博 士 学 位 論 文

Doctoral Dissertation

Engineering the design of miniaturized biosensors with optimized fabrication strategies ideal for point-of-care (POC) testing

（ポイントオブケア検査のための小型バイオセンサの作製に関する研究）

東北大学大学院環境科学研究科

Graduate School of Environmental Studies, Tohoku University

Department of Frontier Sciences for Advanced Environment Applied Environmental Chemistry	専攻 major／ コース course
学籍番号 Student ID No.	B9GD3003
氏　名 Name	SITI MASTURAH BINTI FAKHRUDDIN

<table>
<tr><td>指　導　教　員
Supervisor at Tohoku Univ.</td><td colspan="2">珠玖　仁　教授</td></tr>
<tr><td>研 究 指 導 教 員
Research Advisor at Tohoku Univ.</td><td colspan="2">井上　久美　准教授</td></tr>
<tr><td rowspan="4">審　査　委　員
（〇印は主査）
Dissertation Committee Members
Name marked with
“○” is the Chief Examiner</td><td colspan="2">〇　珠玖　仁　教授</td></tr>
<tr><td>1　壹岐　伸彦　教授</td><td>2　中山　亨　教授
（工学研究科）</td></tr>
<tr><td>3</td><td>4</td></tr>
<tr><td>5</td><td>6</td></tr>
</table>

Contents

Chapter 1 Introduction

Early detection and early treatment of diseases are essential for a patient's survival and for ensuring their quality of life. For long-term diseases, an effortless and undemanding way to enable regular health monitoring is also crucial to allow them a normal standard of living. Biosensors are ideally portable, low-cost analytical devices that can be used for the rapid detection of various biological pathogens, chemical molecules, and other analytes. The global biosensor market was valued at USD 25.5 billion in 2021 and is projected to reach approximately USD 36.7 billion by 2026 with an ever-increasing consumer pool and burgeoning demands from various fields of applications such as environmental monitoring, quality control assessment, healthcare, and more, at both industrial- and consumer-level. However, most biosensor research are limited to laboratory-scale developments due to challenges in adapting them for commercial use because of their complexity, cost, or unfeasibility for real-world use. In this thesis, I strive to develop biosensors that can be implemented in early detection of diseases, especially in point-of-care testing.

1.1 Biosensors

A biosensor is a device that integrates a biological receptor to an electronic transducer to produce signals that can be detected or analyzed. An official IUPAC nomenclature states that 'an electrochemical biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element'[1]. Research in the biosensing field has increased greatly since the development of the first biosensor by Clark et al.[2] in 1962, which was an amperometric oxygen electrode immobilized with glucose oxidase. Since then, development

of biosensors has expanded to include biosensor types such as electrochemical, optical, and piezoelectric.

While biosensors have seen applications in diverse fields that include agriculture, environmental monitoring, food safety, and even biodefense, the most notable field of interest for biosensors is the healthcare field (clinical diagnostics and health monitoring) as shown by the first biosensor developed for blood glucose detection. The conventional method of health check-ups is usually at centralized medical laboratories or hospitals. This is because these tests require highly expensive medical equipment with complicated testing procedures that need specialized personnel to carry out the tests and to operate the equipment. The test results themselves usually take time to get back to the patients, which when included into the travel time needed to get to the hospital, means that the diagnostic results may be too late for effective treatment. However, conventional laboratory testing methods give accurate results and continue to be held as the gold standard for most medical diagnoses. On the other hand, point-of-care testing (POCT) are capable of bedside or patient-side testing with relatively quick results and cheaper costs. POCT biosensors available commercially include glucose monitoring devices, pregnancy testing, infectious disease testing and cardiac markers biosensors. They have played an important role in early disease detection, health monitoring and even in emergency departments where fast medical decisions need to be made. In light of recent events such as the Ebola and MERS infectious diseases and the current COVID-19 pandemic, biosensors that offer point-of-care testing are increasingly vital to increase patients' survival rates and prevent infection outbreaks. POCT biosensors are also important in countries with an aging population and require consistent health monitoring to enable early diagnosis for early treatment of diseases, or in developing countries where the medical infrastructure may be sparse. The World Health Organization (WHO) has also defined a set of guidelines in developing POCT biosensors, referred to as ASSURED (affordable, sensitive, specific, user-

friendly, rapid and robust, equipment-free, and deliverable to end-users)[3,4]. Therefore, the ideal POCT biosensor is required to have rapid results to enable patients to receive the appropriate follow-up treatment right after. The biosensor is also required to have accurate and quantitative results comparable to standard laboratory-based test results in order to get reliable diagnosis. And the POCT biosensor needs to run on an easy-to-use system that can be operated even by a non-expert.

1.2 Types of bioreceptors used in biosensors

The biological recognition element, or bioreceptor, is an important part of a biosensor since it plays a role in determining the biosensor's sensitivity and selectivity. Different types of biological molecules can be applied as bioreceptors including enzymes, proteins, nucleic acids, or cells (Fig. 1-1). To ensure the biosensor's selectivity, specificity and lifetime, the appropriate immobilization method should be chosen for each case. Bioreceptor immobilization methods are mainly either physical adsorption (involves van der Waals force), covalent bonding (bonding between a functional group in the bioreceptor to the supporting substrate), crosslinking (uses bifunctional molecules such as glutaraldehyde) or entrapment (uses polymers or gels).

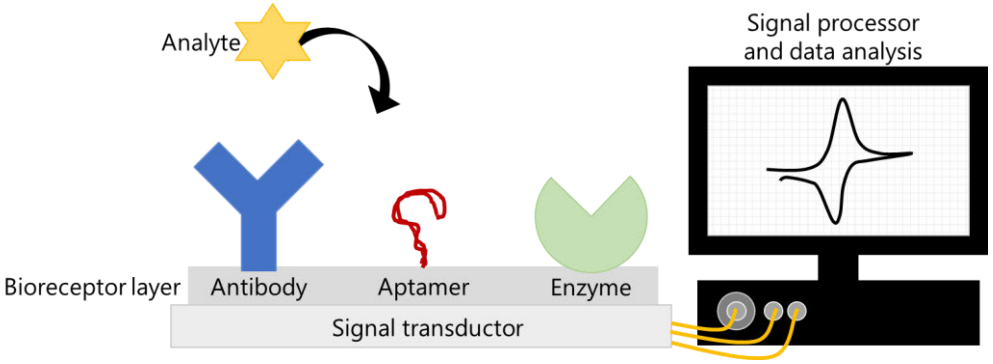

Figure 1-1 Types of bioreceptors used in a biosensor system that is made up of a bioreceptor layer, signal transducer, and a signal processor and data analysis platform.

1.2.1 Enzymes

Enzymes are proteins that catalyze specific chemical reactions *in vivo*, meaning that they accelerate the reaction rate of a particular substrate without being consumed in the process. The structure of an enzyme is formed from a single polypeptide chain of about 13 - 50 kDA folded in a precise and complex way. Within this folded structure is a region of about 5 - 10 amino acids arranged in a specific conformation that interacts with the substrate of the enzyme known as the active site. Many enzymes have their active sites within their protein structure to promote substrate specificity and to prevent non-specific interactions by inhibitor molecules. Some enzymes have more than one active site that allow binding to another substrate or a cofactor (a non-protein organic chemical compound required to maximize the efficiency of the enzymatic catalysis). The early theory describes the formation of the enzyme-substrate complex as the lock-and-key model which portrayed the enzyme as conformationally rigid and only binds to substrates that exactly fit its active site. However, further research led to the proposal of induced fit model[5] by Koshland in 1958 which describes that when an enzyme binds with its substrate, it optimizes the interface through physical interactions to form the final complex structure. Once the enzyme-substrate complex is formed, the active site promotes redistribution of electron density within the substrate by forming ionic or hydrogen bonding interactions to create a better fit. These interactions that occur after the complexation help lower the activation energy of the reaction, thus increasing the rate of reaction. After the enzyme-substrate is converted into product, product then dissociates from the active site to regenerate the enzyme's original structure. Enzymatic activity is measured by the amount of substrate converted to product per unit weight of enzyme per unit time at defined conditions. Whereas turnover number of the enzyme is the number of substrates transformed per molecule of enzyme per second.

Enzymes are among the most widely used bioreceptors in biosensors. Biosensors mainly use enzymes that are of the oxidoreductase class which includes oxidases, peroxidases, oxygenases, or dehydrogenases. Oxidoreductases catalyze the oxidation or reduction of substrate through hydrogen or electron transfer. Some of these enzymes require coenzymes (a type of cofactor that is a non-protein molecule and binds loosely to the enzyme) such as nicotinamide adenine dinucleotide (NAD), or flavin adenine dinucleotide (FAD). Oxidase enzymes also exhibit oxygen dependence as they need oxygen as a secondary substrate. Usually only one enzyme is used in an enzymatic biosensor, however research development has allowed multi-enzyme modification on one biosensor platform.

There are multiple reasons why enzymes are popular choices as bioreceptors. Compared to chemical catalysts, enzymes have a higher level of specificity due to their active sites. A model enzyme, glucose oxidase, will preferentially oxidize glucose even in the presence of equimolar amounts of maltose and fructose. However, acid phosphatase will catalyze the hydrolysis of different types of organic phosphate esters (producing an alcohol and inorganic phosphate). Therefore, both enzymes with wide and narrow specificity ranges can be applied in biosensors. Furthermore, the sensitivity of enzymatic biosensors is dependent on the catalytic activity of the enzyme which is high in optimized conditions. Using enzymes also offer a wide array of detectable species with flexibility in detection methods. Highly pure enzymes are also commercially available.

However, enzymes also present problems or limitations for biosensor applications. This is especially true for POCT biosensors. Enzymes have limited lifetime, shortening the biosensors' shelf-life. Enzymatic activity is, as mentioned before, is high only in optimized conditions. When the environmental pH, temperature or solution composition is not optimal, the enzyme will not work at maximum activity and can even get denatured in extreme

conditions, thus compromising the biosensors' long-term stability. Furthermore, their cost of production is often expensive.

