The Development of a Rapid Immunosensor for the Quantification of Insulin

by

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Thesis presented in partial fulfilment of the requirements for the degree of Master of Engineering (Mechatronic) in the Faculty of Engineering at Stellenbosch University

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Co-supervisor: Prof. WJ. Perold

April 2019

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Abstract

The Development of a Rapid Immunosensor for the Quantification of Insulin

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Thesis: MEng (Mech)

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Diabetes is a serious, chronic disease that occurs when the pancreas fails to produce enough insulin (in type 1 diabetes), or when the body cannot use the insulin, produced by the pancreas, effectively (in type 2 diabetes). Measuring and diagnosing type 2 diabetes is currently performed by conventional immunoassay techniques which require sophisticated equipment, are extremely time consuming and expensive. This increases the need for a rapid point-of-care device which will enable diabetic patients to monitor insulin levels on a more regular basis and help with the compliance in following strict diets and also improve overall medical healthcare. Immunosensors are compact, analytical devices that detect and measure a specific antigen (e.g. proteins) by using biological receptors, such as antibodies, and specific transducing mechanisms to generate a measurable signal. The Organic Electrochemical Transistor (OECT) has been developed as a promising alternative to conventional immunoassays. The electrochemical signal produced by OECT-based immunosensors is generated as a result of the antigen-antibody binding. OECT-based immunosensors present real-time, rapid results and exhibit high sensitivity and selectivity towards the antigen. In this project an unique inexpensive rapid OECT-based immunosensor was developed, capable of detecting and quantitatively measuring insulin. As part of the research a thorough literature review and background study was undertaken.

Uittreksel

Die Ontwikkeling van 'n Sorgtoestel om Insulien Vinnig te Kwantifiseer

("The Development of a Rapid Immunosensor for the Quantification of Insulin")

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April 2019

Diabetes is 'n ernstige, chroniese siekte wat voorkom wanneer die pankreas nie genoeg insulien produseer nie (in die geval van tipe 1-diabetes), of wanneer die liggaam nie die insulien wat deur die pankreas geproduseer word, effektief gebruik nie (in die geval van tipe 2-diabetes). Die meting en diagnose van tipe 2-diabetes word tans uitgevoer deur konvensionele immunotoetse wat gesofistikeerde toerusting benodig, uiters tydrowend en duur is. Dit verhoog die behoefte aan 'n sorgtoestel wat diabetes in staat stel om insulienvlakke op 'n gereelde basis te monitor, en sodoende te help met die nakoming van 'n streng dieet en ook algemene mediese gesondheidsorg te verbeter. Immunosensors is kompakte, analitiese toestelle wat 'n spesifieke antigeen (bv. proteïene) kan opspoor en meet deur biologiese reseptore, soos teenliggaampies, en spesifieke opneemmeganismes te gebruik om 'n meetbare sein te genereer. Die Organiese Elektrochemiese Transistor (OECT) is ontwikkel as 'n belowende alternatief vir die konvensionele immunotoets. Die elektrochemiese sein wat deur OECT-gebaseerde immunosensors gegenereer word, word gegenereer as gevolg van die antigeen-teenliggaambinding. OECT-gebaseerde immunosensors bied vinnige resultate en vertoon hoë sensitiwiteit en selektiwiteit teenoor die antigeen. In hierdie projek is 'n unieke, koste-effektiewe OECT-gebaseerde immunosensor ontwikkel wat in staat is om insulien te meet en vinnig te kwantifiseer. As deel van die navorsing is 'n deeglike literatuuroorsig en agtergrondstudie onderneem.

Dedications

This thesis is dedicated to my parents:
Kobus and Arlyna van Dyk
For their unconditional love and support

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Nomenclature

Constants

e	=	$1.60\text{x}10^{-19}$ Coulombs
k	=	$1.38\text{x}10^{-23}$ J.K^{-1}

Variables

AR	Area Ratio	[Unitless]
A_{ch}	Channel Area	[m]
A_g	Gate area	[m]
C_c	Channel Capacitance	[F/m^2]
C_g	Gate Capacitance	[F/m^2]
Da	Daltons	[g/mol]
g_m	Transconductance	[S]
I_{CH}	Channel Current	[A]
I_D	Drain Current	[A]
I_G	Gate Current	[A]
K_D	Dissociation Constant	[Unitless]
OD	Optical Density	[Unitless]
R_D	Drain Resistor	[Ω]
R_G	Gate Resistor	[Ω]
R_S	Sheet Resistance	[Ω]
V_D	Drain Voltage	[V]
V_{DS}	Drain-Source Voltage	[V]
V_G	Gate Voltage	[V]
V_{GS}	Gate-Source Voltage	[V]
V_G^{eff}	Effective Gate Voltage	[V]

Greek Symbols

γ	Capacitance Ratio	[Unitless]

Chapter 1

Introduction

In this thesis the proposal for the design and development of a device for the project called, "The Development of a Rapid Immunosensor for the Quantification of Insulin" is discussed.

1.1 Problem Statement

According to the Global Report on Diabetes set out by the World Health Organization (WHO), an estimated 422 million adults globally were diagnosed with diabetes in 2014, compared to the 108 million in 1980 [1]. This increasing number in diagnosed patients reflects an increase in risk factors associated with diabetes such as obesity and high blood pressure. Adding to the risks, 1.5 million deaths occurred in 2012 due to diabetes. [1]. Another 2.2 million deaths occurred caused by high blood glucose, which increased the risks of other diseases, of one which is cardiovascular (heart) disease. From these 3.7 million deaths, 43% were of people under the age of 70 years.

Diabetes is a serious, chronic disease that occurs either when the pancreas fails to produce enough insulin (type 1 diabetes), or when the body cannot use the insulin, produced by the pancreas, effectively (type 2 diabetes) [1]. The incidence of type 2 diabetes can lead to complications in many parts of the body including morbid obesity affecting the psychological health status, hypertension, stroke disorders and vascular insufficiencies to organs such as the eye, kidneys, lower limbs and brain [1]. Diabetes can also lead to premature fatal death during pregnancy. Due to pancreatic exhaustion, the patient will develop type 1 diabetes requiring insulin which will speed up the disease profile mentioned above.

It is currently extremely difficult to distinguish between type 1 and 2 diabetes without the use of expensive and sophisticated facilities and equipment. It is also expensive to regularly measure insulin levels due to the cost and availability of the equipment. The current method and duration of the insulin tests also present great challenges as it is extremely time-consuming to perform

these tests.

1.2 Aims and Objectives

Current methods of diagnosing diabetes and measuring insulin is with the use of immunoassays which are sensitive analytical tests performed by highly skilled personnel and equipment. Immunoassays also require large sample sizes of blood, have long analysis durations and are non-portable. A solution to this that has received much attention during the last couple of years is the use of point-of-care (POC) devices. POC devices require minimal training of personnel, have an inherit rapid response rate, is inexpensive and it can measure multiple biomarkers simultaneously in the case of spreading epidemic.

Point-of-care testing has been integrated into the health care system to offer the ability for health care testing outside of these laboratories. There is therefore an opportunity for these point-of-care devices to substitute the sophisticated instrumentation due to the improvement in repeatability, increase in patient compliance, improved efficiency, effective portability and improved patient outcomes.

The aim of this project is to develop a unique low-cost immunosensor that will be able to detect and measure insulin quantitatively. The advantage of such a device will be an inexpensive, readily available and user-friendly device which can be used as a rapid-diagnostic device to provide better treatment outcomes. The patient will be able to monitor insulin levels more often as it is important for diabetic patients to lower their insulin levels in order to eliminate the extreme health complications.

The objectives of this project are as follows:

1. To develop a rapid immunosensor strip that is capable of measuring recombinant human insulin protein in low concentrations

2. The immunosensor strip should be able to provide results in no more than 5 minutes

3. The immunosensor strip should be easily manufacturable using low-cost fabrication solutions and not exceed a unit cost of \$0.1 per strip

4. The immunosensor should be simple, highly sensitive and specific and reach a limit of detection of 8 μIU$/ml$ (0.3 ng/ml)

Chapter 2

Literature Review

This literature review starts with describing Diabetes Mellitus and how it is diagnosed and controlled. Next, the attention is turned to insulin and how it is secreted and currently measured. Thereafter a detailed description is given of antibodies and how they are applied. Finally immunoassays and immunosensors are discussed and compared with reference to Organic Thin Film Transistors (OTFTs) and printed immunosensors.

2.1 Diabetes Mellitus

Type 2 Diabetes Mellitus, also referred to as non-insulin-dependent or insulin-resistant diabetes, is the subject of the research described in this thesis.

2.1.1 Global Burden of Diabetes

Diabetes imposes a large burden on the global health-care economic system as well as the wider global economy together with the devastating loss of lives. The costs involved with diabetes include the direct and indirect costs, premature mortality (measure of unfulfilled life expectancy) and negative effect of diabetes on the nation's gross domestic product (GDP).

The direct costs associated with diabetes encompasses the expenditures for treating and preventing diabetes e.g. emergency care, medical supplies such as injection devices, self-monitoring devices and costs related to long-term care. The International Diabetes Federation (IDF) estimated the total global health-care direct costs to be more than 827 billion US dollar in 2016, as a result of an increase in diagnosed patients [1]. While the primary diabetes cost drivers are hospital inpatient and outpatient care which are expected to continue to increase, one other contributing factor is the expenditure increase on patented, branded medicines for the treatment of diabetic patients. The indirect costs are related with productivity loss.

Besides the burden on the health-care system, diabetes also imposes a burden on the affected families in terms of higher out-of-pocket health-care payments and loss of family income due to the nature of the disability and premature loss of life [1]. There is also a strong relationship between diagnosed patients with diabetes and the risk of catastrophic medical expenditure. Especially in lower-income countries, health-care payments not paid by medical aid scheme result in increasing medical expenditure.

2.1.2 Causes of Type 2 Diabetes

Type 2 diabetes is caused by impaired glucose tolerance (IGT) which is a prediabetic state of hyperglycemia, an abnormally high blood glucose (sugar) levels in the blood [2]. IGT is caused as a result of insulin resistance with ensuing insulin deficiency impacting skeletal muscle, liver and adipose tissue (fat tissue). Diabetes is also associated with low-grade chronic inflammation that is due to the activation of the innate immune system which is the non-specific defense mechanisms of the human body [2]. The interaction of genetic and metabolic factors determine the risks of type 2 diabetes. Other aspects that influence the severity of type 2 diabetes are ethnicity, family history of diabetes, unhealthy dietary plans, lack of physical activity, smoking and the two strongest risk factors, overweight and obesity.

Obesity is complicated by metabolic dysregulation including hypertension and dyslipidemia (collectively known as the metabolic syndrome) and is the leading precursor to type 2 diabetes [2]. The dyslipidemia involves high levels of circulating fatty acids originating from the patient's diet, and direct exposure of muscle cells to these fatty acids impairs insulin-mediated glucose uptake and leads to insulin resistance [2].

A current method to determine obesity is by calculating the Body Mass Index (BMI). The BMI calculates the relationship between the patient's height and weight and is an indication whether the patient is obese (overweight) for his/her respective height [3]. The BMI is used by health professionals to assess the risk for any chronic disease such as diabetes. The BMI is calculated by dividing the patient's weight (in kilograms) by his/her height squared (in meters squared). A BMI lower than 25 kg/m^2 indicates a healthy patient, a BMI between 25 kg/m^2 and 30 kg/m^2 indicates the patient is overweight and a BMI greater than 30 kg/m^2 indicates serious obesity. Over time diabetes causes damage to the heart, eyes, kidneys and nerves which increases the risk of heart disease and stroke [1]. The damage to the heart can impair blood flow to important body components and together with nerve damage can lead to limb amputation.

2.1.3 Diabetes Measurement and Control

Screening and early detection of type 2 diabetes is essential and enables initiation of patient-centered management to improve glycaemic control and minimize future complications [4]. The optimal management for such patients includes lifestyle interventions such as increased physical activity, healthy diets, weight loss, terminating smoking habits, decrease alcohol intake and glucose-lowering therapies to reach desired blood sugar targets [4].

Diabetes is either diagnosed by measuring the glucose level in a blood sample while the patient is in a fasting state (a willing abstinence from food for a period of time). An alternative method is by measuring glycated haemoglobin (HbA1c). The difference between these two methods is that HbA1c reflects the average glucose concentration over a few weeks and not the glucose concentration at that moment in time. However, HbA1c does not require the patient to be in a fasting state but is more expensive than blood glucose measurements. According to private patient prices of PathCare, a network of pathology laboratories that provide testing of patients' blood (or other specimens) for medical diagnostic purposes, a HbA1c test costs R280 and a glucose measurement costs R71.

Diabetes is diagnosed according to World Health Organization criteria [5] and these criteria include when:

- A fasting plasma glucose is equal to or greater than 7 mmol/L (126 mg/dL).
- The 2-h postchallenge plasma glucose is equal to or greater than 11.1 mmol/L (200 mg/dL).
- HbA1c greater than 6.5% using a DCCT aligned assay.

It is important that the accurate and precise measurement of glucose-concentration is upheld for the management of diabetes. It is assumed that measurement of glucose in blood is straightforward; however, it is influenced by numerous factors such as origin of the sample (arterial, capillary or venous), sample preparation, analytical method and whether plasma, whole blood or serum is used [6].

Glucose is normally measured from venous plasma or capillary whole blood and diagnostic criteria frequently provide equivalence estimates for these two methods [6]. Venous plasma measurements include inserting a Venflon catheter into an antecubital vein and extracting a small amount of blood. Capillary whole blood measurements include using a fine lancet to pierce the skin to extract a drop of blood from the patient's finger tips that can be used to test the glucose concentration from commercial blood glucose monitor devices.

The most common technique for diabetes measurement is the capillary whole blood measurements using the fine lancet and glucose monitor devices. This method is ideal because there are numerous patients who fear needles

which makes venous plasma measurements difficult. Another advantage would mean more regular testing due to the efficiency and simplicity of these devices because they are developed to be used as home tests.

The preferred method of diagnosing diabetes is the fasting insulin test which involves analyzing a blood sample taken from a patient that has fasted for at least eight hours. The main disadvantage of the fasting insulin test is that it can only be performed in health care environments or laboratories that have access to expensive testing facilities and equipment such as liquid chromatography-tandem mass spectrometry. Appendix A presents an ultra-senstive insulin immunoassay test procedure that exhibits the typical procedure for specimen collection and preparation, product information and warnings, required materials and equipment, and finally the testing procedure. It is clear from this document that the current method of measuring insulin requires generally inaccessible equipment and materials which makes it almost impossible to monitor insulin levels at home or without health care facilities.

Another disadvantage is that due to the tests having to be analyzed in laboratories by lab technologists, the test results take extremely long to reach the patient which makes it ineffective in a situation where the insulin level results are required immediately. The cost of a fasting insulin test for a private patient at PathCare is R240 which is more expensive compared to the fasting glucose test. This increases the need for a point-of-care device that is able to provide rapid results at a more suitable price.

2.1.4 Managing Diabetes

The first and most important step to maintaining a good quality of life with diabetes is early diagnosis. If a patient lives undiagnosed, it worsens the health outcomes drastically. It is therefore crucial for all people to have easy access to basic diagnostics which should be available in any health-care setting. The only small advantage of type 1 diabetes over type 2 diabetes is that it presents symptoms that prompt the patient to get help from health-care services. Some of these type 1 diabetes symptoms include thirst and weight loss. Type 2 diabetes, however, does not present these symptoms and the only reason why patients would get help from health-care services is when some severe complications already occur such as vision loss or heart attack.

Some factors of type 2 diabetes are unmodifiable such as genetics, ethnicity and age, while other factors such as being overweight (obese), having an unhealthy diet, a lack of physical activity and smoking can however be managed by the patient through a change in behavior and environment. Type 2 diabetes is generally first treated by the lifestyle modifications such to reduce weight, to improve glycemic control and also to reduce risk factors and health complications [7]. Weight loss is achieved by decreasing the total intake of energy and by increasing effective physical exercise which leads to desired energy expenditure.

When lifestyle modifications are not effective, various anti-obesity drugs can help with diabetes measurement and control. These drugs either stimulate insulin secretion or improve patient sensitivity to insulin by altering the patient's appetite or absorption of calories to ensure the necessary weight loss [8]. It is however not ideal to manipulate one of the main functions of the human body, therefore an appropriate solution would be to have the best dietary recommendations and plans. The focus of dietary plans should be on quality of fat and carbohydrates rather than quantity alone, together with balancing energy intake and expenditure.

There is a progressive decline in β-cell function and insulin sensitivity in type 2 diabetes, which results on deteriorating glycemic control and the constant need to intensify treatment [7]. Dietary adjustments and exercise are sufficient to achieve adequate glycemic control in $<10\%$ of type 2 diabetic patient's therefore an oral anti-diabetic hypoglycemic agent is generally introduced to help with diabetes management [7].

When a patient is diagnosed with diabetes, the patient should have access to systematic, ongoing and organized care, administered by skilled health-care providers [1]. These providers must be able to intervene with medication, health education and follow-up counseling which should improve the outcome of the diagnosis. The effectiveness of the programme depends on the recommendations and treatment given by die health-care providers. These providers should be highly skilled but should also convey to the patient's need to understand the principles and importance of a healthy diet, sufficient physical activity, commitment to medication and the importance of constant assessment of the presence or progression of any complications.

2.2 Insulin

Insulin is a hormone secreted by the pancreas that allows the body to use blood glucose (blood sugar) from carbohydrates in the food intake for energy or to store glucose for when needed in the future [9]. Insulin is the key that unlocks the glucose channel and enables glucose to enter the cell and to be used as energy. Therefore insulin prevents the blood glucose (sugar) level from getting too high or too low.

The pancreas produces more insulin in response to a sugar level spike caused by eating a high energy meal. After insulin opens the channels of the cells and allows the glucose to be used as energy, insulin also encourages the storage of excess glucose as glycogen in the liver, muscle and fat cells [10]. Other functions of insulin include the ability to enhance growth of muscle, enhance learning and memory of the brain functions and modify the activity of enzymes [10].

In the absence of insulin, the body is not able to utilize the glucose as energy which results in the glucose remaining in the bloodstream and leads to high

blood sugar, known as hyperglycemia. The opposite of high blood sugar is low blood sugar which is known as hypoglycemia. Insulin is considered to be the most powerful hormone in the body and it is therefore critical to understand the concept and structure of insulin and how it is secreted inside the body. By diagnosing insulin levels, it is easier to predict the type of diabetes or insulinoma [11]. Even in healthy conditions, insulin levels in blood serum can be in the order of picomolar.

2.2.1 History of Insulin

The discovery of insulin is indisputably one of the greatest achievements in modern medicine. Insulin was discovered at the University of Toronto in 1921 to 1922 after a series of dramatic events in the history of treating diabetes [12]. It all began in Germany in 1889 when Oskar Minkowski and Joseph von Mering discovered that removing the pancreas (pancreatectomy) from experimental animals (specifically a dog) resulted in severe diabetes mellitus which later resulted in death. This lead to the speculation that the pancreas had two functions. The one being digestion and the second was to produce a mysterious substances responsible for metabolic control. During that time people hypothesized that an "internal solution" of the pancreas controls carbohydrate metabolism [12].

Although the development of purifying and modifying insulin took a further 30 years, the discovery of insulin can be attributed to the Canadian surgeon Frederick Grant Banting, a senior physiology professor at the University of Toronto, John James Rickard Macleod, a summer student at the University of Toronto, Charles Best and finally James Bertram Collip who had a PhD in biochemistry from the University of Alberta. Refer to the article by Joshi et. al. for the complete history, biochemistry, physiology and pharmacology of insulin [12].

2.2.2 Insulin Structure

As mentioned earlier, insulin is an essential protein-based hormone secreted by the islet β-cells in the pancreas that allows the body to use blood glucose (sugar) for immediate energy or as storage for future use. Insulin is the key which unlocks the cell to allow sugar to enter the cell and be used for energy. The other functions of insulin include monitoring our metabolism and utilizing the energy from other ingested nutrients. Insulin also stimulates the uptake of glucose by muscle and adipose tissue (body fat), stimulates protein synthesis and glycolysis (the process that is critical to aerobic and anaerobic cellular respiration).

The pancreas is long shaped gland that is situated deep in the abdomen between the stomach and the spine [13]. Most people are uninformed about the importance of the pancreas because it is not felt in our daily lives; how-

ever, the pancreas is a vital part of the digestive system and important for the control of blood glucose (sugar) levels. The pancreas is divided into two functional components. The first functional component is the "exocrine" cells that produce enzymes to help with food digestion. The second functional component is the "endocrine" pancreas that is composed of small islands of cells, called the Islets of Langerhans [13]. These endocrine cells do not release their secretions into the pancreatic ducts such as the "exocrine" cells, alternatively they release insulin hormones into the blood stream which controls the blood glucose levels [13].

Insulin has two precursor molecules namely preproinsulin and proinsulin and is illustrated in Figure 2.1. Preproinsulin is a single amino acid chain prohormone synthesized at the outer (cytosolic) side of the endoplastic reticulum (ER), where it undergoes translational translocation across the ER membrane [14]. The ER has many functions such as synthesis and modification but the most important is that it serves as the intracellular transport system of the cell, primarily transporting proteins. Preproinsulin encodes only 110 amino acids which makes it a very small protein. A graphical illustration of these 110 amino acids of preproinsulin is given in Figure 2.2. The gene for preproinsulin is located on chromosome 11 and therefore also the gene for insulin [7].

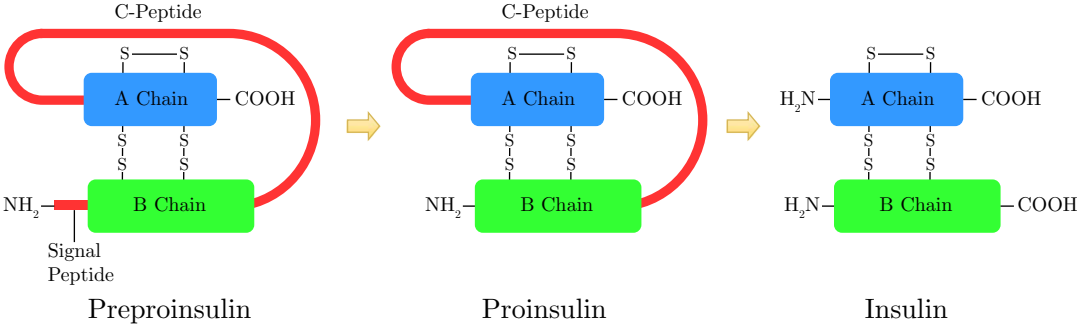

Figure 2.1: Process of insulin synthesis [12]

During this translational translocation across the ER membrane, the precursor preproinsulin loses its encoding signal peptide sequence (24 amino acids) and folds to form three oxidized disulfide bonds (S) to produce human proinsulin (hPI) (refer to Figure 2.1 and 2.2). Proinsulin consists of a polypeptide B chain (30 amino acid), a polypeptide A chain (21 amino acid) and a connecting C-peptide (31 amino acid) [12]. The proinsulin is then transported to the Golgi Apparatus (GA) of the β-cell where it is packaged into immature secretory vesicles or vacuoles. The connecting C-peptide is then excised to form fully bioactive two-chain insulin to be stored in mature vesicles for insulin secretion [14].

Mature insulin has 51 amino acids, 35 fewer amino acids than proinsulin due to the removal of the C-peptide and the excess 4 amino acids (refer to

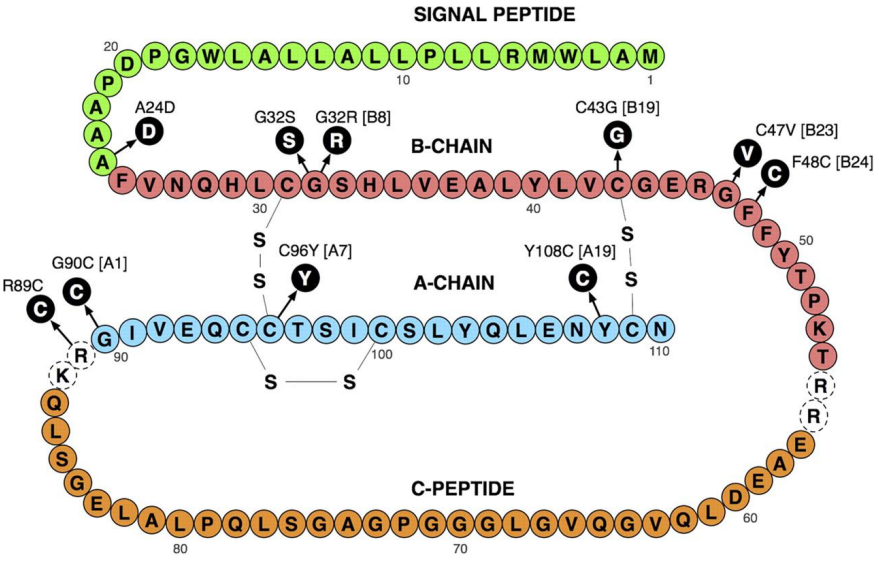

Figure 2.2: Preproinsulin structure [15]

Figure 2.2). The mature insulin also contains the three disulfide bonds (S) between residue A6-A11, A7-B7 and A20-B19 (refer to Figure 2.3). Due to the small size of the insulin structure, insulin only has a molecular weight of 5808 Daltons [9] and can be seen in Figure 2.3. Daltons, also known as the unified atomic mass unit, is the standard unit of measure to quantify mass on a molecular scale. 1 Dalton is also numerically equivalent to 1 g/mol.

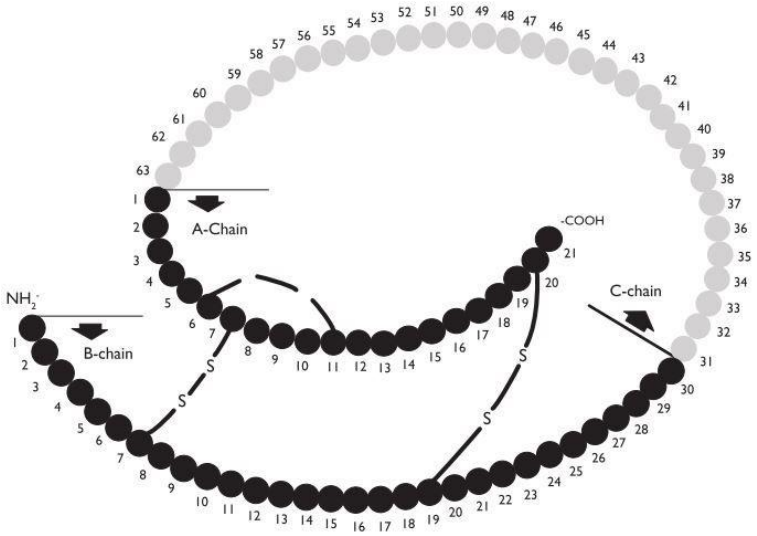

Figure 2.3: Mature insulin structure [12]

A common standard for medication dosage is either milligrams or grams, due to it being the standard quantity of mass. Insulin, however, is not dosed in milligrams or grams and therefore to understand the units of insulin, the basic understanding of an international unit (IU) is required. The international unit is an unit of measurement that is internationally accepted as the amount of

substance. One international unit of insulin is defined as biologically equivalent to 0.0347 mg of insulin (1 IU = 6nmol = 34.7 μg of insulin)[16]. According to Iwase et al. [17] the normal insulin level in living humans between meals ranges from 8 - 11 μIU/ml (0.3 - 0.41 ng/ml) and can increase up to 60 μIU/ml (2.25 ng/ml) after meals. A constant average insulin level greater than 11 μIU/ml indicates the start of Type 2 diabetes and becomes critical to monitor.

2.2.3 Process of Insulin Secretion

The main stimulant of insulin secretion is elevated concentrations of blood glucose and occurs in a typical biphasic pattern [7]. The drastic 'first phase' only lasts a few minutes and is due to secretion of preformed insulin, followed by the sustained 'second phase' which reflects the significant amount of newly synthesized insulin that is released from the β-cell. Other stimuli that promote insulin secretion include increased blood concentration of other fuel molecules (e.g. fatty acids), taste of food and sight. Figure 2.4 illustrates the biphase glucose-stimulated release of insulin.

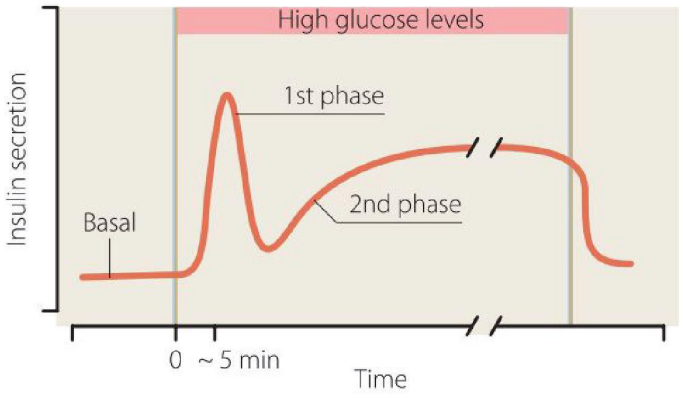

Figure 2.4: Biphasic glucose-stimulated release of insulin [7]

Elevated concentrations of glucose in the extracellular fluid is transported into the β-cell by facilitated diffusion with the help of the GLUT-2 transporter [7]. The glucose is then phoshorylated by glucokinase which acts as a 'glucose sensor' and couples insulin secretion to the prevailing glucose level [7]. This results in the generation of ATP energy (Adenosine Triphosphate) which is transferred back to the cytosol and increases ATP/ADP (Adenosine Diphosphate) ratio. The increased ATP/ADP ratio leads to plasma membrane depolarization which cause ATP-sensitive potassium (KATP) channels to close which causes an influx of extracellular calcium through voltage-gated channels. The increase in cytosolic calcium triggers the above mentioned translocation and exocytosis of insulin [7]. The process of glucose-stimulated insulin secretion from the β-cell is illustrated in Figure 2.5.

The activity of glucokinase also determines the amount of insulin secretion by governing the rate-limiting step for glucose metabolism in the β-cell [7]. Glucose levels below 5 mmol/L do not trigger insulin release whereas glucose levels at about 8 mmol/L results in half-maximal stimulation [7]. Therefore when the β-cell is appropriately stimulated, insulin is then secreted into islet capillary blood and the connecting C-peptide is also secreted into blood but has no known biological activity.

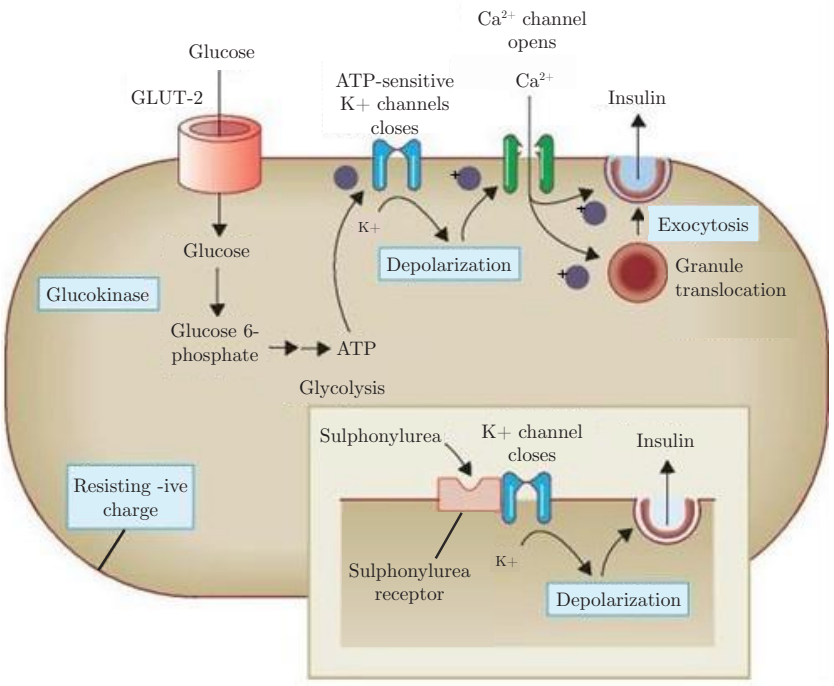

Figure 2.5: Process of glucose-stimulated insulin secretion from the β-cell [7]

2.3 Antibodies

Antibodies (Abs) are highly specific glycoproteins produced by plasma β-cells of the immune system in response to pathogenic antigens and belong to the immunoglobulin supergene family. The primary function of an antibody is to bind to its specific antigen and label it for destruction by the immune system. Antibodies are therefore used to control and stop pathogens and to assist in an immune response. An antibody is also called an immunoglobulin (Ig) and was first referenced in 1890 by Emil von Behring and Shibasabura Kitasato [18]. The potential for treatment in humans was immediately apparent after a publication showed the transfer of therapeutic serum from immunized animals could cure infected animals from a serious bacterial infection named Diphtheria [18]. After years of development, antibodies inherit three important and unique characteristics [8] namely:

- Antibodies can bind to an extremely wide range of chemicals, cells, biomolecules and viruses due to the nature of proteins and their binding sites.
- Antibodies have exceptional specificity to the target substance it binds to which enables extremely small volumes of analyte to be detected.
- Antibodies have high binding affinity and strength to the target analyte.

