Electrical bio-sensing based on non-antibody recognition elements

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ELECTRICAL BIO-SENSING BASED ON NON-ANTIBODY RECOGNITION ELEMENTS

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2016

**ELECTRICAL BIO-SENSING APPROACHES BASED ON
NON-ANTIBODY RECOGNITION ELEMENTS**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological University
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Statement of Originality

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

17/5/2017

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Date

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Abstract

This study focuses on monitoring of sports fatigue related biomarkers using electrical assays (i.e., field-effect transistor sensing and tunable resistive pulse sensing) that are based on non-antibody recognition elements. The main hypothesis is that assays based on synthetic receptors can deliver sensing performances comparable to that of conventional antibody based ones, while providing advantages such as excellent stability in harsh environments, facile synthesis process, and small molecular sizes. Three specific hypotheses have been proposed. First, it is proposed that sports fatigue related biomarkers can be measured quantitatively in real time using carbon nanomaterial based FET sensors. Second, it is proposed that synthetic receptors such as liposomes and polypeptides can be used in combination with carbon nanomaterial based FET sensors for the monitoring of sports fatigue related biomarkers. Third, it is proposed that aptamers can be used in combination with tunable resistive pulse sensing for bio-sensing applications.

In the first part, detection of interleukin-6, which is a typical sports fatigue related biomarker, using a portable field-effect transistor biosensor is demonstrated. This sensing assay exhibits superior sensitivity ($\text{LOD} = 1.37 \text{ pg/mL}$) in virtue of the reduced tube-to-tube contact resistance, good selectivity as a result of the highly specific interaction between interleukin-6 and its receptor, and excellent stability in virtue of the strong adhesion of CNT to the quartz substrate and good horizontal alignment of these tubes. This section suggests that sports fatigue related biomarkers can be measured quantitatively in real time using carbon nanomaterial based FET sensors.

In the second part, detections of two different analytes (phospholipase A_2 and matrilysin) using field-effect transistor biosensors based on non-antibody recognition elements are demonstrated, indicating that it is viable to combine the field-effect transistor sensing platform with non-antibody recognition elements. Protein detection

using rGO-based FET sensor with liposomes or polypeptides as recognition element is demonstrated to be viable and the sensing performance is comparable (in terms of sensitivity and specificity) with assays using antibodies as recognition element.

In the third part, an aptamer sensor using a Dipstick and Tunable Resistive Pulse Sensing for rapid and label free detection of human cardiac troponin I is proposed. The cTnI aptamers were immobilized on a slide (used as a dipstick), which enabled the immobilization of nanoparticles modified with partially complementary sequences. The presence of the target protein caused a conformational change to the aptamer and the release of immobilized nanoparticles into solution. The concentration of nanoparticles released was proportional to the concentration of the target protein. Optimization of the partially complementary sequence produces an assay that was reliable and easy to setup with a LOD of 6.7 ng/mL in buffer. The outcome of this part verifies the hypothesis that aptamers can be used in combination with tunable resistive pulse sensing for bio-sensing applications.

The results obtained demonstrated all three hypotheses proposed in the beginning. Hence, monitoring of sports fatigue related biomarkers using electrical assays (i.e., field-effect transistor sensing and tunable resistive pulse sensing) that are based on non-antibody recognition elements is demonstrated.

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journey a worthwhile one. Also, to expand the horizon of knowledge brings me joy and sense of achievements that can never be measured by money.

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Abbreviations

AChE	Acetylcholinesterase
AFM	Atomic Force Microscope
APTES	(3-Aminopropyl)Triethoxysilane
AptaTRPS	Aptamer Based Tunable Resistive Pulse Sensing
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
CNTs	Carbon Nanotubes
CRP	C-Reactive Protein
cTnI	Human Cardiac Troponin I
CVD	Chemical Vapor Deposition
D-AptaTRPS	Aptamer Sensor Based on Dipstick and Tunable Resistive Pulse Sensing
DCM	Dichloromethane
DIPEA	Diisopropylethylamine
DLS	Dynamic Light Scattering
DMF	Dimethylformamide
DNA	Deoxyribonucleic Acid
<i>E. coli</i>	<i>Escherichia Coli</i>
EIS	Electrochemical Impedance Spectroscopy
ELISA	Enzyme-Linked Immunosorbent Assay
eNPs	Polymeric Expansile Nanoparticles
Fab' units	Antibody Fragment-Antigen Binding Units
FESEM	Field Emission Scanning Electron Microscope
FET	Field-Effect Transistor
Fmoc	Fluorenylmethoxycarbonyl
FRET	Förster Resonance Energy Transfer
GO	Graphene Oxide
GOx	Glucose Oxidase
HIV-1 Tat protein	Human Immunodeficiency Virus Type 1 Trans-Activator Transcription
HPLC	High Performance Liquid Chromatography

I _d	Source-Drain Current
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL-6	Interleukin-6
IL-6R	Interleukin-6 Antibody
LOD	Limit of Detection
MMP-1	Matrix Metalloproteinase 1
MMP-7	Matrilysin
MOSFET	Metal–Oxide–Semiconductor Field-Effect Transistor
MWCNTs	Multi-walled Carbon Nanotubes
PBS	Phosphate-buffered Saline
PBSE	1-Pyrenebutanoic Acid Succinimidyl Ester
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PDMS	Polydimethylsiloxane
PEG	Polyethylene Glycol
PLA ₂	Phospholipase A ₂
PNPG	4-Nitrophenyl β-D-Glucuronide
POPC	1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphatidylcholine
PSA	Prostate Specific Antigen
rGO	Reduced Graphene Oxide
RPS	Resistive Pulse Sensing
scFv	Single-Chain Fv Fragments
SELEX	Systematic Evolution of Ligands by Exponential Enrichment
SEM	Scanning Electron Microscope
SPB	Superparamagnetic Bead
SPR	Surface Plasmon Resonance
ssDNA	Single-Stranded DNA
SWCNTs	Single-Walled Carbon Nanotubes
TBTU	Tetramethyluronium Tetrafluoroborate
TEM	Transmission Electron Microscopy

TFA	Trifluoroacetic Acid
TNP	2,4,6-Trinitrophenol
TRPS	Tunable Resistive Pulse Sensing
V_d	Source-Drain Voltage
V_g	Gate Voltage
VEGF	Vascular Endothelial Growth Factor

Chapter 1

Introduction

This chapter serves as a preview of the thesis. Three hypotheses (1. Sports fatigue related biomarkers can be measured quantitatively in real time using carbon nanomaterial based FET sensors; 2. Synthetic receptors such as liposomes and polypeptides can be used in combination with carbon nanomaterial based FET sensors for the monitoring of sports fatigue related biomarkers; 3. Aptamers can be used in combination with tunable resistive pulse sensing for bio-sensing applications) were proposed, the motivation and objectives were described, and an overview of all projects involved in this dissertation was presented. Also, findings and outcomes from the research projects achieved were summarized and presented in alignment with the hypotheses proposed.

1.1 Hypothesis

This study focuses on monitoring of sports fatigue related biomarkers using electrical assays (i.e., field-effect transistor sensing and tunable resistive pulse sensing) that are based on non-antibody recognition elements. The main hypothesis is that assays based on synthetic receptors can deliver sensing performances comparable to that of conventional antibody based ones, while providing advantages such as excellent stability in harsh environments, facile synthesis process, and small molecular sizes. Three specific hypotheses are proposed, as follows:

1.1.1 Hypothesis 1 - Sports fatigue related biomarkers can be measured quantitatively in real time using carbon nanomaterial based FET sensors.

1.1.2 Hypothesis 2 - Synthetic receptors such as liposomes and polypeptides can be used in combination with carbon nanomaterial based FET sensors for the monitoring of sports fatigue related biomarkers.

1.1.3 Hypothesis 3 - Aptamers can be used in combination with tunable resistive pulse sensing for bio-sensing applications.

1.2 Objectives and Scope

The ultimate objective is the development of miniaturized biosensors based on non-antibody recognition elements for detections of sports fatigue related biomarkers (e.g., interleukin-6). More specifically, carbon nanomaterials are deployed as the material for the transducing layer in field-effect transistor sensors; the functionality of carbon nanomaterials as the transducing layer is verified by interleukin-6 detections. In virtue of their advantages in chemical stability, synthesis, and molecular size, liposomes and polypeptides are used instead of antibodies for detection of different analytes. Eventually, biomarker detection using aptamer-based tunable resistive pulse sensing is investigated.

1.3 Dissertation Overview

The thesis addresses biomarker detection using carbon nanomaterials based field-effect transistor sensors with non-antibody recognition elements. Multiplexed detection of biomarkers using aptamer-based tunable resistive pulse sensing is also investigated.

Chapter 1 provides a rationale for the research and outlines the goals and scope. Hypotheses are proposed and motivations and objectives are clarified. Also, an overview of the thesis (alternatively a summary of the contents of all chapters included in this thesis) is presented.

Chapter 2 reviews the literature concerning working principles of biosensors, properties of carbon nanomaterials and their applications in bio-sensing, and synthetic molecules as the recognition elements of biosensors. Firstly, the basic elements and working principles of biosensors are described. Then, applications of carbon nanomaterials (i.e., carbon nanotube and reduced graphene oxide) in bio-sensing are discussed. After that, non-antibody recognition elements that have been used in sensing assays are introduced and a rationale of synthetic molecules as recognition element is given.

Chapter 3 discusses the experimental procedures of sample preparation, sample characterization, device fabrication and kinetic measurement. The principles underlying the characterization techniques employed and the methods of data analysis are discussed. Also, equipment and instruments used in this study are elaborated. Briefly, preparations of samples used in different projects are clarified in the first part, followed by an introduction of techniques used for sample characterization. Device fabrications are then described, as well as kinetic measurements.

Chapter 4 elaborates detection of interleukin-6, a key sports related biomarker, using a FET sensor based on horizontally aligned CNT as a demonstration of quantitative and real-time detection of sports fatigue related biomarkers using carbon nanomaterials based sensors.

Chapter 5 introduces detection of Phospholipase A₂ (PLA₂) using rGO-based FET sensor with liposomes as the recognition element. The results demonstrated that protein detection using rGO-based FET sensor with liposomes as the recognition element was viable and the sensing performance was comparable with assays with antibodies as the recognition element in terms of sensitivity and specificity.

Chapter 6 presents detection of matrilysin (MMP-7) using rGO-based FET sensor with polypeptides as the recognition element. The results revealed that protein detection using rGO-based FET sensor with liposomes as the recognition element was viable and the sensing performance was comparable with assays with antibodies as the recognition element in terms of sensitivity and specificity. Chapter 5 and 6 indicated that protein detections can be achieved by rGO-based FET sensor in combination with different synthetic receptors.

Chapter 7 proposes an aptamer sensor using a Dipstick and Tunable Resistive Pulse Sensing (D-AptaTRPS) for quantitative protein. Detection of cTnI, which is a key myocardial infarction biomarker, in buffer and human serum were demonstrated, indicating great potential of the proposed assay for clinical applications.

Chapter 8 focuses on preliminary observations that are not included in Chapter 4 and outstanding problems for future studies.

1.4 Findings and Outcomes

This research has led to the following findings and outcomes:

1.4.1 Development of a FET Sensor Based on Horizontally Aligned CNT for Detection of Sports Fatigue Related Biomarkers

In the first part, an FET biosensor based on horizontally aligned CNT for detection of IL-6, a key sports fatigue biomarker was developed. IL-6R, the antibody to IL-6, was used as the recognition element. Quantitative detection of IL-6 was achieved using the proposed sensor, demonstrating that bio-markers related to sport and exercise science can be measured using carbon nanomaterials based FET sensors in a facile and sensitive way. Additionally, excellent long-term robustness of the sensor proposed has been demonstrated, making this sensor suitable for *in situ* measurements. This outcome verifies the hypothesis that sports fatigue related biomarkers can be measured quantitatively in real time using carbon nanomaterial based FET sensors.

1.4.2 Demonstration of Protein Detection Using rGO Based Sensors using Liposomes and Polypeptides as Recognition Elements

In the second part, an rGO based FET sensor using reporter-encapsulated liposomes as the recognition element is proposed for enzymatic sensing. Detection of PLA₂ was achieved by the proposed sensor via real time monitoring of I_d responses of the rGO-FET upon rupture of reporter-encapsulated POPC liposomes triggered by the PLA₂. 2,4,6-trinitrophenol (TNP) is used as the reporter. It was observed that the change in I_d is proportional to analyte concentration. The limit-of-detection (LOD) achieved was much lower than the circulating level of PLA₂ and superior to that of most assays reported elsewhere. Besides the liposome based sensor, an rGO based FET sensor using *de novo* designed synthetic polypeptide as the recognition element is proposed for rapid and sensitive detection of matrilysin. The hydrolysis of JR2RC facilitated by MMP-7 leads to reduction of size and net charge (from -5 to -1, as shown in Figure 6.2) of the polypeptide. As a result, I_d of the rGO-FET device decreased drastically and the change in I_d could be readily detected in real time. It was observed that the change in I_d is dependent on the concentration of MMP-7, thus enabling a quantitative monitoring of the concentration of this protease. Therefore, the outcome of this part demonstrates that synthetic receptors can be used in combination with carbon nanomaterial based FET sensors for bio-sensing applications.

1.4.3 Demonstration of Protein Detection Using D-AptaTRPS Sensors using Aptamers as Recognition Element

In the third part, an aptamer sensor using a Dipstick and Tunable Resistive Pulse Sensing (D-AptaTRPS) for rapid and label free detection of human cardiac troponin I (cTnI), a myocardial infarction biomarker, is proposed. The cTnI aptamers were immobilized on a slide (used as a dipstick), which enabled the immobilization of nanoparticles modified with partially complementary sequences. The presence of the target protein caused a conformational change to the aptamer and the release of immobilized nanoparticles into solution. The concentration of nanoparticles released was proportional to the concentration of the target protein. Optimization of the partially complementary sequence produces an assay that was reliable and easy to setup with a LOD of 6.7 ng/mL in buffer. More importantly, quantitative detection of cTnI in serum using the proposed assay has also been demonstrated and the excellent sensitivity makes the proposed assay highly promising for clinical applications. The outcome of this part verifies the hypothesis that aptamers can be used in combination with tunable resistive pulse sensing for bio-sensing applications.

Chapter 2

Literature Review *

This chapter introduces the fundamental principles of biosensors and describes some of the remarkable works related to this study. Two electrical sensing approaches field-effect transistor (FET) sensors and tunable resistive pulse sensing (TRPS) assays, both of which are based on electrical sensing approaches, are focused on as both were employed in the research projects involved in this study. The sensing mechanisms of FET and TRP are discussed and characteristics of non-antibody receptors (liposome, peptide and aptamer) used in this study are described. In FET sensors, binding of electrically charged molecules/ions to receptors on the transducing layer triggers changes of I_d , which is a detectable signal; in TRP sensors, molecules passing through the nanopore triggers blockades whose parameters are dependent on the size, concentration, and charge of these molecules. Questions to be answered (1. Are carbon nanomaterial based FET biosensors applicable to detection of sports fatigue related biomarkers? 2. Can antibodies in biosensors be substituted by synthetic molecules) were proposed and Ph.D in context of literature was clarified.