1.2.2 Antibodies

Antibodies are serum proteins produced by memory B-cells and plasma cells, in response to a foreign substance (antigen). Antibody-antigen interaction has a very high affinity constant and low cross-reactivity, giving antibodies high specificity. Antibodies (also referred to as immunoglobulins, Ig) has a Y-shaped structure consisting of four polypeptide subunits that are two heavy chains and two light chains. The regions that have the antigen binding sites are called fragment antigen-binding (Fab) regions, consisting of a light chain and a segment of the heavy chain. Fab fragments are sometimes used as the bioreceptor instead of the whole antibody to reduce nonspecific binding and for better control of the bioreceptors orientation during immobilization[6]. An antibody can be classified into monoclonal, polyclonal, or recombinant. Monoclonal antibodies are from a single B-cell parent clone and have specificity for only one epitope per antigen. This is made possible by fusing the B-cells with hybridoma cells which allows for long-term generation of highly homogenous monoclonal antibodies. Polyclonal antibodies are a heterogenous mixture of antibodies that were derived from multiple B-cells and each one has a specificity for a different epitope of the same antigen. Therefore, polyclonal antibodies have higher batch-to-batch variability and cross-reactivity than monoclonal antibodies, since no mixture of antibodies will be the same. On the other hand, recombinant (monoclonal) antibodies consist of identical antibody chains that target the same epitope and are developed in vitro using synthetic genes. This process involves cloning antibody gene libraries into phage vectors and infecting a host cell with the phage vectors. The host cells then produce daughter phages that express the recombinant antibodies on their surfaces.

Antibodies are widely used for biosensing applications, either as a direct or indirect immunosensors. Furthermore, antibodies can be modified to include labels such as fluorophores or electroactive indicators, allowing flexibility in detection methods. They also have relatively better long-term stability even under slight environmental changes compared to enzymes. Due to their high sensitivity and specificity, antibodies are also popular bioreceptor candidates for POCT biosensors. However, their high cost of production, especially for monoclonal antibodies, and long turnaround time from antigen preparation to antibody harvesting (2 – 4 months for polyclonal, 6 months for monoclonal) can be significant disadvantages. Furthermore, while polyclonal has higher risk of batch-to-batch variability and cross-reactivity, monoclonal antibodies can be too specific for certain applications, for example drug screening tests.

1.2.3 Aptamers

Aptamers are small single-stranded DNA (ssDNA) or RNA sequences, produced in vitro using systematic evolution of ligands by exponential enrichment (SELEX), which does not need a living organism unlike enzymes or antibodies. Aptamers can fold into secondary and 3D shapes which allows them to bind with various types of target analyte (includes metal ions, small molecules, peptides, proteins, viruses and even cells) with high specificity and binding affinity. Since their first report in 1990 by two separate research groups[7,8], aptamers have been applied in fields such as diagnostics, drug delivery, biomarker discovery and biosensing. Aptamers have various advantages compared to antibodies and enzymes. As mentioned earlier, they can be obtained through an in vitro process using SELEX without needing living organisms such as cells or animals. This enables tailoring the aptamers to recognize any target even inorganic ions and small molecules. The tailoring of aptamers also includes easy modification and synthesis of aptamers with functional groups, fluorophores, biotin, or nanomaterials. Once an aptamer sequence has been selected from the SELEX process,

it can be easily prepared using polymerase chain reaction (PCR), making it more time-efficient compared to the preparation of antibodies and without the risk of batch-to-batch variations. Aptamers are also non-immunogenic and more stable in harsh conditions than antibodies that are sensitive to temperature and will undergo irreversible denaturation. The small size of aptamers also allows a higher density of immobilization on biosensing surfaces. Therefore, aptamers are slowly but surely replacing antibodies as bioreceptors for many biosensing applications.

1.3 Bipolar electrode (BPE)-based biosensor

Bipolar electrochemistry has received much attention, because when applied in analytical tools, it provides numerous useful properties as highlighted in various reviews[9–16]. Bipolar electrochemistry itself has been around since 1969, when Backhurst et al. described the concept of fluidized bed electrodes, in which a voltage applied between two driving electrodes promotes electrochemical reactions at discrete conducting particles[17]. A bipolar electrode (BPE) refers to a conducting material that is placed in an electrolyte with two other driving electrodes, and potential is applied between the two driving electrodes. Anodic and cathodic reactions occur at either end of the BPE when the potential between the driving electrodes is sufficient, even in the absence of a direct Ohmic contact. Bipolar electrochemistry has seen an increase in research development in various fields, spanning from photoelectrochemical cells and batteries to material synthesis and electrochemical analysis.

1.3.1 Open and closed BPEs

Open bipolar electrode system

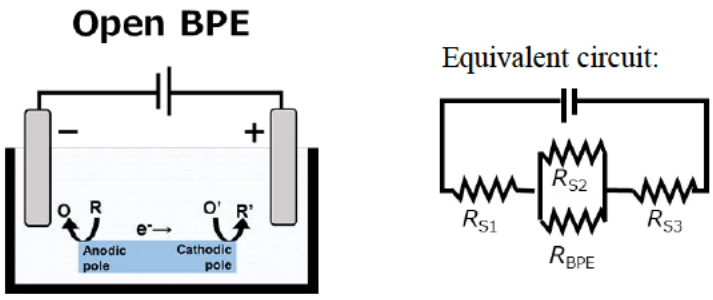

Figure 1-2 Open BPE system. Current path: BPE and electrolyte.

In the open bipolar electrode configuration, the conductive substrate is suspended in an electrolytic solution between two driving electrodes without direct physical connection between the substrate and the power supply (potentiostat), as illustrated in Fig. 1-2.

In Fig. 1-2, the driving electrodes apply a uniform electric field across the electrolyte solution and the resulting faradaic electrochemical reactions at the bipolar electrodes are shown occurring at the anodic and cathodic poles of the bipolar electrode. The interfacial potential difference between the solution and the bipolar electrode is highest at the ends of the electrode, therefore faradaic processes are always observed there first.

The voltage applied between the two driving electrodes (E_{tot}) results in an electric field in solution that causes the bipolar electrode to float to an equilibrium potential (E_{elec}) that depends on its position in the field and the solution composition. Because the bipolar electrode is a conductor, its potential (E_{elec}) is relatively the same throughout the surface of the electrode. However, the interfacial potential difference between the bipolar electrode and the solution varies along its length due the presence of an electric field in the solution. These anodic and cathodic overpotentials drive the electrochemical reactions at the bipolar electrode poles. The magnitudes of the overpotentials depend on the magnitude of E_{tot} and the bipolar electrode

length. In contrast, the working electrode in a conventional three-electrode configuration has relatively uniform interfacial potential difference.

Based on Fig. 1-2, in the absence of a bipolar electrode, the current flowing through the circuit is entirely ionic and the magnitude is influenced by the applied potential E_{tot} and the resistance of the solution, R_s. However, when faradaic reactions occur at a bipolar electrode, electrons then travel through the electrode and a second current path is formed. Hence, the bipolar electrode's total resistance to electronic current, R_{bpe} is defined. If the resistance of the solution above the bipolar electrode (R_{S2}) is lower than R_{bpe}, then most of the cell current passes through the solution rather than the bipolar electrode. However, when R_{S2} is more than R_{bpe}, which can be achieved by lowering the electrolyte concentration, substantial current flows through the BPE. This can cause either an increase or decrease in the local electric field strength in solution, which is proportional to $I_{tot}R_{S2}$ (I_{tot} is the total current flowing in the cell), thus resulting in a nonlinear electric field in the solution above the bipolar electrode (faradaic depolarization).

Closed bipolar electrode system

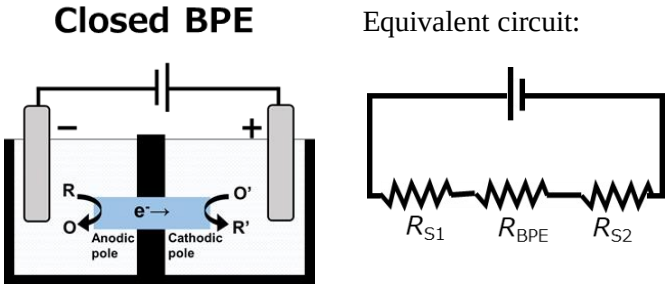

Figure 1-3 Closed BPE system. Current path: BPE only.

In a closed bipolar electrode configuration, the solutions in contact with the anode and cathode of the bipolar electrode are physically separated from one another (Fig. 1-3). A pair of driving electrodes connected to an external power supply is also placed in each solution. Since they are connected by electron transport, two different redox reactions occur simultaneously at the anodic and cathodic poles of the BPE when a sufficient driving potential is applied. The

current efficiency of the closed BPE is theoretically 100%, since the only current path between the two solutions is through the BPE. As the reporting and sample cells are physically separated, crosstalk between the sample and reporting solutions is prevented.

1.3.2 Applications

Although the charge flow in a BPE can be quantitatively studied through direct electrical measurements, optical readouts have become formats that are able to make full use of the wireless and high-throughput features of a BPE. The operating principle of a BPE-based device relies on the electrical coupling between the sensing and reporting poles attributable to the electroneutrality across the BPE. The reporting signal is due to charge-transfer reactions that occur in the presence of the analyte and is dependent on the analyte concentration. Consequently, a diverse range of imaging BPE-based devices has been developed, including electrochemiluminescence (ECL) [18,19], fluorescence[20,21], electrochromic[22] and even by metal film dissolution[23]. Furthermore, one of the advantages of bipolar electrochemistry is that multiple BPEs can be driven simultaneously with just a single power supply and a pair of driving electrodes, allowing fabrication of large imaging array devices[24–27]. However, electrochemical-based biosensors have higher accuracy and sensitivity, are insensitive to environmental illumination conditions and additional imaging apparatus (cameras) or software are unnecessary.

BPE-based biosensors have been used to detect various kinds of analyte such as adenosine in cancer cells[28], prostate specific antigen (PSA)[29], tetracycline (antibiotic)[30], mRNA[31], carcinoembryonic antigen (CEA)[32], adenosine triphosphate (ATP) and tobramycin[33]. These biosensors mostly used ECL as the reporting signal due to ECL not requiring excitation light thus it does not have background signals from scattered light or sample autofluorescence, giving it low background signals. To put it briefly, ECL is when an electroactive luminophore goes into an excited state due to the electrochemical reaction

sequence initiated by electron transfer at one end of the BPE pole. ECL-based BPE biosensors typically use anodic-type ECL which uses either the tris(2,2′-bipyridine)ruthenium ($Ru(bpy)_3^{2+}$) and tri-n-propylamine (TPA) or 3-aminophthalhydrazide (luminol) and hydrogen peroxide (H_2O_2) pairs. Recent research and development have enabled cathodic ECL that can also be applied to BPE biosensors[34]. BPE-based biosensors have even seen paper-based fabrication[9,35–38] and multiplexed[35,39–43] detection application to make them more attractive for POC testing applications.

