence; ANPs are subsequently utilized for amplified detection of

miRNA. PT was injected simultaneously into two chambers mounted with cleaned QCM crystals.

Figure 6.1: Schematic illustration of the mir21 assay on QCM crystals. Quartz crystal is sequentially exposed to (a) PT; (b) BSA for gold substrate passivation; (c) RNA and duplex formation; (d) b-PNA and triplex formation; and (e) ANP for signal amplification.

Upon injection of PT, a two-step frequency response (Figure 6.2(a) was observed) due to changes in the morphology of PT upon buffer exchange between DI water and PBST (PBS with 0.002 % tween), in agreement with previous reports [278]. 5 mg ml⁻¹ BSA in PBST was then injected for substrate passivation in order to minimize physisorption of ANP and other interfering species present in the complex matrices on Au surfaces (Figure 6.2(b)) if any. 10 μM of mir21 and 10 μM of non-complementary (Nc) sequences were then injected into chambers 1 and 2, respectively. Identical resonance frequency shifts of ~5 Hz (Figure 6.2(c)) were obtained for both mir21 and Nc sequences indicating that the synthesized PT captures both Nc and mir21 sequences. 10 μM b-PNA complementary to mir21 was subsequently injected into both channels, followed by injection of ANP. Although no obvious frequency or dissipation shifts (0.5 Hz and 0.27E⁶, respectively) were noticed upon b-PNA hybridization with mir21 (Figure 6.2 (d)), significant frequency and dissipation shifts (20 Hz and 10E⁶, respectively) were observed upon injection of 0.1 mg ml⁻¹ ANP (Figure 6.2(e)). PNA–mir21–PT triplex formation is therefore ascertained as only a negligible shift was recorded for the Nc–PT duplex, indicating the successful capture of miRNA by PT immobilized on the Au surface and selective recognition of the mir21–PT duplex by b-PNA. As shown in Figure 6.2, approximately 40-fold signal amplification, a shift of 20 Hz upon ANP addition for mir21 compared to ~0.5 Hz for Nc sequence, was achieved by injection of ANP.

Figure 6.2: QCM kinetic measurements: frequency (df) and energy dissipation (D) at the third overtone for (A) mir21 and (B) Nc sequence illustrating responses after the injection of (a) PT; (b) BSA; (c) 10 μM mir21/Nc sequence; (d) b-PNA; and (e) 0.1 mg ml^{-1} ANP.

Concentration dependent responses (Figure 6.3) reveal that frequency shifts were observed only when mir21 is captured by PT immobilized on the QCM surface. Almost negligible responses were obtained for all test concentrations of Nc sequence (curve Nc) indicating minimum interaction and binding between Nc and b-PNA (complementary to mir21)/ANP. A frequency shift of ~ 5 Hz was obtained for 100 nM of mir21 compared to ~ 0.5 Hz for 100 nM of the Nc sequence, illustrating that incorporation of capture and amplification methodologies enables selective detection of miRNA. Quantification of miRNA is subsequently achieved by correlating frequency responses with mir21 concentrations. As observed from Figure 6.3, frequency responses were obtained for 1 nM to 10 μM mir21, with a limit of detection of 400 pM, calculated using $3\sigma/S$, [279] where σ is the standard deviation and S is the slope of the concentration response, derived at a lower linear response range [between 1 nM and 10 nM, Figure 6.3]. Repeatable frequency responses for all test concentrations of mir21 and Nc ($n = 3$) were obtained indicating the homogeneity in PT adsorption on QCM surfaces. The proposed methodology therefore illustrates the potential for analysis of complex samples containing miRNA in the clinically relevant nM concentration ranges [280-282]. The mir21 assay was then carried out using plasma for demonstrating the applicability of the proposed approach for complex matrices. Plasma samples were spiked with 1 μM of mir21 and Nc to mimic clinical

samples. Commercially available extraction kits were then used for miRNA isolation, in order to minimize the potential interference associated with physisorption of plasma components. The inset in Fig. 4 shows a negligible shift for the plasma sample spiked with mir21 and Nc sequences upon injection of b-PNA as shown in Figure 6.3(a). Following the injection of 0.1 mg ml^{-1} ANP, a frequency shift of $\sim 2 \text{ Hz}$ was observed for mir21 spiked plasma samples (Figure 6.3(b)) compared to a negligible shift for the plasma samples spiked with the Nc sequence. The experimental results indicate that amplification enhancement could be achieved upon increasing the concentration of ANP. A further increase in the frequency response $\sim 4 \text{ Hz}$ (Figure 6.3(c)) was observed upon injection of 0.5 mg ml^{-1} ANP to the plasma sample spiked with mir21.

Figure 6.3: Concentration dependent responses for mir21 and Nc sequences ($n = 3$). Inset: QCM kinetic measurement for mir21 and Nc sequences isolated from plasma (a) b-PNA; (b) 0.1 mg ml^{-1} ANP; and (c) 0.5 mg ml^{-1} ANP.

