FABRICATION OF LATERAL FLOW IMMUNOSENSOR FOR THE DETECTION OF HEMOGLOBIN A1C (HBA1C)

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FACULTY OF SCIENCE

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ABSTRACT

The purpose of the study was to provide an alternative point-of-care device for efficient management of diabetic status for type 2 diabetic patients in a resource-limited setting. The study focused on developing functional lateral flow immunosensor for the detection of hemoglobin A1c (HbA1c), the biomarker for the management of type 2 diabetes mellitus. Because human whole blood is a complex matrix with different glycan moieties, a highly selective biosensor towards the specific glycan moiety (HbA1c) in erythrocytes is desirable. In the study, specific pair of antibodies was selected to perform the "sandwich" immunoassay on the paper-based surface, where the assay was performed with lateral flow principle. Besides finding the right pair of antibodies that is highly selective towards HbA1c against all other glycated species in blood, the ideal condition to form stable antibodies-functionalized colloidal gold to transduce the signal, the blocking reagent to minimize the background on the immunosensor, the test line format for visual interpretation and semi-quantitative measurement of HbA1c level, the potential interference from blood, and the dilution factor necessary to lyse the erythrocytes and reduce the viscosity of whole blood on the lateral flow immuno-sensing platform were investigated. Later, with a calibration curve established, the immunosensor was proved to be able to perform a quantitative analysis on the HbA1c level in human blood, validated by comparing the measurement of HbA1c level by the developed lateral flow immunosensor to that of the standard method and existing point-of-care device commonly used in a clinical setting. Using the established optimized parameters, a new design of sandwich immunoassay format was developed to detect both the total hemoglobin and hemoglobin A1c simultaneously within a test run in a single step. In conclusion, both of the developed prototypes of lateral flow immunosensors have been proven to be clinically operational efficient for measuring HbA1c level, hence, both of them can serve as alternatives for diabetes management in resource-limited setting.

ABSTRAK

Kajian ini adalah bertujuan untuk membekalkan alat penjagaan alternatif untuk pengurusan berkesan terhadap pesakit berstatus diabetis jenis 2 dalam keadaan sumber yang terhad. Fokus kajian kepada pembangunan *immunosensor* aliran sisian ini adalah untuk mengesan hemoglobin A1c (HbA1c), *biomarker* (protein penanda) bagi pengurusan diabetis melitus jenis 2. Disebabkan darah manusia adalah matriks kompleks dengan moieti glikan yang berbeza, *biosensor* yang sangat selektif terhadap moieti glikan tertentu (HbA1c) di dalam eritrosit adalah diperlukan. Dalam penyelidikan ini, sepasang antibodi tertentu telah dipilih untuk melaksanakan *"sandwich"* immunoasai di permukaan berasaskan kertas, di mana asai dilaksanakan dengan prinsip aliran sisian. Selain daripada mencari pasangan antibodi yang hanya selektif terhadap HbA1c berbanding lain-lain spesis glikan di dalam darah, keadaan yang ideal untuk menghasilkan fungsian-antibodi koloidal emas yang stabil untuk penyampaian isyarat, reagen penyekatan untuk meminimumkan gangguan pada *immunosensor* tersebut, format garis ujian untuk tafsiran visual dan pengukuran separa-quantitatif terhadap tahap HbA1c, potensi gangguan dari darah, dan faktor pencairan yang diperlukan untuk memecahkan eritrosit dan mengurangkan kelikatan keseluruhan darah pada platform *immunosensor* aliran sisi telah dikaji. Seterusnya, dengan menggunakan keluk penentuukuran yang dijana, telah dibuktikan bahawa *immunosensor* tersebut dapat melaksanakan analisis kuantitatif untuk menentukan tahap HbA1c di dalam darah manusia, disahkan dengan membandingkan pengukuran tahap HbA1c menggunakan *immunosensor* aliran sisi yang dibangunkan terhadap kaedah piawai dan alat penjagaan lain yang biasa digunakan dalam persekitaran klinikal. Dengan menggunakan parameter optimum, *sandwich* immunoasai format baru telah dibangunkan bagi mengesan jumlah hemoglobin dan hemoglobin A1c secara serentak dalam satu langkah jalanan ujian. Kesimpulannya, kedua-dua pembangunan prototaip

immunosensor aliran sisi telah terbukti dapat digunakan dengan cekap dalam persekitaran klinikal untuk mengukur tahap HbA1c, oleh itu, ianya boleh berfungsi sebagai alat penjagaan alternatif untuk pengurusan diabetis dalam keadaan sumber yang terhad.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

α	:	Alpha subunit/chain
β	:	Beta subunit/chain
γ	:	Gamma subunit/chain
δ	:	Delta subunit/chain
AACC	:	American Association for Clinical Chemistry
ADA	:	American Diabetes Association
ADAG	:	A1c-Derived Average Glucose
AG	:	Average Glucose
AGE	:	Advanced Glycated Products
BMI	:	Body Mass Index
BSA	:	Bovine Serum Albumin
CAP	:	College of American Pathologies
CDA	:	Canadian Diabetes Association
CL	:	Central Lab
CLIA	:	Clinical Laboratory Improvement Amendments
CV	:	Coefficient Variants
DCCT	:	Diabetes Control and Complications Trials
DCDR	:	Drop-Coating Deposition Raman
eAG	:	Estimated Average of Glucose Level
EDTA	:	Ethylenediaminetetraacetic acid
ELISA	:	Enzyme-Linked Immunosorbent Assay
FAO	:	Fructosyl Amine Oxidase
FDA	:	Food and Drug Administration

FPG	:	Fasting Plasma Glucose
FV	:	Fructosyl Valine
Hb	:	Hemoglobin
HbA1c	:	Hemoglobin A1c
HPLC	:	High Performance Liquid Chromatography
IFCC	:	International Federation of Clinical Chemistry
IFG	:	Impaired Fasting Glucose
Ig	:	Immunoglobulin
IGT	:	Impaired Glucose Tolerance
JDS	:	Japan Diabetes Society
LDL	:	Low-Density Lipoprotein
LOD	:	Limit Of Detection
NC	:	Nitrocellulose
NGSP	:	National Glycohemoglobin Standardization Program
NIDDK	:	National Institute of Diabetes and Digestive and Kidney Diseases
OGTT	:	Oral Glucose Tolerance Test
POC	:	Point-of-Care
PVA	:	Polyvinyl Alcohol
PVP	:	Polyvinylpyrrolidone
ROC	:	Receiver Operating Characteristic
RPG	:	Random Plasma Glucose
Rpm	:	Revolutions per Minute
SAM	:	Self -Assembled Monolayer
SI unit	:	International System of unit
SPR	:	Surface Plasmon Resonance
SRLs	:	Secondary Reference Laboratories

T2DM	:	Type 2 Diabetes Mellitus
UKDPS	:	UK Prospective Diabetes Study
UMMC	:	University of Malaya Medical Center
UV	:	Ultraviolet
w/v	:	Weight/Volume
WBR	:	Western Blocking Reagent
WC	:	Waist Circumference
WHO	:	World Health Organization

LIST OF APPENDICES

CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

Type 2 diabetes mellitus is a disease where patients suffer from elevated glucose level in bloodstream due to a variety of etiologies. Because of the high mortality associated to the disease, and with the lifelong morbidities that require intensive medical care, which in return could impact the daily lives; type 2 diabetes mellitus is therefore a dreadful disease that constantly poses high socioeconomic burden to a nation. The society has long attached to the plasma-specific tests which require long fasting period that decreases patients' compliance for routine check-ups at clinics and hospitals. Hence, with compromised patients' compliance to routine check-ups, patients can have incomplete recollection about the potential symptoms, or when physicians are hesitant to prescribe more intensive treatment than lifestyles modifications at the screening stage of prediabetes, it often leads to undiagnosed cases of type 2 diabetes for a prolonged time (Karve & Hayward, 2010). Therefore, there's a need to create the awareness in the society about the alternative test that does not require long fasting time - the hemoglobin A1c (HbA1c) test, and to encourage them for scheduled visits at clinics or hospital for diabetic status check-ups.

Hemoglobin A1c is a stable amadori compound that is found as a result of a spontaneous reaction between glucose in blood stream and the hemoglobin in the red blood cells. It can provide a "weighted" average of glucose level in plasma over the preceding four months (NGSP, 2010b). With studies discovering that the incidence of diabetic retinopathy increases steeply ≥6.5% HbA1c (ADA, 2011), the role of HbA1c in diagnosing type 2 diabetes mellitus is slowly gaining attention. On the other hand, HbA1c has long been used as a surrogate of personal lifestyle for the pass 2 to 3 months to aid with clinical decision upon therapy modalities for type 2 diabetic patients.

Because of its role in reflecting the glycemic status and its important value in clinical evaluation of diabetic status, HbA1c was eventually recognized as one of the diagnosis tests for type 2 diabetes in 2011 by WHO (WHO, 2011). A lot of attempts were committed into the standardization of the market available HbA1c methods; however, because of the lack of a valid reference method, few countries started their own national standardization programs. With a global consensus reached for the valid reference method in 2012 (Hanas *et al.*, 2013), it allowed traceability of results generated using different HbA1c methods to important findings established on type 2 diabetes, hence aid for better communication from the bench top to the end users and to the patients; therefore, better diabetic care. Owing to the absence of valid reference method recognized worldwide for HbA1c test, different notions emerge to aid with the communication of HbA1c results; the % HbA1c, the estimated average of glucose level (eAG), and HbA1c level in mmol mol^{-1} are the established units to define HbA1c results. After year 2012, the SI unit for HbA1c level is recognized to be mmol mol^{-1}, nonetheless, the % HbA1c still remained the common unit in clinical setting. These units were inter-convertible using master equations, and the calculator can be easily assessed on NGSP (POC) website (NGSP, 2010a). To avoid confusion, throughout the study, "% HbA1c" or "HbA1c level" will be used to describe the level of HbA1c, where the results will be reported in both the SI unit (mmol mol^{-1}) and NGSP percentage (%) unit.

In general, methods to detect HbA1c can be divided based on operational principles, whether the detection of HbA1c is performed *via* structural or charges differences. Cation-exchange HPLC (high performance liquid chromatography) and capillary electrophoresis are amongst the commonly used methods that detect HbA1c based on charges differences; while affinity chromatography and immunoassays are the typical methods that recognize HbA1c based on structural differences. Because of the bulkiness

of the instruments, on-site facilitation for diabetes care was often impeded. To meet the demand of high mobility, point-of-care devices/analyzers are designed. However, the stability of the analytical performance of point-of-care (POC) devices are often in doubt, therefore they are not suitable for the diagnosis of type 2 diabetes (Sacks *et al.*, 2011). Nonetheless, they still remain very useful for diabetes care. While the mobility of the POC devices have increased the efficiency of the diabetes care, the test can only be performed and results be interpreted by professional personnel. Also, the installation cost and cost per test remains a challenge for populations in resource-limited setting where the impact of type 2 diabetes is often devastating.

While there have been increasing effort to create more stable POC devices *via* development of different types of biosensors for HbA1c, most of them still require professional interpretation and involve great cost (H. Liu & Crooks, 2012). To resolve the issues associated with creating POC devices suitable for underdeveloped and developing areas which have limited budget allocation for healthcare, this Ph.D. project aims to create a sustainable personalized POC device that is inexpensive, rapid, stable, and user-friendly to measure HbA1c level in whole blood. The objectives identified for this study include:

a) To develop a functional lateral flow strip that could directly detect HbA1c in human whole blood based on immunoassay principle without involving pretreatment.
b) To ensure the fabricated immuno-sensing platform detect specifically HbA1c and signal is observed only with the presence of HbA1c, but not from other interfering agents.
c) To observe a pattern for signal generated on the immunosensor and to relate them to distinctive groups of ranges of HbA1c levels (normal, under control, or elevated) (semi-quantitative measurement).

d) To perform quantitative measurement of HbA1c level with calibration curve established and to apply the developed immunosensor in a clinical setting, later, to compare the measurement of HbA1c using the immunosensor to that of the standard method and also a recognized POC device.

e) To create a new immunoassay design (2nd prototype) that could simultaneously measure total hemoglobin and HbA1c within single-step test (quantitative analysis) and evaluate the analytical performance of the 2nd prototype.

In order to achieve the above-mentioned objectives in the project, a spectrum of optimization works were conducted to enhance the performance and the functionality of the immunosensor in complex biological matrix such as whole blood without elaborated pretreatment stage. Besides using paper that is comparatively inexpensive as a material to develop the lateral flow immunosensor, the sampling and testing procedure that only requires simple washing step is very user-friendly, where non-professional individuals can perform the test and interpret the result easily based on the signal generated on the lateral flow test strip; which in the long run, will decrease the cost of diabetes care substantially.

Throughout the project, the lining of the antibodies on the strips was performed using manual pipetting, and the washing of the strips was done with just dipping the strips into the 96-well microplate filled with washing buffer. Measurement of signal generated on the strip was performed using an ESEQuant lateral flow reader. In fact, the fabrication of the sensing surface that is specific towards HbA1c is vital, therefore the project was started with the search of the right pair of antibodies to be used as the capturing and detecting antibodies in a sandwich type immunoassay.

During the development of the first and 2nd prototypes of lateral flow immunosensor, searching for the correct pair of antibodies is very challenging as not every clone of

antibody worked with each other to give visually interpretable signal. Also, the fact that the glycation site at N-terminal valine in β subunits that is characteristic to HbA1c is only a small site on the hemoglobin (protein) that consists of four sub-units, makes the selection of the right pair of antibodies more difficult. After determining the right pair of antibodies, optimizations were performed to create stable, solvable gold conjugates, to minimize non-specific proteins binding by employing suitable blocking reagents, to study the potential interference from other species of hemoglobin such as HbA0, glycated HbA0, and HbA2; and to determine the test line formats. With the optimization studies involved purified human HbA1c or lysed packed human red blood cells (HbA1c calibrators), it is critical to make sure that the lateral flow immunosensor worked in whole blood, a more complex matrix, without much pretreatment. Optimization of the washing buffers and the reagents to lyse the red blood cells for HbA1c detection was conducted in search of the ideal condition for a blood sample to flow through the strip easily and homogeneously without compromising the assay time. Later, analytical performance of the developed lateral flow immunosensor was assessed and quantitative analysis was performed in a clinical setting. Method validation with the standard method and a POC device was also performed. Other than creating a lateral flow immunosensor that measure absolute % HbA1c, the study moved on to enhance the analysis capability of the lateral flow immunosensor with a new immunoassay format that detect both the HbA1c and the total hemoglobin within a single test run. With the improved format, the detection format for the new design was optimized; the potential interference from variants, the storage stability of the blood samples, and the capability to perform the quantitative analysis were investigated.

1.2 Chapters' Description

In this thesis, chapter 2 is a literature review that relates the role of HbA1c to the long used plasma-specific tests (that involve long fasting duration), explaining about the potential interference and concern associated to HbA1c tests and discussing on the technologies and methods reported for HbA1c measurement in details. Chapter 3 delineates the concept of developing the 1st prototype of HbA1c lateral flow immunosensor that operated based on lateral flow principle in details, where it includes a variety of optimization works to generate visual interpretable signal for semi-quantitative purpose. In chapter 4, the focus is shifted to the real applications in a clinical setting where the analytical performance of the 1st prototype of HbA1c lateral flow immunosensor was evaluated and the quantitative analysis was conducted. Comparison to standard HPLC method and a commercially available POC device was also included in the chapter for method validation. To move on, in chapter 5, a new immunoassay design (2nd prototype) was established to measure % HbA1c as a fraction of the total hemoglobin, where it allowed the simultaneous detection of total hemoglobin and HbA1c within a single-step test on the lateral flow test strip. To relate the ratio of signals obtained for HbA1c/total hemoglobin to % HbA1c, a calibration curve was generated. The reproducibility, the selectivity towards HbA1c against all glycan moieties, the potential interference of variants and the extended storage duration of blood samples with the 2nd prototype were investigated in Chapter 5. Chapter 6 is a conclusion chapter where all the findings and future perspectives of the invention reported in the thesis will be discussed.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Type 2 diabetes mellitus (T2DM) is a pandemic health issue. As a result of rapid population growth, with longer life expectancy and lifestyle changes, T2DM incidence was estimated to leap to 54% worldwide by 2030 (Shaw *et al.*, 2010). Approximately 7.7% of the world's adult population aged 20-79 years (439 million adults) are estimated to be afflicted with diabetes by 2030 (Shaw *et al.*, 2010). While the high prevalence of the chronic disease itself is worrisome, the medical complications and socioeconomic impacts associated with diabetes are as fearful as the disease itself. In fact, diabetic patients have higher risk of developing microvascular (for example, retinopathy, nephropathy, and neuropathy) and macrovascular (especially stroke and coronary artery) diseases (Greenberg & Sacks, 2002). In cases of acute episodes such as diabetic ketoacidosis, hyperosmolar coma, and severe hypoglycemia, patients could suffer from the associated morbidities and mortality (Greenberg & Sacks, 2002), which would then lead to a socioeconomic burden.

By definition, diabetes is a metabolic disorder which comprises of multiple etiologies, characterized by chronic hyperglycemia (elevated glucose level) with disturbances in the carbohydrate, fat and protein metabolism as a result of the defects in insulin secretion, insulin action, or both (Alberti & Zimmet, 1998). Owing to the variations in diabetic symptoms, it can be very complicated to diagnose diabetes. Some diabetic patients can remain asymptomatic, whereas others would show evident symptoms. Here, the diagnostic tests for type 2 diabetes mellitus are divided into two distinct categories, the plasma-specific tests and the whole blood hemoglobin A1c (HbA1c) test. While plasma-specific tests have long been used as the diagnostic tests for type 2 diabetes, the HbA1c test has been the subject of multiple controversies, where the precision and

accuracy associated with different commercialized detection methods are often the topic for dispute (Erna Lenters-Westra & Slingerland, 2010). Nonetheless, the role of HbA1c as a distinctive biomarker for type 2 diabetes mellitus is undeniable. Therefore, it is very critical to understand the role of HbA1c from the biological standpoint before a further discussion on the detection methods.

2.2 Biological Challenges in Hemoglobin A1c Tests: HbA1c and the Variants

Biological interference of HbA1c assays includes the presence of the hemoglobin variants, morbidities that affect the red blood cells' turnover rate, and the genetic and dietary factors that influence the glycation rate of hemoglobin (for a detailed description please refer to Hare *et al.*, 2012 (Hare *et al.*, 2012)). A healthy individual hemoglobin pool can be subdivided into the following three categories: HbA (adult hemoglobin, 97%), HbA2 (2.5%) and HbF (fetal hemoglobin, 0.5%) (Erna Lenters-Westra *et al.*, 2013). Approximately 94%, a majority of HbA is not glycated, while 6% of the HbA can be categorized as glycated hemoglobin (Erna Lenters-Westra *et al.*, 2013). As the main glycated component (HbA1a and HbA1b together contribute approximately 1%), HbA1c contributes to approximately 5% of the total hemoglobin in a healthy individual (B. Chen *et al.*, 1998).

Glycation is a spontaneous and non-enzymatically process that occurs when glucose reacts with the amine groups in proteins to form the Amadori compound, a stable ketoamine (refer to Figure 2.1). Further oxidation and rearrangement of the amadori compound will lead to the formation of a more reactive species, the advanced glycation end-products (AGEs), which are presumed to play a role in causing the diabetic complications (Q. Zhang *et al.*, 2008). Of all the glycated proteins species, the hemoglobin A1c, HbA1c, was recognized as an indicator for glycemic control in

diabetic patients because of its stability in the Amadori form. HbA1c forms when glucose attaches specifically to the N-terminal valine of the β subunit of hemoglobin. However, only 60% of glucose is attached to valines at the N-terminal of the β-chain of hemoglobin. There are chances where glycation can occur on the lysine side chains either on the α or β chains (Goodall, 2005), (Lapolla *et al.*, 2013).

Hemoglobin variants exist as a result of congenital disorders of globin chain synthesis, called "hemoglobinopathies", which can have rather high prevalence in certain heritages (please refer to Figure 2.2 for the grouping of hemoglobin types). Variants can pose direct and indirect interference to the HbA1c tests (R. R. Little & Roberts, 2009). Some variants interfere by causing premature turnover of red blood cells; however, in cases where an individual is heterogeneous, the individual will most probably remain asymptomatic and have normal red cell survival. For example, homozygosity of HbS results in sickle cells anemia, which leads to premature turnover of red blood cells; however, for those who are heterozygous for the sickle cell allele, they are asymptomatic (Bain, 2011). Because variants can lead to over or underestimation of hemoglobin A1c level, therefore, screening for hemoglobinopathies is necessary before performing HbA1c definitive tests, especially for the populations of Mediterranean, African or Southeast Asian heritages, who are found to exhibit a higher prevalence of hemoglobinopathies (National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)).

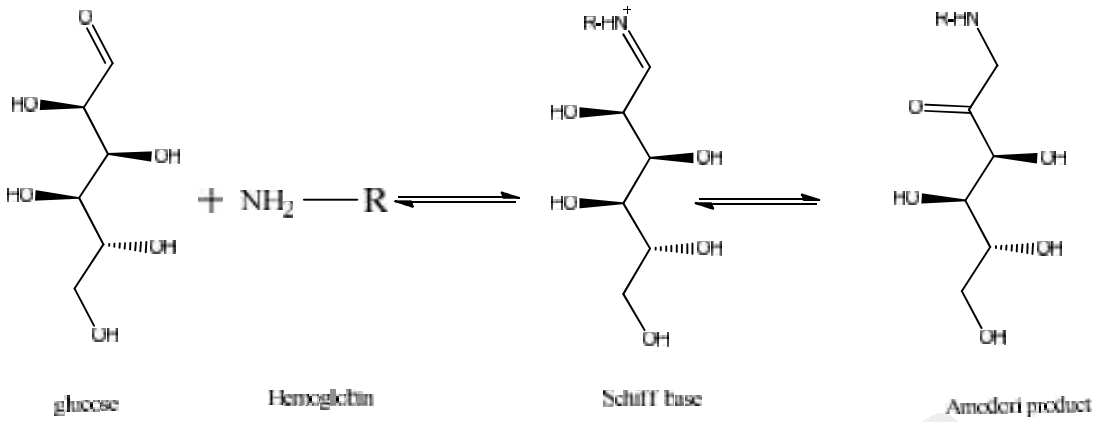

Figure 2.1: Formation of Hemoglobin A1c (Erna Lenters-Westra *et al.*, 2013).

In the lifespan of a red blood cell (120 days), the hemoglobin is constantly in contact with glucose in the blood stream. Glucose reacts non-enzymatically with the hemoglobin amine group, and when it specifically binds to valine of β chain of hemoglobin, it will undergo the intermediate stage (Schiff base) and later become a stable Amadori product, which is known as hemoglobin A1c (HbA1c). There is a spectrum of membrane proteins that undergo the same glycation reactions in the blood stream, most of them undergo further oxidation and rearrangement to become a more reactive species, advanced glycation end-products (AGEs) that are perceived to be responsible for the long term diabetes complications.

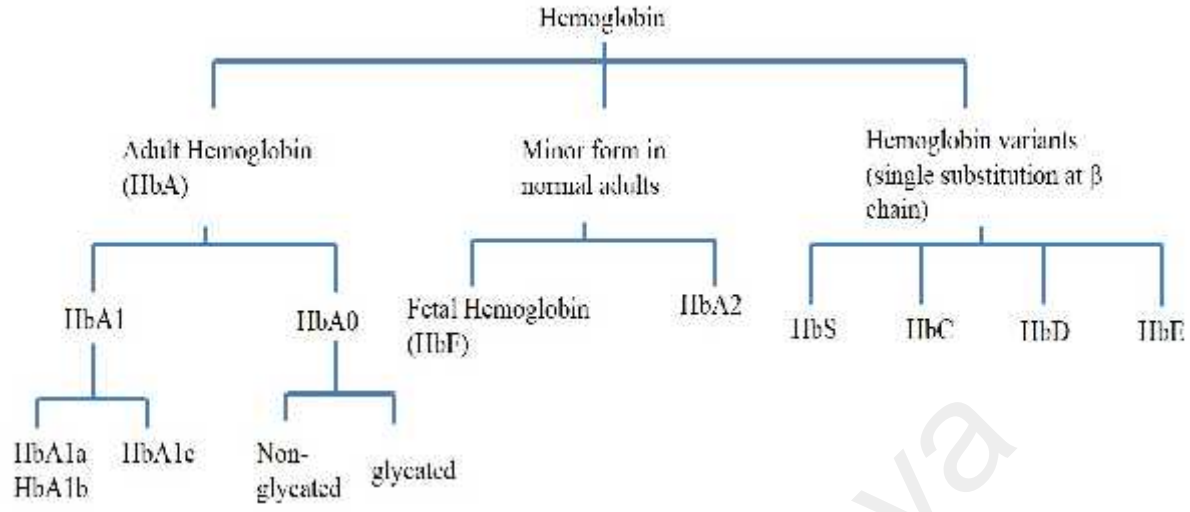

Figure 2.2: Hemoglobin and its Variants (Erna Lenters-Westra *et al.*, 2013).

Hemoglobin exists in mainly three forms (HbA, HbF, HbA2) in healthy adults. HbA contributes to around 97% of the whole pool, while the rest of hemoglobin can exist in HbF or HbA2 forms. HbA, HbF, and HbA2 are unique with the different subunits. HbA has two α and β subunits (ααββ), HbF is made up of two α and γ subunits (ααγγ), while HbA2 is comprises of two α and δ subunits (ααδδ) (Erna Lenters-Westra *et al.*, 2013). On the other hand, hemoglobin variants can exist at the expense of single amino acid substitution at the beta chain of HbA. The variants shown in Figure 2.2 are the commonly found worldwide; there are a variety of variants that do not affect red blood cells lifespan and patients remain asymptomatic (e.g. heterozygous for sickle cell allele). Glycation occurs mostly on HbA1 species and some on HbA_0 species; where the majority of the glycated HbA1 species is the HbA1c fraction (Erna Lenters-Westra *et al.*, 2013; R. R. Little & Roberts, 2009). Glycation can also occur on the variants as long as the glucose-binding moiety is not affected by the amino acid substitution.

2.3 Plasma-specific Tests versus HbA1c Test for Diagnosis

There are multiple tests that can be performed for the measurement of glucose levels. Diabetes diagnostic tests measuring glucose in clinical settings are very dependent on the clinical decisions, hence, the variation in tests or methods conducted is common across different health systems. Conventionally, plasma-specific tests include the fasting plasma glucose (FPG), 2-hour post glucose loading with oral tolerance test (OGTT) and random plasma glucose (RPG), in which glucose level in the plasma is measured (ADA, 2011).

The fasting plasma glucose (FPG) and the 2-hour plasma glucose by the oral glucose tolerance test (OGTT) are the most widely accepted tests for diagnosing type 2 diabetes mellitus (ADA, 2011). Not all patients exhibit symptoms of diabetes, thus, the use of a random plasma glucose test which is commonly conducted on patients that have apparent symptoms (for example, unexplained weight loss, increased thirst, and a high level of glycosuria) is not a practical test for diagnosing diabetes in all cases (ADA, 2011). While both FPG (diagnostic of diabetes at plasma glucose level ≥ 126 mg dL^{-1} or 7.0 mmol L^{-1} (ADA, 2011)) and OGTT (diagnostic of diabetes at plasma glucose level ≥ 200 mg dL^{-1} or 11.1 mmol L^{-1} (2011)) are common plasma-specific glucose diagnostic tests for type 2 diabetes, the low cost and the popularity of automated laboratory machines rendered FPG the preferred plasma-specific glucose test. Nevertheless, FPG is susceptible to biological variations, pre-analytical variations such as prolonged incubation time of the blood samples where glycolysis tend to occur, and analytical variations in which the serum instead of plasma glucose concentration is measured (Sacks, 2011). While analytical variations can occur when glucose measurement was performed in serum instead of plasma, studies have concluded that the glucose measurement in serum and plasma do not differ extensively (Sacks *et al.*, 2011). Anyhow, clinical organizations do not encourage the measurement of serum glucose in

place of plasma glucose to perform the diagnosis of diabetes, because the use of plasma allows samples to be spun down immediately to avoid any further glycolysis (Sacks *et al.*, 2011).

Despite its long established role as one of the diagnostic test for diabetes, when compared with FPG, OGTT is less favored as a plasma glucose test in clinical settings (Sacks *et al.*, 2011). The WHO discouraged the use of the OGTT to diagnose diabetes because of its inconvenience, high cost, and poor reproducibility (WHO, 2006). Owing to its principle of examining the efficiency of carbohydrates metabolism, patients who undergo the OGTT are required to go through intensive preparation prior to the test. Other than a 10- to 16- hour fast, the test has to be conducted in the early morning between 7 to 9 am, which can be very exhausting for both patients and nurses (Sacks, 2011). On the other hand, with the casual plasma glucose (random plasma glucose, RPG), individuals can be diagnosed as diabetic when the plasma glucose is ≥ 200 mg dL^{-1} (11.1 mmol L^{-1}) in the presence of apparent classical diabetes symptoms. Even though the random plasma glucose test can be conducted at any time point, it is very insensitive as a screening tool for diabetes (Johnson *et al.*, 2005). To maximize the test's sensitivity for detecting type 2 diabetes in a population, a 3-year epidemiological study suggested that the cutoff should be adjusted to RPG ≥ 130 mg dL^{-1} (7.2 mmol L^{-1}) to minimize false positives (Herman, 2007). Some may argue that casual plasma glucose is very convenient to be conducted at any time point and to avoid a long fast; however, the standardized HbA1c test that remains convenient while offsetting the sensitivity issues associated with RPG could offer a better alternative as a useful diagnostic test for type 2 diabetes.