The above mentioned characteristics are the reason why antibodies are considered ideal for therapeutic interventions and medical diagnosis. This section discusses the basic structure of an antibody, the two types of antibodies and elaborates on the three unique characteristics.

2.3.1 Antibody Structure

Antibodies are large Y-shaped molecules that consists of two distinct regions and have a molecular weight of approximately 150kDa [19]. The upper-half is used as the antigen-binding fragment (Fab) and the lower-half is a crystallizable fragment (Fc). The upper-half consists of two identical "heavy" (H) polypeptide chains and two identical shorter "light" (L) chains folded into constant (C) and variable (V) domains and is shown in Figure 2.6. The two variable domains form the variable fragment (Fv) and provides the antigen specificity and the antigen binding site. The two constant domains provides the structural framework.

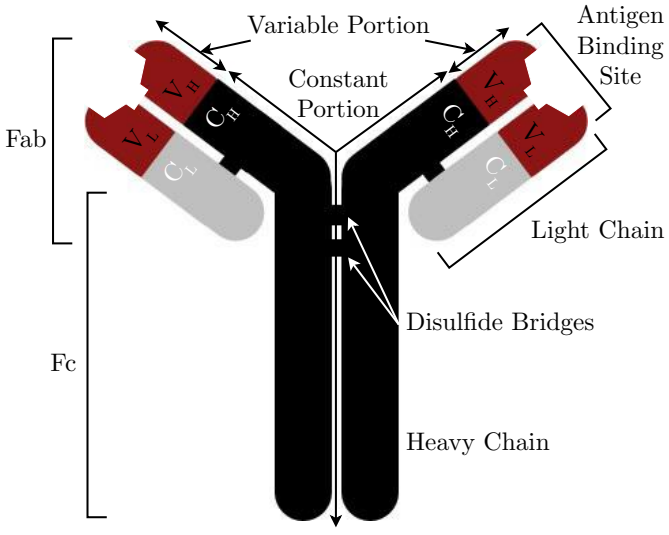

Figure 2.6: Antibody Structure [20]

Every antibody has a different type of heavy chain which categorizes antibodies (immunoglobulin) in five classes namely IgG, IgA, IgD, IgE and IgM,

which differ on the basis of size, charge, amino acid composition and carbohydrate content [8]. All five classes are secreted by the activated β-cells as glycoproteins [18]. In IgG, IgA, IgD antibody classes, the Fab is separated from the Fc by a flexible hinge region (not illustrated in Figure 2.6) whereas in IgE and IgM classes an extra constant domain replaces the hinge which makes it less flexible [18].

IgG antibodies are the most abundant class of antibody and used almost exclusively in immunoassays. IgG antibodies are the highest produced antibodies in response to immunization, have the highest binding affinity and are the most stable during the isolation and purification processes.

2.3.2 Types of Antibodies

There are millions of different proteins in a single cell, therefore the use of an antibody is extremely favorable due to its high specificity to antigens. There are three different types of antibodies namely monoclonal, polyclonal and recombinant antibodies. These antibodies have frequently been selected for a wide variety of applications, including immunodiagnostics and biomarker detection [21].

A monoclonal antibody (mAb) is a single antibody produced by single antibody producing β-cells that only bind to unique sites on the antigen and causes the antibody to only recognize a particular antigen [22]. The specific monoclonal antibody and antigen binding is discussed in Section 2.3.3. Monoclonal antibodies are produced using traditional hybridoma-based technologies which involves the injection of a specific antigen into a host species (typically a mouse) in order to provoke an immune response. The β-cell then produce these monoclonal antibodies that bind to the injected antigen where after it is harvested from the mouse's spleen and fused with tumor cells, also called myeloma cells to produce a hybrid cell line called a hybridoma. The hybridoma has both the antibody-producing ability of the β-cell and the exaggerated longevity and reproductivity of myeloma cells.

Polyclonal Antibodies (pAbs) are antibodies secreted from different β-cells that recognize multiple binding cites on the same antigen [22]. The inherent nature of polyclonal antibodies means that a selection of different epitopes may often be recognised on a single cell; however, as discussed in Section 2.3.3, polyclonal antibodies are also produced using traditional hybridoma-based method when cultured in rabbits, goats or sheep [21]. Polyclonal antibodies are inexpensive to produce, but can have a higher chance of cross-reactivity.

Recombinant antibodies (rAbs) are monoclonal antibodies which are not produced using traditional hybridoma-based technologies, but produced in vitro using synthetic genes and therefore do not require a host species. This is a preferred method due to the significant pain and discomfort endured by the animals. Due to the absence of an immunization step, recombinant antibodies have a faster production rate and are smaller in size, but require experienced

personnel for the production. For diagnostic manufacturing and therapeutic drug development that require large volumes of antigen specificity, monoclonal antibodies are the ideal solution [22]. However, for general research applications, the advantages of polyclonal antibodies outweigh those of monoclonal antibodies [22].

2.3.3 Antibody Specificity

The antibody-antigen interaction is a non-covalent specific chemical reaction between these antibodies and the antigens during an immune reaction. There are two main components that facilitate these interactions namely epitopes (antigen) and paratopes (antibody) and Figure 2.7 illustrates these components together with their respective carrier.

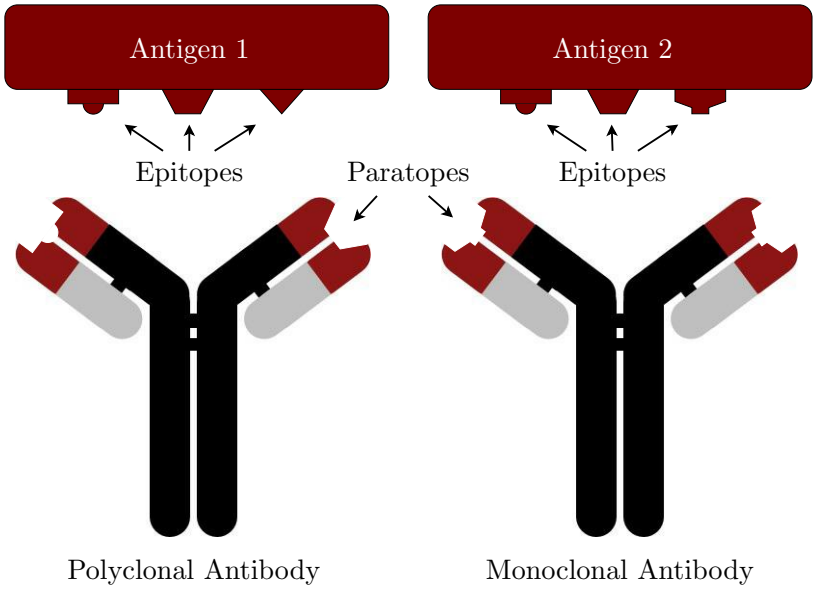

Figure 2.7: Antibody-antigen binding differences between polyclonal and monoclonal antibodies [23]

An epitope refers to the specific target on the antigen onto which an individual antibody bind [22]. This means that the binding between the antibody and the antigen does not occur between the entire full-length antigen, but involves only the specific segment. On any given antigen (protein) there are multiple unique epitopes onto which specific antibodies can bind. The binding between the antibody and the epitope occurs at the antibody binding site, which is also called a paratope and is located at the tip of the variable fragment of the antibody [22]. Refer to Figure 2.6 and 2.7 for a graphical representation of the epitopes and paratopes.

The critical feature of a paratope is that it can only bind with one unique epitope which enhances the specificity of these bindings. The specificity allows precise detection of a target antigen while avoiding detection of unrelated antigens that are not of interest [22]. Figure 2.7 illustrates two different antigens and a polyclonal and a monoclonal antibody. It can be noted that both antigens possess three epitopes (only for illustration purposes) where two of the epitopes are similar on both antigens, and the third epitope differs on both. The polyclonal antibody possesses two different paratopes on the ends of the variable fragment (Fv) and the monoclonal antibody possesses similar paratopes at both ends of the variable fragment.

It can therefore be noted that the polyclonal antibody is able to bind to both antigen 1 and antigen 2 due to the available epitopes on the antigens and the nature of the paratopes on the antibody. However, the monoclonal antibody is only able to bind to antigen 2 due to the nature of the paratopes and the lack of the appropriate epitope on antigen 1. It can therefore be concluded that monoclonal antibodies are more antigen specific than polyclonal antibodies.

2.3.4 Antibody Binding Affinity

Antibody binding affinity refers to the strength with which the epitope binds to an individual paratope on the antibody [22]. Antibodies with high binding affinity tend to bind quickly to the antigen, permit greater sensitivity and maintain its bond more readily under difficult conditions. In contrast, antibodies with low binding affinity bind weakly to the antigen and often do not detect the desired antigen. The binding between an antibody an its antigen is a reversible process, and the rate of the binding reaction is proportional to the concentrations of the reactants is expressed in Equation 2.1 below:

$$[Ab] + [Ag] \rightleftharpoons [AbAg] \tag{2.1}$$

where [Ab] respresents the antibody concentration and [Ag] represents the antigen concentration, either in bound ([AbAg]) or free ([Ab],[Ag]) state.

Antibody binding affinity is typically measured and indicated by the dissociation constant (K_D) which is used to rank and evaluate biomolecular interaction strengths. The K_D constant is the ratio of the antibody dissociation rate (k_{off}) which is how quickly the antibody dissociates from its antigen, to the antibody association rate (k_{on}) which is how quickly the antibody binds to its antigen. The equilibrium dissociation constant is expressed in Equation 2.2 below:

$$K_D = \frac{k_{off}}{k_{on}} = \frac{[Ab][Ag]}{[AbAg]} \tag{2.2}$$

The dissociation constant is inversely related to the affinity therefore the smaller the K_D value, the higher the affinity. The dissociation constant is a

unitless constant which can be correlated with a molar concentration (sensitivity) ratio that ranges from micromolar (10^{-6}) to femtomolar (10^{-15}) values. Typical antibodies have K_D values in the low micromolar to nanomolar (10^{-9}) range whereas antibodies with high afinity have K_D values in the picomolar range (10^{-12}).

2.4 Immunoassays

Immunoassays are sensitive analytical tests that measure either the presence or concentration of an analyte through an antibody-antigen binding. The basic principle of an immunoassay is to produce a measurable signal in response to the binding event [24]. The target molecule detected by an immunoassay is called the analyte and is isolated using the appropriate antibody.

Immunoassays were first expounded in 1959 by Rosalyn Yalow and Solomon Berson, and since then there has been a continuous growth, not only in the range of applications, but also in the number of assay designs [8]. The continuous improvements led to more sensitive assays that have opened up new horizons of clinical research and diagnosis. Immunoassays are currently still used for medical and research purposes and remains an invaluable tool for pharmaceutical analysis such as clinical diagnosis of diseases, drug discovery and development, and therapeutic drug monitoring.

The advantage of immunoassays is that it enables the above-mentioned industries to measure low concentrations of low molecular weight drugs, macromolecular biomolecules, metabolites and biomarkers which indicate and aid disease diagnosis or prognosis [25]. The significance of immunoassays are attributed due to their specificity, high-throughput and sensitivity.

2.4.1 The Basic Working Principle of Immunoassays

The reagents required for immunoassays are antibodies, signal-generating labels and separation matrices. The most important component of an immunoassay is, however, the antibody, and the reaction between the antibody and the analyte is the driving force of immunoassays. This reaction can vary considerably and is of profound significance to an effective assay [8]. These reactions can take from a few seconds to few hours, depending of the nature of the reaction and various factors. During the initial development of immunoassays, overnight incubations were necessary to allow the reaction to reach full equilibrium. Since then, immunoassays have developed significantly to allow for shorter incubations and more efficient assays.

Important factors to take into consideration when choosing the appropriate antibodies include the type of antibody (monoclonal, polyclonal or recombinant), purified or native, whether to purchase or manufacture antibodies and whether the antibodies should be fragmented, bispecific or fusion proteins and

most importantly the cost. During immunoassay development for pharmaceutical analysis purposes, monoclonal antibodies are preferred due to their high binding affinity and specificity towards the analyte [25].

Immunoassays can be performed in two different formats namely competitive or non-competitive, and the choice of immunoassay format is based on the nature of the analyte, labeling availability and the required analytical parameters such as sensitivity, specificity, accuracy and dynamic range [25]. In competitive immunoassays, the sample analyte is mixed together with a labelled analyte, which both compete for a limited number of antibody-binding sites [20]. Figure 2.8 graphically shows the different immunoassay formats. In the antigen-capture format (Figure 2.8a), the competitive reaction occurs between the labelled and unlabeled analyte.

In non-competitive immunoassays, the target analyte is captured by an excess of capture antibodies which is then separated from the analytes left in the solution [20]. The captured analyte is then exposed to an excess of detection antibodies which will only bind to available epitopes. Non-competitive immunoassays are also referred to as "two-site" or "sandwich" immunoassays and is shown schematically in Figure 2.8b. The one antibody is immobilized onto a solid support (e.g. a well in a microtiter plate) to capture the target analyte and is called the primary or capture antibody. The second antibody is used as the basis of the signal generation component and is labeled with a tag which facilitates the detection and purification. This antibody is referred to as the secondary or detection antibody.

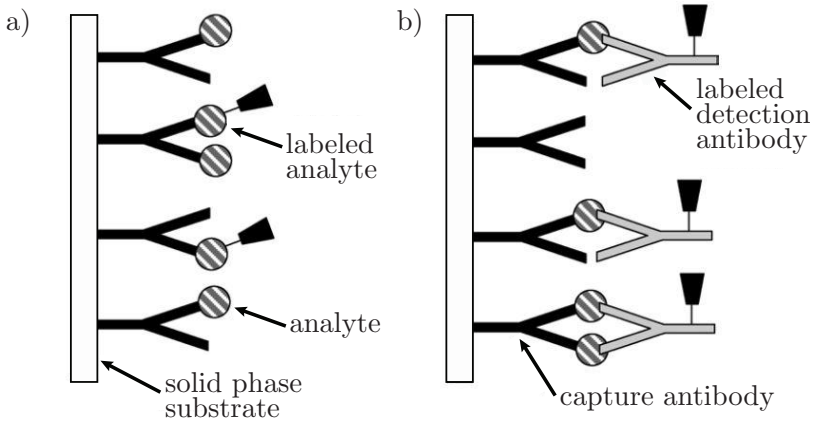

Figure 2.8: Schematic diagram of the a) competitive and b) non-competitive immunoassay configuration [20]

2.4.2 Immunoassay Detection Techniques

The main objective of an immunoassay is to produce a measurable signal in response to the antibody-antigen interaction. The signal is produced by a detectable label that is employed by the assays and is also the second most

important reagent on which the success of any immunoassay depends. The desired label is generally chemically linked or conjugated to the secondary antibody or antigen (depending on the type of immunassay format). A standard curve representing the measured signal as a function of the concentration of the analyte in the sample is constructed and can be used to determine the concentration of the unknown analyte [25].

As the field of biotechnology and immunoassays advances, new techniques are constantly developed, but for the purpose of this study only four techniques are discussed. These methods and techniques are Radioimmunoassay (RIA), Enzyme-linked Immunosorbent assay (ELISA), Electrochemiluminescent Immunoassay (ECLIA) and Chemiluminescent Immunoassay (CLIA). Other techniques include Counting Immunoassay (CIA), Fluorescent Immunoassay (FIA), Liposome Immunoassy (LIA), and Magnetic Immunoassay (MIA), but are not discussed in this thesis.

2.4.2.1 Radioimmunoassay

The radioimmunoassay (RIA) is the oldest type of immunoassay and is also the first technique used to measure and quantify insulin in the late 1950s by Berson and Yalow [26]. In RIAs, a radioisotope is conjugated to a known concentration of a desired antigen and bound with its complementary antibody. When the sample solution is added, the target antigen competes with the radioactive antigen and replaces it on the binding spot. The unbound antigens are then washed away and the radioactivity of the sample is measured. The radioactive signal is therefore inversely related to the amount of target antigen that is bound to the antibodies.

The advantages of RIAs include the accuracy, low detection limit and extreme sensitivity which can not be achieved by the other techniques [25]. The disadvantage of the technique is the health hazard of radioactive substances and the requirement for a license to handle radioactive material. RIAs also require highly trained personnel and storage facilities.

2.4.2.2 Enzyme-linked Immunosorbent Assay

Enzyme-linked Immunosorbent Assays (ELISAs) are analogous to RIAs; however, the main difference is the enzymatic label instead of the radioisotope [25]. In ELISA tests, the enzyme molecule is coupled onto the antigen (Ag), or antibody, and catalyses the chemical reaction. After the separation of unbound factions and adding an additional desired substrate, the assay possesses measurable physical and chemical differences. One approach is to have a colorless chromogenic substrate which is converted to a colored product due to the reaction of the enzyme [25]. The color change can be measured with a spectrophotometer and the signal is directly correlated to the analyte concentration.

Commonly used enzymes are Horseradish Peroxidase (HRP) and Alkiline Phosphatase (ALP). Although ELISA tests have evolved significantly to the point where it is the most common assay test today, the main disadvantage is that it is only possible to measure a single analyte at a time due to the nature of the ELISA plates. There are four common ELISA configurations based on their structure namely direct ELISA, indirect ELISA, competitive ELISA and non-competitive ELISA (sandwich ELISA), illustrated in Figure 2.9 below.

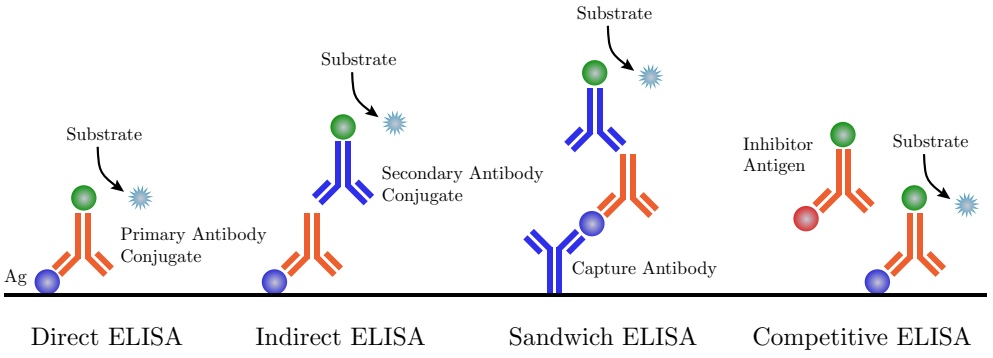

Figure 2.9: Comparison between ELISA configurations [8]

The objective of a direct ELISA test is to detect and measure antibodies instead of antigen. In direct-ELISA tests, a known concentration of antigen is fixed to the surface of the microtitre plate rendering it immobile. The advantages include the use of a single antibody which makes it inexpensive and quicker to gather results. The cross-reactivity of the secondary antibody is also eliminated. The disadvantages include the limited signal amplification, the time consumed by labeling primary antibodies for each ELISA-test and the cost.

In indirect-ELISA tests, the objective is also to detect antibodies therefore a known concentration of antigen is coated onto the microtitre plate. The advantages of indirect-ELISA tests include high sensitivity due to the use of a labeled secondary antibody which can bind to various epitopes on the target antibody allowing for signal amplification and the high flexibility due to wide variety of secondary antibodies available. Another advantage is the possibility to use various different visualization labels. The main disadvantages include the possibility of cross-reactivity due to the secondary antibody and that the use of a secondary antibody adds additional incubation steps making it more time consuming and more expensive.

Competitive ELISA tests make use of a competitive binding process executed by the original antigen and add-in antigen. Competitive ELISA tests are very specific due to the use of two antibodies. It can also be used in complex samples since the antigen does not require any purification before any measurements. One major disadvantage of competitive ELISA tests is due to

the inverse rule, past a certain limit of detection, the signal strength becomes unreadable.

The most common type of ELISA test is the sandwich ELISA test that measures the presence or concentration of an antigen between two layers of antibodies (i.e. capture and detection antibodies). The sandwich ELISA technique is an extremely sensitive and accurate assay due to the use of two antibodies. The only challenge is to find the correct antibody pairs that do not interfere and match due to their clonality. When monoclonal antibodies are used, it is critical to have different epitopes they bind to. Other advantages of ELISA tests include the versatility of possible analytes, high specificity and sensitivity, no radiation hazards, ease of process, widely available equipment and the fact that ELISAs produce qualitative and qualitative results. Another potential advantage of ELISAs is the possibility of amplifying the signal in order to get the desired sensitivity for the analysis [25]. The limitations of ELISA tests include possible false positives or false negatives and when using the sandwich configuration, can be expensive and time-consuming.

2.4.2.3 Chemiluminescent Immunoassay (CLIA)

Chemiluminescent Immunoassays are also analogous to ELISAs and RIAs, but it uses a chemiluminescent substance as a label for signal generation [25]. Luminescence is the emission of light to due an electron being in a higher energy level and emitting a photon as it comes down to normal energy level. CLIAs are similar to Flourescent Immunoassys (FIA), but FIAs utilizes the effect with certain frequencies of light whereas CLIAs uses a chemical reaction. CLIA signals are measured using photodetectors that detect or respond to incident light using the individual photon electrical effect [27]. CLIAs are ultra-sensitive, can detect small amounts of the biological molecule and has a wide dynamic range.

2.4.2.4 Electrochemiluminescent Immunoassay (ECLIA)

Electrochemiluminescenct Immunoassays (ECLIA) are also based on luminescence however, the signal is generated by a specific electrochemical reaction. When electrochemically generated intermediates are stimulated by electricity, they undergo reactions that release energy which also emits lights upon relaxation back to a lower energy level. ECLIA signals can therefore be measured without a photodetector which makes it an extremely attractive technique due to the inherit luminescence and electrochemical properties [27].

The advantages of ECLIAs include high sensitivity, possibility to amplify signals and enhance light levels and a broad dynamic range. ECLIAs are also relatively easy to use, has great flexibility when the labels are stable, non-radioactive, and conveniently conjugated to the target biological molecules, and lastly, ECLIAs has unsurpassed performance and quality that inherits

a highly successful detection system that achieves clinical quality data in a variety of sample types.

2.4.3 Blood, Pooled Plasma and Serum

The most common specimen samples used in immunoassays are blood, plasma, serum, urine, milk and saliva, each with various desirable analytes. Blood consists of four components namely red blood cells which carry oxygen through the body, white blood cells which are the cells of the immune system to protect the body against infectious diseases and foreign substances, platelets which prevent excessive bleeding and plasma, a yellowish liquid that carry nutrients, hormones and proteins through the body. Blood is the most desirable sample for clinical diagnosis and prognosis of serious infectious diseases as the plasma possess millions of biomarkers and proteins [28].

Biomarkers are naturally occurring molecules or genes by which diseases (such as diabetes) or physiological process can be identified. The quality of the biomarkers present in blood depends on biological factors such as the physical condition and age of the patient, but also depends on technical factors such as sample collection and preparation [28]. Although blood is the most common sample collected from patients, the plasma has to be separated from the other components and prepared for immunoassay testing. In bad separation procedures, blood cells can cause contamination and degrade the sample. Currently blood plasma separation (BPS) relies on a bench-top centrifuge to produce plasma. It is therefore critical to improve microscale blood plasma separation to achieve a reliable point-of-care device.

Plasma is the yellowish liquid portion remaining after centrifuging blood with anti-coagulants (also referred to as blood thinners) that prevent or reduce the coagulation (clotting) of blood. Serum is however the undiluted, extracellular portion of blood remaining after coagulation (clotting). Serum is therefore the plasma excluding the clotting substances.

Current point-of-care devices avoid BPS due to the inconsistencies introduced during the separation, resulting in lower concentration of target analytes, inaccurate measurement and reduced assay sensitivity [28]. There is however a trade-off between using blood in point-of-care devices that has inaccuracies due to blood cells, or using integrated BPS membranes that has inaccuracies due to the separation effectiveness. The quality of the analytical methods does however not always guarantee the diagnostic success. According to Mielczarek et al. [28], an important but overlooked issue relates to the variability in sample collection, storage and processing, accounting for up to 70% of all laboratory errors.

2.4.4 Immunoassay Performance Parameters

As the main objective of immunoassays is to provide prompt, accurate and reliable data, the validation method should be an inherent part of the development process. A number of validation and performance characteristics have been defined to emphasize the importance of the quality of the analytical process [29]. Some of these parameters include reusability, clinical stability, reproduceability, selectivity, robustness and measuring range. The success of immunoassays is generally attributed to the four main parameters sensitivity, specificity, accuracy and precision [8].

Sensitivity

Sensitivity refers to the quantitative measurement of the concentrations of the desired analyte. Analytical sensitivity refers to the ability of a test to detect a target analyte expressed as the minimum detectable concentration of that desired analyte. The approach is to assay the zero concentration calibrator repeatedly to define the limit of sensitivity (through a calibration curve) as the concentration that falls 2 or 3 standard deviations above the mean [8]. The values at this limit are not necessarily reliable as one assumes that the fitted calibration curve is an accurate reflection of the true dose-response, which in real clinical situations might differ.

Specificity

Assay specificity refers to the ability of an antibody to produce a measurable signal in response to only the desired analyte [8]. Cross-reactivity is a measurement of the response of an antibody to substances other than the target analyte. Many proteins are closely related owing to their structures and can lead to cross-reactivity. The level of cross-reactivity can be monitored by either using the right antibodies in a competitive assay design or by using a sandwich design with the highly specific antibodies.

Accuracy

Accuracy refers to how close the average measured signal is to the true value through the use of reference standards and calibration curves from laboratories and previously established reference data. This can be a difficult task as for many analytes, no true reference methods or data exists to compare measured data against. It is therefore critical to make sure that all possible causes of variations are eliminated from assay designs. This is also a key characteristic of POC devices where multiple electronic devices can cause interference and noise in the measured signal. It is important that a well defined concentration standard and reference material is available during assay tests.

Precision

Precision refers to the repeatability and minimal (standard) variation between measurements of an analytical technique [8]. Imprecision is the opposite of precision and refers to an estimate of errors in the analytical technique, therefore high precision also refers to low imprecision. Assay precision is affected by multiple contributors due to variation, separation and detection errors. Other contributors include ill-defined antibody concentrations, poor antibody immobilization techniques, temperature fluctuations or spikes, ionic strength and instrumentation variations and inefficiencies.

2.4.5 Immunoassay Considerations

Immunoassay techniques have been the flagships for drug monitoring and analyte quantification, however since the development of POC devices, the disadvantages of immunoassays relative to POCs became apparent. The major drawback of immunoassays is the fact that it requires expensive equipment and trained staff and that it is extremely expensive and time consuming. The need for these rapid analytical POC devices is increasing as millions of people in third world countries are deprived of proper medical resources and care.

2.5 Immunosensors

Immunosensors are compact analytical devices that detect specific analytes by using biological receptors such as antibodies, enzymes, cellular receptors, nucleic acids, microorganisms or artificial biomemetric materials [30]. More specifically, immunosensors are aimed to detect the presence of specific antigens and antibodies that are important for the diagnosis of diseases.

The advantage of immunosensors is that the clinical testing process is extremely fast compared to that of the generally used immunoassays. In immunoassay testing, many parties are involved and this influences the efficiency of processes such as testing of the patient, transport of samples, analysis, validation of results, reporting of results. Only then the patient can be contacted. Point-of-care devices, such as immunosensors, implement four basic steps during the procedure. These steps are: making the decision to test, sampling from the patient, performing the test and immediately obtaining results to inform the patient. This is however only one of the reasons to improve POC devices. The other reasons are that it requires minimal training or personnel, it is fast, cost-effective and able to diagnose multiple antigens simultaneously in the case of a rapidly spreading epidemic [31].

Immunosensors consists of three main components that work together in an integrated fashion to deliver successful results. These components are: a sensitive biological element, a transducer and a signal processor. An optional,

but not essential, component can be a display unit. Figure 2.10 shows the components of an immunosensor.

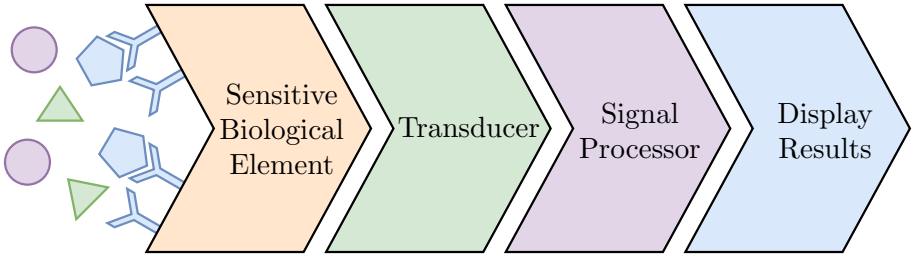

Figure 2.10: Components of an immunosensor [32]

The sensitive biological element functions as the recognition system and detects the analyte via the formation of antigen-antibody complexes in body fluids, especially in serum. This antigen-antibody complex generates chemical signals due to physical and chemical changes which are converted into an electrical signal by means of the transducer [31]. The final component is signal processing where the electrical signal is received, analysed, processed and returned to an optional display.

Various transducing mechanisms are used in immunosensors, based on the signal generation or property changes following the antigen-antibody reaction. Signal transducing can be carried out by various means, but this research focuses on immunosensors that measure electrons, photons and mass changes. Other types of transducing mechanisms include thermal changes and pH variations but are omitted from this thesis.

Optical Immunosensors

In optical immunosensors, the biological recognition element responds to the interaction with the target analyte (antibody) and generates an optical signal, such as fluorescence, or undergoes changes in optical properties, such as absorption, reflectance, emission, refractive index, and optical path [31]. The optical signal is then collected by a photodetector which converts the perceived light signals to electrical signals that can be digitally processed.

Piezoelectric Immunosensors

Piezoelectric immunosensors measure the change in mass due to antigen-antibody complex formations, compared to the mass of the antigen and antibodies separately. Two of the most popular piezoelectric immunosensors are quartz crystal microbalances and microcantilevers, which vibrate at a certain frequency. Piezoelectric immunosensors detect a vibration frequency shift as a result of the formation of antigen-antibody complexes.

Electrochemical Immunosensors

Electrochemical immunosensors react to the formation of antigen-antibody complexes and generate an electrical signal which can then be digitally interpreted and processed for analysis. The three main electrochemical transducers are: amperometric, potentiometric and conductimetric (impedimetric) [31]. Amperometric immunosensors measure current or the change in current, whereas potentiometric or voltammetric immunosensors measure electrode potential or voltage differences while impedimetric immunosensors measure the conductivity or resistance. Electrochemical immunosensors are fast, sensitive and inexpensive and are commonly used in point-of-care devices.

2.6 Organic Thin Film Transistors

Organic Thin Film Transistors (OTFTs) have been a popular research topic in the scientific community in recent decades and have received a fair amount of attention due its ability to be used as immunosensors in POC devices. OTFTs are highly sensitive, highly selective, have inherent low detection limits, low production and fabrication costs, and are extremely flexible [33]. OTFTs have the desirable ability to be miniaturized and integrated into portable electronic devices due to the nature of their design. In addition, the use of organic materials is considered to be compatible with the highly sensitive biological elements. This also allows tailoring of the organic material and the adjustment of its chemical and physical properties to fit the need of the application. Transistor-based sensors obtain their high sensitivity due to inherent combination of a sensor and an amplifier into the design, therefore, OTFTs show promising applications in chemical and biological sensors. OTFTs are also used in a variety of applications such as light sensing, environmental monitoring, drug delivery, food safety and medical diagnostics [34].

OTFTs are three-terminal electrical devices (similar to the popular conventional MOSFET, Metal-Oxide-Semiconductor Field-Effect Transistor) that enables for the control of a current that flows between two electrodes (the source and drain), through the modulation of an applied voltage at the third electrode (the gate) [33]. The measured current is referred to as the drain current (I_D) and can either flow from drain to source or vice versa, depending on the transistor setup and applied voltages. The critical active channel between the source and drain electrodes, consists of a thin film of an organic semiconductor which establishes electrical contact between the source and drain electrodes while they do not have physical contact. OTFTs have been used in a variety of gas sensors, cell sensors, chemical sensors (such as humidity, ions and pH) and biological sensors (such is DNA, enzyme, antibodies, tissues, micro-organisms, glucose and dopamine) [34]. Highly sensitive biological and chemical sensors are in great demand especially for environmental monitoring, food safety tests,

medical analysis and health-care products.

The organic semiconductor acts as the channel for current to flow and the magnitude thereof is modulated by the gate voltage [35]. This enables the sensors to detect the target analyte due to the physical or chemical changes in the semiconductor [35]. Therefore OTFTs are used in immunosensors as the transducing mechanism (or sensing platform). OTFTs use an electrochemical approach where the biological elements are immobilized onto the sensor in order to give an electrical signal due to the chemical change in the semiconductor. However, an OTFT can also be used as an optical immunosensor where a colorimetric probe can be conjugated to the biological element which generates an optical signal.