This work was published in Analyst. I provided the help in the developing the assay strategy, experiments in plasma, discussion of result and writing of the paper.

This section (including phrases and figures) is adapted/reproduced from our published article “Polythiophene derivative on quartz resonators for miRNA capture and assay Analyst 140.23 (2015): 7912-7917”

6.2 Visual Detection of Aluminium Ions using Polythiophene

6.2.1 Introduction

Metal ions play an important role in living organisms since they are involved in many biological processes such as osmotic regulation, catalysis, metabolism, biomineralization and signalling [283]. However, abnormal concentration levels of metal ions might cause detrimental effects, for example, trivalent aluminium (Al^{3+}) in aqueous solution has been reported to be highly toxic; main source of which is from food and drinking water. The maximum acceptable Al^{3+} concentration in drinking water is $200\text{ }\mu\text{g L}^{-1}$ ($7\text{ }\mu\text{M}$) according to European Economic Community (EEC) standards [284]. Excess amount of Al^{3+} is believed to potentially cause brain diseases such as dementia, Parkinson's disease and Alzheimer's disease [285, 286]. The poisoning risk could also exist with the accumulation of lower level Al^{3+} in the brain [287]. Therefore, developing a method to sensitively and selectively detect Al^{3+} is crucial. Herein, polythiophene random copolymer (CP) was incubated with negatively charged ATP to form a complex based on the electrostatic self-assembly [288, 289]. The color of resulting solution changed immediately (from yellow to purple), and the fluorescence intensity was quenched to around 15% of its original intensity. Addition of Al^{3+} resulted in the recovery of color (from purple to yellow) and fluorescence intensity due to the disassembly of CP-ATP complex as a result of strong affinity between Al^{3+} and ATP [290]. Al^{3+} ions binds stronger with ATP than other metal ions because of high stability constant [291]. This higher affinity is due to the combination of Al^{3+} ions small size and high charge resulting a polarizing effect on electrons of oxygen atoms in phosphate groups [292]. The formation of Al^{3+} -ATP complex triggered the transformation from a planar π -stacking morphology of CP to a random coiled morphology, thus resulting in recovery of color and fluorescence of CP. The proposed methodology enabled selective and sensitive visual detection of Al^{3+} without sophisticated instrumentation with a limit of detection of $\sim 4\text{ }\mu\text{M}$ in tap water.

6.2.2 Detection Mechanism of Al^{3+} ions

The assay schematic of Al^{3+} is demonstrated as shown in the Figure 6.4. The color and fluorescence intensity of CP solution ($80\text{ }\mu\text{M}$) changed (from yellow to purple) immediately upon the addition of $16\text{ }\mu\text{M}$ ATP due to electrostatic complexation of CP-ATP. This complexation promotes planarization of the CP backbone, and above a critical concentration efficient π - π stacking interaction between

CP backbones is induced by hydrophobic interaction [31]. The color recovered upon addition of Al^{3+} due to disassembly of the CP-ATP complex and formation of more energetically favourable Al^{3+} -ATP complex. Al^{3+} was stabilized by Al^{3+} -phosphate coordination thereby inducing disassembly of CP-ATP complex to random coiled CP. The assay was tested in various concentrations of Al^{3+} (from 40 nM to 400 μM) using fluorescence and UV-Vis spectroscopy.

Figure 6.4: Schematic illustration of colorimetric detection of aluminum ion based on cationic polythiophene with different conformation with ATP in the presence and absence of aluminum ions.

The fluorescence intensity of solution containing 80 μM CP and 16 μM ATP gradually increases with increasing concentration of Al^{3+} as shown in Figure 6.5(a) which is attributed to the substitution of CP in CP-ATP complex with Al^{3+} , resulting in formation of ATP-Al^{3+} complexes. In the UV-Vis spectrum analysis shown in Figure 6.5(b), the peak intensity around 400 nm, associated to random coiled CP morphology increases with increasing concentrations of Al^{3+} , whereas the vibronic peaks at 544 and 590 nm associated with CP-ATP planar π -stacking morphology decreases in intensity with increasing concentration of Al^{3+} . Figure 6.5(c) shows the optical image of CP-ATP vials incubated with various concentrations of Al^{3+} (40 nM to 400 μM , from right to left), recorded using a digital camera. The naked eye detection limit was around 4 μM , which is below the Al^{3+} ions maximum acceptable concentration limit in drinking water according to the EEC standards [284]. Furthermore, the proposed approach ascertained detection of Al^{3+} ions in the low μM regime via a simple RGB analysis as shown in Figure 6.5(d). The RGB analysis was performed via software ImageJ, where 2000 pixels of each Al^{3+} concentration were chosen to read the average RGB value. The RGB result is in agreement with naked eye observation, suggesting that the proposed approach could be used in combination with ubiquitous image

processing devices such as digital cameras and smart phones, for highly sensitive detection of Al^{3+} ions. We have also correlated the normalized fluorescence intensity derived from I/I_{CP} , where I and I_{CP} represent integrated fluorescence intensity from 450 to 700 nm for the tested assay and CP, respectively, for different concentrations of Al^{3+} ions in drinking and tap water. As shown in Figure 6.5 (e), the assay yielded a linear response on a log-scale with a corresponding regression equation: $\log Y = 0.942 + 0.281 \log X$, and the dynamic range of Al^{3+} detection ranges from 4 μM to 400 μM .