Although FPG has been considered as the preferred plasma-specific T2DM diagnostic test, the high 14% of biological variability of FPG indicates that the test is neither perfectly stable nor free of laboratory variability (Sacks *et al.*, 2002). In addition, study

conducted found no evidence that FPG is superior to the HbA1c test when comparing both the FPG and HbA1c tests to the OGTT (Bennett *et al.*, 2007). Because there is no specific evidence to endorse the superiority of the FPG test over HbA1c test in diagnosing type 2 diabetes, the wide implementation of the HbA1c test as a diagnostic test for type 2 diabetes should be encouraged.

2.3.1 Current Trends in HbA1c Tests in Diagnosis of Type 2 Diabetes

The HbA1c test prevails as one of the useful approaches to diagnose diabetes because of the ease of testing without extensive patient preparation, low intra-individual biological variability (stable in the presence of sudden glycemic variations to better reflect the plasma glycemic status over the past 2-3 months), and greater reproducibility (Ma *et al.*, 2013). The shift of trend in adapting the HbA1c test into health systems as one of the diagnosis tests for type 2 diabetes has a huge impact on the public health sector, which has long attached to the conventional plasma-specific tests like FPG test.

In fact, the ADA (American Diabetes Association) endorsed HbA1c test as a diagnostic criteria for diabetes only by 2010 (ADA, 2010). To diagnose type 2 diabetes using HbA1c test, the ADA had selected a result of 6.5% as the diagnostic value, determined using the rate or risk of developing diabetic retinopathy, which was found to increase steeply at ≥ 6.5% HbA1c (ADA, 2010). In 2011, by setting a yardstick that all the measurements of HbA1c values have to be performed using standardized HbA1c methods that have passed the stringent quality assurance tests, the HbA1c test was finally endorsed by the WHO as one of the diabetes diagnostic tests, in 2011 (WHO, 2011).

Albeit its role as a diabetic biomarker has long been recognized, there are doubts and disputes on HbA1c's role as the diagnosis test for type 2 diabetes due to initial

difficulties in standardizing HbA1c methods. For example, the China Guideline in 2010 did not recommend the HbA1c test for the diagnosis of type 2 diabetes because of the inconclusive results in the Chinese population and the lack of a standardized HbA1c methods nationwide (Ma *et al.*, 2013). To identify the demographic and ethnic factors that may potentially contribute to complications in using HbA1c tests to diagnose type 2 diabetes, extensive longitudinal epidemiological studies have to be conducted. In fact, after the official recognition on the role of HbA1c in diagnosing diabetes by WHO, arguments associated to widely adapt HbA1c test in health systems continued; most of the concerns were on the fluctuations in % HbA1c due to genetic and biological variations, coexisting morbidities, potential assay interference and high implementation costs (Dagogo-Jack, 2011). Instead of debating over the plausibility of adapting HbA1c as a diagnostic test for type 2 diabetes, it is more practical to focus on resolving the problems and adjust the diagnostic cutoffs according to demographic, anthropometric, and laboratory measurements to maintain consistency of the results generated in HbA1c tests for better communication from lab bench to end users.

On the other hand, studies were conducted to mitigate the disputes by combining the results of the HbA1c test with the fasting plasma glucose test (FPG). In fact, an eight-year longitudinal prospective study of the Chinese population reported that, in clinical practice, the HbA1c test has better capability in predicting the incidence of diabetes as compared to the fasting plasma glucose test; hence, the study proposed the use of FPG, HbA1c and WC (waist circumference) in combination to diagnose diabetes accurately (Law *et al.*, 2011). Also encouraging the use of both HbA1c test and FPG test, a study conducted in Tokyo found that with the combination of FPG at 6.1- 6.9 mmol L^{-1} and 6.0- 6.4% HbA1c level, it gave a prediction of an absolute progression to type 2 diabetes over the course of five years (Heianza *et al.*, 2012); indicating that the

combination of plasma glucose test and HbA1c test can be used to improve the diagnosis efficiency for type 2 diabetes.

In short, HbA1c test plays a critical role in the diagnosis and screening of type 2 diabetes. Despite of the initial obstacles, intensive efforts invested to standardize HbA1c methods have once again fortified the role of HbA1c test as a diagnosis test for type 2 diabetes. Later, when all HbA1c methods are standardized against the IFCC (International Federation of Clinical Chemistry) reference methods, a wider implementation of HbA1c test in clinical setting as a diagnosis test for type 2 diabetes is to be expected.

2.3.2 The Path Towards Standardizing HbA1c Tests

When the Diabetes Control and Complications Trial (DCCT) (1993) and the UK Prospective Diabetes Study (UKPDS) (Stratton *et al.*, 2000) reported a good relationship between glycemic control and reduced outcome risks, the precise and accurate measurement of % HbA1c was highly sought after. While there are a variety of methods to perform HbA1c measurement, the lack of a primary reference method often lead to inconsistent interpretation of the % HbA1c, hence risking the judgments on the diabetic status of the patients; which will later lead to mis-prescription of therapy modules. In the absence of an international standard reference method, few countries had initiated national standardization programs to set a reference method to standardize the HbA1c methods available in the market. For example, Mono-S ion-exchange chromatography was the standard reference method used for standardizing the HbA1c tests available in Sweden, while the Japan Diabetes Society (JDS) employed two calibrators with JDS-assigned values (Randie R. Little & Rohlfing, 2009) to standardize the HbA1c methods. In an attempt to standardize HbA1c methods, the NGSP (National

Glycohemoglobin Standardization Program) as the subcommittee of the AACC (American Association for Clinical Chemistry) was established in 1996, where it held responsibility in managing a network of reference laboratories that were calibrated against the DCCT reference values (Randie R. Little & Rohlfing, 2009). On the other hand, Secondary Reference Laboratories (SRLs) under the NGSP work specifically with manufacturers, aiming to standardize the HbA1c kits or methods and compare the data collected directly with the DCCT results for method certification (NGSP, 2010c). Unlike NGSP, the IFCC (International Federation of Clinical Chemistry and Laboratory Medicine) Working Group was formed to perfect reference methods for higher-level calibration systems. In 2001, the IFCC has established a higher-order reference method (please refer to section 2.4 for a discussion of the IFCC reference method) for HbA1c test. Although both the IFCC and NGSP managed distinctive laboratory networks that were established to serve different purposes in standardization of HbA1c tests, continuous monitoring within and between IFCC and NGSP laboratory networks has resulted in the harmonization of HbA1c results worldwide (Randie R.Little, 2011). To oversee the effectiveness of the NGSP in harmonizing the HbA1c results for more effective conveyance of HbA1c results from lab benches to the patients, College of American Pathologists (CAP) was responsible to evaluate the NGSP networks twice per annum (2010d). Under constant assessment and stringent control of the certification criteria, the CVs (coefficient variants) within and between laboratories have decreased, justifying the routine use of HbA1c tests in the clinical setting for diabetes management and diagnosis (Randie R.Little, 2011).

Nonetheless, the absolute numbers reported are different between the IFCC and NGSP because of different reference methods utilized to standardize the HbA1c tests (the NGSP network reports in % HbA1c, and the IFCC network reports in mmol HbA1c/mol Hb). Typically, the IFCC reference system's measurements are 1.5-2.0% units lower

than the NGSP values (2010d), conceivably due to the higher specificity of IFCC reference methods. Although they are more accurate, IFCC methods are not meant for routine clinical usage because they consume more time and are comparatively higher in cost. Therefore, the NGSP values are considered a better approach for assessment in diabetes diagnosis and management (due to the traceability to UKPDS and DCCT), and most clinical settings report HbA1c values in NGSP units (in % HbA1c). To allow better communication between systems, a master equation was established (Eq. 2.1) to relate the IFCC results to the NGSP results. On the other hand, Japan and Sweden that owned the independent national standardization programs have also established master equations with different conversion numbers (please refer to Equations 2.2 and 2.3) (Randie R. Little & Rohlfing, 2009).

$$NGSP\ (\%) = 0.09148 \times IFCC\ \left(\frac{mmol\ HbA1c}{mmol\ Hb}\right) + 2.512 \qquad \text{(Eq. 2.1)}$$

$$JDS\ (Japan\ Diabetes\ Society, in\ \%) = 0.0927 \times IFCC + 1.73 \qquad \text{(Eq. 2.2)}$$

$$MonoS\ (Sweden, in\ \%) = 0.0989 \times IFCC + 0.88 \qquad \text{(Eq. 2.3)}$$

To encourage better patient compliance in routine self-monitoring for better diabetes management, the A1c-Derived Average Glucose (ADAG) Study Group found a more user-friendly way to deliver the result from A1C assays to patients (Nathan *et al.*, 2008). By establishing a linear relationship between HbA1c and average glucose (AG) levels, the ADAG proposed to report HbA1c as eAG (estimated average glucose, in mg dL^{-1} or mmol L^{-1}) (Nathan *et al.*, 2008), and the conversion equation between eAG and HbA1c % is shown below (2010d).

$$eAG\ \left(\frac{mg}{dL}\right) = (28.7 \times HbA1c\ \%) - 46.7 \qquad \text{(Eq. 4)}$$

With the reference method developed by the IFCC, it was a global consensus that the calibration in clinical laboratories has to be performed with the IFCC reference method

(Hanas *et al.*, 2013). To facilitate communication of HbA1c values from lab bench to the public in different institutes and systems, three standards were set for the presentation of HbA1c results to clinicians, patients, and scientific journals. First, the HbA1c SI unit should be expressed in IFCC unit (mmol mol^{-1}, with no decimals), and both the IFCC-and NGSP-derived units (%, with one decimal) are to be reported in clinical laboratories across the globe (Hanas *et al.*, 2013). Second, to ease the delivery of the HbA1c results to patients, the HbA1c conversion table with the IFCC and NGSP units is to be easily accessible to the diabetes community. Third, journals are recommended to require the submitted manuscripts to report HbA1c in both SI (IFCC) and NGSP units (Hanas *et al.*, 2013). As a result of global standardization of HbA1c test, countries such as Sweden and Japan, which are famous for their independent HbA1c reference methods, have switched and adapted these standards. For example, Sweden, which used to employ Mono S as the national reference method, agreed to report HbA1c results using the IFCC units (mmol mol^{-1}) starting in January 2011 (Mona Landin-Olsson, 2010). Instead of continuing with the national JDS values, the Japan Diabetes Society had agreed to switch and start to report HbA1c results using NGSP unit (%) in 2013 (Kashiwagi, 2013).

Overall, the effort to standardize HbA1c tests has led to a global consensus to convey HbA1c results for more effective diabetes care. Still, the choice of reporting in IFCC, NGSP, or eAG units varies by country; however, with the recognition of a common language for HbA1c results, the implementation of HbA1c tests worldwide is not too far away.

2.4 Limitations of HbA1c Tests in Laboratory Settings

In 2001, the IFCC working group first described a higher-order reference method which was accepted worldwide as the valid reference method later (Jeppsson *et al.,* 2002). The IFCC reference method includes the following three phases: (1) hemoglobin cleavage with endoproteinase, (2) separation of the β-chain glycated and non-glycated N-terminal hexapeptides, and (3) quantification with either the combination of HPLC and electrospray ionization mass spectrometry or the combination of HPLC and capillary electrophoresis with UV (for a detailed description, please refer to the IFCC publication) (Jeppsson *et al.,* 2002). HbA1c methods can be classified into two main groups: detection based on charge differences (cation exchange matrices and capillary electrophoresis) and detection based on structural differences (affinity chromatography and immunoassays). The HbA1c methods commonly employed in the laboratory settings often involve long turnaround time in order to generate the results.

The process of glycation is an irreversible reaction where the glucose adduct formed at the N-terminus of the β chain contributes to additional negative charges, and the extra negativity enables HbA1c to flow through negatively-charged cation-exchange matrices (Goodall, 2005). Then, spectrophotometric analysis of the eluents will give results in percentage for each hemoglobin species detected in the samples (Curt L. Rohlfing *et al.*, 2002). In general, cation-exchange chromatography is a method of detecting % HbA1c with high precision under rapid hemoglobin separation (Randie R.Little, 2011). Besides, it was found that with a buffer system at pH 6.2 or below, cation-exchange assays are sufficiently sensitive to quantify αβ dimers, which could be glycated on both β chains (Finke *et al.*, 1998). While some cation-exchange HPLC methods are sensitive enough to identify the presence of hemoglobin variants, they lack sufficient resolution to recognize specific hemoglobin variants. In cases of which the method fails to resolve the peaks, different HPLC methods have distinctive responses. For example, the Bio-

Rad Variant II thalassemia method is able to recognize the additional peaks as specific HbA1c variants (Lahousen *et al.*, 2002), whereas other HPLC methods, such as HA-8140 HPLC, would describe the additional peaks in a chromatogram as "variant Hb" (John *et al.*, 1997).

In general, most cation-exchange HPLC methods are able to resolve the most common HbA1c variants now and they are amongst the most widely employed methods in clinical settings (R. R. Little & Roberts, 2009). Nonetheless, they are still being affected by the high prevalence of hemoglobinopathies, particularly in certain heritages. By then, other detection methods such as affinity separation and immunoassays can be the alternatives to cation-exchange HPLC method (R. R. Little & Roberts, 2009). Affinity chromatography that utilizes the reaction between boronic acid and the cis-diol groups of glucose (bound to hemoglobin), measures the total glycated hemoglobin (R. R. Little & Roberts, 2009). Therefore, HbA1c measurement using affinity chromatography is less affected by the presence of variants (R. R. Little & Roberts, 2009). However, affinity chromatography assays do not distinguish amongst the glycated hemoglobin species; thus, they tend to overestimate the HbA1c level when the total glycated hemoglobin value is used in place of the value of HbA1c (Miedema, 2004). As a result, affinity separation methods commonly yield 40-50% higher HbA1c values in relative to that of the HbA1c values measured with cation-exchange assays (Goodall, 2005). In order to harmonize HbA1c results and to avoid confusion in communication, using the guidelines established by NACB (National Academy of Clinical Biochemistry), all affinity assays have been calibrated to report the HbA1c equivalents (Sacks *et al.*, 2011). In addition, the seemingly non-specific boronate affinity method can be advantageous in cases where hemoglobin variants are present. In fact, it is especially useful as a comparative method when cation- exchange HPLC (the DCCT reference method is Bio-Rex 70 resin cation-exchange HPLC, NGSP employs the same reference method

(2010d)) gives bizarre chromatograms ("abnormal separation", additional peaks, too low HbA1c concentrations or those above the nondiabetic range) (Thevarajah *et al.*, 2009).

Immunoassays, especially the immunoturbidity assays, involve an agglutinator and antibodies-coated latex particles (Curt L. Rohlfing *et al.*, 2008). HbA1c will react with the antibodies to inhibit agglutination, hence lowering the turbidity of the sample. The drawback of these types of immunoassays is that they are sensitive to HbF interference (Curt L. Rohlfing *et al.*, 2008). A high concentration of HbF (due to hereditary persistence (National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)) has been reported to cause more than 20% underestimation (a clinically significant value) of the HbA1c values when the measurement was performed using a DCA 2000 analyzer, one of the immunoturbidity assays (Curt L. Rohlfing *et al.*, 2008). While HbA tends to be glycated at the β chain terminal valine, HbF that comprises of γ chain in place of β chain (with glycine instead of valine at its terminus) is more prone to acetylation rather than glycation; which in return leads to the underestimation of glycated hemoglobin (Curt L. Rohlfing *et al.*, 2008). Note: please refer to the table on the NGSP website, for a more comprehensive discussion on the interference of variants in particular methods (NGSP, 2013).

Other than the constant effort to standardize well-established lab methods for HbA1c measurement, attempts to achieve more effective measurements of HbA1c in lab settings go on. For example, a drop-coating deposition Raman (DCDR)- spectroscopy was reported with a detection limit approximately 15 folds lower than the lowest physiological HbA1c concentrations associated with clinical settings, providing an alternative for highly sensitive measurement of HbA1c in laboratory (Barman *et al.*, 2012). Additionally, to enhance the accuracy of HbA1c detection, novel systems such as

a microfluidic magnetic bead-based immunoassay with the incorporation of nanotechnologies have been reported (S.-P. Chen *et al.*, 2011).

In short, end-users should be aware of the potential interference posed by HbA1c variants, especially if the assays utilized can under- or overestimate HbA1c values in a clinically significant way (Erna Lenters-Westra & Slingerland, 2010). Other than understanding the potential interference to be able to provide a particular population with the right choice of assays, it is as important to monitor the analytical performance of the assay constantly for quality control. To achieve the goals of intra-laboratory (CV < 2%) and inter-laboratory (CV < 3.5%), the essence of monitoring and quality control is to run tests on at least two control materials with different mean values at the beginning and at the end of the day's run (Sacks *et al.*, 2011). Besides maintaining the standardization efforts for the NGSP-certified methods, the effort to develop different principles-based detection methods should be encouraged.

2.4.1 Challenges of Point-of-Care (POC) Technologies as Diagnostic Tools

Laboratory detection methods, such as cation-exchange HPLC and capillary electrophoresis, are not very useful when it comes to performing HbA1c detection *in situ* (please refer to Table 2.1 for a list of available POC analyzers). The bulky machines, laborious preparations and long turnaround time are not a good match for point-of-care technologies, which are more convenient because of their high mobility and short turnaround time. Undeniably, with good mobility, POC devices are attractive for nursing practitioners because % HbA1c can be obtained *in situ* and thus allow better on-site medical facilitation and patient care. However, the adaption of POC analyzers in clinical settings is fairly controversial due to the continuous doubt about their accuracy (Erna Lenters-Westra & Slingerland, 2010).

While there are a variety of commercially available POC devices, only few devices are calibrated precise enough to acquire NGSP certification. For example, the DCA Vantage (Siemens Medical Solutions Diagnostics, New York) and Afinion (Axis-Shield, Norway) are the only devices that are stable enough to meet the NGSP criteria with imprecision of <3% CV within two different reagent lots; an indication that their analytical performance is equivalent to that of laboratory-based methods (Erna Lenters-Westra & Slingerland, 2010). In general, central laboratory methods are prone to positive bias, whereas results generated using POC devices tend to be negatively biased (Holmes *et al.*, 2008). Therefore, the relative biases gap of POC and CL (central lab) methods is large and would potentially be unacceptable in cases where different principle-based methods are used interchangeably (Holmes *et al.*, 2008). While utilizing different methods can result in complications when it comes to data analysis, but the 18% false negatives obtained by HbA1c POC devices is a pressing concern, because it can lead to high incidence of missed diagnosis of diabetes (Schwartz *et al.*, 2009).

In fact, POC devices are susceptible to reagent variations across different batches of production (E. Lenters-Westra & Slingerland, 2009). Therefore, POC devices are often perceived to not be on par with standard laboratory tests when it comes to HbA1c measurements in clinical settings. Owing to the unstable analytical performance of some POC analyzers, clinical practices are advised to always compare the HbA1c results by POC devices to that of the laboratory methods before adapting POC methods in routine practice (Schwartz *et al.*, 2009). Although the 18% false negative results obtained using POC devices is a concern, the potential advantages entailed with the incorporation of the POC devices in clinical practice should not be overlooked. Hence, attention should be given to the ongoing effort to improve the accuracy and to minimize the variability between batches of commercialized POC devices. However, owing to the waived status of the assessment of POC methods by the Clinical Laboratory Improvement

Amendments (CLIA), there is concern that the quality of POC methods may be compromised. Clinical Laboratory Improvement Amendments was established in 1988 to assure the accuracy and reliability of a method regardless of the places where the test is performed (Bode *et al.*, 2007). Under the law, waived tests, by definition, are laboratory examinations cleared by the Food and Drug Administration (FDA) and safe for household use (Bode *et al.*, 2007). Once the CLIA-waived status is obtained, POC devices are not required to undergo further assessment under other schemes, such as CAP surveys (refer to section 2.3.2 for detailed discussion). When the analytical performance consistency of POC devices is not assessed from time to time, it would render the personal diabetes care in the public to be less effective. As of today, the ADA (American Diabetes Association) does not encourage the use of POC analyzers for the diagnosis of diabetes (NGSP, 2015).

With advances of technology, the analytical performance of POC devices can be enhanced; also, by revising the CLIA-waived status to create the obligation to participate in external quality control schemes, POC devices have the capability to become reliable diagnosis tools in the near future. Nonetheless, because clinicians often rely on % HbA1c to decide therapy modality, the negative bias of POC analyzers could potentially affect clinical decisions. Therefore, pharmacists and medical practitioners should be cautious to rely solely on POCs for deciding the treatment modality for patients. While most CLIA-waived POC devices reflect a satisfactory correlation to laboratory methods when it comes to HbA1c measurement, it is often advisable to perform a POC test and a laboratory test simultaneously to avoid misjudgments in practice. It probably requires some time before POC devices are ready for diabetic diagnosis purposes; however, POC devices are reliable for diabetic care and management purposes (Sánchez-Mora *et al.*, 2011), apart from being more cost-effective (Carter, 2008), POC devices are also perceived to yield a higher patient-

satisfaction level because of the decentralized system for rapid and same-visit results generation (Sanchez-Mora *et al.*, 2011).

Table 2.1: Point-Of-Care A1c Analyzers[1].

Point-of-Care instruments for A1c tests	Manufacturer	Types of methods/ Assay time (mins)	Detection Range and Coefficient Variants (CV)	References
In2it*	Bio-rad, California	Affinity separation/ 10 min	HbA1c (10 µL whole blood): 4% to 14% (20 mmol mol^{-1} to 130 mmol mol^{-1}), CV 2.4-3.9%	(Erna Lenters-Westra & Slingerland, 2010)
DCA Vantage *	Siemens Medical Solutions Diagnostics, New York	Immunoassays/ 6min	HbA1c (1 µL whole blood): 2.5% to 14 % (4 mmol mol^{-1} to 130 mmol mol^{-1}) (Siemens), CV <3%	(Erna Lenters-Westra & Slingerland, 2010)
Afinion*	Alere, California	Affinity separation/ 3 min	HbA1c (1.5 µL whole blood): 4% to 15% (20 mmol mol^{-1} to 140 mmol mol^{-1}), CV <3%	(Alere™, 2015a)
Nyocard	Alere, California	Affinity separation/ 3 min	HbA1c (5 µL whole blood): 4% to 15% (20 mmol mol^{-1} to 140 mmol mol^{-1}),CV <5%	(Alere™)
GDX/Micromat II*	Bio-Rad, California	Affinity separation/ 5min	HbA1c (10 µL whole blood) : 4% to 15% (20 mmol mol^{-1} to 140 mmol mol^{-1}), CV 2.93-4.65%	(Erna Lenters-Westra & Slingerland, 2010)
Clover	Infopia, Korea	Affinity separation/ 5 min	HbA1c (4 µL whole blood) : 4% to 14% (20 mmol mol^{-1} to 130 mmol mol^{-1}), CV <3%	(Ltd)
InnovaStar	DiaSys, Germany	Agglutination/ 7 min	HbA1c: 4% to 15% (20 mmol mol^{-1} to 140 mmol mol^{-1}), CV <3%	(DiaSys, 2015)
A1CNow+*	Metrika, Bayer, California	Immunoassay/ 5 min	Accurate between 7% to 8.5% (53 mmol mol^{-1} to 69 mmol mol^{-1})	(Carter, 2008)
Quo-Test	Quotient Diagnostics, UK	Affinity separation/ 4 min	HbA1c: 4% to 15% (20 mmol mol^{-1} to 140 mmol mol^{-1}), CV <3%	(EKFDiagnostics, 2015)

[1] Point-of-Care (POC) play a very significant role in aiding the patience compliance of self-monitoring and also allow the practitioners to perform routine checking in situ on patients who are not convenient in moving around for blood testing. POC analyzers which are in par with laboratory machineries in HbA1c testing provide a more practical way for better clinical monitoring in order to perform better personalized treatments (* CLIA-waived POC technologies).

2.4.2 Novel Inventions for HbA1c Tests: The Biosensors

The development of HbA1c biosensors typically involves a layer of biological recognition elements that give high specificity and sensitivity towards HbA1c. Because the designs of HbA1c biosensors usually involve miniaturized set-ups, it is fair to anticipate a revolutionary development of more accurate and cost-effective HbA1c biosensors to be transformed into useful POC devices in clinical settings.

HbA1c biosensors can be grouped based on the detection mode, whether the detection of HbA1c is performed direct or indirectly. For those designed to detect HbA1c directly, they can be further categorized based on detection principles, such as amperometric, potentiometric, piezoelectric biosensors, and biochips (Pundir & Chawla, 2014). Electrochemical biosensors that detect HbA1c directly often involve anti-HbA1c antibodies as the biological recognition element, so they are also known as immunosensors. For example, by combining the principle of piezoelectricity and electrochemistry, a HbA1c immunosensor was developed using antibodies labeled *in situ* with ferroceneboronic acid (redox label) for the electrochemical signal amplification (Halámek, Wollenberger, Stöcklein, Warsinke, *et al.*, 2007). By utilizing nanotechnology, Xue *et al.* (2010) reported a miniaturized mixed SAM (Self Assembled Monolayer)-wrapped nanospheres potentiometric HbA1c immunosensor. The prototype was then tested for its consistency in a clinical setting to determine the practical usage of HbA1c biosensors as a POC device (Xue *et al.*, 2011). Because of the constant urge to improve the sensitivity of HbA1c biosensors, a sandwich immunoassay on microarrays was proposed to enhance the sensitivity of the HbA1c detection down to the nanomolar level (H.-H. Chen *et al.*, 2012). Expensive instrumentation, such as SPR (surface plasmon resonance) spectroscopy, has also been employed to develop highly sensitive HbA1c biosensors for the robust detection of HbA1c (J.-T. Liu *et al.*, 2008). While conducting detection with high sensitivity is important, method like antibody

microarrays was reported to involve 2 hours incubation time before the test can be performed (H.-H. Chen *et al.*, 2012); thus, it would also be wise to consider the turnaround time involved so that the rapidity of the HbA1c measurement would not be compromised.

Boronic acid has long been used in the affinity chromatography method for HbA1c detection (R. R. Little & Roberts, 2009). As an excellent biomimetic molecule, it is also famous as the biological recognition element used in HbA1c biosensors to selectively bind to the sugar moiety of HbA1c. For example, by manipulating a boronic acid-modified electrode in combination with a GO*x* (glucose oxidase) backfilling assay, Song *et al.*(2009) was able to quantitatively measure HbA1c level (Song & Yoon, 2009). Also utilizing the biomimetic nature of boronic acid, Hsieh *et al.*(2013) reported an affinity biosensor to measure HbA1c level based on impedance (Hsieh *et al.*, 2013). With the low-cost design that only requires low-volume sample (20 μL), the HbA1c biosensor was claimed to be feasible as a sensitive point-of-care device (Hsieh *et al.*, 2013). However, because boronic acid binds to the cis-diol of any glycan moiety (HbA1c is only one of the glycan moieties), studies attempting to develop HbA1c biosensors using boronic acid as the biological recognition element should be careful to not compromise specificity by using the boronic acid in place of antibodies.

Fructosyl valine (FV), product generated by the decomposition of glycated hexapeptides as a result of HbA1c proteolysis, has been highly exploited in the development of HbA1c biosensors (Pundir & Chawla, 2014). Using the enzyme fructosyl amine oxidase (FAO) as the biological recognition element, FV can be further catalyzed to yield hydrogen peroxide for electrochemical detection (Pundir & Chawla, 2014). Owing to the different organisms where FAO can be extracted; either from marine yeast or bacteria such as *Escherichia coli* and *Arthrobacter* sp., the altered enzymatic activity of FAO can often influence the sensitivity and reproducibility of the FV biosensors,

besides the operational potential and temperature (L. Fang *et al.*, 2009). To overcome the weaknesses of FV biosensors, Fang *et al*. designed a disposable iridium-modified FV biosensor to detect HbA1c (L. Fang *et al.*, 2009). By absorbing nanotechnology in the fabrication of the FV biosensor, Chawla and Pundir (2012) had successfully developed an amperometric biosensor for HbA1c with improved operational efficiency, higher stability and enhanced sensitivity (Chawla & Pundir, 2012). Note: please refer to Table 2.2 for examples of different types of HbA1c biosensors and their reported analytical performance.