2.6.1 Organic Thin Film Transistor Architectures

The name OTFT is generally used to describe most types of organic transistors, but they can also be classified into two types of transistors according to the operation principles and device structures, namely, Organic Electrochemical Transistors (OECTs) and Organic Field-Effect Transistors (OFETs) [34].

Recently, Electrolyte-Gated Organic Field-Effect Transistors (EGOFETs), a new type of transistor architecture, has attracted much attention due to the low operating voltages compared with OFETs gated with solid dielectrics [36]. EGOFETs are similar to OECTs in that the gate is separated from the semiconductor by an electrolyte, but it is similar to OFETs in that it does not rely on ion transfer to modulate the current. An Ion Sensitive Organic Field-Effect Transistor (ISOFETs) is another sub-architecture of OTFTs where the basic structure is similar to EGOFETs, with only the addition of an insulator (dielectric layer) that separates the electrolyte and the semiconductor. EGOFETs, ISOFETs and OFETs use an organic semiconductor where electrons (or holes) are the main charge carriers. OECTs use an organic electrically conductive polymer (ECP) which has ions and electrons (or holes) as the charge carriers. The architecture, structure and respective channels (indicated in red) of OECTs, OFETs, EGOFETs and ISOFETs are illustrated in Figure 2.11.

Organic Electrochemical Transistors

The first Organic Electrochemical Transistor (OECT) was developed in the early 80's when White et al. [37] used an organic material as the active component of a transistor [36]. Since then, OECTs have been extensively investigated as a promising platform for a variety of chemical and biological sensing applications such as ions, pH, glucose, dopamine, bacteria, cells, and tissues, etc. [38].

OECTs are three-terminal devices in which the source and drain is connected via an electrically conducting polymer (ECP). OECTs can either operate in accumulation mode or depletion mode. The operating principle of an

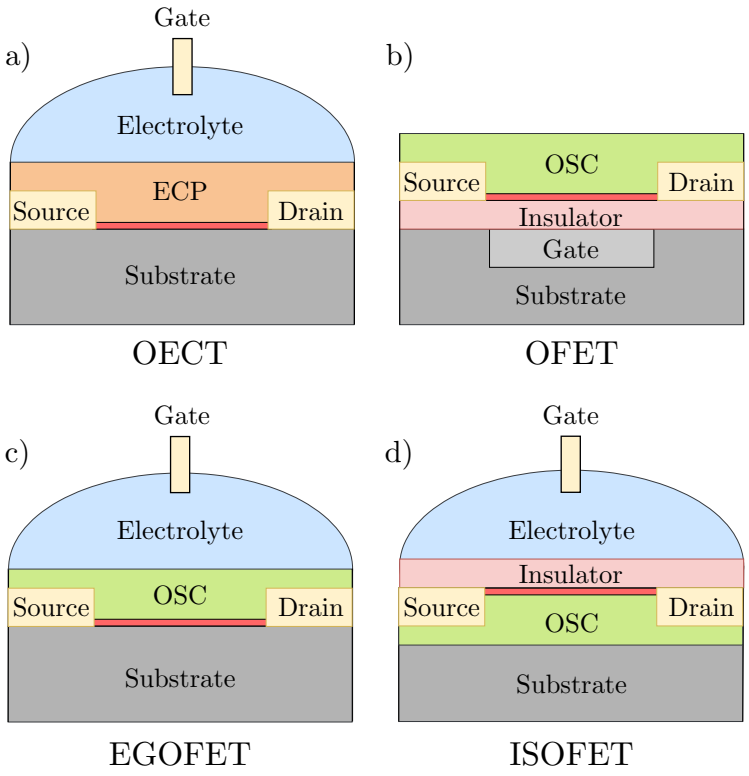

Figure 2.11: Comparison between different Organic Thin Film Transistor configurations [36]

OECT relies on the doping or de-doping of the conductive polymer (semiconductor) which changes its conductivity due to the migration of mobile ions into or out of the semiconductor from the electrolyte [36]. In accumulation mode, applying a gate voltage, switches on the OECT by injecting ions into the semiconductor, thus increasing the carrier concentration. In depletion mode, applying a gate voltage switches off the OECT by injecting ions into the semiconductor which compensates for native dopants and reduces the carrier concentration.

One advantage of using an organic ECP is the fact that the doping/de-doping processes are reversable and therefore can be switched by the applied gate voltage [35]. Another advantage is the ease of fabrication since the gate electrode and the channel are separated. It is consequently more convenient to fabricate an array of OECTs or to integrate them into microfluidic channels [34]. The most common organic ECP used is poly(3,4-ethylenedioxythiophene) polystyrene sulfonate (PEDOT:PSS) [36], however PEDOT:PSS and various other polymers are discussed in detail in Section 2.6.2. PEDOT:PSS is a depletion mode p-type semiconductor which uses holes as the charge carriers. OECTs that use PEDOT:PSS are normally-on devices which causes current to flow without the application of any gate voltage, due to the holes being the charge carrier. Therefore PEDOT:PSS based OECTs are depletion mode

transistors. One major disadvantage of OECTs are their low switching speed due to the intrinsically low mobility of the ions in the electrolyte and the restricted speed of the redox reaction [39].

OECTs are generally characterized using an electrolyte called Phosphate Buffered Saline (PBS). PBS is a balanced water-based salt solution commonly used in biological research. PBS inherits the desirable ability to maintain a constant pH (generally pH 7.4) and also has osmolarity and ion concentrations matching those of the human body. PBS is formulated by two salts named calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$) which facilitate the ion transfer in OECTs.

Upon the application of a positive gate voltage, the magnesium and calcium cations (positively charged ions) are extracted from the electrolyte and penetrate the conductive polymer and accumulate inside the ECP and replaces the native holes inside the ECP. The positively charged gate electrode then attracts the negatively charged chlorine (Cl^{2-}) anions as shown in Figure 2.12. This causes a decrease in hole density which reduces the conductivity of the polymer because fewer holes exist to carry charge. This is known as de-doping of the polymer and causes the current to decrease. Applying a negative gate voltage will dope the polymer, causing the cations to be removed from the ECP thus causing an increase in hole carriers and the drain current. The ion transfer as a result of a biased gate voltage is illustrated in Figure 2.12 below.

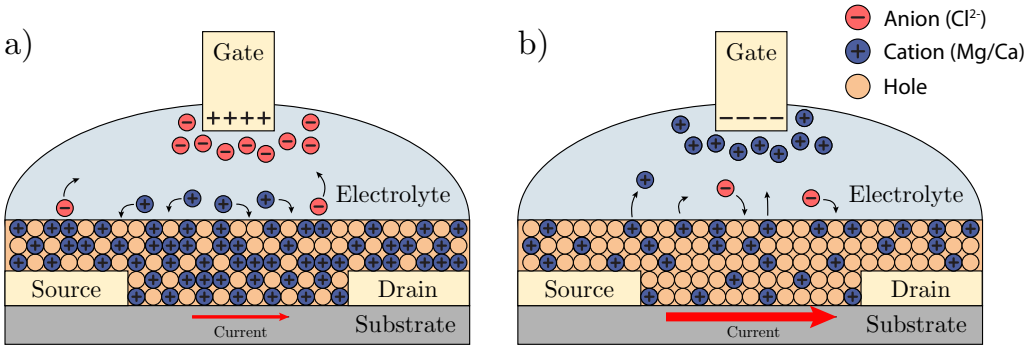

Figure 2.12: Graphic illustration of the OECT ion transfer upon an applied gate voltage

Due to the doping and de-doping caused by ion transfer, an OECT converts ionic current into an electronic current and is therefore ideal for monitoring biological phenomena. Another advantage of OECTs is that they operate at extremely low voltages (less than 1 V) which is ideal for biosensing in aqueous media, where low voltages are required to avoid undesired redox reactions and to prevent the risk for hydrolysis during operation [36]. In short, applying a voltage to the OECT gate electrode makes it possible to modulate the conductivity of the active conductive polymer material "channel" between

the transistor's source and drain electrodes by electrochemically doping or de-doping the semiconductor [40].

The schematic in Figure 2.13 shows that the ionic gate current (I_G) contributes to the channel current (I_{CH}) by the interaction between electronic and ionic charge carriers in the channel. Also shown are the drain current (I_D) the source current (I_S) the drain voltage (V_D) and the gate voltage (V_G). The variable name for the gate current is a lower-case letter to emphasize that it is a transient current.

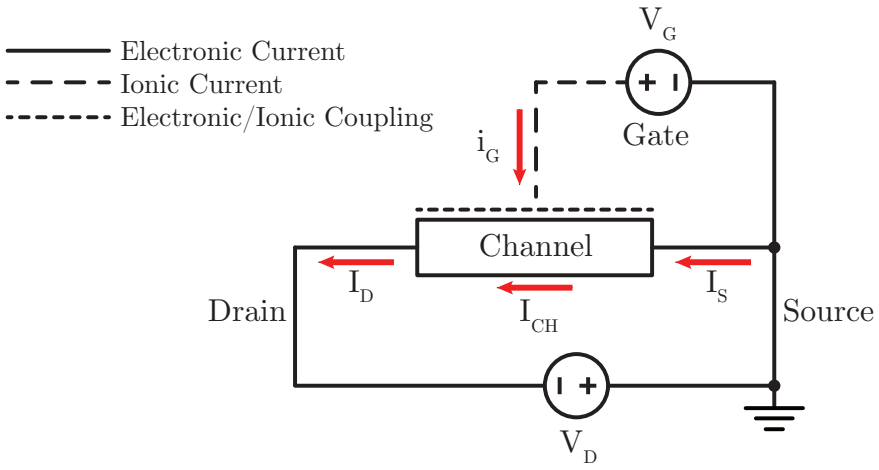

Figure 2.13: Schematic illustration of the electronic and ionic current in OECTs [40]

The effective gate voltage (V_g^{eff}) represents the potential drop at the channel-electrolyte and gate-electrolyte interfaces [41]. Upon the immobilization of antibodies or proteins on the OECT channel, it forms a negatively charged layer which attracts positively charged ions in the electrolyte. This leads to a decrease in V_g^{eff} which means fewer ions are dedoping the semi-conductive channel which decreases the drain current and threshold voltage [42]. When no proteins are immobilized, V_g^{eff} equals V_G and therefore the channel current is only affected by the ionic transfer into the semiconductor. The effective gate voltage can be described by Equation 2.3 as follows:

$$V_g^{eff} = V_g + 2.30(1+\gamma)\frac{kT}{ne} log[C_{Insulin}] + A \tag{2.3}$$

where V_g is the applied gate voltage; γ the capacitance ratio (defined as $\gamma = C_c/C_g$, where C_c and C_g are the channel and gate capacitance, respectively); k the Boltzmann's constant; T the temperature, e the elementary charge; n (=2) the number of electrons transferred during the electro-oxidation of insulin; and A is a constant related to other factors. The V_g^{eff} can be modulated by changes in sheet resistance, electrolyte resistance, charge transfer and sensing of external signals such as protein immobilization.

Organic Field-Effect Transistors

Organic Field-Effect Transistors (OFETs) are somewhat similar to OECTs in basic architecture, however they differ in that the gate is further separated from the organic semiconductor by an insulator that acts as a dielectric (refer to Figure 2.11). Different dielectrics can be used for the insulator e.g. polymers, oxides, vacuum, etc. OFETs also do not require an electrolyte, instead it uses the insulator to provide the field effect doping.

When the gate electrode is negatively biased in a p-channel device, free holes in the organic semiconductor are drawn closer to the semiconductor-insulator boundary to compensate for an equivalent negative charge at the gate-insulator boundary [36]. This build-up of charge leads to the formation of a conducting channel at the semiconductor-insulator boundary. Thereafter, when a negative voltage is applied between the source and drain, holes are injected from the source and current flows inside the channel.

The threshold voltage (the gate voltage required to switch the transistor from its "off" to its "on" state) of an OFET is greatly affected by the semiconductor-insulator boundary properties, along with the charge carrier mobility and the charge current modulation ratio [36]. When a sufficient gate voltage (above the threshold voltage) is applied, a conductive transistor channel is established for the flow of current.

Electrolyte-Gated Organic Field-Effect Transistors

Electrolyte-Gated Organic Field-Effect Transistors (EGOFETs) have recently gained much attention due to the minimalist architecture and required components. As mentioned earlier, the distinguishing characteristic of an EGOFET is that the gate is separated from the semiconductor by an electrolyte. Interestingly, it does not require an insulator (dielectric) layer. EGOFETs typically have a top gate, bottom contact configuration [43]. EGOFETs are similar to OECTs, the main difference lying in the interface between the channel and the electrolyte. In EGOFETs, the ions of the electrolyte create an electrical double layer (EDL) with the charges of the channel. This implies ions do not penetrate into the semiconductor, but rather builds up at the surfaces (interfaces) [42].

EGOFETs appear to be particularly suitable for use as the sensing platform for immunosensors due to their simple architecture combined with the low operating voltage. This can lead to inexpensive sensors having a low power consumption. EGOFETs also tend to have faster response times than OECTs due to fast switching times and the fact that the process does not depend on ion transfer.

Ion Sensitive Organic Field-Effect Transistors

Ion Sensitive Organic Field-Effect Transistors (ISOFETs) are similar to EGOFETs, with one distinguishing component which is the insulating layer between the electrolyte and organic semiconductor. The drain current (I_D) is driven by the potential of the electrolyte-insulator boundary and the channel. In the case of an immunosonsor, the sensitive biological elements are immobilized onto the insulator which modulates the drain current.

2.6.2 Organic Semiconductors and Conductive Polymers

The organic semiconductors (OSC) and electrically conductive polymers (ECP) are essential to OTFT-based immunosensors as it provides the charge transport to conduct the effective output current [34]. Conductive polymers and organic semiconductors also improve stability and sensitivity in OTFTs [42]. Several active organic semiconductors have been used in OTFT-based sensors such as polyaniline (PANI), polythiophenes (PTs), polypyrrole (PPy), Pentacene, poly(triaryl amine)(PTAA), DDFTTF, poly(3-hexylthiophene)(P3HT), poly (3,4-ethylenedioxythiophene):poly(styrene sulfonic acid)(PEDOT:PSS), etc.

The ability to provide effective charge-transport properties is affected by various intrinsic and extrinsic factors such as interaction, material purity, orientation, etc. [44]. It is important to note that high purity is desirable as impurities result in defects and degrade the transport profile which affects performance, causes inferior mobility and instability [44]. Another performance factor is OSC and ECP film thickness. For very thin layers ($t_{SC} < 5$ nm) stable continuous films are difficult to maintain and charge transport is hampered. Very thick layers ($t_{SC} > 100$ nm) may suffer from extreme surface roughness, poor finish and may have a high sheet resistance due to the long distance for vertical charge transport [44].

PEDOT:PSS is an electrically conductive polymer (ECP) and is considered the golden standard and is frequently used in OECTs [36]. PEDOT:PSS consists of poly(3,4-ethylenedioxythiophene)(PEDOT) combined with a high-molecular-weight counter-ion named poly(styrene sulfonate)(PSS), having the molecular structure illustrated in Figure 2.14. The combination of PEDOT and PSS forms a stable polyelectrolyte complex suspension which can easily be printed and coated onto flexible substrates. OECTs rely on the characteristics of PEDOT:PSS as ions are injected into the PEDOT:PSS to change its doping state and hence alter its conductivity.

A unique characteristic of PEDOT:PSS is that it influences the OECT to become a 'normally-on' device which means that without the presence of a gate voltage, PEDOT:PSS is in a conducting state and can induce a drain current [36]. Upon application of a positive gate bias, cations (positiviely charged ions) in the electrolyte penetrate the organic semiconductive polymer

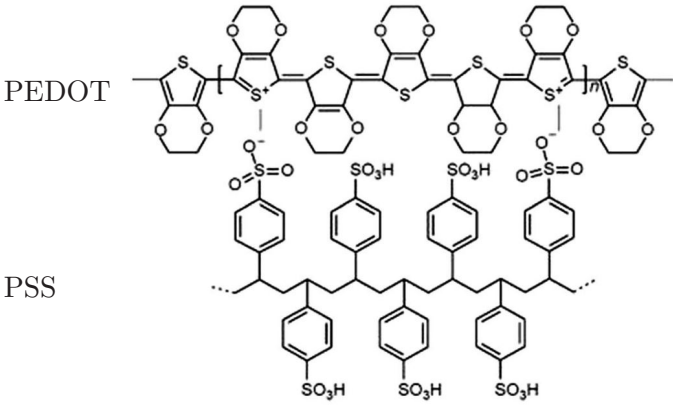

Figure 2.14: Molecular structure of PEDOT:PSS [45]

and takes the place of the holes to compensate the negatively charged sulfonate moieties on the PSS backbone [36]. As a consequence, the hole density reduces and decreases the polymer conductivity and therefore lowers the current that passes through the channel.

PEDOT:PSS is therefore a p-type semiconductor which has holes as charge carriers and these holes are extremely mobile and in the presence of a voltage drop across the polymer, the holes can easily move to carry charge. When the polymer is de-doped, heavy (less mobile) cations from the electrolyte penetrate the polymer and replaces the holes which decreases conductivity and subsequently reduces the current flow through the polymer. The relevant electrochemical reaction of PEDOT:PSS subject to an electrolyte given in Equation 2.4 below:

$$PEDOT : PSS + M^+ + e^- \Leftrightarrow PEDOT + M : PSS \tag{2.4}$$

where M^+ represents a cation present in the electrolyte that enters the polymer film [46]. PEDOT:PSS undergoes oxidation with an applied voltage greater than 0.8 V and undergoes reduction with an applied voltage less than -0.8 V.

One of the main advantages of PEDOT:PSS is that it has the potential of introducing a large number of carboxyl (-COOH) groups on the surface when mixed with a modified bipolar carboxymethelated dextran (CMD) [47]. The carboxyl groups are ideal for antibody immobilization through covalent bonding using NHS/EDC or bioaffinity interactions. Antibodies are immobilized onto PEDOT:PSS with the use of a buffer solution for 30 to 60 minutes to allow for incubation and complete immobilization. Berezhetska et al. [47] discovered that mixing pristine dextran (0.2% w/v) into the buffer solution, the dextran acted as a blocking agent towards the non-specific adsorption of the capture antibodies which increased the immobilization.

PEDOT:PSS is also extremely stable in aqueous conditions, allows for electronic and ionic ions transport, processable by a variety of methods and is readily available [48]. Another advantage of PEDOT:PSS is that the electrical

conductivity can also be further enhanced by mixing it with certain solutions. Glycerol, a simple colorless polyol compound, is a popular conductivity enhancer because of its low toxicity, ready availability at low cost and ease of fabrication and processing [47]. Other popular conductivity enhancers are Dimethyl sulfoxide (DMSO) and Ethanol.

2.6.3 Immobilization Techniques

The immobilization of the antibodies onto the surface of the transducer is a critical step in the construction of assays and immunosensors [49]. Some assays and immunosensors require maximum sensitivity, while others require more consistency or lower cost [50]. Many factors influence the efficiency of these sensors, but the correct orientation of immobilized antibodies have become critical for antigen detection. Many studies have shown that properly oriented antibodies, with their binding sites well exposed to the solution phase, exhibit higher binding efficiency [50]. The proper selection of linker molecules also reduces non-specific binding.

The correct technique also indirectly determines the number of binding sites available for antibody-antigen binding. A typical success rate of immobilization techniques ranges from 20% - 80% [8]. Various antibody immobilization approaches are available in literature with different degrees of complexity, but the three most common immobilization techniques used in immunosensors are: adsorption, covalent bonding and non-covalent bonding.

Adsorption

Physical adsorption is the simplest, most straightforward and most frequently used technique for protein immobilization [51]. Adsorption offers rapid, minimal steps and minimal usage of chemical reagents, being some of the main reasons for its popularity. The limitations of physical adsorption include unstable binding which leads to easy desorption of antibodies, no control over random oriented antibodies and being prone to significant loss of sensitivity and functionality [49]. In ELISA tests, the capture antibodies are generally adsorbed to the bottom the plate.

Covalent Bonding

A Covalent bond refers to the chemical (molecular) bond that involves the sharing of electron pairs between atoms and the stable balance of forces between the atoms are known as covalent bonding. Covalent bonding is irreversable with a high immobilization density between the antibody and the functional groups on the surface [51]. Covalent bonding is also extremely stable, flexible and exhibits a strong binding strength and low desorption from the surface [49].

During covalent bonding, it is common practice to immobilize amine (NH_2) terminated DNA probes (antibodies) with several functional groups such as carboxyl ($COOH$), thiol (SH), aldehyde (CHO), sulfonic ($SO_3{}^-$), epoxy and isothiocyanate (NCS) groups, leading to highly specific attachments which may prevent non-specific binding [49]. Some of the most popular covalent linkage agents include gluteraldehyde, carbodiimide and succinimide. Although covalent bonds are the strongest from of immobilization technique, they may result in randomly oriented antibodies which decrease the availability of functional active sites (paratopes) and decrease the overall efficiency [52].

Non-covalent Bonding

Research into the development of orientation-controlled immobilization techniques have recently received considerable attention. An interesting solution to this problem is to increase the number of functional antibodies by introducing bioaffinity pairs which can bind to the Fc region of antibodies to optimize the orientation. Affinity refers to the degree to which a substance tends to combine with another for which it has a natural preference. An antibody-antigen pair is an example of an affinity pair.

One of the most common and widely used bioaffinity immobilization approaches is the use of the biotin-streptavidin interaction. The binding affinity of biotin to streptavidin is the strongest non-covalent interaction known, with a dissociation constant (K_D) in the femtomolar range [49]. This interaction is highly specific, has a rapid on-rate, highly resistant to changes in temperature or pH, and is extremely robust and stable. This interaction, however, requires multiple surface functionalization steps which increases the complexity, production time and cost. The interaction between biotin and streptavidin can be seen as a weight (biotin) and gravity (the affinity interaction). The biotin is bound to the Fc region of the antibody and the "gravitational forces" optimizes the orientation of the antibodies and the density of bound antibodies [50].

2.6.4 Chemical Crosslinkers

As mentioned previously, chemical crosslinkers are vital to the immobilization of antibodies. Ates et al. [51] compared two covalent and two bioaffinity (non-covalent) antibody immobilization crosslinkers and compared each crosslinker in respect to its capture efficiency and selectivity. These crosslinkers are APTES, NHS/EDC, Glutaraldehyde and Streptavidin and are discussed below.

APTES

APTES ((3-Aminopropyl)triethoxysilane) is an aminosilane commonly used for silanization, the covering of a surface with organofunctional alkoxysilane molecules. Therefore APTES is used as a surface functionalizer to generate a NH_2 terminated monolayer after which the surface can be treated with a carboxyl activation step before antibody immobilization [49].

NHS/EDC

NHS/EDC consists of two carbodiimide reagents namely N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and is a commonly used coupling reagent to covalently bond carboxyl groups to amines (NH_2) terminated DNA probes (antibodies) [49]. Therefore NHS/EDC can be utilized as the carboxyl activation step.

Glutaraldehyde

Gluteraldhyde is a frequently used bifunctional linker between amine functionalized electrodes and NH_2 terminated DNA probes to form covalent amide bonds [49]. Gluteraldehyde can be used as a substitute for NHS/EDC to bind carboxyl and amine groups. The main disadvantage of Gluteraldehyde that it is extremely sensitive and can cause non-specific binding.

Streptavidin

As mentioned earlier, the non-covalent affinity between streptavidin and biotin is extremely high, nearly to a point of a covalent bond. Streptavidin is a large tetrameric protein (with molecular weight of 70 kDa) which provides binding sites for four biotin molecules as illustrated in Figure 2.15. This complex is commonly used to immobilize antibodies onto solid electrode surfaces(e.g ELISA tests) [49]. This is done by conjugating the antibodies with a biotin molecule and later introducing it to the streptavidin-coated substrate. Streptavidin is commonly adsorbed to the substrates (e.g. ELISA wells) as it forms a monolayer which increases the binding-efficiency of the biotin-streptavidin complex.

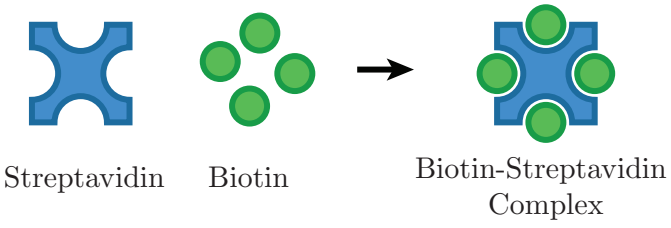

Figure 2.15: Biotin-Streptavidin complex

2.6.5 Antibody Conjugation Labels

A conjugated antibody refers to any antibody that is linked with a label to provide a means of measurement for the desired signal. Conjugated labels can either be conjugated to the primary antibody (direct detection) or to the secondary antibody (indirect detection). It is also seen as an excellent alternative to using secondary antibodies, but it can also be used together with a secondary antibody to amplify the signal or to produce a colored (fluorescent) readout to help with the detection of the target antigen. Other advantages of directly labeled antibodies include fewer incubation and wash steps, increased signal quality and better reproducibility. A wide variety of conjugation labels or tags are available; however, the most commonly used conjugated tags utilize enzymes or proteins such as Horseradish Peroxidase (HRP) or Alkaline Phosphatase (ALP). Other labels that can be used include dyes, Biotin, fluorescent proteins and gold nanoparticles. This section discusses some of the tags in greater detail.

Horse Radish Peroxidase

Horseradish peroxidase (HRP) is an popular enzyme used to amplify photometric (the measurement of light) signals in immunoassays by catalyzing the conversion of chromogenic or chemiluminescent substrates for the detection of target proteins. HRP enzymes are commonly utilized in ELISA tests and are used as conjugates to determine the presence of the target protein or molecule. HRP is ideal because it is smaller, more stable and less expensive than alternative enzymes such as Alkaline Phosphatase (ALP). Another chromogenic substrate, Tetramethyl Benzidine (TMB), is used in conjunction with HRP. TMB in its reduced form is colorless, but in the presence of HRP and H_2O_2, the TMB is oxidized and turns blue which indicates the signal strength.

Biotin

Biotin is a water-soluble vitamin (also known as vitamin B7 or vitamin H) and has a molecular weight of 2.4431 g/mol and chemical formula $C_{10}H_{16}N_2O_3S$. Antibodies are generally conjugated (labeled) with Biotin through the binding with available amine groups. The direct labeling of antibodies eliminates the need for secondary reagents (e.g. HRP conjugated antibodies) in immunoassays, thus removing tedious extra incubation and wash steps in applications such as ELISAs and Western Blotting.

Gold Nanoparticles

Gold nanoparticles (Au NP's) are extremely popular conjugation labels due to their large surface areas, straightforward synthesis methods, good biocompatibility and optical properties when conjugated with antibodies. Gold nanopar-

ticles are preferred for sandwich-type immunoassays because of the inherent signal amplification properties, enlarged dynamic range, improved sensitivity, stability an selectivity [53]. On-site signal amplification is preferred over hardware signal amplification (e.g. operational amplifiers) as the latter induce undesirable noise and interference in the signal, affecting the immunosensor accuracy and sensitivity.

2.6.6 Device Geometry and Structure

Despite the growing interest in OECTs, their device physics is not well understood. Two critical considerations for the optimization of the device sensitivity and amplification efficiency, is channel geometry and the gate electrode material and size. The device geometry also has a substantial influence on the transistor speed and response times [39]. One unique characteristic of OECTs is that the gate electrode does not have to be placed close to the channel area. The reason for this is that the electrolyte increases the variability of possible allowable device geometries such as planar device architectures [54].

The area ratio (AR) is defined as the ratio between the channel area and the gate area (AR = A_{ch}/A_g) and is a critical parameter to take into consideration when designing OTFTs and OECTs in particular. Cicoira et al. [54] evaluated various area ratios for different gate voltages and concluded that OECTs with smaller area ratios (e,g. AR = 0.1) possessed the best current modulation ($|I - I_O|/I_O$), lowest background signal and highest sensitivity. Hutter et al. [39] discovered that thicker PEDOT:PSS layers and a smaller gap between the gate and channel area, increased the drain current and sensitivity even further.

2.6.7 Substrate

The substrate not only serves as mechanical support but greatly affects the performance of OTFTs. The ideal substrate surface should be clean and have low surface energy (the energy associated with the intermolecular forces at the interface between the surface and the deposited material) [44]. Some fabrication techniques require the substrate to be hydrophobic (water resistant) whereas other techniques require the substrate to by hydrophilic (water loving). The substrate itself should also be chemically resistant to some solvents, where desired, to aid in the subsequent processes. Some popular substrates used for OTFTs are glass, silicon wafers (Si/SiO_2), plastic, metal foil and paper (e.g. film or photo paper).

2.6.8 Electrodes

The most commonly used materials for electrodes in OTFTs are Silver (Ag), Platinum (Pt) and Gold (Au), where each material has its respective advantages and disadvantages. Tarabella et al. [55] investigated the current mod-

ulation in an OECT and found that the current modulation is larger with silver gate electrodes in comparison to platinum gate electrodes. The interface between the electrode and OSC is also extremely important in order to allow for a simple and large charge injection area. The working electrodes are biologically functionalized and should therefore be chosen to optimize the OTFT characteristics and performance.

As mentioned earlier, the gate electrode modulates the current in the channel therefore it is essential to the operating principle of OTFTs [44]. The conductivity of the gate electrode affects the OTFT performance therefore much attention should be paid to choosing to optimal material for the gate electrode. According to Contat-Rodrig et al [56] the gate electrode should be larger than the channel in order to exhibit higher current modulation and amplification as well as decreasing the area ratio.

2.6.9 Performance Criteria

To evaluate OTFT performance, it is important to understand the related performance criteria in order to develop high-performance OTFTs.

Mobility

Mobility refers to the ability to move freely, therefore with respect to OTFTs, mobility refers to the movement of charge carriers, where a higher mobility provides a larger output current as the holes or electrons move more easily [44]. When a thin OSC film is used as the active layer, the mobility is affected by a great number of factors such as charge injection, purity of OSC, surface roughness of OSC-interfaces, charge trapping inside the OSC and applied voltages [44]. When a thick OSC film is used, theoretically it should result in a higher mobility as more charge carriers are present. As mentioned in Section 2.6, upon the application of a positive gate voltage, ions penetrate the electrically conducting polymer in a OECT causing a decrease in mobile charge carriers resulting in a decrease in mobility and subsequently the channel current.

Threshold Voltage

The threshold voltage (V_T) refers to the minimum gate-source voltage (V_{GS}) that is required to create a conducting path between the source and drain terminals through the channel and therefore to switch the OTFT "on". High-performance OTFTs always demand small threshold voltages, since the desirable operating voltages of OTFTs are less than 1 V [44]. OTFTs with small threshold voltages are also ideal as this minimizes the power consumption of OTFTs, which is important especially when used with portable devices powered with batteries. The threshold voltage is influenced by a number of factors

such as the OSC mobility, OSC thickness, gate dielectrics and OSC-interfaces; however, these can be fine tuned and optimized during the device fabrication process prior to any antibody immobilization.

Threshold voltage shifts are frequently reported in the literature. This phenomenon is similar to that which occur during the application of a constant gate voltage (V_G) for an extended period of time[57]. This bias stress causes instabilities that lead to either hysteresis or degradation. The direction of the shift is such that a fully turned on OTFT is able to slowly turn itself off or vice versa [57].

Sheet Resistance

The sheet resistance is the resistance between the source and drain terminals due to the thin OSC layer prior to the addition of an aqueous medium and is measured in ohm (Ω). Sheet resistance (R_S), also referred to as contact resistance (R_C), is a critical figure-of-merit in developing high-performance OTFTs, since a high sheet resistance degrades transistor mobility and impedes device down-scaling for high-speed operation [44]. Modern MOSFETs have sheet resistances of 0.1 Ω whereas the typical series resistances of OTFTs often tend to be in the kΩ range.

Some of the main contributors to the sheet resistance are the OSC material and design. The charge injection in OECTs occurs at the contact interface of the electrolyte and the OSC, therefore a small injection area may limit injection efficiency and raise R_S. Other factors that influence the sheet resistance include OSC microstructure, OSC purity and the OSC film thickness.

Hysteresis

Hysteresis is the unwanted effect in which the OECT parameters (specifically I_D) depend on the applied gate voltage sweep range, direction and time. Hysteresis can also be described as irreversible electrical disabilities and should ideally be minimized or eliminated. According to Xu et al. [44], hysteresis is not a device parameter, however it reflects the device quality, stability and reliability. The hysteresis effect is verified using cyclic transfer or output characteristics where the drain voltage (V_D) is swept at specific gate voltages (V_G) resulting in varying forward and backward currents.

Lower backward sweep current hysteresis is very often attributed to charge carrier trapping close to the channel interface, whereas higher backward sweep current hysteresis is usually caused by mobile ions in the dielectric [57]. Hysteresis often depends on the sweep rate, the start and end voltage of the sweep, the step width, the delay time, the hold time and the step delay time, therefor it is extremely important to clearly define all these parameters in order to compare different OECTs [57].