Figure 6.5: (a) Fluorescence spectra, (b) UV-Vis spectra, and (c) color change upon addition of various concentrations of Al^{3+} . (d) RGB analysis. (e) Correlation curve between normalized fluorescence intensity and concentration of Al^{3+} in DI water (black line) and tap water (red line).

The limit of detection (LOD) was estimated to be $3.7 \mu\text{M}$ ($S/N = 3$). In order to evaluate the sensor for practical applications, various concentration of Al^{3+} was spiked in tap water. As shown in Figure, the curve profile of tap water was similar to that of DI water. However, the normalized fluorescence intensity of assay in tap water was higher than the assay in DI water, which is attributed to the lower fluorescence intensity of I_{CP} . A possible explanation could be that there were other anions existing in tap water compared to DI water, and that the electrostatic force imposed by these anions contributed to formation of planar π -stacking phase, further lowering the fluorescence intensity of CP (I_{CP}). Al^{3+} addition recovered the fluorescence not only from the CP-ATP, but also from CP-anions, thus the normalized fluorescence intensity was higher in tap water than in DI water. The regression equation of Al^{3+} assay in tap water was $\log Y = -0.959 + 0.347 \log X$, and the LOD was calculated to be $3.6 \mu\text{M}$ ($S/N=3$), which is comparable to the estimated LODs obtained using naked eye and RGB detection strategies.

Figure 6.6(a) illustrates the selectivity of CP-ATP complex with addition of 16 metal ions ($400 \mu\text{M}$). The image indicates color recovery (to original yellow color) upon addition of Al^{3+} , whereas addition of $400 \mu\text{M}$ of other metal ions yielded almost negligible recovery, indicating minimum interferences.

Figure 6.6: (a) Selective detection of Al^{3+} ion. (b) Fluorescence recovery of metal ions.

Figure 6.6(b) summarizes the fluorescence recovery for 16 metal ions, where the fluorescence recovery is defined as $(I - I_{\text{ATP}})/(I_{\text{CP}} - I_{\text{ATP}})$, where I_{ATP} represents integrated fluorescence intensity of CP-ATP complex. For selective Al^{3+} detection, two methods were evaluated; (1) PT ($80 \mu\text{M}$) was incubated with ATP ($16 \mu\text{M}$) first, followed by addition of Al^{3+} ; (2) Al^{3+} was incubated with ATP, followed by addition of PT. For developments of the colorimetric sensor, method 1 was utilized for all

experiments yielding colorimetric responses upon addition of Al^{3+} . Method 2 was used to investigate the mechanism of interaction between Al^{3+} and ATP. Higher fluorescence recovery was recorded for method 2, probably due to the better complexation of Al^{3+} with ATP, thereby decreasing the amount of free ATP in the solution, leading to lesser fluorescence quenching upon CP-ATP electrostatic interactions. The absorbance ratio between 400 nm and 544 nm was used to estimate the amount of random coiled and planar π -stacked CPs.

This work was published on *Analytica Chimica Acta*. I provided the help in the developing the assay strategy, optimization of polymer experiments and writing of the paper.

This section (including phrases and Figures) is adapted/reproduced from our published article "Visual detection of Al^{3+} ions using conjugated copolymer-ATP supramolecular complex." *Analytica Chimica Acta* 912 (2016): 105-110".

6.3 Vapour Induced Chromism and Luminescence of Polythiophene Derivatives

6.3.1 Introduction

Vapochromic and vapoluminescent materials are capable of detecting volatile organic compounds (VOCs) via changes in color and luminescence. These materials have attracted increasing attention because of their potential for VOCs detection. Detection of volatile organic compounds has attracted significant interest for human and environmental safety as VOCs are continuously released into the environment from multiple sources such as transportation, agriculture and household products [293]. Among various VOCs detection principles and transduction techniques reported [294-296], colorimetric detection of VOCs is of particular interest due to its potential for naked eye detection without requiring sophisticated instrumentation [297].