In short, the effort invested to develop HbA1 biosensors gives a positive impact in stimulating more innovations for point-of-care analyzers with improved analytical performance in the future. While the stability of biomolecules as the recognition element has always been a challenge, with the advent of nanotechnology, more effective and stable biosensors with consistent analytical performance on par with laboratory HbA1c methods are to be anticipated

Table 2.2: HbA1c Biosensors[2]

Compound Detected	Characteristics	Types of Biosensors	Detection range/ limits	Sensitivity	Limitations	References
Fructosyl valine (FV)	-Detect: End product from decomposition of glycated hexapeptides - Biological recognition element: Enzyme fructosyl amine oxidase (FAO)	Electrochemical (amperometric) with magnetic nanoparticles	-Detection range: 0 to 2 mM FV -Detection limit: 0.1 mM for FV	Not Available	-Lack of reproducibility and stability	(Chawla *et al.*, 2011)
		Electrochemical (amperometric) with zinc oxide nanoparticles-polypyrrole film	-Detection range: 0.1 to 3 mM FV -Detection limit: 50 μM FV	38.42 μA mM^{-1}		(Chawla *et al*, 2012)
		Disposable Iridium-modified electrochemical biosensor (amperometric)	-Detection range: 0 to 2 mM of FV - Detection limit: Not Available	21.5 μA mM^{-1} cm^{-2}		(Fang *et al.*, 2008)
	-Detect: Fc-IgG-HbA1c immunocomplexes -Biological Recognition element: anti-HbA1c antibody	On-chip electrochemical flow immunoassay	-Detection range: up to 500 μg mL^{-1} -Detection limit: Not Available	Not Available	-Laborious pretreatment	(Tanaka *et al.*, 2007)

[2] Table shows examples of fabricated HbA1c biosensors. HbA1c biosensors can be grouped under two main groups: the Fructosyl valine (FV) and HbA1c biosensors. The FV biosensors measure the end product from the decomposition of glycated hexapeptides (after proteolysis of HbA1c), while the HbA1c biosensors measure concentrations of HbA1c directly. The HbA1c biosensors typically involve immunoglobulins as the biological recognition elements, and some of them utilize the biomimetic ability of boronic acid to selectively measure HbA1c.

Table 2.2, Continued**.**

Compound Detected	Characteristics	Types of Biosensors	Detection range/ limits	Sensitivity	Limitations	References
Hemoglobin A1c	- Detect: HbA1c - Biological recognition element: Anti-HbA1c antibody	Sandwich Immunoassays on Polydimethylsiloxane-based antibody microarrays	- Detection range: 10-100 ng mL^{-1} - Detection limit: 3.58 ng mL^{-1}	4-5 orders of magnitude higher	- Long incubation time (2 hours)	(Chen *et al.*, 2012)
	Boronic acid-based HbA1c Biosensors					
	- Detect: HbA1c - Biological Recognition element: Ferroceneboronic acid	Electrochemical (piezosensor) Immunoassay	- Detection range: 0 to 20% - Detection limit: >5% (Standard Deviation 20%)	Not available	- Low sensitivity - Low specificity	(Halamek *et al.*, 2007)
	- Detect: HbA1c - Biological Recognition element: Ferroceneboronic-acid labeled anti-HbA1c antibody	Electrochemical (piezosensor) Immunoassay	- Detection range: 4 to 13% - Detection limit: Not Available	Sensitivity was increased at three fold compared to without antibodies	- Low Sensitivity	(Halamek *et al.*, 2007)
	- Detect: Wide range of glycoproteins, for example HbA1c - Biological recognition element: Boronic-acid	Disposable Biochip	Not available	Not Available	- Laborious pretreatment on sample matrix. - High sample volume was required (50 μL)	(Son *et al.*, 2006)
	- Detect: Wide range of glycoproteins, for example HbA1c - Biological recognition element: Boronic-acid - Coupled with GOx (glucose oxidase) backfilling assay	Electrochemical	- Detection range: 2.5 to 15% HbA1c - Detection limit: Not available	Not available	- Lack of specificity to HbA1c	(Song *et al.*, 2009)

Table 2.2, Continued**.**

Compound Detected	**Characteristics**	**Types of Biosensors**	**Detection range/ limits**	**Sensitivity**	**Limitations**	**References**
Hemoglobin A1c	- Detect: Glycoproteins - Biological recognition element: self-assembled monolayer (SAM) thiophene-3-boronic acid	Affinity Biosensors with Impedance measurement	- Detection range: 1 to 100 ng μL^{-1} - Detection limit: 1 ng μL^{-1} of HbA1c	Not available	- Lack of stability	(Hsieh *et al.*, 2013)
	- Detect: HbA1c - Biological recognition element: Phenylboronic acid	SPR (surface plasmon resonance) biosensor	- Detection range: 0.43 to 3.49 $\mu g\ mL^{-1}$ - Detection limit: 0.01 $\mu g\ mL^{-1}$	Not available	- Expensive instrumentation	(Liu *et al.*, 2008)

2.5 HbA1c Tests in the Determination of Prediabetes and Its Role as a Screening Tool

Before one becomes hyperglycemic, one can be asymptomatic and remain dysglycemic for a period of time that could persist for years (ADA, 2011). Dysglycemia is a state where the blood glucose level is higher than the normal level but does not reach the diabetic cutoff. This period of time is when the prediabetic stage appears and becomes part of the continuum of dysglycemia. Prediabetes can be known as IFG (impared fasting glucose with FPG at 100-125 mg dL^{-1} or 5.6-6.9 mmol L^{-1}) or IGT (impaired glucose tolerance with 2- hour oral glucose test at 140-199 mg dL^{-1} or 7.8-11.0 mmol L^{-1}). With an A1c of 5.7 to 6.4% (ADA, American Diabetes Association prediabetes diagnostic range) (ADA, 2011), one is considered prediabetic. Because type 2 diabetes is a progressive disease, when patients remain asymptomatic for certain duration, it could result in delayed intervention and therapy; which in return can lead to morbidities and premature death. In fact, studies have reflected that prediabetic patients are associated with a 20% increase in cardiovascular diseases, other than higher prevalence of neuropathy, chronic kidney disease and microvascular complications (Echouffo-Tcheugui *et al.*, 2011). With the Diabetes Prevention Project revealed that only 4.8% of individuals are properly diagnosed as prediabetic, and the majority of patients remain undiagnosed (Karve & Hayward, 2010), it is an alarming situation especially when this particular population have increased risk of being afflicted with other morbidities. Therefore, an effective screening tool to recognize such high-risk populations is highly sought after.

Depending on demography, ethnicity, age, and the detection methods chosen in the health system, HbA1c cutoff sensitivity can be affected. Hence, it is not surprising that different countries may have variable values set for the detection of prediabetes in the populations. For example, instead of utilizing the ADA cutoff (5.7 to 6.4% HbA1c), the

CDA (Canadian Diabetes Association) set 6.0 to 6.4% of HbA1c (42-46 mmol mol^{-1}) to define prediabetes (Ekoé *et al.*, 2013). To set the prediabetic ranges with the % HbA1c, Suzuki *et al.* performed a ROC (Receiver Operating Characteristic) curve analysis to demonstrate that A1C ≥ 5.7% has better screening power for prediabetes as compared to A1C ≥ 6.0% in Japanese patients (Hiroshi Suzuki, 2011).

However, compared to its counterparts of plasma-specific tests, HbA1c appears to have lower screening power for prediabetes. With the ADA recommended HbA1c values for prediabetes, it was found that the HbA1c test is less sensitive to detect the prediabetic patients as compared to the FPG and OGTT tests in non-Hispanic whites society (Lorenzo *et al.*, 2010). In support of Lorenzo *et al.'s* findings, Zhou *et al.* (2010) also concluded that FPG has better screening power as compared to the HbA1c test based on studies in Qingdao, China (Zhou *et al.*, 2010). In contrast to the findings by Zhou *et al.* and Lorenzo *et al.*, a Bulgarian population-based study reported a significant positive correlation between HbA1c and both the fasting plasma glucose test and OGTT by imposing the HbA1c cutoff at ≥ 5.5% (Tankova *et al.*, 2012), demonstrating that the HbA1c test can be an equally effective screening tool. Also, in an attempt to evaluate the screening power of HbA1c, a review of 63 studies had reached a conclusion that the HbA1c test, despite of contradicting findings in different heritages and populations, is as effective as FPG as a screening tool (Bennett *et al.*, 2007). More recently, the HbA1c test was perceived to be at least equally good, often better, as a screening tool when compared with FPG, in clinical practice (Mannarino *et al.*, 2013). Although the evidence is lacking, HbA1c was recently reported to have similar screening power as OGTT for prediabetes incidence (Mannarino *et al.*, 2013). To improve the screening power for more efficient recognition of individuals who are most likely to develop diabetes, Heianza *et al.* (2012) suggested the use of both the HbA1c and FPG tests to screen for prediabetes incidence (Heianza *et al.*, 2012).

In summary, the HbA1c test can be an effective screening tool for prediabetes incidence provided that the cutoffs are adjusted based on ethnicity, demography, gender, and age. To accurately detect the prediabetic individuals, additional plasma glucose tests (namely the FPG and OGTT tests) are highly recommended to be conducted hand in hand with HbA1c test (Fajans *et al.*, 2011). With increased efficiency in detecting prediabetes at early stage, individuals can receive early intervention, hence stalling the progression to type 2 diabetes. Although the idea of implementing HbA1c as the sole screening tool remains premature, under the constant surveying and standardization of HbA1c methods with adjusted cutoffs, the HbA1c test has high potential to become a reliable independent screening tool for the prediabetes and undiagnosed diabetes.

2.6 HbA1c Tests in Long Term Management of Diabetes

Because of the impacts of T2DM on society, it is critical to implement the most suitable treatments to postpone the progression of diabetes. There are a variety of treatments available to cater to different groups of prediabetic or diabetic patients. Treatment modalities like monotherapy with hypoglycemic drugs, combinations of drugs, insulin therapy and lifestyle changes, each of which can be initiated and prescribed to patients based on the glycemic status and patient age, pre-existing medical complications, and/or diabetic symptoms. To initiate treatment, clinicians often refer to the glycemic status reflected in the HbA1c tests.

To balance up the side effects and effectiveness of delaying the progress of the disease, therapy modality prescribed for each T2DM patient has to be personalized. For example, administration of metformin is as effective as practicing life-style modification in individuals aged 24 to 44 years old or in those with BMI $\geq$ 35 kg m^{-2}; however, these categories of diabetic patients only involve a small group of diabetic population

("Prevention or Delay of Type 2 Diabetes," 2004). In reality, because of the fear of hypoglycemic complications or uneasiness associated with initiating a new therapy, clinicians may be skeptical to treat patients aggressively (Cole *et al.*, 2009). In fact, the rule of "ABC" (A1C, Blood Pressure, Cholesterol) outlines the treatment goals for A1c (< 7%, depending on the modification based on individuals' adverse effects during glycemic management), blood pressure (≤ 129/79 mm Hg), and cholesterol (LDL <100 mg dL^{-1} in patients with ≥ 40 years of vascular disease) (Cole *et al.*, 2009).

Although the role of the HbA1c test in the diabetic management and treatment has long been established, different treatment goals with HbA1c test was practiced in different regions of the world to mitigate the cases of over- and under-treatment. For example, the ADA (USA) suggested a treatment goal of HbA1c < 7% (ADA, 2011), whereas the Asia Pacific region practice 6.5% for a successful diabetes treatment (Asian-Pacific Type 2 Diabetes Policy Group, 2005). Because HbA1c values give useful insights to aid with clinical decision on appropriate modalities, it is important to practice specific treatment goals for the HbA1c level in order to provide more personalized and effective treatment.

2.7 Conclusions and Future Perspectives

While there were many debates concerning the utilization of HbA1c test as one of the diagnosis tests for type 2 diabetes mellitus initially, it has now been recognized as a reliable diagnostic biomarkers for type 2 diabetes. With the establishment of IUPAC unit (IFCC unit, mmol mol^{-1}) for HbA1c, commercial methods are to be standardized in order to maintain traceability to IFCC reference methods. Although laboratory HbA1c methods have been utilized to perform HbA1c measurements in clinical setting, the bulky machines that involve long turnaround time to generate HbA1c result to the

patients can decrease patients' compliance, thus lowering the effectiveness of diabetes management. On the other hand, because of the biases associated with POC analyzers, clinical practitioners remain skeptical to rely on POC analyzers as a stand-alone HbA1c method to make clinical decision on diagnosing type 2 diabetes. In short, the quality control for CLIA-waived POC analyzers is very critical, because they can be very beneficial to diabetes management in resource-limited setting that does not have sufficient health budget allocations for the installation of HbA1c laboratory tests nationwide. According to one survey, to initiate the set-up for the laboratory HbA1c test, the costs required are 13.6 fold greater than those for the plasma glucose measurement (Gomez-Perez *et al.,* 2010). Therefore, POC analyzers that are on par with laboratory methods are not only cost-effective but also convenient and particularly useful in these underdeveloped regions. In order to resolve the implementation cost for HbA1c tests, attention should be directed at the use of cost-effective materials in the fabrication of the HbA1c biosensors. With more stable design using cost-effective materials, HbA1c tests can be transformed to become more convenient, user-friendly, accurate, and cost-effective diabetic diagnosis and management tool for better containment of the world-wide diabetes incidence.

CHAPTER 3: THE CONCEPT OF DEVELOPING A LATERAL FLOW IMMUNOSENSOR TO DETECT HEMOGLOBIN A1C SEMI-QUANTITATIVELY IN WHOLE BLOOD

3.1 Introduction

To postpone the progression of Type 2 diabetes, rapid and continuous monitoring on hemoglobin A1c (HbA1c) levels of patients is highly required. Because the routine checkup is needed for management of diabetic status, it has become a challenge in underdeveloped regions that are short of laboratory infrastructures, where the social burden of the disease is often overwhelming (Gomez-Perez *et al.*, 2010). Nonetheless, constant testing and monitoring under these conditions can still be practiced with the help of point-of-care devices. However, pre-existing HbA1c point-of-care devices often involve trained personnel and complicated equipment, therefore, they are deficiently suited in these limited-resources settings.

To improve the on-site facilitation of Type 2 diabetes management in limited-resources settings, efforts directed at inventing HbA1c biosensors for inexpensive point-of-care devices is increasing. In fact, biosensors utilizing biomimetic molecule, boronic acid as the bio-recognition element were reported to detect HbA1c at low cost. For example, a lateral flow strip was developed utilizing zinc and reporter-labelled boronic acid conjugate to bind and precipitate hemoglobin (non-glycated and all glycated species) in solution. In the studies, the pretreated blood sample was transferred onto the developed strip, then, the absorbance of HbA1c at a specific wavelength was measured (Sundrehagen, 2014). The extensive sample pretreatment procedure can be hampering to unskilled end-users, therefore the design is not very useful for economically challenged settings. On the other hand, McCroskey described another boronic acid-based lateral flow assay that manipulated pH to induce preferential binding of boronic acid to non-glycated or glycated hemoglobin on the chemically modified separation

matrix on the strip (McCroskey & Melton, 2010). In consideration of the potential shift in dissociation due to changes in temperature (Promega, 2015) that could change the pH of the buffers, the separation matrix lateral flow strip that operates based solely on the pH could be compromised if the test is performed in underdeveloped regions of the world that have huge shift in temperature and weather. Further, because boronic acid can bind to any glycan moiety (HbA1c is only one of the glycan moieties) pre-existing in whole blood, the selectivity of the developed HbA1c boronic acid-based assays can be a concern.

Alongside with the growing interest to detect a variety of biomarkers indicative of chronic diseases, biosensing applying the immunoreaction between target analytes and specific clones of antibodies has become more clinically relevant nowadays (Dutton, 2013). Immunoassays have been integrated with various sensing platforms such as microarrays (H.-H. Chen *et al.*, 2012), electrodes (Xue *et al.*, 2011), and biochips (Tanaka *et al.*, 2007) in order to detect hemoglobin A1c, a biomarker for Type 2 diabetes diagnosis and management. Electrochemical immunoassays were the highly favored method commonly utilized to fabricate the biosensors for the detection of HbA1c (Halámek, Wollenberger, Stöcklein, & Scheller, 2007; Tanaka *et al.*, 2007) because of the plausibility for miniaturization. Nonetheless, the selectivity studies on HbA1c against all other glycated species of hemoglobin coexisting in whole blood remained elusive in the reported studies. To fulfill the demand for devices with high selectivity and good sensitivity in the detection of HbA1c, a lateral flow assays operated based on immunoreaction using a highly selective anti-HbA1c antibody was developed in this project. Also, because the HbA1c biosensor was made of paper, the cost of the test strip can fall within the range of $0.10 to $3.00 (depending on the antibodies production cost) with large-scale production (Rosen, 2009), which makes the test strips relatively inexpensive. Designed and developed to function as simple as the urine

pregnancy test strip, the lateral flow immunosensor was aimed to be operating in a user-friendly way with relatively easy sampling, testing, and subsequently interpreting procedure. Defined volume of diluted blood sample was dispensed onto and let flowed through the lateral flow assay matrix. When HbA1c was captured at the lateral flow test lines, the immunoreaction with anti-HbA1c antibodies, and subsequently with the anti-hemoglobin antibodies functionalized-colloidal gold will generate visible test line(s).

This chapter focuses on discussing the concept of developing a functional HbA1c lateral flow immunosensor with emphasis on optimization works to investigate the ideal blocking reagents, the potential interference from the color of the blood sample, the test line format, and the selectivity of the assay. In addition, the optimal dilution factor to improve the feasibility to perform measurement of HbA1c level in actual blood sample in a semi-quantitative way using the developed lateral flow immunosensor was demonstrated.

3.2 Materials and Methods

3.2.1 Reagents and Materials

All the antibodies used in this study, including the capturing antibody (monoclonal anti-hemoglobin A1c IgG1 antibody) and the detecting antibody (monoclonal anti-hemoglobin IgG1 antibody), were purchased from Fitzgerald Industries International (Acton, MA, USA). The secondary antibody (polyclonal goat-anti-mouse IgG antibody) was obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The purified HbA1c, HbA0, glycated HbA0, and HbA2 were purchased from Fitzgerald Industries International (Acton, MA, USA); gold nanoparticles (40 nm) were purchased from Kestrel Biosciences Co., Ltd. (Pathumthani, Thailand); bovine serum albumin was obtained from Amresco LLC (Solon, OH, USA); and 10% Western Blocking Reagent was purchased from Roche Diagnostics (Selangor, Malaysia). The calibrators and

hemolysis reagent were obtained from Kamiya Biomedical Company (Seattle, WA, USA). All other chemicals were purchased from Sigma-Aldrich (Selangor, Malaysia). Phosphate buffer (containing Na_2HPO_4 and NaH_2PO_4) was prepared using Milli-Q water with a resistivity of 18.2 MΩ cm, adjusted to pH 7.4 using diluted HCl. The pH of the phosphate buffer was adjusted to pH 7.4 to correspond to the normal physiological pH range between 7.35 and 7.45 in human blood (Y.-X. Huang *et al.*, 2013). On the other hand, solutions of NaCl, K_2CO_3, and HCl were prepared using Milli-Q water. Because of different matrices that involved in the optimization study, distinctive reagents were needed to perform dilutions. To dilute gold conjugates, blocking reagents and purified proteins (i.e. antibodies, bovine serum albumin, and purified hemoglobin species) that are sensitive to pH fluctuations, the pH-adjusted phosphate buffer that resembles physiological pH was used. On the other hand, to lyse the erythrocytes completely, in study that involved whole blood (the interference study and real sample analysis), hemolysis reagent (distilled water with stabilizers) was used. In order to maintain the matrices similarity between whole blood and calibrators purchased, hemolysis reagent was also used to perform dilution for calibration curve generation. Laminated nitrocellulose membrane card (ref. HF135MC100), cellulose fiber pads (ref. CFSP173000) and glass fiber pads (ref. GFCP083000) were purchased from Merck Millipore (Selangor, Malaysia). Informed consent was obtained from patients recruited and the study protocol was implemented in accordance to the institutional medical ethics board committee for studies that involved blood testing.

3.2.2 Apparatus

The absorbance of the gold conjugates pre- and post-treatment with 10% NaCl was measured at a wavelength of 530 nm using an Infinite® M200 PRO microplate reader (Tecan Group Ltd., Männedorf, Switzerland). On the other hand, signals on the

immunosensor were measured using an ESEQuant lateral flow reader (Qiagen Lake Constance GmbH, Stockach, Germany). Before the manual lining of capture reagents (ie. anti-HbA1c antibody and goat anti-mouse antibody) on strips using a pipette, the lateral flow strips were cut using Matric 2360 programmable shear from Kinematic Automation (Twain Harte, USA).

3.2.3 Construction of Lateral Flow Strips

3.2.3.1 Conjugation of Gold Nanoparticles to Antibodies

Using the flocculation assay as described by Thobhani *et al.*, the optimization of the gold nanoparticle-conjugated anti-hemoglobin-antibody was conducted (Thobhani *et al.*, 2010). In the assay, the ideal pH and the minimal antibody concentration required to stabilize the gold conjugates were investigated. First, the stability of gold conjugates was tested with gold nanoparticles adjusted to pH in the range of pH 5.0 to 9.0 (adjusted using K_2CO_3), under a constant antibody concentration of 30 μg mL^{-1}. An Infinite® M200 PRO microplate reader (Tecan Group Ltd., Männedorf, Switzerland) was used to measure the absorbance of the gold conjugates before and after treatment with 10% NaCl at a wavelength of 530 nm. The minimal concentration of antibody that solvates the gold nanoparticles was determined using gold nanoparticles with adjusted pH obtained in the first part of the study. The pH-adjusted gold nanoparticles were then submitted for titration using a range of antibody concentrations. The stability of the gold conjugates across the range of antibody concentration was assessed as described above. With the ideal concentration of antibody and pH determined, gold conjugates were prepared in a large scale and diluted to OD (optical density) of 8 to be deposited onto the conjugate pad of the strips. The preparation of gold conjugates at OD 8 was aimed to generate visually interpretable lines without delaying the release time (time being

mobilized) from the conjugate pad to the NC membrane in order to complete the immunoreactions.

3.2.3.2 Optimization of Blocking Reagents

Blocking reagents play an important role to remove the nonspecific protein binding on the NC membrane and thus, help to minimize the interference from the background noise contributed by the nonspecific binding on the test strip. Industrial polymers such as polyvinylpyrrolidone (PVP) and polyvinyl alcohol (PVA), carrier proteins such as casein in the western blocking reagent (WBR), and bovine serum albumin (BSA) were tested to determine the suitable blocking reagent for the lateral flow immunosensor. In the study, positive (tested with purified HbA1c) and negative sets (tested with phosphate buffer) of fully assembled strips were prepared. Ten microliters of purified HbA1c (0.1 mg mL^{-1}) (purchased from Fitzgerald Industries Inc, Acton, MA, USA) was dispensed onto positive test strips, and to determine the background generated, a corresponding negative control strip (blocked with the same blocking reagent) was tested with 10 μL of phosphate buffer in place of the purified HbA1c. The signals generated from positive test strips and corresponding negative control strips were then measured using an ESEQuant lateral flow reader (Qiagen Lake Constance GmbH, Stockach, Germany). The ratio of signal to background was calculated by dividing the background (signals on negative control strips) with the signals generated on positive test strips.

3.2.3.3 Preparation of Lateral Flow Strips

The lateral flow strip was made up of four overlaying components, namely the buffer application pad, the conjugate pad, the laminated nitrocellulose membrane, and the absorbent pad (Figure 3.1). The lateral flow strip was 4 mm in width, where all four components on the strips overlaid each other at 2 mm in length. With the fully assembled strip, the test is to be run by capillary reaction until the excess reagents reach the absorbent pad. The nitrocellulose membrane is the reaction zone which contains three test lines and one control line. Test lines are where the capturing antibody (monoclonal anti-HbA1c IgG1 antibody, 1 μL, 1.0 mg mL^{-1}) will be deposited at, while polyclonal goat-anti-mouse IgG antibody (1 μL, 1.0 mg mL^{-1}) was used to line the control line (Figure 3.1). The lined strips were dried for at least 30 min in a desiccator prior to the application of blocking reagents. Later, the blocked strips were let dried in desiccator for at least an hour. Full lateral flow immunosensor were then assembled with the strips lined with the capture antibodies and blocked with optimized blocking reagent (please see section 3.2.3.2 for optimization of blocking reagents), the dried conjugate pads, and the sample pads. 10 μL of the diluted samples (calibrators, whole blood, or purified antigens) were dispensed directly onto the laminated nitrocellulose membrane. Upon the application of the washing buffer (50 mM phosphate buffer, pH 7.4, with 5% Tween-20), the gold conjugates on the conjugate pad were solvated and mobilized to react with the antigen (HbA1c) and capture antibodies on the nitrocellulose membrane. The excess gold conjugates and antigens (HbA1c) will then be mobilized to the absorbent pad to complete the test.

3.2.4 Selectivity and Interference Test

Glycated species of HbA0, HbA0, HbA2 and HbA1c were diluted to 0.1 mg mL^{-1} with phosphate buffer (0.01 M, pH 7.4) prior to the selectivity test. Then, 10 μL of HbA1c, HbA0, and glycated species of HbA0 were dispensed directly onto the NC membrane. Later, washing buffer (50 mM phosphate buffer, pH 7.4, contained 5% Tween-20) was applied to the sample pad to mobilize the gold conjugates and antigens (glycated species of HbA0, HbA0, or HbA1c) to complete the assay.

In the interference test, a control strip was assembled using a conjugate pad dried with 1 mg mL^{-1} monoclonal anti-hemoglobin antibody (IgG1, without conjugation to gold nanoparticles). Then, 10 μL of whole blood (1:5 dilution with hemolysis reagent) was dispensed onto the control strip and test strip (with the conjugate pad containing gold-conjugated-monoclonal anti-hemoglobin antibody). Both control and test strips were then washed with washing buffer.

3.2.5 Optimization on Test Lines and Dilution Factors

The number of test lines was optimized to allow identification of different HbA1c level (% HbA1c) in blood samples using just visual interpretation. Strips were lined with one, two, or three test lines containing monoclonal anti-HbA1c antibody alongside with one control line consisting of polyclonal goat-anti-mouse antibody. Three levels of HbA1c were selected for testing. In the preliminary study of test lines optimization, the 4.6%, 6.0%, and 7.6% HbA1c calibrators were diluted ten times (with the hemolysis reagent), and 5 μL of the diluted HbA1c calibrators were dispensed directly onto the half dipsticks (without the buffer application pad). Then, the half dipsticks were immersed into 10 μL of gold conjugates-anti-hemoglobin antibody, followed by 100 μL of phosphate buffer to complete the test.

3.2.6 Real Sample Analysis

Because of the high viscosity of the whole blood samples, the dilution factor was very critical not only to lyse the erythrocytes in whole blood to release HbA1c, but also to improve the fluidity of whole blood on the test strip in order to increase the rapidity of the assay. The whole blood samples used in the study were collected from the University of Malaya Medical Center (UMMC), and manual dilution on the whole blood samples was performed using a hemolysis reagent (distilled water with blood stabilizers). The diluted whole blood (10 µL) was then dispensed directly onto the NC membrane, phosphate buffer containing Tween-20 was then used to wash the strip. The pattern of the signal generated on the lateral flow strips was observed.

3.3 Results and Discussion

3.3.1 The Concept of the Lateral Flow Immunosensor

Hemoglobin is a protein that consists of four subunits, and HbA1c is formed *via* glycation reaction that specifically occurs at the terminal valine residue in β subunit(s) (John *et al.*, 2007). In fact, HbA1c constitutes only a small fraction (approximately 5%) of the total hemoglobin (B. Chen *et al.*, 1998) (for a detailed description on the biological aspect of the hemoglobin pool, please refer to chapter 2), to amplify the immunoreaction from the "sandwich" formed in the presence of HbA1c, the anti-hemoglobin antibody was chosen as the detecting antibody. On the hand, to maintain the selectivity of our assay towards HbA1c, anti-hemoglobin A1c (HbA1c) antibody was used as the capturing antibody on the NC membrane (Figure 3.1). As the sample flows through the assay, the capturing antibody binds selectively to the glycated valine at N-terminal of β subunits, exposing the non-glycated sites or non-specific glycation sites (non-N-terminal valine residues in β subunits or other residues in any hemoglobin subunits). The unbound parts of the captured HbA1c will then be recognized by the gold

nanoparticle (40 nm)-conjugated anti-hemoglobin detecting antibody (bind to human hemoglobin), forming “sandwich” immuno-complexes that would be transduced as red line (s) on the NC membrane. Then, excess or unbound HbA1c will move to the subsequent test lines where the binding sites of capturing antibody are still available.

Comparing to the reported sandwich immunoassay on microarray (H.-H. Chen *et al.*, 2012), the sandwich design on the lateral flow immunosensor developed in this part of the project was switched. With the combination of the anti-HbA1c antibody-functionalized gold nanoparticles and immobilized anti-hemoglobin antibody on the strip (Chen *et al.*’s reported sandwich format), no signal was observed on the test lines. Hence, it was presumed that the different orientations of the HbA1c and steric hindrance by the pre-formed immuno-complexes (between hemoglobin or HbA1c and the immobilized anti-hemoglobin antibodies) on the strip has impeded the subsequent binding of the gold conjugated-anti-HbA1c antibody to the HbA1c that was bound to the immobilized anti-hemoglobin antibody on the strip (Figure 3.2). As a result, no signal was observed when the reported format was used. To resolve the issue, the only approach to create a functional sandwich immunoassay on the lateral flow strip was by employing a different sandwich format than the reported one (Figure 3.1).