1.4 Field-effect transistor (FET)-based biosensor

The FET-based biosensor was first reported by Bergveld[44] in 1970 as an ion-sensitive FET biosensor based on the traditional semiconductor FET structure. Then a proof-of-concept report of enzyme FETs were made in 1976[45] followed by a FET-based biosensor for penicillin array in 1980[46] by Janata et al. From there on, development of FET-based biosensors experienced great progress in research and development considering their advantages for point-of-care testing applications. They offer label-free and direct electronic measurement with fast responses, not to mention a mature fabrication process that enables miniaturization and low-cost mass-production, making them a highly competitive candidate for POCT biosensor application.

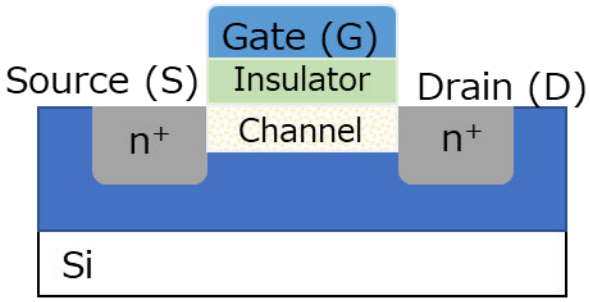

Figure 1-4 General schematic of a field-effect transistor (FET)

In general, a FET has three semiconductor devices, the source, drain and gate (Fig. 1-4). There is no physical contact between the source and drain, instead a conduction channel connects them. A FET biosensor will have its gate electrode surface modified with a bioreceptor layer that will interact or bind with the target analyte. These interactions will then modulate the channel conductivity between the source and drain terminals, which results in threshold voltage shifts. FETs are generally divided into n-type and p-type devices. In n-type devices, the main charge carriers are electrons, therefore when the target analyte is positively charged, the binding interaction will cause an increase in electrons in the channel, thus increasing channel conductance. On the other hand, when the target analyte is negatively charged, the binding interaction causes electrostatic repulsion of electrons, decreasing the channel conductance. In p-type devices where the main charge carriers are holes, the opposing phenomenon occurs. Positively charged analyte will decrease the channel conductance whereas negatively charged analyte will increase channel conductance. Hence, FET measurements will depend on the charge density of the analyte on the gate surface.

1.4.1 Metal oxide semiconductor FET (MOSFET)

The MOSFET was invented by Mohamed M. Atalla and D. Kahng in 1959 and is a type of insulated-gate FET fabricated by the controlled thermal oxidation of a semiconductor that is usually silicon. MOSFET is also the main semiconductor device in digital and analog integrated circuits for switching and amplifying electronic signals. As its name implies, MOSFET has a metal-insulator (oxide)-semiconductor structure with a metal electrode on top of an insulating oxide layer. MOSFET biosensors have been used in the detection of proteins[47], charged polymers[48], C-reactive protein (CRP)[49–51] and DNA hybridization[52,53].

1.4.2 Ion-sensitive FET (ISFET)

ISFET is a MOSFET with the metal gate electrode replaced with an ion-sensitive gate (insulator) electrode, a reference electrode, and electrolyte solution. In other words, the gate of an ISFET is a sample solution with a reference electrode and an ion-sensitive insulating layer. Thus, in an ISFET, the gate-insulating mechanism of a MOSFET is substituted with an electrochemical gating effect. In ISFETs that have a dielectric insulator such as SiO_2 or other oxide dielectrics, the capacitance is dominated by the gate dielectric and the capacitance of the electrical double layer (EDL) is negligible. In ISFETs that do not have the gate dielectric such as the case for carbon and metal oxide-type ISFETs, the capacitance is dominated by the EDL instead, arising from the redistribution of ions near the surface which then influences the channel[54]. The sensitivity of an ISFET depends on the insulating dielectric layer capacitance because it directly affects the quantity of induced charges per potential change [55]. The typical oxides used for the gate insulating material are SiO_2, Si_3N_4, Al_2O_3, or Ta_2O_5. It should be noted that in a measurement result of surface buffer capacity (the ability of the oxide surface to deliver or take up protons) for the four typical oxides used for ISFETs (SiO_2, Si_3N_4, Al_2O_3, Ta_2O_5), Ta_2O_5 has the largest surface buffer capacity such that variation in the value of the double-layer capacity hardly had any influence on the ISFET response[56]. Therefore, Ta_2O_5 has the highest pH sensitivity among these four oxides. Hence, the sensitivity and selectivity of the ISFET are completely controlled by the properties of the electrolyte/insulator interface[55]. ISFET biosensors have been applied in sensing a variety of bio-molecules, including the detection of C-reactive protein (CRP)[57], L-carnitine[58], cholesterol[59] (an extended-gate configuration was used, which adds an additional serial capacitance to the ISFET, limiting its sensitivity), maltose[60], glucose[61], human IgG[62], food pathogens[63], horseradish peroxidase (HRP), green fluorescent protein (GFP), DNA[64] and DNA hybridization[65].

1.4.3 Nanowire FET(NW-FET)

Nanomaterials such as quantum dots, nanoparticles and nanotubes have received great attention due their high surface-to-volume ratio that allows for ultra-sensitivity, among other characteristics, enabling various novel miniaturized biosensor designs. As such, it was originally thought that nanowire FET sensors also have improved sensitivity due to the increased surface area-to-volume ratio. Instead, nanowire FET biosensors have increased sensitivity due to the nanoscale surface geometry. When a nanowire which has a convex shape is on an insulating substrate, the corners create a concave surface. Since the counter-ion screening is weaker near concave surfaces compared to convex surfaces, the screening effect will thus vary depending on the overall concavity of the electrode structure. Hence, in the case of nanowire FET sensors, weaker Debye screening and smaller capacitance density in the corners between the nanowire and the substrate contributed to the sensor having a larger charge density[66] and therefore a higher sensitivity. The first report of a silicon NW-FET biosensor was by Cui et al[67] and included demonstration of detecting streptavidin down to 10 pM, reversible antibody binding (monoclonal anti-biotin), and even detection of calcium ion, Ca^{2+}. Fabrication of SiNW-FETs uses either a top-down or a bottom-up method. The bottom-up approach uses a metal-cluster-catalyzed vapor-liquid-solid growth method that has the problem of difficulty in precise positioning of individual devices through solution-processing methods which is a disadvantage for mass production of the SiNW-FETs and for integration with external circuitry. On the other hand, the top-down approach uses the well-established complementary metal-oxide-semiconductor (CMOS) fabrication technique which enables mass-production of SiNW-FETs that can be easily integrated with external circuitry, however, lithography techniques with nanometer scale resolution are costly. NW-FETs have undergone extensive research and development to optimize their sensing capabilities considering their great potential in the field of biosensors[55,66,68–73]. NW-FET biosensors have been used to

detect C-reactive protein (CRP)[74–76], uric acid[77], influenza A virus[78], antigen-specific T-cell[79], pH-sensing array[80], prostate-specific antigen (PSA)[81,82], avian influenza virus[83], amyloid beta-40 (Alzheimer's disease biomarker)[84], hepatitis B virus[85], dopamine released from PC12 cells[86], and even to study the effect of anti-cancer drug (topotecan hydrochloride) on tumor cells[87].

1.4.4 Graphene FET (GFET)

Graphene is a single atom-thick two-dimensional material that consists of a honeycomb lattice of carbon atoms covalently linked through sp^2 hybridization that exhibits high electron/hole mobility, high conductivity, good biocompatibility, and mechanical strength. Graphene is also a zero-bandgap semiconductor with ambipolar field-effect in a FET structure with the advantage of having a high surface area with each carbon atom available on the surface for sensing, giving it ultra-sensitivity. Synthesis of graphene can be done through the top-down exfoliation process of graphite or the bottom-up chemical vapor deposition (CVD) method exploiting the catalytic and carbon-saturated properties of specific metals when exposed to hydrocarbon gases at high temperatures. However, graphene is expensive and difficult to fabricate at a mass-scale and its sensitivity is easily compromised since every carbon atom is easily exposed to buffer ions and non-specific binding which gives it high background noise. The lack of a bandgap and low current on/off ratio also affects its sensitivity. GFET biosensors are often biofunctionalized using noncovalent modifications such as π-π stacking and hydrophobic interactions since covalent bonding will alter the carbon bonding from sp^2 to sp^3 which decreases carrier mobility. GFET biosensors have been used to detect DNA hybridization[88], SARS-CoV-2[89] and SARS-CoV-2 antibody[90], prostate-specific antigen/α-antichymotrypsin (PSA-ACT)[91], *E. coli* bacteria[92], interferon gamma[93], human IgE[94], adenosine triphosphate (ATP)[95], and tumor necrosis factor-α (TNF- α)[96,97]. However, the detecting mechanism for some GFET biosensors should be approached with care[98,99].

1.5 Gravimetric biosensor

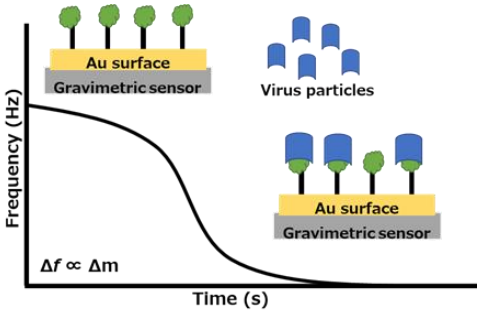

Figure 1-5 Simplified detection principle of a gravimetric biosensor.

Gravimetric, or mass-sensitive, biosensors operate based on the principle of changes in resonant frequency when there is a change in mass on the biosensor (Fig. 1-5). Since they offer label-free, direct, and real-time detection of analytes in both gas- and liquid-phase medium, these mass-sensitive biosensors have high potential in POC biosensing, especially since they can also be easily integrated into various types of smart devices.