Conjugated polymers such as polydiacetylene, polypyrrole, polythiophene and poly(phenylene vinylene) etc. has been widely studied as sensor materials due to their unique electrical and optical properties [298]. However, the main drawback to their application in VOCs sensing are low sensitivity and selectivity due to mass transfer resistance between analyte vapors and polymers [299]. Amongst conjugate polymers, conjugated polyelectrolytes such as cationic polythiophenes (PTs) have been explored for chemo and biosensing due to superior solid state optical properties, high

conductivity, high chemical stability and solubility in water [33]. Although previous studies have utilized PT derivatives for VOCs sensing, mainly non-naked eye detection methods such as gravimetric, electrochemical and spectroscopy have been explored [300-302]. Most of these methodologies requires sophisticated instrumentation for analysis and transduction. In this study, we have investigated the application of PT vapochromic and vapoluminescent properties for the naked eye detection of VOCs.

PTs on PVDF membrane exhibits vapochromic and vapoluminescent properties where organic vapors are able to induce a significant change in emission shift and intensity within the visible spectrum that are perceivable by naked eye. In this study, PTs with varying pendant groups such as trimethylamine and 1-methyl imidazole in varying stoichiometric ratios was investigated to maximize and tune PTs optical responses to varying VOCs.

6.3.2 VOC Assay

Optical transition of PT-film relies mainly on the planar-nonplanar conformation of the backbone and extent of π -electron delocalization within the conjugated system induced by external stimuli, such as temperature, pH, electric field and interaction with molecules that may induce chirality in the supramolecular structure [169, 303, 304]. PTs on PVDF membranes shows a rapid vapochromic response from orange to green with a concurrent increase in luminescence upon exposure to VOCs. The vapochromism is modulated by varying pendant groups of PTs, owing to their intermolecular attraction/affinity with VOCs. PTs on PVDF membranes also exhibits a rapid and reversible optical response upon introduction and removal of gaseous analyte which can be understood in terms of order-disorder phenomena [305], where the open porous structure combined with a low rigidity polymer backbone which allows for a reversible transition between its planar-nonplanar states. The rapid recovery of original baseline observed upon purging with ambient air at room temperature suggests that VOCs mainly interact via a physisorption instead of chemisorption.

A vapochromic array consisting of 6 different PTs and VOCs such as dichloromethane, acetone, chloroform, tetrahydrofuran, hexane, ethanol and toluene is developed to demonstrate the feasibility of differentiating VOCs is shown in Figure 6.7.

Figure 6.7: Saturated vapor colorimetric response with different PT derivatives.

Currently, this project is in proof of concept stage. The main advantage of this assay compared to the existing assays are in terms of reversibility and instant response time. In the current phase, assay needs improvement in both sensitivity and selectivity. Therefore, there is on-going work in progress for this project to create a dual-sensing platform utilizing both optical and electrical detection to improve sensitivity as well as analyte differentiation capability via analyte electron doping mechanism where analyte differentiation could be achieved via an increase/decrease of conductivity.

Manuscript is under preparation for this work. I initiated and started this project, optimize the polymer coating and conducted preliminary experiments and I'm currently helping in the writing of paper.

CHAPTER 7: CONCLUSION AND FUTURE OUTLOOK

7.1 Summary

This thesis focused on the design and development of point of care diagnostics for carbonyl/oxidative stress biomarkers with high selectivity and specificity for the earlier detection of T2D and related metabolic diseases. Cationic polythiophenes (PT) were used as luminescent reporters for the development of colorimetric assay. The assay was developed for three different types of carbonyl/oxidative stress biomarkers such as mir21 (nucleic acid based), 8-OHdG (a small molecule due to DNA damage) and Gly-AGEs (sugar molecules attached to proteins).

Although there are reports on fluorescent and colorimetric biosensors using conjugated polymers for nucleic acid detection such as miRNA in solution-based sensing, there is still a lack of discussion in the literature on how the sensing capacity of a PT may vary with structure of the pendant group. To understand this, two homopolymers Poly(3-alkoxythiophene) polyelectrolytes (hPT1 and hPT2) with different functional groups (i.e. triethylamine and 1-methyl imidazole) and their random copolymers (cPT1, cPT2 and cPT3) in different monomer ratios of 1:1, 1:5, 5:1, respectively, were designed and synthesized by oxidative polymerization. Even though, all the polymers gave good responses for the mir21 assay, I demonstrated that copolymer of PT (cPT3) yielded better optical responses for the mir21 detection in comparison to those of their homopolymers in solution state implying tuning of pendant group could improve the sensitivity and selectivity.

PTs are predominantly studied in the solution state. However, solution-based assays are not robust as compared to paper-based assays and often suffers from low selectivity. So, the main focus of my work has been on the translation of solution-based sensing assays into a paper-based assay using the 5 polymers/co-polymers. While, in case of solution platform, cPT3 worked better, the contrary was found in paper-based platform where hPT1 showed better performance.