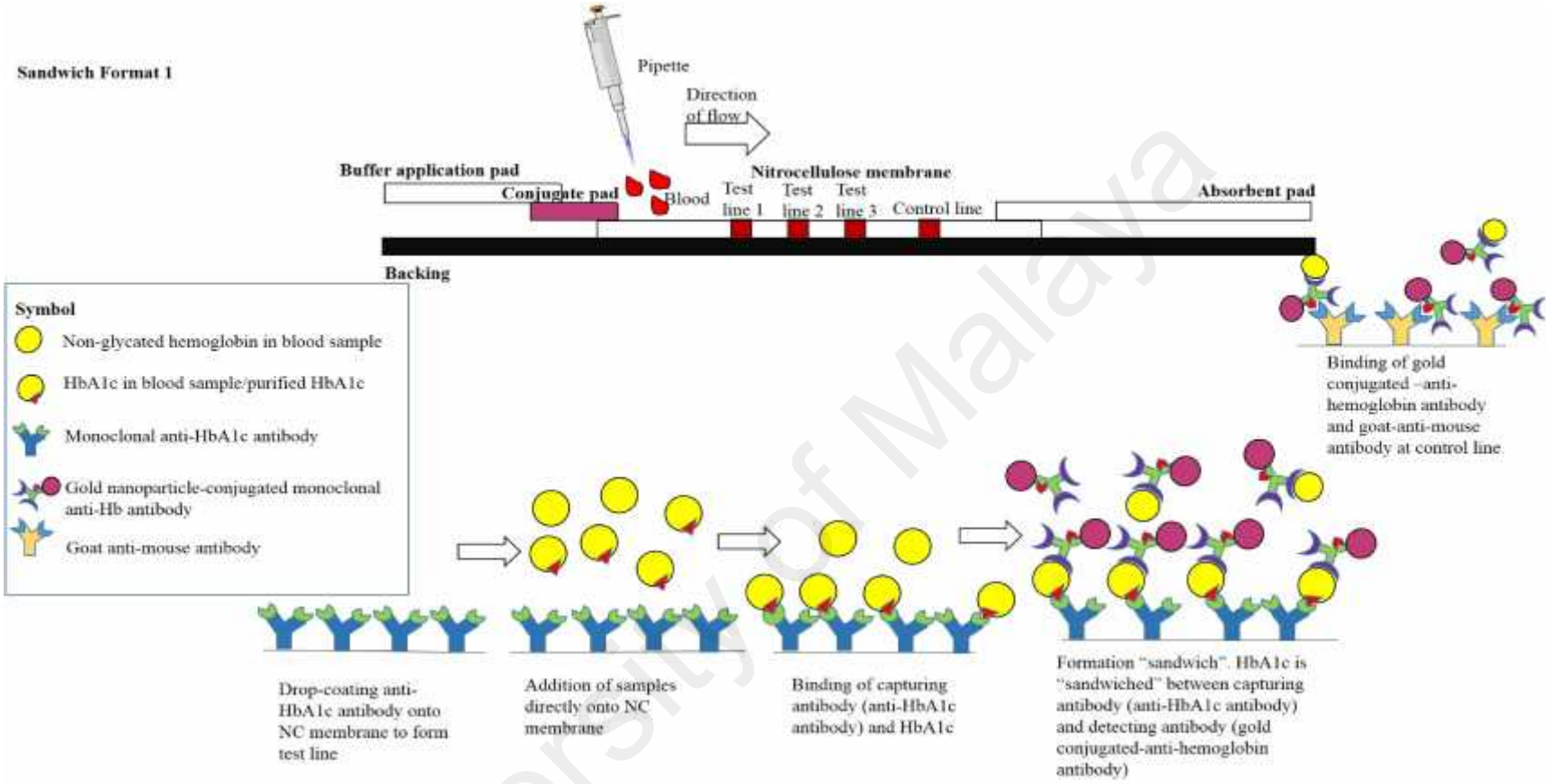

Figure 3.1: Scheme of the Fabrication of HbA1c Lateral Flow Immunosensor.

Fabrication of a HbA1c lateral flow immunosensor. Drop-coating of capturing antibody, monoclonal anti-HbA1c antibody at the test lines, and secondary antibody, goat-anti-mouse at the control line was conducted using pipette.

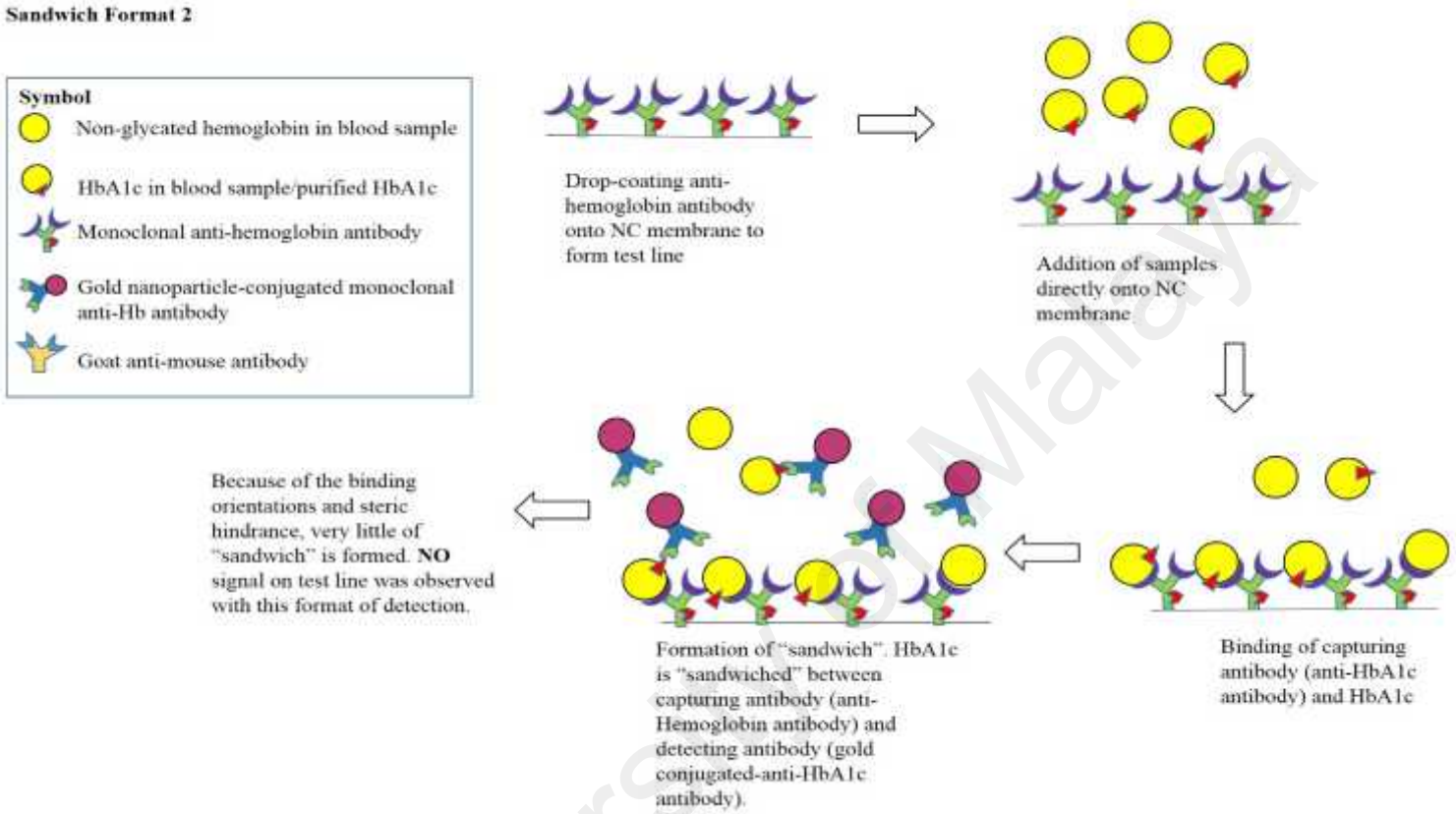

Figure 3.2: Sandwich Format 2.

This format resembles that of the sandwich immunoassay reported in Chen *et al* (H.-H. Chen *et al.*, 2012). On the lateral flow strip, no signal was observed using the shown sandwich format 2.

3.3.2 Selectivity and Interference Tests

Owing to the concept of our hemoglobin A1c immunosensor that took advantage of the amplification power of the anti-hemoglobin antibody-gold nanoparticles conjugates, it was thus very important to ensure that the amplification of signal only occurs specifically in the presence of HbA1c, when HbA1c was bound to the capturing antibodies (monoclonal anti-HbA1c antibody, IgG1) on the NC membrane that will subsequently result in the formation of "sandwich". HbA0, the glycated species of HbA0, and HbA2 were tested as the potential cross reactants in the study. Because anti-hemoglobin antibody bind to all types of hemoglobin, therefore, it is critical to ensure that the non- glycated species such as HbA0 and HbA2 that could form immuno-complexes with the free-flowing anti-hemoglobin antibodies-functionalized colloidal gold did not form "sandwich" with the anti-HbA1c antibody on the test lines. On the other hand, to demonstrate that the chosen anti-HbA1c antibody was highly selective towards the glycan moiety only at the β chain-N-terminal valine, the glycated species of HbA0 (consisted of other glycation sites in place of the glycation site at β chain-N-termini-valine) was tested.

Little to no significant interference was observed from the cross-reactants tested (Figure 3.3a). Such observations demonstrated that the "sandwich" can only be formed, and signal to be seen when HbA1c was present in the samples. In addition, the anti-HbA1c antibody as the capturing antibody at the test lines was shown to be highly selective towards the glycation site at the N-terminal valine in the β chain. In fact, other glycation sites on hemoglobin will not be recognized by the capturing anti-HbA1c antibodies to give signals in the hemoglobin A1c immunosensor. Hence, it was concluded that when the free-flowing gold conjugated-anti-hemoglobin antibody (detecting antibody) would bind to the cross-reactants, the formed complexes will not be able to interact with the

capturing antibodies and will be washed away from the NC membrane; therefore, the signal observed on the immunosensor only originated from the bound HbA1c.

On the other hand, because the whole blood samples are red in color, there was concern that the signals observed from the test lines were possibly originated from the redness of the whole blood sample. While the reflectance measurement of the test lines was performable by the ESEQuant lateral flow reader, a portable small size instrument designed specifically to detect only gold conjugates, the visual interpretation can be impeding for household end-users that do not own the reader if blood will interfere with the color generated on the test lines. From the study, it was found that the color of whole blood did not contribute to the background or noises that interferes the color observed on the test line (refer to Figure 3.3b). Hence, it can be concluded that the color generated on the test line was exclusively originated from the bound gold conjugates that form immuno-complexes with the capturing antibody (anti-HbA1c antibodies) in the presence of HbA1c. In short, it was confirmed that the developed lateral flow immunosensor will show visible signals on the test lines if and only if gold conjugates react with HbA1c and formed “sandwiches” with the capturing antibody (monoclonal anti-HbA1c antibody) at the test lines.

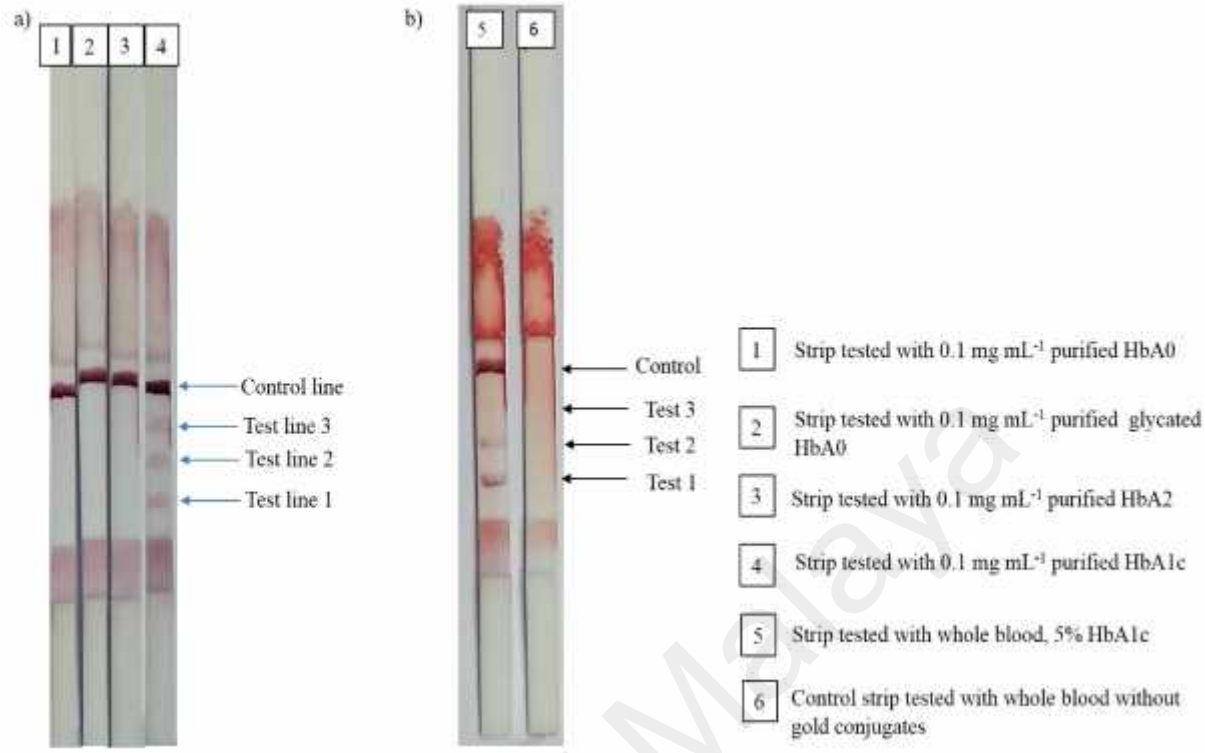

Figure 3.3: Selectivity Test and Interference Test

(a) Selectivity Test. No cross-reactivity observed from the non-glycated hemoglobin (HbA0) (strip 1), glycated species of HbA0 (strip 2), and HbA2 (strip 3), indicating that the immunosensor developed was very specific to HbA1c. (b) Interference test. Strip 5 is the developed hemoglobin A1c immunosensor while strip 6 was the control strip with anti-hemoglobin antibody without conjugation to gold nanoparticles. No signal was observed from the control strip without gold conjugates, demonstrating that color of the whole blood did not contribute to the signal observed on the lateral flow strip.

3.3.3 Optimization Experimental Parameters

3.3.3.1 Optimization of Gold Conjugates

To improve the detection sensitivity and specificity of the lateral flow immunoassay, the condition to generate stable gold conjugates need to be optimized. The flocculation assay was performed as described in section 3.2.3.1 to determine the ideal condition for the synthesis of stable gold conjugates. In the flocculation assay, electrolytes such as sodium chloride were commonly used to mask the charges on the surfaces of gold nanoparticles, hence disrupting the attraction and repulsive forces, causing the gold nanoparticles to collapse and aggregate, which would then lead to visual changes in color (Thobhani *et al.*, 2010). However, with the presence of the proteins, the aggregation of gold nanoparticles can be inhibited when the proteins absorb to the surface of the gold nanoparticles (Thobhani *et al.*, 2010). Therefore, the smaller the difference in absorbance before and after the addition of 10% NaCl (termed as absorbance decrease), the better the conjugation of proteins onto the surface of the gold nanoparticles, hence, the higher the stability of the gold conjugates generated. Using the flocculation assay, it was found that the gold conjugates formed at pH 8.0 with a concentration of 30 μg mL^{-1} of anti-hemoglobin antibody, had the smallest absorbance difference (Figure 3.4), indicating that the gold conjugates generated were very stable under the specific conditions. The ideal conditions for generating stable gold conjugates were then applied in the later part for large-scale preparation of gold conjugates, which will then be deposited onto conjugate pads for the assembly of a full lateral flow immunosensor.

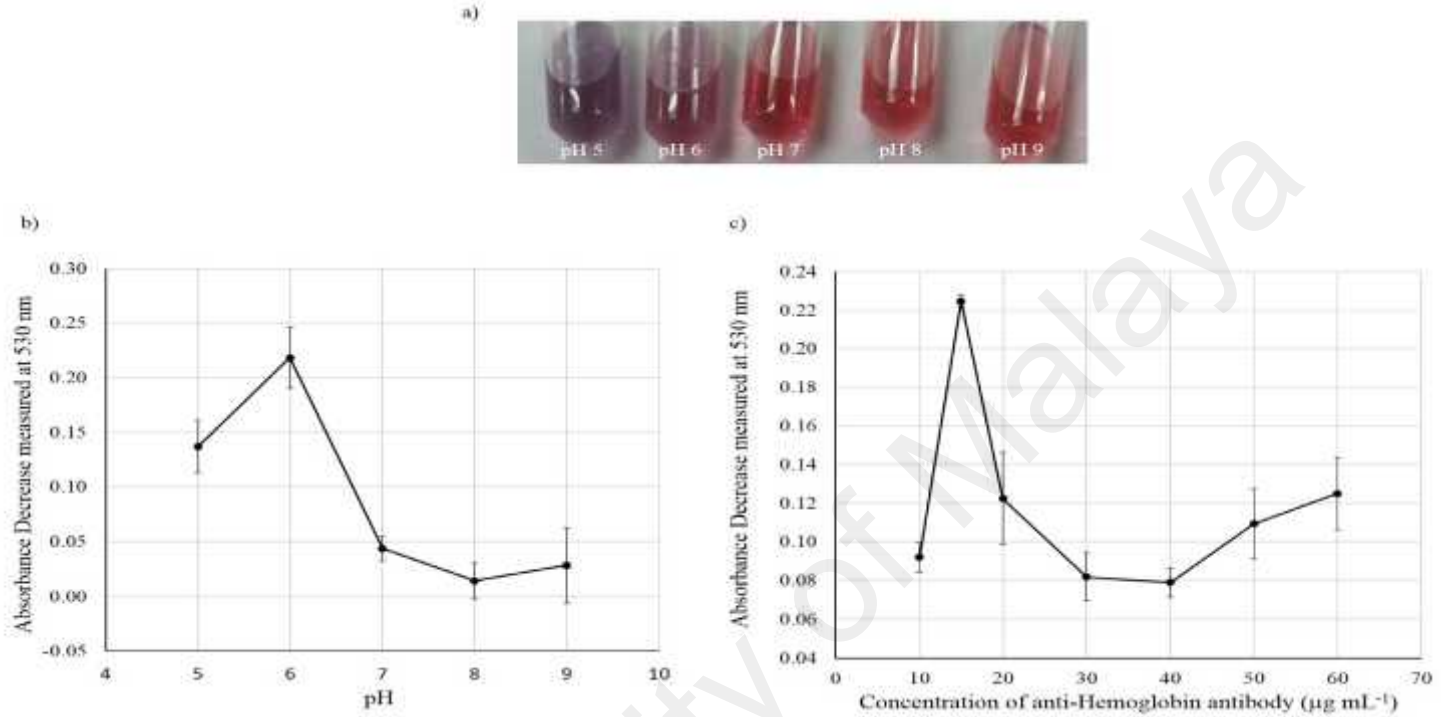

Figure 3.4: The Flocculation Assay.

(a) The variation of the colors across a range of pH, after the treatment of 10% NaCl. The gold conjugates formed at pH 5 and pH 6 were unstable, indicated by the purplish blue color. (b) Absorbance decrease versus pH (measured at 530 nm). The optimum pH to solvate and stabilize gold conjugates was identified at pH 8.0. (c) Absorbance decrease versus detecting antibody concentrations (measured at 530 nm). 30.0 μg mL^{-1} of anti-hemoglobin antibody was determined to be the least concentration of antibody needed to form stable gold conjugates at pH 8.0. Error bars display the standard deviation of triplicates for each data point.

3.3.3.2 Optimization of Blocking Reagents

To determine the blocking reagent that can suppress the background effectively, different types of blocking reagents were tested. An optimal blocking buffer should be able to minimize the background noise while enable the detection of HbA1c in a HbA1c level-dependent detection. This study showed that 1% of the western blocking reagent (WBR) containing casein, was able to block the unwanted non-specific signal efficiently. Little to no background was observed on the negative control strips (strips tested with phosphate buffer in substitution of purified HbA1c) blocked with 1% WBR. On the other hand, the unblocked strips and strips blocked with other blocking reagents (polyvinyl pyrrolidone (PVP) and polyvinyl alcohol (PVA), bovine serum albumin (BSA), and a mixed blocking reagent that consisted of 1% BSA, 0.02% polyvinyl pyrrolidone (PVP), 0.05% casein, 0.002% Tween-20 in 1X Tris-buffered saline (pH 7.4)) generated high signal in negative control strips, suggesting that the signal observed on the positive test strips (strips tested with purified HbA1c) did not necessarily originate from the binding of the HbA1c (Figure 3.5). In most of the reported lateral flow studies, BSA (bovine serum albumin) was commonly used to block the remaining protein-binding sites on the NC membrane (C. Fang *et al.,* 2011), (Wang *et al.,* 2005), (Anfossi *et al.*, 2013); however, in this study, high background noise (non-specific binding) was observed when the strips were blocked with BSA. In short, BSA and other blocking reagents did not block the protein-binding sites on the strips sufficiently while 1% WBR was the best blocking reagent for the test strips, similar to the observation by Ang *et al.* (Ang *et al.,* 2012). By optimizing the blocking reagents, it can then be confirmed that the signal generated from the lateral flow immunosensor blocked with 1% WBR was specifically originating from the HbA1c "sandwiched" between the gold conjugated-anti-hemoglobin antibody (detecting antibody) and anti-HbA1c antibody (capturing antibody).

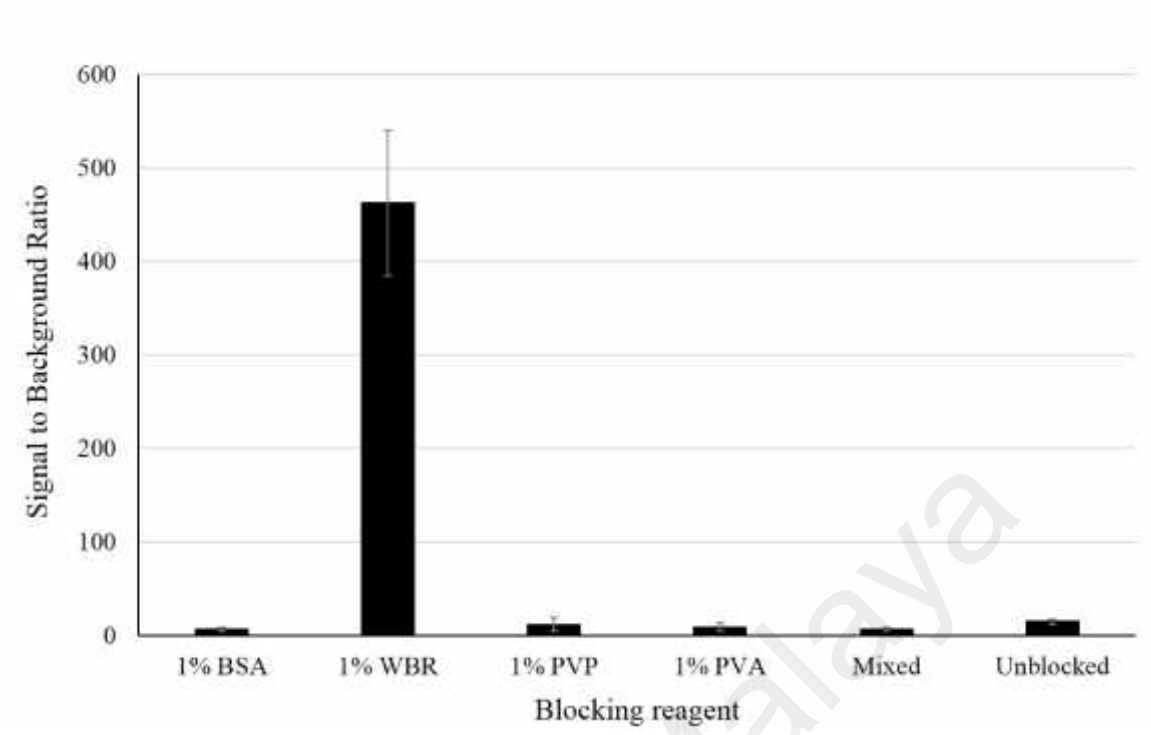

Figure 3.5: Signal to Background Ratio vs. Blocking Reagents.

All the blocking reagents were prepared in dilution using 0.01M phosphate buffer, pH 7.4. Mixed blocking buffer was prepared by 1% BSA, 0.02% polyvinylpyrolidone (PVP), 0.05% Casein, 0.002% Tween-20 in 1XTri-buffered Saline (pH 7.4). All the other blocking buffers, except 1% WBR (Western Blocking Reagent), resulted in visually detectable signal on strips. 1% WBR was thus selected as the best blocking buffer for our fabricated HbA1c immunosensor. Error bars represent standard deviation triplicates associated to each data point.

3.3.3.3 Optimization of Test Lines

To enable visual interpretation of HbA1c level (% HbA1c) according to the number and the intensity of the test lines shown, the NC membrane was designed to accommodate three test lines (anti-HbA1c-antibody) with a control line (goat-anti-mouse antibody). The idea was that, when the first test line was saturated, the remaining HbA1c will then move to the subsequent test lines, where they again be involved in the immunoreaction by getting sandwiched between the capturing and detecting antibodies; hence, the HbA1c level in a sample can be assessed by the number and the color intensity of the lines observed in the test zone(s). Optimization of test lines involved testing on different test line formats to determine which format would yield sufficient signal gaps between different HbA1c levels for a more user-friendly semi-quantitative interpretation, without the requirement of a lateral flow reader for quantitative measurement. Figure 3.6a to 3.6c show different test lines formats across three HbA1c levels (4.6%, 6.0%, 7.6%). While the interpretation using merely signal intensity can be very subjective, the number of lines will better aid the visual interpretation. One- (Figure 3.6a) and two-line formats (Figure 3.6b) showed a gradient of intensity across an increasing HbA1c level; however, it was subjective to tell which strip has the more intense test line(s) with either one- or two-line format. The three-line format, on the other hand, allowed a clearer interpretation of which strip tested with higher level of HbA1c. The least concentrated HbA1c (4.6%) not only had a less intense first test line, but the 2^{nd} and 3^{rd} test lines were faint and close to invisible, giving a good intensity gap between the subsequent higher level (6.0%) of HbA1c tested (Figure 3.6c). To differentiate between the 6.0% and 7.6% HbA1c in the three-line format, the response observed on third line was the determining one. When all three lines showed up, the intensity of the 3^{rd} line was the determining line to judge which strip was tested with a higher level of HbA1c. The third line showed up clearly with a higher intensity on the strip when a higher level of HbA1c

was tested. Because of the clear intensity gap associated with a range of HbA1c level, the three-line format was chosen as the ideal format for a more straight forward visual interpretation in the household setting.

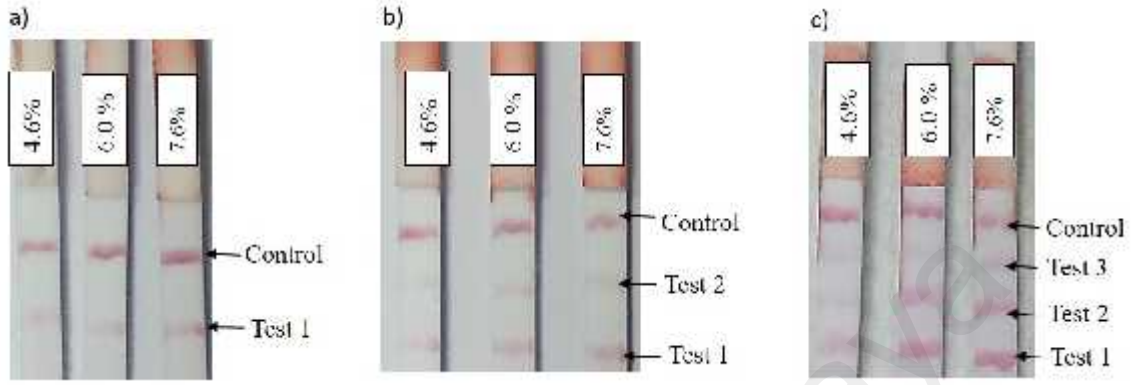

Figure 3.6: Optimization of Test lines Format.

(a) Strips with 1-test line format tested with increasing HbA1c level. (b) Strips with 2-test lines format tested with increasing HbA1c level. (c) Strips with 3-test line format tested with increasing HbA1c level.

3.3.4 Real Sample Analysis (HbA1c in Whole Blood)

Figure 3.7a shows at a 1:2 dilutions, whole blood remained reasonably viscous, and it was relatively slow to flow through the assay *via* capillary reaction. Compared to whole blood at 1:2 dilutions, whole blood at a 1:10 dilution flowed relatively easily across the assay; however, because of the swift flow across the strip, the immunoreaction was not able to occur thoroughly, hence, the test lines generated were too weak for visual interpretation. At a dilution factor of 1:5, the whole blood sample was shown to yield a HbA1c level-dependent signal. Therefore, a 1:5 dilution was decided to be the ideal dilution factor for whole blood to flow readily across the assay with minimized background, while still be able to generate visually interpretable signal within a reasonable assay time of approximately 20 min.