1.5.1 Quartz crystal microbalance (QCM)

A QCM is typically made of a thin AT-cut quartz crystal with metal electrodes on opposite sides, connected with an oscillator circuit which induces the QCM's resonant frequency. Its crystal surface will be modified with a bioreceptor layer on one side which then binds with the target analyte. It is one of the most widely used gravimetric biosensors, ever since Sauerbrey established the relation between resonant frequency change and change in surface mass density deposited onto the sensing surface. QCM measuring methods include electrochemical QCM (EQCM) or QCM with dissipation (QCM-D). QCM-D is used to measure viscoelastic layers which QCM itself cannot since it can only evaluate rigid layers. QCMs are commercially available and have been used in the detection of various analytes including C-reactive protein (CRP)[100–102], viruses[103–106], and even genetically modified organisms (GMO)[107,108]. However, they have sensitivities that are limited by their operational frequencies and have a brittle biosensor structure, making them inappropriate for POC use. The

manufacturing process can be complicated and hence, costly, which is also another disadvantage.

1.5.2 Magnetostrictive cantilever

The sensing principle of magnetostrictive cantilever-type biosensors is also based on a change in resonant frequency when there is a change in mass on the sensing surface. The biosensor is made of magnetoelastic materials such as Fe, Co, or Ni that exhibit the magnetostrictive effect which is when a magnetic field is applied using a driving coil, it induces an oscillation of the cantilever. This oscillation emits a magnetic flux that is then read by the pickup coil. The magnetoelastic cantilever has the advantage of a simpler fabrication process and a sturdier biosensor structure than QCM, capable of wireless operation in gas- or liquid-phase. They have been used to detect pathogens such as bacteria[109–111], classical swine fever virus[112] and its antibody[113], carcinoembryonic antigen (CEA)[114], and human serum albumin (HSA)[115,116].

1.6 Device fabrication and modification methods

1.6.1 Photolithography

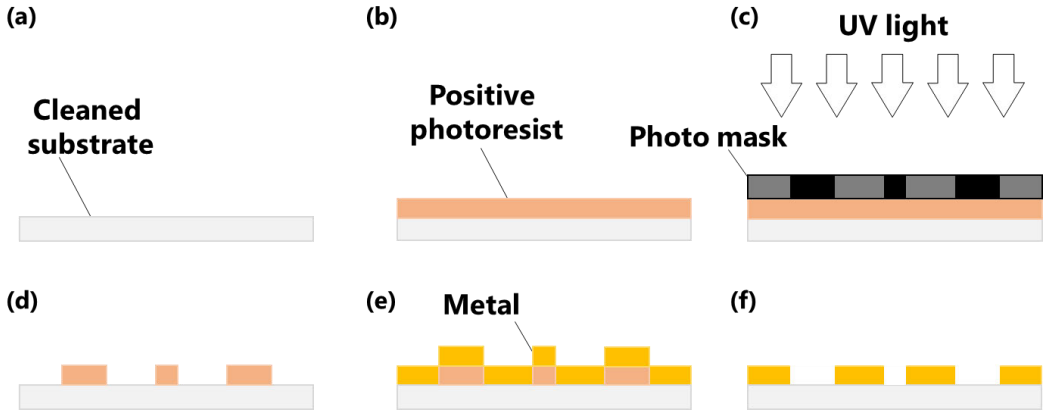

Figure 1-6 Photolithography process with positive photoresist. (a) The substrate (glass, silicon, or others) is cleaned. (b) Spin coating positive photoresist onto the substrate. (c) UV light exposure through aligned photomask. (d) Development of photoresist where the unexposed part is dissolved. (e) Deposition of metal thin film layer. (f) Lift-off process to remove photoresist and forming the desired pattern.

Photolithography (or optical lithography or UV lithography) is the standard method for fabricating microelectromechanical systems (MEMS) devices or semiconductor devices. The process defines and transfers a pattern onto a thin film layer on a wafer or substrate (typically silicon, glass, or other flat material). Photolithography has the advantage of being highly controllable and reproducible over other mechanical and template method and can reach nanometer scale. The general flow for photolithography (Fig. 1-6) is first, substrate surface cleaning using standard degrease protocol and additional oxygen plasma cleaning to remove organic residues. Then, photoresist (negative or positive photoresist) is spin coated onto the cleaned substrate, forming a thin and uniform layer of photoresist. To improve photoresist adhesion, a primer layer is usually spin coated first. The thickness of the photoresist film depends on the type, density, spin coating speed and duration. Afterwards, pre-bake (soft bake) is done to remove the solvent from the photoresist, either using an oven or a hot plate, to improve photoresist adhesion and uniformity. Next, a photomask is aligned onto the photoresist-coated substrate, and it is exposed to UV light to transfer the photomask pattern onto the photoresist. For negative photoresist, the UV light polymerizes and hardens the exposed area, enabling the developer to strip off the unexposed parts later. Whereas for positive photoresist, the opposite occurs, and the unexposed area will remain while the exposed area gets stripped. UV light exposure strength (wavelength) and time will influence the quality of the developed photoresist. Then, development using a chemical developer suited to the photoresist is used to dissolve the exposed/unexposed parts of the photoresist. Post-bake (hard bake) is then carried out to stabilize and harden the developed photoresist before moving to the next step. However, post-bake is unnecessary if there is a lift-off process involved since post-bake will make photoresist removal harder. Further substrate processing by metal thin film deposition then etching or lift-off followed by photoresist stripping is carried out to form the

desired pattern. Lift-off is typically over a range of a few minutes to a day, although using ultrasonic agitation (carefully) can help shorten this time.

1.6.2 Metal sputtering

While there are many methods of thin film deposition that can be either physical (evaporation methods) or chemical (gas- and liquid-phase deposition methods) or both (glow-discharge and reactive sputtering processes), the basic sputtering system consists of a vacuum chamber containing a metallic anode and cathode and the chamber is pumped to a base pressure of 1 x 10^{-4} Pa or lower followed by the introduction of a noble gas (usually argon) into the vacuum chamber, increasing the pressure to about 10 Pa, thus when a high voltage is applied between the cathode and anode, a glow-discharge is obtained. Two common types of sputtering are direct current (DC) sputtering that is usually used with electrically conductive target materials and radio frequency (RF) sputtering that is usually for most dielectric materials[117].

1.6.3 Silanization

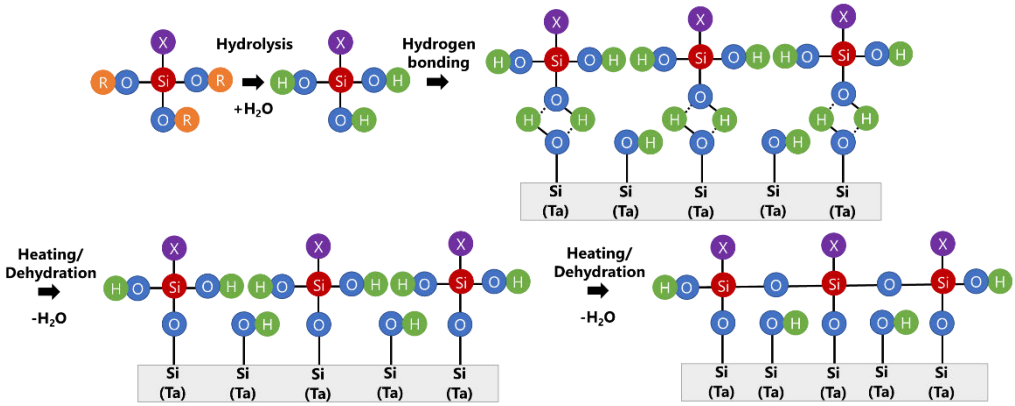

Figure 1-7 General mechanism of silanization, R: leaving groups, X: functional groups.

In general, silanization of hydroxyl-terminated substrate (SiO_2 or other metal oxides) is often used for chemical modification and biomolecule immobilization of the substrate surface. Silane self-assembled monolayer (SAM) properties depend heavily on the silane structure, the density of -OH groups available on the substrate surface and the physical structure of the surface. The exact mechanism of silanization is not well-understood but it is generally

accepted that liquid-phase silanization involves three steps (Fig. 1-7). First, hydrolyzation of the silanes in the presence of water to form silanetriols on the surface or in the solvent. These silanetriols then attach onto the substrate surface through hydrogen bonding. And finally, the silanol groups react with the free -OH groups on the surface to form siloxane groups. When the density of -OH groups is low, crosslinking between the siloxane groups occur, creating a stronger attached silane SAM. However, crosslinking between monoalkoxy- and monochlorosilanes are not possible. Well-defined SAM formation with silanes requires precise consideration of experimental variables such as reaction time, temperature, humidity, among others. Trialkoxysilanes are among the most used silanes for immobilizing biomolecules, however their short carbon chain can induce 3D silane "islands" or if the silane has a functional group such as amino groups, the amino group might form hydrogen bonds with the -OH groups on the substrate instead.

1.6.4 Bioreceptor immobilization

Commonly used methods include physical adsorption, covalent bonding, crosslinking, and hydrogel entrapment. Physical adsorption is the easiest and fastest method that does not need any chemicals and instead makes use of hydrogen bonds, van der Waals force or electrostatic interactions. However, this method has weak attachment and the bioreceptors are easily desorbed by environmental factors such as pH, temperature, or changes ins solution composition. On the other hand, covalent coupling forms covalent bonds between the functional groups present on the bioreceptor and on the substrate. This includes formation of amide bonds between aminated or carboxylated substrate surfaces and the amino- or carboxyl-group of the enzyme and thiol-disulphide bond that is often used for immobilization of proteins onto thiolated substrate. Covalent bonding allows for a stronger attachment of the bioreceptor onto the substrate and has a diverse applicability. However, the covalent bonding might cause the bioreceptor to undergo conformational change thus affecting its functionality. Entrapment

(or encapsulation) is constraining the bioreceptors within a (usually) hydrogel matrix while still allowing the analyte to diffuse/penetrate through. While this method usually will not cause conformational change of the bioreceptors, there are the disadvantages of biofouling of the hydrogel or bioreceptor leaking from the matrix. Crosslinking involves bi- or multifunctional reagents or ligands such as glutaraldehyde that is often used to attach the amino groups on substrates to the amino groups of the bioreceptors. Since it is a kind of covalent bonding too, it can cause conformational changes which can affect the activity of the bioreceptor, but it offers stable bonds that will not desorb easily.