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2.1 Overview

As a discipline that focuses on how a healthy human body works during exercises [1], sport and exercise science has attracted increasing attention due to the drastically increasing interest within the sporting world on achieving optimized results and popularity of physical exercises among the public. To date, various issues in sport and exercise science have been investigated and discussed. Particularly, considerable efforts have been made to minimize the over-training issue, which has been demonstrated to contribute to injuries and fatigue [2]. To achieve that, real time and *in situ* monitoring of athlete body using a portable and sensitive biosensor is required to facilitate the decisions of coaches and consultants.

A biosensor is defined as an analytical device used for detection of various biological analyte, including glucose, DNA, protein, etc. [3]. The exquisite sensitivity and specificity of biological interactions is utilized in conjunction with physicochemical transducers to deliver bioanalytical measurements [4]. First elucidated by Leyland Clark in 1962 [5], biosensors have been intensively investigated and applied in a variety of fields, including environmental monitoring [6, 7], food safety [8, 9], and healthcare [10-12]. As shown in Figure 2.1a, publications related to biosensor in this field have exponentially increased over the past decade due to intensive studies in this field. On the other hand, the biosensor market is also rapidly increasing. Indeed, the global market value of commercial biosensors exceeded 13 billion US dollars in 2014 [13] and is estimated to reach 22 billion US dollars by 2020 [14], indicating an urgent demand of further studies related to biosensors to facilitate their commercialization.

Generally, a biosensor consists of three major components [15]: (1) receptor, a sensitive biological element that can selectively interact with the target analyte; (2) transducer, a detecting element which transforms the interaction of the analyte with the biological element into signals that can be easily measured and quantified; (3) reader, a device that can display the results in a user-friendly way. The working principle of biosensors is as shown in Figure 2.1b. Briefly, the bio-receptor recognizes and interacts with target

analyte, generating certain kinds of signals that can be captured and translated into measurable signals by the transducer. Then, signals from the transducer is processed and converted to discernible results by the reader [16]. In the sensing process, the specific interaction between receptor and the target analyte is considered as the key step as it has a dominating effect on the sensitivity and selectivity of biosensors in most cases.

Figure 0.1 (a) Searching results of the term “biosensor” on Google Scholar (2005-2014). (b) Working principles of biosensors: binding of target analyte with bio-receptors resulted in signals that can be captured by the transducer, and these signals were converted to discernible results that can be shown on the reader in a user-friendly way.

To date, receptor-analyte interactions used in biosensors include antibody/antigen interactions, enzymes interactions, nucleic acids/DNA interactions, cellular structures/cells interactions, and biomimetic materials interactions [17]. Additionally, the detection process by the transducer is also a key factor for the device performance.

In this chapter, biosensor was discussed based on the transducer (field-effect transistor and tunable resistive pulse) used and bio-receptor (liposome, peptide, and aptamer) involved.

2.1.1 Transducer and Sensing Mechanism

Depending on its transducer and sensing mechanism, biosensors can be further categorized as electrical sensor, optical sensor, and magnetic sensor [18]. Among different sensors, electrical biosensors exhibit excellent sensitivity [19, 20], good reliability [21] and potential of miniaturization [22]. Therefore, electrical biosensors have been regarded as ideal candidate for *in situ* measurements in sport and exercise science. Two electrical sensing approaches, including field-effect transistor (FET) based sensing and tunable resistive pulse sensing (TRPS), have been investigated in this study.

2.1.1.1 Field-Effect Transistor Sensing

FET is a device that achieves the control of channel conductivity within a semiconductor material by an electric field. As shown in Figure 2.2, it consists of three terminals: source, drain, and gate. With a constant source-drain voltage (V_d), the current from source to drain (I_d) is modulated by the voltage applied on gate (V_g). FET biosensor is a structural analog of a metal–oxide–semiconductor field-effect transistor (MOSFET) that has been widely used to detect molecules and atoms in the electrolyte.

In the sensing process, a constant potential difference is established between the source electrode and the drain electrode to generate a detectable I_d . Without alterations to its surface, the conductance of the transducer layer stays constant and I_d is determined by V_g

only. In the presence of target analyte (e.g., a ligand) binding in the gate region, the equivalent V_g varied and a change in the conductance of the transducer layer is observed, resulting in a change in I_d . This change, if detectable, is regarded as an electrical signal. Therefore, it is believed that the sensing performance is dominated by the transducer in most cases.

Figure 0.2 Schematic illustration of FET biosensor. Binding of target analyte in gate region causes a change in the conductance of the transducer layer, which is indicated by a change in I_d .

Among various transducer materials proposed, carbon nanomaterials are regarded as promising candidates for. Carbon nanomaterials have attracted great attention in the past 60 years due to three distinctive advantages over conventional macroscopic materials [23]. The first advantage is the unprecedented electronic properties. Appropriately prepared carbon nanomaterials exhibit ballistic transport with extremely high electron

mobility [24]. The second advantage is the ultra-large surface area due to their single-atom thickness. This feature is of great significance for sensing as molecular bindings can only take place on the surface. The third advantage is the excellent biocompatibility [25], which makes them excellent candidates for implantable biosensors. Among various carbon nanomaterials, carbon nanotubes (CNTs) and graphene are most widely applied in FET biosensing, in virtue of their ultrahigh conductivity, cost-effectiveness. In this study, CNT and reduced graphene oxide (rGO, an analog of graphene) have been employed as transducers in FET biosensors.

Carbon Nanotubes as the Transducer

Carbon nanotubes are defined as well-ordered, hollow graphitic nanomaterials made of sp^2 -hybridized carbon atoms [23]. Due to their unique structures, CNTs possess distinctive electrical and physical characteristics, such as ultrahigh length-to-diameter ratio (up to 13,000,000:1) [26] and superior carrier mobility [27]. CNTs can be classified as single-walled carbon nanotubes (SWCNT) or multi-walled carbon nanotubes (MWCNT). Discovered by Iijima in 1993 [28], SWCNT are described as cylindrical tubes seamlessly wrapped by single sheets of graphene. The chirality plays a key role in determining various properties of nanotubes. For instance, the bandgap varies from zero to ~ 2 eV and the electrical conductivity can be either metallic or semiconducting, depending on the chirality [29]. MWNT consists of several graphene cylinders that are concentrically nested like rings of a tree trunk [29] with an interlayer spacing of 3.4 Å and a diameter typically on the order of 10–20 nm [30].

To date, CNTs have emerged as a promising class of materials for biosensing applications and real time detections of various biomolecules [31], chemical species [32], and gas molecules [6] using functionalized CNT-based transistors have been reported. However, random networks of SWCNT were employed in most cases, resulting in degraded sensing performances compared with those incorporated individual tubes. This can be attributed to inherent resistances at the tube/tube contacts in the networks [33]. In order to improve the sensing performance, various configurations have been proposed for

CNT-based FET sensors. In 2002, Joselevich *et al.* reported the horizontal alignment for single-wall carbon nanotubes [34]. As shown in Figure 2.3a, horizontally aligned were grown on the surface by patterning of catalyst nanoparticles and application of a local electric field parallel to the substrate. More specifically, the origin of nanotube growth was defined by the nanoparticle patterns and the direction of nanotube growth was determined by the electric field applied. Then, Kocabas *et al.* proposed a thin-film transistor with horizontally aligned arrays of SWCNT as the semiconductors [33]. The large-scale, horizontally aligned arrays of SWCNT were synthesized on single-crystal quartz substrates by chemical vapor deposition (CVD), as shown in Figure 2.3b. The SWCNT synthesis was facile and cost-effective, while the transistor proposed was demonstrated to be a high-performance one. Therefore, the transistor proposed exhibited a great potential in sensing applications. In virtue of the synthesis routes reported, horizontally aligned SWCNT have been applied in various sensors. For instance, Palaniappan *et al.* introduced a liquid-gated electrical biosensor based on horizontally aligned SWCNT on quartz substrate [20], as shown in Figure 2.3c. The SWCNT were synthesized by CVD in a facile and effective way. Detection of prostate specific antigen (PSA, a potential biomarker of prostate cancer [35]) using the biosensor proposed is demonstrated. Herein, the specific binding of PSA with the immobilized antibody led to a detectable change in I_d , and the LOD achieved was 30 pM, which is within the clinically relevant concentration regimes. Recently, Palaniappan *et al.* reported a FET sensor in which the horizontal alignment of CNTs was further improved by patterning the catalyst nanoparticles using a stripe-patterned polydimethylsiloxane (PDMS) stamp [19]. Direct electronic detection of epsilon toxin was demonstrated using this approach. In virtue of the perfect alignment of SWCNT, the sensing performance of FET sensor was significantly improved due to the reduced resistance of SWCNT as a semiconductor (as shown in Figure 2.3d) and the sensitivity achieved was comparable to enzyme-linked immunosorbent assay (ELISA).

In summary, CNTs, especially horizontally aligned SWCNT, have been intensively studied and applied in FET biosensors due to their superior mechanical, electrical, and physical characteristics. Continuous improvements in the sensing performance (e.g.,

LOD) have been observed for CNT-based FET biosensors. Therefore, FET based on horizontally aligned SWCNT was employed as the sensing platform for detection of interleukin-6 (IL-6), a key bio-marker related to over-training issues, in the first project.

Figure 0.3 (a) Growth of horizontally aligned SWCNT from patterned catalyst nanoparticles [34]. (b) SEM images of horizontally aligned SWCNT on quartz substrates [33]. (c) Liquid-gated biosensor based on horizontally aligned SWCNT on quartz substrate [20]. (d) Performances (I_d vs. V_g) of FET sensors based on SWCNT with perfect alignment, partial alignment, and random network structure [19].

Reduced Graphene Oxide as the Transducer

As a 2D carbon nanomaterial whose existence was predicted in the 1960s, graphene had never been successfully isolated until the milestone work reported in 2004 [36]. This material consists of hexagonal lattices made of carbon atoms (see Figure 2.4) and it serves as basic structural element of other carbon allotropes (e.g., graphite and CNTs). Despite its relatively short history, graphene has attracted significant attentions, owing to

its extraordinary physiochemical properties, including high mechanical strength [37], superior carrier mobility (ballistic transport) [38], ultra-large specific surface area [39], and good transparency [40]. Indeed, over 2 billion US dollars have been invested to investigate graphene [41]. Nevertheless, the semi-metal nature of pristine graphene indicates that no band gaps are expected in the electronic structure of this material. Hence, modifications are needed for graphene to be used in applications such as electronics and sensing.

Figure 0.4 (a) Schematic illustration of the atomic structure of graphene: graphene consists of hexagonal lattices made of carbon atoms. (b) Electronic structure of graphene [42]: the conduction band and the valence band meet at the Dirac point, resulting in no band gaps.

Currently, post-processing (e.g., lateral confinement and strain engineering) is commonly used to create a band gap in graphene [43]. Among various approaches proposed, utilization of graphene oxide (GO) has attracted particular attention due to the facile, solution-based, and cost-effective synthesis routes available. GO is a soft matter with non-stoichiometric molecular formula and large internal degree of freedom [44]. Hence, the chemical structure of GO has not been fully understood. However, the presence of epoxy groups, hydroxyl groups, and carboxyl groups in the chemical structure of GO have been demonstrated. The GO structural model (see Figure 2.5) proposed by Lerf *et al.* [45] suggested that the basic units of GO include aliphatic six-membered rings and un-oxidized benzene rings, and these two structures compete with each other. With subsequent modifications and improvements, this model has been widely recognized and applied.

Figure 0.5 GO structural model [45]. In this model, GO is a mixture of aliphatic six-membered rings and un-oxidized benzene rings, with epoxy and hydroxyl groups attached to the basal planes and carboxyl groups attached to the edges.

However, GO is limited by several intrinsic drawbacks. For instance, GO has been demonstrated to be an electrically insulating material in most cases (under ambient conditions). This can be attributed to the fact that ordered sp^2 conductive clusters isolated within sp^3 insulating matrix. Therefore, a reduction step, in which the sp^3 structures could be converted to sp^2 clusters, is usually required to restore its electrical conductivity, although a pure sp^2 structure is not achievable by this method. The electrical properties (e.g., on/off ratio and electrical conductivity) of rGO prepared in this way can be tuned by varying the degree of reduction (see Figure 2.6) to be used for different applications [46]. More specifically, mildly-reduced GO sheets exhibit high on/off ratio but low electrical conductivity as the internal electrical transport is dominated by tunneling and/or hopping between sp^2 clusters, both of which are highly dependent on the voltage applied. As the degree of reduction increased, oxygen moieties are removed and some of the π -bonds are restored. Hence, percolation pathways between existing sp^2 domains are generated. As a result, the on/off ratio decreases while the electrical conductivity increases.

Figure 0.6 Conductivity of thermally reduced rGO vs. the fraction of sp^2 carbon atoms [46]. Insert shows the structural models of GO at different stages. The upper triangle, the lower triangle and the square indicate the conductivity of doped graphene, intrinsic graphene and polycrystalline graphite, respectively. Two different regimes are observed for electrical transport of rGO. At sp^2 carbon fraction < 0.6 , tunneling and/or hopping plays a dominant role in the transport; at sp^2 carbon fraction > 0.6 , percolation dominates the electrical transport.

To date, FETs based on thermally or chemically reduced GO have been widely applied for detections of DNA hybridization and protein binding. For instance, Stine *et al.* [47] demonstrated the detection of ssDNA using DNA-functionalized rGO-FET with a LOD of 10 nM. Mao *et al.* [48] reported a FET rGO with AuNP-antibody conjugates on the surface for detection of protein binding events. In this assay, AuNPs were involved to facilitate aggregations of probe molecules in the gate area. The LOD achieved was on the order of ng/mL. He *et al.* [49] introduced an rGO-FET biosensor and demonstrated its

application in detection of hormonal catecholamine molecules and their dynamic secretion from living cells.

In summary, rGO, an analogue of graphene, has been widely utilized in biosensing applications in virtue of its superior physiochemical properties. The tunable electrical properties, biocompatibility and cost-effectiveness make rGO a versatile material. Hence, rGO-FET was employed as the sensing platform for protein detections in this study.

2.1.1.2 Tunable Resistive Pulse Sensing

Resistive pulse sensing (RPS) is a technique to measure the size, concentration and surface charge of particles individually within their natural environment. In this approach, two reservoirs, which are filled with electrolyte and each containing an electrode, are separated by a pore. Particles in the electrolyte passing through a micro-sized or nano-sized pore are detected individually as a transient change in the ionic current flow as the particle is displacing a certain volume of electrolyte, thus increasing the resistance. This change is defined as blockade event [50].