Then, a preliminary test was conducted using the whole blood samples that were tested to have different HbA1c levels (5.5%, 6.5%, and 9.9% HbA1c, determined by Bio-Rad

Variant II HPLC). In Figure 3.7b, strip 1 tested with 5.5% HbA1c showed up to have only two test lines, a clear implication that it had the lowest HbA1c values tested. To distinguish which strip was tested with higher HbA1c values, strip 3 tested with 9.9% HbA1c showed up with three test lines that were relatively more intense compared to strip 2 that was tested with 6.5% HbA1c. Hence, for diabetic patients with poorly controlled blood glucose, especially for those with HbAlc levels > 6.5% (Asian-Pacific Type 2 Diabtes Policy Group, 2005) (or >7% in the United States (ADA, 2011)), the lateral flow HbA1c immunosensor will show intense dark red colour in all of the test zones, signifying that immediate medical attention is needed. With unique patterns observed on the strip tested with distinctive ranges of HbA1c levels in whole blood samples, the developed lateral flow immunosensor demonstrated promising potential as a useful point-of-care device that meets the demand of user-friendly and inexpensive technologies for better diabetic care in resource-limited settings.

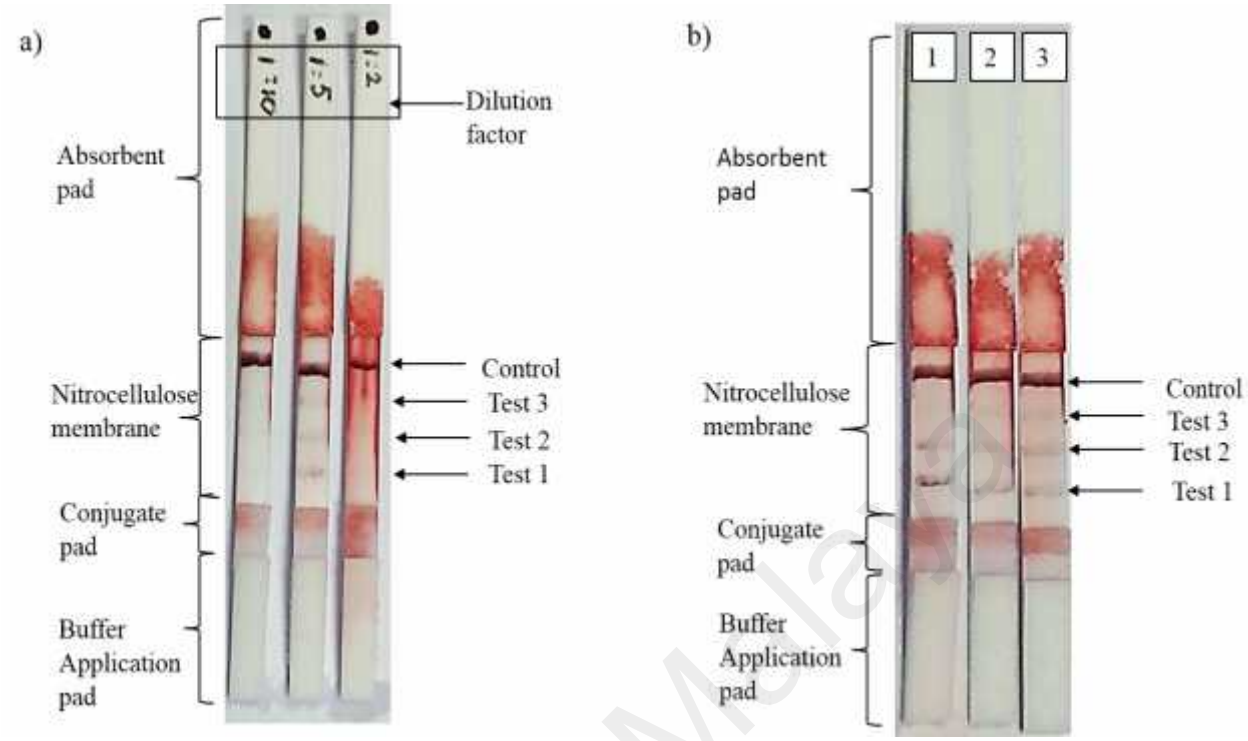

Figure 3.7: Optimization of Dilution Factors for Whole Blood and Whole Blood Analysis

(a) Dilution factors 1 to 2, 1 to 5, and 1 to 10 were tested to determine the ideal dilution factor for the whole blood. Note: picture shown after 20 min of washing. (b) The Hemoglobin A1c immunosensor tested with whole blood at 1 to 5 dilution factor, with three different concentrations (strip 1: 5% HbA1c; strip 2: 6.5% HbA1c; strip 3: 9.9% HbA1c).

3.4 Conclusions

In summary, a functional HbA1c immunosensor operated on the basis of lateral flow that is simple, economical, and portable was developed. The HbA1c lateral flow immunosensor was optimized so that stable gold conjugates were generated at pH 8.0 with 30 μg mL^{-1} of anti-Hb antibodies. Also, the ideal blocking reagent for the immunosensor was investigated and 1% western blocking reagent (containing casein) was found to be the best blocking reagent to eliminate the background noises while allowing signal to be generated in a HbA1c-dependent way. In addition, the immunosensor was found to be highly specific towards HbA1c, where blood color did not interfere or contributed to the color shown on the test lines and control line. To allow quantification of HbA1c level in the whole blood samples, calibration curve was generated and optimized where 1:5 dilutions was found to be the ideal dilution factor to yield a calibration curve with good fit and high sensitivity.

By performing simple manual dilution on finger-prick blood, this developed prototype can be used with a standard images set (a similar concept to the pH paper color scale) for defined ranges of HbA1c levels (normal range <6.0% HbA1c, under control range 6.5% to 7.0% HbA1c, elevated and require medical attention range >7.0% HbA1c) where the patients can compare the color intensity or pattern of signals generated on the strips for accurate visual interpretation. With specific pattern of signal observed associated with distinctive ranges of HbA1c levels, alongside with the simple sampling and testing method affordable at relatively low cost, this lateral flow immunosensor developed in this chapter have potential to become useful point-of-care device to aid with personal diabetes management, especially in the resource-limited setting.

CHAPTER 4: QUANTITATIVE ANALYSIS AND EVALUATION OF THE ANALYTICAL PERFORMANCES OF HEMOGLOBIN A1C LATERAL FLOW IMMUOSENSOR

4.1 Introduction

The HbA1c lateral flow immunosensor was optimized to be functional in detecting HbA1c level in whole blood via semi-quantitative interpretation, without pretreatment in previous chapter 3. Although lateral flow assays are commonly designed to perform qualitative detection in which a yes/no signal is sufficient (X. Huang *et al.*, 2016), in this part of the project, with the aid of lateral flow reader, the quantitative measurement on HbA1c was made possible. The focus in this chapter was on investigating the quantitative ability of the developed lateral flow immunosensor, besides evaluating the analytical performances of the immunosensor such as the limit of detection, the reproducibility, and the stability of the immunosensor. In the previous chapter 3, the semi-quantitative mode of detection on HbA1c level is helpful for untrained end-user to interpret their diabetic status. However, it is very important that the developed immunosensor is able to measure HbA1c level in a quantitative way, because the numerical values of HbA1c levels allow more effective diabetes care in a clinical setting. To achieve the quantitative measurement on HbA1c level, signal generated on the developed lateral flow immunosensor was performed using the ESEQuant lateral flow reader. Purchased HbA1c calibrators (lysed packed human red blood cells with assigned HbA1c levels) were used to generate a calibration curve, where effects of dilution factors on the calibration curves generated were investigated as well.

Later, method validation with a standard method, Bio-Rad HPLC Variant II, and a CLIA-waived point-of-care device, DCA Vantage, was conducted to verify the accuracy of quantitative measurement of the developed lateral flow immunosensor. In the method

validation study, Bio-Rad Variant II HPLC was chosen because of the traceability to DCCT study (please refer chapter 2 for discussion on DCCT study) (Bio-Rad, 2015), also, it is the standard method used in the clinical setting of UMMC (University of Malaya Medical Center) for routine measurement of HbA1c levels. Bio-Rad Variant II HPLC operates based on chromatographic separation of HbA1c on cation-exchange catridge, where the separation is optimized to eliminate interferences from hemoglobin variants and other species of hemoglobin. On the other hand, DCA Vantage (or DCA 2000®+ Analyzer) was chosen to be compared with the developed lateral flow immunosensor because it is one of the CLIA-waived point-of-care devices (please refer to chapter 2 for detailed discussion on CLIA status on point-of-care device) (Bode *et al.*, 2007) that operates on immunoturbidity assay. In the reagent cartridges purchased in order to run the test with the DCA Vantage analyzer, inhibition of latex agglutination assay was used to measure HbA1c, where the agglutination reaction between the agglutinator (synthetic polymer with multiple copies of immunoreactive portions of HbA1c) was inhibited with the presence of HbA1c that would react with HbA1c specific mouse antibody coated on the latex particles (information and principles can be found in the manual with the purchase of DCA Vantage). By comparing to the standard method and the choice of commercialized POC device of similar immunoassay principle, it gave solid verification on the performance of the developed lateral flow immunosensor when it comes to the quantitative analysis in whole blood samples.

4.2 Materials and Methods

4.2.1 Chemicals and materials

HbA1c calibrators and hemolysis reagent (distilled water with added blood stabilizers) were purchased from Kamiya Biomedical Company (Seattle, WA, USA). Blood samples were collected from University of Malaya Medical Center (UMMC) with informed consent from participants and the protocol was conducted with the approval of the UMMC Medical Ethical Committee. The Variant II Turbo HbA1c Kit (including Elution buffer A, B, and Wash/Diluent solution enough for 2500 test runs) was used to run test in Bio-Rad HPLC Variant II. Reagent cartridges were purchased to run the tests with the DCA Vantage analyzer.

4.2.2 Apparatus

The ESEQuant lateral flow reader was used to measure signal on the developed lateral flow immunosensor. Bio-Rad Variant II HPLC and DCA Vantage analyzer were used to measure HbA1c levels in whole blood samples.

4.2.3 Optimization and Generation of a Calibration Curve

Due to the viscosity of the whole blood samples, the dilution factor used was very critical not only to lyse the cellular content of whole blood to release HbA1c, but also to improve the fluidity of whole blood on the test strip. The hemolysis reagent (distilled water containing blood stabilizers) was used to perform manual dilutions on whole blood and calibrators for 5 min prior to test. Calibrators (lysed packed human red blood cells) with assigned values (4.6%, 6.0%, 7.6%, 9.1%, 10.7 %, and 15.1%) of HbA1c were tested on different dilution factors to determine the quality of the calibration curve

produced. 10 μL of diluted HbA1c (at 1:5 dilutions with hemolysis reagent) calibrators were dispensed directly onto the NC membrane. Washing buffer was then added to the lateral flow immunosensor to mobilize the gold conjugates to the NC membrane and wash off the background. Signals generated on the strips were measured with ESEQuant lateral flow reader. The ideal dilution factor for constructing the calibration curve was identified, assessed by the quality of the calibration curves generated, the same dilution factor was used to determine the ideal dilution factor for whole blood quantitative analysis on the developed immunosensor.

4.2.4 Reproducibility Test

Nine replicates of the developed lateral flow immunosensor were prepared and tested on a diluted HbA1c calibrator (7.6% HbA1c, at 1:5 dilution using hemolysis reagent) to determine the reproducibility of the results generated on the developed HbA1c immunosensor. 10 μL of diluted HbA1c calibrator (7.6% HbA1c) was dispensed directly onto the NC membrane. Washing buffer was the introduced into the assay to complete the immunoreaction and wash off the unbound reagents to the absorbent pad. Signals generated on the strips were measured using the ESEQuant lateral flow reader.

4.2.5 Determination of the Limit of Detection

In occasions when the HbA1c level is too low to form enough “sandwiches” for the signal transmission, the signal generated can be too low and therefore beyond detection by the naked eye. Similar to Parolo *et al.*’s (Parolo *et al.*, 2013), the limit of detection for the developed lateral flow immunosensor was determined by using the degree of visibility of the test lines by naked eye.

Commercialized HbA1c calibrator with 4.6% (27 mmol mol^{-1}) HbA1c were diluted using the hemolysis reagent (distilled water with blood stabilizers) at seven different dilution factors (1:5, 1:10, 1:15, 1:20, 1:25, 1:30, 1:40). Eight hemoglobin A1c lateral flow immunosensors (one was negative control strip) were prepared. Seven of the prepared strips were tested with 10 μL of the diluted HbA1c calibrator at different dilution factors, while for the negative control strip, 10 μL of 10 mM phosphate buffer was tested in place of diluted HbA1c calibrator. All strips were then submitted to washing with phosphate buffer containing Tween-20.

4.2.6 Method Validation – a Comparison Study with Bio-Rad Variant II HPLC and DCA Vantage.

Fresh blood samples added with EDTA anti-coagulant were collected from UMMC (University of Malaya Medical Center), where the same blood samples went through three rounds of testing with the Bio-Rad Variant II HPLC, DCA Vantage, and the developed hemoglobin A1c immunosensor. The EDTA-preserved blood samples (in tubes) were arranged in line and submitted to the sample chamber of the Bio-Rad Variant II HPLC. The automated system picked up the blood from tubes where automatic dilution was performed and the diluted blood was injected into the cation-exchange cartridge where the HbA1c detection was performed. After the testing with the Bio-Rad Variant II system, the blood samples were tested with the DCA Vantage system. Blood samples were first collected from the EDTA tubes using a needle in the test cartridges. Then, the testing was done as an automatic process in the DCA Vantage system. DCA Vantage measured the change of turbidity in the test cartridges - as the concentration of HbA1c increased, the turbidity decreased. Later, the blood samples

were delivered back to the lab for testing on the developed hemoglobin A1c immunosensor.

4.2.7 Stability test

Fourteen aluminum pouches filled with silica gel, each with three hemoglobin A1c lateral flow strips were prepared. Then, seven of the pouches were stored in a 37°C-adjusted oven, while the rest of the pouches were stored in a 4°C fridge for 14 days. On day 0, 1, 2, 5, 7, 9, and 14, pouches stored in oven and fridge were retrieved to be tested with the diluted calibrator 2 (7.6%) (at 1:5 dilutions with hemolysis reagent). After dispensing 10 μL of diluted calibrator directly onto the nitrocellulose membrane, 50 mM phosphate buffer containing Tween-20 was used to mobilize the gold conjugates for reactions on the NC membrane and to wash off the background on the strips for signal measurement in the ESEQuant lateral flow reader. The stability of the immunosensor can be assessed through the minimal loss of signal over the course of 14 days, under both storage conditions at 37°C and 4°C.

4.3 Results and Discussion

4.3.1 Establishing a Calibration Curve for Quantitative Analysis On HbA1c Levels

Figure 4.1a to 4.1c show the detection range of the calibration curves at different dilution factors. For the undiluted calibrators, the calibration curve generated had the lowest coefficient of determination, R^2 of 0.61, indicating that the linear relationship between the HbA1c levels and the signal on the assay was the weakest. Also, the consistently large standard deviations throughout the range of HbA1c level tested was observed (Figure 4.1a). In fact, the discrepancy in the sample composition (matrix effect)

can often affect the accuracy in quantification of analytes using a lateral flow assay (Faulstich *et al.*, 2009). Therefore, it was presumed that the high viscosity of the undiluted calibrators was the contributing factor of the high degree of variability for each test across the HbA1c level (Figure 4.1a). Hence, it was concluded that optimization on the dilution factors was necessary to generate a calibration curve because the assay could be saturated with undiluted HbA1c calibrators. Similar to this study, another study by Chen *et al.* also reported using water to perform dilution on whole blood sample prior to testing (H.-H. Chen *et al.*, 2012). With the dilutions at 1:2 (Figure 4.1b) and 1:5 (Figure 4.1c) (using hemolysis reagent, distilled water with added stabilizers), the calibration curves yielded reasonably good fit within the range of 4.0% (20 mmol mol^{-1}) to 12.0% (108 mmol mol^{-1}) or 14% (130 mmol mol^{-1}) (for calibrators diluted at 1:5) HbA1c. To compare, the 1:2 diluted calibration curve assumed a higher R^2 at 0.95, while the 1:5 diluted curve showed a R^2 of 0.93. However, with the consideration of a shorter assay time (1:2 diluted HbA1c calibrators consumed 45 min to be ready for one complete analysis, while 1:5 diluted HbA1c calibrators took only 20 min to be ready for a quantitative measurement), also for better sensitivity in measurement observed across the HbA1c levels (assessed by the higher slope, reflecting that the signal had a good intensity "gap" between the HbA1c levels and this feature allowed distinctive visually interpretation for end-users (in a semi-quantitative way)), it was found that the calibration curve generated at 1:5 dilutions was the best calibration curve to perform future quantitative analysis. At different dilution factors, the calibration curves generated demonstrated distinct patterns, implying that dilution factors can directly alter the performance of the developed lateral flow immunosensor in terms of sensitivity.

In short, the calibration curve at 1:5 dilutions assumed a reasonably good fit with highest sensitivity to demonstrate a linear relationship between the signal measured on

the strips and HbA1c levels tested, within the range of 4% (20 mmol mol^{-1}) to 14% (130 mmol mol^{-1}). The linear range for HbA1c measurement covered 4% and 12% HbA1c that contained the critical treatment goal values of 6.5%-7% HbA1c, therefore, the developed lateral flow HbA1c immunosensor meets the demands of efficient diabetes control. Nonetheless, there was a restraint for a semi-quantitative analysis via visual interpretation based on the number of test lines and its intensity when the HbA1c level tested was beyond 9.1% with calibrators. With all the three test lines appeared with high similarity in the degree of the intensity after 9.1% HbA1c (Figure 4.1d), it could be challenging for the end user to tell the higher level of HbA1c beyond this point of HbA1c level. However, to tell the higher level of HbA1c apart, the lateral flow reader could be utilized to perform a quantitative measurement. In fact, the signal intensity of the test lines was increased in a HbA1c-dependent way even the degree of signal intensity may look alike beyond the 9.1% of HbA1c level.

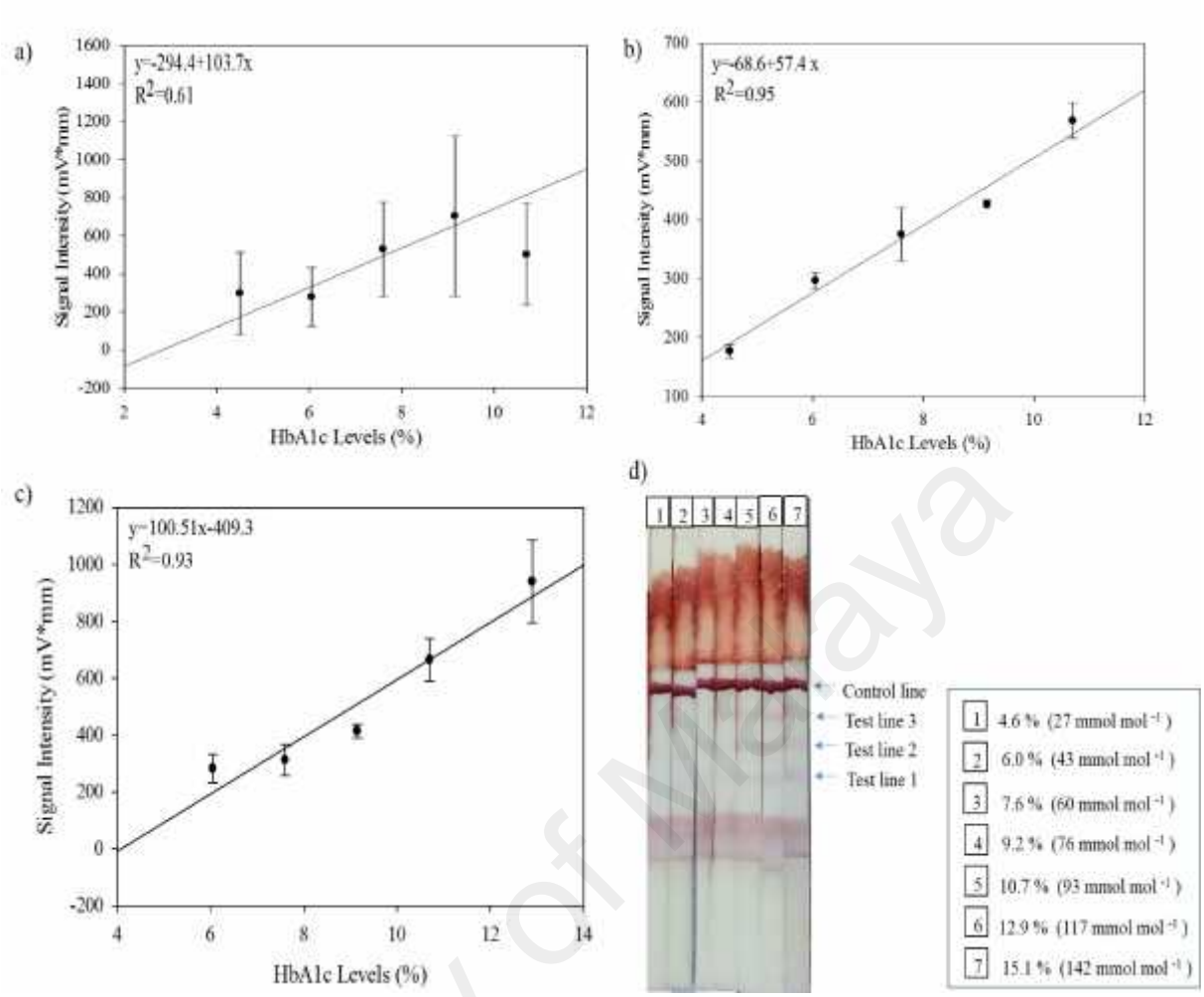

Figure 4.1: Optimization on Calibration Curves

a) Signal intensity of test lines versus HbA1c levels, without dilution. b) Signal intensity of test lines versus HbA1c levels, at 1:2 dilutions. c) Signal intensity of test lines versus HbA1c levels, at 1:5 dilutions. Error bar represents the standard deviation for triplicates of each data point. d) Visual representation of strips tested with calibrator diluted at 1:5 dilution factor. 1 to 7 indicated the increasing HbA1c levels (1: 4.6%; 2: 6.0%; 3: 7.6%; 4: 9.1%; 5: 10.7 %, 6: 12.9%, and 7: 15.1%).

4.3.2 The Reproducibility Study for HbA1c Lateral Flow Immunosensor

Figure 4.2 shows the coefficient of variation (CV) of the lateral flow immunosensor was tested to be 13.45%, demonstrating a fairly good reproducibility on the results observed using the developed lateral flow immunosensor. Hence, the signals generated from the immunosensor with the same HbA1c level are expected to be reasonably consistent.

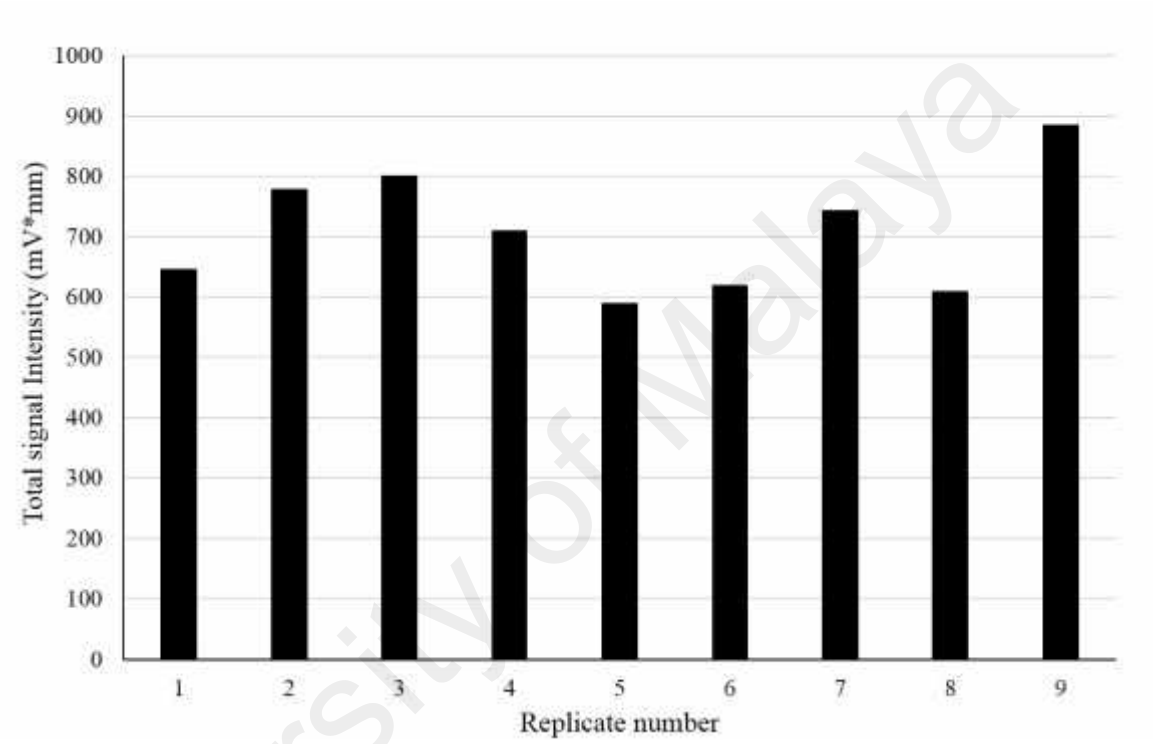

Figure 4.2: Reproducibility Test.

Total Signal versus replicates. With nine replicates, the coefficient of variation (CV) of the developed Hemoglobin A1c lateral flow immunosensor was tested to be 13.45%.

4.3.3 Limit of Detection of the Sandwich Immunoassay

Figure 4.3 shows that the signal intensity on the test strips slowly diminished with a decreasing HbA1c level (from left to right), so as the degree of visibility of the test lines. Judging from the degree of visibility of the test lines, strip 4 tested with 1:20 dilutions (1.35 mmol mol^{-1}, 42.5 μg mL^{-1}) was set as the limit of detection for the developed immunosensor. In fact, the visibility of the test lines was beyond visual interpretation when the strips were tested with HbA1c level was lower than 1.35 mmol mol^{-1}. Also, at 1:20 dilutions on 4.6% HbA1c, the pattern of the signal observed from the immunosensor remained consistently similar to that of the strips tested with 4.6% HbA1c diluted at 1:5 dilutions. In short, with the limit of detection established at 1.35 mmol mol^{-1}, the resultant immunoassay was not only proved to be capable of generating coherent results useful for visual interpretation down to 20 dilutions, also, it suggested that the developed lateral flow immunosensor was reasonably sensitive towards the presence of HbA1c (LOD for commercial ELISA kit was 15.6 μg mL^{-1} (H.-H. Chen *et al.*, 2012)).

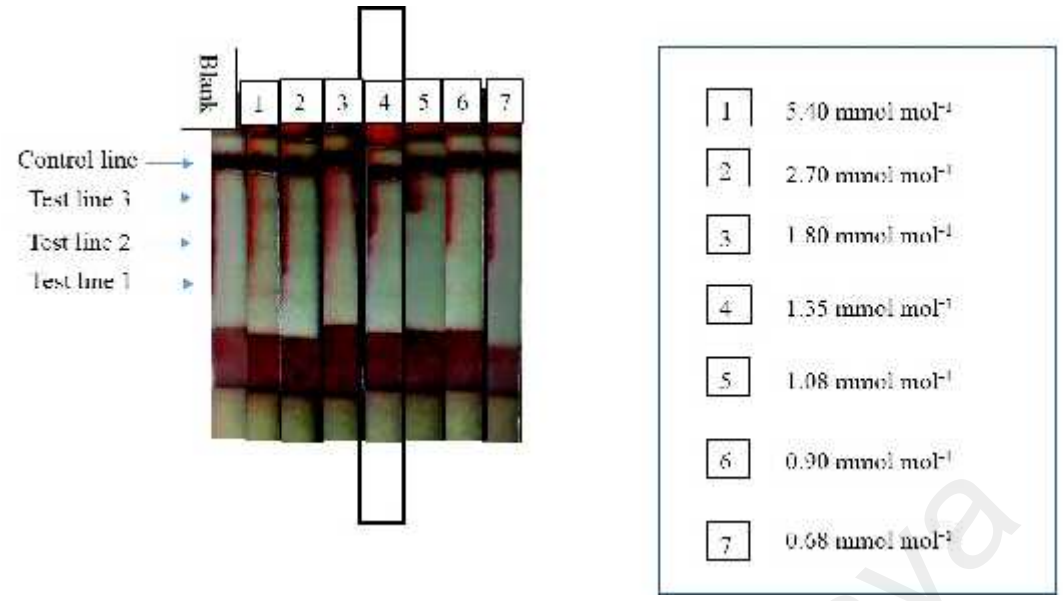

Figure 4.3: Limit of Detection

Determination of limit of detection for the developed hemoglobin A1c immunosensor. 1.35 mmol mol^{-1} (strip number 4) was determined to be the limit of detection for the developed immunosensor, decided by the degree of visibility of test lines on strips.