1.7 Surface evaluation methods

1.7.1 X-ray photoelectron spectroscopy (XPS)

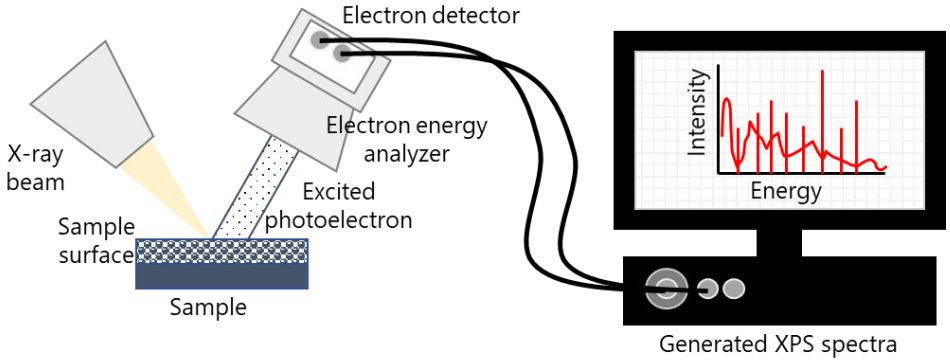

Figure 1-8 Simplified schematic of X-ray photoelectron spectroscopy (XPS).

XPS is an analytical technique used to understand a material's surface chemistry. XPS can also measure the elemental composition and chemical and electronic state of the atoms of the material's surface. XPS spectrum/spectra can be obtained by irradiating a material's surface with an X-ray beam and measuring the kinetic energy of the photoelectrons that are emitted from the material's surface (the top 1 – 10 nm) (Fig. 1-8). The spectra peaks obtained are then used to identify and quantify the surface elements (except for H and He) by looking at the peaks' binding energy and intensity. Shifts in the binding energies can give information on the bonding state or oxidation state of the surface elements.

1.7.2 Fourier-transform infrared spectroscopy (FT-IR)

FT-IR is an analytical technique that uses infrared light to scan substrates and observe its chemical properties. Specifically, the instrument sends infrared radiation through the substrate with some of the radiation absorbed and some passing through the substrate. The substrate molecules then convert the absorbed radiation energy into rotational/vibrational energy which is then picked up as a spectrum which can be used to identify the chemical species available in the substrate. However, FT-IR is more suited for "bulk" analysis, therefore substrate with trace/small concentrations of material cannot be analyzed effectively.

1.7.3 Scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX)

SEM-EDX is an analytical technique that can produce high resolution images of surface topography using a highly focused scanning primary electron beam. The primary electrons enter the substrate surface and generate secondary electrons which can be used to construct surface topography. Backscattered electrons and X-ray are also generated by the primary electrons, which can be correlated to an element's atomic number allowing for qualitative elemental analysis which is also added with information obtained from EDX analysis of the X-rays. EDX analysis can be both quantitative and qualitative since it can be used to identify the type of elements present as well as the percentage of concentration available in the substrate. SEM-EDX requires little sample pretreatment and is a non-destructive analytical technique. However, the depth of its analysis is up till µm-order, making it unsuitable for thin layer (nm-order) analysis.

1.8 Research objectives

To tackle the global need for biosensors that are capable of point-of-care-testing (POCT), my research approach is to engineer the design of miniaturized biosensors with optimized fabrication strategies ideal for POCT. This is while keeping in mind that the POCT biosensor must be able to provide rapid detection, with accurate and quantitative results and when possible, have easy-to-use systems that even a non-expert can operate the biosensor. The research objectives for each chapter are described below:

Chapter 1: Introduction

A brief research background on biosensors and the development of biosensors for POC testing is explained. The types of bioreceptors and the types of biosensors (BPE-based, FET-based and gravimetric-based) that will be used in the latter chapters of this thesis are explained with examples of applications also added. Short explanations on the surface evaluation analytical methods used in this thesis were also included.

Chapter 2: BPE-based biosensor with multiple enzyme modifications

By exploiting bipolar electrochemistry that enables the operation of multiple BPEs with just a pair of driving electrodes, a BPE-based biosensor that is capable of multiplexed and simultaneous sensing of three different kinds of metabolites (glucose, lactate, and uric acid) was developed. Oxidase-type enzymes for each metabolite were immobilized on respective detection electrodes and a redox mediator was added to each sample cell to enable electrochemical detection of each metabolite.

Chapter 3: ISFET-based biosensor with aptamer modification

Field-effect transistor technology, specifically ISFET, was applied to fabricate an ISFET-based aptasensor that is capable of measuring C-reactive protein, an inflammation

biomarker, in a range of 0.002 to 20 μg/mL in physiological ionic strength. Silanization was done to reduce -OH group density on the ISFET gate surface and hence reduce background signals to increase detection sensitivity. This strategy was combined with using aptamers that provide significant surface potential change with their conformational changed before and after binding with their target.

Chapter 4: Gravimetric-based biosensor with antibody modification

In this chapter, mass-sensitive or gravimetric technique was used to develop a gravimetric sensor to detect human coronavirus 229-E (HCoV-229E), a virus that causes the common cold in humans, using a novel biorecognition method. CD13/aminopeptidase N is a protein that is known to bind to HCoV-229E and has been used in cell-based assays, but its use as a bioreceptor layer for HCoV-229E has not been reported. By modifying CD13 onto the gravimetric biosensor's surface, the detection of HCoV-229E was attempted.

Chapter 5: Summary and conclusion

A concise summary of the research results obtained in each chapter is described with the necessary details included.

1.9 Reference

[1] D. R. Thévenot, K. Toth, R. A. Durst, G. S. Wilson, *Biosens. Bioelectron.* **2001**, *16*, 121–131.

[2] L. C. Clark, C. Lyons, *Ann. N. Y. Acad. Sci.* **1962**, *102*, 29–45.

[3] C. S. Kosack, A. Page, P. R. Klatser, **2017**, 639–645.

[4] R. W. Peeling, K. K. Holmes, D. Mabey, **2006**, DOI 10.1136/sti.2006.024265.

[5] D. E. Koshland, *Angew. Chemie Int. Ed.* **1994**, *33*, 2375–2378.

[6] A. A. Karyakin, G. V. Presnova, M. Y. Rubtsova, A. M. Egorov, *Anal. Chem.* **2000**, *72*, 3805–3811.

[7] A. D. Ellington, J. W. Szostak, *Nature* **1990**, *346*, 818–822.

[8] C. Tuerk, L. Gold, *Science (80-.).* **1990**, *249*, 505–510.

[9] C. Renault, K. Scida, K. N. Knust, S. E. Fosdick, R. M. Crooks, **2013**, *4*, 146–152.

[10] N. Shida, Y. Zhou, S. Inagi, *Acc. Chem. Res.* **2019**, *52*, 2598–2608.

[11] L. Bouffier, N. Sojic, A. Kuhn, *Electrophoresis* **2017**, *38*, 2687–2694.

[12] L. Koefoed, S. U. Pedersen, K. Daasbjerg, *Curr. Opin. Electrochem.* **2017**, *2*, 13–17.

[13] Y. Ishiguro, S. Inagi, T. Fuchigami, *J. Am. Chem. Soc.* **2012**, *134*, 4034–4036.

[14] K. L. Rahn, R. K. Anand, **2021**.

[15] F. Mavré, R. K. Anand, D. R. Laws, K. F. Chow, B. Y. Chang, J. A. Crooks, R. M. Crooks, *Anal. Chem.* **2010**, *82*, 8766–8774.

[16] S. E. Fosdick, K. N. Knust, K. Scida, R. M. Crooks, *Angew. Chemie - Int. Ed.* **2013**, *52*, 10438–10456.

[17] J. R. Backhurst, J. M. Coulson, F. Goodridge, R. E. Plimley, M. Fleischmann, *J. Electrochem. Soc.* **1969**, *116*, 1600.

[18] L. Bouffier, S. Arbault, A. Kuhn, N. Sojic, *Anal. Bioanal. Chem.* **2016**, *408*, 7003–7011.

[19] K. Hiramoto, E. Villani, T. Iwama, K. Komatsu, S. Inagi, K. Y. Inoue, Y. Nashimoto, K. Ino, H. Shiku, *Micromachines* **2020**, *11*, 1–22.

[20] W. Xu, C. Ma, P. W. Bohn, *ChemElectroChem* **2016**, *3*, 422–428.

[21] J. P. Guerrette, S. J. Percival, B. Zhang, *J. Am. Chem. Soc.* **2013**, *135*, 855–861.

[22] S. Masturah binti Fakhruddin, K. Ino, K. Y. Inoue, Y. Nashimoto, H. Shiku, *Electroanalysis* **2021**, 1–16.

[23] K. F. Chow, B. Y. Chang, B. A. Zaccheo, F. Mavré, R. M. Crooks, *J. Am. Chem. Soc.* **2010**, *132*, 9228–9229.

[24] K. F. Chow, F. Mavré, J. A. Crooks, B. Y. Chang, R. M. Crooks, *J. Am. Chem. Soc.* **2009**, *131*, 8364–8365.

[25] J. Gao, S. Chen, F. Altal, S. Hu, L. Bouffier, G. Wantz, *ACS Appl. Mater. Interfaces* **2017**, *9*, 32405–32410.

[26] T. J. Anderson, P. A. Defnet, B. Zhang, *Anal. Chem.* **2020**, *92*, 6748–6755.

[27] W. Guo, X. Lin, F. Yan, B. Su, *ChemElectroChem* **2016**, *3*, 480–486.

[28] H. W. Shi, M. S. Wu, Y. Du, J. J. Xu, H. Y. Chen, *Biosens. Bioelectron.* **2014**, *55*, 459–463.

[29] H.-J. Lu, W. Zhao, J.-J. Xu, H.-Y. Chen, *Biosens. Bioelectron.* **2018**, *102*, 624–630.

[30] L. Jin, J. Qiao, J. Chen, N. Xu, M. Wu, *Talanta* **2020**, *208*, 120404.

[31] M.-S. Wu, G. Qian, J.-J. Xu, H.-Y. Chen, *Anal. Chem.* **2012**, *84*, 5407–14.

[32] Q. Zhai, X. Zhang, Y. Xia, J. Li, E. Wang, *Analyst* **2016**, *141*, 3985–3988.

[33] X. Zhang, R. A. Lazenby, Y. Wu, R. J. White, *Anal. Chem.* **2019**, *91*, 11467–11473.