Firstly, the magnitude of the blockade event can be used to determine the analyte size. More specifically, the overall resistance change (ΔR) over the length of the pore (L) can be calculated by Equation 2.1:

$$\Delta R = \rho \int_0^L \frac{dz}{A(z)} - R_i \quad 0.1$$

where ρ is the resistivity of the electrolyte, $A(z)$ is the area of the cross section that is perpendicular to z , and R_i is the intrinsic resistance of the pore, as given by Equation 2.2:

$$R_i = \frac{4L\rho}{\pi D_L D_S} \quad 0.2$$

where D_L and D_S are the maximum and minimum pore diameters, respectively.

The diameter (d) and volume (v) of the particle are related to the pulse magnitude ($\Delta R/R_i$) by Equation 2.3 [51]:

$$\frac{\Delta R}{R_i} = f \frac{v}{V} S\left(\frac{d}{D}\right) \quad 0.3$$

where f is the shape factor, v and d are the volume and diameter of the particle, V and D are the volume and diameter of the pore.

On the other hand, the quantity of blockade events per unit time, namely the blockade frequency, provides information on the concentration of the analyte. More specifically, the concentration of particles (C) can be related to the blockade frequency (J) by the Nernst-Planck equation, as shown in Equation 2.4 [52]:

$$J = \frac{\pi v_s D^2}{4} C \quad 0.4$$

where v_s is the velocity of the particle going through the pore and D is the pore diameter.

Additionally, the zeta potential of the particle is directly related to its surface charge [53]. Zeta potential is a parameter indicating the magnitude of the electrostatic repulsion/attraction between particles. The Smoluchowski approach indicates that zeta potential (denoted as ζ) of a particle can be determined based on the convective forces and electro osmotic forces experienced, as well as its intrinsic electrophoretic mobility, as shown in Equation 2.5 [54]:

$$\zeta = \frac{\eta \mu}{\varepsilon} \quad 0.5$$

where η and ε are the dynamic viscosity and dielectric constant of the electrolyte, respectively, and μ is the mobility of particles.

In virtue of their user-friendly nature, measurement sensitivity and individual single-object readout capability, RPS has attracted increasing attention and various studies have

been reported in this field (see Figure 2.7). Indeed, resistive pulse sensors have been utilized to detect DNA, protein, cellular vesicles, nanoparticles, etc.. Resistive pulse sensors can be traced back to the Coulter counter proposed in the 1950s [55]. This primary platform was used for detection of biological cells and microorganisms. Kasianowicz *et al.* demonstrated ssDNA detection using a α -hemolysin pore [56]. This method exhibited the capability of discriminating slight differences in the size of the four DNA base pairs. Deblois *et al.* reported the detection and characterization of nanoparticles and viruses using a submicron resistive pulse sensor based on a synthetic pore, which shows advantages in bio-compatibility and chemical stability [57, 58]. In virtue of advances in nanofabrication, microfluidic sensor with pore arrays was proposed. Siwy *et al.* introduced a resistive pulse sensor whose pore surface was modified with biological capture probes for ricin detection [59].

Figure 0.7 The evolution of RPS development: from microparticle to single molecule detection [60]. Coulter counter refers to a fixed micropore used for measurement of the concentration and size of large particles; biological nanopore refers to a α -hemolysin pore; synthetics nanopore refer to manufactured pores of fixed size and shape; microfluidic sensors are characterized by multi-channel configuration and hydrodynamic focusing capability; elastic pore is a new generation pore whose size can be tuned to suit target analyte.

However, the fixed size of the pore has been a limiting factor as it narrows the size range of analyte that can be detected and degrades the measurement accuracy. To overcome

that, tunable resistive pulse sensors were developed. TRPS refers to the RPS techniques where the pore size can be tuned by real time stretching according to the size of target analyte so that the measurement accuracy is enhanced and the size range is improved. In virtue of these advantages, TRPS has been intensively studied and widely used for sensing applications.

Willmott *et al.* demonstrated the detection of micro-sized superparamagnetic bead (SPB) aggregation using resistive pulse sensing based on tunable pores on an elastomeric membrane (Figure 2.8a) [61]. Herein, aggregates induced a detectable change in the average magnitude of blockades. Platt *et al.* reported a TRPS approach based on thermoplastic polyurethane elastomeric membrane with a mechanically punctured tunable pore whose size can be altered by up to an order of magnitude (Figure 2.8b) [62]. In this report, aggregation of nanorods induced by analyte was monitored based on the observation that the resistive pulse signal of a rod was distinguishable from that of a sphere of similar sizes.

Figure 0.8 (a) The tunable pore in the central membrane [61]. (b) Schematic illustration of the tunable pore on a thermoplastic and elastomeric polyurethane membrane. The signals associated with nanorods are fundamentally different from those associated with nanospheres [62], thus enabling detection of nanorods.

Colby *et al.* monitored the swelling of polymeric expansile nanoparticles (eNPs) using various techniques, including TRPS, as shown in Figure 2.9 [63]. In a mildly acidic environment, these nanoparticles expanded by up to 10 times (in terms of diameter).

Expanded eNPs exhibited unique translocation behaviors as they could squeeze through a pore whose size is close to their diameters. This phenomenon, together with other differences in resistive pulse signals, enabled discrimination of expanded nanoparticles using TRPS.

Figure 0.9 Schematic illustration of TRPS detection of eNPs [63]. (a) At pH = 7.4, eNPs did not swell and can readily cross a large pore (resulting in a sharp drop in the current) but not a small pore. (b) At pH = 5.0, expanded eNPs could squeeze through a pore whose size is close to their diameters.

Figure 0.10 Schematic illustration of aptamer-protein interaction. Aptamers are immobilized on nanoparticles via streptavidin/biotin interaction, followed by the introduction of the target protein that causes a conformational change of the immobilized aptamer [64].

Figure 0.11 Schematic illustration of multiplexed protein detection using aptamers and tunable resistive pulse sensing (AptaTRPS). Nanoparticles of different sizes are functionalized with two different aptamers that recognize one target protein, respectively. Introduction of the target proteins results in conformational changes of the aptamers, which in turn affects the velocity and frequency of nanoparticles diffusing through the pore [65].

Billinge *et al.* demonstrated the monitoring of aptamer-protein (thrombin and its aptamers) interaction using TRPS (see Figure 2.10) [64]. More specifically, aptamers were immobilized on superparamagnetic beads and the aptamer-immobilized beads were

incubated with thrombin. The protein-binding caused a conformational change of the aptamer (shielding of the polyanion backbone), which in turn led to a change in translocation time and pulse frequency associated with the particles diffusing across the pore.

Nevertheless, approaches mentioned above focused on one specific protein only, while diagnostics require the monitoring of two or more proteins in most cases. To overcome this limitation and facilitate application of TRPS in diagnostics, Billinge *et al.* demonstrated simultaneous detection of two proteins in a one-step analysis using TRPS [65]. In this assay, incubation of hemostatic proteins vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) with two beads of different sizes (300 nm and 120 nm) that were functionalized with aptamers to VEGF and PDGF respectively triggered two signals that can be separated from each other. In this way, independent detections of VEGF and PDGF were achieved without any labels involved.

In summary, RPS is a technique that can measure size, concentration and surface charge of individual nanoparticles in their natural environment. Therefore, RPS has been widely used for sensing applications. TRPS exhibits further enhanced measurement accuracy and increased size range as the pore size can be tuned by real time stretching according to the size of target analyte. Hence, TRPS was used for the detection of cTnI.

2.1.2 Recognition Element

Designed to specifically interact with the target analyte to produce a signal measurable by the transducer, bio-receptor (also known as the sensing element) is one of the key elements of biosensors. Due to its dominant role in analyte recognition, bio-receptor has a determining effect on the sensitivity and specificity of the sensing assay [66]. Currently, various molecules (e.g., antibody, enzyme and nucleic acid) have been utilized as bio-receptors in sensing assays. In this section, bio-receptors that have been involved in projects of this study will be discussed.

2.1.2.1 Antibody

Antibodies are natural biomolecules with binding capabilities for specific molecules (known as ‘antigen’) [67]. In most cases, an antibody is a complex biomolecule consisting of hundreds of individual amino acids and can recognize the antigen by its specific geometrical configuration. In virtue of the superior binding affinity and specificity, antibody/antigen interactions have been widely applied in various sensing approaches. Among all antigen-based approaches reported, assays involving FET platforms have attracted intensive attentions. Figure 2.12a illustrates a typical antibody immobilized on a transducer layer. Binding of analyte with the immobilized antibody triggers changes in certain parameters associated with the transducer layer, resulting in measurable signals.

Figure 0.12 (a) Schematic illustration of a typical antibody immobilized on a transducer layer. The antibody can capture the target analyte, which binds at the binding sites. (b) Schematic illustration of CNT-FET modified with IgG antibodies [68].

Kim *et al.* reported the detection of immunoglobulin G (IgG) using FET sensors functionalized with antibodies or antibody fragments (Figure 2.12b) [68]. Due to their small sizes, antibody fragments enabled higher sensitivity. This assay was further improved by involving spacers to optimize the distance between receptors [69] so that the charged analyte molecules can approach the transducer layer within the Debye length. Afterwards, Palaniappan *et al.* used horizontally aligned CNTs in FET sensors and

demonstrated the measurement of PSA using antibody-functionalized FET sensors [20]. This assay was further improved by optimizing horizontally alignment of CNTs and used for detection of bio-toxins [19]. To facilitate the application of antibody based sensing assays in diagnostics, detection of biomarkers in serum has been studied. Hideshima *et al.* demonstrated the detection of tumor markers in serum using antibody-modified FET [70]. Surface passivation by bovine serum albumin (BSA) was involved to minimize non-specific binding of other molecules in the solution.

Nevertheless, several limitations have been observed for antibody/antigen interactions. Firstly, the functional structures of antibodies tend to be complicated and fragile. Hence, typical working environments of biosensors or long-term storage can easily trigger denaturation/deactivation of these molecules [71]. Meanwhile, the large molecular sizes of antibodies may significantly contribute to the steric hindrance effect on target molecules [72, 73] and limit the quantity of receptors immobilized on surface per unit area [74]. Also, the large molecular sizes of antibodies lead to high immunogenicity and low bioavailability [75]. Additionally, preparations of antibodies typically requires tedious processes and various resources, resulting in poor cost effectiveness of biosensors based on these approaches [76]. As a result, antibody based biosensors have encountered issues such as short shelf-life, limited stability and unacceptable reproducibility. Therefore, assays based on non-antibody receptors have been paid great attention.

2.1.2.2 Non-antibody Recognition Elements

Table 2.1 summarizes most recent achievements and advances in electronic biosensing based on different synthetic recognition elements, including liposomes, polypeptides, and aptamers.

Liposome

Liposomes are microscopic vesicles obtained by self-assembly and hydration of lipid thin film [77, 78]. These artificially-prepared vesicles typically exhibit a structure in which an

inner aqueous compartment is surrounded by a lipid bilayer (see Figure 2.13), resulting in hydrophilic interior and exterior.

Table 0.1 A comparison of electronic biosensing based on different synthetic recognition elements (i.e., liposomes, polypeptides, and aptamers).

Recognition elements	Target analyte	LOD	Methodology
Liposomes	HIV [79]	$6.7 \times 10^{11} / \mu\text{l}$	Impedance spectroscopy
	Nuclei acid sequences [80]	12.5 μM	Amperometric detection
	Glucose [81, 82]	40 mM	Cyclic voltammetry
	Glucose [83]	$8.6 \pm 1.1 \mu\text{M}$	Amperometric detection
	Organophosphate [84]	$0.68 \pm 0.076 \mu\text{g/L}$	Amperometric detection
	<i>E. coli</i> [85]	100 cfu/mL	Amperometric detection
Aptamers	DNA [86]	2-5 nM	Potentiometric sensing
	Adenosine [87]	50 μM	Potentiometric sensing
	IgE [88]	250 pM	Amperometric detection
	HIV-1 Tat protein [89]	< 1 nM	Amperometric detection
	ATP [90]	< 10 nM	Potentiometric sensing
	Adenosine [90]	1-10 nM	Potentiometric sensing
	Lysozyme [91]	12 nM	Potentiometric sensing
	Thrombin [91]	6.7 nM	Potentiometric sensing
	Thrombin [92]	5.5 nM	Potentiometric sensing
	DNA and streptavidin-biotin protein complex [93]	Not specified	Amperometric detection
Peptides	MMP-7 [94]	3.4 pM	Cyclic voltammetry
	Trypsin and α -Thrombin [95]	1 nM	Cyclic voltammetry
	<i>E. coli</i> [96]	100 cfu/mL	Impedance spectroscopy
	CRP [97]	0.1 $\mu\text{g/L}$	Impedimetric sensing

Owing to this unique structure, liposomes are theoretically capable to encapsulate any molecules/nanoparticles with reasonable water solubility. More importantly, rupture of liposomes can be specifically triggered by certain molecules, resulting in the release of encapsulants [98, 99]. Additionally, phospholipids, which are the basic units of liposomes, exhibit distinctive advantages such as non-toxicity, biodegradability, and good biocompatibility. Therefore, liposome is a good candidate of bio-receptor and various sensing approaches involving liposomes have been reported. Attributed to the signal amplification effect achieved by reporter encapsulation, molecular detections using liposomes tend to exhibit ultra-high sensitivity.

Figure 0.13 Basic structure of a typical unilamellar liposome [100]. Hydrophilic molecules are encapsulated in the vesicle, thus being separated from the solvent outside.

Liposome-based assays for detections of various analyte (e.g., viruses, enzymes, and nuclei acids) have been reported. Damhorst *et al.* reported an assay for electronic detection of viruses using ion-encapsulated liposomes [79]. Owing to the portability of the platform used, this assay is a promising candidate for point-of-care diagnostics. Wongkaew *et al.* demonstrated an amperometric detection of chemical molecules using microfluidic channel [80]. Specific nuclei acid sequences were quantified using ferri/ferro hexacyanide-encapsulated liposomes. The reported assay is claimed to be fast

and sensitive. Taylor *et al.* presented a liposomal enzyme electrode based assay for quantitative glucose detection (see Figure 2.14a) [81]. More specifically, glucose molecules were detected using glucose oxidases-encapsulated liposomes and a cyclic voltammetry related methodology. In cases where the glucose concentration is below 40 mM, the response increased linearly with the glucose concentration. Then, Graça *et al.* presented a glucose oxidase (GOx)-encapsulated liposomes based amperometric sensing assay for glucose detection [83]. To preserve their native structures, GOx were encapsulated in liposomes, as shown in Figure 2.14b. As a result, the sensitivity of this assay was reported to be 7.5 times higher than that of most peers using non-encapsulated GOx. Yan *et al.* described an assay for detections of organophosphate pesticides residues [84]. In this assay, acetylcholinesterase (AChE) molecules were encapsulated in phosphatidylcholine liposomes, whose porin-embedded lipid membrane allows in pesticide only. Changes in the current can only be generated by the inhibition reaction of organophosphate pesticides by AChE.

Figure 0.14 (a) Configuration of oxygen electrode that is used in combination with a liposome impregnated membrane [81]. (b) Sensing mechanism of the glucose oxidase (GOx)-encapsulated liposomes based amperometric glucose assay [83].