4.3.4 Quantitative Analysis on HbA1c Levels in Whole Blood using HbA1c Lateral Flow Immunosensor

Figure 4.4 shows the correlation at 0.95 when the measurement of HbA1c levels performed using the DCA Vantage and Bio-Rad Variant II HPLC was compared to each other, confirming that the DCA Vantage yielded a precise and accurate estimation of HbA1c level on par to the Bio-Rad Variant II- HPLC method.

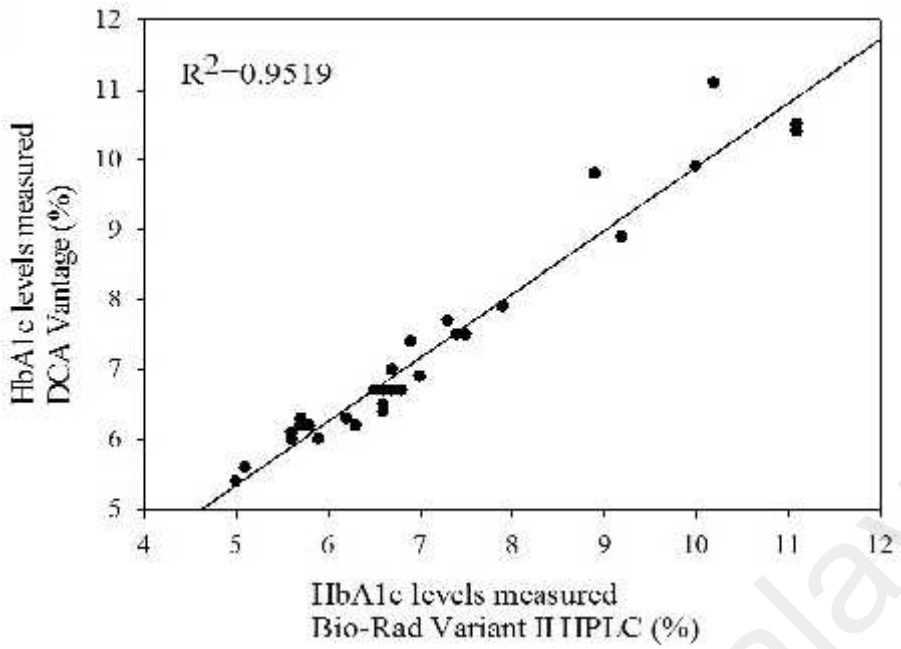

Figure 4.4: Regression Test between DCA Vantage versus Bio-Rad Variant II HPLC

By preforming regression tests for the HbA1c measurements collected from the Bio-Rad Variant II-HPLC and DCA, a good correlation was established with R^2 of 0.95.

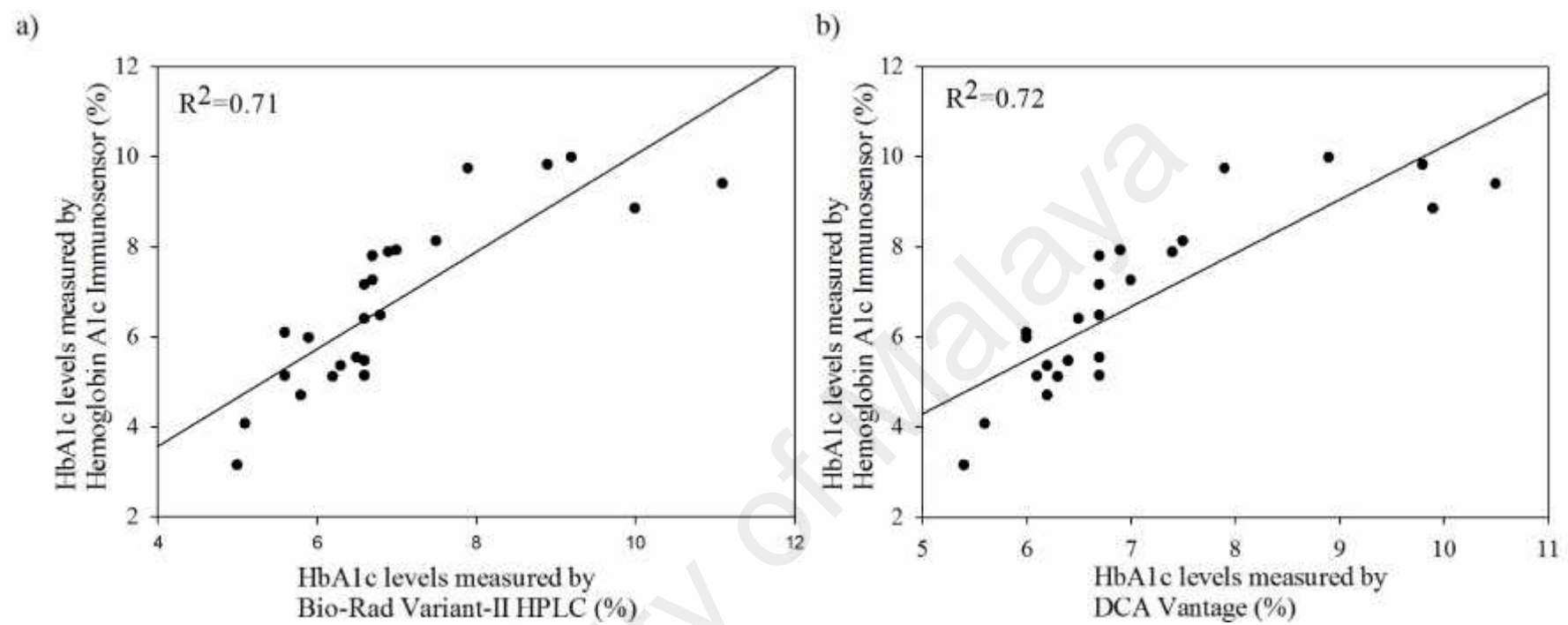

Figure 4.5: Regression Tests

(a) Hemoglobin A1c lateral flow immunosensor versus Bio-Rad Variant II HPLC, across all twenty four blood samples. (b) Regression plot. Hemoglobin A1c lateral flow immunosensor versus DCA Vantage, twenty four blood samples.

A possible explanation for the lower correlation value when compared to that of Bio-Rad HPLC and DCA Vantage (Figure 4.5) was, the developed lateral flow matrix was saturated, hence, it led to the increased steric hindrance for immunoreaction to occur, when the HbA1c level was higher than 7.0%. Instead of adhering to the linear response within the 4% to 14% HbA1c range as shown in the calibration curve (Figure 4.1c), the linear range of HbA1c detection was narrowed down to 4.0 to 7.0% HbA1c in real blood sample analysis. Such observation could be due to the fact that whole blood samples still remained comparatively more viscous than the HbA1c calibrators which consisted only lysed packed human red blood cells, without other co-existing proteins that are likely to contribute to the viscosity in whole blood. The inherently higher viscosity in the whole blood subsequently allowed the diluted whole blood to retain longer, enhancing the interaction with the capturing antibody (monoclonal anti-HbA1c antibody, IgG1) on the test lines, therefore, yielding a higher intensity signal on the strip (as compared to the HbA1c calibrators). Nonetheless, with a longer retention time that allowed enhancement on the interaction of HbA1c with the capturing antibody and gold conjugates to generate more intense signal; the dynamic range of the detection on the developed lateral flow immunosensor was saturated beyond 7% of HbA1c, which the steric hindrance and limited binding sites available were presumed to be the limiting factors. The surplus or unreacted HbA1c in blood samples will then be washed off the sensing platform (NC membrane) and mobilized to the absorbent pad. While the dynamic linear range of detection was narrowed down, it was interesting to note that the saturation of the lateral flow immunosensor did not affect the pattern for visual interpretation (Figure 4.6) (please refer to chapter 3 for detailed discussion on semi-quantitative measurement).

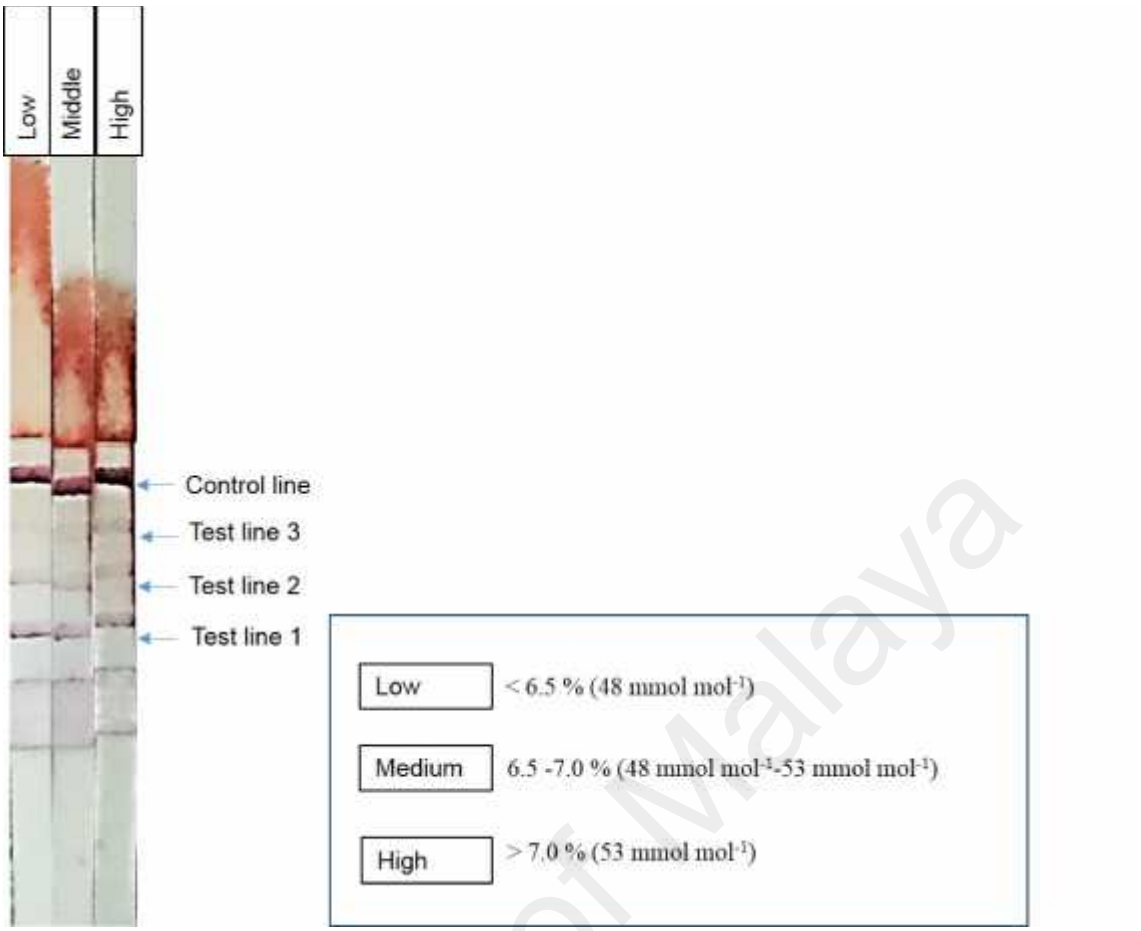

Figure 4.6: Visual Interpretation of HbA1c Level

Three representative strips from each category to show responses in clinical testing (out of thirty testing). In clinical testing, blood samples were divided into three distinct groups, low range (<6.5%), middle range (6.5% to 7.0%) and high (>7.0%). The signal intensity increased when HbA1c levels elevated.

When comparing the developed lateral flow immunosensor to that of Chen *et al.*'s (H.-H. Chen *et al.*, 2012), it was found that the correlation values compared to standard methods were lower. Instead of comparing the measurements to those of Bio-Rad Variant II HPLC and a point-of-care analyzer (DCA Vantage in this study), Chen *et al.* used a different HPLC method (TOSOH) and an ELISA kit for comparison, which could potentially contribute to different correlation values.

To study the relationship between the developed hemoglobin A1c immunosensor, the Bio-Rad Variant II HPLC and the DCA Vantage, a two-tailed Student's t-test was conducted. The null hypothesis was that there was no significant difference between the measurements performed using the developed hemoglobin A1c immunosensor versus the Bio-Rad Variant II HPLC and DCA Vantage. In the Student's t-test between the developed immunosensor and the Bio-Rad Variant II HPLC in (Table 4.1a), it was found that the t statistical value was 0.3883, and it fell within the range of the ± t critical two-tail value (±2.0167). In addition, the p value was determined to be 0.6997, which led to the acceptance of the null hypothesis, where no significant difference was demonstrated between the measurements on HbA1c level performed by the Bio-Rad Variant II HPLC and those from the developed lateral flow immunosensor. Table 4.1b shows the t-test analysis between the DCA Vantage and the hemoglobin A1c immunosensor. Similarly, from the t statistical value (0.6515) that fell within the range of the ± t critical two-tail value (±2.0181), and a p value of 0.5183, it was concluded that there was no significant difference between the measurement made by DCA Vantage and that of the developed lateral flow HbA1c immunosensor. In summary, there was no significant difference observed by comparing the measurement of HbA1c level by the developed immunosensors to those of the Bio-Rad Variant II HPLC, as well as those of DCA Vantage.

Table 4.1: Student's T-Tests.

(a) Two-tailed Student's t-Test between Bio-Rad Variant II HPLC and the developed hemoglobin A1c immunosensor. (b) Two-tailed Student's t-Test between DCA Vantage and the developed hemoglobin A1c.

a)

	Bio-Rad Variant II HPLC	**Hemoglobin A1c Immunosensor**
Mean	6.9625	6.769349613
Variance	2.248532609	3.690051921
Observations	24	24
Hypothesized Mean Difference	0	
df (Degree of Freedom)	43	
t Stat	0.388293152	
P(T<=t) one-tail	0.349857471	
t Critical one-tail	1.681070703	
P(T<=t) two-tail	0.699714943	
t Critical two-tail	2.016692199	

b)

	DCA Vantage	Hemoglobin A1c Immunosensor
Mean	7.083333333	6.769349613
Variance	1.884057971	3.690051921
Observations	24	24
Hypothesized Mean Difference	0	
df (Degree of Freedom)	42	
t Stat	0.651515854	
P(T<=t) one-tail	0.259131435	
t Critical one-tail	1.681952357	
P(T<=t) two-tail	0.518262869	
t Critical two-tail	2.018081703	

By eliminating the extensive sample pretreatment step in the reported zinc-boronic acid conjugates-based lateral flow strip (Sundrehagen, 2014), the developed lateral flow immunoassay that required only manual dilution with distilled water prior to testing improved the rapidity of the assay. On the other hand, the developed lateral flow immunoassay that used buffer only for rinsing purpose was able to resolve the concern on the potential shift of pH that is likely to compromise the device's analytical performance in the reported separation matrix boronic acid-based lateral flow assay (McCroskey & Melton, 2010). Other than being able to quantify the signal generated and subsequently perform estimation on HbA1c level precisely and accurately within the clinically significant range (6.5% to 7.0% of HbA1c level), the developed lateral flow immunosensor was also validated to be able to perform measurement on HbA1c level with no significant difference from the measurements of HPLC method and the existing point-of-care device. Enhanced with the direct and simple sampling and testing procedure, the lateral flow HbA1c immunosensor was sufficiently effective as a

portable device to facilitate on site diabetes care, particularly in the underdeveloped or developing world.

4.3.5 HbA1c Lateral Flow Immunosensor Stability Study

Figure 4.7a and Figure 4.7b show that over the course of 14 days, the developed hemoglobin A1c immunosensors demonstrated consistent performance in generating the signal under both storage conditions. Fig. 4.7e shows that, under accelerated stability test at 37°C, the signal generated from the developed lateral flow HbA1c immunosensors did not fluctuate too much from the mean value, proving that the developed lateral flow HbA1c immunosensors were reasonably stable within 14 days storage. The accelerated stability test at 37°C gave an estimation for at least 1.4 months of shelf life when the lateral flow strips were stored at ambient room temperature (Chua *et al.*, 2011).

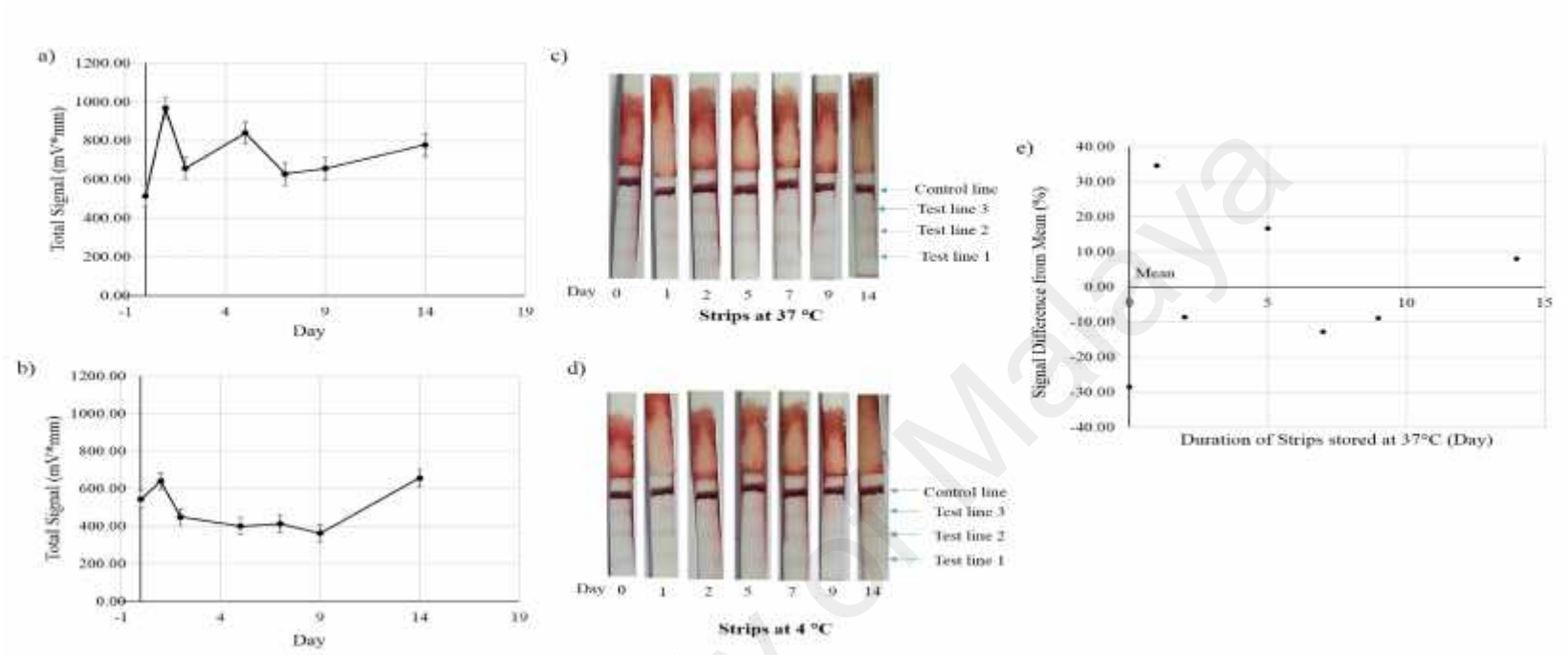

Figure 4.7: Stability Test

(a) Stability test at +37°C, Total Signal versus Day. (b) Stability test at 4°C, Total Signal versus Day. (c) Visual representation of developed immunosensors tested with 1:5 diluted calibrator (7.6% HbA1c) across 14 days, at 37°C. (d) Visual representation of developed immunosensors tested with 1:5 diluted calibrator (7.6% HbA1c) across 14 days, at 4°C. (e) Signal difference from Mean versus Strips stored at 37°C (Days).

4.4 Conclusions

In this chapter, optimization on the dilution factors was demonstrated to affect the performance of the calibration curve generated. The ideal dilution factor to perform the calibration curve was found to be at 1:5 dilutions, using the hemolysis reagent (distilled water added with blood stabilizers). With the calibration curve that related the signal intensity generated on the developed lateral flow immunosensor to the HbA1c levels (%), the quantitative analysis on HbA1c levels was made possible. Tested in a clinical setting and validated as an effective device by performing comparison to the Bio-Rad Variant II-HPLC and DCA Vantage, the developed hemoglobin A1c lateral flow immunosensor was proven to be a potentially useful as a simple point-of-care device for individual diabetic care. With the stable analytical performance and the ease of performing the test without professional personnel, the developed lateral flow immunosensor offers an alternative over the commercialized point-of-care device or HPLC methods that are not only costly but also involved patient compliance to routine visit to clinics and hospitals. In short, the developed lateral flow immunosensor was capable of performing both semi-quantitative (from lines number and intensity of test lines) and quantitative measurements.

CHAPTER 5: DEVELOPMENT OF A NEW DESIGN OF LATERAL FLOW IMMUNOSENSOR FOR SIMULTANEOUS MEASUREMENT OF HEMOGLOBIN A1C AND TOTAL HEMOGLOBIN

5.1 Introduction

In previous chapter 4, quantitative analysis on HbA1c levels was performed in a clinical setting using prototype 1 discussed in chapter 3. With the calibration curve that established the relationship between signals observed and the HbA1c levels, HbA1c levels determined in the clinical setting was absolute level of HbA1c. While prototype 1 yielded good analytical performances and demonstrated practical quantitative capability in a clinical setting, its functionality can be enhanced with the incorporation of the idea that HbA1c is a fraction of total hemoglobin. By measuring the ratio of HbA1c to total hemoglobin, the HbA1c level obtained in percentage is a more useful result. Such occasion is especially useful in heritages where the morbidity such as sickle cell anemia that results in high turnover rate of hemoglobin is prevalent. Prototype 1 in previous chapter 3 that only measure absolute HbA1c level will be potentially affected in the presence of the sickle cell anemia because of the decreased amount of hemoglobin. End-users may encounter false negative results because the HbA1c levels could be falsely low.

To resolve the concern of potential false negatives results while using the developed lateral flow immunosensor, the idea of measuring HbA1c as a fraction to total hemoglobin was incorporated in the chapter. This part of the project was aimed to develop a lateral flow immunosensor to direct detect both total hemoglobin and HbA1c in human whole blood, without sample pretreatment. Unlike the reported lateral flow assays that utilized the biomimetic boronate groups which could bind to all glycan moieties readily co-exist with HbA1c in whole blood, this part of the study will involve

a sandwich immunoassay that was known to be the more selective and sensitive format to probe for multi-epitopes antigens (Ngom *et al.*, 2010) such as hemoglobin. To enable the detection of HbA1c and total hemoglobin to be conducted simultaneously, this part of the project involve test strip that was designed to comprise a Hb line, 3 test lines for specific detection of HbA1c, and a control line. When hemoglobin species (including non-glycated and all other glycated hemoglobin species) did not participate in the sandwich immunoreaction at the HbA1c test lines 1, test lines 2 and test lines 3 (the reaction zone where only HbA1c binds and reacts with capturing and detecting antibodies to show a signal), the hemoglobin species will bind to the polyclonal anti-hemoglobin antibodies (goat) at the Hb line (Figure 5.1).

In this chapter, the optimal detection format for HbA1c measurement as a fraction of total hemoglobin was investigated. Also, the selectivity towards HbA1c against common Hb species (namely HbA0, glycated HbA0, and HbA2), the consistency of the results observed when blood samples were stored for a week, and the potential interference from variants were studied. Prior to the application of this approach, a linear relationship between the ratio of HbA1c to total hemoglobin versus % HbA1c was established, and later, % HbA1c was determined in the whole blood samples based on the established relationship.

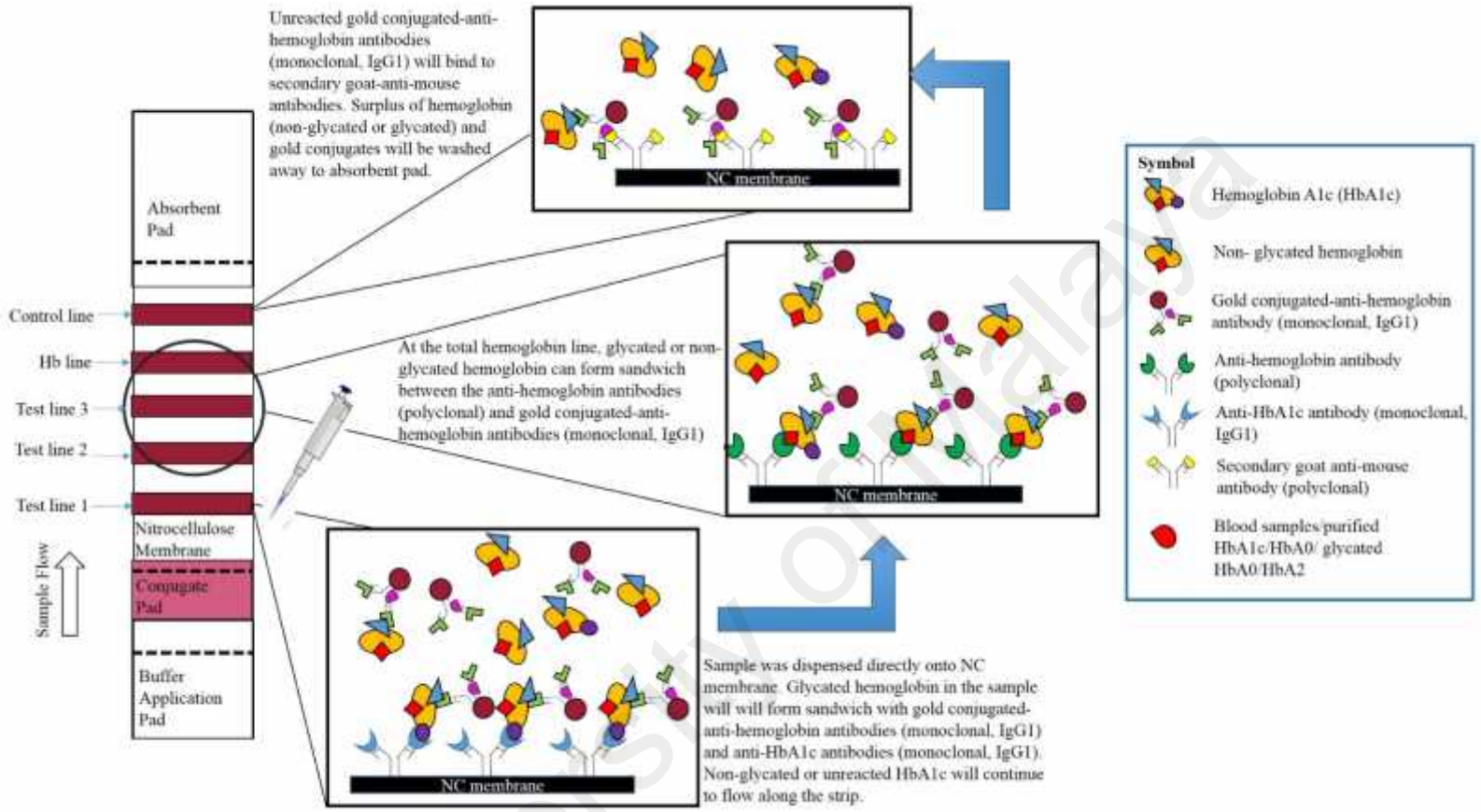

Figure 5.1: Simultaneous Detection of HbA1c and Total Hemoglobin

Determination of HbA1c as a fraction of total hemoglobin in one test run. Only a simple washing procedure was required to complete the test and perform a quantitative and semi-quantitative measurement.

5.2 Materials and Methods

5.2.1 Chemical and Materials

The capturing antibody at test lines 1 to 3, the anti-hemoglobin A1c antibody (mouse, IgG1); the capturing antibody at the Hb line and the detecting antibody (conjugated to gold nanoparticles), the polyclonal anti-hemoglobin A1c antibody (goat); and the secondary antibody at the control line, the polyclonal rabbit-anti-goat antibody; the purified reagents such as HbA0, glycated HbA0, HbA2, and HbA1c, were all purchased from the Fitzgerald Industries International (Acton, MA, USA).

5.2.2 Apparatus

Sigma 4-16k refrigerated table top centrifuge (SciQuip Ltd., Newtown, Wem, Shropshire, SY4 5NU) was used to synthesize gold conjugates in large scale. ESEQuant lateral flow reader was used to measure signal generated on the developed lateral flow immunosensor.