Transconductance

OECTs are characterized by the use of transfer curves which describe modulation effectiveness and the dependence of the drain current (I_D) on applied gate voltage (V_G). The transconductance is represented by the ratio between the change in drain current as a result of a change in gate voltage and is expressed in Equation 2.5 below and is measured in siemens (S).

$$g_m = \frac{\triangle I_D}{\triangle V_G} \tag{2.5}$$

The transconductance can also be calculated as the derivative of the transfer curve in terms of the gate voltage. The transconductance is a proper indication whether an OECT will be able to operate as an immunosensor [48]. The transconductance also evaluates the OECT performance, efficiency, current modulation and amplification effectiveness [58]. High amplification is important to increase signal to noise ratios and to lower the detection limits and increase sensitivity [42]. The transconductance also depends on the channel geometry and biasing conditions and describes the performance of the device at steady-state. According to Friedlein et al. [59] OECT transconductance has a non-monotonic (unpredictable) dependence on the gate voltage which means it decreases at both high and low gate voltages, depending on the geometry and fabrication technique. The transconductance is also dependent on the materials used in the fabrication of the OECT.

Limit of Detection

The limit of detection (LOD), is defined as the lowest concentration or quantity that can be reliably detected with a given analytical method. The LOD can also be determined by the signal-to-noise ratio which can be optimized by decreasing the noise level in the measurement[38]. OTFT-based immunosensors are commonly characterized based on the measurements of DC current responses and should ideally be greater than 3 times higher than the noise levels of the currents. One solution to this is to use AC measurements which can alleviate noise by filter circuits which can lead to more stable data and better signal-to-noise ratios. Wang et al [38] successfully investigated AC measurements of OECT-based immunosensors to detect chemicals with lower LODs by miniaturizing the channel area of the OECT to tens of micrometers.

Uniformity and Stability

High uniformity and stability are also important criteria of OTFT performance and requires all of the preceding parameters to be almost identical for each transistor. Therefore is it extremely important that the fabrication process of the OTFTs is repeatable and robust. However for printed OTFTs, it proves to

be a challenging task, as common varying factors that influence the repeatability are: OSC deposition, surface treatments, printing-related variations in film thickness and misalignment issues [44]. Various articles in the literature discuss annealing temperatures and durations; however, this is different for each fabrication technique and material. Annealing removes residual solvent,removes impurities, absorbs moisture and chemically stabilizes all films and their interfaces [44].

2.6.10 Organic Thin Film Transistor Considerations

Immense progress has been made in the past decade in the development of OTFT-based immunosensors. OTFTs also have the ability to be manufactured at low cost, deployed on flexible substrates and can be disposable which is ideal of low-cost point-of-care devices. Another ideal characteristic is the possibility of tailoring organic material to fit the desired application. OTFT-based immunosensors also have the possibility of using printing techniques for manufacturing as most of the materials are in a liquid form, e.g. silver or gold nanoparticle ink for the electrodes, or PEDOT:PSS and P3HT for the semiconductors.

The main concern regarding the development of OTFT-based immunosensors remains the integration of the biological recognition element into or on the semiconductor without compromising performance and durability [36]. Another concern is the fabrication process which is critical for highly sensitive and accurate immunosensors.

2.7 Printed Immunosensors

Much progress has been made recently in developing methods for the additive and roll-to-roll printing manufacturing of immunosensors. This was stimulated by the ease of large-scale fabrication, low-cost, bio-compatible and environmentally friendly methods [44]. Printing processes are versatile techniques enabling the deposition of various inks and solutions (such as biomolecules, polymers, solvents, etc.) onto different types of substrates (such as polymers, paper, glass, silicon, etc.) [58]. The ability to print high-performance OTFTs directly onto a rigid or flexible substrate could drastically simplify the fabrication process to produce low cost POC devices.

There are two kinds of printing methods based on the printing characteristics, namely direct-wiring printing (where patterning is done by ink ejection without contact) and transfer printing (where the patterned material is transferred from a donor substrate to an acceptor substrate) [44]. Examples of direct-wiring printing methods include inkjet printing, spray printing and screen printing. Examples of transfer printing methods include gravure printing, offset printing, flexography printing and laser printing. The two most

popular methods are inkjet printing and screen printing and will be discussed in detail in this section.

2.7.1 Inkjet Printing

Inkjet printing has recently received a fair amount of interest as method for OTFT fabrication. Inkjet printing can produce high resolution prints and uses ink that can be manufactured easily from a wide range of materials [44]. The versatility of inkjet printing enables it to deposit almost any material that can be formulated as a suspension, solution or dispersion in a liquid form. Inkjet printing also facilitate the deposition on contact-sensitive materials due to the absence of physical contact between the printhead and the substrate [60].

There are two types of inkjet printing technologies, namely the continuous inkjet system and drop-on-demand (DOD) inkjet system; the latter being the most commonly used technology. As the name suggests, continuous inkjet printing uses a high-pressure pump that directs liquid ink from a reservoir through a gun body and a microscopic nozzle, creating a continuous stream of ink droplets while the printhead is moving. In these printers, a piezoelectric crystal creates an acoustic wave as it vibrates within the gun body, causing the stream of ink to break into droplets which are subjected to an electrostatic field created by a charging electrode as they form. The charged droplets passes through an electrostatic field and is deflected by piezoelectric elements that either allow the droplets to fall on the substrate, or allow the droplets into a recycling gutter for re-use.

Drop-on-demand inkjet printing is quite similar to continuous printing, but instead of a continuous stream of ink droplets, droplets are only formed when required. This is done by means of either thermal or piezoelectric excitation inside the printhead. One of the main drawbacks of thermal printheads is that it uses heat to form the droplets which can denature bio-material inks and polymers. Piezoelectric printheads force small drops through the fine nozzles by pressure pulses generated by vibrating piezoelectric crystals [46]. Piezoelectric printheads do not apply heat to the ink [60]. Figure 2.16 shows graphically the different principles of operation of continuous and drop-on-demand inkjet printing systems.

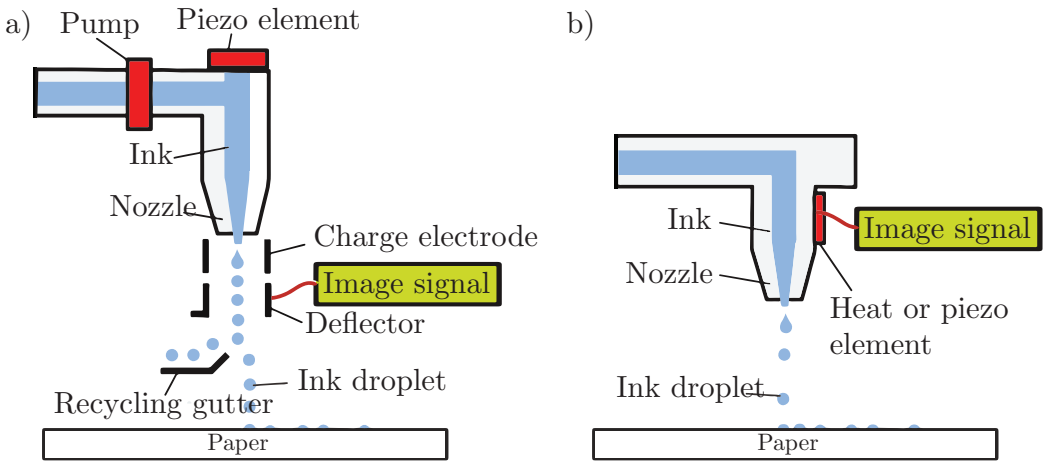

Figure 2.16: Principle of operation of the a) continuous inkjet system and the b) drop-on-demand inkjet printing system [61]

A major challenge of inkjet printing is the uneven surface morphology due to the varying viscosities and drying times of the ink droplets known as the coffee stain effect. The coffee stain effect leads to poor performance, poor uniformity and uneven films [44]. A solution to this is to use a co-solvent with different surface tension in order to counter the coffee stain effect. Another disadvantage of inkjet printing is the tendency of the printhead nozzles to clog due inconsistent ink viscosity, heating, incorrect solution mixture, etc. Inks with viscosities between 1 and 10 mPa.s are considered to be inkjet printable [58]. Apart from these challenges, inkjet printing remains a popular printing technique due to the low cost, resolution and versatility.

2.7.2 Screen Printing

Screen printing is another popular printing technique used for the fabrication of OTFTs. Screen printing involves squeezing a specially prepared ink through a mask (stencil) comprising a fine mesh onto a substrate to form the desired pattern [62]. This requires a more viscous ink compared to inkjet printing [44]. The resolution is determined by the size of the mesh openings and spaces between the openings. Screen printing is ideal for the development of multiple layers and electrodes [44].

Screen printing holds some advantage over inkjet printing owing to the ability of large scale production due to the printing speed. Figure 2.17 illustrates the principle of operation of screen printing and shows how the ink is squeezed through the mask to form the pattern.

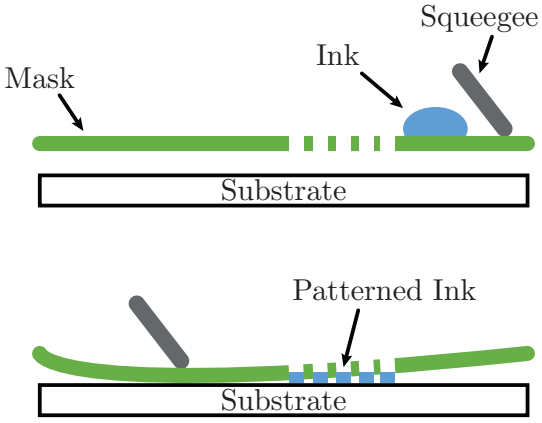

Figure 2.17: Principle of operation of screen printing [63]

Chapter 3

Design Specifications

This chapter presents the design specifications for the proposed immunosensor with reference to the aims and objectives defined in Section 1.2 as well as the contribution of the thorough literature review in Chapter 2.

Structure and Materials:

- The immunosensor should have a three electrode structure to control the flow of the channel current with the use of an external electrode
- The immunosensor should be based on an OECT and include an conducting polymer to facilitate the migration of ions
- The immunosensor should use PEDOT:PSS as conducting polymer
- The immunosensor should use Silver Nano-particle ink for the electrodes to allow for current-flow
- The substrate of the immunosensor should be paper to reduce the cost and increase ease of fabrication

Fabrication:

- The immunosensor should be manufactured using an inkjet printer to reduce cost
- The immunosensor should be annealed to remove moisture in the paper, semiconductor and electrodes

Antibodies:

- The immunosensor should utilize a sandwich antibody configuration to increase the sensitivity and specificity towards the insulin protein

- The immunosensor should employ high affinity monoclonal antibodies with different clones to obtain the highest sensitivity and specificity
- Thorough antibody pairing tests should commence to verify the best possible antibody pair
- The capture antibodies should be conjugated with Biotin to assist the antibody orientation and successful immobilization
- The detection antibodies should be conjugated with gold nano-particles for on-site signal amplification

Immobilization:

- The surface of the immunosensor should be functionalized with Streptavidin to aid antibody orientation and immobilization
- The Biotinylated antibodies should be adsorb to the streptavidin-coated surface to reduce immobilization steps

Measurement:

- The immunosensor should undergo an electrical characterization before any antibody immobilization to verify if the immunsensors function as OECTs
- The immunosensor should be characterized and tested using voltages less than 1 V to prevent oxidation or reduction

Performance:

- The control unfunctionalized immunosensor should have a sheet resistance of no more than 20 Ω to increase the channel current
- The immunosensor sheet resistance should increase after the addition of the antibodies
- The immunosensor should have a limit of detection of 8 μIU$/ml$ (0.3 ng/ml)

Chapter 4

Materials and Methods

This chapter presents all materials used during the duration of the project as well as the methodologies and processes followed in order to obtain accurate results.

4.1 Apparatus and Instrumentation

The apparatus that was used to supply the driving voltages and to measure the desired voltages and currents was the National Instruments (NI) CompactDAQ USB Module chassis (cDAQ-9174) with a 32-Channel C Series Voltage Input Module (NI-9205) and a 16-Channel C Series Voltage Output Module (NI-9264). The apparatus was controlled by the NI LabVIEW 2015 Instrumentation Software to provide constant and periodically varying voltages to the drain and gate electrodes.

A popular benchtop inkjet printer used to manufacture immunosensors is the Dimatix Materials Printer DMP-2850 which trades for R800 000, which is extremely expensive compared to the inkjet printer employed during this project which was the household HP DeskJet InkAdvantage 4535 printer which trades for only R1500 [64]. All OECT designs and layouts were designed in Gimp 2.0 (an open-source raster graphics editor) with a resolution of 1200 pixels per inch (ppi) in order to print at the highest resolution.

4.2 Materials and Reagents

The materials and reagents used during this study is provided in Table 4.1, as well as die company it was purchased from and respective datasheet location. DMSO, Glycerol (99.5+% purity), SU1818 Photoresist and Phosphate Buffer Saline (PBS) (pH 7.4) was donated by the Electrical and Electronic Engineering Department of Stellenbosch University.

Table 4.1: Materials and Reagents used during the study

Material	Purchased From	Datasheet
Monoclonal Anti-Insulin Antibody (A1364)	BioVision	Appendix B
Monoclonal Anti-Insulin Antibody (A1365)	BioVision	Appendix C
Recombinant Human Insulin Protein (ab123768)	Abcam	Appendix D
PEDOT:PSS (Clevios PH1000)	Heraeus Electronics	Appendix E
Silver nanoparticle Ink (NBSIJ-MU01)	Mitsubishi Paper Mills Limited	Appendix F
Mitsubushi Photopaper (NB-RC-3GR120)	Mitsubishi Paper Mills Limited	Appendix G
Lightning Link Rapid Biotin Conjugation Kit	Expedeon	Appendix H
GOLD Conjugation Kit (ab154873)	Abcam	Appendix I
Purified Streptavidin (280302)	BioLegend	Appendix J
Bovine Serum Albumin (BSA), Fraction V	Sigma-Aldrich	Appendix K

4.3 Antibody Pairing Tests

The aim and objectives of the antibody pairing tests is to verify the best antibody pair that would provide the highest antibody binding affinity, widest dynamic range (detectable concentrations) and most stable signal for the detection of human insulin. These tests will also confirm any reaction between the antibodies and the target epitopes and also validate the dynamic range of the antibody pair. The antibody pairing tests were performed at Synexa Life Sciences on a 96-well ELISA microplate using a sandwich ELISA configuration. This section discusses the antibody selection process, required reagents and buffers used during the testing as well as the plate layout and methodology.

4.3.1 Antibody Selection

The two chosen antibodies for the ELISA tests are the Anti-Insulin Antibody A1364 and A1365 from BioVision. These two specific antibodies were chosen because they have the same manufacturer which already performed extensive testing on order to compare the antibodies. These two antibodies are ideal for in vitro diagnostic assay development as both antibodies are mouse monoclonal which will result in the optimal specificity and sensitivity that is desired for these tests.

According to the antibody datasheets (Appendix B and C), the antibody A1364 detects human insulin and slightly recognizes proinsulin, however it has no cross-reactivity with C-peptide. The antibody A1365 detects human Insulin and it has no cross-reactivity with neither Proinsulin or C-peptide. The manufacturer have performed ELISA tests and recommends that the antibody A1364 is used as the capture and the antibody A1365 is used as detection antibody due to the nature of the cross-reactivity and the influence it will have on the sensitivity.

4.3.2 Antibody Pairing Reagents and Buffers

All the required reagents and buffers were prepared at Synexa Life Sciences and are given in Table 4.2 below.

Table 4.2: In-house prepared reagents and buffers for the antibody pairing tests

Reagents and buffers	Preparation
Anti-Insulin A1364 Stock	1 mL Stock Solution (0.5 mg/mL)
Anti-Insulin A1365 Stock	1 mL Stock Solution (1.007 mg/mL)
Recombinant Insulin Protein Solution	Prepare 1 mL Protein Solution (add 5 mg Stock lyophilized powder to 1 mL PBS)
Anti-Insulin A1364 Solution (Capture antibody Solution)	Prepare 6 mL Anti-Insulin A1364 Solution (add 12 μL A1364 Stock to 5988 μL PBS)
Anti-Insulin A1365 Solution (Capture antibody Solution)	Prepare 6 mL Anti-Insulin A1365 Solution (add 6 μL A1365 Stock to 5994 μL PBS)
Wash Buffer	Prepare 100 mL PBS-Tween (0.05%) Solution (Add 0.5 mL Tween-20 to 999.5 mL PBS)
Assay Buffer	Prepare 50 mL 1% BSA Solution (Add 0.5 g BSA to 50 mL PBS)
Blocking Buffer	Prepare 50 mL 3% BSA Solution (Add 1.5 g BSA to 50 mL PBS)
Biotinylated Anti-Insulin A1364 Solution (Detection antibody Solution)	Prepare 6 mL Biotinylated Anti-Insulin A1364 Solution in Assay Buffer
Biotinylated Anti-Insulin A1365 Solution (Detection antibody Solution)	Prepare 6 mL Biotinylated Anti-Insulin A1365 Solution in Assay Buffer
Streptavidin-HRP Solution	Prepare 1:200 dilution of Streptavidin-HRP stock in Assay buffer (Add 60 μL stock to 11940 μL Assay Buffer)
TMB Substrate Solution	Prepare 1:1 dilution of TMB Reagent stock in Assay buffer (Add 13 μL stock to 12987 μL Assay Buffer)
Stop Solution	2N HCL Solution

4.3.3 Antibody Pairing Plate Layout

As mentioned earlier, the manufacturer recommends to use the antibody A1364 as the capture antibody and the antibody A1365 as the detection antibody, however for the purpose of this study, it was chosen to test both combinations (as recommended and vice versa). Figure 4.1 below illustrates the ELISA plate layout for both combinations. For the rest of this report, the antibody pair with the anti-insulin A1364 capture antibody (left in Figure 4.1a) will be referred to as antibody pair 1 and the other pair (right in Figure 4.1a) will be referred to as antibody pair 2.

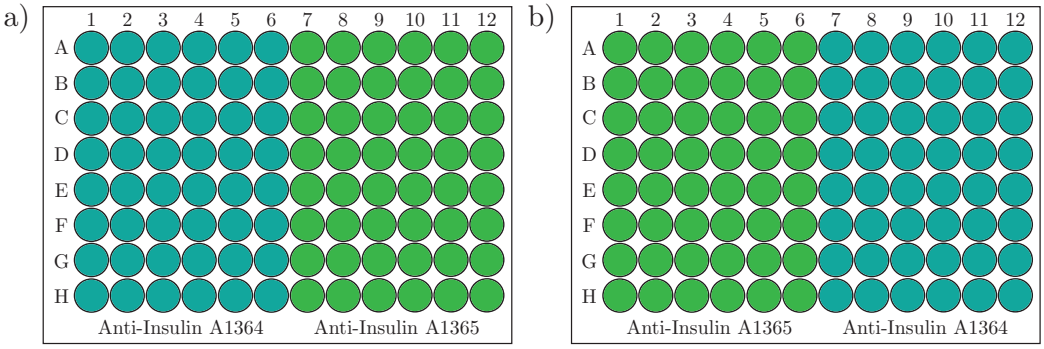

Figure 4.1: ELISA plate layout for the a) capture antibodies and the b) detection antibodies

In order to test the dynamic range of the antibody pairs, the recombinant human-insulin protein solution was diluted in PBS into various concentrations that were added to the ELISA plate using the standard ELISA protocol. Figure 4.2 indicates the ELISA plate layout with the respective designed concentrations and wells. The chosen concentrations covers a wide dynamic range in order to fully test the ability of these antibodies.

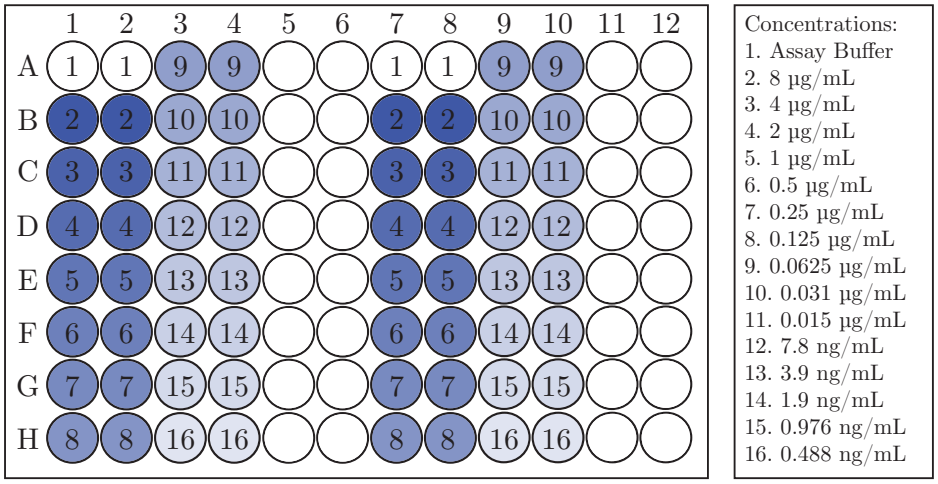

Figure 4.2: ELISA plate layout for the designed insulin concentrations

4.3.4 Antibody Pairing Methodology

The antibody pairing test were performed using a sandwich ELISA configuration as shown in Figure 4.3 below.

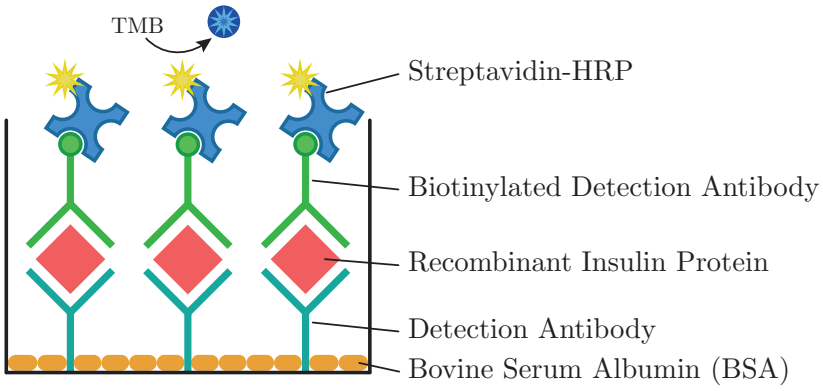

Figure 4.3: Schematic illustration of the sandwich ELISA configuration [49]

The ELISA process is extremely time consuming as each reagent and buffer has to be prepared prior to any test as well as the required wash step between each incubation step. The following general ELISA protocol was followed in order to ensure successful antibody immobilization, protein incubation and wash steps.

1. Prepare all necessary reagents and buffers as tabulated in Table 4.2
2. Add 100 μL/well of Capture Antibody Solution (A1364 and A1365, respectively) to the relevant wells as illustrated in Figure 4.1
3. Incubate ELISA plate (sealed) overnight in the dark at 4 °C
4. Wash plate three times with 300 μL/well Wash Buffer using a microplate washer and remove remaining Wash Buffer by inverting plate and blot against clean paper towel
5. Add 300 μL Blocking Buffer to each well and incubate at 1 hour at room temperature with gentle shaking at 350 rpm
6. Wash plate three times with 300 μL/well Wash Buffer using a microplate washer and remove remaining Wash Buffer by inverting plate and blot against clean paper towel
7. Add 100 μL/well of Recombinant Human Insulin Dilutions, QCs and Blanks (PBS) to relevant wells
8. Incubate plate (sealed) for 1 hour at room temperature with gentle shaking at 350 rpm
9. Wash plate three times with 300 μL/well Wash Buffer using a microplate washer and remove remaining Wash Buffer by inverting plate and blot against clean paper towel

10. Add 100 μL/well of Biotinylated Detection Antibody Solution (A1364 and A1365, respectively) to relevant wells as illustrated in Figure 4.1
11. Incubate plate (sealed) for 1 hour at room temperature with gentle shaking at 350 rpm
12. Wash plate three times with 300 μL/well Wash Buffer using a microplate washer and remove remaining Wash Buffer by inverting plate and blot against clean paper towel
13. Add 100 μL/well of Streptavidin-HRP Solution to relevant wells
14. Incubate plate (sealed) for 1 hour at room temperature with gentle shaking at 350 rpm
15. Wash plate three times with 300 μL/well Wash Buffer using a microplate washer and remove remaining Wash Buffer by inverting plate and blot against clean paper towel
16. Add 100 μL/well TMB Substrate Solution to all the wells
17. Incubate plate (unsealed) for 30 minutes at room temperature with gentle shaking at 350 rpm and check regularly to prevent over-development f color
18. Add 50 μL/well of Stop Solution to all the wells
19. Read plate absorbance at 450 nm using an ELISA plate reader

4.4 Device Fabrication Procedure

The fabrication of the OECTs were performed at the Electrical and Electronic Engineering Department of Stellenbosch University. The source and drain electrodes were printed with silver nanoparticle ink (Appendix F) and the gate electrode was cut from a sheet sterling silver. For the preparation of the PEDOT:PSS ink, the Clevious PH1000 solution was mixed with water (60 wt.%), DMSO (5 wt.%) and Glycerol (5 wt.%) in order to obtain the correct viscosity and conductivity. The viscosity could however not be measured due to the lack of equipment. The PEDOT:PSS mixture was then sonicated in order to remove any impurities and kept on a magnetic mixer to evenly distribute particles and improve uniformity. A cross-sectional view of the fabricated OECT is shown in Figure 4.4. The fabricated OECT serves as the basis for the control OECTs (used for the electrical characterization) and the functionalized OECTs (used for the final insulin measurement tests).

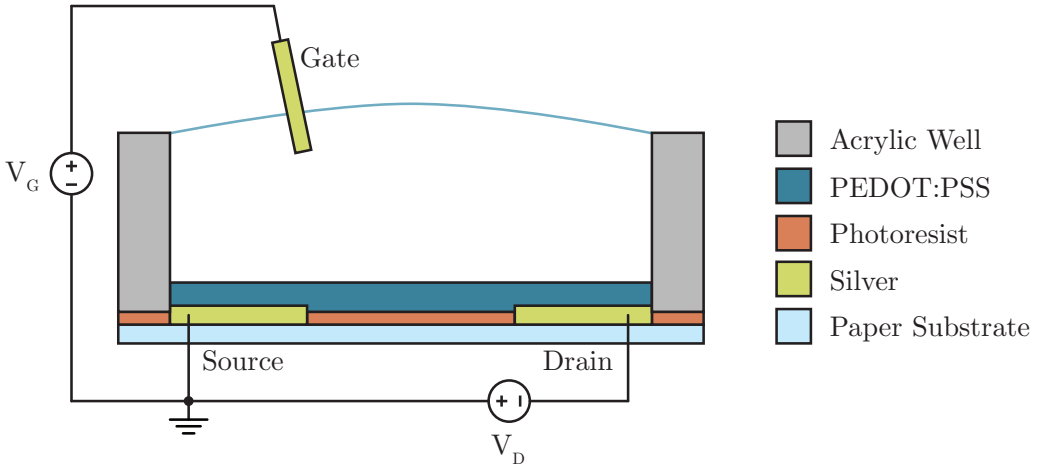

Figure 4.4: Cross-sectioned schematic diagram of the OECT and the wiring system for device operation [38]

4.4.1 OECT Fabrication

The following procedure was followed in order to ensure repeatable functioning OECTs:

1. Print silver source and drain electrodes on Mitsubishi paper using the HP DeskJet InkAdvantage 4535 printer
2. Spincoat 1 mL of Photoresist-Actone mixture (1:4 wt.%) at 600 rpm for 30 seconds followed by 2000 rpm for 10 seconds onto the printed paper.
3. Place a 5 μl drop of PEDOT:PSS mixture over of interdigitated source and drain electrodes with a pipette
4. Anneal sensors at 90 - 100 °C for 2 hours in an oven
5. Fix the Acrylic well to the substrate with the use of double-sided tape

4.4.2 Antibody Immobilization

The OECTs were biologically functionalized with the antibodies in order to test its ability to act as immunosensors. The functionalization configuration is given in Figure 4.5 below.

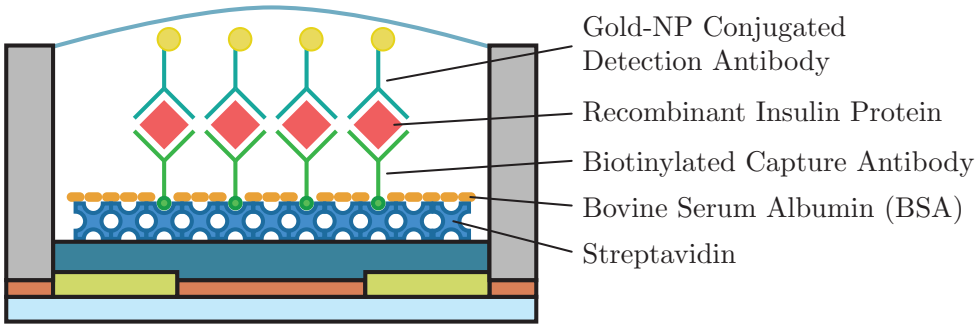

Figure 4.5: Schematic illustration of the sandwich OECT configuration [49]

The reagents and buffers used for the immobilization and functionalization of the antibodies and proteins onto the OECTs, are given in Table 4.3.

Table 4.3: In-house prepared reagents and buffers for final immunoassay tests

Reagents and buffers	Preparation
Biotynalated Anti-Insulin A1365 Stock	Prepare 200 μL Biotinylated Anti-Insulin A1365 Stock using Lightning-Link Rapid Biotin Conjugation Kit
Biotinylated Anti-Insulin A1365 Solution (Capture Antibody Solution)	Prepare 600 μL of Biotynalated Anti-Insulin A1365 Solution (add 5 μL of Biotinylated Anti-Insulin A1365 Stock to 595 μL Assay Buffer)
Recombinant Human-Insulin Solution	Prepare 1 mL Insulin Protein Solution (add 5 mg Stock lyophilized powder to 1 mL PBS)
Streptavidin Coating Solution	Prepare 1 mL of Streptavidin Coating Solution (Add 10 μL Purified Streptavidin to 990 μL PBS)
Gold Nanoparticle Conjugated Anti-Insulin A1364 Stock	Prepare 100 μL Gold nanoparticle Conjugated Antibodies using the ab154873 Gold Conjugation Kit
Gold Nano-Particle Conjugated Anti-Insulin A1364 Solution (Detection Antibody Solution)	Prepare 500 μL Gold nanoparticle Conjugated Anti-Insulin A1364 Solution (add 100 μL Gold Nanoparticle Conjugated Anti-Insulin A1364 Stock to 400 μL PBS)
Wash Buffer	Prepare 50 mL of PBS
Assay Buffer (50 mL)	Prepare 1% BSA Solution (Add 0.5 g BSA to 50 mL PBS)
Blocking Buffer (50 mL)	Prepare 3% BSA Solution (Add 1.5 g BSA to 50 mL PBS)

The following procedure was followed in order to successfully immobilize the antibodies on the PEDOT:PSS layer of the OECT.

1. Prepare all necessary reagents and buffers listed in Table 4.3
2. Add 16 μL of Streptavidin Solution to each well
3. Incubate sensor (sealed) for 120 minutes 4 °C

4. Wash sensors three times with 16 μL/well Wash Buffer by gently pipetting up and down and remove remaining Wash Buffer by inverting sensor and blot against clean paper towel
5. Add 16 μL of Biotinylated Capture Antibody Solution to each well
6. Incubate sensors (sealed) overnight in the dark at 4 °C
7. Wash sensors three times with 16 μL/well Wash Buffer by gently pipetting up and down and remove remaining Wash Buffer by inverting sensor and blot against clean paper towel
8. Add 16 μL Blocking Buffer to each well
9. Incubate sensors (sealed) for 60 minutes at room temperature (20 - 25 °C
10. Wash sensors three times with 16 μL/well Wash Buffer by gently pipetting up and down and remove remaining Wash Buffer by inverting sensor and blot against clean paper towel
11. Add 16 μL Recombinant Human Insulin Concentrations to relevant wells
12. Incubate sensors (sealed) for 60 minutes at room temperature (20 - 25 °C
13. Wash sensors three times with 16 μL/well Wash Buffer by gently pipetting up and down and remove remaining Wash Buffer by inverting sensor and blot against clean paper towel
14. Add 16 μL Gold Nano-Particle Conjugated Detection Antibody Solution to relevant wells
15. Incubate sensors (sealed) for 60 minutes at room temperature (20 - 25 °C
16. Wash sensors three times with 16 μL/well Wash Buffer by gently pipetting up and down and remove remaining Wash Buffer by inverting sensor and blot against clean paper towel

4.5 Measurement Procedure

All measurements were performed using the NI DAQ (cDAQ-9174), Analog to Digital Converter (ADC) Module (NI-9205), Digital to Analog Converter (DAC) Module (NI-9264) and the NI LabView 2015 Instrumentation Software. The measurement setup is shown in Figure 4.6. A 147 Ω resistor (R_D) was

connected in series with the drain electrode, a 148 Ω resistor (R_G) was connected in series with the gate electrode and the source electrode was connected to ground as illustrated in Figure 4.7. The driving voltages (V_{DD} and V_{GG}) were supplied via the DAC module. In order to obtain current measurements, output and transfer characteristics, four ADC channels were used to measure required voltages (V_{DD}, V_{DS}, V_{GG} and V_{GS}). The collected data was analyzed with a custom Matlab Script in order to provide accurate graphs.