Nevertheless, liposomes were not used as receptors in this assay. Instead, they were either tagged with a reporter probe that is capable to hybridize with the target or employed as carriers only. Recently, considerable efforts have been made in the development of liposome-based assays without additional receptors. Zhang *et al.* reported an amperometric detection of *Escherichia coli* (*E. coli*), with 4-nitrophenyl β -D-glucuronide

(PNPG) used as the receptor [85]. β -D-glucuronide released from *E. coli* could catalyze the PNPG hydrolysis, which generates 4-nitrophenyl. As the quantity of β -D-glucuronide molecules is proportional to the concentration of *E. coli*, quantitative detection of *E. coli* in the range of $1.5 \times 10^2 \sim 1.0 \times 10^6$ cfu/mL was demonstrated using this assay and the LOD achieved was 100 cfu/mL.

However, it is worth noting that liposome-based assays suffer from time-consuming selection/modification processes and some of them exhibit limited on/off ratio due to encapsulant leakage [101]. Therefore, alternative synthetic recognitions elements should be investigated and considered.

Peptide

Peptides are short-chain molecules consisting of amino acid oligomers that are linked by peptide bonds. Indeed, peptides have been widely used in clinical and diagnostic applications as substitutes of proteins [102], owing to the fact that they share the same building blocks with proteins and other distinctive advantages over antibodies, such as excellent affinity to particular analytes [103], good chemical stability, and multiple interactions in most binding sites of peptides. The affinity can be further enhanced by facile modifications [104]. Additionally, the short-chain structures of peptides lead to extraordinary intrinsic stability of these molecules in harsh environments [105]. Therefore, peptide-based biosensors show significantly advantages in shelf-life over antibody based devices [96]. Also, peptides can be easily and cost effectively synthesized using chemical methods, provided that the sequence information has been isolated from the phage display (see Figure 2.15). The solid phase synthesis introduced by Merrifield [106, 107] enabled standardized and highly reproducible peptide synthesis, resulting in intensive studies and wide applications of these synthetic molecules. Liu *et al.* presented a cyclic voltammetry assay for quantitative detection of MMP-7 [94]. In this assay, peptides immobilized on Au electrodes via self-assembly were cleaved in the presence of MMP-7 (see Figure 2.16), resulting in detectable current changes. Facile yet sensitive, this assay can be easily extended to detections of infectious agents. Adjánian *et al.*

proposed a similar assay for rapid enzymatic measurements [95]. Herein, peptides were immobilized on Au electrodes using polyethylene glycol (PEG) linkers (see Figure 2.17) and detections of proteases were achieved on the peptide-functionalized electrodes via cyclic voltammetry. This assay was exceptionally rapid, which is believed to be a result of the flexible structure of PEG linkers that allow easy access of protease molecules to cleavage sites.

Figure 0.15 A typical phage display cycle for peptides [106]. The target is immobilized on a microtiter plate and phages that bind to the target molecule are eluted and then multiplied, while other phages and molecules are removed. In this way, a phage mixture enriched with relevant phages is obtained and peptides can be easily and cost effectively synthesized via chemical routes.

Mannoor *et al.* presented an impedance spectroscopy biosensor for the detection of pathogenic bacteria [96]. Herein, arrays of microelectrodes that were functionalized with peptides were used to capture pathogenic bacteria based on impedance changes generated by binding events (see Figure 2.18). The LOD achieved using this assay was within the

clinically relevant range of the analyte, indicating potential for diagnostics. Additionally, the specificity of the detection was demonstrated using similar bacteria.

Figure 0.16 Detection of matrilysin using peptide-functionalized Au electrodes [94]. Herein, immobilized peptides are cleaved in the presence of target analyte, which lead to detectable current changes.

Figure 0.17 Detection of protease using peptide-functionalized Au electrodes. Herein, peptides labelled with reporters were end-grafted on electrodes. Cleavage of the peptides was triggered by trypsin and thrombin [95].

Figure 0.18 Detection of bacteria using peptide-functionalized inter-digitated microelectrode array. In this assay, the target cells bind to peptides that are immobilized on substrate [96].

Figure 0.19 Detection of CRP using peptide-based electrochemical impedance spectroscopy (EIS) assay [97].

Johnson *et al.* proposed a peptide-based impedimetric assay for the detection of C-reactive protein (CRP), which is a key cardiac biomarker [97]. In this assay, peptide-

functionalized electrodes were used for surface plasmon resonance (SPR) based detection of CRP, electrochemical impedance spectroscopy (EIS) and cyclic voltammetry. Additionally, antibody based assay was also demonstrated and compared with its peptide-based peer. The results revealed that the small size of peptides enabled a larger gate accessible to the probes (see Figure 2.19), resulting in a sharp reduction of the charge transfer resistance.

Nevertheless, it should be noticed that the specificity of peptide-based assays has been demonstrated to be insufficient for bio-sensing applications and further improvements are needed before practical applications of these assays. The relatively poor specificity may be attributed to the semi-selective nature of peptides [108]. Additionally, some peptide-based assays requires signal marker labelling, which is considered a negative effect.

Aptamer

Aptamers refer to single-stranded nucleic acid ligands (DNA or RNA) with high binding affinity and specificity to specific analyte [109]. Owing to the folding capability upon binding [110], the binding affinity and specificity of aptamers are comparable to that of antibodies. Therefore, nucleic acid aptamer has been termed as “chemical antibody” [111, 112].

Currently, most aptamers are obtained by the systematic evolution of ligands by exponential enrichment (SELEX) process (see Figure 2.20) [113, 114]. Briefly, a huge nuclei acid library is created and then incubated with the target molecule for a sufficient period. After removal of unbound sequences and molecules, bound nuclei acids are eluted and amplified by polymerase chain reaction (PCR) to develop an enriched library. This new library is then used as the starting library in the next cycle. After 6–12 consecutive cycles, the final library is cloned and sequenced. Hence, SELEX is an in vitro technique for aptamer selection and in vivo immunization of animals, as in the case of antibody production, is not required for aptamer. Additionally, aptamers can be synthesized in an easy and reproducible manner using chemical methods as long as the sequence is

obtained using SELEX. Upon target binding, aptamers exhibit significant conformational changes, which facilitate the detection of target analyte at low concentrations. Additionally, interactions between aptamers and target molecules are reversible (via intermolecular hybridizations) and can be used repeatedly. Unlike natural biomolecules (i.e., antibodies), aptamers are highly stable and not subject to denaturation, even in harsh environments. In virtue of unprecedented advantages mentioned, aptamers have attracted increasing attention in diagnostics and healthcare [115, 116], including biosensing.

Figure 0.20 The procedures of systematic evolution of ligands by exponential enrichment (SELEX) process. A large library of nuclei acids is created and then incubated with the target molecule. After removal of unbound sequences and molecules, bound ones are eluted and amplified by polymerase chain reaction (PCR) to develop an enriched library, which is used as the starting library in the next cycle. Generally, 6 to 12 consecutive cycles are performed for each target before the cloning and sequencing of the library [117].

Shin *et al.* demonstrated preliminary detection of DNA and protein molecules using a novel FET-based biosensor, although the sensitivity and selectivity of this assay were not fully investigated [93]. Fritz *et al.* described a DNA sensing assay where complementary DNA was immobilized on micro-fabricated FET and used as the recognition element [86]. The LOD achieved by this assay was 2-5 nM and its specificity was excellent as the detection of a single base mismatch within 12-mer oligonucleotides was demonstrated. Zayats *et al.* monitored the binding of adenosine to its corresponding aptamer [87]. In this

study, nucleic acid 5 was used as the aptamer and nucleic acid 6 was hybridized with the aptamer. Adenosine triggered the displacement of nucleic acid 6, resulting in changes of the local charge associated with the gate potential (Figure 2.21a). This assay exhibited high specificity yet relatively poor sensitivity (detection limit = 50 μM).

Figure 0.21 (a) FET-based aptasensor for adenosine [87]. Amino-functionalized nucleic acid 5 was used as an aptamer for adenosine and nucleic acid 6 was hybridized with the aptamer to amplify the signal. (b) Aptamer modified CNT-FET sensor for the detection of IgE. The smaller size of aptamer enabled a significant effect of bound proteins on the equilibrium carrier distribution in the nanotubes as the distances between these molecules and nanotubes were less than the Debye length [88].

Maehashi *et al.* reported an aptamer-modified CNT-FET for the detection of immunoglobulin E (IgE) (Figure 2.21b) [88]. Presence of IgE caused I_d decreases that were proportional to the concentration of target molecules and the LOD achieved was 250 pM. More importantly, this study demonstrated superior sensing performance of aptamer-modified devices compared to antibody-modified ones under similar conditions. This could be attributed to the small size of aptamers, which reduced the distances between bound IgE molecules and the nanotubes, thus enabling a larger effect of bound IgE molecules on the equilibrium distribution of mobile carriers in the nanotubes.

Clinical sample detection, which is essential for diagnostic applications, has also been demonstrated. Ruslinda *et al.* demonstrated the detection of human immunodeficiency virus type 1 Trans-activator transcription (HIV-1 Tat protein) in clinical sample using RNA aptamers on FET (see Figure 2.22) [89]. Incubations with the target protein at concentrations of 100 nM, 10 nM and 1 nM resulted in 91 mV, 49 mV and 20 mV of V_g shifts in the negative direction, indicating feasible quantitative measurements. The LOD achieved using this assay was beyond 1 nM. Additionally, the RNA aptamers used were demonstrated to be highly specific to the target and cyclic detections were achieved, suggesting good reusability of the proposed sensor.

Figure 0.22 Changes in gate potential induced by detection of real sample of HIV-1 Tat protein on diamond FET in 1 mM PBS (pH = 7.4) [89].

More recently, Goda *et al.* conducted a series of studies in the field of FET-based biosensors using aptamers as the recognition element. In 2012, an assay using hairpin-structured DNA aptamer-functionalized FET sensor with a gold electrode as extended

gate was reported [90]. As a proof-of-concept, detections of adenosine triphosphate (ATP) and adenosine were demonstrated. Capture of the target molecules led to a structural switching of the aptamer from closed loop to open-loop conformations and the release of the preloaded DNA binder (DAPI, positively charged) into the solution (Figure 2.23a). The sensitivity achieved using this assay (< 10 nM for ATP and 1–10 nM for adenosine) was significantly improved compared with previous assays. Then, this platform was used for protein detection [91]. More specifically, binding of intrinsically charged target protein led to an alternation in the density of charges at the gate/solution interface (Figure 2.23b). Label-free detections of lysozyme and thrombin in the dynamic ranges of 15.2 – 1040 nM and 13.4 – 1300 nM were demonstrated and LOD achieved for lysozyme and thrombin were 12.0 nM and 6.7 nM, respectively.

Figure 0.23 (a) Conformational switching of the short hairpin (sh)-aptamer accompanied by the release of the cationic DNA binder upon detection of ATP on an FET-based sensing platform [90]. (b) Aptamer-induced binding of protein target (e.g., lysozyme and thrombin) in an electrical double layer at the gate–solution interface [91]. (c) Electrical thrombin sensing using dual aptamer surfaces. Improved affinity between aptamers and thrombin due to multiple binding contacts results in enhanced sensitivity [92].

In 2015, Goda *et al.* developed a novel aptamer-functionalized potentiometric biosensor with improved affinity for the target protein by employing a multiple-contact configuration (Figure 2.23c) [92]. Herein, two different aptamers that recognize different epitopes in thrombin were immobilized (in parallel or serial manners) to capture the target via multiple contacts. In virtue of the multiple-contact configuration, the sensitivity of this assay was further enhanced ($\text{LOD} = 5.5 \text{ nM}$) compared to previous assays.

Nevertheless, it should be pointed out that aptamers, especially RNA ones, are readily digested by nucleases [118]. Therefore, concentrations of nucleases must be reduced to a negligible level for proper functioning of aptamer-based assays. Additionally, specificity of aptamers needs to be further improved.

2.2 Question to Be Answered: Are Carbon Nanomaterial Based FET Biosensors Applicable to Detection of Sports Fatigue Related Biomarkers?

As a key part in athletes' daily life, training is regarded as the most effective and efficient way for athletes to keep their body conditions at a relatively high level [119] and make further performance improvements. Hence, athletes and coaches tend to maximize training loads whenever possible to seek for better performances in coming sports events. However, recent studies revealed that training loads over a threshold level had a negative effect on athlete performance due to physiological and psychological fatigue [120]. In most cases, the threshold level is expressed by levels of several biomarkers. To maximize the effectiveness of training and avoid over-training, real time and *in situ* monitoring of physiological conditions of athletes using a portable and sensitive biosensor must be achieved as coaches and consultants can never make appropriate/informed decisions on the duration, intensity, and frequency of training sessions without real time information about physiological and psychological conditions of athletes.

Aimed at portable biosensors for *in situ* monitoring of athletes, we propose that FET biosensors are promising sensing platforms owing to their miniaturization potential. As mentioned in Chapter 4, quantitative detection of IL-6, a key biomarker related to sports

fatigue, has been achieved using an FET biosensor based on horizontally aligned CNT as a demonstration. The proposed assay shows excellent sensitivity (LOD = 1.37 pg/mL, which is comparable to that of ELISA and other assays) and good specificity. Additionally, the developed biosensor exhibits great long-term robustness (no significant performance degradation after storage under ambient conditions for up to 3 months), thus are suitable for *in situ* measurements. Based on the results obtained in this project, it can be concluded that carbon nanomaterial based FET biosensors are applicable for real time detection of sports fatigue related biomarkers.

2.3 Question to Be Answered: Can Antibodies in Biosensors Be Substituted by Synthetic Molecules?

Owing to their superior binding affinity and specificity, natural biomolecules such as antibodies are widely used as recognition element in biosensors. However, biosensors using antibodies as recognition element exhibit several limitations:

- Antibodies are limited by complicated and fragile functional structures. As a result, antibodies are readily deactivated/denatured in typical working environments of biosensors [71].
- Antibodies are limited by steric hindrance effect caused by large molecular sizes [72, 73]. As a result, the quantity of receptors allowed per unit surface area is relatively low.
- Antibodies are limited by tedious preparation procedures, resulting in poor cost effectiveness of biosensors [76]. As a result, issues such as short shelf life, limited stability, and unacceptable reproducibility have been observed in antibody based biosensors.

Therefore, a question has been proposed by researchers: “can antibodies be substituted by synthetic molecules?” As a response, great efforts have been made over the past decades to explore sensing approaches using synthetic molecules as recognition element. Among various options, liposomes, polypeptides, and aptamers are three promising candidates.

This study explores the viability of electrical biosensors using synthetic molecules as recognition element.