5.2.3 Conjugation of Detecting Antibodies to Gold Nanoparticles

In this part of the project, instead of conjugating gold nanoparticles to monoclonal anti-hemoglobin antibody (IgG1) in previous chapter 3, polyclonal anti-hemoglobin antibody (detecting antibody) was chosen to be conjugated to the gold nanoparticles (40 nm). In order to generate stable gold conjugates for later part immunoreactions, pH and final concentration of the detecting antibody (polyclonal anti-hemoglobin antibody) were optimized according to the ideal condition established in chapter 3. Gold conjugates were prepared on a large scale using 10 mL of colloidal gold nanoparticles adjusted to pH 8.0 and polyclonal anti-hemoglobin antibody at a final concentration of

30.0 μg mL^{-1}. To allow the coupling reaction between colloidal gold and the added antibody (polyclonal anti-hemoglobin antibody), the gold conjugates were incubated for 1 hour with gentle shaking (100 rpm) at room temperature. Next, BSA at a final concentration of 1% (w/v) was added to the gold conjugates. An additional hour of gentle shaking was allowed for optimal mixing of gold conjugates with BSA. The gold conjugates were centrifuged at 10,000 rpm for 30 min at 4 °C to yield a soft pellet, and the supernatant was discarded. The purified gold conjugate pellet was then resuspended in 10 mM of phosphate buffer containing 1% (w/v) BSA and was stored at 4 °C for future use.

5.2.4 Construction of Hemoglobin A1c Lateral Flow Immunosensor

The hemoglobin A1c lateral flow immunosensor was constructed with the assembly of a buffer application pad, conjugate pad, laminated nitrocellulose membrane, and an absorbent pad (Figure 5.1). Unlike the prototype developed in chapter 3, this prototype was designed to comprise of five lines, which included using three types of capturing antibodies. The three test lines closest to buffer application pad were coated with the capturing antibodies specific towards HbA1c, the anti-HbA1c antibodies (monoclonal, IgG1). The fourth line, the Hb line, was coated with another capturing antibody, the anti-hemoglobin antibodies (polyclonal, goat). The line to the end of the nitrocellulose membrane (closest line to absorbent pad) was coated with the third capturing antibody, the secondary polyclonal rabbit-anti-goat antibody. The antibodies were lined manually using a pipette. Later, the lined strips were dried in a desiccator for 30 min. Then, the dried strips were blocked with 1% (w/v) western blocking reagent in 10 mM phosphate buffer. The gold conjugated-polyclonal anti-hemoglobin antibody (goat) (used as the detecting antibody) were diluted to the optical density of 8 in 10 mM phosphate buffer

supplemented with 10 % (w/v) sucrose, and were deposited onto the glass fiber conjugate pad to dry overnight in a desiccator. The full lateral flow immunosensor was then assembled with the cut buffer application pad, dried conjugate pad, and laminated nitrocellulose membrane with an absorbent pad (in 4 mm width). Overlap between the conjugate pad and nitrocellulose (NC) membrane was optimized at 1 mm to allow sufficient time for immuno-complexes formation (between HbA1c and monoclonal anti-HbA1c antibody) before the gold conjugates reached the test lines, while the NC membrane and absorbent pad overlapped at 2 mm.

5.2.5 Optimization of the Detection Format for the Sandwich Immunoassay

The detection format of the testing zone (on NC membrane) was optimized to develop a functional sandwich immunoassay that could detect both the total hemoglobin and HbA1c simultaneously. Two types of formats were tested, one with an Hb line at the first (closest to the conjugate pad), while the other with an Hb line towards the end of the strip (closest to the control line). Two formats were tested with 3 different % HbA1c blood samples (5.3%, 6.5%, and 9.0% of HbA1c, diluted 1:5 with hemolysis reagent). After depositing the diluted blood onto the NC membrane, washing buffer was added to complete the assay. Patterns of signals generated on the strips were observed to determine the ideal detection format that allows the detection of the total hemoglobin and HbA1c simultaneously.

5.2.6 Selectivity Test

Because the polyclonal anti-Hb antibody (detecting antibody)-functionalized colloidal gold could easily bind to all kinds of glycated and non-glycated hemoglobin species, it is critical to ensure that the capturing anti-HbA1c antibodies only recognize the glycation site that is specific to HbA1c, the valine at N-terminal of β subunits, where the transduced signal originated only from the bound HbA1c.

The assembled hemoglobin A1c immunosensor was tested for its selectivity towards HbA1c using purified HbA0, glycated species of HbA0, HbA2 and HbA1c. All purified reagents were prepared in 10 mM of phosphate buffer to the final concentration of 0.1 mg mL^{-1}. The developed lateral flow HbA1c immunosensor was tested in triplicate for 10 μL of diluted purified HbA1c. After directly dispensing the purified HbA1c at the NC membrane, the test strips were washed with washing buffer (50 mM phosphate buffer containing Tween-20) to mobilize the gold conjugates to the NC membrane. Further washing was performed to eliminate background and to flush away the surplus of reagents (or unreacted ones) to the absorbent pad. The ESEQuant lateral flow reader was then used to quantitatively measure the signal generated on the strips.

5.2.7 Investigation of the Potential Interference from Variants

Three variants blood samples, HbE, HbJ, and HbH, with the anticoagulant, EDTA, were collected from the hematology lab after the variant types were being verified using the Bio-Rad Variant II HPLC. The three samples were collected and tested on the 7^{th} day after the withdrawal from patients or donors. The blood sample with elevated HbF was discovered incidentally during the study and was tested on the same day right after the withdrawal from the patient. All four samples (three variants and the elevated HbF sample) were diluted at 1:10 with hemolysis reagent. Then, 10 μL of diluted blood was

dispensed directly onto the NC membrane. All strips were washed with phosphate buffer containing Tween-20. The patterns generated on the strips were observed and the signal on the strips was measured with ESEQuant lateral flow reader.

5.2.8 Storage Stability study

Fresh blood samples with EDTA anticoagulant were collected from the University of Malaya Medical Center (UMMC) and tested on the same day (denoted as Day 1 in the study). One blood sample with 6% HbA1c was collected from UMMC and stored at 4°C over the course of 7 days. During the week, the same blood sample was retrieved from the storage and was tested on the fabricated strips on different days (day 1, 2, 3, 4 and 7). Throughout the study, the sample was diluted at 1:5 with hemolysis reagent. The diluted sample was dispensed directly onto the NC membrane. Later, the strips (triplicates for day) were washed with 50 mM phosphate buffer containing Tween-20. The signal generated on the strips were observed and measured using the lateral flow reader. A two-tailed t-test was performed to determine the significance of the difference in signal observed during the course of 7 days.

5.2.9 Reproducibility Study

Nine lateral flow immunosensors were tested with the diluted blood sample of 5.0% HbA1c (determined by Bio-Rad Variant II HPLC) to determine the reproducibility of the hemoglobin A1c immunosensor. Blood samples were diluted at 1:5 with hemolysis reagent (distilled water with blood stabilizers) and were dispensed directly onto the NC membrane. Washing buffer was added as the diluted blood and gold conjugates moved to the NC membrane. Signals were obtained from the nine replicates using the ESEQuant lateral flow reader.

5.2.10 Generation of Calibration Curve

The calibration curve was generated based on the HbA1c/ total hemoglobin ratio as a function of % HbA1c. To factor in all pre-existing interfering agents in the whole blood, the calibration curve in this part of the project was constructed using whole blood samples (EDTA as the anti-coagulant) collected from UMMC. Signal intensity for the test lines and Hb line was measured for each sample collected and the ratio was calculated. All whole blood samples were diluted with the hemolysis reagent (distilled water with blood stabilizers) and 10 μL of the diluted blood sample was deposited directly onto the NC membrane, where the washing buffer will mobilize the gold conjugates to complete the immunoreactions. Signals generated were measured with the ESEQuant lateral flow reader. Calculated ratio of HbA1c/total hemoglobin was then plotted against the % HbA1c predetermined using the Bio-Rad Variant II HPLC.

5.3 Results and Discussions

5.3.1 Principle and Designs of Sandwich Immunoassay

Figure 5.2 shows two different types of detection formats. By applying the alternate position of the Hb line as either before or after the three test lines, the pattern observed on the strips was shown to be differed significantly. The efficiency of the two designs in distinguishing different levels of HbA1c was tested by using 10 μL of diluted whole blood samples (1:5 dilution with hemolysis reagent) at 5.3%, 6.5%, and 9.0% HbA1c. In Figure 5.2b, a type 1 design with the Hb line allocated before the three HbA1c test lines, showed an increased intensity with increased % HbA1c in blood samples. However, all test lines coated with anti-HbA1c antibodies (test line 1 to 3, Figure 5.2a) were too pale to be observable. From the observations, it was presumed that, with the Hb line set as the first line before the three HbA1c test lines on NC membrane, the assay

captured most of the hemoglobin in blood samples, inclusive of HbA1c. When most of the HbA1c was captured at the first line coated with polyclonal anti-Hb antibodies, only a negligible amount of HbA1c passed through the immuno-complexes formed on the Hb line to reach the subsequent three HbA1c test lines for further reaction with anti-HbA1c antibodies (monoclonal, IgG1). In contrast, with the type 2 design, which the Hb line was placed closest to the control line, an increase in intensity and number of HbA1c test lines appeared in response to the increase in % HbA1c. When the Hb line was positioned after the three HbA1c test lines, HbA1c in the whole blood will encounter and subsequently react with the anti-HbA1c antibodies first. The consequent saturation of the binding sites at the first test line allowed the unbound HbA1c to migrate to the following second and third test lines to involve in further immunoreactions. All the unreacted hemoglobin (non-glycated and glycated) is then flushed to the last line, the Hb line, where binding occurs with the polyclonal anti-Hb antibodies. From the detection format optimization studies, it was noticed that the intensity gaps were sufficiently large with increasing % HbA1c, therefore, allowing visual interpretation for the recognition of distinct HbA1c ranges (normal, less than 6.5% HbA1c; under control, 6.5%- 7.0% HbA1c; and elevated, > 7.0% HbA1c); an observation similar to what was reported in chapter 3. To summarize, type 2 format with the Hb line positioned after the three HbA1c test lines, was chosen as the ideal detection format to allow for simultaneous detection of HbA1c and total hemoglobin.

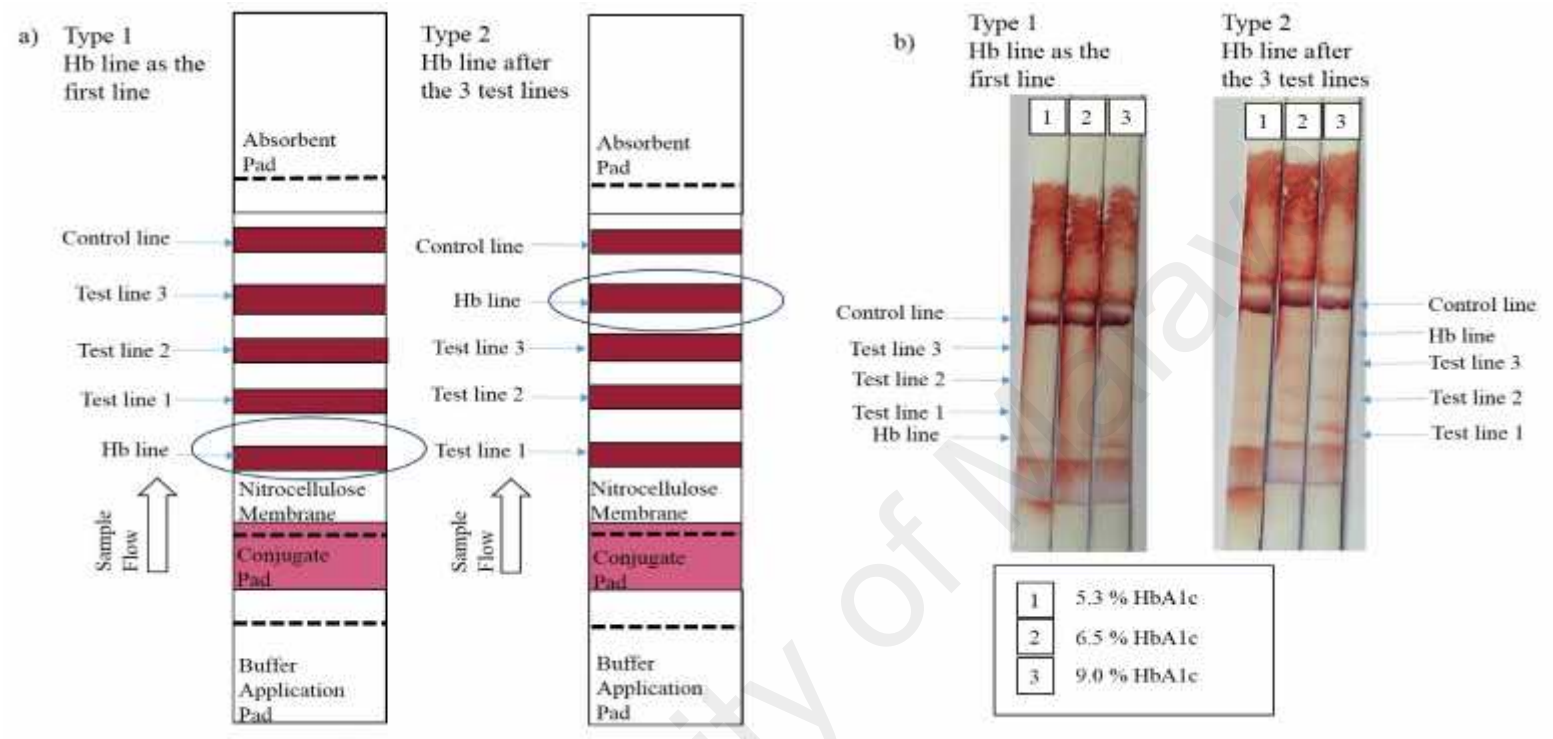

Figure 5.2: Format Design Optimization

a) Designs of the Type 1 and Type 2 detection format, with the Hb line positioned either before or after the three HbA1c test lines. b) Whole blood testing with increasing % HbA1c using the type 1 and type2 design. Type 2 design shows increasing signal intensity with an increase in HbA1c levels, allowing the recognition of distinctive HbA1c groups (<6.5%, 6.5%-7.0%, and >7.0% HbA1c), which are in good agreement with the observations of a previous study *via* visual interpretation (please refer to chapter 3).

5.3.2 Selectivity Study

In the selectivity studies, Figure 5.3 shows only purified HbA1c showed responses on test lines 1 till 3, where monoclonal anti-HbA1c antibodies were coated on the strip. The rest, tested with HbA0, glycated HbA0, and HbA2, yielded no results on test line 1 till 3. These observations verified that the design of a sandwich immunoassay was highly selective towards HbA1c which contains glycation sites on valine residue of the N-terminal β chains. Other glycation sites on hemoglobin were not recognized and a "sandwich" was not formed even with the binding and the formation of immuno-complexes with the polyclonal anti-Hb antibodies (detecting antibody) - functionalized colloidal gold.

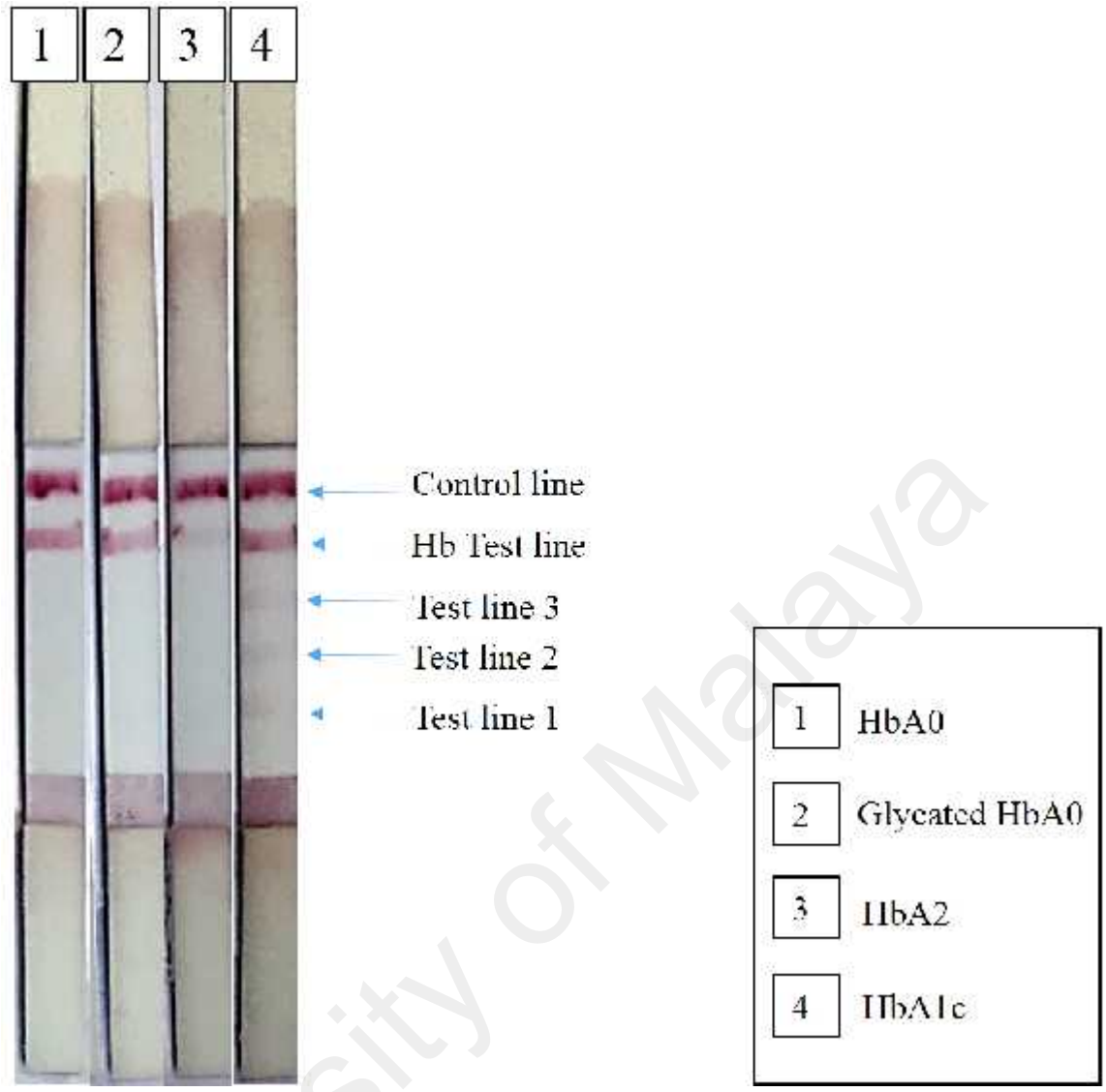

Figure 5.3: Selectivity study.

Four purified cross-reactants were tested on the developed lateral flow HbA1c immunosensor. The only strip tested with purified HbA1c showed responses on the test lines, indicating a high selectivity towards HbA1c against HbA0, glycated HbA0, and HbA2.

5.3.3 Determination of the Potential Interference from Variants

Figure 5.4 demonstrates that at same HbA1c level (5.5% HbA1c, strip 1), the Hb line on strip 2 (tested with sample of elevated HbF, 5.5% HbA1c) was relatively more intense (483% higher in intensity), and the test line 1 till 3 were comparatively paler (80.2% paler than normal 5.5% HbA1c) than strip 1. These observations were in concordance with those reported previously by Rohlfing *et al.* on DCA 2000, an immunoturbidimetric method (immunoassay), where the presence of elevated HbF artificially lowers the % HbA1c (Curt L. Rohlfing *et al.*, 2008). In fact, HbF comprises $\alpha\gamma$ subunits where glycan moieties differ from HbA1c, and studies have shown that glycine at the γ subunit was more readily acetylated than glycated (Curt L. Rohlfing *et al.*, 2008), suggesting that the observation of lower intensity on test line 1 till 3 was expected, especially when the monoclonal anti-HbA1c antibodies used were proven to be highly selective towards the glycation sites at β subunit, N-terminus valine residue. Also, the sample with elevated HbF yielded a significantly higher intensity on the Hb line than the control (strip 1, Figure 5.4), which was similarly tested with 5.5% HbA1c blood sample (measured by Bio-Rad Variant II HPLC). While the elevated HbF artificially lowered the HbA1c level detectable on the strip, the population of HbA still constituted a relatively large portion of the whole hemoglobin population in the sample, therefore the higher intensity at the Hb line was observed.

Three more variants, HbH, HbE, and HbJ were also tested on the developed lateral flow HbA1c immunosensor to observe a distinctive pattern associated with different variants. With HbJ tested on strip 3, test lines 1 till 3 remained visible. When compared to strip 2 that was tested with elevated HbF, the intensity of the Hb line on strip 3 tested with HbJ was lower. HbJ is a variant that contains $\alpha\beta$ chains such HbA, except it consists of a mutation that could result in the gain of negative charge on the hemoglobin molecules, thereby affecting the cation-exchange method and resulting in artificially low HbA1c

values (Bhat *et al.*, 2012). Similarly, Tsai *et al.* reported falsely low HbA1c levels in the presence of HbJ, when the detection of HbA1c was performed with the cation-exchange method (Tsai *et al.*, 2001). This prototype of lateral flow immunosensor, which utilizes highly selective monoclonal anti-HbA1c antibodies, was shown to be partially affected by the presence of HbJ, resulting in a falsely low HbA1c level when measured using the pre-established calibration plot (section 5.3.6).

On the other hand, a strip tested with HbE showed relatively similar intensity on the Hb line as compared to strip 3 tested with HbJ and strip 5 tested with HbH. With the intensity of test lines 1 till 3 observed was considerably less intense (presumably due to pre-analysis handling) than the normal whole blood sample, it was presumed that HbE contributed to partial interference on the strip. In fact, HbE possessed a mutation farther away from the N-terminal of β chain (R. R. Little & Roberts, 2009) that, in return, could lead to structural incongruence with the anti-HbA1c antibodies to a certain extent. Similar observations also applicable on strip 5 tested with HbH, a variant that possess a mutation on the α subunit. The HbA1c level measured in ratio was falsely low with the HbH, similar to the observation by Pravatmuang *et al.* (Pravatmuang *et al.*, 2001). While Pravatmuang *et al*. presumed that a low HbA1c level in the presence of HbH variant was owing to the polymerization of β_4 that was eluted as the non-quantitating area in HPLC method, in this study, it was speculated that the polymerization of β_4 could potentially lead to structural changes where the glycated site on the β chain could be harder to probe for. In addition, because the HbJ, HbE, and HbH samples were collected and tested on the 7^{th} day after blood withdrawal from the patients, it was believed that the pre-analysis storage, handling, and transportation time could contribute to the lower intensity that was observed.

Nonetheless, based on the findings of the specimen storage stability test (section 5.3.4), which indicated that the ratio remains constant across 7 days, it was concluded that all three variants interfere with HbA1c measurement, resulting in a falsely low HbA1c level. While the pattern on the test lines somehow remained intact with the presence of variants (end user can still judge the HbA1c level from visual interpretation of test lines that are visible), the intensity of test lines 1 to 3 could be affected. On the other hand, while the presence of the variants was shown to interfere with the lateral flow HbA1c immunosensor, this prototype allows end-users to distinguish potential variants and samples with elevated HbF from normal blood samples (particularly when samples have elevated HbF, as indicated by a distinctively intense Hb line).

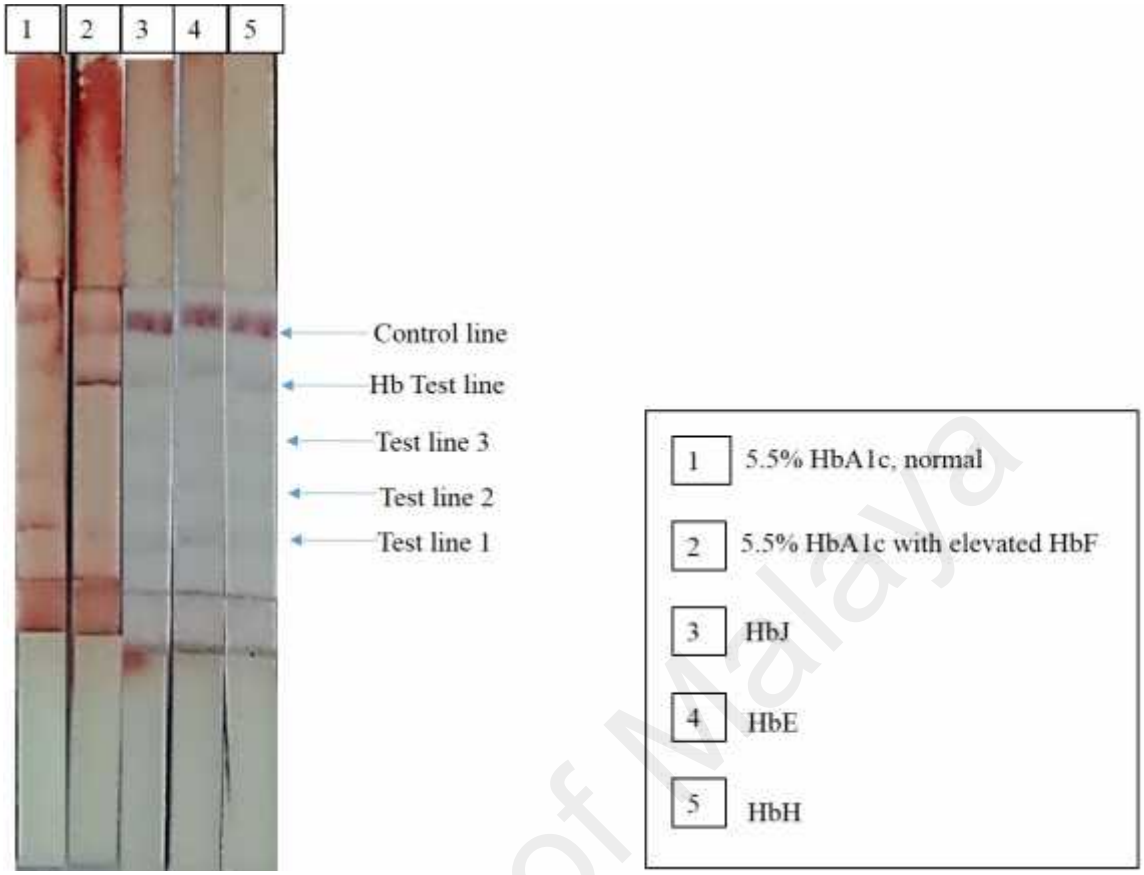

Figure 5.4: Variant Interference study.

Variant interference study conducted with a sample of elevated HbF, HbJ, HbE, and HbH. All the variants interfered with the measurement of HbA1c, resulting in a falsely low HbA1c level.

5.3.4 Storage Stability Test

To investigate the effect of the storage period of blood sample on the fabricated immunosensor, the storage stability test was conducted. Figure 5.5 shows that the signal generated on the strips using the same 6% HbA1c blood sample, over the course of 7 days, showed reasonably consistent results *via* visual interpretation. By measuring the signal generated on the strips, a slight decrease in signal intensity was recorded starting from day 4, indicating that HbA1c in the sample started to deteriorate. To investigate the significance of the signal intensity differences observed during the study, a two-tailed t-test was performed, comparing each point of the testing day to that of day 1. From the two-tailed t-test, it was found that there was no significant difference (all t Statistical values fell within the range of the ± t critical two tail) in the HbA1c measurement between day 1 and each testing day for 7 days (Table 5.1a to 5.1d), similar to that of the observation by Rohlfing *et al*. (C. L. Rohlfing *et al.*, 2012). While the signal intensity was comparatively lower (Figure 5.5b) than the first day of testing, the ratio of HbA1c to total hemoglobin remained reasonably constant. From the ratio obtained, the % HbA1c was determined using the calibration curve established, where the % HbA1c was found to lie within 5.2%-6.2% HbA1c, as compared to the real value of 6% HbA1c (Fig. 5.5a). After storing the sample for 7 days, measurement of % HbA1c provided a good estimation, and no false positive results (≥ 6.5% HbA1c) were determined. These observations reflected that the sample remained stable at 4°C storage and there would be little to no effects on the measurement of % HbA1c if the test was conducted within 7 days with the blood withdrawn from patients and stored at 4°C. Therefore, to assure precise and accurate measurement of HbA1c levels, a 7-day window was suggested for whole blood samples stored at 4°C to be tested on the developed HbA1c lateral flow immunosensor. Unlike Lakshmy and Gupta (Lakshmy & Gupta, 2009) that reported HbA1c to be stabled for up to 15 days in dried blood samples

(as measured with an immunoturbidimetric method), and Rohlfing *et al.* that reported a storage stability of HbA1c in whole blood for as long as 14 days when stored at 4°C (C. L. Rohlfing *et al.*, 2012), 7 days of storage at 4°C was suggested because it was a practice that blood samples were kept for as long as 7 days in the UMMC before they were discarded.