[34] J. Miranda, N. Humphrey, R. Kinney, R. O'Sullivan, B. Thomas, I. E. Mondaca Medina, R. Freedman, E. Fahrenkrug, *ACS Sensors* **2021**, *6*, 4136–4144.

[35] Q. Feng, H. Chen, J. Xu, *Sci. China Chem.* **2015**, *58*, 810–818.

[36] E. Rafatmah, B. Hemmateenejad, *Microchim. Acta* **2019**, *186*, DOI 10.1007/s00604-019-3793-y.

[37] H. Liu, X. Zhou, W. Liu, X. Yang, D. Xing, *Anal. Chem.* **2016**, *88*, 10191–10197.

[38] S. Ge, J. Zhao, S. Wang, F. Lan, M. Yan, J. Yu, *Biosens. Bioelectron.* **2018**, *102*, 411–417.

[39] Y. Xiao, L. Xu, L. W. Qi, *Talanta* **2017**, *165*, 577–583.

[40] K. Y. Inoue, M. Ikegawa, T. Ito-Sasaki, S. Takano, H. Shiku, T. Matsue, *ChemElectroChem* **2018**, *5*, 2167–2170.

[41] W. Xu, K. Fu, P. W. Bohn, *ACS Sensors* **2017**, *2*, 1020–1026.

[42] M. S. Wu, Z. Liu, H. W. Shi, H. Y. Chen, J. J. Xu, *Anal. Chem.* **2015**, *87*, 530–537.

[43] C. Liu, D. Wang, C. Zhang, *Sensors Actuators, B Chem.* **2018**, *270*, 341–352.

[44] P. Bergveld, *IEEE Trans. Biomed. Eng.* **1970**, *BME-17*, 70–71.

[45] J. Janata, S. D. Moss, *Biomed. Eng. (NY).* **1976**, *11*, 241–245.

[46] S. Caras, J. Janata, *Anal. Chem.* **1980**, *52*, 1935–1937.

[47] S. H. Han, S. K. Kim, K. Park, S. Y. Yi, H. J. Park, H. K. Lyu, M. Kim, B. H. Chung, *Anal. Chim. Acta* **2010**, *665*, 79–83.

[48] J. Lee, J. Jang, B. Choi, J. Yoon, J. Y. Kim, Y. K. Choi, D. Myong Kim, D. Hwan Kim, S. J. Choi, *Sci. Rep.* **2015**, *5*, 1–6.

[49] H.-K. Lyu, Y.-S. Choi, J.-K. Shin, J.-H. Kim, in *Smart Biomed. Physiol. Sens. Technol. VI* (Eds.: B.M. Cullum, D.M. Porterfield), SPIE, **2009**, p. 73130S.

[50] H. Yuan, H. C. Kwon, S. H. Yeom, D. H. Kwon, S. W. Kang, *Biosens. Bioelectron.* **2011**, *28*, 434–437.

[51] H. M. Jeong, J. B. Kwon, H. C. Kwon, J. S. Kim, B. Xu, D. H. Kwon, S. W. Kang, *IEEE*

Trans. Electron Devices **2018**, *65*, 243–250.

[52] D. S. Kim, Y. T. Jeong, H. J. Park, J. K. Shin, P. Choi, J. H. Lee, G. Lim, *Biosens. Bioelectron.* **2004**, *20*, 69–74.

[53] E. Souteyrand, J. P. Cloarec, J. R. Martin, C. Wilson, I. Lawrence, S. Mikkelsen, M. F. Lawrence, *J. Phys. Chem. B* **1997**, *101*, 2980–2985.

[54] V. Pachauri, S. Ingebrandt, *Essays Biochem.* **2016**, *60*, 81–90.

[55] K. Shoorideh, C. O. Chui, *IEEE Trans. Electron Devices* **2012**, *59*, 3104–3110.

[56] P. Bergveld, *Sensors Actuators, B Chem.* **2003**, *88*, 1–20.

[57] O. Kutova, M. Dusheiko, N. I. Klyui, V. A. Skryshevsky, *Microelectron. Eng.* **2019**, *215*, DOI 10.1016/j.mee.2019.110993.

[58] M. S. Andrianova, E. V. Kuznetsov, V. P. Grudtsov, A. E. Kuznetsov, *Biosens. Bioelectron.* **2018**, *119*, 48–54.

[59] Y. Ishige, M. Shimoda, M. Kamahori, *Biosens. Bioelectron.* **2009**, *24*, 1096–1102.

[60] H. J. Park, S. K. Kim, K. Park, H. K. Lyu, C. S. Lee, S. J. Chung, W. S. Yun, M. Kim, B. H. Chung, *FEBS Lett.* **2009**, *583*, 157–162.

[61] S. D. Caras, D. Petelenz, J. Janata, *Anal. Chem.* **1985**, *57*, 1920–1923.

[62] M. Yano, K. Koike, K. Mukai, T. Onaka, Y. Hirofuji, K. I. Ogata, S. Omatu, T. Maemoto, S. Sasa, *Phys. Status Solidi Appl. Mater. Sci.* **2014**, *211*, 2098–2104.

[63] C. Duarte-Guevara, V. V Swaminathan, B. Reddy, J.-C. Huang, Y.-S. Liu, R. Bashir, *RSC Adv.* **2016**, *6*, 103872–103887.

[64] D. Gonçalves, D. M. F. Prazeres, V. Chu, J. P. Conde, *Biosens. Bioelectron.* **2008**, *24*, 545–551.

[65] F. Uslu, S. Ingebrandt, D. Mayer, S. Böcker-Meffert, M. Odenthal, A. Offenhäusser, *Biosens.*

Bioelectron. **2004**, *19*, 1723–1731.

[66] K. Shoorideh, C. O. Chui, *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 5111–5116.

[67] Y. Cui, Q. Wei, H. Park, C. M. Lieber, *Science (80-.).* **2001**, *293*, 1289–1292.

[68] W. P. Hu, C. C. Tsai, Y. S. Yang, H. W. H. Chan, W. Y. Chen, *Sci. Rep.* **2018**, *8*, 1–8.

[69] S. Regonda, R. Tian, J. Gao, S. Greene, J. Ding, W. Hu, *Biosens. Bioelectron.* **2013**, *45*, 245–251.

[70] Y. Kutovyi, I. Zadorozhnyi, H. Hlukhova, V. Handziuk, M. Petrychuk, A. Ivanchuk, S. Vitusevich, *Nanotechnology* **2018**, *29*, DOI 10.1088/1361-6528/aaaf9e.

[71] E. Stern, R. Wagner, F. J. Sigworth, R. Breaker, T. M. Fahmy, M. A. Reed, *Nano Lett.* **2007**, *7*, 3405–3409.

[72] A. Vacic, J. M. Criscione, N. K. Rajan, E. Stern, T. M. Fahmy, M. A. Reed, *J. Am. Chem. Soc.* **2011**, *133*, 13886–13889.

[73] C. Park, K. Kim, M. Meyyappan, D. Kim, N. Hong, J. S. Lee, *IEEE-NANO 2015 - 15th Int. Conf. Nanotechnol.* **2015**, 1335–1338.

[74] J. H. Ahn, J. Y. Kim, M. Im, J. W. Han, Y. K. Choi, in *14th Int. Conf. Miniaturized Syst. Chem. Life Sci. 2010, MicroTAS 2010*, **2010**, pp. 1301–1303.

[75] S. M. Kwon, G. B. Kang, Y. T. Kim, Y. H. Kim, B. K. Ju, *J. Nanosci. Nanotechnol.* **2011**, *11*, 1511–1514.

[76] M.-H. Lee, D.-H. Lee, S.-W. Jung, K.-N. Lee, Y. S. Park, W.-K. Seong, *Nanomedicine Nanotechnology, Biol. Med.* **2010**, *6*, 78–83.

[77] X. Liu, P. Lin, X. Yan, Z. Kang, Y. Zhao, Y. Lei, C. Li, H. Du, Y. Zhang, *Sensors Actuators, B Chem.* **2013**, *176*, 22–27.

[78] F. Patolsky, G. Zheng, O. Hayden, M. Lakadamyali, X. Zhuang, C. M. Lieber, *Proc. Natl.*

Acad. Sci. U. S. A. **2004**, *101*, 14017–14022.

[79] E. Stern, E. R. Steenblock, M. A. Reed, T. M. Fahmy, *Nano Lett.* **2008**, *8*, 3310–3314.

[80] D. Rani, V. Pachauri, A. Mueller, X. T. Vu, T. C. Nguyen, S. Ingebrandt, *ACS Omega* **2016**, *1*, 84–92.

[81] N. Lu, A. Gao, P. Dai, H. Mao, X. Zuo, C. Fan, Y. Wang, T. Li, *Anal. Chem.* **2015**, *87*, 11203–11208.

[82] Y. W. Huang, C. S. Wu, C. K. Chuang, S. T. Pang, T. M. Pan, Y. S. Yang, F. H. Ko, *Anal. Chem.* **2013**, *85*, 7912–7918.

[83] P. L. Chiang, T. C. Chou, T. H. Wu, C. C. Li, C. Da Liao, J. Y. Lin, M. H. Tsai, C. C. Tsai, C. J. Sun, C. H. Wang, J. M. Fang, Y. T. Chen, *Chem. - An Asian J.* **2012**, *7*, 2073–2079.

[84] Y. Kutovyi, H. Hlukhova, N. Boichuk, M. Menger, A. Offenhäusser, S. Vitusevich, *Biosens. Bioelectron.* **2020**, *154*, DOI 10.1016/j.bios.2020.112053.

[85] M. Shariati, *Biosens. Bioelectron.* **2018**, *105*, 58–64.

[86] B. R. Li, C. W. Chen, W. L. Yang, T. Y. Lin, C. Y. Pan, Y. T. Chen, *Biosens. Bioelectron.* **2013**, *45*, 252–259.

[87] A. Susloparova, D. Koppenhöfer, X. T. Vu, M. Weil, S. Ingebrandt, *Biosens. Bioelectron.* **2013**, *40*, 50–56.

[88] N. Mohanty, V. Berry, *Nano Lett.* **2008**, *8*, 4469–4476.

[89] G. Seo, G. Lee, M. J. Kim, S. H. Baek, M. Choi, K. B. Ku, C. S. Lee, S. Jun, D. Park, H. G. Kim, S. J. Kim, J. O. Lee, B. T. Kim, E. C. Park, S. Il Kim, *ACS Nano* **2020**, *14*, 5135–5142.