2.4 PhD in context of literature

According to literature, over-training is regarded as a key contributor to fatigue and injury in sports. As a key part in athletes' daily life, training is the most effective and efficient way for athletes to keep their body conditions at a relatively high level and, if possible, make further improvements in their sports performances. Hence, coaches tend to maximize training loads of athletes, especially those in their early stage of career. However, recent studies revealed that increasing training loads did not always lead to improved performances. Instead, over-high training loads may have a severe negative effect on athlete performance. In most cases, the threshold level is quantified by levels of several biomarkers. Therefore, a portable and sensitive biosensor that can realize real time and *in situ* monitoring of physiological conditions of athletes is urgently needed as coaches and consultants can never make appropriate decisions on the duration, intensity, and frequency of training sessions without real time information about athletes. Aimed at portable biosensors for *in situ* monitoring of athletes, we propose that FET biosensors are promising sensing platforms owing to their miniaturization potential. As a demonstration, quantitative detection of IL-6, a key biomarker related to sports fatigue, has been achieved using an FET biosensor based on horizontally aligned CNT. The proposed assay shows excellent sensitivity and good specificity. Additionally, the developed biosensor exhibits great long-term robustness.

On the other hand, most biosensors reported are based on natural biomolecules such as antibodies. Despite the superior binding affinity and specificity of antibodies, biosensors using antibodies as recognition element are limited by several factors. First, antibodies are readily denaturized in typical working environments of biosensors owing to their complicated and fragile functional structures. As a result, shelf-life and long-term reliability of antibody based biosensors could be an issue in some cases. Second, the quantity of receptors allowed per unit surface area in antibody based biosensors is

relatively low, which can be attributed to the steric hindrance effect caused by large molecular size of antibodies. Third, the tedious preparation procedures make the device fabrication of antibody based biosensors a prolonged and costly process. Therefore, conventional biosensors that are based on natural biomolecules are not suitable for *in situ* monitoring of athletes. For this reason, protein detections using rGO FET sensors using liposomes and polypeptides, both of which are synthetic molecules, as recognition element are investigated in the second part of this thesis. The results revealed that the performances (in terms of sensitivity) of the proposed sensors were comparable to their antibody based peers, demonstrating that it is viable to combine electrical sensing platforms and synthetic molecules for protein detections.

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Chapter 3

Experimental Methodology

This chapter illustrates the experimental methods and instruments involved in FET-based sensing approaches and TRPS approaches. Firstly, a rationale for selection of materials and methods was presented. Then, synthesis of CNT and rGO by chemical vapor deposition and preparation of POPC liposomes and synthetic polypeptides were described. Characterization techniques used in this study, including FESEM (for sample morphology), AFM (for sample morphology), Raman spectroscopy (for elemental analysis), and dynamic light scattering (for determination of liposome size), were introduced. Also, fabrication of liquid-gated FET sensors was described. Finally, processes of kinetic sensing using CNT-based FET sensors, rGO-based FET sensors, and TRPS sensors were described.

3.1 Rationale for Selection of Methods and Materials

3.1.1 Rationale for Material Selection

3.1.1.1 Rationale for Selection of CNT

Carbon nanotubes are defined as well-ordered, hollow graphitic nanomaterials made of sp^2 -hybridized carbon atoms. Due to their unique structures, CNTs possess distinctive electrical and physical characteristics, such as ultrahigh length-to-diameter ratio (up to 13,000,000:1) [1] and superior carrier mobility [2]. To date, CNTs have emerged as a promising class of materials for biosensing applications and real time detections of various biomolecules [3], chemical species [4], and gas molecules [5] using functionalized CNT-based transistors have been reported.

Generally, CNTs are classified as single-walled nanotubes (SWNT) or multi-walled nanotubes (MWNT).

Single-walled nanotubes. Discovered by Iijima in 1993 [6], SWCNT are described as cylindrical tubes seamlessly wrapped by single sheets of graphene. The chirality plays a key role in determining various properties of nanotubes. For instance, the bandgap can vary from zero to about 2 eV and their electrical conductivity can be either metallic or semiconducting, depending on the chirality [7].

Multi-walled nanotubes. MWNT consists of several graphene cylinders that are concentrically nested like rings of a tree trunk [7] with an interlayer spacing of 3.4 Å and a diameter typically on the order of 10–20 nm [8].

However, random networks of SWCNT were employed in most of these cases, resulting in degraded sensing performances compared with those incorporated individual tubes. This can be attributed to inherent resistances at the tube/tube contacts in the networks [9]. In order to improve the sensing performance, various configurations have been proposed for CNT-based FET sensors. In 2002, Joselevich *et al.* reported the horizontal alignment for single-wall carbon nanotubes [10]. Herein, horizontally aligned were grown on the

surface by patterning of catalyst nanoparticles and application of a local electric field parallel to the substrate. More specifically, the origin of nanotube growth was defined by the nanoparticle patterns and the direction of nanotube growth was determined by the electric field applied. Then, Kocabas *et al.* proposed a thin-film transistor with horizontally aligned arrays of SWCNT as the semiconductors [9]. The large-scale, horizontally aligned arrays of SWCNT were synthesized on single-crystal quartz substrates by CVD. The SWCNT synthesis was facile and cost-effective, while the transistor proposed was demonstrated to be a high-performance one. Therefore, the transistor proposed exhibited a great potential in sensing applications.

In virtue of the synthesis routes reported, horizontally aligned SWCNT have been applied in various sensors. For instance, Palaniappan *et al.* introduced a liquid-gated electrical biosensor based on horizontally aligned SWCNT on quartz substrate [11]. The SWCNT were synthesized by CVD in a facile and effective way. Detection of prostate specific antigen (PSA, a potential biomarker of prostate cancer [12]) using the biosensor proposed is demonstrated. Herein, the specific binding of PSA with the immobilized antibody led to a detectable change in I_d , and the limit-of-detection (LOD) achieved was 30 pM, which is within the clinically relevant concentration regimes. Recently, Palaniappan *et al.* reported a FET sensor in which the horizontal alignment of CNTs was further improved by patterning the catalyst nanoparticles using a stripe-patterned PDMS stamp [13]. Direct electronic detection of epsilon toxin (a common food toxin produced by clostridium perfringens) was demonstrated using this approach. In virtue of the perfect alignment of SWCNT, the sensing performance of FET sensor was significantly improved due to the reduced resistance of SWCNT as a semiconductor and the sensitivity achieved was comparable to ELISA, a reliable biosensing approach that has been widely applied.

3.1.1.2 Rationale for Selection of rGO

As a 2D carbon nanomaterial whose existence was predicted in the 1960s, graphene had never been successfully isolated until the milestone work reported in 2004 [14]. Despite its relatively short history, graphene has been paid great attentions in virtue of its superior

properties such as high mechanical strength [15], excellent carrier mobility (ballistic transport) [16], ultra-large specific surface area [17], and good transparency [18]. However, the semi-metal nature of pristine graphene indicates that no band gaps are expected in the electronic structure of this material. Hence, modifications are needed for graphene to be used in applications such as electronics and sensing.

Currently, post-processing has been employed to open up a band gap in graphene [19]. Among various approaches proposed, utilization of GO has attracted particular attention due to the facile, solution-based, and cost-effective synthesis routes available. GO is a soft matter with non-stoichiometric molecular formula and large internal degree of freedom [20]. Hence, the chemical structure of GO has not been fully understood. However, the presence of epoxy groups, hydroxyl groups, and carboxyl groups in the chemical structure of GO have been demonstrated. The GO model proposed by Lerf *et al.* [21] suggested that the basic units of GO include aliphatic six-membered rings and un-oxidized benzene rings, and these two structures compete with each other. With subsequent modifications and improvements, this model has been widely accepted.

However, GO has been demonstrated to be an electrically insulating material in most cases. This can be attributed to the fact that ordered sp^2 conductive clusters isolated within sp^3 insulating matrix. Hence, a reduction step, in which the sp^3 structures could be converted to sp^2 clusters, is usually required to restore its electrical conductivity, although a pure sp^2 structure is not achievable by this method. The electrical properties (e.g., on/off ratio and electrical conductivity) of rGO prepared in this way can be tuned by varying the degree of reduction to be used for different applications [22]. More specifically, mildly-reduced GO sheets exhibit high on/off ratio but low electrical conductivity as the internal electrical transport is dominated by tunneling and/or hopping between sp^2 clusters, both of which are highly dependent on the voltage applied. As the degree of reduction increased, oxygen moieties are removed and some of the π -bonds are restored. Hence, percolation pathways between existing sp^2 domains are generated. As a result, the on/off ratio decreases while the electrical conductivity increases. To date, FETs based on thermally or chemically reduced GO have been widely applied for

detections of DNA hybridization and protein binding. For instance, Stine *et al.* [23] reported detection of ssDNA using DNA-functionalized rGO-FET with LOD of 10 nM.

3.1.2 Rationale for Method Selection

3.1.2.1 Rationale for Selection of FET platform

FET is a device that uses an electric field to control the conductivity of a channel within a semiconductor material. A typical FET consists of three terminals: source, drain, and gate. With a constant V_d , I_d is modulated by V_g . FET biosensor is a structural analog of MOSFET that has been widely used to detect molecules and atoms in electrolyte.

In the sensing process, a constant potential difference is established between the source electrode and the drain electrode to generate a detectable I_d . Without alterations to its surface, the conductance of the transducer layer stays constant and I_d is determined by V_g only. In the presence of target analyte binding in the gate region, the equivalent V_g varied and a change in the conductance of the transducer layer is observed, resulting in a change in I_d . This change, if detectable, is regarded as an electrical signal. Therefore, it is believed that the sensing performance is dominated by the transducer in most cases.

Among various transducer materials proposed, carbon nanomaterials are regarded as promising candidates for. Carbon nanomaterials have attracted considerable attention in the past 60 years due to three distinctive advantages over conventional macroscopic materials [24]. The first advantage is the unprecedented electronic properties. Appropriately prepared carbon nanomaterials exhibit ballistic transport with extremely high electron mobility [25]. The second advantage is the ultra-large surface area due to their single-atom thickness. This feature is of great significance for sensing as molecular bindings can only take place on the surface. The third advantage is the excellent biocompatibility [26], which makes them excellent candidates for implantable biosensors.

3.1.2.2 Rationale for Selection of TRPS

As a sensing technique to measure the size, concentration and surface charge of particles individually within their natural environment, RPS involves two reservoirs separated by a pore. Particles in the electrolyte passing through a micro-sized or nano-sized pore are detected one at a time as a transient change in the ionic current flow as the particle is displacing a certain volume of electrolyte, thus increasing the resistance. This change is a defined as blockade event [27].

In virtue of their user-friendly nature, measurement sensitivity and individual single-object readout capability, RPS has attracted increasing attention and various studies have been reported in this field. Indeed, resistive pulse sensors have been utilized to detect DNA, protein, cellular vesicles, nanoparticles, etc.. Resistive pulse sensors can be traced back to the Coulter counter proposed in the 1950s [28]. This primary platform was used for detection of biological cells and microorganisms. Kasianowicz *et al.* demonstrated ssDNA detection using a α -hemolysin pore [29]. This method exhibited the capability to discriminate slight differences in the size of the four DNA base pairs. Deblois *et al.* reported the detection and characterization of nanoparticles and viruses using a submicron resistive pulse sensor based on a synthetic pore, which shows advantages in bio-compatibility and chemical stability [30, 31]. In virtue of advances in nanofabrication, microfluidic sensor with pore arrays was proposed. Siwy *et al.* introduced a resistive pulse sensor whose pore surface was modified with biological capture probes for ricin detection [32].

However, the fixed size of the pore has been a limiting factor as it narrows the size range of analyte that can be detected and degrades the measurement accuracy. To overcome that, tunable resistive pulse sensors were developed. TRPS refers to RPS techniques where the pore size can be tuned by real time stretching according to the size of target analyte so that the measurement accuracy is enhanced and the size range is improved. In virtue of these advantages, TRPS has been intensively studied and widely used for sensing applications. Willmott *et al.* demonstrated the detection of micro-sized SPB aggregation using resistive pulse sensing based on tunable pores on an elastomeric membrane [33]. Herein, aggregates induced a detectable change in the average magnitude

of blockades. Platt *et al.* reported a TRPS approach based on thermoplastic polyurethane elastomeric membrane with a mechanically punctured tunable pore whose size can be altered by up to an order of magnitude [34]. In this report, aggregation of nanorods induced by analyte was monitored based on the observation that the resistive pulse signal of a rod was distinguishable from that of a sphere of similar sizes. Colby *et al.* monitored the swelling of polymeric eNPs using various techniques, including TRPS [35]. In a mildly acidic environment, these nanoparticles expanded by up to 10 times. Expanded eNPs exhibited unique translocation behaviors as they could squeeze through a pore whose size is close to their diameters. This phenomenon, together with other differences in resistive pulse signals, enabled discrimination of expanded nanoparticles using TRPS.

3.2 Synthesis

3.2.1 Chemical Vapor Deposition

Figure 0.1 Schematic illustration of CVD mechanism.

As a material processing technique involving chemical processes, CVD has been widely employed for generation of conformal solid thin-films on the augment substrate surfaces via chemical reactions occurring on or in the vicinity of the substrate surface [36]. CVDs can effectively generate films that cannot be achieved by conventional surface modification techniques. As shown in Figure 3.1, the volatile precursors are transported by the carrier gas to the substrate surface and react and/or decompose to produce the desired deposit. Also, the volatile by-products, which are observed in some cases, are removed by the gas flow through the reaction chamber. Particularly, CVD is extremely useful in the process of atomic layer deposition at depositing extremely thin layers of material.

3.2.1.1 CNT Growth by CVD

In this project, the horizontally aligned CNTs were grown on quartz substrates using a modified method based on previous reports [37]. $\text{Co}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 4\text{H}_2\text{O}$ (cobalt(II) acetate tetrahydrate, ACS reagent, $\geq 98\%$), the catalyst for CNT growth, was purchased from Sigma Aldrich, Singapore, and used without further purification. The ST-cut quartz substrates were purchased from Hoffman Materials Inc., U.S.. The 99% absolute ethanol used for catalyst dissolution and CVD synthesis was obtained from Merck, Singapore.

The quartz substrates were firstly sonicated in acetone, isopropyl alcohol and deionized water for 5 min each to remove surface contaminants [38]. The substrates were then annealed with a 4 h ramp from room temperature, held at 850 °C for 8 h before cooling down to room temperature in 4 h. To fabricate the PDMS stamps utilized for micro-contact printing, siloxane was cured over a silicon mask with the desired pattern [39]. The Cobalt(II) acetate tetrahydrate catalyst was dissolved in absolute ethanol solution at a concentration of 0.4 mg/ml and applied on the quartz substrate using the PDMS stamp. The catalyst, which was first deposited onto a flat piece of Si, was deposited on the quartz surface using the stamp. More specifically, the stamp was brought into conformal contact with the substrate and kept for 2 min, as illustrated in Figure 3.2. To optimize the horizontal alignment of CNT, the catalyst was deposited in a direction perpendicular to

the growth direction of CNT. Finally, the stamp was removed and the catalyst-deposited substrate was dried naturally.

Figure 0.2 Deposition of catalyst on quartz substrate using PDMS stamp. The catalyst was dissolved in absolute ethanol and applied on the quartz substrate using a PDMS stamp. The catalyst, which was first deposited onto a flat piece of Si, was deposited on the quartz surface using the stamp. More specifically, the stamp was brought into conformal contact with the substrate and kept for 2 min. Eventually, the stamp was removed and the catalyst-deposited substrate was dried naturally.