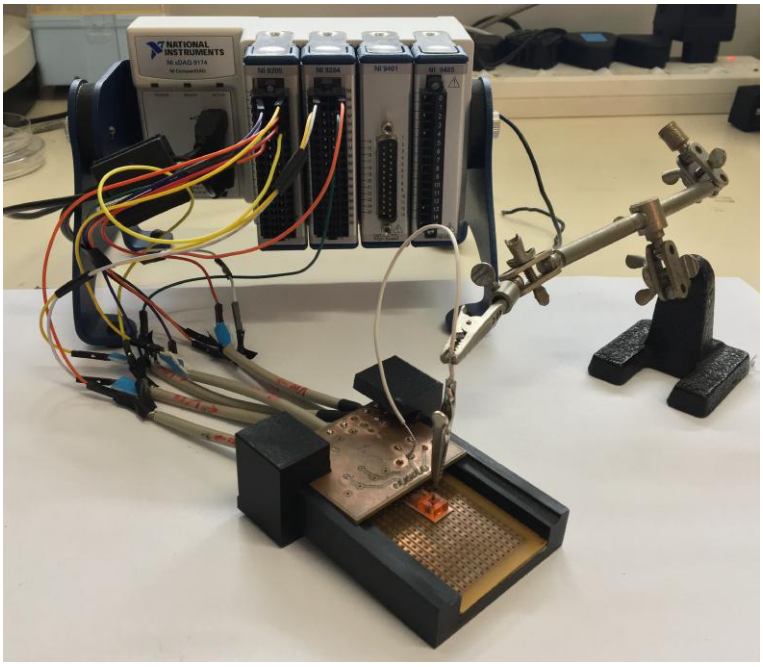

Figure 4.6: A picture of the measurement device and suspended gate electrode

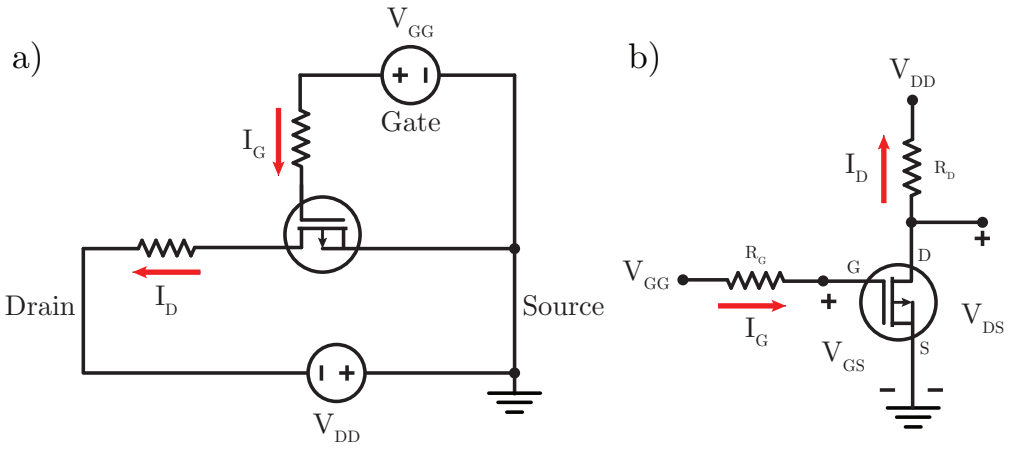

Figure 4.7: Graphical illustration of the a) device setup and b) circuit layout for the OECT tests [35]

The formulas for calculating the drain and gate currents are given in Equation 4.1 and 4.2 below:

$$I_D = \frac{V_{DS} - V_{DD}}{R_D} \tag{4.1}$$

$$I_G = \frac{V_{GG} - V_{GS}}{R_G} \tag{4.2}$$

Chapter 5

Results

This section discusses the results obtained for the antibody pairing tests, the OECT fabrication, OECT electrical characterization and lastly the final functionalized immunosensor tests. All the data was processed using Matlab, a numerical computing environment, in order to prepare the graphs and results. The Matlab script is given in Appendix M.

5.1 Antibody Pairing

All the antibody pairing tests were performed following the methodology and protocol discussed in Section 4.3. Succeeding the preparation of the ELISA tests, immobilizing the respective antibodies and adding the designed insulin protein concentrations, the TMB Substrate Solution was added. The TMB reacts with the HRP and produces a solid blue reaction product. Figure 5.1 shows the physical color change. It is clear that the antibody pair 2 produces a more gradual color change compared to the almost saturated color change on the left (antibody pair 1). Therefore antibody pair 1 can be said to be more specific, however the antibody pair 2 could be more sensitive as any form of insulin present generated a significant signal.

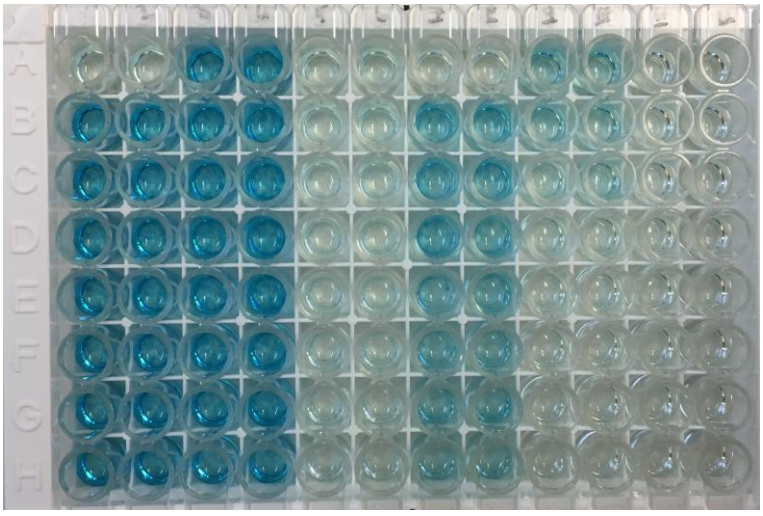

Figure 5.1: A picture of the color change during the antibody pairing tests for the ELISA plate

After the addition of the sulfuric acid Stop Solution, the color changed to yellow, enabling accurate measurement of the intensity at 450 nm using an ELISA plate reader. Figure 5.2 and 5.3 below provides the generated graphs for the optical density (OD) signals for the respective antibody pairs at the designated concentrations.

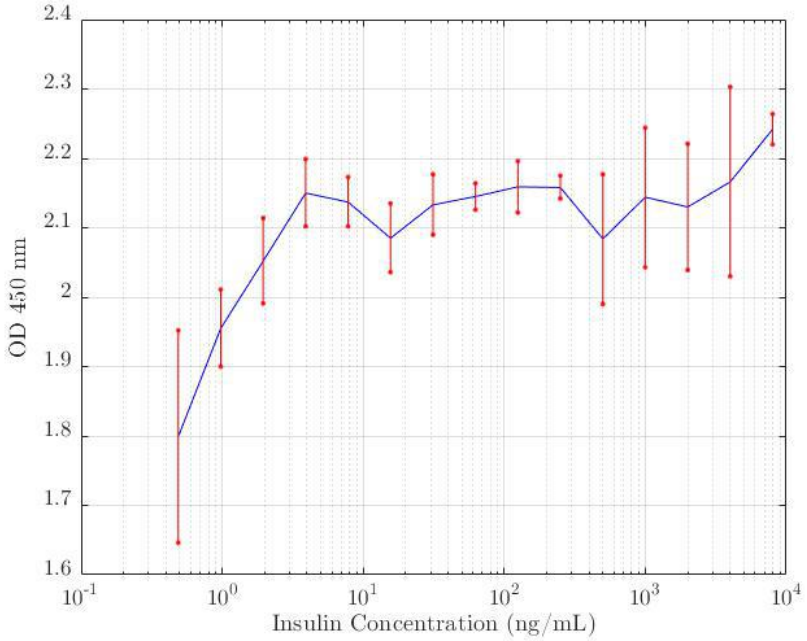

Figure 5.2: ELISA test results for antibody pair 1 (Capture Antibody A1364)

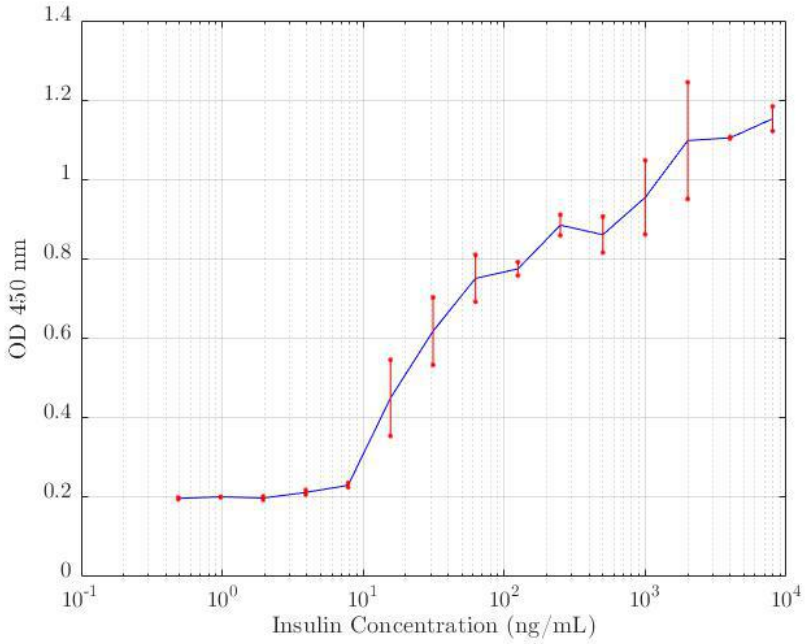

Figure 5.3: ELISA test results for antibody pair 2 (Capture Antibody A1365)

The results supports the hypothesis that the second antibody pair produces a more gradual signal over the tested concentration range. By inspecting the results for antibody pair 1, it can be seen that above 4 ng/ml, the signal strength appears to be saturated which contributes to a single logarithmic dynamic range (0.5 - 4 $/ml$). The second antibody pair provides a 3 logarithmic dynamic range (from 10 ng/ml to 8 $\mu g/ml$) which is superior to that of the antibody pair 1. It can be noted that the ELISA protocol is extremely time-consuming and was performed over 2 days, giving impetus to the need for a rapid POC immunosensor.

5.2 Analysis of Device Fabrication

A total of over 500 transistors were developed and tested with an extremely low yield rate due to the poor repeatability and alignment of the inkjet printer. However, after placing a drop of the PEDOT:PSS instead of printing the semiconductor, the yield rate rose to over 90% which increased the repeatability and reproducibility. This resulted in more consistent transistors with regard to sheet resistance and drain currents, and enabled the production of the final OECTs including the immobilized antibodies and functionalized protein.

A total of 40 OECTs were developed, handled and stored under the same conditions: 8 were left unfunctionalized to serve as controls, 4 were immobilized with capture antibodies and the other 28 were immobilized with Biotinylated capture antibodies and gold nanoparticle conjugated detection antibodies to-

gether with the designed insulin concentrations. This section discusses the analysis of the 8 fabricated control OECTs.

5.2.1 Silver Electrodes

The dimensions (in μm) of the interdigitated silver electrodes and uniform gaps are shown in Figure 5.4. The channel area (the white space between the electrodes) is therefore calculated to be 1.11 mm^2. The size of the suspended gate electrode that was in contact with the PBS solution was roughly 2 x 3 mm, which resulted in an area ratio of 0.18.

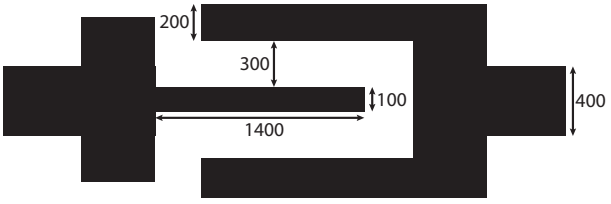

Figure 5.4: Interdigitated electrode design dimensions of the OECT

Two magnification photos of separate printed silver electrodes are shown in Figure 5.5. Figure 5.5a illustrates less effective printed electrodes as can be seen from the number of holes in the silver, whereas Figure 5.5b illustrates superior printed silver electrodes. The holes can be attributed to a number of factors such as clogged nozzles, inaccurate print speeds, incorrect viscosities, ambient temperatures, etc. Due to the nature of the individual drops, it is extremely difficult to measure the actual film thickness.

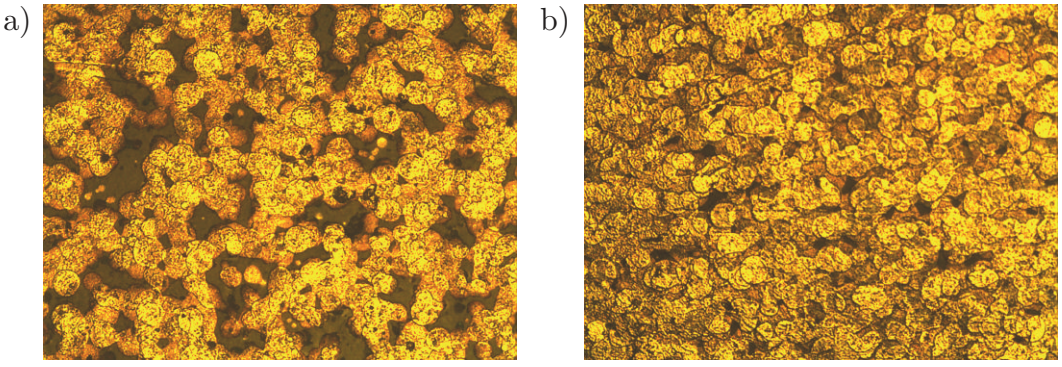

Figure 5.5: A microscopic picture of a) less effective printed silver electrodes and b) superior printed silver electrodes

Figure 5.6 illustrates the difference in printing quality before and after replacing a clogged printhead. Less effective printed electrodes negatively affect the sheet resistance, amplification capabilities and responsiveness of the OECT.

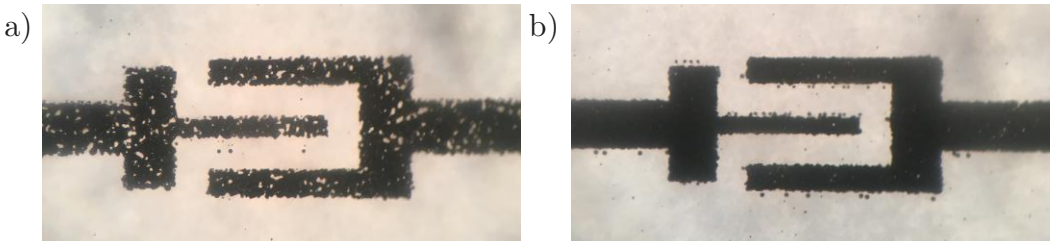

Figure 5.6: A picture of the printing quality of a) less effective printed silver electrodes before and b) superior printed silver electrodes after the replacement of a printhead having clogged nozzles

The final printed source and drain electrodes are shown in Figure 5.7a below.

5.2.2 Semiconductor Characteristics

The semiconductor plays and vital role in order to obtain good electrical contact between the source and drain electrodes. The effectiveness of the semiconductor is also influenced by the fabrication technique. For inkjet printing, it is common practice to print multiple layers of semiconductor ink on top of one another to obtain the desired channel thickness. Here, the PEDOT:PSS layer could however not be printed due to misalignment issues caused by the low-cost printer. The solution was to drop a 5 μl drop of PEDOT:PSS onto the interdigitated electrodes (shown in Figure 5.7b) instead of printing the semiconductor. This resulted in higher mobility and decreased sheet resistance due to the increased layer thickness. Table 5.1 shows the variation of sheet resistances of the fabricated functionalized and unfunctionalized OECTs.

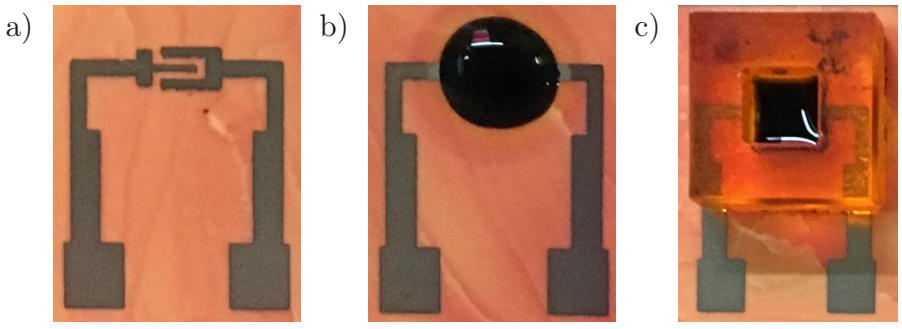

Figure 5.7: A picture of the manufactured a) spin coated electrodes, b) 5 μl PEDOT:PSS Drop and c) the attached acrylic well

Table 5.1: Sheet resistance measurements for unfunctionalized OECTs, OECTs immobilizaed with capture antibodies and completely functionalized OECTs

Developed OECT	Sheet Resistance
Control (Unfunctionalized)	7.05 Ohm
Control (Only Capture Ab)	8.33 Ohm
Concentration 1 (2000 $\mu g/ml$)	9.98 Ohm
Concentration 2 (1000 $\mu g/ml$)	10.4 Ohm
Concentration 3 (500 $\mu g/ml$)	11.65 Ohm
Concentration 4 (250 $\mu g/ml$)	12.63 Ohm
Concentration 5 (125 $\mu g/ml$)	10.28 Ohm
Concentration 6 (62.5 $\mu g/ml$)	12.85 Ohm
Concentration 7 (31.25 $\mu g/ml$)	11.95 Ohm

It is clear from Table 5.1 that the sheet resistance increased after the immobilization of the streptavidin and biotinylated capture antibodies as well as the addition of the insulin protein concentrations and gold-nanoparticle conjugated detection antibodies.

5.3 Measurement Device Validation

The NI CompactDAQ and Modules together with the customized LabVIEW software was validated using a LND150 N-Channel Depletion-Mode MOSFET. The LND150 MOSFET is a N-channel MOSFET which uses electrons as the mobile charge carriers, compared to holes in p-channel OECTs which therefore required positive drain voltages. Figure 5.8 below illustrates the output characteristics of the LND150 MOSFET. Compared to the supplied output characteristics curve given in the datasheet (Appendix L) the results corresponds closely which shows shows that the measurement device is functioning as desired.

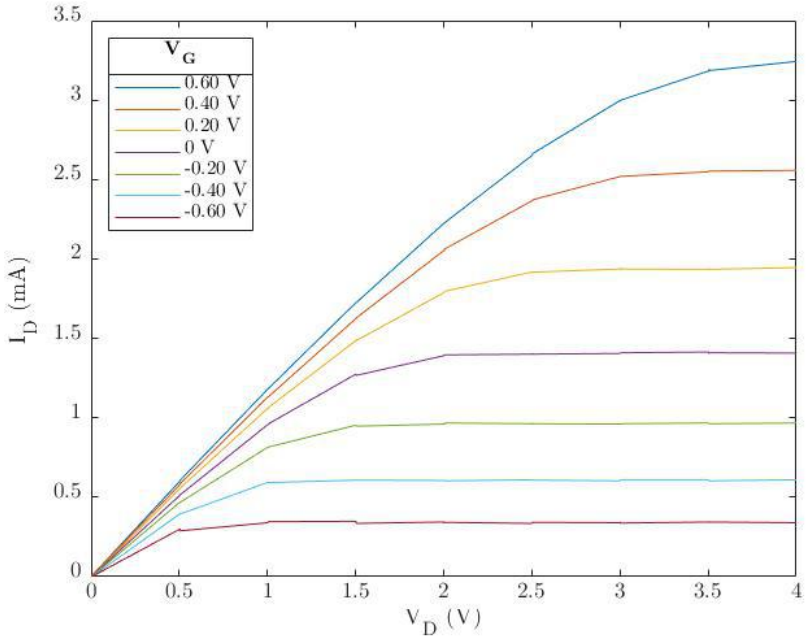

Figure 5.8: Output characteristics (I_D versus V_D) of an LND150 N-Channel MOSFET under gate voltages (V_G) varying from -0.6 to 0.6 V and sweeping V_D from 0 to 4 V

5.4 OECT Electrical Characterization

For electrical characterization, a 20 μl drop of phosphate buffer saline (PBS) solution was put on the surface of the PEDOT:PSS layer, inside the acrylic well. The Ag gate (a strip of sterling silver) was suspended from the top to make contact with the PBS droplet (refer to Figure 4.6). The output characteristics were recorded by sweeping V_D voltage from 0 V to -0.8 V and the gate voltage was incrementally switched from 0.4 V to 0.7 V in steps of 0.05 V. Figure 5.9 shows the output characteristics (I_D versus V_D).

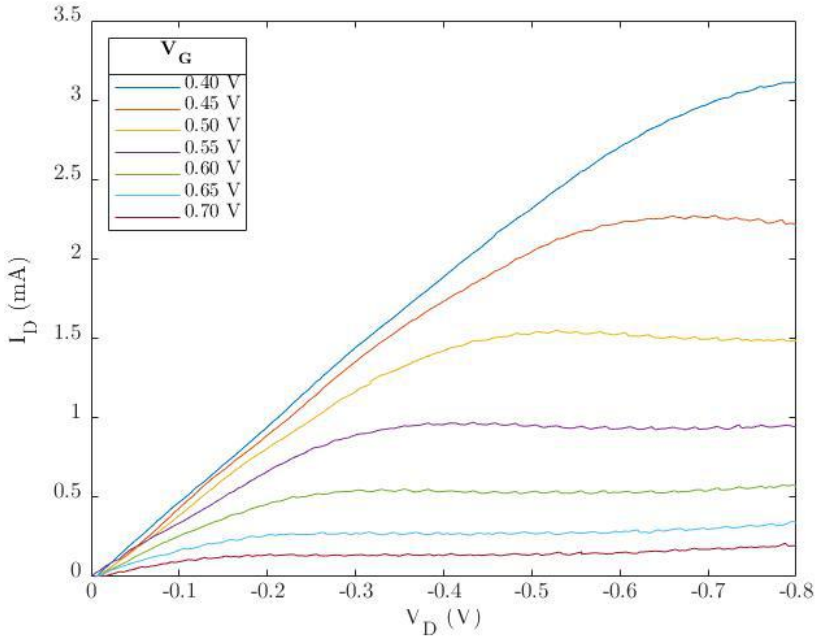

Figure 5.9: Output characteristics (I_D versus V_D) of an OECT under gate voltages (V_G) varying from 0.4 to 0.7 V and sweeping V_D from 0 to -0.8 V

The OECTs were biased at a constant V_D = -0.4 V and V_G = 0.4 V in order to test the accuracy of the sensor at a fixed biased point. From Figure 5.9, when biased at the above mentioned values it should result in a drain current of 1.9 mA. Figure 5.10 demonstrates the response of the OECTs after 30 seconds at the biased point. It is clear that the current settles at 1.926 mA after 20 seconds, which is a typical settling time of OECTs due to the slow transfer rate of ions.

Figure 5.11 illustrates the transfer characteristic (I_D versus V_G) and resulting transconductance (g_m versus V_g) for the OECT biased at V_D equals -0.4 V and sweeping V_G from 0.7 down to 0.1 V.

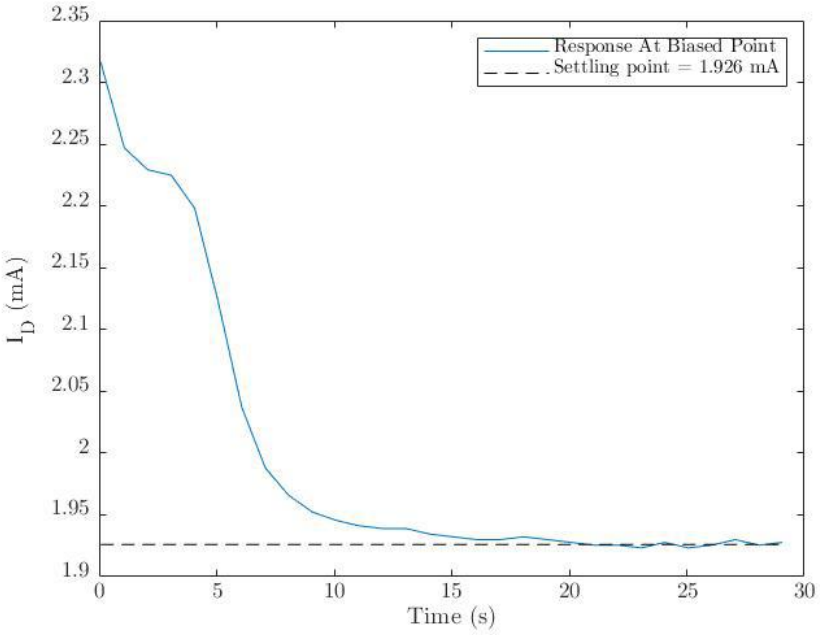

Figure 5.10: OECT response curve (I_D versus Time (s)) biased at a gate voltage (V_G) of 0.4 V and a drain voltage (V_D) of -0.4 V

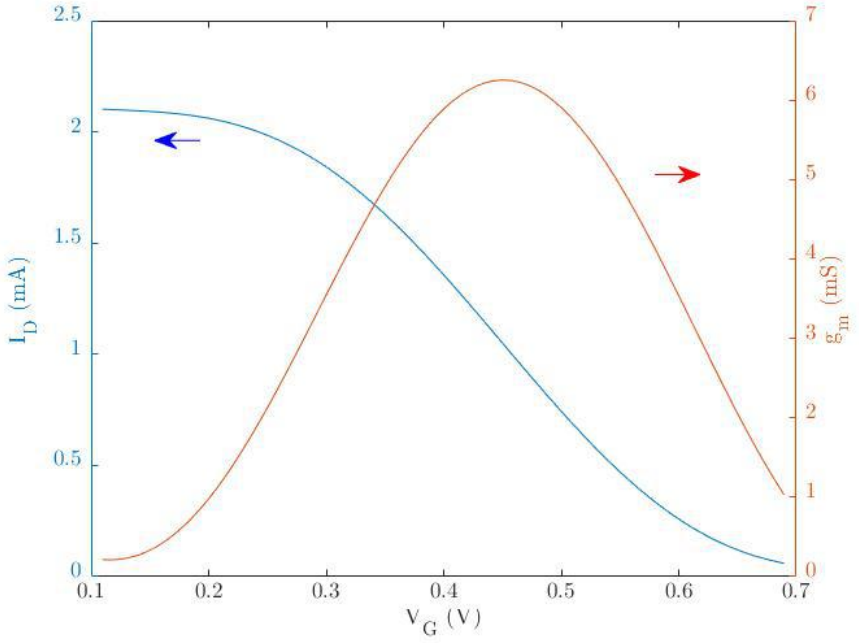

Figure 5.11: Transfer characteristic (blue line, I_D versus V_G) and resulting transconductance (red line, g_m versus V_G) derived from mathematical differential biased at V_D = -0.4 V

The hysteresis output curves of an OECT under gate voltages (V_G) varying from 0.4 to 0.7 V and sweeping V_D from 0 to -0.8 V is given in Figure 5.12. It can clearly be noted that the backward sweep current is lower than the forward sweep current, which is caused by the charge carrier trapping at the channel interface.

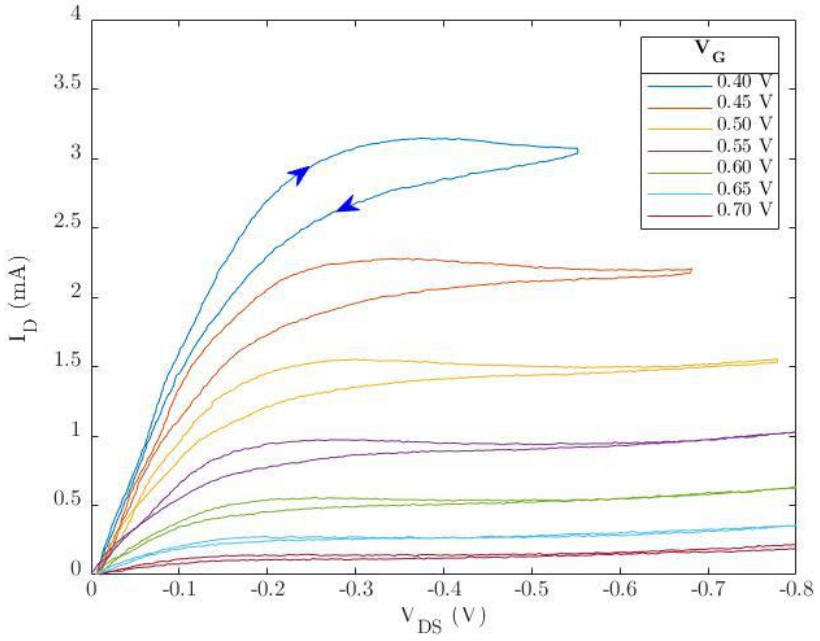

Figure 5.12: Hysteresis output curves (I_D versus V_{DS}) of an OECT under gate voltages (V_G) varying from 0.4 to 0.7 V and sweeping V_D from 0 to -0.8 V

5.5 Final Immunosensor tests

After the immobilization and functionalization of the antibodies and insulin proteins, the OECTs now function as immunosensors. The PBS containing the varying recombinant human insulin concentrations were prepared and immobilized prior to the measurements. The transfer characteristics measurements were carried out by sweeping V_G from 1 V down to 0 V and biasing V_D at -0.4 V. Figure 5.13 shows the immunosensor transfer curves at the different designed insulin concentrations ranging from 31.25 ng/ml to 2000 ng/ml.

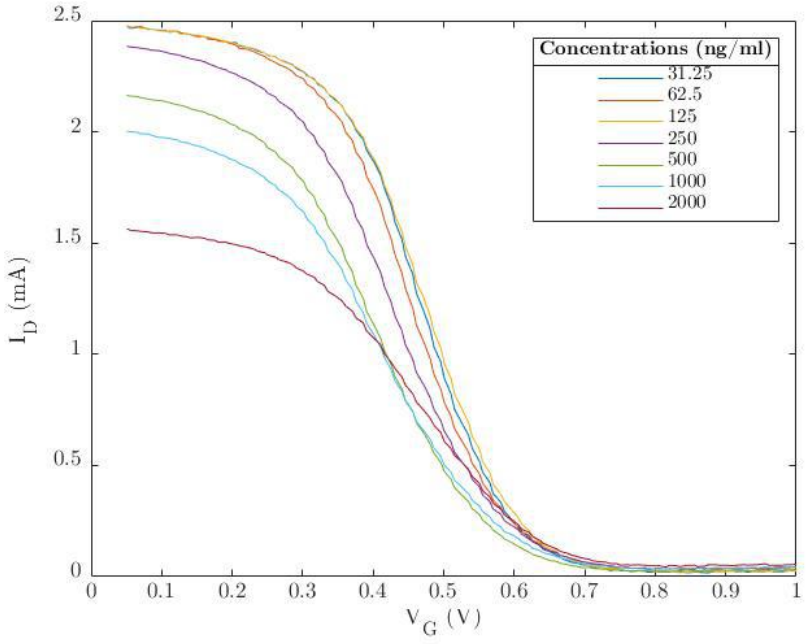

Figure 5.13: V_G dependent transfer characteristics (I_D versus V_G, V_D = -0.4 V) of OECTs functionalized with the designed insulin concentrations

It is clear from Figure 5.13 that the addition of the antibodies, blocking buffer and insulin proteins resulted in a decrease in channel current as the insulin concentration increased. Since the transconductance (g_m) is a function of the effective gate voltage (V_g^{eff}), the transconductance will show a horizontal shift due to the addition of an insulin concentration. Figure 5.15 illustrates this horizontal shift in the transconductance after the addition of insulin at a concentration of 500 ng/ml proving that the sensors in fact function as OECTs.