Then, a multiplexed flow-through paper-based colorimetric device is developed for the detection of nucleic acids such as mir21 and HBV-DNA in plasma sample at concentrations spanning five orders of magnitude. The integration of FUSION 5 filter papers with the optical reporter (PT) incorporated PVDF membranes enabled sample pre-treatment and detection in the same platform, with both qualitative and quantitative analysis. The assay yields colorimetric detection of nucleic acids with

limits of detection of 600 pM and 2 nM in DI and plasma, respectively. Colorimetric responses from the flow-through device is then utilized to develop a logic gate circuit that possesses a great potential for POC diagnostics.

Similarly, the developed flow-through device is tested for the detection of oxidative DNA damage molecule in artificial urine. The established methodology has been validated for 8-OHdG detection using luminescent reporter (PT) impregnated PVDF membranes with its aptamer. The assay yields optical responses for 8-OHdG in artificial urine with a limit of detection of 500 pM, indicating the potential use in clinical applications. The fabricated platform could be ideal in POC applications for early diagnosis of oxidative stress related diseases.

Furthermore, a simple solution-based assay for the naked eye detection of AGEs is developed utilizing PT and aptamer. This methodology has been validated for the detection of Gly-AGEs (a biomarker related to T2D) in plasma. The obtained colorimetric responses yield a limit of detection of 1 μ g/ml in plasma. This solution-based assay could be ideal for POC management framework for detection of carbonyl stress and could be used in nutrition and metabolic fields for controlling food intake of diabetic patients.

In conclusion, this thesis showed the development of colorimetric sensor for various biomarkers such as mir21, 8-OHdG and Gly-AGEs using cationic polythiophenes. Also, the developed flow-through (vertical flow) device have very simple components such as FUSION 5 filter paper as sample pad/filtration, a PVDF membrane for analysis, and a plastic lamination sheath for protection from contamination as well as for handling. This device has the following advantages such as (a) low sample volume (in microliters) requirement (b) sample pre-treatment and analysis in the same platform, (c) can be used for multiplexing, (d) low cost, ease of use, portable and robust, (e) use capillary flow (does not require external pump) (f) can be disposed easily and (g) the device could be integrated with mobile phone for telemedicine and thus enabling off-site diagnosis.

The overall performance for the colorimetric assays are good in terms of sensitivity and selectivity for both 8-OHdG and Gly-AGEs as they can detect the clinical threshold limits in urine and plasma as mentioned in Table 1.1, whereas for mir21 assay, further improvement in sensitivity/LOD is required as current developed assay can only detect up to $\sim$ 5 ng/ml in DI.

7.2 Future Perspectives

Currently, for the evaluation of colorimetric responses of nucleic acids, a 10-fold dilution of plasma is required. So, further optimization is needed in flow-through device architecture and PT deposition protocols to enable assaying in undiluted plasma samples. Future studies will also emphasize on new and old PTs with controlled composition and stoichiometric ratios of pendant groups to improve the limit of detection of mir21 for naked eye detection. Additionally, while I have demonstrated the performance of the flow-through device on spiked samples of plasma, a detailed analysis of device performance on clinical/patient samples is required.

Similarly, testing of flow-through device on clinical urine samples for 8-OHdG is needed. A blind study has to be conducted to find out the performance of the device to see whether the assay can be used for differentiating T2D patients from healthy people by quantitatively analyzing the 8-OHdG levels in the urine sample. The obtained responses from these samples would be then compared with current analytical procedures such as ELISA, chromatography and mass spectroscopy to validate the flow-through assay. Currently, such studies are ongoing. Here, a blind study where 50 urine samples are collected (contains unknown samples of both T2D patients and healthy donors). The objective is to classify and quantify these samples based on T2D and healthy samples by measuring 8-OHdG levels. Then, these samples will be tested using ELISA to measure the 8-OHdG concentrations. The idea is to see whether by measuring 8-OHdG could help in classification of the sample based on the disease and also to find out the performance (false positives/negatives) of the developed flow-through device in a clinical setting. The preliminary experiments showed some challenges due to the high autofluorescence and pigments in urine. Due to this, when urine is added to the device without aptamer, PT is blue-shifted. Presently, experiments are conducting to neutralize this fluorescence.

Currently, the solution-based Gly-AGEs assay in plasma is just a proof of concept. Further experiments are required to determine the specificity of this assay with respect to various AGEs other than Gly-AGEs and Glu-AGEs. Also, this solution-based assay has to be translated into the flow-through paper-based assay as described in previous sections. Moreover, the AGEs assay has to be tested with food samples/matrices, as they also contain different types of AGEs and could be beneficial in finding out various levels of AGEs in commonly consumed beverages and foods. By determining the amount of AGEs in food samples helps to intake a low AGEs diet

as dietary AGEs is another source for circulating AGEs and contributes to T2D. So, a simple control of food intake (dietary intervention) helps to protect against premature aging and diseases such as diabetes.