Figure 0.3 Schematic illustration of the CVD set-up used for CNT growth. Ar was used as the carrier gas, H₂ was used for the reduction of cobalt(II) acetate tetrahydrate and absolute ethanol was used as the carbon source.

The catalyst-coated substrates were heated up to 850 °C under a mixture flow of Ar (200 sccm) and H₂ (100 sccm) and kept for 5 min to allow oxidation of catalysts, which were then reduced to pure Co at 925 °C under a mixture flow of Ar (40 sccm) and H₂ (20 sccm) for 10 min. The CVD set-up for CNT growth is shown in Figure 3.3.

3.2.1.2 rGO Growth by CVD

The rGO was prepared by extended growth of GO on SiO₂ substrates, as reported previously [40]. Briefly, GO flakes in aqueous solution were synthesized and attached to SiO₂ substrates functionalized with (3-Aminopropyl)Triethoxysilane (APTES) [41]. Then, these substrates were placed on a Si holder and inserted into a quartz tube. Before growth process, the reaction tube was purged with Ar at 300 sccm for 40 min to avoid oxidation of rGO. Subsequently, a mixture gas of Ar (100 sccm) and H₂ (20 sccm) was introduced into an ethanol bubbler, before entering the reaction tube. The bubbler and reaction tube were kept at room temperature and 950 °C respectively for 2 h, followed by cooling to room temperature.

3.2.2 Preparation of POPC Liposomes

TNP (used as the reporter molecule) and PLA₂ were purchased from Sigma-Aldrich Inc. and used without further treatment. POPC was purchased from Avanti Polar Lipids and the POPC liposomes were fabricated by a self-assembly method reported elsewhere [42, 43].

The dried POPC films obtained from 5 mg/mL POPC were rehydrated with 1.0 mL of 1 mM TNP in PBS. This is followed by multiple extrusion cycles (20 times) through the membranes with 200 nm pores. Non-encapsulated TNP molecules were removed by centrifugation with an ultracentrifuge (Sorvall Legend Micro 21 R, provided by Thermo Scientific) for 20 min at 10000 g at 15 °C [44]. After removal of the supernatant, the residual liposomal pellet was re-suspended in 1 mL of phosphate-buffered saline (PBS), and the centrifugation was repeated for another three times. The final liposomal pellet

was re-suspended in 0.5 mL of PBS to obtain a liposome solution with a concentration of 13.2 mM. The size distribution and polydispersity of liposomes fabricated were checked by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZS (Malvern, UK). A 20× diluted liposome sample (in PBS) was measured at a fixed scattering angle of 173 °; three measurements, each consists of 3 runs of 30 s duration, were recorded at room temperature.

3.2.3 Preparation of Synthetic Peptides

MMP-7 was purchased from Merck Pte Ltd, Singapore and used without further purification. Pyrene maleimide and all chemicals used for rGO preparation were obtained from Sigma Aldrich Inc.. Human plasma (GTX73265, normal human plasma) was purchased from Genetex Inc., USA.

The polypeptides (JR2EC) were synthesized as reported previously [45, 46] and the electrostatic potential of JR2EC was calculated at pH = 7. More specifically, the polypeptides were synthesized on an automated peptide synthesizer using standard fluorenylmethoxycarbonyl (Fmoc) chemistry with O-(7-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) as the activating reagent. An Fmoc-Gly-PEG-PS resin and a four-fold excess of amino acid used in each coupling. The resin was then washed with 20 mM diethyldithiocarbamic acid in dimethylformamide (DMF), 30 mM diisopropylethylamine (DIPEA) in DMF, DMF and dichloromethane (DCM), respectively, followed by desiccation. Peptides were cleaved from the resin by incubation with a mixture of trifluoroacetic acid (TFA), ethanedithiol, water, and triisopropylsilane (94: 2.5: 2.5: 1, v/v/v/v) for 2 h at room temperature. After filtration and evaporation of TFA, polypeptides were precipitated (upon addition of cold diethyl ether), centrifuged, re-suspended in diethyl ether, and lyophilized. The products were then purified by reversed-phase high performance liquid chromatography (HPLC) [47] and peptides were eluted with 0.1% TFA30%–50% and aqueous 2-propanol.

3.3 Characterization

3.3.1 Field Emission Scanning Electron Microscopy

Figure 3.4 shows a schematic illustration of FESEM. Briefly, electrons generated by the electron gun are accelerated by two condenser lens before reaching the sample surface. Upon interactions of electrons and the sample, secondary and back scattered electrons are generated and collected by respective detectors. Currently, advanced FESEM can achieve sub-nanometer resolution as electron microscopes are not limited by the diffraction limit [48] and the vacuum environment in which electrons travel is excellent. A typical SEM image of horizontally aligned CNT sample is shown in Figure 3.5. As can be seen, horizontal alignment of CNT can be readily observed and the density of nanotubes on the substrate can be evaluated.

Figure 0.4 Schematic illustration of FESEM. FESEM is not limited by the diffraction limit as it relies on the interaction of electrons with sample atoms, thus.

Figure 0.5 A typical FESEM image of horizontally aligned CNT sample obtained. Excellent horizontal alignment of CNT is observed and the density of nanotubes on the substrate can be estimated.

The as-prepared CNT devices and rGO devices were characterized using field emission scanning electron microscopy (FESEM, JSM-7600F in this study). FESEM is an electron microscope that has been widely used for the examination of microstructural characteristics of solid objects [49]. The sample, which is usually an ultra-thin specimen, is scanned with a focused beam of electrons, which interact with the atoms of the sample. An image is obtained from the interaction of electrons transmitted through the specimen, magnified, and then focused onto an imaging device on a layer of photographic film or to be detected by a sensor. In this way, signals containing information about surface morphology (secondary electron imaging) and elemental composition (back scattered electron imaging) of the sample can be obtained. Due to the small de Broglie wavelength of electrons, TEMs exhibit significantly higher resolution than light microscopes. Theoretically, the maximum resolution that can be achieved by a light microscope is limited by the wavelength of photons that are used to probe the sample and the numerical aperture of the system.

3.3.2 Atomic Force Microscopy

The as-prepared CNT devices and rGO devices were characterized using atomic force microscopy (AFM, Dimension 3100 in this study). As shown in Figure 3.6, a laser beam from the emitter is reflected on the photo diode by the cantilever. In response to any cantilever displacement caused by the interaction between the tip and the surface, the reflection of the laser beam on the surface of the photodiode is displaced. This displacement is received by the detector and control system as a signal [50].

Figure 0.6 Diagram of AFM using beam deflection detection. In response to the cantilever displacement caused by the interaction between the tip and the surface, the reflection of the laser beam on the surface of the photodiode is displaced, which is received by the detector and control system as a signal.

AFM is characterized by three key capabilities: force measurement, imaging, and manipulation. First, forces between the probe and the sample as a function of their mutual separation can be measured to facilitate force spectroscopy. Second, the reaction of the probe to the forces imposed by the sample can be employed to generate a high resolution image showing the topography (usually displayed as a pseudo-color plot) of sample surface. This is achieved by raster scanning the position of the sample with respect to the tip and recording the height of the probe corresponding to a constant probe-sample interaction. Third, the forces between tip and sample can be used to alter sample properties in a controlled way. In this study, CNT and rGO on SiO_2 substrate were examined using AFM to generate images that can visualize surface topology of the sample. Figure 3.7 shows the AFM image and corresponding section analysis of the synthesized nanotubes. Based on that, the average thickness and density of nanotubes can be obtained.

Figure 0.7 AFM image and corresponding section analysis of the synthesized nanotubes. The average thickness and density of synthesized nanotubes can be obtained.

3.3.3 Raman Spectroscopy

The Raman spectroscopy is a spectroscopic technique commonly used to provide a fingerprint by which molecules can be identified [51]. This spectroscopy relies on the inelastic scattering (also known as the Raman scattering) of monochromatic light (from a laser, in most cases) in the visible, near infrared, or near ultraviolet range (see Figure 3.8).

More specifically, the laser light interacts with molecular vibrations, phonons or other excitations in the system, resulting in the energy shift (up or down) of the laser photons. The energy shift provides information about the vibrational modes, which are specific to the chemical bonds and symmetry of molecules in the system.

Figure 0.8 Schematic illustration of energy levels involved in Raman scattering. The Rayleigh scattering, which is usually strong, should be filtered out.

Raman shifts are usually reported in wavenumbers ($\Delta\omega$), which have units of inverse length and can be related to the wavelength by the following equation [52]:

$$\Delta\omega = \frac{1}{\lambda_0} - \frac{1}{\lambda_1} \quad 0.1$$

where λ_0 and λ_1 denote the excitation wavelength and the Raman spectrum wavelength, respectively.

In Raman spectroscopy, electromagnetic radiation from the spot illuminated with a laser beam is collected with a lens and sent through a monochromator. The elastic scattered radiation (Rayleigh scattering) at the wavelength corresponding to the laser line is filtered out, while the rest of the collected light is dispersed onto a detector.

3.3.4 Dynamic Light Scattering

Figure 0.9 Schematic illustration of dynamic light scattering of large and small particles. A monochromatic light source is shot through a polarizer and into the sample. The scattered light then goes through a second polarizer where it is collected by a photomultiplier.

The size distribution and polydispersity of POPC liposomes synthesized were examined using dynamic light scattering (DLS). DLS is a physical technique that is widely used to

determine the size distribution profile of small particles in suspension or polymers in solution [53]. Temporal fluctuations in scattered light intensity are usually analyzed by means of the intensity or photon auto-correlation function. The dynamic information of the particles is derived from an autocorrelation of the intensity trace recorded during the experiment.

3.4 Fabrication of Liquid-Gated Sensors

2 mm wide, 100 nm thick Au source and drain electrodes (spacing = 200 μm) were developed on the samples by evaporation. A silicon rubber reservoir was generated in the vicinity of the electrodes to confine the solution and silver paint was coated for the convenience of probing. Figure 3.10 illustrates a typical device fabricated using the proposed methodology.

Figure 0.10 A typical liquid-gated FET biosensor fabricated using the proposed methodology.

3.5 Kinetic Measurement

3.5.1 Bio-sensing Using FET Based on Horizontally Aligned CNT

Before the kinetic detections, surface passivation was achieved by incubation with 1% (v/v) PEG in PBS for 30 min to minimize non-specific binding. Then, antibodies (IL-6R in this case) were immobilized on the nanotubes using 1-pyrenebutanoic acid succinimidyl ester (PBSE) as linkers [54]. The immobilization was achieved by incubation with 5 mM PBSE (in phosphorous buffer, pH = 7.4) for 1 h, followed by incubation with 5 µg/mL IL-6R. Subsequently, the device was rinsed with PBS for several times to remove loosely bound antibodies. A voltage bias of 10 mV was applied between the source electrode and the drain electrode, while the gate potential was applied via a reference electrode (3M KCl) (FLEXREF from World Precision Instruments). The devices were incubated with testing solutions (with different concentrations of IL-6R) for 15 min each, followed by removal of the solution and incubation with PBS. The source-drain current was monitored (liquid-gated measurement) continuously during the entire process (in testing solutions and buffer). LOD was calculated by the $3\sigma/S$ approach [55]:

$$\text{LOD} = \frac{3\sigma}{S} \quad 0.2$$

Where σ is the standard deviation of device responses to PBS and S is the sensitivity, which is defined as the slope of the linear sensor response range. S was calculated by:

$$S = \frac{I_d' - I_d}{C' - 0} \quad 0.3$$

where C' = the concentration tested, I_d' = drain current corresponding to C' , I_d = drain current corresponding to pure buffer/serum.

3.5.2 Bio-sensing Using FET Based on rGO

3.5.2.1 Detection of PLA₂ Using Reporter-Encapsulated Liposomes

TNP-encapsulated liposomes of equal quantity were mixed with solutions with different concentrations of PLA₂ and kept for 30 min to allow complete reaction. Afterwards, the as-prepared devices were incubated with a mixed solution (starting from PLA₂ concentration = 100 pM) for 10 min, followed by removal of the solution and electrical measurements in buffer. Then, the device was rinsed by buffer and incubated with a mixed solution with higher concentration, followed by removal of the solution and electrical measurements in buffer. This process was repeated for concentrations with PLA₂ concentration = 1 nM and 10 nM. Notably, all measurements were conducted under ambient conditions (25 °C and atmosphere pressure).

3.5.2.2 Detection of MMP-7 Using Synthetic Peptides

The polypeptides were immobilized on rGO using pyrene maleimide as linkers. The pyrene groups adhere to rGO film via π - π interaction, while the maleimide groups bind to the polypeptides at the 22th amino acid via introduction of a cysteine residue [56]. Firstly, the devices were incubated with 1 mM pyrene maleimide (in ethanol) for 30 min, followed by rinsing with buffer. Then, the pyrene maleimide-immobilized devices were incubated with 1% polyethylene glycol (PEG) in phosphate buffer solution (PBS, 0.1 % Tween) for 30 min to achieve surface passivation and minimize non-specific binding. Subsequently, the passivated devices were incubated with 5 μ M JR2EC (in PBS) for 30 min to achieve polypeptide functionalization. The I-V characteristics of the transistors fabricated were obtained using a methodology similar to a previous report [57]. With a small voltage bias (V_d) of 10 mV applied between the source electrode and the drain electrode, the current (I_d) flowing through the transducing layer was recorded while sweeping the gate voltage (V_g) from - 600 mV to 0 mV. After rinsing thoroughly with PBS for several times (to remove loosely bound polypeptides), the I_d of the device (measured in PBS) was allowed to stabilize and the stabilized I_d was taken as the baseline for kinetic measurements. The transfer characteristics (I_d vs. V_g) of the devices were

obtained using a methodology reported elsewhere [42]. For kinetic measurements, MMP-7 at concentrations of 0.01 $\mu\text{g/mL}$, 0.05 $\mu\text{g/mL}$, 0.1 $\mu\text{g/mL}$, 0.5 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$ were tested individually. The device was incubated with MMP-7 solution (in PBS) until I_d stabilization. In this process, I_d of the device was monitored in real-time (liquid-gated measurement). Upon I_d stabilization, the MMP-7 solution was removed and the device was incubated in buffer. Notably, all measurements were conducted under ambient conditions (25 $^{\circ}\text{C}$ and atmosphere pressure). LOD was calculated by the $3\sigma/S$ approach, as mentioned above.