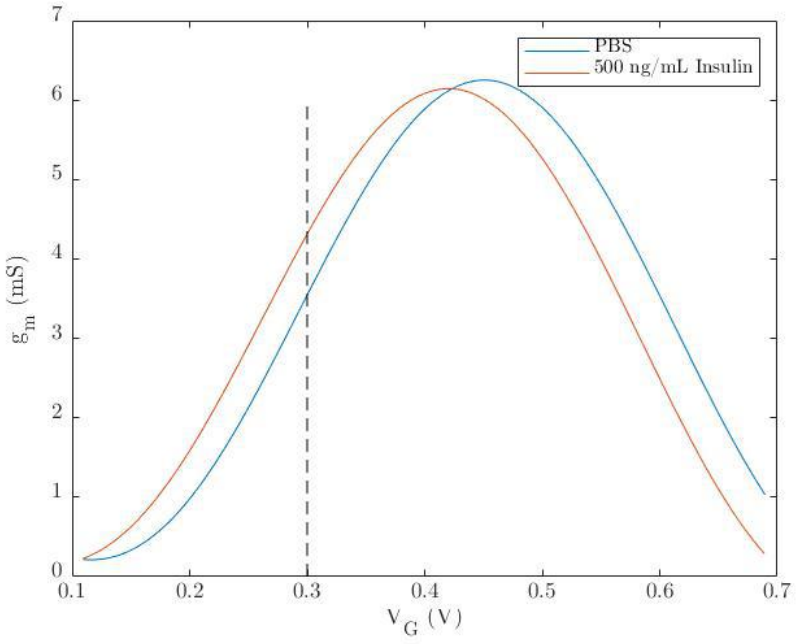

Figure 5.14: V_G dependent transconductance shift(g_m versus V_G, V_D = -0.4 V) of an OECT measured in PBS solution (pH = 7.4) before and after the addition of insulin with the concentration of 500 ng/mL

For comparison, the different transfer curves for the functionalized and unfunctionalized OECTs are given in Figure 5.15. It is clear that there is also a horizontal shift caused by the change in effective gate voltage due to the addition of insulin concentrations. It is also clear that the addition of only antibodies and the insulin concentrations, cause the threshold voltage to decrease which is expected with OECTs. The threshold voltage decreased from roughly 0.85 V (for unfunctionalized OECTs) to 0.7 V (for OECTS with insulin concentrations added) resulting in lower operating voltages as the necessary turn-on voltage has decreased.

In order to verify the limit of detection (LOD) and sensitivity of the fabricated immunosensors, the final tests involved biasing the immunosensors at V_G at 0.4 V and V_D at -0.4 V and measuring the respective drain currents (I_D) after 30 seconds at the various insulin concentrations. Figure 5.16 provides the resulting current versus concentration (logarithmic) results that can be used as an immunosensor characterization curve. This characterization curve clearly shows an exponential regression line, to a certain degree, gained from the drain current values at the respective concentrations. The fabricated immunosensors exhibit great sensitivity towards the immobilizaiton and functionalization of proteins and antibodies. The immunosensors also display excellent selectivity for the designed concentrations and provides a wide dynamic range. This provides a proper alternative to the conventional immunoassays and could assist

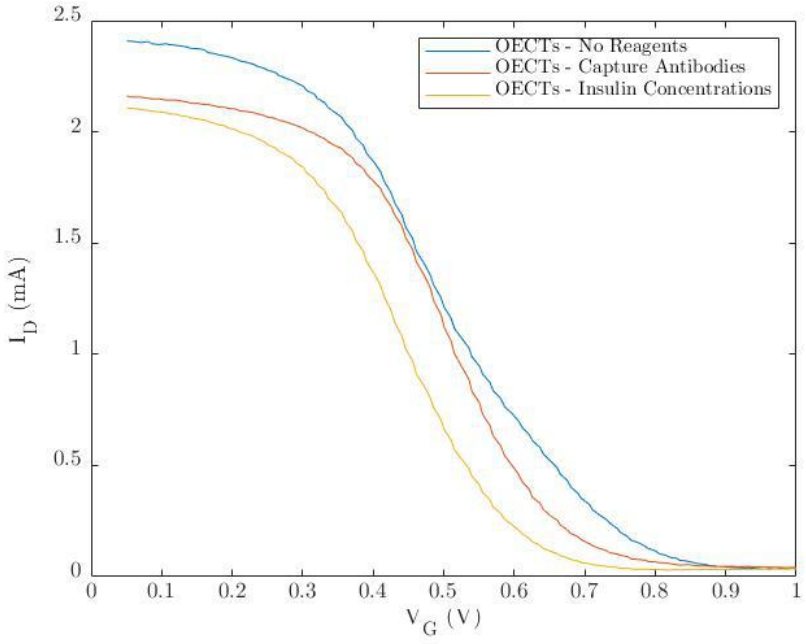

Figure 5.15: V_G dependent transfer characteristics (I_D versus V_G, V_D = -0.4 V) of functionalized and unfunctionalized OECTs

in the measurement and control of diabetes to prevent or eliminate the extreme health complications.

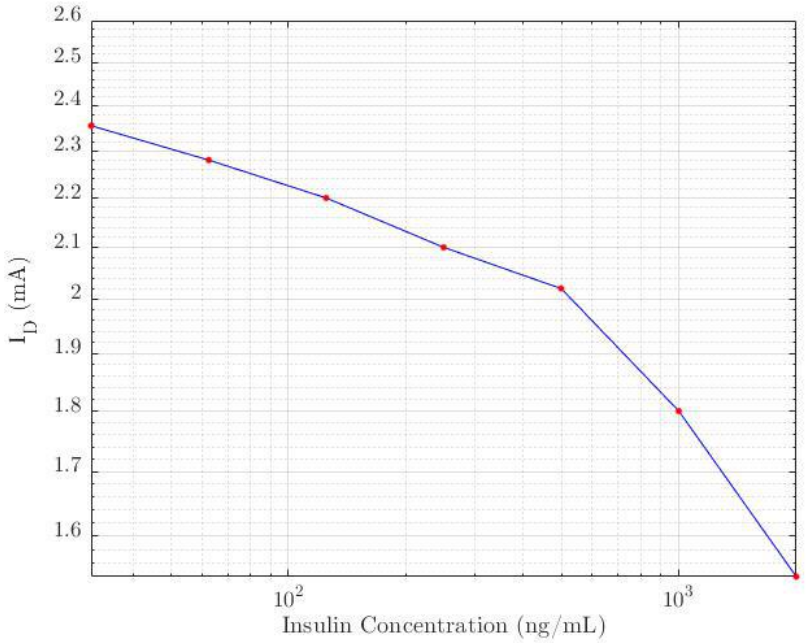

Figure 5.16: Immunosensor characterization curve for the designed concentrations

Chapter 6

Discussion and Conclusion

This thesis presented the development of a rapid immunosensor for the quantification of insulin based on a paper-based inkjet printed OECT. The proposed immunosensor is inexpensive compared to current methods of insulin measurement due to the use of a paper substrate and a low-cost inkjet printer. This methodology reduces fabrication costs and improves the ability for large-scale fabrication. The immunosensor is highly sensitive and specific towards insulin due to the use of two high affinity monoclonal antibodies in a sandwich configuration.

The developed immunosensor presented favourable sheet resistances of 7 - 12 Ω, which was much lower than the designed specification (20 Ω). The immunosensor also exhibited very few hysteresis which is highly desirable for this type of sensors. A high transconductance was achieved through the use of top-quality materials and device geometry. The limit of detection was 31.25 ng/ml that does not meet the desired 0.3 ng/ml. This was attributed to the limitations of the antibody pair as observed during the antibody testing.

One of the major limitations of this research study was the poor alignment observed by the inkjet printer, rendering it impossible to print multiple layers of the semiconductor on top of each other and led to the substitution of the printed layer for a drop of the semiconductor. Further research and work into highly accurate low-cost inkjet printers would make a big contribution to this technology. The oxidation of the gate electrode posed a limitation and required the use of the acrylic well and external gate electrode as the printed gate electrode dissolved due to the applied voltages. The final major limitation was caused by PBS seepage through the paper substrate which resulted in a short circuit between the source and drain electrodes. This was solved after the addition of the acrylic well that possessed an inner surface area smaller than the surface area of the semiconductor drop preventing the PBS to seep past the drop into the paper.

This study provided an inexpensive and innovative solution to quantify insulin which can be manufactured using a low-cost household inkjet printer. This would be extremely valuable for third world countries as it would be

inexpensive, readily available and provide better treatment outcomes for diabetic patients that do not have direct access to health-care facilities. The succeeding point-of-care device will also aid the distinguish between type 1 and type 2 diabetes without the need for sophisticated equipment i.e. liquid chromatography-tandem mass spectrometry.

Other suggestions for future work include optimizing the device geometry for even higher transconductances, sourcing of appropriate materials for the electrodes to prevent oxidation, and finally the use of alternative materials for the substrate such as ceramic or plastics to prevent seepage. The proposed immunosensor met all other design specifications. The findings in this thesis is in agreement with related research in this field and permits future development of a point-of-care device which can be used for rapid monitoring of insulin.

Appendices

Appendix A

Access Ultrasensitive Insulin

BECKMAN COULTER CE

ACCESS
Immunoassay Systems

Instructions For Use

Access Ultrasensitive Insulin

REF 33410

FOR PROFESSIONAL USE ONLY

Rx Only

ANNUAL REVIEW

Reviewed by	Date	Reviewed by	Date

PRINCIPLE

INTENDED USE

The Access Ultrasensitive Insulin assay is a paramagnetic particle, chemiluminescent immunoassay for the quantitative determination of insulin levels in human serum and plasma (EDTA) using the Access Immunoassay Systems.

SUMMARY AND EXPLANATION

Insulin is a hormone secreted by the beta cells of the pancreas. Insulin regulates the uptake and utilization of glucose, and is also involved in the regulation of protein synthesis and triglyceride storage.[1,2,3] An increase in the amount of glucose in circulation stimulates insulin secretion. Insulin in turn stimulates the uptake of glucose into the tissues and inhibits the breakdown of glycogen in the liver. As the glucose level comes back to baseline so does insulin.

One of insulin's primary clinical uses is in the diagnosis and management of diabetes mellitus, a disease arising when glucose is not adequately taken up into the tissues. The result is chronic hyperglycemia. Diabetes can have severe complications including renal failure, heart disease, nerve damage, blindness, and gangrene. Severe hyperglycemic episodes can cause ketoacidosis and coma.

Diabetes has been divided into 2 major categories based on the secretion of insulin. The first is insulin dependent diabetes mellitus (IDDM) or Type I diabetes. It is brought on by the autoimmune destruction of the insulin secreting beta cells in the pancreas.[4] Insulin secretion gradually declines to an insignificant level as ultimately all beta cells are destroyed. The patient must receive insulin injections in order to survive.

The second category is non-insulin dependent diabetes mellitus (NIDDM) or Type II. This disorder arises by an entirely different mechanism. Here, the beta cells can still secrete insulin but the body has developed resistance to the hormone.[3] When the concentration of glucose in circulation rises, the insulin response is slow and of insufficient magnitude. As the disease progresses more and more insulin may be required to obtain the same level of glucose control. The patient may need to be placed on drugs which stimulate insulin secretion or be supplemented with insulin, depending on their degree

of glucose control. Type II diabetes is associated with genetic factors, obesity, sedentary life-style, and other unknown factors. It can often be controlled by proper diet and adequate exercise.

The measurement of insulin is used in the following investigations:

- Diagnosis of diabetes:
  Insulin levels under basal conditions or after glucose administration are useful for assessing the ability of the pancreas to secrete insulin. Insulin levels are normally low in patients with insulin-dependent diabetes mellitus (IDDM) and are normal or elevated in patients with non-insulin dependent diabetes mellitus (NIDDM).
- Early detection of diabetes:
  The insulin response to the administration of glucose may be blunted well before the onset of clinical manifestations.
- Follow-up and stabilization of insulin-treated diabetics:
  Insulin assays can be useful at the onset of insulin therapy to evaluate the duration of action of various insulin preparations.
- Predicting complications of Type II diabetes (NIDDM):
  The persistent elevation of insulin is a risk factor for the development of coronary disease.[5]
- Diagnosis of insulinoma:
  Pancreatic beta-cell tumors may produce a state of hyperinsulinism leading to hypoglycemia.[6]

METHODOLOGY

The Access Ultrasensitive Insulin assay is a simultaneous one-step immunoenzymatic (“sandwich”) assay. A sample is added to a reaction vessel along with mouse monoclonal anti-insulin alkaline phosphatase conjugate and paramagnetic particles coated with mouse monoclonal anti-insulin antibody.[7,8,9] The serum or plasma insulin binds to the antibody on the solid phase, while the conjugate reacts with a different antigenic site on the insulin molecule. After incubation in a reaction vessel, materials bound to the solid phase are held in a magnetic field while unbound materials are washed away. Then, the chemiluminescent substrate Lumi-Phos* 530 is added to the vessel and light generated by the reaction is measured with a luminometer. The light production is directly proportional to the concentration of insulin in the sample. The amount of analyte in the sample is determined from a stored, multi-point calibration curve.

SPECIMEN

SPECIMEN COLLECTION AND PREPARATION

1. Serum and plasma (EDTA) are the recommended sample types and should not be used interchangeably. Literature has indicated that differences in insulin levels may be seen for some individual matched serum and plasma samples.[10,11,12] Therefore, each laboratory should consistently use either serum or plasma for patient care or research. Use of a single sample type will ensure proper insulin interpretations for those samples that are tracked over time. Each laboratory should determine the acceptability of its own blood collection tubes and serum separation products. Variations in these products may exist between manufacturers and, at times, from lot-to-lot.
2. Observe the following recommendations for handling, processing, and storing blood samples:[13]
   - Collect all blood samples observing routine precautions for venipuncture.
   - Allow serum samples to clot completely before centrifugation.
   - Keep tubes stoppered at all times.
   - Physically separate serum or plasma from contact with cells as soon as possible.
   - Store samples tightly stoppered at room temperature (15 to 30°C) for no longer than eight hours.
   - If the assay will not be completed within eight hours, refrigerate the samples at 2 to 8°C.
   - If the assay will not be completed within 24 hours, or for shipment of samples, freeze at -20°C or colder.
   - Thaw samples only once.

3. Use the following guidelines when preparing specimens:
   - Ensure residual fibrin and cellular matter has been removed prior to analysis.
   - Follow blood collection tube manufacturer's recommendations for centrifugation.
4. Each laboratory should determine the acceptability of its own blood collection tubes and serum separation products. Variations in these products may exist between manufacturers and, at times, from lot-to-lot.
5. Do not use hemolyzed samples, as hemolysis releases enzymes which degrade insulin.[14]

REAGENTS

PRODUCT INFORMATION

Access Ultrasensitive Insulin Reagent Pack

Cat. No. 33410: 100 determinations, 2 packs, 50 tests/pack

- Provided ready to use.
- Store upright and refrigerate at 2 to 10°C.
- Refrigerate at 2 to 10°C for a minimum of two hours before use on the instrument.
- Stable until the expiration date stated on the label when stored at 2 to 10°C.
- Stable at 2 to 10°C for 28 days after initial use.
- Signs of possible deterioration are a broken elastomeric layer on the pack or control values out of range.
- If the reagent pack is damaged (i.e., broken elastomer), discard the pack.
- All antisera are polyclonal unless otherwise indicated.

R1a:	Mouse monoclonal anti-insulin coupled to paramagnetic particles, TRIS buffer, bovine serum albumin (BSA) matrix, < 0.1% sodium azide, and 0.1% ProClin** 300.
R1b:	Mouse monoclonal anti-insulin conjugated to bovine alkaline phosphatase, TRIS buffer, BSA matrix, < 0.1% sodium azide, and 0.1% ProClin 300.
R1c:	Mouse IgG in HEPES buffer, BSA matrix, < 0.1% sodium azide, and 0.5% ProClin 300.

**ProClin™ is a trademark of The Dow Chemical Company (“Dow”) or an affiliated company of Dow.

WARNING AND PRECAUTIONS

- For *in vitro* diagnostic use.
- Patient samples and blood-derived products may be routinely processed with minimum risk using the procedure described. However, handle these products as potentially infectious according to universal precautions and good clinical laboratory practices, regardless of their origin, treatment, or prior certification. Use an appropriate disinfectant for decontamination. Store and dispose of these materials and their containers in accordance with local regulations and guidelines.
- For hazards presented by the product refer to the following sections: REACTIVE INGREDIENTS, GHS HAZARD CLASSIFICATION and EU HAZARD CLASSIFICATION.

REACTIVE INGREDIENTS

> ⚠ **CAUTION**
>
> **Sodium azide preservative may form explosive compounds in metal drain lines. See NIOSH Bulletin: Explosive Azide Hazard (8/16/76).**
> **To avoid the possible build-up of azide compounds, flush wastepipes with water after the disposal of undiluted reagent. Sodium azide disposal must be in accordance with appropriate local regulations.**

GHS HAZARD CLASSIFICATION

Insulin PMP (Compartment R1a)	WARNING	
		
	H317	May cause an allergic skin reaction.
	P280	Wear protective gloves, protective clothing and eye/face protection.
	P333+P313	If skin irritation or rash occurs: Get medical advice/attention.
	P362+P364	Take off contaminated clothing and wash it before use.
		reaction mass of: 5-chloro-2-methyl-4-isothiazolin -3-one [EC# 247-500-7] and 2-methyl-4-isothiazolin-3-one [EC# 220-239-6](3:1) < 0.05%
Insulin Conjugate (Compartment R1b)	WARNING	
		
	H317	May cause an allergic skin reaction.
	P280	Wear protective gloves, protective clothing and eye/face protection.
	P333+P313	If skin irritation or rash occurs: Get medical advice/attention.
	P362+P364	Take off contaminated clothing and wash it before use.
		reaction mass of: 5-chloro-2-methyl-4-isothiazolin -3-one [EC# 247-500-7] and 2-methyl-4-isothiazolin-3-one [EC# 220-239-6](3:1) <0.05%
Insulin Blocker (Compartment R1c)	WARNING	

H317	May cause an allergic skin reaction.
H402	Harmful to aquatic life.
P273	Avoid release to the environment.
P280	Wear protective gloves, protective clothing and eye/face protection.
P333+P313	If skin irritation or rash occurs: Get medical advice/attention.
P362+P364	Take off contaminated clothing and wash it before use.
	Ethoxylated alkyl alcohol 0.1 - 0.5%
	reaction mass of: 5-chloro-2-methyl-4-isothiazolin -3-one [EC# 247-500-7] and 2-methyl-4-isothiazolin-3-one [EC# 220-239-6](3:1) <0.05%

SDS	Safety Data Sheet is available at techdocs.beckmancoulter.com

EUROPEAN HAZARD CLASSIFICATION

Insulin PMP (Compartment R1a)	Xi;R43	
	R43	May cause sensitization by skin contact.
	S28	After contact with skin, wash immediately with plenty of soap and water.
	S37	Wear suitable gloves.
Insulin Conjugate (Compartment R1b)	Xi;R43	
	R43	May cause sensitization by skin contact.
	S28	After contact with skin, wash immediately with plenty of soap and water.
	S37	Wear suitable gloves.
Insulin Blocker (Compartment R1c)	Xi;R43	
	R43	May cause sensitization by skin contact.
	S28	After contact with skin, wash immediately with plenty of soap and water.
	S37	Wear suitable gloves.

MATERIALS NEEDED BUT NOT SUPPLIED WITH REAGENT KIT

1. Access Ultrasensitive Insulin Calibrators
   Provided at zero and approximately 1.0, 10, 50, 150 and 300 µIU/mL
   (7.0, 70, 350, 1,050 and 2,100 pmol/L).
   Cat. No. 33415
2. Quality Control (QC) materials: commercial control material.
3. Access Sample Diluent A
   Vial Cat. No. 81908
   Diluent Pack Cat. No. A79783 (For use with the UniCel DxI system onboard dilution feature.)
4. Access Substrate
   Cat. No. 81906
5. Access Wash Buffer II, Cat. No. A16792
   UniCel DxI Wash Buffer II, Cat. No. A16793

EQUIPMENT AND MATERIALS

R1	Access Ultrasensitive Insulin Reagent Packs

CALIBRATION

CALIBRATION INFORMATION

An active calibration curve is required for all tests. For the Access Ultrasensitive Insulin assay, calibration is required every 28 days. Refer to the appropriate system manuals and/or Help system for information on calibration theory, configuring calibrators, calibrator test request entry, and reviewing calibration data.

QUALITY CONTROL

Quality control materials simulate the characteristics of patient samples and are essential for monitoring the system performance of immunochemical assays. Because samples can be processed at any time in a “random access” format rather than a “batch” format, quality control materials should be included in each 24-hour time period.[15] Include commercially available quality control materials that cover at least two levels of analyte. More frequent use of controls or the use of additional controls is left to the discretion of the user based on good laboratory practices or laboratory accreditation requirements and applicable laws. Follow manufacturer's instructions for reconstitution and storage. Each laboratory should establish mean values and acceptable ranges to assure proper performance. Quality control results that do not fall within acceptable ranges may indicate invalid test results. Examine all test results generated since obtaining the last acceptable quality control test point for this analyte. Refer to the appropriate system manuals and/or Help system for information about reviewing quality control results.

TESTING PROCEDURE(S)

PROCEDURAL COMMENTS

1. Refer to the appropriate system manuals and/or Help system for a specific description of installation, start-up, principles of operation, system performance characteristics, operating instructions, calibration procedures, operational limitations and precautions, hazards, maintenance, and troubleshooting.
2. Mix contents of new (unpunctured) reagent packs by gently inverting pack several times before loading on the instrument. Do not invert open (punctured) packs.

3. Use twenty (20) µL of sample for each determination in addition to the sample container and system dead volumes. Use fifty (50) µL of sample in addition to the sample container and system dead volumes for each determination run with the DxI system onboard dilution feature. Refer to the appropriate system manuals and/or Help system for the minimum sample volume required.
4. The system default unit of measure for sample results is µIU/mL. To change sample reporting units to the International System of Units (SI units), pmol/L, refer to the appropriate system manuals and/or Help system. To manually convert concentrations to the International System, multiply µIU/mL by multiplication factor 7.0.

PROCEDURE

Refer to the appropriate system manuals and/or Help system for information on managing samples, configuring tests, requesting tests, and reviewing test results.

RESULTS INTERPRETATION

Patient test results are determined automatically by the system software using a smoothing spline math model. The amount of analyte in the sample is determined from the measured light production by means of the stored calibration data. Patient test results can be reviewed using the appropriate screen. Refer to the appropriate system manuals and/or Help system for complete instructions on reviewing sample results.

REPORTING RESULTS

EXPECTED RESULTS

1. Each laboratory should establish its own reference ranges to assure proper representation of specific populations.
2. Sera from 67 healthy fasting subjects were assayed to establish an expected range. A non-parametric estimate at the 95% confidence level yields the following range:

Units	Normal Range
µIU/mL	1.9-23
pmol/L	13.0-161

PROCEDURAL NOTES

LIMITATIONS

1. Samples can be accurately measured within the analytic range of the lower limit of detection and the highest calibrator value (approximately 0.03-300 µIU/mL [0.21-2,100 pmol/L]).
   - If a sample contains less than the lower limit of detection for the assay, report the results as less than that value (i.e., < 0.03 µIU/mL [< 0.21 pmol/L]). When the DxI system onboard dilution feature is used, the system will report results as less than 255 µIU/mL (1,785 pmol/L).
   - If a sample contains more than the stated value of the highest Access Ultrasensitive Insulin Calibrator (S5), report the result as greater than that value (i.e. > 300 µIU/mL [> 2,100 pmol/L]). Alternatively, dilute one volume of sample with 9 volumes of Access Ultrasensitive Insulin Calibrator S0 (zero) or Access Sample Diluent A. Refer to the appropriate system manuals and/or Help system for instructions on entering a sample dilution in a test request. The system reports the results adjusted for the dilution. The DxI system onboard dilution feature automates the dilution process, using one volume of sample with 9 volumes of Access Sample Diluent A, allowing samples to be quantitated up to approximately 3,000 µIU/mL (21,000 pmol/L). The system reports the results adjusted for the dilution.

2. For assays employing antibodies, the possibility exists for interference by heterophile antibodies in the patient sample. Patients who have been regularly exposed to animals or have received immunotherapy or diagnostic procedures utilizing immunoglobulins or immunoglobulin fragments may produce antibodies, e.g. HAMA, that interfere with immunoassays. Additionally, other heterophile antibodies such as human anti-goat antibodies may be present in patient samples.[16,17]

   Such interfering antibodies may cause erroneous results. Carefully evaluate the results of patients suspected of having these antibodies.

3. The Access Ultrasensitive Insulin results should be interpreted in light of the total clinical presentation of the patient, including: symptoms, clinical history, data from additional tests and other appropriate information.

4. Patients on insulin therapy are prone to the development of anti-insulin antibodies. These antibodies may interfere with the assay.[18,19,20,21,22]

PERFORMANCE CHARACTERISTICS

PERFORMANCE CHARACTERISTICS

METHODS COMPARISON

A comparison of 153 serum insulin values using the Access Ultrasensitive Insulin assay on the Access Immunoassay system and a commercially available immunoassay kit gave the following statistical data:

n	Range of Observations (µIU/mL)	Intercept (µIU/mL)	Slope	Correlation Coefficient (r)
153	1.4-219	-0.42	0.88	0.996

A comparison of 59 values obtained by assaying clinical samples of serum and plasma (EDTA) using the Access Ultrasensitive Insulin assay kit on the Access Immunoassay System gave the following statistical data:

n	Range of Observations (µIU/mL)	Intercept (µIU/mL)	Slope	Correlation Coefficient (r)
59	2.5-100	0.53	1.03	0.988

DILUTION RECOVERY (LINEARITY)

Multiple volumetric dilutions of three samples containing various insulin levels with Access Ultrasensitive Insulin Calibrator S0 (zero) resulted in the following data:

Sample 1	Expected Concentration (µIU/mL)	Determined Concentration (µIU/mL)	Recovery (%)
Neat	N/A	258.0	N/A
1:1.4	184.0	184.0	100
1:2	129.0	126.0	97
1:4	64.5	61.7	96
1:8	32.3	36.3	112
1:16	16.1	16.7	104
		Mean % Recovery	**102**

Sample 2	Expected Concentration (µIU/mL)	Determined Concentration (µIU/mL)	Recovery (%)
Neat	N/A	146.0	N/A
1:1.4	104.0	110.0	106
1:2	73.0	78.5	108
1:4	36.5	37.3	102
1:8	18.3	19.5	107
1:16	9.1	9.2	101
		Mean % Recovery	**105**

Sample 3	Expected Concentration (µIU/mL)	Determined Concentration (µIU/mL)	Recovery (%)
Neat	N/A	86.8	N/A
1:1.4	62.0	63.2	102
1:2	43.4	42.9	99
1:4	21.7	19.7	91
1:8	10.9	10.5	96
1:16	5.4	5.2	96
		Mean % Recovery	**97**

SPIKING RECOVERY

Addition of four different levels of insulin to two serum samples with low insulin resulted in the following data:

Sample 1 (µIU/mL)	Expected Concentration (µIU/mL)	Determined Concentration (µIU/mL)	Recovery (%)
Neat	N/A	11.5	N/A
5	16.5	15.8	96
50	61.5	57.0	92
100	112.0	105.0	94
220	232.0	242.0	104
		Mean % Recovery	**97**

Sample 2 (µIU/mL)	Expected Concentration (µIU/mL)	Determined Concentration (µIU/mL)	Recovery (%)
Neat	N/A	2.9	N/A
5	7.9	7.1	90
50	52.9	49.4	93

Sample 2 (µIU/mL)	Expected Concentration (µIU/mL)	Determined Concentration (µIU/mL)	Recovery (%)
100	103.0	105.0	102
220	223.0	199.0	89
		Mean % Recovery	**94**

IMPRECISION

This assay exhibits total imprecision of less than 10% across the assay range. One study, performed by running three replicates of each sample per assay and two assays per day provided the following data, analyzed via analysis of variance (ANOVA).[23,24]

Control	Grand Mean (n=60) (µIU/mL)	Within Run (%CV)	Total Imprecision (%CV)
1	0.15	4.2	5.6
2	0.30	2.7	4.0
3	0.93	2.6	4.5
4	12.90	2.0	3.5
5	37.40	2.0	3.3
6	99.30	2.1	3.1

ANALYTICAL SPECIFICITY / INTERFERENCES

Samples containing up to 10 mg/dL (171 µmol/L) bilirubin and lipemic samples containing the equivalent of 1,800 mg/dL (20.32 mmol/L) triglycerides do not affect the concentration of insulin assayed. The addition of 5 g/dL of human albumin to samples does not affect the concentration of insulin assayed.

Proinsulin and C-peptide were spiked into the S2 calibrator which contains 70 pmol/L (10 µIU/mL) insulin. Bovine and porcine insulin were spiked into the zero calibrator. The following cross-reactivity data was obtained:

Substance	Analyte Added (pmol/L)	Cross-reactivity (%)
Proinsulin	4,000	-0.26
C-peptide	20,000	Not detectable
Bovine insulin	350	30
Porcine insulin	350	97

ANALYTICAL SENSITIVITY

The lowest detectable level of insulin distinguishable from zero (Access Ultrasensitive Insulin Calibrator S0) with 95% confidence is 0.03 µIU/mL (0.21 pmol/L). This value is determined by processing a complete six point calibration curve, controls, and ten replicates of the zero calibrator in multiple assays. The analytical sensitivity value is calculated from the curve at the point that is two standard deviations from the mean measured zero calibrator signal.

ADDITIONAL INFORMATION

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* Lumi-Phos is a trademark of Lumigen, Inc., a subsidiary of Beckman Coulter, Inc.

SYMBOLS KEY

Glossary of Symbols is available at techdocs.beckmancoulter.com (document number C02724)

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Tel: +41 (0)22 365 36 11

Beckman Coulter, Inc., 250 S. Kraemer Blvd., Brea, CA 92821 U.S.A.

Appendix B

Anti-Insulin Antibody (A1364) Datasheet

BioVision

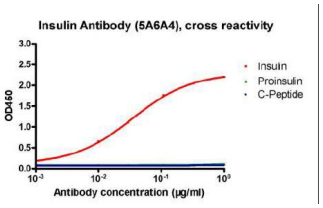
06/17

For research use only

Anti-Insulin Antibody (6E9F1)

CATALOG NO:	A1364-1000
AMOUNT:	1 mg
IMMUNOGEN:	Human recombinant Insulin expressed in yeast
CLONALITY:	Monoclonal
CLONE:	6E9F1
HOST/ISOTYPE:	Mouse IgG2a, κ
PURIFICATION:	Protein A purification
FORM:	Liquid
CONCENTRATION:	0.5 mg/ml
FORMULATION:	In PBS buffer, pH 7.4, containing 0.02% sodium azide
STORAGE CONDITIONS:	For long term storage, aliquot and store at -20°C or below. Avoid repeated freezing and thawing cycles.
SPECIFICITY:	Insulin Antibody (6E9F1) detects human Insulin and slightly recognizes proinsulin, it has no cross-reactivity with C-peptide.
DESCRIPTION:	Insulin is one of the major regulatory hormones of intermediate metabolism throughout the body. It regulates the cellular uptake, utilization, and storage of glucose, amino acids, and fatty acids and inhibits the breakdown of glycogen, protein, and fat. Proinsulin is the prohormone precursor to insulin made in pancreas. It is processed by a series of proteases to form mature insulin. Mature insulin has 35 fewer amino acids; 4 are removed altogether, and the remaining 31 form the C-Peptide. The C-Peptide is abstracted from the center of the proinsulin sequence; the two other ends (α and β chains) remain connected by disulfide bonds. Deficiency of insulin results in diabetes mellitus, one of the leading causes of morbidity and mortality in the general population. Insulin is also present in tumors of B cell origin such as insulinoma. Insulin Antibody (6E9F1) is produced from the hybridoma resulting from fusion of SP2/0-Ag14 myeloma and B-lymphocytes obtained from mouse immunized with human recombinant Insulin expressed in yeast
APPLICATION:	These antibodies are perfect choice for in vitro diagnostic assay development. **They are prepared for non-clinical research use only.** The recommended pairs are based on our R&D results.

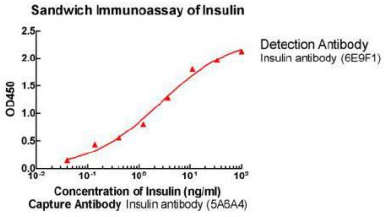
FOR RESEARCH USE ONLY! Not to be used on humans.



Cross-reactivity of Insulin monoclonal antibodies by Indirect ELISA

Assay protocol for sandwich ELISA:

1. Microplate was coated with insulin, proinsulin or C-peptide respectively, followed by 3 washing cycles.
2. Incubation with mouse anti-insulin antibody followed by 3 washing cycles.
3. Incubation with goat anti-mouse IgG conjugated to peroxidase, followed by 3 washing cycles.
4. Colorimetric determination of bound peroxidase activity.



Assay protocol for Sandwich ELISA:

1. Microplate was coated with a capture antibody against insulin, followed by 3 washing cycles.
2. Incubation with insulin followed by 3 washing cycles.
3. Incubation with peroxidase conjugated detection antibody against insulin, followed by 3 washing cycles.
4. Colorimetric determination of bound peroxidase activity.