Also, the development of multiplexed flow-through device for the detection various carbonyl/oxidative stress related T2D markers in addition with mir21, 8-OHdG and Gly-AGEs simultaneously could give a holistic approach for monitoring diabetes and its complications. Additionally, the incorporation of carbon nanotubes (CNTs) with PTs in flow-through device would enable dual detection (both optical and electrical) and could help in improving the limit of detection further especially for mir21 assay.

7.3 List of Publications

1. Gopal Ammanath, Umit Hakan Yildiz, Alagappan Palaniappan, and Bo Liedberg. "Luminescent Device for the Detection of Oxidative Stress Biomarkers in Artificial Urine." *ACS applied materials & interfaces* 10, no. 9 (2018): 7730-7736.
2. Deepa Rajwar, Gopal Ammanath, Jamal Ahmed Cheema, Alagappan Palaniappan, Umit Hakan Yildiz, and Bo Liedberg. "Tailoring conformation-induced chromism of polythiophene copolymers for nucleic acid assay at resource limited settings." *ACS applied materials & interfaces* 8, no. 13 (2016): 8349-8357.
3. Tu, Meng-Che, Deepa Rajwar, Gopal Ammanath, Palaniappan Alagappan, Umit Hakan Yildiz, and Bo Liedberg. "Visual detection of Al^{3+} ions using conjugated copolymer-ATP supramolecular complex." *Analytica chimica acta* 912 (2016): 105-110.
4. Alagappan Palaniappan, Jamal Ahmed Cheema, Deepa Rajwar, Gopal Ammanath, Liu Xiaohu, Lim Seng Koon, Wang Yi, Umit Hakan Yildiz, and Bo Liedberg. "Polythiophene derivative on quartz resonators for miRNA capture and assay." *Analyst* 140, no. 23 (2015): 7912-7917.
5. Gopal Ammanath, Sanjida Yeasmin, Yuvasri Srinivasulu, Mukti Vats, Jamal Ahmed Cheema, Fairuz Nabilah, Rohit Srivastava, Umit Hakan Yildiz, Alagappan Palaniappan, and Bo Liedberg. "Flow-through Colorimetric Assay for Visual Detection of Nucleic Acids in Plasma." *Analytical Chemistry, Under Review*

6. Gopal Ammanath, Vineeth Sama, Umit Hakan Yildiz, Alagappan Palaniappan, and Bo Liedberg. "Colorimetric Screening Assay for Advanced Glycation End Products.", *In preparation*
7. Lee Chao Qun, Gopal Ammanath, Umit Hakan Yildiz, Alagappan Palaniappan, and Bo Liedberg. "Vapour Induced Chromism and Luminescence of Polythiophene Derivatives.", *In preparation*
8. Jamal Ahmed Cheema, Gopal Ammanath, Muge Yucel, Umit Hakan Yildiz, Alagappan Palaniappan, and Bo Liedberg "Pixelated Colorimetric Paper-based Nucleic Acid Detection with Smart Phone Algorithm.", *In preparation*

7.4 Conferences

The works shown in this thesis were presented in the following conferences.

1. Gopal Ammanath, Umit Hakan Yildiz, Alagappan Palaniappan, and Bo Liedberg. Point of Care Diagnostics for Age Related Diseases in Low Resource Settings; Select Biosciences Point-of-Care Diagnostics, Global Health and Emerging Viral Diseases World Congress: 02-04 October, 2017, Coronado Island, California, USA (Oral Presentation)
2. Gopal Ammanath, Meng Che Tu, Umit Hakan Yildiz, Alagappan Palaniappan, and Bo Liedberg. Conjugated Polymer Assays for Sensing Applications; Symposium on Advances in Molecular Sensory Engineering (SAMSE): 27-28 July, 2017, Singapore (Poster Presentation).
3. Gopal Ammanath, Meng Che Tu, Umit Hakan Yildiz, Alagappan Palaniappan, and Bo Liedberg. Conjugated Polymer Assays for Sensing Applications; NTU-NITHM-IIT Bombay Symposium for Healthcare and Medical Technologies: 06-07 March, 2017, Singapore (Poster Presentation).
4. Gopal Ammanath, Umit Hakan Yildiz, Alagappan Palaniappan, and Bo Liedberg. Point of Care Diagnostics for Age Related Diseases in Low Resource Settings; IITB-NTU Singapore Joint Symposium on Healthcare Technologies: 26-28 September, 2016, Mumbai, India (Poster Presentation).
5. Gopal Ammanath, Bernhard Boehm, Yusuf Ali, Umit Hakan Yildiz, Alagappan Palaniappan, and Bo Liedberg. Novel Diagnostic Tools for Age Related Monitoring; Ageing Research Institute for Society and Education (ARISE) launch: 06 May, 2016, Singapore (Poster Presentation).