3.5.3 Bio-sensing Using D-AptaTRPS

40 μL of the collected solution was transferred into the upper fluid cell of the TRPS instrument and the data was captured for 10 min. An appropriate voltage was selected for all experiments to enable the detection of the peaks above the level of noise ($< 10 \text{ pA}$). A bandwidth filter of 1 kHz was applied during measurements, and averages obtained from data histograms are used in all plots. In all experiments, 80 μL of electrolyte buffer was placed in the lower fluid cell. After each measurement cycle, the fluid cell and the pore were rinsed until no particles were observed. DNA aptamers against cTnI [58] and their complementary sequences were purchased from Thermo Fisher Scientific UK and Sigma Aldrich UK, respectively; their full sequences are listed in Table 3.1. cTnI was purchased from Abcam Ltd. (Cambridge, UK). 125 nm streptavidin-modified SPBs were purchased from Ademtech (Pessac, France) and used without further purification. The qNano system for TRPS measurements and SPBs of known concentration and size were sourced from Izon Science. Avidin (A9275) and BSA (A2153) were obtained from Sigma Aldrich UK. Human serum samples were collected and prepared at Peterborough City Hospital Pathology Laboratory, U.K.. Briefly, serum samples gathered using blood from a healthy volunteer donor were collected into Sarstedt monovette/collection tubes, followed by centrifugation (3000 rpm, 6 min). The supernatants from each sample were transferred into separate vials and stored at -80°C .

Table 0.1 Sequence of aptamer and complementary DNA.

DNA	Full Sequence
Aptamer	5'GCCTGTTGTGAGCCTCCTAACTACATGTT CTCAGGGTGTAGGCTGGATGCGATGGTGG CATGCTTATTCTTGTCTCCC'3
Half complementary DNA	5'TCAACCCTGAGAACATGTA'3
First 15 bases complementary DNA	5'CCCTGAGAACATGTA'3
First 10 bases complementary DNA	5'AGAACATGTA'3
Middle 15 bases complementary DNA	5' CAGCCTCAAC'3

3.5.3.1 DNA Modification of SPB

A solution containing streptavidin-modified SPBs (150 μL , concentration = 1×10^{10} particles/mL) were mixed with solutions containing biotin-modified DNA sequences that are complementary to aptamers (50 μL in buffer, concentration = 10 μM) and left for 30 min. DNA-functionalized SPBs were then isolated using a magnet and re-dissolved in buffer. The SPBs were examined using TRPS before and after DNA functionalization to determine changes in their diameters and surface charges.

3.5.3.2 Preparation of the Dipstick and Assay

Aptamer-immobilized slides were incubated with DNA-functionalized SPBs for overnight and rinsed with buffer for three times. Afterwards, SPB-immobilized slides were incubated with 50 μL of cTnI at different concentrations (100 ng/mL, 1 $\mu\text{g/mL}$ and 10 $\mu\text{g/mL}$) for 6 h. The initial solution (50 μL) was removed and placed into a centrifuge tube, and the chamber was washed once with buffer (40 μL), which was then removed and added to the initial hyb solution, creating a 90 μL solution. For cTnI in human serum, free SPBs collected after protein incubation were isolated and re-dissolved in buffer of equal volume. As a control group, SPB-immobilized slides were incubated with 50 μL of BSA solution at 100 $\mu\text{g/mL}$ for a comparable time period.

3.6 Overview of Methodologies

In summary, CVD has been used for growth of CNT and rGO on quartz substrates. In this study, FESEM has been used to investigate surface morphology of as-prepared samples, AFM has been used to examine CNT samples in terms of horizontal alignment, Raman spectroscopy has been used to identify CNTs based on characteristic peaks, and DLS has been used to investigate the sizes of POPC liposomes prepared using the method mentioned above. Electrical bio-sensing using FET and TRPS have been elaborated and involved.

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Chapter 4

Bio-Sensing Using Horizontally Aligned CNT Based FET *

This chapter elaborates detection of interleukin-6, a key sports related biomarker, using a FET sensor based on horizontally aligned CNT as a demonstration of quantitative and real-time detection of sports fatigue related biomarkers using carbon nanomaterials based sensors. The proposed sensing assay exhibits superior sensitivity ($LOD = 1.37$ pg/mL) in virtue of the reduced tube-to-tube contact resistance, good selectivity (no responses to bovine serum albumin and cysteine were observed and detection of target molecules in serum was achieved) as a result of the highly specific interaction between IL-6 and IL-6R, and excellent stability (no significant degradation in the electronic performance after storage under ambient conditions for up to 3 months) in virtue of the strong adhesion of CNT to the quartz substrate and good horizontal alignment of these tubes.

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4.1 Introduction

Figure 0.1 Schematic illustration of the liquid-gated FET sensor based on horizontally aligned SWCNT for protein detections. IL-6R molecules were immobilized on carbon nanotubes to capture IL-6, the analyte in this study.

As a preliminary study of carbon nanomaterials based FET sensing platforms, the first assay proposed was an FET sensor based on horizontally aligned CNT for detection of IL-6 using its antibody, IL-6R, as the receptor. Quantitative detection of the target analyte using the platform proposed was achieved, indicating that bio-markers related to sport and exercise science can be measured in a facile and sensitive way using carbon nanomaterials based FET sensors. Additionally, excellent long-term robustness of the sensor proposed has been validated, indicating its great potential for *in situ* measurements. In this chapter, we describe a sensitive, label-free sensing assay using liquid-gated FET based on horizontally aligned SWCNTs for real time protein detection. As a

demonstration, IL-6 was detected using the proposed platform [1]. Figure 4.1 depicts the horizontally aligned SWCNTs based liquid-gated FET sensor.

The reported liquid-gated FET biosensors offer real-time monitoring of biomolecules with superior sensitivity and device stability. The proposed sensors showed excellent sensitivity (LOD = 1.37 pg/mL, which is comparable to that of ELISA and other assays) and good specificity. Detection of target molecules in diluted serum (rabbit serum diluted by 100 times with buffer) was also achieved. 1 pg/mL IL-6 in serum triggered a detectable I_d response, indicating that the sensitivity of the serum testing is comparable to that of the buffer testing. Additionally, the devices developed exhibited good stability under ambient conditions for up to 3 months as no major degradation in electronic performance was observed. Considering the cost-effectiveness of the raw materials involved and the facile synthesis routes available for the growth of horizontally aligned CNT on quartz substrates in virtue of the significant advances recently, this IL-6 assay exhibits a great potential for *in situ* measurements in the future.

IL-6 is a pleiotropic cytokine that regulates cell growth and differentiation of various tissues. It is known particularly for its role in the immune response and acute phase reactions [2]. As a major mediator of the inflammatory response [3], IL-6 plays a key role in the inflammatory process by acting as both pro-inflammatory cytokine and anti-inflammatory myokine [4]. The anti-inflammatory effect of IL-6 is mediated through its inhibition on tumor necrosis factor (TNF- α) and interleukin 1 (IL-1), and activations of interleukin-1 receptor antagonist (IL-1RA) and interleukin-10 (IL-10) [5]. It has been demonstrated that the dysregulation (in most cases, over-expression) of IL-6 is involved in the pathogenesis of a variety of diseases such as rheumatoid arthritis [6], myeloma [7] and even cancers [8]. More recently, IL-6 has been related to sport and exercise science as the circulating level of IL-6 increases in a near-exponential pattern (it precedes the appearance of other cytokines in the circulation) with the intensity and the duration of exercise and training [9], and the working skeletal muscle has been identified as the major source [10]. This can be explained by the fact that IL-6 level is elevated in response to muscle contractions. For this reason, IL-6 can be used as an indicator of the

glycogen level in the muscles and a stimulator of glucose metabolism when the glycogen level is low [11]. Furthermore, non-invasive capillary blood sampling, which is more applicable for real-time and point-of-care testing, has been proven to be of the same effectiveness as conventional venous blood sampling for the measurement of plasma IL-6 [12]. This phenomenon is of great significance as it indicates a great potential of IL-6 for real-time monitoring of fatigue that is related to exercises and trainings.

Owing to its diverse biological roles, IL-6 has been targeted by a variety of assays [13, 14]. As shown in Table 4.1, different immunosensing assays targeting at the detection of IL-6 have been reported, including electrochemical assays [15], fluorescence-based assays [16] and surface plasmon resonance based assays [17]. Among all assays reported, ELISA [18] and the western blot method [19] are most widely used for detections of IL-6. ELISA can provide superior sensitivity ($\text{LOD} < 1 \text{ pg/mL}$), while the western blot method exhibits excellent selectivity and specificity. However, ELISA is a resource intensive method (expensive equipment and well-trained personnel are typically required), thus not ideal for practical applications; the western blot method is a non-quantitative method which provides limited information on the concentration of the analyte targeted [20].

Table 0.1 A comparison of assays reported for detection of IL-6.

Assay	Sensitivity	Advantages	Drawbacks
ELISA	$< 1 \text{ pg/mL}$	Superior sensitivity	Intensive resources required
Western blot	N.A.	Good selectivity	Non-quantitative assay
Electrochemical immunoassay [15]	1 pg/mL	Label-free detection Good sensitivity	Sophisticated procedure needed
Fluorescence-based immunoassay [16]	20 pg/mL	Simple assay design	Labelling required
SPR immunoassay [17]	700 pg/mL	Label-free detection Rapid response	Low sensitivity

4.2 Experimental Methods

4.2.1 Synthesis

The quartz substrates were firstly sonicated in acetone, isopropyl alcohol and deionized water for 5 min each to remove surface contaminants [21]. The substrates were then annealed with a 4 h ramp from room temperature, held at 850 °C for 8 h before cooling down to room temperature in 4 h. To fabricate the Polydimethylsiloxane (PDMS) stamps utilized for micro-contact printing, siloxane was cured over a silicon mask with the desired pattern [22]. The Cobalt(II) acetate tetrahydrate catalyst was dissolved in absolute ethanol solution at a concentration of 0.4 mg/ml and applied on the quartz substrate using the PDMS stamp. The catalyst, which was first deposited onto a flat piece of Si, was deposited on the quartz surface using the stamp. More specifically, the stamp was brought into conformal contact with the substrate and kept for 2 min. To optimize the horizontal alignment of CNT, the catalyst was deposited in a direction perpendicular to the growth direction of CNT. Finally, the stamp was removed and the catalyst-deposited substrate was dried naturally. The catalyst-coated substrates were heated up to 850 °C under a mixture flow of Ar (200 sccm) and H₂ (100 sccm) and kept for 5 min to allow oxidation of catalysts, which were then reduced to pure Co at 925 °C under a mixture flow of Ar (40 sccm) and H₂ (20 sccm) for 10 min.

4.2.2 Characterization

To investigate the horizontal alignment of CNTs produced, samples were examined using FESEM and the result is shown in Figure 4.2a. It can be observed that CNTs in good horizontal alignment were grown on quartz substrates by the methodology proposed. Devices based on horizontally aligned CNT exhibited improved transconductance, sensitivity and ultimately the overall performance as biosensors [23] as it has been revealed that good horizontal alignment can significantly reduce the tube-to-tube contact resistance [24], which is one of the key problems associated with random networks of carbon nanotubes.

Then, samples were characterized by Raman Spectroscopy and the result is shown in Figure 4.2b. The SWCNT samples obtained were characterized by a confocal Raman spectroscopy (WITec Alpha 300 R) employing a Nd:YAG 532 nm laser at 30 μW with a spot size of $\sim 1 \mu\text{m}^2$ and a CCD detector with 3 cm^{-1} resolution. The laser was focused using a $20\times$ Nikon air objective ($\text{NA} = 0.4$). As can be seen, the peak at 207 cm^{-1} is associated with the quartz substrate [25], while the peak at 359 cm^{-1} is associated with SWCNT [26].

Figure 0.2 (a) FESEM image of horizontally aligned CNT on quartz; (b) Raman spectrum of horizontally aligned CNT on quartz substrate (The peak at 207 cm^{-1} corresponds to the quartz substrate and the peak at 359 cm^{-1} is associated with SWCNT).

Figure 0.3 (a) AFM image of horizontally aligned CNT on quartz and (b) its corresponding section analysis.

Additionally, samples were examined using AFM. Figure 4.3 a and b show the AFM image of the nanotubes grown on quartz substrates and its corresponding section analysis, respectively. According to Figure 4.3a, the density of nanotubes on the quartz surface is approximately 3-5 tubes/ μm , which is comparable to other protocols reported previously [25]. The section analysis shown in Figure 4.3b reveals that the diameter of nanotubes generated ranges from 1.7 nm to 2.0 nm, while the average tube diameter is measured to be 1.9 nm.

Figure 4.4 (a) and (b) show the I_d vs. V_g at a drain voltage of 10 mV and I_d vs. V_d at different gate voltages (- 400 mV, - 300 mV, - 200 mV and - 100 mV), respectively. As observed from Figure 4.4 (a), the device shows a typical semiconducting behavior in the range of - 600 mV to 0 mV, indicating good functionality as a transistor [27]. Additionally, the linear relation between I_d and V_d illustrates Ohmic contacts between the carbon nanotubes and the Au electrodes.

Figure 0.4 (a) I_d vs. V_g at a fixed bias voltage (10 mV); (b) I_d vs. V_d at different V_g (ranging from - 400 mV to - 100 mV).

4.3 Principle Outcomes

4.3.1 Kinetic Measurements

Figure 0.5 (a) Kinetic measurements of IL-6 (concentrations ranging from 1 pg/mL to 100 pg/mL) by the proposed sensor; (b) average I_d responses of devices from different batches (n>3).

Figure 4.5 shows the results of kinetic measurements of IL-6 using the proposed immunosensor. Herein, the normalized I_d was defined as:

$$\frac{I_d \text{ at different } V_g}{I_d \text{ at } V_g = 0 \text{ mV}} \times 100\% \quad 0.1$$

As shown in Figure 4.5a, I_d remained unaffected upon rinsing with buffer solution but dropped drastically upon injection of IL-6. More importantly, the I_d response increased with concentration of IL-6 in the testing solution in a logarithmic pattern, as shown in Figure 4.5b. The results suggested that the as-prepared CNT-FET devices are capable of quantitatively measuring IL-6 via the interaction of this molecule with its corresponding antibody (IL-6R in this case). LOD calculated was 1.37 pg/mL, which is significantly lower than the circulating level of IL-6 (10-12 pg/mL [28]) and comparable to assays previously reported such as ELISA and electrochemical assays.

4.3.2 Selectivity

The selectivity of as-prepared devices was evaluated by tracking I_d of the immunosensor in response to 100 pg/mL BSA and 100 pg/mL cysteine.

As shown in Figure 4.6, the I_d responses triggered by 100 pg/mL BSA and 100 pg/mL cysteine were 2.42% and 2.15% respectively, while the responses triggered by 1 pg/mL and 100 pg/mL IL-6 were 4.05% and 18.7% respectively. Neither BSA nor cysteine triggered significant responses compared with IL-6, indicating excellent selectivity of the proposed immunosensor. The superior selectivity can be attributed to the highly specific interaction between IL-6 and its antibody, as well as successful surface passivation prior to kinetic measurements.

Figure 0.6 I_d responses of the proposed immunosensor to 100 pg/mL BSA and 100 pg/mL cysteine as compared to responses triggered by IL-6 ($n > 3$).

4.3.3 Serum test

Figure 0.7 Measurements of IL-6 in diluted serum using the proposed assay ($n>3$).