[90] H. Kang, X. Wang, M. Guo, C. Dai, R. Chen, L. Yang, Y. Wu, T. Ying, Z. Zhu, D. Wei, Y. Liu, D. Wei, *Nano Lett.* **2021**, *21*, 7897–7904.

[91] D. J. Kim, I. Y. Sohn, J. H. Jung, O. J. Yoon, N. E. Lee, J. S. Park, *Biosens. Bioelectron.* **2013**,

41, 621–626.

[92] C. García-Aljaro, L. N. Cella, D. J. Shirale, M. Park, F. J. Muñoz, M. V. Yates, A. Mulchandani, *Biosens. Bioelectron.* **2010**, *26*, 1437–1441.

[93] S. Farid, X. Meshik, M. Choi, S. Mukherjee, Y. Lan, D. Parikh, S. Poduri, U. Baterdene, C. E. Huang, Y. Y. Wang, P. Burke, M. Dutta, M. A. Stroscio, *Biosens. Bioelectron.* **2015**, *71*, 294–299.

[94] X. Wang, Y. Zhu, T. R. Olsen, N. Sun, W. Zhang, R. Pei, Q. Lin, *Electrochim. Acta* **2018**, *290*, 356–363.

[95] S. Mukherjee, X. Meshik, M. Choi, S. Farid, D. Datta, Y. Lan, S. Poduri, K. Sarkar, U. Baterdene, C. E. Huang, Y. Y. Wang, P. Burke, M. Dutta, M. A. Stroscio, *IEEE Trans. Nanobioscience* **2015**, *14*, 967–972.

[96] Z. Hao, Z. Wang, Y. Li, Y. Zhu, X. Wang, C. G. De Moraes, Y. Pan, X. Zhao, Q. Lin, *Nanoscale* **2018**, *10*, 21681–21688.

[97] Z. Wang, Z. Hao, X. Wang, C. Huang, Q. Lin, X. Zhao, Y. Pan, *Adv. Funct. Mater.* **2021**, *31*, 1–10.

[98] J. Janata, *ECS Solid State Lett.* **2012**, *1*, 29–32.

[99] A. Purwidyantri, T. Domingues, J. Borme, J. R. Guerreiro, A. Ipatov, C. M. Abreu, M. Martins, P. Alpuim, M. Prado, *Biosensors* **2021**, *11*, DOI 10.3390/bios11010024.

[100] K. Noi, A. Iwata, F. Kato, H. Ogi, *Anal. Chem.* **2019**, *91*, 9398–9402.

[101] A. Kowalczyk, J. P. Sęk, A. Kasprzak, M. Poplawska, I. P. Grudzinski, A. M. Nowicka, *Biosens. Bioelectron.* **2018**, *117*, 232–239.

[102] M. Jarczewska, R. Ziółkowski, Ł. Górski, E. Malinowska, *Electroanalysis* **2018**, *30*, 658–664.

[103] R. Schirhagl, P. A. Lieberzeit, F. L. Dickert, *Adv. Mater.* **2010**, *22*, 2078–2081.

[104] S. R. Hong, H. Do Jeong, S. Hong, *Talanta* **2010**, *82*, 899–903.

[105] T. Wangchareansak, A. Thitithanyanont, D. Chuakheaw, M. P. Gleeson, P. A. Lieberzeit, C. Sangma, *Medchemcomm* **2014**, *5*, 617–621.

[106] C. H. Lu, Y. Zhang, S. F. Tang, Z. Bin Fang, H. H. Yang, X. Chen, G. N. Chen, *Biosens. Bioelectron.* **2012**, *31*, 439–444.

[107] I. Karamollaoğlu, H. A. Öktem, M. Mutlu, *Biochem. Eng. J.* **2009**, *44*, 142–150.

[108] I. Mannelli, M. Minunni, S. Tombelli, M. Mascini, *Biosens. Bioelectron.* **2003**, *18*, 129–140.

[109] K. Zhang, L. Fu, L. Zhang, Z. Cheng, T. Huang, *Biotechnol. Bioeng.* **2014**, *111*, 2229–2238.

[110] S. Li, L. Fu, J. M. Barbaree, Z. Y. Cheng, *Sensors Actuators, B Chem.* **2009**, *137*, 692–699.

[111] M. L. Johnson, J. Wan, S. Huang, Z. Cheng, V. A. Petrenko, D. J. Kim, I. H. Chen, J. M. Barbaree, J. W. Hong, B. A. Chin, *Sensors Actuators, A Phys.* **2008**, *144*, 38–47.

[112] X. Guo, S. Gao, S. Sang, A. Jian, Q. Duan, J. Ji, W. Zhang, *Biosens. Bioelectron.* **2016**, *82*, 127–131.

[113] X. Guo, S. Sang, J. Guo, A. Jian, Q. Duan, J. Ji, Q. Zhang, W. Zhang, *Sci. Rep.* **2017**, *7*, 1–8.

[114] J. Wang, X. Guo, R. Liu, J. Guo, Y. Zhang, W. Zhang, S. Sang, *Nanotechnology* **2020**, *31*, DOI 10.1088/1361-6528/ab4506.

[115] S. Sang, Y. Li, X. Guo, B. Zhang, X. Xue, K. Zhuo, C. Zhao, W. Zhang, Z. Yuan, *Biosens. Bioelectron.* **2019**, *141*, 111399.

[116] X. Guo, R. Liu, H. Li, J. Wang, Z. Yuan, W. Zhang, S. Sang, *Sensors* **2020**, *20*, 5286.

[117] J. E. Greene, *J. Vac. Sci. Technol. A Vacuum, Surfaces, Film.* **2017**, *35*, 05C204.

Chapter 2 Bipolar electrode-based biosensors with multiple enzyme modifications

This chapter is currently in writing to be submitted as an article with the title "Investigation of an Oxidase-based Biosensor based on a Liquid Junction-less Closed Bipolar Electrode System". Therefore, only the chapter's summary is written here.

2.1 Summary

Bipolar electrodes are capable of simultaneous reduction and oxidation reactions on either electrode pole when an adequate potential is applied to it via driving electrodes. Using bipolar electrodes to connect multiple sample cells to one auxiliary (reference) cell, sufficient potential can be applied simultaneously to the driving electrode in the auxiliary cell and in each sample cell and thus, a BPE-based biosensor with multiplexing capability was fabricated.

The BPE sensor was fabricated using standard photolithography and metal sputtering techniques to deposit and shape the layout of the Ti/Pt/Au electrodes. An insulating layer was added to protect the connecting lines before enzyme modifications were carried out. After optimization, glucose oxidase was modified using glutaraldehyde and bovine serum albumin (BSA) crosslinking method, lactate oxidase with chitosan immobilization method, and uric acid oxidase with glutaraldehyde crosslinking method. A combination of these enzyme-modified detection electrodes on one BPE device with 8 sample cells allowed for the multiplexed and simultaneous detection of two of the three metabolites, glucose, lactate and uric acid. These metabolites were chosen since they are all important biomarkers for various diseases, making them highly relevant for health monitoring.

An interesting phenomenon was observed during enzymatic measurements using the BPE device where the supposed oxidation peak for the redox mediator, ferrocenemethanol was

observed at a more negative potential. Further investigation of the sensing mechanism of the enzyme modified BPE device revealed that the dissolved oxygen concentration in the sample cell had decreased due to oxidase enzymatic reaction. Therefore, to power the oxidation of ferrocenemethanol, the next redox candidate, hydrogen peroxide, was reduced at the driving electrode in the sample cell instead, thus inducing the negative potential shift for ferrocenemethanol oxidation. The effect is negligible when the appropriate potential range was used. Therefore, this phenomenon should also be considered when setting the measurement parameters for BPE-based biosensors.

Chapter 3 Ion-sensitive field-effect transistor (ISFET)-based biosensor with aptamer modification

This chapter is currently under review to be submitted as an article with the title "C-reactive protein detection using an ion-sensitive field-effect transistor (ISFET)-based aptasensor with a chemically modified gate surface for higher sensitivity". Therefore, only the chapter's summary is written here.

3.1 Summary

C-reactive protein (CRP) is a well-established biomarker for inflammation in the body and CRP levels in blood have been correlated with cardiovascular risk (<1 μg/mL as low risk, 1—3 μg/mL as medium risk and >3 μg/mL as high risk). Its short half-life means that it is necessary to have real-time and long-term monitoring of CRP level in patients for accurate diagnosis. Field-effect transistor (FET)-based biosensors are useful in point-of-care (POC) settings for their ease of miniaturization, label-free measurement, fast response time and a mature fabrication process that enables mass manufacturing. One of the most used FET biosensors is the ion-sensitive FET (ISFET) and its mechanism is based on the potentiometric detection of changes in charge density at the (usually biofunctionalized) gate electrode, where the interactions between target and probe biomolecules occur. The change in charge density at the gate electrode induces the change in the channel conductivity between the source and drain which is then translated into electric signals by the ISFET. However, ISFET sensors have limitations such as the Debye screening effect and detection difficulties for low or neutral charged biomolecules. To tackle these limitations, the simple method of using commercial ISFET sensors and applying a silane modification step to the ISFET gate surface to reduce -OH group density and thus lower background signal noise was used. Silane optimization results showed that epoxysilane was more effective in reducing the overall pH

sensitivity compared to the commonly used aminosilane. Additionally, aptamers were immobilized onto the gate surface, providing significant surface potential change when they bind to CRP (that is only slightly negatively charged in pH 7.4 solution) on the ISFET gate surface. Thus, the ISFET aptasensor was able to measure $0 - 20$ µg/mL CRP in 1x PBS with a higher sensitivity compared to non-modified FET sensors with their original pH sensitivity. Furthermore, the FET aptasensor has the advantages of easy and low-cost fabrication, does not need nanomaterial modifications, expensive external equipment, or complicated materials to achieve its current sensitivity and measurement range. Future research and development with the combination of microneedle patch technology can pave the path for a wearable POC CRP real-time monitoring device.

Chapter 4 Gravimetric-based biosensor with antibody modification

This chapter is currently under review to be submitted as an article with the title "Self-Powered Batteryless Wireless Communication and Human Coronavirus Detection with Magnetostrictive Fe–Co/Ni Clad Plate". Therefore, only the chapter's summary is written here.