6. Gopal Ammanath, Umit Hakan Yildiz, Alagappan Palaniappan, and Bo Liedberg. Point of Care Diagnostics for Nucleic Acid Biomarkers in Low Resource Settings; From Materials to Life: Multidisciplinary Challenges, Kyoto Winter School: 15-26 February, 2016, Japan (Poster Presentation).

APPENDIX I

Synthesis of Cationic Polythiophenes

All the reagents and solvents were obtained from Sigma Aldrich and used without further purification. PTs were synthesized by oxidative polymerization of their monomers; NMR spectra were recorded on a Bruker Advance 400 MHz spectrometer.

Synthesis of precursor 1 (3-methoxy-4-methylthiophene)

To a dry round-bottom flask, 1-methyl-2-pyrrolidinone (6.7 mL, 69.80 mmol) and sodium methoxide/MeOH (25 wt%; 17.8 mL, 78.50 mmol) were added. After heating this mixture to 110 °C, 3-bromo-4-methylthiophene (5 g, 28.20 mmol) and CuBr (2.47 g, 17.20 mmol) were added. The round-bottom flask was then equipped with a water-cooled condenser and refluxed for 1 day at 110 °C (Figure A1(a)). The reaction mixture was cooled to room temperature and then a solution of 1 g of sodium bromide in 40 mL of water was added and stirred vigorously for 1 h. The mixture was then filtered and extracted with 50 mL diethyl ether five times. The combined organic layers were washed with water at least three times and dried over MgSO₄. Solvent was then removed by rotary evaporator to yield a light-yellow oil. This light-yellow oil was further purified with column chromatography to yield 3-methoxy-4-methylthiophene (2.28 g (63%)). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 6.84 (1H, d), 6.18 (1H, d), 3.84 (3H, s), 2.11 (3H, s) (Figure A1(b)).

Figure A1: (a) Synthetic route to 3-methoxy-4-methylthiophene (b) ¹H NMR spectrum of 3-methoxy-4-methylthiophene (400 MHz, CDCl₃).

Synthesis of precursor 2 (3-(3-Bromo) propoxy-4-methylthiophene)

A mixture of 3-methoxy-4-methylthiophene (precursor 1) (0.84 g, 6.60 mmol), 3-bromo-1-propanol (2.00 g, 14.4 mmol), and NaHSO₄ (123 mg, 0.900 mmol) in 15 mL of toluene was added to a two neck round bottom flask. This reaction mixture was heated at 100 °C for 24 hours under nitrogen atmosphere (Figure A2(a)); meanwhile the produced methanol was distilled off from the reaction flask. The reaction mixture was allowed to cool to room temperature. Toulene was removed by rotory evaporator and the remaining reaction mixture was extracted five times with diethyl ether, subsequently washed with water and dried over magnesium sulfate. Diethyl ether was removed by rotory evaporator. The crude product was purified with column chromatography (silica gel, hexane) to give 1.0 g (65%) 2 as colorless oil. ¹H NMR (400 MHz, CDCl₃) δ (ppm): 6.83 (1H, d), 6.19 (1H, d), 4.08 (2H, m), 3.59 (2H, m), 2.34 (2H, m), 2.09 (3H, s) (Figure A2(b)).

Figure A2: (a) Synthetic route to 3-methoxy-4-methylthiophene (b) ¹H NMR spectrum of 3-(3-Bromo) propoxy-4-methylthiophene (400 MHz, CDCl₃).

Synthesis of monomer 1

To a round bottom flask 3-(3-Bromo)propoxy-4-methylthiophene (0.27 g, 1.15 mmol), triethylamine (26.45 mmol, 3.7 mL) and THF (30 mL) were added. The mixture was stirred at 72 °C for two days (Figure A3(a)). The crude Product was washed with THF to give 0.30 g (77%) monomer 1. ¹H NMR (400 MHz, DMSO-d₆) δ (ppm): 7.09 (d, 1H), 6.53 (d, 1H), 4.02 (t, 2H), 3.29 (m, 8H), 2.08 (m, 2H), 2.03 (s, 3H), 1.20 (t, 9H). ¹³C NMR (100 MHz, DMSO-d₆) δ (ppm): 155.41, 128.56, 121.09, 97.85, 66.61, 53.60, 52.52, 21.88, 12.92, 7.53 (Figure A3(b)).

Figure A3: (a) Synthetic route to monomer 1 (b) ^1H NMR spectrum of monomer 1 (400 MHz, DMSO- d_6).