Table 5.1: Student's T-Test for Blood Stability Stored at 4°C Over Seven Days.

a) t-test on strips tested with blood stored at 4°C on Day 1 and Day 2. b) t-test on strips tested with blood stored at 4°C on Day 1 and Day 3. c) t-test on strips tested with blood stored at 4°C on Day 1 and Day 4. d) t-test on strips tested with blood stored at 4°C on Day 1 and Day 7.

a)

	Day 1	Day 2
Mean	0.749639732	0.795628283
Variance	0.005100754	8.82375E-05
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	-1.105780355	
P(T<=t) one-tail	0.192017845	
t Critical one-tail	2.91998558	
P(T<=t) two-tail	0.384035691	
t Critical two-tail	4.30265273	

b)

	Day 1	Day 3
Mean	0.749639732	0.772827069
Variance	0.005100754	0.002749457
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	-0.453284462	
P(T<=t) one-tail	0.336922525	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.673845049	
t Critical two-tail	2.776445105	

c)

	Day 1	Day 4
Mean	0.749639732	0.708256357
Variance	0.005100754	0.002924729
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	0.800112311	
P(T<=t) one-tail	0.234234508	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.468469017	
t Critical two-tail	2.776445105	

d)

	Day 1	Day 7
Mean	0.74964	0.731711
Variance	0.005101	0.001997
Observations	3	3
Hypothesized Mean Difference	0	
df	3	
t Stat	0.368591	
P(T<=t) one-tail	0.368454	
t Critical one-tail	2.353363	
P(T<=t) two-tail	0.736908	
t Critical two-tail	3.182446	

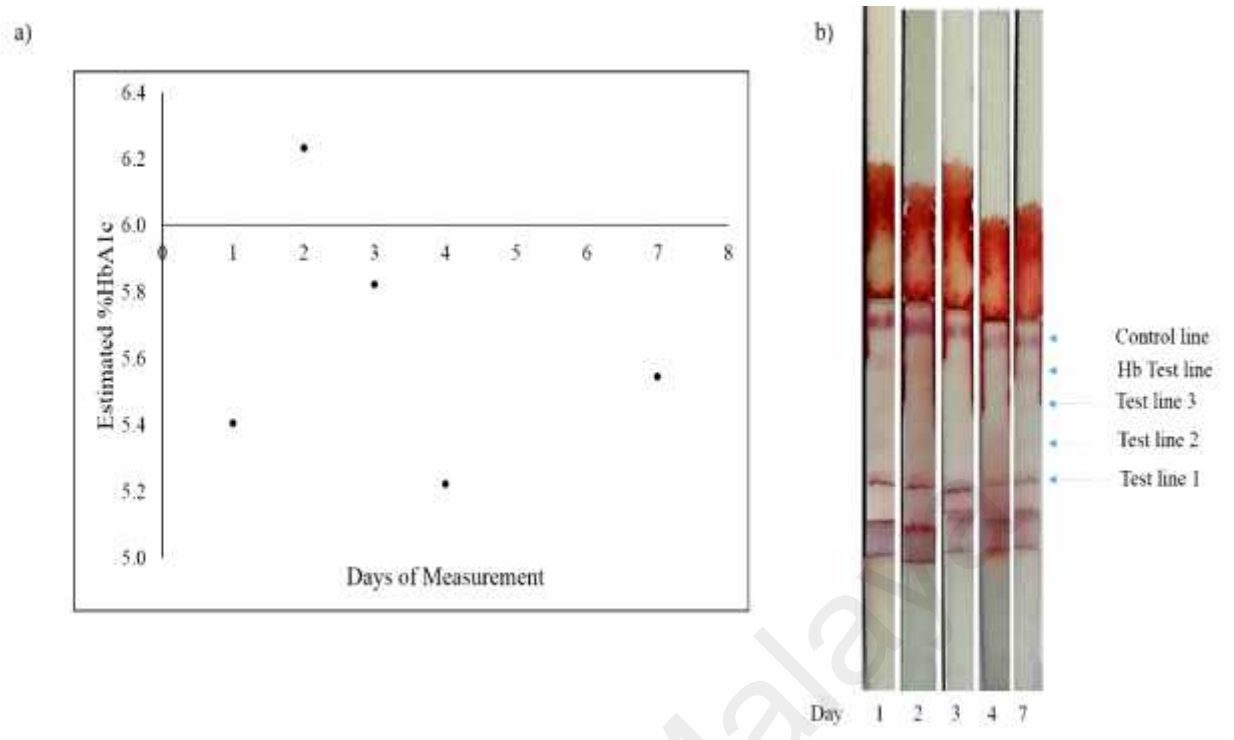

Figure 5.5: Storage Stability Test

A study was conducted over the course of 7 days to determine the consistency of the signal generated signal from the same sample after storing at 4°C for a week. (a) The estimated % HbA1c versus Days of Measurement. The estimated % HbA1c over the course of 7 days was stable. Little to no effect was observed on the estimated % HbA1c using our immunosensor if the sample was stored at 4°C for a week and tested within that week. (b) No significant difference (Table 5.1) was found between the signals generated on the first day of collection versus the signal generated at every point of the testing day.

5.3.5 Reproducibility

Figure 5.6 shows the coefficient of variation (CV) of the lateral flow immunosensor was found to be 6.02% in the reproducibility test, an improvement compared to previous prototype as discussed in chapter 4. Therefore, signals generated from the immunosensor with the same % HbA1c are expected to be consistent and precise. Compared to other reported biosensors such as immunoassay microarrays (reported to be 0.19% to 0.82%), (H.-H. Chen *et al.*, 2012) the CV of the 2nd prototype of lateral flow immunosensor was found to be relatively higher. The smaller CV values in immunoassay microarrays reported could be attributed to a smaller number of replicates being tested in the study (n=3). Nevertheless, this prototype of lateral flow immunosensor offered rapidity in measurement and test (20 min) compared to the microarray immunoassay (where 2 hour of incubation is required), demonstrating a more practical application for better on-site facilitation of diabetic care. While comparing the potentiometric method reported (H. Liu & Crooks, 2012), this prototype not only requires less sample volume for testing (2 µL), but also has high selectivity for HbA1c detection. Moreover, unlike the reported potentiometric method, which required pretreatments to remove any interference from redox species and diols that are present in whole blood, this prototype does not require any sample pretreatment. Compared to the separation matrix lateral flow strip, this prototype does not require multistep measurement to obtain a signal for both HbA1c and total hemoglobin; owing to the optimized design for simultaneous detection of both HbA1c and total hemoglobin, signal measurement can be performed in a single step within one test run. Table 5.2 presents a summary of the overall analytical performance of the developed lateral flow immunosensor in comparison to other reported biosensors.

Table 5.2: Comparison of Analytical Performance of the Developed Lateral Flow Immunosensor to those of the Reported HbA1c Biosensors

	HbA1c Lateral Flow Immunosensor (this study)	**Microarray Immunoassay (H.-H. Chen *et al.*, 2012)**	**Potentiometric Method (H. Liu & Crooks, 2012)**	**Separation Matrix (McCroskey & Melton, 2010)**
Bio-recognition material	Antibodies	Antibodies	Boronic acid	Boronic acid
Sample Volume required	2 μL	4 μL	50 μL	2-5 μL
Clinical Testing	Yes	Yes	No	Yes
Pretreatment	No	No	Yes	No
Rapidity of Test	Approximately 20 min	2 hour incubation prior to Test	Approximately 20 min	Approximately 5 min
Sensitivity	NA*	0.20 ng mL^{-1}	NA	NA
Reproducibility	6.02%	0.19- 0.82%	NA	1.7%
Linear dynamic range	4.0–7.0% HbA1c	NA	NA	5–16% HbA1c

**NA-Not Available in the Study*

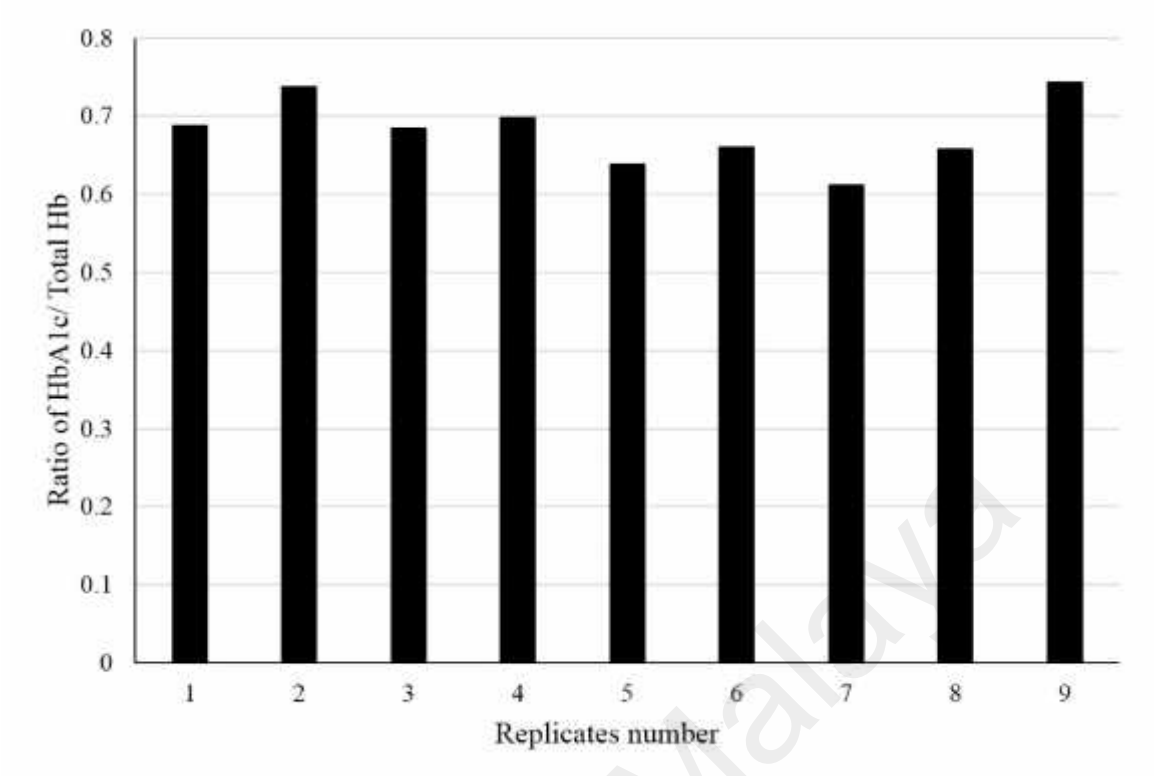

Figure 5.6: Reproducibility Test.

Using this prototype for simultaneous measurement of hemoglobin and HbA1c, we observed a good reproducibility of 6.02%.

5.3.6 Calibration Curve and Quantitative Measurement

Figure 5.7 shows that the linear dynamic range established using whole blood fell ranged from 4% (20 mmol mol^{-1}) to 7% (53 mmol mol^{-1}) of HbA1c, with a coefficient of determination (R^2) of 0.95, indicating a strong linear relationship between HbA1c concentration and the rationalized signal (HbA1c/Total Hb) generated on the lateral flow HbA1c immunosensor. The small value of the slope established with the calibration curve suggested that the changes in HbA1c levels were subtle compared the total hemoglobin concentration, which was consistently constant. While the dynamic range of detection was narrow, the critical treatment goal values of 6.5%-7% HbA1c falling within the linear dynamic range of the immunosensor (4% to 7.0% of HbA1c)

rendered the developed lateral flow immunosensor a useful and potential POC device for diabetes care. For diabetic patients with a poorly controlled glycemic status, especially those with HbAlc levels >6.5% (Asian-Pacific Type 2 Diabtes Policy Group, 2005) (or >7% in the United States) (ADA, 2012)**,** the immunosensor will be able to give close estimation of % HbA1c to indicate medical attention is needed. On the other hand, points marked as 1, 2, 3 and 4 indicate responses obtained for unknown whole blood samples (Figure 5.7). Using the calibration plot, the HbA1c level in the unknown blood sample can be estimated with the signal measured using ESEQuant lateral flow reader. Samples 1, 2, 3, and 4 were tested to be 4.6%, 4.8%, 5.4%, and 5.8% HbA1cHbA1c, while the Bio-Rad Variant II HPLC measured HbA1c levels were 4.5%, 4.7%, 6.0%, and 6.0% HbA1c for sample 1, 2, 3, and 4 respectively. With a two-tailed t-test, the significance of the difference in measurement of HbA1c levels using the developed lateral flow immunosensor versus the Bio-Rad Variant II HPLC was determined. With a p value of 0.79, and t statistical value of -0.28 that fell within ±2.57, it can be concluded that there was no significant difference in the measurement of HbA1c levels using the Bio-Rad Variant II HPLC and the developed lateral flow immunosensor. However, when the estimated 5.4% HbA1c level of unknown sample 3 was compared to HPLC-measured value of 6.0% HbA1c, the 0.6% difference in measurement could be concerning when it comes to diagnosing diabetes. It was believe that with further experimentation on larger sample size (n ≥30), outliers like unknown sample 3 could be addressed and so to improve and tighten the variance between the developed assay and HPLC method to a clinically relevant variance of 3-5% (Sacks *et al.*, 2011).

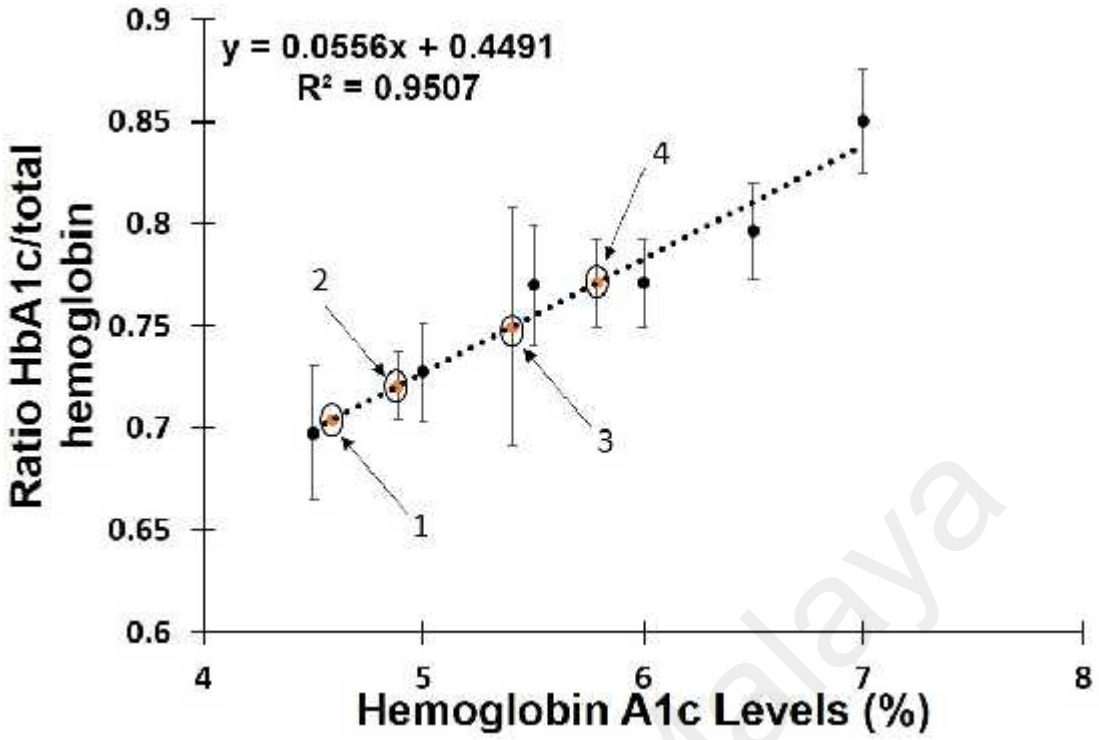

Figure 5.7: HbA1c/total hemoglobin ratio versus % Hemoglobin A1c.

A dynamic range within 4.0% - 7.0% of HbA1c level was established with R^2 of 0.95. Pointer 1, 2, 3, and 4 are unknown samples. Error bars indicated standard deviations with n=3.

5.4 Conclusions

Here, a robust and promising lateral flow HbA1c immunosensor was designed to allow hemoglobin A1c to be measured as a fraction of total hemoglobin within a single test run, involving just washings and single-step measurement. Because of the idea of measuring HbA1c as a fraction of total hemoglobin, a reduction in the immunoreaction between anti-Hb antibodies and hemoglobin, in return, was compensated with the parallel reduction in HbA1c binding for stable measurement of the signal ratio of signals to calculate % HbA1c. The results indicated higher stability of the measurement and accuracy in the calculation of % HbA1c, improving the reproducibility of results.

Moreover, a storage stability test was conducted to validate that the measurement of % HbA1c can be performed in whole blood samples stored up to 7 days at 4°C. Furthermore, the anti-HbA1c antibodies immobilized at the test lines were shown to be highly selective towards HbA1c against all other glycated proteins (HbA0, glycated HbA0, and HbA2). On the other hand, with this prototype, blood samples with the normal hemoglobin were able to be distinguished from that with elevated HbF, judging from the apparent high intensity at the Hb line. However, the presence of hemoglobin variants (HbJ, HbE, and HbH) and elevated HbF affects the measurement of % HbA1c. Therefore, a pre-screening for the presence of variants is highly recommended before performing the definitive test.

With the constructed calibration curve, quantitative analysis of the % HbA1c can be conducted alongside a lateral flow reader. Estimation of the calibration curve on % HbA1c in blood samples analyzed using the developed immunosensor showed no significant difference compared to the Bio-Rad Variant II HPLC. With the more useful relationship established by performing simultaneous detection on HbA1c and total hemoglobin, this 2nd prototype has demonstrated promising role as a simple, useful point-of-care device to aid with diabetes care in under-developed regions of the world.

CHAPTER 6: CONCLUSIONS AND FUTURE PERSPECTIVE

In the project, two prototypes of functional lateral flow immunosensors that could detect and measure the hemoglobin A1c (HbA1c) level in whole blood without elaborated pretreatment were developed. The invented immunosensors were simple, relatively inexpensive, and user-friendly to suit the demand for effective diabetes care in underdeveloped regions of the world.

The developed prototypes of immunosensors operated under the principle of lateral flow; where one was developed to detect absolute levels of HbA1c, while the other was designed to detect and measure HbA1c and total hemoglobin to give a more useful result of HbA1c as a fraction to total hemoglobin. A span of optimization works was involved to get to the stage where the lateral flow immunosensor can be functional in complex biological matrix like blood. Both designs were tested using whole blood and their capabilities to perform quantitative analysis were evaluated. With all the studies discussed in this thesis, it was demonstrated that, both the developed lateral flow HbA1c immunosensor designs have huge potential to be useful as a point-of-care device for better and more effective diabetes care in the near future.

The narrow detection range (4% to 7% HbAc) in both prototypes for whole blood analysis, while inclusive of important treatment goals for diabetes, is a challenging issue. In the attempts to resolve the narrow detection range presumably due to steric hindrance and matrices effects, a more advanced separation matrix for NC membrane and lining with dispensing machine could possibly improve the detection range for the prototypes of lateral flow immunosensors developed in this thesis. Also, to increase the sensitivity of the immunosensors developed, a hierarchiral flowerlike gold nanoparticles instead of the conventional spherical gold nanoparticles can be used (L. Zhang *et al.*, 2015). Other than that, to enhance the signal and further lower the detection limit, different strategies of modification on gold nanoparticles such as amplication method by

means of gold deposition post-assay, rehydration of dry reagents stored in porous media, or by taking advantage on the quenching effect of gold ions on quantum dots (crystalline nanoparticles that fluoresce) (Quesada-González & Merkoçi, 2015).

To resolve the cost issue for a lateral flow reader, it was anticipated that the developed lateral flow immunosensor can be integrated with the smart-phone technologies to provide an all-time facilitation to the end-users, and hence allow a wider implementation of the test in the limited-resources setting for better health care directed to the diabetic population, who often suffer from the lack of proper treatment because of the neglect in constant monitoring of their diabetic status.

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SUPPLEMENTARY

LIST OF PUBLICATIONS

1) Ang, S.H., Thevarajah, M., Alias, Y., Khor, S.M., 2015a. Current aspects in hemoglobin A1c detection: A review. Clinica Chimica Acta 439(0), 202-211.

2) Ang, S.H., Yu, C.Y., Ang, G.Y., Chan, Y.Y., Alias, Y.b., Khor, S.M., 2015b. A colloidal gold-based lateral flow immunoassay for direct determination of haemoglobin A1c in whole blood. Analytical Methods 7(9), 3972-3980.

3) Shu Hwang Ang, Musalman Rambeli, T Malathi Thevarajah, Yatimah binti Alias, Sook Mei Khor. 2016. Quantitative, single-step dual measurement of hemoglobin A1c and total hemoglobin in human whole blood using a gold sandwich immunochromatographic assay for personalized medicine. Biosensors and Bioelectronics. 78: 187-193.

PRESENTATIONS

1) Fabrication of a Potential Point-of-Care Device for Hemoglobin A1c: A Lateral Flow Immunosensor – IICBEE, Feb 11-12, 2015, Penang, Malaysia

2) Hemoglobin A1c Laboratory Instrumentations, Point-of-care Devices, and Biosensors: Challenges and the Role as Useful Diagnostic Tools for Type 2 Diabetes Mellitus – ICMIB, Mar 7-8, 2015, Malacca, Malaysia

3) Exploring Challenges and Issues associated with Hemoglobin A1c as Diagnostic Tool for Type 2 Diabetes Mellitus – ICETA, Apr 22-24, 2015, Taiwan

APPENDIX A

Clinica Chimica Acta 439 (2015) 202–211

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Invited critical review

Current aspects in hemoglobin A1c detection: A review

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is a pressing health issue that threatens global health and the productivity of populations worldwide. Despite its long-recognized role in diabetes management, glycated hemoglobin (HbA1c) only received WHO endorsement as a T2DM diagnostic tool in 2011. Although conventional plasma-specific tests have long been utilized to diagnose T2DM, the public should be informed that plasma-specific tests are not markedly better than HbA1c tests particularly in terms of variability and convenience for diagnosing diabetes. In the midst of the debates associated with establishing HbA1c as the preeminent diabetes diagnostic tool, increasing efforts to standardize HbA1c tests have played an integral part in achieving more efficient communication from laboratory to clinical practice and thus better diabetes care. This review discusses the current status of HbA1c tests in the diagnosis, prevention, treatment and management of T2DM across the globe, focusing on increasing the recognition of glycated hemoglobin variants with effective utilization of different HbA1c methods, updating the current status of HbA1c standardization programs, tapping into the potential of POC analyzers to establish a cost-effective HbA1c test for diabetes care, and inspiring the advancement of HbA1c biosensors for future clinical usage.

Contents

1. Introduction: significance of the study

Type 2 diabetes mellitus (T2DM) is a global epidemic health issue. Approximately 439 million adults (7.7% of the world's adult population aged 20–79 years) are estimated to be afflicted with diabetes by 2030 [1]. As a consequence of population growth, longer life expectancy, and lifestyle changes, the estimated 54% increase in T2DM incidence worldwide by 2030 is concerning [1]. Although the spreading of the chronic disease itself is worrisome, the medical complications and socioeconomic impacts associated with diabetes are as fearful as the disease itself. Individuals with diabetes are at increased risk of developing

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APPENDIX B

Analytical Methods

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A colloidal gold-based lateral flow immunoassay for direct determination of haemoglobin A1c in whole blood

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We developed an immunosensor that operates based on the lateral flow principle designed to detect haemoglobin A1c (HbA1c), a biomarker for type 2 diabetes mellitus in human blood samples. Two different clones of antibodies were used to form a "sandwich" when HbA1c was present. Functionalization of colloidal gold with antibodies was carefully optimized to generate stable gold conjugates to amplify the signal from the formed "sandwich" for the immunoassay. The ideal blocking reagent to minimize background noises, the test line format on the strip, the selectivity of the assay towards HbA1c against HbA0 and glycated species of HbA0, and the potential interference contributed by the colour of the blood sample were investigated. Captured HbA1c on the lateral flow immunosensor can be distinguished based on the number and intensity of the test lines shown; visual detection of the lines shown then indicated the distinctive groups of normal, under control, and elevated levels of HbA1c. Also, a calibration curve that covered the detection range of 4% (20 mmol mol^{-1}) and 12% (108 mmol mol^{-1}) HbA1c was reported, indicating that the prototype can be used for future quantification utilizing a lateral flow reader. The resultant immunosensor was found to report results that were easier to be interpreted and relatively inexpensive compared to electrochemical biosensors developed for the detection of HbA1c.

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1. Introduction

Haemoglobin A1c (HbA1c) has long been used as a biomarker for diabetes management. Haemoglobin in the red blood cells interacts with blood glucose to form glycated haemoglobin (HbA1c), a stable Amadori adduct that serves as a reliable indicator of individual glycemic status over 120 days.[1] Because of its usefulness as a good gauge for diabetes care, HbA1c levels should be constantly monitored. In fact, the methodology for HbA1c detection differs between laboratory and point-of-care testing. Laboratory-based HbA1c detection methods, such as cation-exchange HPLC, affinity chromatography, and capillary electrophoresis,[2] involve bulky and expensive instruments and require a long turnaround time. In contrast, advances in technology that resulted in fast, easy-to-use, point-of-care (POC) HbA1c analyzers offer a better alternative for on-site facilitation to reduce patient inconvenience for better diabetes management.

To enhance the on-site facilitation for diabetes care, the attempt to develop POC devices for HbA1c detection never ceases. In general, HbA1c biosensors can be categorized into biosensors that directly detect HbA1c or fructosyl valine (FV, a peptide generated from HbA1c proteolysis)-based biosensors. With the advent of nanotechnology, more HbA1c biosensors were designed and reported to possess outstanding analytical performance. For example, a FV biosensor (which uses enzymes as the biorecognition element) utilizing a zinc-oxide nanoparticles-polypyrrole film for indirect detection of HbA1c was reported to have enhanced stability and sensitivity compared to previously reported FV biosensors.[3] On the other hand, biosensors that directly detect HbA1c can be further categorized as amperometric, potentiometric, and piezoelectric biosensors and biochips.[4] In fact, with the discovery of more biomarkers for different diseases, efforts to increase the specificity and sensitivity to detect the biomarkers have led immunoassay-based devices to become more clinically relevant.[5] Hence, most of the fabricated biosensors reported that operate based on the electrochemical principle (owing to the ease of miniaturizing the devices at high sensitivity) typically involve anti-HbA1c antibodies as the bio-recognition element. For example, a potentiometric HbA1c immunosensor using mixed SAM (Self Assembled Monolayer)-wrapped nanospheres was reported to possess good consistency in a clinical setting, in a miniaturized form.[6] Other than electrochemical immunosensors, SPR (surface plasmon resonance) biosensors[7] and antibody

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APPENDIX C

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Quantitative, single-step dual measurement of hemoglobin A1c and total hemoglobin in human whole blood using a gold sandwich immunochromatographic assay for personalized medicine

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Single-step dual measurement

ABSTRACT

We describe a gold nanoparticle based sandwich immunoassay for the dual detection and measurement of hemoglobin A1c (HbA1c) and total hemoglobin in the whole blood (without pretreatment) in a single step for personalized medicine. The optimized antibody-functionalized gold nanoparticles immunoreact simultaneously with HbA1c and total hemoglobin to form a sandwich at distinctive test lines to produce visible signals. The applicability of this method as a personal management tool was demonstrated by establishing a calibration curve to relate % HbA1c, a useful value for type 2 diabetes management, to the signal ratio of captured HbA1c to all other forms of hemoglobin. The platform showed excellent selectivity (100%) toward HbA1c at distinctive test lines when challenged with HbA0, glycated HbA0 and HbA2. The reproducibility of the measurement was good (6.02%) owing to the dual measurement of HbA1c and total hemoglobin. A blood sample stability test revealed that the quantitative measurement of % HbA1c was consistent and no false-positive results were detected. Also, this method distinguished the blood sample with elevated sHb from the normal samples and the variants. The findings of this study highlight the potential of a lateral flow immunosensor as a simple, inexpensive, consistent, and convenient strategy for the dual measurement of HbA1c and total Hb to provide useful % HbA1c values for better on-site diabetes care.

1. Introduction

Hemoglobin A1c (HbA1c) plays a central role in the diagnosis and monitoring of type 2 diabetes. A current report from the WHO estimates that 80% of global type 2 diabetes-related deaths occur in low- and middle-income countries (WHO, 2015) in which the market for HbA1c tests is limited by the scarcity of the resources. In these countries, inexpensive HbA1c monitoring tools for personalized medicine are crucial in the provision of in-time treatments that slow disease progression and minimize devastating health, social, and economic burdens in afflicted individuals. The development of inexpensive, user-friendly methods for HbA1c testing reduces implementation costs, increases public access to HbA1c test, and improves disease management through by monitoring the % HbA1c.

Ion-exchange high-performance liquid chromatography (HPLC) and affinity chromatography are generally used to perform HbA1c tests, but the long turnaround time and high implementation cost are incompatible with on-site disease management. Although commercially available point of care devices are designed to be mobile for on-site use, their implementation costs limit the use in underdeveloped countries.

The demand for relatively inexpensive point of care devices for HbA1c measurement in resource-limited regions has driven ongoing attempts to develop more rapid, consistent, easy-to-use, and cost-effective biosensors for HbA1c measurement. Compared with various HbA1c biosensors such as microarrays (Chen et al., 2012), surface plasmon resonance (Liu et al., 2008), and chemiluminescence flow cells (Ahn et al., 2016), electrochemical biosensors are highly favored for HbA1c detection (Xue et al., 2011; Song and Yoon, 2009; Hsieh et al., 2013) because of their compatibility with miniaturization into portable point of care devices (Liu et al., 2012). However, the multiple steps of results interpretation and measurement that require professional personnel increase the implementation costs of this method. Such limitations have driven

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APPENDIX D

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APPENDIX E

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APPENDIX H