RELATED PRODUCTS:

- ProInsulin (human) ELISA Kit **(Cat. No. K7433)**
- Proinsulin Antibody (Clone HPI-B5) **(Cat. No. 3106)**

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155 S. Milpitas Boulevard, Milpitas, CA 95035 USA

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www.biovision.com | tech@biovision.com

Appendix C

Anti-Insulin Antibody (A1365) Datasheet

BioVision

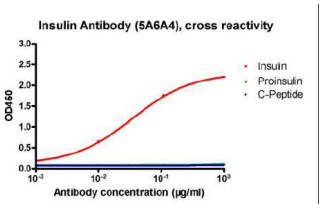
06/17

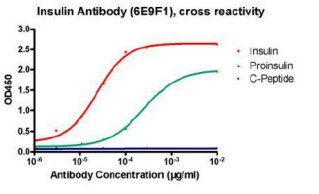
For research use only

Anti-Insulin Antibody (5A6A4)

CATALOG NO:	A1365-1000
AMOUNT:	1 mg
IMMUNOGEN:	Human recombinant Insulin expressed in yeast
CLONALITY:	Monoclonal
CLONE:	5A6A4
HOST/ISOTYPE:	Mouse IgG2b, κ
PURIFICATION:	Protein A purification
FORM:	Liquid
CONCENTRATION:	0.5 mg/ml
FORMULATION:	In PBS buffer, pH 7.4, containing 0.02% sodium azide
STORAGE CONDITIONS:	For long term storage, aliquot and store at -20°C or below. Avoid repeated freezing and thawing cycles.
SPECIFICITY:	Insulin Antibody (5A6A4), mAb, Mouse detects human Insulin and it has no cross-reactivity with Proinsulin and C-Peptide.
DESCRIPTION:	Insulin is one of the major regulatory hormones of intermediate metabolism throughout the body. It regulates the cellular uptake, utilization, and storage of glucose, amino acids, and fatty acids and inhibits the breakdown of glycogen, protein, and fat. Proinsulin is the prohormone precursor to insulin made in pancreas. It is processed by a series of proteases to form mature insulin. Mature insulin has 35 fewer amino acids; 4 are removed altogether, and the remaining 31 form the C-Peptide. The C-Peptide is abstracted from the center of the proinsulin sequence; the two other ends (α and β chains) remain connected by disulfide bonds. Deficiency of insulin results in diabetes mellitus, one of the leading causes of morbidity and mortality in the general population. Insulin is also present in tumors of B cell origin such as insulinoma. Insulin Antibody (6E9F1) is produced from the hybridoma resulting from fusion of SP2/0-Ag14 myeloma and B-lymphocytes obtained from mouse immunized with human recombinant Insulin expressed in yeast
APPLICATION:	These antibodies are perfect choice for in vitro diagnostic assay development. **They are prepared for non-clinical research use only.** The recommended pairs are based on our R&D results.

FOR RESEARCH USE ONLY! Not to be used on humans.





Cross-reactivity of Insulin monoclonal antibodies by Indirect ELISA

Assay protocol for sandwich ELISA:

1. Microplate was coated with insulin, proinsulin or C-peptide respectively, followed by 3 washing cycles.
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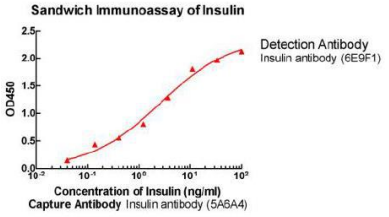
3. Incubation with goat anti-mouse IgG conjugated to peroxidase, followed by 3 washing cycles.
4. Colorimetric determination of bound peroxidase activity.



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2. Incubation with insulin followed by 3 washing cycles.
3. Incubation with peroxidase conjugated detection antibody against insulin, followed by 3 washing cycles.
4. Colorimetric determination of bound peroxidase activity.

RELATED PRODUCTS:

- ProInsulin (human) ELISA Kit **(Cat. No. K7433)**
- Proinsulin Antibody (Clone HPI-B5) **(Cat. No. 3106)**

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Appendix D

Recombinant Human Insulin Protein (ab123768) Datasheet

abcam

Product datasheet

Recombinant human Insulin protein ab123768

2 Abreviews

Overview

Product name	Recombinant human Insulin protein
Protein length	Full length protein

Description

Nature	Recombinant
Source	Escherichia coli

Amino Acid Sequence

Accession	P01308
Species	Human
Sequence	GIVEQCCTSIC SLYQLENYCN FVNQHL CGSHLVEALY LVCGERGFFY TPKT
Molecular weight	6 kDa
Additional sequence information	Recombinant Human Insulin produced in E. coli is a two chain, non-glycosylated polypeptide chain containing 51 amino acids and having a molecular mass of 5.81 kDa.

Specifications

Our Abpromise guarantee covers the use of **ab123768** in the following tested applications.

The application notes include recommended starting dilutions; optimal dilutions/concentrations should be determined by the end user.

Biological activity	ab123768 is fully biologically active when compared to World Health Organization (WHO) reference standard which is 28 units/mg.
Applications	Functional Studies SDS-PAGE HPLC
Purity	> 98 % SDS-PAGE. Purity is greater than 98% as determined by HPLC and SDS-PAGE. ab123768 is purified by proprietary chromatographic techniques. Endotoxin Level: <0.1 ng/µg of Insulin.
Form	Lyophilised

Preparation and Storage

Preparation and Storage

Stability and Storage	Shipped at 4°C. Store at -20°C. Store under desiccating conditions. This product is an active protein and may elicit a biological response in vivo, handle with caution.
Reconstitution	Reconstitute in 5-10 mM HCl to a concentration of 1.0 mg/ml. The solution can then be diluted to other aqueous buffers. Upon reconstitution ab123768 should be stored at 4°C for 2-7 days. For long-term storage, it is recommended to add a carrier protein (0.1% HSA or BSA) and store aliquots at –20°C or –70°C. Avoid freeze-thaw cycles.

General Info

Function	Insulin decreases blood glucose concentration. It increases cell permeability to monosaccharides, amino acids and fatty acids. It accelerates glycolysis, the pentose phosphate cycle, and glycogen synthesis in liver.
Involvement in disease	Defects in INS are the cause of familial hyperproinsulinemia (FHPRI) [MIM:176730]. Defects in INS are a cause of diabetes mellitus insulin-dependent type 2 (IDDM2) [MIM:125852]. IDDM2 is a multifactorial disorder of glucose homeostasis that is characterized by susceptibility to ketoacidosis in the absence of insulin therapy. Clinical fetaures are polydipsia, polyphagia and polyuria which result from hyperglycemia-induced osmotic diuresis and secondary thirst. These derangements result in long-term complications that affect the eyes, kidneys, nerves, and blood vessels. Defects in INS are a cause of diabetes mellitus permanent neonatal (PNDM) [MIM:606176]. PNDM is a rare form of diabetes distinct from childhood-onset autoimmune diabetes mellitus type 1. It is characterized by insulin-requiring hyperglycemia that is diagnosed within the first months of life. Permanent neonatal diabetes requires lifelong therapy. Defects in INS are a cause of maturity-onset diabetes of the young type 10 (MODY10) [MIM:613370]. MODY10 is a form of diabetes that is characterized by an autosomal dominant mode of inheritance, onset in childhood or early adulthood (usually before 25 years of age), a primary defect in insulin secretion and frequent insulin-independence at the beginning of the disease.
Sequence similarities	Belongs to the insulin family.
Cellular localization	Secreted.

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- Replacement or refund for products not performing as stated on the datasheet
- Valid for 12 months from date of delivery
- Response to your inquiry within 24 hours
- We provide support in Chinese, English, French, German, Japanese and Spanish
- Extensive multi-media technical resources to help you
- We investigate all quality concerns to ensure our products perform to the highest standards

If the product does not perform as described on this datasheet, we will offer a refund or replacement. For full details of the Abpromise, please visit https://www.abcam.com/abpromise or contact our technical team.

Appendix E

PEDOT:PSS (Clevios PH1000) Datasheet

Heraeus

Heraeus Deutschland GmbH & Co. KG
Leverkusen

Number 81076212
Issue 2016-09-13

CLEVIOS™ PH 1000

Description of Product Aqueous dispersion, blue liquid

Synonyms / Abbreviations PEDT / PSS, PEDOT / PSS

Physical Characteristics[1)]

	Min		Max	Unit
Solid content	1.0	-	1.3	%
Specific conductivity*	850			S/cm
Viscosity	15	-	60	mPas

*After the addition of 5% Dimethyl sulfoxide. Measured on the dried coating.

 1) Information on test methods is available on request.

Appendix F

Silver Nano Particle Ink (NBSIJ-MU01) Datasheet

MATERIAL SAFETY DATA SHEET

Effective Date: Jan. 20. 2014
Product Name: NBSIJ-MU01 (Silver Nano Particle Ink)

1. CHEMICAL PRODUCT AND COMPANY IDENTIFICATION

Product Name: NBSIJ-MU01 (Silver Nano Particle Ink)

Company Name: MITSUBISHI PAPER MILLS LIMITED
10-14, RYOGOKU 2-CHOME SUMIDAKU, TOKYO 130-0026, JAPAN
Phone: +81-3-5600-1475

2. HAZARDS IDENTIFICATION

EMERGENCY OVERVIEW

Liquid, contact may cause skin & eye irritation. Ingestion may cause gastric distress. Hazard symbols for this product-none, Risk Phrase-Not classified.

Hazard communication elements	Hazard category
Skin corrosion/irritation	3
Eye damage/irritation	2A
Sensitization-skin	1
Germ cell mutagenicity	1B
Toxic to reproduction	1A
Specific target organ toxicity (Single exposure) (respiratory, kidneys, central nervous system, heart)	1
Specific target organ toxicity (Repeated exposure) (eye, respiratory organs, central nervous system, heart)	1
Specific target organ toxicity (Repeated exposure) (liver)	2

Signal word: Danger

Hazard statement:

Causes mild skin irritation.
Causes serious eye irritation.
May cause an allergic skin reaction.
May cause genetic defects.
May damage fertility or unborn child.
Causes damage to organs. (respiratory, kidneys, central nervous system, heart)
Causes damage to organs through prolonged or repeated exposure.
(eye, respiratory organs, central nervous system, heart)
May causes damage to organs through prolonged or repeated exposure. (liver)

Prevention

Keep container tightly closed.
Wear protective gloves/eye protection/face protection.
Wash hand thoroughly after handling.
Do not breathe fume/gas/mist/vapors/spray.
Contaminated work clothing should not be allowed out of the workplace.

Appendix G

Mitsubushi Photopaper (NB-RC-3GR120) Datasheet

ARTICLE INFORMATION SHEET

MITSUBISHI PAPER MILLS LTD.
10-14, RYOGOKU 2-CHOME, SUMIDA-KU
TOKYO 130 0026 JAPAN
Information provided by:
Division: Technology & Environmental Dept.
Contact: Graphic Systems Dept.
Imaging Media Division.
Telephone number: +81-3-5600-1475

ISSUE DATE: Nov. 14. 2012

1. Product: NB-RC-3GR120 (Special Media)

2. Composition:

All products in this category are manufactured by coating silver nanoparticle ink-accepting materials composed of polyvinyl alcohol, Aluminium Oxide, etc., onto resin-coated paper.
The important ingredients are listed below.

Important ingredients

Wood Free Pulp, Titanium Dioxide, Polyethylene, Aluminium Oxide, Polyvinyl alcohol

3. Health and safety hazards:

This product does not cause health and safety hazards with normal handling and use.

4. Fire hazards and fire fighting measures:

This product is not classified as a dangerous material defined in the Japanese Fire Service Law.
Combustion gases: Carbon dioxide, carbon monoxide, fumes and small amount of nitrogen oxides, sulfur oxides, etc. will be generated depending on the burning condition.
Fighting measures: Water spray, carbon dioxide, extinguishing powder or foam is used as an extinguishing medium. While fire fighting, wear protective equipments such as self-contained breathing apparatus depending on the fire situation.

5. Disposal considerations:

In general, it is recommended to bring product into any available recycling system or appropriate waste disposal in accordance with the regional regulations.
When the waste product is disposed of, incineration treatment or landfill through a licensed hauling company is recommended.

6. Transportation and storage considerations:

This product is not subject to dangerous material classification and labeling pertaining to transportation and storage.

7. Other information:

None.

PRINTED DATE: December 2, 2013

Appendix H

Lightning-Link Rapid Biotin Conjugation Kit Datasheet

PROTOCOL

Lightning-Link® Rapid Biotin Conjugation Kit (Type A*)

*Optimized for assays using a streptavidin-labeled detection reagent

Applicable to: 370-0030 3 x Ab labelings (up to 20ug) 370-0005 1 x Ab labeling (up to 200µg)
370-0010 3 x Ab labelings (up to 200µg) 370-0015 1 x Ab labeling (up to 2mg)

Release 1 © EXPEDEON. 07/03/2018

INTRODUCTION

Lightning-Link® conjugation technology works by targeting amine groups (e.g. lysines) and is widely used to label antibodies.

The Lightning-Link® Biotin kit allows Biotin conjugations to be set up in less than 30 seconds, simply by adding a solution of the antibody to a lyophilized mixture containing a proprietary activated Biotin ligand (Figure 1).

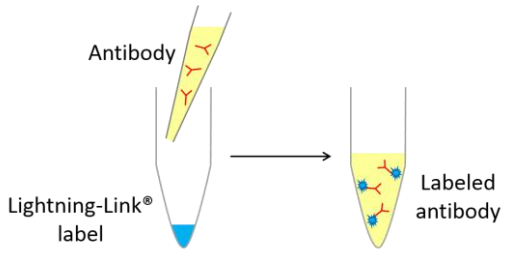


Figure 1. Lightning-Link® antibody conjugation

By circumventing the desalting or dialysis steps that commonly interrupt traditional antibody conjugation procedures, Lightning-Link® technology can be used to label both small (e.g. 10 *µg*) and large quantities of primary antibodies with ease. Batch-to-batch variation upon scale up is minimal as the process is so simple, and recoveries are always 100%.

Directly labeled primary antibodies are advantageous as they eliminate the need for secondary reagents in immunoassay procedures, thus removing a tedious extra cycle of incubation and wash steps in applications, such as ELISA and Western blotting.

Lightning-Link® technology can also be used to label proteins, peptides and other biomolecules (See 'the What can I label using Lightning-Link®?' Section of the protocol for further information).

KIT CONTENTS

- 1 or 3 glass vial(s) of Lightning-Link® Rapid mix
- 1 vial of LL Rapid Modifier reagent
- 1 vial of LL Rapid Quencher reagent

SHIPPING CONDITIONS

The kit is shipped at ambient temperature in a tamper-evident polypropylene container. Store the kits at -20^{0} C upon receipt.

Please note that the modifier and quencher FD after initial thawing can be stored at either 4^{0} C or -20^{0} C.

BUFFER CONSIDERATIONS

Please see the below table for recommended buffer conditions and components:

Buffer Components	
pH	6.5-8.5
Amine free buffer *(e.g. MES, MOPS, HEPES, PBS)*	✓
Non-buffering salts *(e.g. sodium chloride)*	✓
Chelating agents *(e.g.EDTA)*	✓
Sugars	✓
Glycerol	<50%
Thiomersal / Thimerosal	✗
Merthiolate	✗
Sodium Azide[1]	<0.1%
BSA[1,2]	<0.1%
Gelatin[1,2]	<0.1%
Tris	<50mM
Glycine	✗
Proclin	✗
Borate buffer	✓
Nucleophilic components *(Primary amines e.g. amino acids or ethanolamine and thiols e.g. mercaptoethanol or DTT)*	✗

[1] *Please note that individually the concentrations shown should not affect the reaction. However in combination with additional compounds that are not recommended above a certain concentration, the reaction may be affected.*

[2] *If intending to use this kit for immunohistochemistry, it is recommended that there be no gelatin or BSA present.*

AMOUNT AND VOLUME OF ANTIBODY

Prod. Code	Recommend amount of antibody	Maximum amount of antibody	Maximum Conjugation volume
370-0030	10µg	20µg	10µl
370-0005 370-0010	100µg	200µg	100µl
370-0015	1mg	2mg	1ml

Superior conjugates are normally generated using the recommended amount of antibody. Using the maximum amount of antibody will still generate quality conjugates.

Antibodies less than 1mg/ml can still be used to generate good conjugates provided the maximum conjugation volume is not exceeded. Adding less than the recommended maximum amount of antibody may result in unbound label post conjugation. This excess label will be deactivated by the quencher and removed during the first

wash step of any application. We would recommend that antibodies below 0.5mg/ml are concentrated prior to use.

Please contact our technical support team for more advice.

INSTRUCTIONS

Setting up your conjugation reaction

1. Before you add antibody to the Lightning-Link® Rapid mix, add 1*µl* of LL Rapid Modifier reagent for each 10 *µl* of antibody to be labeled. Mix gently.
2. Remove the screw cap from the vial of Lightning-Link® Rapid mix and pipette the antibody sample (with added LL Rapid Modifier) directly onto the lyophilized material. Resuspend gently by withdrawing and re-dispensing the liquid once or twice using a pipette.
3. Replace cap on the vial and leave standing for 15 minutes at room temperature (20-25ºC). Alternatively, and sometimes more conveniently, conjugations can be set up and left overnight at room temperature. Longer incubation times have no negative effect on the conjugation.
4. After 15min incubation, add 1 *µl* of LL Rapid Quencher reagent for every 10 *µl* of antibody used. The conjugate can be used after 5 minutes. The conjugates do not require purification.

STORAGE OF CONJUGATES

Your biotin conjugate can be stored at 4^0 C for up to 18 months. For longer storage the conjugate can be stored at -20ºC with a cryoprotectant such as 50% glycerol.

The best storage conditions for any particular conjugate must be determined by experimentation.

WHAT CAN I LABEL USING LIGHTNING-LINK®?

Lightning-Link® technology works by targeting free amine groups. It can be used to label antibodies, peptides, proteins and other molecules with free amine groups.

The protocol provided here is optimized for labeling IgGs, when labeling other molecules please contact our technical support team at: www.expedeon.com/contact/

Our custom conjugation service team will also be happy to generate your conjugate for you. Please contact our sales team for a quote. www.expedeon.com/contact/

WHAT IF MY BUFFER DOESN'T FIT THE REQUIREMENTS?

The AbSelect™ purification kit range allows you to quickly and simply purify your antibody using our conjugation friendly buffers.
The appropriate kit to use depends on your particular sample. Please view our AbSelect ™ range of products on our website to select the best kit.

If your antibody is already purified but its concentration is too low, you can concentrate it by using our AbSelect™ Antibody Concentration and Clean Up Kit (product code 861-0010). This kit can also be used to remove low molecular weight contaminants such as azide, Tris or glycine.

If your antibody contains BSA, you can use our AbSelect™ BSA removal kit (product code 820-0100) to purify your antibody in one simple step.

Our AbSelect™ kits are fully compatible with Lightning-Link® and are designed to work with IgG antibodies. The only exception is the Concentration and Clean Up kit (product code 861-0010) which will work with any molecule greater than 10kDa.

TECHNICAL SUPPORT

For technical enquiries get in touch with our technical support team at: www.expedeon.com/contact/

Appendix I

Gold Conjugation Kit (ab154873) Protocol

ab154873

Gold Conjugation Kit

(40nm, 20OD)

Instructions for Use

For the Covalent Conjugation of Antibodies or Proteins to Gold

This product is for research use only and is not intended for diagnostic use.

Version 2 Last Updated 11 September 2018

Table of Contents

1. Introduction

Abcam's Gold Conjugation Kit allows antibodies or proteins to be covalently attached to ultra-stable Gold nanoparticles at very high OD quickly and easily. The hands-on time for the Gold conjugation procedure is about 2 minutes and the conjugate is ready to use within 15 minutes.

The Gold nanoparticles in this kit are supplied as a freeze dried mixture. The conjugation reaction is initiated simply by adding a solution of the antibody, which becomes attached (via lysine residues) to the gold surface.

The resulting covalent conjugates are more stable than those prepared by passive adsorption methods. Moreover, unlike passive methods, the coating procedure is not dependent on the isoelectric point of the antibody, and extensive trials at different pH values are not required; all antibodies react at a single fixed pH.

The 3 and 10 test Conjugation Kits are designed to label 12 µl per vial.

The 1 test Conjugation Kit is designed to label 120 µl per vial.

2. Kit Contents

Components	Amount			Storage
	3 Tests	10 Tests	1 Test	
Amount of Antibody/Test	**12 µl**	**12 µl**	**120 µl**	
Gold Reaction Buffer	1 vial	1 vial	1 vial	-20°C
Gold Antibody Diluent	1 vial	1 vial	1 vial	-20°C
Gold	3 vials	10 vials	1 vial	-20°C
Gold Quencher	1 vial	1 vial	1 vial	-20°C

3. Storage and Handling

For storage temperatures please see the Table

For handling refer to Safety Datasheet

4. Additional Materials

Microfuge Tubes (0.5 or 1.5 ml)

Microfuge

5. General Guidelines

A. Prior to Labeling

- Antibody must be purified
- Avoid amino acids (e.g. glycine)
- Avoid other primary amines (e.g. Tris)
- Avoid thiols (e.g. mercaptoethanol, DTT)
- Avoid carboxylic acids (e.g. EDTA)

Stock antibodies at concentrations of >1mg/ml are recommended; as this allows potentially interfering substances in the antibody preparation to be diluted out (see Step 6.2). You should pay particular attention to the composition of the stock antibody if you require less than a 1 in 5 dilution in step 6.2 of the protocol. If you are in any doubt about the suitability of buffers/additives in your preparation of antibody please contact our technical team for advice.

The kit is compatible with PBS, MES, MOPS, HEPES, sugars, salts and detergents.

B. Amount of Antibody

The optimum amount of antibody (which will influence the number of antibody molecules per particle) may be application-dependent and you may need to conjugate different amounts of antibody to optimize your assay. The initial amount of antibody recommended corresponds to 10 µg antibody per ml of 10 OD gold, which is about half of that normally used for passive (non-covalent) conjugations. However lower or higher concentrations can be explored as there is no risk of aggregation because of the protective surface coating.

6. Labeling Protocol

Note: *The following protocol is for 1 vial from the 3 or 10 test Conjugation Kit. Adjust volumes as required for the larger 1 test Conjugation Kit.*

1. Allow all of the reagents to warm to room temperature.
2. Dilute your stock antibody with the Gold antibody diluent provided in the kit to 0.1 mg/ml.

   Note: *If you wish to examine the effect of varying the amount of antibody, make additional stocks at 0.05 and 0.2 mg/ml in the first instance. Do not change the* ***volume*** *of antibody added in order to vary the amount of antibody added, you must change the* ***concentration*** *of the stock antibody and use a fixed volume.*

3. For each reaction: In a clean 0.5 ml or 1.5 ml microfuge tube add 42 µl of Gold reaction buffer and then 12 µl of your diluted antibody from Step.2. Mix thoroughly.
   (Note: 420 µl Gold reaction buffer and 120 µl antibody for large volume kit).
4. Transfer 45 µl of the mixture to a vial of Gold. Reconstitute the freeze dried mixture by gently pipetting up and down. Allow to stand at room temperature for 15 minutes.
   (Note: 450 µl Gold for large volume kit).
5. Add 5 µl of EL Gold Quencher and mix gently. You now have 50 µl of 20 OD conjugate. Dilute further as required for your application.
   (Note: 50 µl Gold Quencher for large volume kit – final volume 500 µl of 20 OD conjugate).

Note: *For a conjugate 100% free from unbound antibody we recommend washing the particles and adding 10 times the volume of the quencher diluted 1:10 in water to the conjugate (i.e. 1ml 1:10 diluted quencher to 100µl of conjugate) and then centrifuge it in a microfuge at 9,000 g 10 minutes. Carefully remove the supernatant, gently tap the pellet and add the quencher diluted 1:10 in water for long term storage in the fridge (up to 1 year) or 1:10 diluted quencher with addition of 0.5 - 2% BSA for LFA or your preferred buffer.*

Note: *While you should avoid thiols (e.g. DTT or mercaptoethanol) the other interfering substances noted in section 5 have no negative effect once the conjugate has been formed.*

Gold Labeled antibody should be stored at +4°C

7. Frequently Asked Questions

What can I do if my antibody preparation contains interfering substances?

The simplest procedure is to dialyse the antibody against a suitable buffer. Relatively weak buffers (e.g. 20mM) are preferred so that the pH conditions of the covalent reaction are not significantly altered upon addition of the antibody. See also the comments in Section 5 of the protocol. If dialysis is not practical you can use other popular methods of buffer exchange if required e.g. desalting columns.

Does the antibody bind to the metal surface?

No. The protective surface coat completely shields the metal surface and prevents direct metal-antibody interactions. For this reason, you cannot use Gold Conjugation Kits for passive conjugation of antibodies.

Can antibodies from different species be used?

Yes, the system has been tested with antibodies from a variety of species including mouse, rabbit, goat and sheep.

What type of linkage to gold is formed?

The antibody becomes covalently and irreversibly attached via lysine residues to the GOLD surface.

Can antibody fragments be conjugated?

Yes. One of the advantages of the protective coat is that it is less likely than a bare metal surface to cause denaturation and loss of affinity of the antibody fragments.

What if I need bulk material?

The kit that you have purchased is a convenience product for rapid production of small quantities of conjugate for screening purposes. Abcam offers the kit in a range of sizes. Please enquire.

Is the kit suitable for conjugating analytes and other small molecules?

Yes, but please contact technical services. Depending on the functional groups on your analyte you may need a different type of surface or different chemistry for optimal results.

Appendix J

Purified Streptavidin (280302) Datasheet

Version: 2 Revision Date: 10/19/2016

Purified Streptavidin

Catalog# / Size	280302 / 250 µg
Ave. Rating	★★★★★ 0 reviews
Description	Streptavidin is a 52.8 kD tetrameric protein obtained from *Streptomyces avidinii*. It binds to biotin with a very high affinity and is one of the strongest interactions in nature with a dissociation constant of 10^{-14} mol/L. It is used in a wide range of applications including ELISA, flow cytometry, molecular biology, and bionanotechnology.

Product Details

Formulation	Phosphate-buffered solution containing 0.09% sodium azide.
Concentration	1.0 mg/ml
Storage & Handling	Store between 2°C and 8°C.
Application	FC - Quality tested ELISA Capture, ELISA Detection, IF, IHC-F, IHC-P, IP, WB - Reported in the literature
Recommended Usage	To coat one 96-well plate dilute 24 µl of Purified Streptavidin into 11.976ml of 1X Coating Buffer (Cat# 421701, Coating Buffer 5X concentrate). Final concentration in the coated plate is 2 µg/ml. This recommendation is to perform the protocol for HLA class I ELISA to evaluate Flex-T™ peptide exchange. For other applications the reagent needs to be optimized.

Antigen Details

Structure	52.8 kD tetrameric protein.
Distribution	Streptavidin binds to biotin with high affinity.
Ligand Receptor	Biotin.
Antigen References	1. Dundas CM, et al. 2013. Appl. Microbiol. Biotechnol. 97:9343. 2. Zhao X, et al. 2013. J. Anal. Methods Chem. 2013:581093. 3. Kaplan DL, et al. 1999. Biomol. Eng. 16:135. 4. Wilbur DS, et al. 1999. Biomol. Eng. 16:113. 5. Sano T, et al. 1998. J. Chromatogr. B. Biomed. Sci. Appl. 715:85.

Related Protocols

Flex-T™ HLA Class I ELISA Protocol

Cell Surface Immunofluorescence Staining Protocol

BioLegend Inc., 9727 Pacific Heights Blvd, San Diego, CA 92121 www.biolegend.com
Toll-Free Phone: 1-877-Bio-Legend (246-5343) Phone: (858) 768-5800 Fax: (877) 455-9587

Appendix K

Bovine Serum Albumin (BSA) Datasheet

For life science research only. Not for use in diagnostic procedures.

Bovine Serum Albumin, Fraction V

Lyophilizate

Cat. No. 10 735 078 001	50 g
Cat. No. 10 735 086 001	100 g
Cat. No. 10 735 094 001	500 g
Cat. No. 10 735 108 001	1 kg

Version 08
Content version: March 2013

Store at +2 to +8°C

1. What this Product Does

Contents

Lyophilizate

Storage and Stability

The lyophilizate is stable at +2 to +8°C until the expiration date printed on the label.

Application

- Buffer component (in immunochemistry, biochemistry, cell biology, molecular biology)
- Reducing agent
- Protein blocker (saturation of all protein binding sites in ELISAs, Southern blots and western blots)
- Stabilizer
- Media supplement (for media that require addition of protein)
- Carrier protein
- Standard protein (gel electrophoresis, protein determination)

2. Additional Information on this Product

Solubility

Albumin is readily soluble at concentrations of 50 – 100 mg/ml. To prevent foaming, always use gentle stirring to dissolve the protein. Adding a non-ionic detergent to the solution will also minimize its tendency to foam.

Appearance

Albumin solutions can vary from colorless to light yellow or light greenish-yellow; the color of the solution does not affect its function.

Molecular weight

68,000 Da (583 amino acids; see Ref. 3 for amino acid sequence)

pH

7.0 ± 0.2

Ligand binding

Albumin binds many substances reversibly and therefore can serve as a transport or carrier protein in the body. The most important function of albumin in the body is the transport of lipids and free fatty acids.

The ligand binding properties of albumin serves two purposes in the laboratory. On the one hand it can release bound components into the reaction mixture, while on the other hand it can adsorb impurities from the medium. Since albumin binds many anions and cations, it can also be used in ion binding studies.

The affinity of albumin for ligands depends on the hydrophobic character of the molecules and their charge. Molecules with long alkyl chains and negatively charged groups are bound very firmly, while molecules with short chains and positively charged groups are bound less firmly (1). Ligands that are bound by albumin include fatty acids, cationic and neutral detergents, acetylcholine, ascorbic acid, penicillin, thyroxin, digitonin, hormones, metal ions, bilirubin, sugars and drugs (2).

Analysis

Albumin	⩾98% (gel electroph.)
Protein	⩾95%
H_2O	<5%
Sodium (flame photometry)	<0.5%
Potassium (flame photometry)	<0.006%
Lithium (flame photometry)	<0.0003%
Calcium (o-cresolphthalein)	<0.02%
Magnesium (xylidyl blue)	<0.003%
Heavy metals (as Pb)	<0.003%
Iron (bathophenanthraline)	<0.001%
P (inorganic)	<0.002%
Chloride (mercurom.)	<0.15%
Glucose (enzym.)	<0.05%
Glycerol (enzym.)	<0.005%
L-Lactate (enzym.)	≤0.1%
Microorganisms	<100 organism/g

References

1 Spector, A.A. et al. (1971) *Biochemistry* **10**, 3229.
2 Skipski, V.P. (1972) in: Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism (Nelson, G.J., ed.). New York, Wiley-Interscience, pp. 471–583.
3 Hirayama, K. Akashi, S., Furuya, M. & Fukuhara, K. (1990) *Biochem. Biophys. Res. Commun.* **173**, 639.

Appendix L

LND150 N-Channel Depletion-Mode MOSFET Datasheet

N-Channel Depletion-Mode DMOS FET

Features

- Free from secondary breakdown
- Low power drive requirement
- Ease of paralleling
- Excellent thermal stability
- Integral source-drain diode
- High input impedance and low C_{ISS}
- ESD gate protection

General Description

The LND150 is a high voltage N-channel depletion mode (normally-on) transistor utilizing Supertex's lateral DMOS technology. The gate is ESD protected.

The LND150 is ideal for high voltage applications in the areas of normally-on switches, precision constant current sources, voltage ramp generation and amplification.

Applications

- Solid state relays
- Normally-on switches
- Converters
- Power supply circuits
- Constant current sources
- Input protection circuits

Ordering Information

Part Number	Package Options	Packing
LND150K1-G	TO-236AB (SOT-23)	3000/Reel
LND150N3-G	TO-92	1000/Bag
LND150N3-G P002	TO-92	2000/Reel
LND150N3-G P003	TO-92	2000/Reel
LND150N3-G P005	TO-92	2000/Reel
LND150N3-G P013	TO-92	2000/Reel
LND150N3-G P014	TO-92	2000/Reel
LND150N8-G	TO-243AA (SOT-89)	2000/Reel

-G denotes a lead (Pb)-free / RoHS compliant package

Product Summary

BV_{DSX}/BV_{DGX} (V)	$R_{DS(ON)}$ (max)	I_{DSS} (min)
500	1.0kΩ	1.0mA

Absolute Maximum Ratings

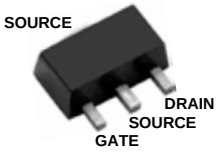
Parameter	Value
Drain-to-source	BV_{DSX}
Drain-to-gate	BV_{DGX}
Gate-to-source	±20V
Operating and storage temperature	-55°C to +150°C

Absolute Maximum Ratings are those values beyond which damage to the device may occur. Functional operation under these conditions is not implied. Continuous operation of the device at the absolute rating level may affect device reliability. All voltages are referenced to device ground.