4.1 Summary

Gravimetric-based biosensors offer label-free measurement since the mass-sensitive biosensors produce a direct shift in resonant frequency as the mass of the biosensor increases from receptor-analyte interaction. The magnetoelastic sensor used in this chapter is a cantilever made of iron-cobalt/nickel. By applying a magnetic field to the cantilever, the magnetoelastic material will induce oscillation which is detected by a pickup coil as resonant frequency reading. The resonant frequency of the cantilever will decrease as the mass of analyte bound onto the cantilever increased. Using CD13 as a novel biorecognition layer for the human coronavirus 229-E (HCoV-229E), the viability of this capture method was first confirmed using fluorescent microscopy and aminosilane-treated glass slides (glutaraldehyde crosslinking method was used to immobilize CD13 onto the glass slide surface). Alexa Fluor 488-conjugated anti-His tag antibody was used as the fluorescent label, which then binds to the His-tagged CD13-modified glass slide surface. Steric hindrance from the virus will prevent the fluorescent label from binding, which means that a decrease in fluorescence signal indicate successful virus binding. Results obtained confirmed that the target capture method was viable for detection of HCoV-229E. The same experiment was carried out using the magnetoelastic FeCo/Ni cantilever (CD13 was immobilized using self-assembled monolayer of -COOH groups forming amide bonds with the -NH2 groups on CD13) and the feasibility of HCoV-229E sensing with a magnetoelastic biosensor was confirmed.

Chapter 5 Summary and conclusion

A biosensor is a device that integrates a biological receptor to an electronic transducer to produce signals that can be detected or analyzed. While biosensors have seen applications in various fields that include agriculture, environmental monitoring, food safety, and even

biodefense, the most notable field of interest for biosensors is the healthcare field (clinical diagnostics and health monitoring) as shown by the first biosensor developed for blood glucose detection. Furthermore, biosensors that are capable of point-of-care-testing (POCT) have played an important role in early disease detection, health monitoring and even in emergency departments where fast medical decisions need to be made, proving that they are increasingly vital to increase patients' survival rates and prevent infection outbreaks. They are also important in developed countries with an aging population and require consistent health monitoring to enable early diagnosis for early treatment of diseases, or in developing countries where the medical infrastructure may be sparse. To tackle the global need for POCT biosensors, my research approach is to engineer the design of miniaturized biosensors with optimized fabrication strategies ideal for POCT. This is while keeping in mind that the POCT biosensor must be able to provide rapid detection, with accurate and quantitative results and when possible, have easy-to-use systems that even a non-expert can operate the biosensor. The fabrication and modification strategies applied were also easy to scale up for mass-production. The research results obtained for each chapter are summarized below:

5.1 Chapter 1: Introduction

The background of this research regarding biosensors and development of point-of-care (POC) biosensors were described, including the fundamentals and examples of applications in various fields for bipolar electrode (BPE)-based, ion-sensitive field-effect transistor (ISFET)-based, and gravimetric-based biosensors. Short explanations were included for the types of

bioreceptors, device fabrication, modification, and evaluation methods. And lastly, the research strategies used in this thesis were emphasized.

5.2 Chapter 2: BPE-based biosensor with multiple enzyme modifications

Bipolar electrochemistry was applied to develop a biosensor capable of simultaneous and multiplexed detection of various metabolites useful for health monitoring and are biomarkers for many diseases, specifically glucose, lactate, and uric acid. Oxidase-type enzymes for each metabolite were modified onto the detection electrodes of the 8-cell BPE-based biosensor and simultaneous detection of glucose, lactate, and uric acid were obtained in the 0 – 10 mM range (0 – 1 mM for uric acid). An investigation of the oxidase-type enzyme-influenced biosensing mechanism was also carried out. It was found that potential-determining mechanism of the biosensor was influenced by the reaction on the driving electrode in the sample cell. Under the condition that an oxidase enzyme was modified onto a detection electrode that is the rate-limiting step, when dissolved oxygen concentration decreased due to the oxidase enzyme, this will affect the reaction that occurs on the driving electrode in the sample cell, which is what induced the appearance of the two current peaks. This is negligible when the potential range during measurement takes account of this occurrence, as confirmed by the multiplexed detection of the three metabolites that were taken at the same time. With further optimization, the BPE-based biosensor is expected to contribute to the medical and environmental monitoring fields in clinical diagnostic devices and other point-of-care devices.

5.3 Chapter 3: ISFET-based biosensor with aptamer modification

Ion-sensitive field-effect transistor (ISFET)-based biosensors are useful in POC settings for their ease of miniaturization, label-free measurement, fast response time and a mature fabrication process that enables mass manufacturing. However, ISFET sensors have limitations such as ion screening effect which limits signal detection to the Debye length of the electrolyte, and detection difficulties for low or neutral charged biomolecules. In this chapter, an easy surface modification method by gas-phase GPTMS silanization is carried out to improve a commercial ISFET sensor's detection capability, by decreasing the pH sensitivity of the gate material which then decreases background signals, thus overcoming the Debye screening effect. Then C-reactive protein (CRP)-binding aptamers are used as the biorecognition layer which provide additional signal amplification. CRP is a vital biomarker for inflammation and tissue damage in the body (concentrations of less than 1 μg/mL as low risk, 1—3 μg/mL as medium risk and more than 3 μg/mL as high risk of cardiovascular diseases). Using the modified ISFET, a CRP biosensor capable of sensitive detection of CRP (0 — 20 μg/mL) in 1x PBS solution (physiological ionic strength) with a higher signal sensitivity compared to other (modified or non-modified) FET sensors. Furthermore, the ISFET-based aptamer-modified biosensor did not need expensive nanomaterial modifications or complicated nanostructure fabrication to achieve its current sensitivity and measurement range, compared to other FET sensors for CRP detection.

5.4 Chapter 4: Gravimetric-based biosensor with antibody modification

Airborne transmission of pathogens from the environment to human lungs can cause significant adverse effects such as allergic reactions, infections, or even serious respiratory diseases. Inhalation of pathogens through the lung is more susceptible for infection such as

influenza virus (influenza A, B, or C), coronaviruses (severe acute respiratory syndrome), norovirus (vomiting and diarrhea), rubeola (measles), or varicella-zoster virus (chickenpox). Therefore, early onsite detection of these airborne pathogens such as viruses are vital for preventing infection outbreaks and for early treatment of patients. Gravimetric, or mass-sensitive, devices are an attractive alternative for point-of-care (POC) biosensors because they provide label-free detection of any analyte since mass is a fundamental property of any analyte and have the potential for both gas-phase and liquid-phase detection. The magnetostrictive cantilever-type biosensor has the advantages of cheaper fabrication cost, sturdier durability, simpler configurations, and the capability for energy-harvesting allowing the potential of a self-powered biosensor. In this chapter, a gravimetric-based biosensor modified with a CD13, a known receptor for the human coronavirus 229-E (HCoV-229E) was used in a novel method to detect HCoV-229E. After obtaining results that confirmed the feasibility of using CD13 as a bioreceptor for detecting HCoV-229E using fluorescent microscopy and a pseudo-competitive binding assay method, it was then applied to the magnetostrictive FeCo/Ni cantilever for magnetostrictive measurement. The results showed that the novel biorecognition method of CD13 using the magnetostrictive cantilever biosensor was viable. With further optimization and technology development, a point-of-care magnetostrictive airborne virus biosensor that is simple to use, cheap and easy to produce, with good durability and even capable of energy harvesting can be expected in the future.

5.5 Conclusion

To conclude, in order to address the demand for POC biosensors in the clinical diagnostics field and health monitoring fields, bipolar electrochemistry, FET technology and gravimetry were applied to develop miniaturized biosensors with optimized fabrication

strategies. The fabricated biosensors were then used for multiplexed and simultaneous sensing of various metabolites, for sensitive detection of an inflammation biosensor in physiological ionic strength solution, and for the novel detection method of HCoV-229E using CD13 as the bioreceptor layer. These miniaturized biosensors also have relatively uncomplicated operation with simple modification procedures that can be easily scaled-up for mass-fabrication. Future application as POC biosensors in the clinical diagnostics and health monitoring fields can be expected.

Acknowledgements

Alhamdulillah, praise to God for this accomplishment. Truly it would not have been possible without God's will and the constant prayers I have received from so many people.

Firstly, I would like to express my sincerest gratitude to Professor Shiku Hitoshi, whose expertise, motivation, enthusiasm, and wide scientific knowledge have enriched my student life. It has been a great honor to be able to enter his laboratory and receive his continuous support and teachings throughout my PhD journey. I would also like to express my deepest appreciation to my graduate committee members, Professor Iki Nobuhiko and Professor Nakayama Toru, for having accepted to be the referees of the thesis and for their invaluable input throughout.

I would also like to thank Professor Inoue Y. Kumi, whose guidance and research methodology has tremendously helped with my research and beyond. Her experience and dedication in her fields of study will always an inspiration to me. Her patience, motivation, and guidance throughout the six years I was under her tutelage, from an undergraduate to a postgraduate student, were priceless. I attribute much of my researching capabilities to her.

I am also deeply indebted to Professor Esashi Masayoshi, Professor Narita Fumio, Professor Osana Shion, and Professor Kurita Hiroki for their invaluable input and teachings regarding field-effect transistors, gravimetric biosensors, and virus experimental know-how. From zero knowledge in these new fields, I was able to learn from the very basic to being able to complete this thesis.

I am grateful to Professor Ino Kosuke, Professor Nashimoto Yuji for the many discussions and helpful advice that gave me insight to parts of my research. Their insight have been highly educational.

I would like to express my gratitude and appreciation of the financial support from Mitsubishi Gas Chemical Memorial Foundation and Tohoku University Global Hagi Scholarship for Doctoral Students. Without their support, I would not have been able to finish my studies.

I would like to thank the many members of the Shiku laboratory, from my colleagues (Iwama Tomoki, Taira Noriko, Hiramoto Kaoru), to my juniors that are too many to list down, including those that have graduated and will graduate for their warm friendship, and to the secretary Ms. Takano Satomi for her invaluable clerical support.

Special thanks to my cats, my family and in-laws, my friends from different nationalities, and fellow Malaysians living in Japan. For their words of encouragement that provided me with emotional support, their delicious food that gave me physical sustenance and their advice that kept me motivated. And finally, I would like to thank my husband, Muhamad Arif Ihsan, for always supporting me with warmth and affection, and a constant supply of sweet snacks.