Synthesis of monomer 2

To a solution of 3-(3-Bromo)propoxy-4-methylthiophene (0.27 g, 1.150 mmol) in CH_3CN (35 mL) in a round bottom flask, 1-methylimidazole (1 mL) was added. The mixture was stirred 48 h at 70 $^\circ\text{C}$, and then evaporated to dryness (Figure A4(a)). After evaporation of the solvent, the crude product was washed subsequently with warm EA and diethyl ether to give 0.27 g (80 %) monomer 2. ^1H NMR (400 MHz, CDCl_3) δ (ppm): 10.12 (s, 1H), 7.51 (s, 1H) 7.46 (s, 1H), 6.74 (d, 1H), 6.51 (d, 1H), 4.51 (t, 2H), 3.98 (m, 5H), 2.39 (m, 2H), 1.97 (s, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ (ppm): 155.10, 137.30, 128.61, 123.58, 122.54, 120.33, 97.27, 66.21, 47.33, 36.81, 29.84, 12.75 (Figure A4(b)).

Figure A4: (a) Synthetic route to monomer 2 (b) ^1H NMR spectrum of monomer 2 (400 MHz, DMSO- d_6).

Synthesis of hPT1

A solution of 3-(4-Methyl-3'-thienyloxy)propyltriethylammonium bromide (monomer 1) (270 mg, 0.910 mmol) in 5 mL dry CHCl_3 was added dropwise to a solution of anhydrous FeCl_3 (590 mg, 3.640 mmol) in 25 mL of dry CHCl_3 , and stirred for 24 h at room temperature under N_2 atmosphere, then evaporated to dryness (Figure

A5(a)). The crude product was washed with CHCl_3 and diethyl ether subsequently many times to get rid of FeCl_3 and starting material. The collected polymer was dried in vacuum for 24 h. ^1H NMR is shown in Figure A5(b).

Figure A5: (a) Synthetic route to hPT1 by oxidative polymerization of monomer 1 (b) ^1H NMR spectrum of hPT1 (400 MHz, D_2O - DMSO).

Synthesis of hPT2

80 mg (0.260 mmol) of monomer 2 in 5 ml CHCl_3 was added dropwise to a solution of 175 mg (1.080 mmol) of anhydrous FeCl_3 in 10 ml CHCl_3 and stirred for 48 h at room temperature under N_2 atmosphere, then evaporated to dryness (Figure A6(a)). The crude product was washed with CHCl_3 and diethyl ether subsequently many times to get rid of FeCl_3 and starting material. The collected polymer was dried in vacuum for 24 h. ^1H NMR is shown in Figure A6(b).

Figure A6: (a) Synthetic route to hPT2 by oxidative polymerization of monomer 1 (b) ^1H NMR spectrum of hPT2 (400 MHz, D_2O - DMSO).

Synthesis of cPT1 (monomer 1: monomer 2 = 1:1 ratio)

A mixture of monomer 1 (0.063 mmol, 21 mg) and monomer 2 (0.063 mmol, 20 mg) in 5 ml CHCl_3 was added dropwise to a solution of anhydrous FeCl_3 of (0.523

mmol, 85 mg) in 10 ml CHCl₃ and stirred for 48 h at room temperature under N₂ atmosphere, then evaporated to dryness (Figure A7(a)). The crude product was washed with CHCl₃ and diethyl ether subsequently many times to get rid of FeCl₃ and starting material. The collected polymer was dried in vacuum for 24 h. ¹H NMR is shown in Figure A7(b).

Figure A7: (a) Synthetic route to cPT1 via oxidative polymerization of monomer 1 and monomer 2 (with varying ratios of m1 and m2) (b) ¹H NMR spectrum of cPT1 (400 MHz, DMSO-d₆) (c) ¹H NMR spectrum of cPT2 (400 MHz, DMSO-d₆) (d) ¹H NMR spectrum of cPT3 (400 MHz, DMSO-d₆).

Synthesis of cPT2 (monomer 1: monomer 2 = 1:5 ratio)

A mixture of monomer 1 (0.050 mmol, 16 mg) and monomer 2 (0.250 mmol, 84 mg) in 10 ml CHCl₃ was added dropwise to a solution of anhydrous FeCl₃ (1.240 mmol, 201 mg) in 20 ml CHCl₃ and stirred for 48 h at room temperature under N₂ atmosphere, then evaporated to dryness (Figure A7(a)). The crude product was washed

with CHCl_3 and diethyl ether subsequently many times to get rid of FeCl_3 and starting material. The collected polymer was dried in vacuum for 24 h. ^1H NMR is shown in Figure A7(c).

Synthesis of cPT3 (monomer 1: monomer 2 = 5:1 ratio)

A mixture of monomer 1 (0.253 mmol, 81 mg) and monomer 2 (0.051 mmol, 17 mg) in 10 ml CHCl_3 was added dropwise to a solution of anhydrous FeCl_3 (1.250 mmol, 203 mg) in 20 ml CHCl_3 and stirred for 48 h at room temperature under N_2 atmosphere, then evaporated to dryness (Figure A7(a)). The crude product was washed with CHCl_3 and diethyl ether subsequently many times to get rid of FeCl_3 and starting material. The collected polymer was dried in vacuum for 24 h. ^1H NMR is shown in Figure A7(d).

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