Pin Configuration

TO-92

TO-236AB (SOT-23)



TO-243AA (SOT-89)

Product Marking

W = Code for Week Sealed
_____ = "Green" Packaging

TO-236AB (SOT-23)

YY = Year Sealed
WW = Week Sealed
_____ = "Green" Packaging

TO-92

W = Code for Week Sealed
_____ = "Green" Packaging

TO-243AA (SOT-89)

Packages may or may not include the following marks: Si or

Thermal Characteristics

Package	I_D (continuous)† (mA)	I_D (pulsed) (mA)	Power Dissipation @T_A = 25°C (W)	θ_{ja} (°C/W)	I_{DR} (mA)	I_{DRM} † (mA)
TO-236AB (SOT-23)	13	30	0.36	203	13	30
TO-92	30	30	0.74	132	30	30
TO-243AA (SOT-89)	30	30	1.6‡	133	30	30

Notes:

† I_D *(continuous) is limited by max rated* T_j.

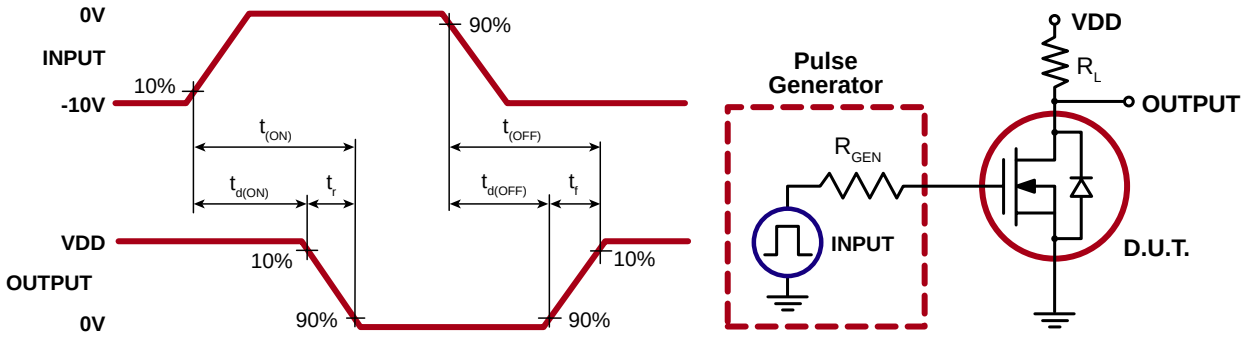
Electrical Characteristics *(T_A = 25°C unless otherwise specified)*

Sym	Parameter	Min	Typ	Max	Units	Conditions
BV_{DSX}	Drain-to-source breakdown voltage	500	-	-	V	V_{GS} = -10V, I_D = 1.0mA
$V_{GS(OFF)}$	Gate-to-source off voltage	-1.0	-	-3.0	V	V_{GS} = 25V, I_D = 100nA
$\Delta V_{GS(OFF)}$	Change in $V_{GS(OFF)}$ with temperature	-	-	5.0	mV/°C	V_{GS} = 25V, I_D = 100nA
I_{GSS}	Gate body leakage current	-	-	100	nA	V_{GS} = ± 20V, V_{DS} = 0V
$I_{D(OFF)}$	Drain-to-source leakage current	-	-	100	nA	V_{GS} = -10V, V_{DS} = 450V
		-	-	100	µA	V_{DS} = 0.8V Max Rating, V_{GS} = -10V, T_A = 125°C
I_{DSS}	Saturated drain-to-source current	1.0	-	3.0	mA	V_{GS} = 0V, V_{DS} = 25V
$R_{DS(ON)}$	Static drain-to-source on-state resistance	-	850	1000	Ω	V_{GS} = 0V, I_D = 0.5mA
$\Delta R_{DS(ON)}$	Change in $R_{DS(ON)}$ with temperature	-	-	1.2	%/°C	V_{GS} = 0V, I_D = 0.5mA
G_{FS}	Forward transductance	1.0	2.0	-	m℧	V_{DS} = 0V, I_D = 1.0mA
C_{ISS}	Input capacitance	-	7.5	10	pF	V_{GS} = -10V, V_{DS} = 25V, f = 1.0MHz
C_{OSS}	Common source output capacitance	-	2.0	3.5		
C_{RSS}	Reverse transfer capacitance	-	0.5	1.0		
$t_{d(ON)}$	Turn-on delay time	-	0.09	-	µs	V_{DD} = 25V, I_D = 1.0mA, R_{GEN} = 25Ω
t_r	Rise time	-	0.45	-		
$t_{d(OFF)}$	Turn-off delay time	-	0.1	-		
t_f	Fall time	-	1.3	-		
V_{SD}	Diode forward voltage drop	-	-	0.9	V	V_{GS} = -10V, I_{SD} = 1.0mA
t_{rr}	Reverse recovery time	-	200	-	ns	V_{GS} = -10V, I_{SD} = 1.0mA

Notes:

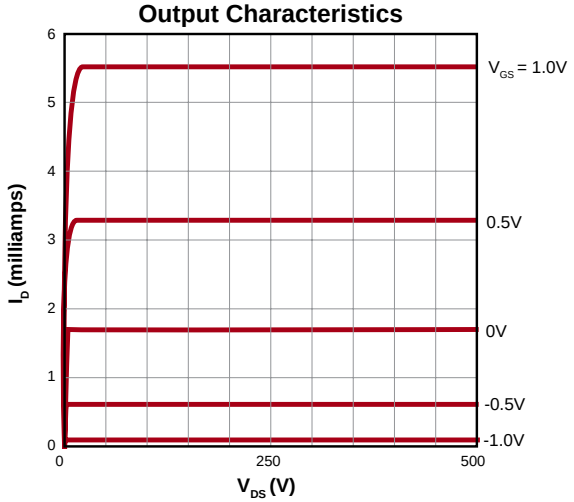
1. *All D.C. parameters 100% tested at 25°C unless otherwise stated. (Pulse test: 300µs pulse, 2% duty cycle.)*
2. *All A.C. parameters sample tested.*

Switching Waveforms and Test Circuit

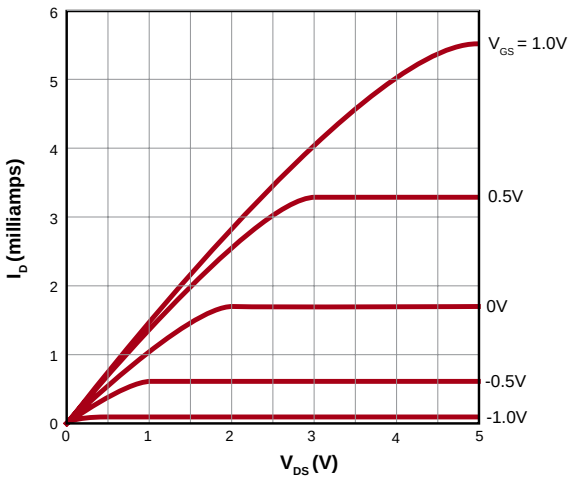


Typical Performance Curves

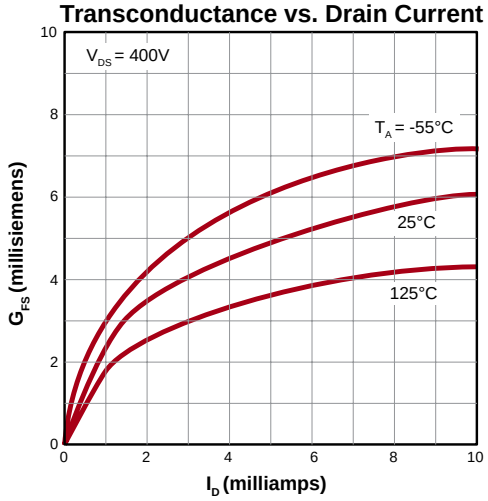
Output Characteristics



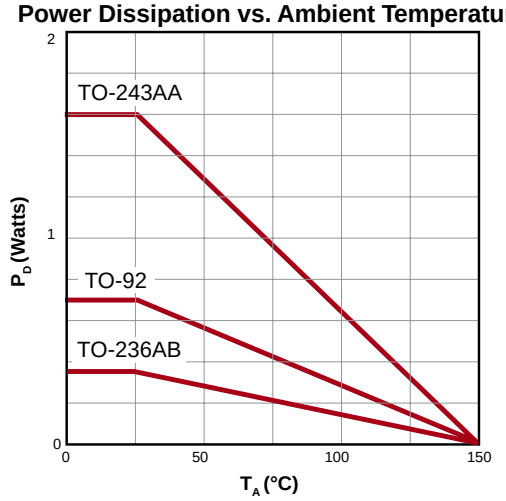
Saturation Characteristics



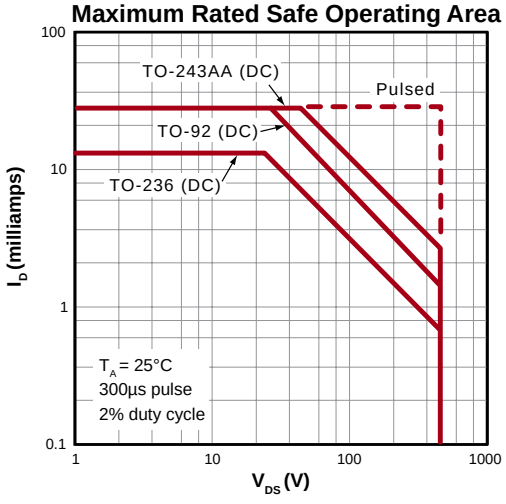
Transconductance vs. Drain Current



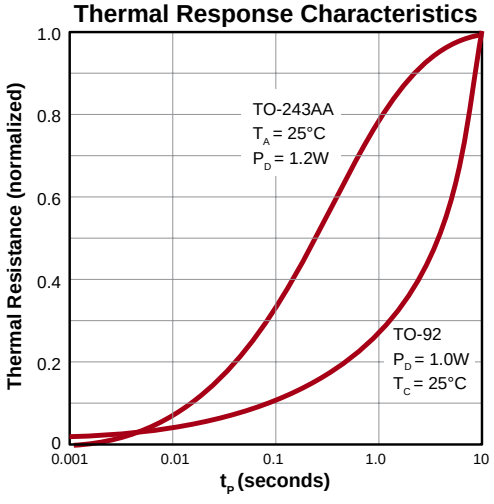
Power Dissipation vs. Ambient Temperature



Maximum Rated Safe Operating Area

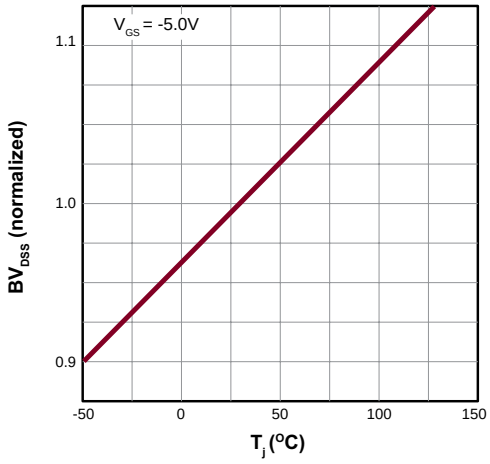


Thermal Response Characteristics

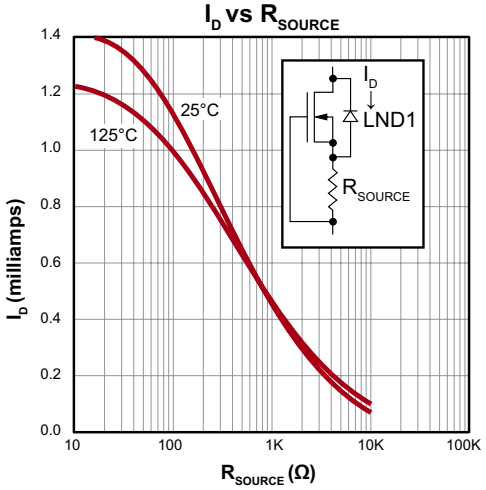


Typical Performance Curves *(cont.)*

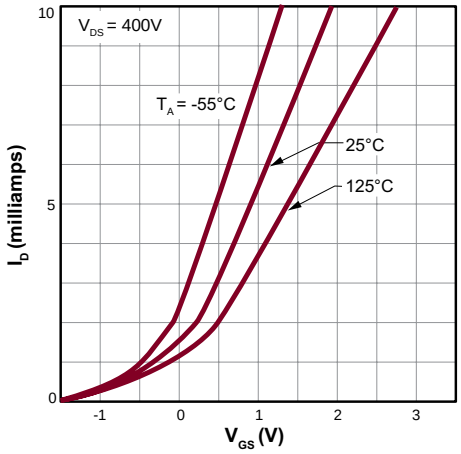
BV_{DSS} Variation with Temperature



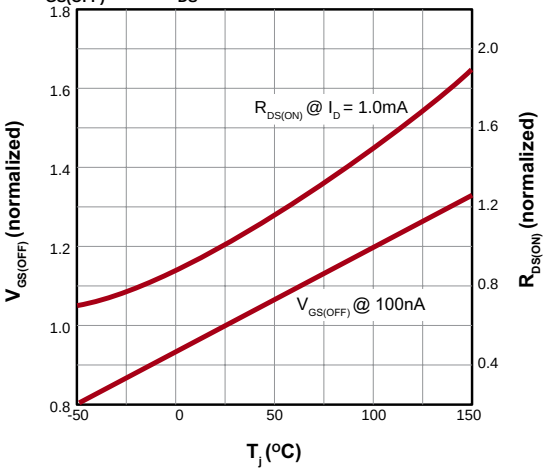
I_D vs R_{SOURCE}



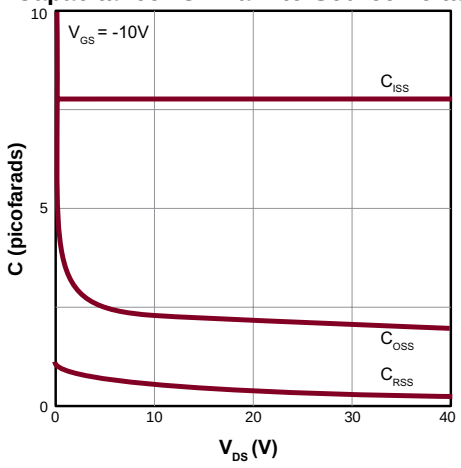
Transfer Characteristics



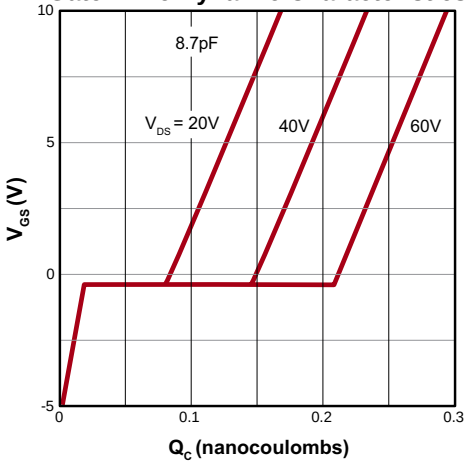
$V_{GS(OFF)}$ and R_{DS} Variation with Temperature



Capacitance vs. Drain-to-Source Voltage



Gate Drive Dynamic Characteristics



Appendix M

Matlab Script

```
% Final Matlab Script
% This section provids the graphs for the Antibody Pairing tests

CAP1364 = xlsread('CapA1364.xlsx','A2:D16');    % Read Data
Cap1364conc = CAP1364(:,1);                     % Allocate Variables
Cap1364odmean = CAP1364(:,2);                   % Allocate Variables
Cap1364odmax = CAP1364(:,3);                    % Allocate Variables
Cap1364odmin = CAP1364(:,4);                    % Allocate Variables
CAP1365 = xlsread('CapA1365.xlsx','A2:D16');    % Read Data
Cap1365conc = CAP1365(:,1);                     % Allocate Variables
Cap1365odmean = CAP1365(:,2);                   % Allocate Variables
Cap1365odmax = CAP1365(:,3);                    % Allocate Variables
Cap1365odmin = CAP1365(:,4);                    % Allocate Variables

figure      % New Figure
semilogx(Cap1364conc,Cap1364odmean,'b'); hold on;
semilogx(Cap1364conc,Cap1364odmax,'r.',Cap1364conc,Cap1364odmin,'r.');
for i = 1:1:15
    semilogx([Cap1364conc(i) Cap1364conc(i)], [Cap1364odmin(i)
 Cap1364odmax(i)],'r','MarkerSize',10); hold on
end
xlabel('Insulin Concentration (ng/mL)');
ylabel('OD 450nM');
title('ELISA results Capture A1364');
grid on;
set(gcf,'color','white')

figure      % New Figure
semilogx(Cap1365conc,Cap1365odmean,'b'); hold on;
semilogx(Cap1365conc,Cap1365odmax,'r.',Cap1365conc,Cap1365odmin,'r.');
for i = 1:1:15
    semilogx([Cap1365conc(i) Cap1365conc(i)], [Cap1365odmin(i)
 Cap1365odmax(i)],'r','MarkerSize',10); hold on
end
xlabel('Insulin Concentration (ng/mL)');
ylabel('OD 450nM');
title('ELISA results Capture A1365');
grid on;
set(gcf,'color','white')

% This section generates the OEC Output Graphs (Id vs Vds)

outputo = dlmread('OECT_output_404.lvm'); % Read OECT Data
outputm = dlmread('OECT_output_306.lvm'); % Read MOSFET Data

oVgg = outputo(:, 1);           % Channel 1 ADC Input
oVgs = outputo(:, 2);           % Channel 2 ADC Input
oVdd = outputo(:, 3);           % Channel 3 ADC Input
oVds = outputo(:, 4);           % Channel 4 ADC Input
oVsd = -oVds;                   % Allocate Variables
oVsg = -oVgs;                   % Allocate Variables
mVgg = outputm(:, 1);           % Channel 1 ADC Input
```

```
mVgs = outputm(:, 2);           % Channel 2 ADC Input
mVdd = outputm(:, 3);           % Channel 3 ADC Input
mVds = outputm(:, 4);           % Channel 4 ADC Input
mVsd = -mVds;                   % Allocate Variables
mVsg = -mVgs;                   % Allocate Variables

oN = 0:1:length(oVgg)-1;        % Number of samples
mN = 0:1:length(mVgg)-1;        % Number of samples
Rd = 147;                       % Drain Resistor Value (Ohm)
Rg = 148;                       % Gate Resistor Value (Ohm)

oId = (oVds - oVdd)/Rd;         % OECT Drain Current Formula
mId = (mVdd - mVds)/Rd;         % MOSFET Drain Current Formula
oNGV = 12;
oipt = findchangepts(oVgg, 'MaxNumChanges', oNGV);  % Detect Falling
 Edges
mNGV = 9;
mipt = findchangepts(mVgg, 'MaxNumChanges', mNGV);  % Detect Falling
 Edges

odiff = (oipt(2)-oipt(1))/2;
mdiff = (mipt(2)-mipt(1))/2;

for i = 1:1:(oNGV-4) % OECT Array Development
    oVddArrayForward(i,:) = oVdd(oipt(i):100:oipt(i) + odiff);
    oVdsArrayForward(i,:) = oVds(oipt(i):100:oipt(i) + odiff);
    oIdArrayForward(i,:) = oId(oipt(i):100:oipt(i) + odiff);
end

for l = 1:1:(mNGV-1) % MOSFET Array Development
    mVddArrayForward(l,:) = mVdd(mipt(l):100:mipt(l) + mdiff);
    mVdsArrayForward(l,:) = mVds(mipt(l):100:mipt(l) + mdiff);
    mIdArrayForward(l,:) = mId(mipt(l):100:mipt(l) + mdiff);
end

for k = 1:1:(oNGV-4)
   IdArrayFull(i,:) =  oId(oipt(i):oipt(i+1));
   VddArrayFull(i,:) =  oVdd(oipt(i):oipt(i+1));
   VdsArrayFull(i,:) =  oVds(oipt(i):oipt(i+1));
end

% Plot the OECT output characteristics Id vs Vds

figure
set(gcf,'color','white');
for i = (oNGV-4):-1:1
    plot(oVdsArrayForward(i,:),oIdArrayForward(i,:)*1000); hold on;
end
xlabel('V_{DS} (V)');
ylabel('I_{D} (mA)');
title('Output Characteristics: OECT');
title(legend,'V_{G}');
legend('0.40 V','0.45 V','0.50 V','0.55 V','0.60 V','0.65 V','0.70
 V','0.75 V','Location','northeast');
```

```
set(gca, 'xdir','reverse');
axis([-0.8 0 0 4 ]);

% Plot the MOSFET output characteristics Id vs Vdd

figure      % New Figure
set(gcf,'color','white');
for i = (mNGV-2):-1:1
    plot(mVddArrayForward(i,:),mIdArrayForward(i,:)*1000); hold on;
end
xlabel('V_{D} (V)');
ylabel('I_{D} (mA)');
title('Output Characteristics: LND150');
title(legend,'V_{G}');
legend('0.60 V','0.40 V','0.20 V','0 V','-0.20 V','-0.40 V','-0.60
 V','Location','northwest'); % '-0.80 V',
% set(gca, 'ydir','reverse');
axis([0 4 0 3.5 ]);

% Plot the OECT output characteristics Id vs Vds

figure      % New Figure
set(gcf,'color','white');
for i = (oNGV-4):-1:2
    plot(oVddArrayForward(i,:),oIdArrayForward(i,:)*1000); hold on;
end
xlabel('V_{DD} (V)');
ylabel('I_{D} (mA)');
title('Output Characteristics: OECT');
title(legend,'V_{G}');
legend('0.40 V','0.45 V','0.50 V','0.55 V','0.60 V','0.65 V','0.70
 V','Location','northwest'); %
set(gca, 'xdir','reverse');
axis([-0.8 0 0 3.5 ]);

% Plot the OECT Hysteresis output characteristics Id vs Vds

figure      % New Figure
set(gcf,'color','white');
% plot(oVds(32188:80:288868),oId(32188:80:288868)*1000);
for i = (oNGV-4):-1:2
    plot(oVds(oipt(i):80:oipt(i+1)-1),oId(oipt(i):80:oipt(i
+1)-1)*1000);hold on;
end
xlabel('V_{DS} (V)');
ylabel('I_{D} (mA)');
title('Output: Hysteresis Id vs Vds');
set(gca, 'xdir','reverse');
title(legend,'V_{G}');
legend('0.40 V','0.45 V','0.50 V','0.55 V','0.60 V','0.65 V','0.70
 V','Location','northeast');
axis([-0.8 0 0 4 ]);
xarrow1 = [0.36 0.37];
yarrow1 = [0.7 0.71];
```

```
annotation('textarrow',xarrow1,yarrow1,'Color','b');
xarrow2 = [0.41 0.4];
yarrow2 = [0.65 0.644];
annotation('textarrow',xarrow2,yarrow2,'Color','b');

% Plot the Transfer and Transconductance Characteristics (Id vs Vg)

transfer1 = dlmread('OECT_transfer_28.lvm'); % Read Data
transfer2 = dlmread('OECT_transfer_40.lvm'); % Read Dara

tVgg1 = transfer1(:, 1);        % Channel 1 ADC Input
tVgs1 = transfer1(:, 2);        % Channel 2 ADC Input
tVdd1 = transfer1(:, 3);        % Channel 3 ADC Input
tVds1 = transfer1(:, 4);        % Channel 4 ADC Input
tVsd1 = -tVds1;                 % Allocate Variables
tVsg1 = -tVgs1;                 % Allocate Variables
tVgg2 = transfer2(:, 1);        % Channel 1 ADC Input
tVgs2 = transfer2(:, 2);        % Channel 2 ADC Input
tVdd2 = transfer2(:, 3);        % Channel 3 ADC Input
tVds2 = transfer2(:, 4);        % Channel 4 ADC Input
tVsd2 = -tVds2;                 % Allocate Variables
tVsg2 = -tVgs2;                 % Allocate Variables

tN1 = 0:1:length(tVgg1)-1;      % Number of samples
tN2 = 0:1:length(tVgg2)-1;      % Number of samples
Rd = 147;                       % Drain Resistor Value (Ohm)
Rg = 148;                       % Gate Resistor Value (Ohm)

tId1 = (tVds1 - tVdd1)/Rd;      % Drain Current Formula
tIg1 = (tVgg1 - tVgs1)/Rg;      % Gate Current Formula
tId2 = (tVds2 - tVdd2)/Rd;      % Drain Current Formula
tIg2 = (tVgg2 - tVgs2)/Rg;      % Gate Current Formula

% Transconductance Calculations
startrange = 50000;   % Startrange = 400;
endrange = 225000;     % Endrange = 820;
order = 9;          % Polynomial order
fit1 = 0;
fit2 = 0;

Fit_array1 =
 polyfit(tVsg1(startrange:endrange),tId1(startrange:endrange),order);
Fit_array2 =
 polyfit(tVsg2(startrange:endrange),tId2(startrange:endrange),order);

h = 0.001;
xVsg1 = tVsg1(startrange):h:tVsg1(endrange);
xVsg2 = tVsg2(startrange):h:tVsg2(endrange);

for i = 1:order+1
   fit1 = fit1 + Fit_array1(i)*xVsg1.^((order+1) - i);
   fit2 = fit2 + Fit_array2(i)*xVsg2.^((order+1) - i);
end
gm1 = diff(fit1);   % Transconductance
```

```
gm2 = diff(fit2);   % Transconductance

%Plot the Transfer curves Id vs Time

figure      % New Figure
set(gcf,'color','white');
yyaxis left
plot(-xVsg1(420:1000),fit1(1,420:1000)*1000);
xlabel('V_{G} (V)');
ylabel('I_{D} (mA)');
title('Transfer: Transfer curve and resulting transconductance');
axis([0.1 0.7 0 2.5 ]);
xarrow1 = [0.25 0.2];
yarrow1 = [0.75 0.75];
annotation('textarrow',xarrow1,yarrow1,'Color','blue');

yyaxis right
plot(-xVsg1(420:1000),gm1(420:1000)*1000000);
ylabel('g_{m} (mS)');
axis([0.1 0.7 0 7 ]);
xarrow2 = [0.75 0.80];
yarrow2 = [0.7 0.7];
annotation('textarrow',xarrow2,yarrow2,'Color','red');

figure      % New Figure
set(gcf,'color','white');
plot(-xVsg1(420:1000),gm1(1,420:1000)*1000000); hold on
plot(-xVsg2(420:1000),gm2(1,420:1000)*1000000);
plot([0.3 0.3],[0 6],'k--','MarkerSize',7);
axis([0.1 0.7 0 7 ]);
xlabel('V_{G} (V)');
ylabel('g_{m} (mS)');
title('Transfer: Shift in Transconductance');
legend('PBS','500 ng/mL Insulin');

% Plot the Time dependant Characteristics (Id vs Time)

response = dlmread('OECT_response_13.lvm'); % Read Data

rVgg = response(:, 1);          % Channel 1 ADC Input
rVgs = response(:, 2);          % Channel 2 ADC Input
rVdd = response(:, 3);          % Channel 3 ADC Input
rVds = response(:, 4);          % Channel 4 ADC Input
rVsd = -rVds;                   % Allocate Variables
rVsg = -rVgs;                   % Allocate Variables

time = (1:15:length(rVgg))/15;
const(1,1:length(time)) = 1.926;
Rd = 147;                       % Drain Resistor Value (Ohm)
Rg = 148;                       % Gate Resistor Value (Ohm)
rId = (rVds - rVdd)/Rd;         % Drain Current Formula
rIg = (rVgg - rVgs)/Rg;         % Gate Current Formula

figure      % New Figure
```

```
set(gcf,'color','white');
plot(time,rId(1:15:450)*1000); hold on;
plot(time,const,'k--');
xlabel('Time (s)');
ylabel('I_{D} (mA)');
legend('Response At Biased Point','Settling point = 1.926 mA');
title('Response: Id vs Time');
axis([0 30 1.9 2.35 ]);

% Plot the Current Differences between various concentrations

CurrConc = xlsread('test_list.xlsx',5,'A1:B28');    % Read Data
Averages = xlsread('test_list.xlsx',5,'D1:E7');     % Read Data
best = xlsread('test_list.xlsx',5,'H1:I7');         % Read Data

Conc = CurrConc(:,1);           % Allocate Variables
Curr = CurrConc(:,2)/1000;      % Allocate Variables
ConcAvg = Averages(:,1);        % Allocate Variables
Avg = Averages(:,2)/1000;       % Allocate Variables
bestx = best(:,1);              % Allocate Variables
besty = best(:,2)/1000;         % Allocate Variables

figure      % New Figure
set(gcf,'color','white');
loglog(ConcAvg, Avg,'b-'); hold on
for i = 1:4:28
        loglog(Conc(i:i+3,1), Curr(i:i+3,1),'r-');
        loglog(Conc(i:i+3,1), Curr(i:i+3,1),'r.','MarkerSize',9);
end
grid on;
xlabel('Insulin Concentration (ng/mL)');
ylabel('I_{D} (mA)');

figure      % New Figure
set(gcf,'color','white');
loglog(bestx, besty,'b-'); hold on
loglog(bestx, besty,'r.','MarkerSize',9);
grid on;
xlabel('Insulin Concentration (ng/mL)');
ylabel('I_{D} (mA)');
axis([0 2000 0 2.6 ]);

% Plot Multiple Transfer Graphs (Id vs Vds)

transfer1 = dlmread('OECT_transfer_53.lvm'); % Read Data
transfer2 = dlmread('OECT_transfer_54.lvm'); % Read Data
transfer3 = dlmread('OECT_transfer_55.lvm'); % Read Data
transfer4 = dlmread('OECT_transfer_56.lvm'); % Read Data

start = 50000;
step = 1000;
stop = 225000;

Vgg1 = transfer1(start:step:stop, 1);       % Channel 1 ADC Input
```

```
Vgs1 = transfer1(start:step:stop, 2);       % Channel 2 ADC Input
Vdd1 = transfer1(start:step:stop, 3);       % Channel 3 ADC Input
Vds1 = transfer1(start:step:stop, 4);       % Channel 4 ADC Input
Vsd1 = -Vds1;                               % Allocate Variables
Vsg1 = -Vgs1;                               % Allocate Variables
Vgg2 = transfer2(start:step:stop, 1);       % Channel 1 ADC Input
Vgs2 = transfer2(start:step:stop, 2);       % Channel 2 ADC Input
Vdd2 = transfer2(start:step:stop, 3);       % Channel 3 ADC Input
Vds2 = transfer2(start:step:stop, 4);       % Channel 4 ADC Input
Vsd2 = -Vds2;                               % Allocate Variables
Vsg2 = -Vgs2;                               % Allocate Variables
Vgg3 = transfer3(start:step:stop, 1);       % Channel 1 ADC Input
Vgs3 = transfer3(start:step:stop, 2);       % Channel 2 ADC Input
Vdd3 = transfer3(start:step:stop, 3);       % Channel 3 ADC Input
Vds3 = transfer3(start:step:stop, 4);       % Channel 4 ADC Input
Vsd3 = -Vds3;                               % Allocate Variables
Vsg3 = -Vgs3;                               % Allocate Variables
Vgg4 = transfer4(start:step:stop, 1);       % Channel 1 ADC Input
Vgs4 = transfer4(start:step:stop, 2);       % Channel 2 ADC Input
Vdd4 = transfer4(start:step:stop, 3);       % Channel 3 ADC Input
Vds4 = transfer4(start:step:stop, 4);       % Channel 4 ADC Input
Vsd4 = -Vds4;                               % Allocate Variables
Vsg4 = -Vgs4;                               % Allocate Variables

n = length(Vgg1);                % Number of samples
N = 0:1:length(Vgg1)-1;          % Samples Vector
Rd = 147;                        % Drain Resistor Value (Ohm)
Rg = 148;                        % Gate Resistor Value (Ohm)

Id1 = (Vds1 - Vdd1)/Rd;         % Drain Current Formula for OECT 1
Ig1 = (Vgg1 - Vgs1)/Rg;         % Gate Current Formula for OECT 1
Id2 = (Vds2 - Vdd2)/Rd;         % Drain Current Formula for OECT 2
Ig2 = (Vgg2 - Vgs2)/Rg;         % Gate Current Formula for OECT 2
Id3 = (Vds3 - Vdd3)/Rd;         % Drain Current Formula for OECT 3
Ig3 = (Vgg3 - Vgs3)/Rg;         % Gate Current Formula for OECT 3
Id4 = (Vds4 - Vdd4)/Rd;         % Drain Current Formula for OECT 4
Ig4 = (Vgg4 - Vgs4)/Rg;         % Gate Current Formula for OECT 4
IdAverage = (Id1 + Id2 + Id3 + Id4)/4;

figure      % New Figure
set(gcf,'color','white');
plot(Vgg1,Id1*1000); hold on;   % Multiply with 1000 to get in mA
plot(Vgg2,Id2*1000);            % Multiply with 1000 to get in mA
plot(Vgg3,Id3*1000);            % Multiply with 1000 to get in mA
plot(Vgg4,Id4*1000);            % Multiply with 1000 to get in mA
plot(Vgg1,IdAverage*1000);      % Multiply with 1000 to get in mA
xlabel('V_{G} (V)');
ylabel('I_{D} (mA)');
title('Transfer: I_{D} vs V_{G}');
legend('OECT 1','OECT 2','OECT 3','OECT 4');
axis([0 1 0 2.5 ]);
```

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