Measurements of IL-6 in serum (rabbit serum diluted with PBS by 100 times) were achieved using the developed devices and the results are shown in Figure 4.7. As can be seen, I_d responses triggered by IL-6 in serum were detectable and correlated to the concentration of IL-6. The results revealed that detections of IL-6 by the proposed immunosensor is highly specific, thus exhibiting a great potential for point-of-care applications.

4.3.3 Stability

Additionally, the stability of the developed devices stored under ambient conditions (25 °C and atmosphere pressure) was evaluated. More specifically, the electrical performances (I_d vs. V_g) of as-prepared devices being stored for different durations were

recorded and compared with freshly prepared devices. As shown in Figure 4.8, ΔI_d in response to the same variation of V_g (- 600 mV to 0 mV) were 15 %, 14.2 %, 13 % and 12 % for devices after storage for 1 day, 1 week, 1 month and 3 months, respectively. As observed, the degradation of sensitivity (in terms of I_d vs. V_g) of the device developed dropped by approximately 20 % after storage under ambient conditions for 3 months. Therefore, it can be concluded that the CNT-FET devices developed by the proposed assay exhibit no significant performance degradation after storage under ambient conditions for up to 3 months, indicating excellent stability of these devices. The good stability under ambient conditions makes the proposed device a promising sensing platform for practical applications.

Figure 0.8 I_d vs. V_g of devices after storage under ambient conditions for different durations (1 d, 1 week, 1 month and 3 months).

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Chapter 5

Detection of PLA₂ Using POPC Liposomes and rGO Based FET *

This chapter introduces detection of PLA₂ using rGO-based FET sensor with liposomes as the recognition element. The results demonstrated that protein detection using rGO-based FET sensor with liposomes as the recognition element was viable and the sensing performance was comparable with assays using antibodies as the recognition element in terms of sensitivity and specificity. More specifically, rupture of reporter-encapsulated liposomes triggered by target molecules introduces changes of I_d , which is a detectable signal. The LOD achieved was 80 pM, which is superior to most assays reported previously and much lower than the cut-off level of circulating secretory PLA₂. The signal enhancement effect realized by the excess concentration of TNP in liposomes is believed to be the main reason for the significantly enhanced sensitivity of the proposed assay, indicating great potential for further improvement in the sensitivity by increasing the reporter concentration..

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5.1 Introduction

A novel approach for enzymatic assay using reporter-encapsulated liposomes on graphene FET is proposed. This approach involves real time monitoring of I_d responses of the rGO-FET upon rupture of reporter-encapsulated POPC liposomes triggered by enzymes. In virtue of its excellent chemical stability [1] and benzene ring structure, which enables its stacking on rGO via π - π interactions [2, 3], TNP is used as reporter for specific detection PLA₂, a key enzyme in various membrane related physiological processes. PLA₂-triggered rupture of liposomes with TNP molecules leads to the release of these molecules, which subsequently adsorb on rGO, thereby modulating the conductance of rGO. A schematic of the enzymatic assay proposed is shown in Figure 5.1.

Figure 0.1 Schematic illustration of reporter-encapsulated liposomes on rGO field effect transistor for PLA₂ detection. TNP molecules released from the liposomes stack on the graphene surface via π - π interaction.

Experimental results revealed that I_d increased with PLA₂ concentration and this can be attributed to the presence of electron withdrawing NO₂ groups in these TNP molecules. In virtue of the signal enhancement effect realized by the excess concentration of TNP in liposomes, the LOD achieved by the proposed approach was 80 pM, which is superior to most assays reported previously and much lower than the cut-off level of circulating secretory PLA₂ (2.07 nM). The sensitivity may be further improved by increasing the

concentration of TNP. Besides the high accuracy of electronic detection methodology, the signal enhancement effect realized by the excess concentration of TNP (approximately 1 mM) in liposomes is believed to be the main reason for the significantly enhanced sensitivity of the proposed assay, indicating a great potential for further improvement in the sensitivity by increasing the concentration of TNP. In addition, the proposed approach is rapid (incubation time ≤ 10 min) and label-free, thus showing a great potential for *in situ* measurements. Furthermore, to the best of our knowledge, this is the first report on an electronic platform for enzymatic assays using reporter-encapsulated liposomes.

PLA₂ is a superfamily of enzymes that degrade phospholipids to produce free fatty acids and lysolipids, achieved by cleavage of the sn-2 acyl ester bond in glycerophospholipids [4]. Dysregulation of PLA₂ can be an indicator for various pathological conditions including atherosclerosis [5], acute sepsis [6] and certain forms of cancers [7]. Owing to its diverse biological roles, PLA₂ has been targeted by various assays. As shown in Table 5.1, commercially available ELISA sensors may not be ideal candidates for practical applications as they are resource intensive (expensive equipment and well-trained personnel are typically required) and can only provide limited information on the enzyme activity, in spite of its excellent sensitivity [8]. Therefore, Aili *et al.* reported a colorimetric approach for the detection of phospholipase concentration and activity using Au nanoparticles (AuNP) and polypeptides [9]. In this approach, naked eye detection is achieved (no measuring equipment required), while the lag time is relatively long (around 20 min). Another assay reported involved hybrid nano-constructs as label-free optical probes for SPR-based detection of PLA₂ [10]. In this approach, the specificity is excellent and the assay time is significantly reduced (less than 2 min), while the sensitivity is relatively low (LOD = 1.82 nM). Fluorescent-based detections of phospholipases using dye-encapsulated liposomes have also being reported previously [11]. The fluorescence from dyes is quenched drastically when encapsulated at high concentrations within liposomes [12]. Nevertheless, these assays are limited by the cross-talk between different dye molecules, yielding a relatively poor spectral discrimination of the fluorescence emission [13]. Moreover, sophisticated instrument such as the fluorescence spectrometer

is required for these assays, in contrast to the proposed methodology that could be miniaturized in form of a small electronic circuitry.

Table 0.1 A comparison of assays reported for detection of PLA₂.

	Advantages	Limitations
ELISA	Ultra-high sensitivity	Resource intensive nature Limited information on enzyme activity
Colorimetric assay	Naked eye detection	Long lag time (≈ 20 min)
SPR-based assay	Lag time free (< 2 min)	Low sensitivity (1.82 nM)

5.2 Experimental Methods

5.2.1 Synthesis

TNP and PLA₂ were purchased from Sigma-Aldrich Inc. and used without further treatment. POPC was purchased from Avanti Polar Lipids and the POPC liposomes were fabricated by a self-assembly method reported elsewhere [14, 15].

The dried POPC films obtained from 5 mg/mL POPC were rehydrated with 1.0 mL of 1 mM TNP in PBS. This is followed by multiple extrusion cycles (20 times) through the membranes with 200 nm pores. Non-encapsulated TNP molecules were removed by centrifugation with an ultracentrifuge (Sorvall Legend Micro 21 R, provided by Thermo Scientific) for 20 min at 10000 g at 15 °C [16]. After removal of the supernatant, the residual liposomal pellet was re-suspended in 1 mL of phosphate-buffered saline (PBS), and the centrifugation was repeated for another three times. The final liposomal pellet was re-suspended in 0.5 mL of PBS to obtain a liposome solution with a concentration of 13.2 mM. The size distribution and polydispersity of liposomes fabricated were checked by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZS (Malvern, UK). A 20 $\times$ diluted liposome sample (in PBS) was measured at a fixed scattering angle of 173 °; three measurements, each consists of 3 runs of 30 s duration, were recorded at room temperature.

5.2.2 Characterization

FESEM image of rGO grown on SiO₂ substrate is shown in Figure 5.2. The bright regions are SiO₂ substrate while the dark regions are deposited rGO flakes.

Figure 0.2 FESEM image of rGO grown on SiO₂ substrate. The bright regions are SiO₂ substrate while the dark regions are deposited rGO flakes.

Figure 0.3 (a) I_d vs. V_g at $V_d = 10$ mV; (b) I_d vs. V_d at different V_g .

Figure 5.3 (a) and (b) show the I_d vs. V_g at $V_d = 10$ mV and I_d vs. V_d at different V_g , respectively. As observed, the device exhibits typical semiconducting behaviors in the range from -600 mV to 0 mV, indicating its good functionality as a transistor. Meanwhile, the linear relation between I_d and V_d illustrated that the contact between the rGO films and the Au electrode was a good Ohmic contact.

The size distribution and polydispersity of liposomes fabricated were checked by DLS and the results are shown in Figure 5.4(a). As can be seen, both liposomes (with and without TNP) exhibited narrow size distributions (the polydispersity index was measured to be 0.195 and 0.21 for liposomes with and without TNP, respectively), indicating good monodispersity of both liposomes [17]. The average sizes of liposomes with and without TNP were measured to be 188 and 179 nm, respectively, indicating that encapsulation of TNP has no significant effect on the self-assembly process of POPC liposomes. Additionally, both liposomes remained stable for at least 5 d at room temperature as no significant aggregations were observed, which is consistent with previous reports [18]. The effect of TNP physisorption on the conductance of proposed rGO-FET was investigated in order to validate the role of TNP in this assay. As shown in Figure 5.4(b), I_d increased proportionally with concentration of TNP (up to 100 μ M), which is in agreement with previous reports [2]. This suggests that the interaction between TNP at micro-molar levels and rGO is detectable by the device proposed.

Figure 0.4 (a) Size distribution of liposomes with and without TNP obtained by DLS; (b) effects of TNP physisorption on the conductance of rGO-FET.

5.3 Principal Outcomes

5.3.1 Kinetic Measurements

Since TNP at 1 mM leads to a significant I_d response (1.2% increase in I_d), the concentration of TNP used for encapsulation process was set to be 1 mM. Kinetic measurements of PLA₂ were conducted and the results are shown in Figure 5.5(a). 10 min incubation in mixed solutions of liposomes (concentration = 0.5 mg/mL) and PLA₂ resulted in a detectable increase in I_d , indicating that PLA₂ is active and yields detectable I_d responses. More importantly, the I_d response increased with concentration of PLA₂ in the mixed solution in a logarithmic pattern up to 10 nM, as shown in Figure 5.5(b), indicating that the concentration of PLA₂ can be measured quantitatively by the proposed methodology.

Figure 0.5 (a) Kinetic measurements of PLA₂ at different concentration levels; (b) I_d response of rGO devices vs. PLA₂ concentration in kinetic measurements.

The LOD obtained was 80 pM, which is comparable to most PLA₂ assays reported previously. Furthermore, the proposed assay can be used for detection of PLA₂ at clinically relevant concentration ranges as the cut-off level of circulating secretory PLA₂ is 2.07 nM [19]. Besides the high accuracy of electronic detection method, the enhanced sensitivity of the proposed methodology can be attributed to the signal enhancement effect by the excess concentration of TNP (approximately 1 mM, provided no significant leakage occurs) in liposomes. In virtue of the excess concentration of TNP encapsulated

in the liposomes, a considerable quantity of reporter is released upon rupture of a small amount of liposomes, resulting in significantly enhanced signals.

5.3.2 Specificity

Two control experiments were conducted to investigate the effects of the enzyme and liposome on the rGO-FET responses. Liposomes without TNP were incubated with active enzymes (denoted as control group A) and liposomes with TNP were incubated with deactivated enzymes (denoted as control group B). The deactivation was achieved by heating the enzymes at 45 °C for 30 min. As shown in Figure 5.6, negligible I_d responses as compared to the responses in kinetic measurement were obtained for both control measurements (0.01 % and 0.02 % at 10 nM of PLA₂ for control groups A and B respectively, while that for the kinetic measurement was 0.55 %), indicating that neither the PLA₂ nor the POPC liposomes significantly influences the sensor response. Furthermore, liposomes with TNP in control group B led to negligible I_d responses, indicating that these liposomes are stable (no leakage of TNP) in the absence of active enzymes. A comparison between I_d responses in control measurements and that in the kinetic measurement indicates ultra-high signal-to-noise ratios (defined as the ratio of normalized I_d in kinetic measurement to normalized I_d in control measurements at the same PLA₂ level) of the proposed assay. For instance, signal-to-noise ratio at 10 nM of PLA₂ is 50. Despite the slight decreases in I_d (as observed in control measurements) due to non-specific binding of liposomes and/or enzymes [20], the control measurements confirmed that the signal was indeed generated by the interaction between rGO and TNP. Additionally, control group B suggested that activity of PLA₂ as an enzyme is detectable by the proposed methodology.

Figure 0.6 Real time I_d responses of control groups A (liposomes without TNP + active enzymes) and B (liposomes with TNP + deactivated enzymes).

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Chapter 6

Detection of MMP-7 Using Synthetic Polypeptides and rGO Based FET *

This chapter presents detection of MMP-7 using rGO-based FET sensor with polypeptides as the recognition element. The results revealed that protein detection using rGO-based FET sensor with liposomes as the recognition element was viable and the sensing performance was comparable with assays with antibodies as the recognition element in terms of sensitivity and specificity. Chapter 5 and 6 indicated that protein detections can be achieved by rGO-based FET sensor in combination with different synthetic receptors.

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6.1 Introduction

A novel assay for rapid (lag time < 5 min) and sensitive detection of MMP-7 using rGO-FET functionalized with *de novo* designed synthetic polypeptide (JR2EC, sequence shown in Figure 6.1) is reported [1].

Figure 0.1 The working principle of the proposed assay. The presence of target molecules (MMP-7) leads to cleavage of polypeptides immobilized on the rGO surface, resulting in reduced size and net charge of the immobilized polypeptides. Therefore, decreases in I_d could be detected. The cleavage sites for MMP-7 are the 11th and the 26th amino acids.

Consisting of 42 amino acids, this polypeptide exhibits two recognition and digestion sites (Ala-Leu and Ala-Gln-Leu) for MMP-7 [2]. Therefore, MMP-7 can specifically digest negatively charged JR2EC immobilized on rGO, thereby modulating the conductance of rGO-FET. More specifically, the hydrolysis of JR2RC facilitated by MMP-7 leads to reduction of size and net charge (from -5 to -1, as shown in Figure 6.2) of the polypeptide [3]. As a result, I_d of the rGO-FET device decreased drastically and the change in I_d could be readily detected in real time. It was observed that the change in I_d is dependent on the concentration of MMP-7, thus enabling a quantitative monitoring of the concentration of this protease. The principle of the proposed assay is illustrated in Figure 6.1. The LOD achieved for MMP-7 in buffer was 10 ng/mL (400 pM), which was one order of magnitude higher than the LOD of the colorimetric detection based on gold nanoparticles functionalized with the same polypeptide [3]. Indeed, the sensitivity of the proposed assay is comparable to ELISA assay [4] and optical assays (e.g., surface enhanced Raman scattering-based assay [5] and the fluorescence-based assay [6]). Also, a LOD of 40 ng/mL was achieved upon detection of MMP-7 in human plasma.

Additionally, this assay exhibited excellent specificity as matrix metalloproteinase 1 (MMP-1), a protease of the same family, did not trigger any significant changes in I_d as compared to MMP-7. In virtue of these advantages, the proposed assay shows a great potential for development into a diagnostic tool used for tumor detection, cancer diagnostic and other point-of-care applications.

Figure 0.2 Electrostatic potential distribution of JR2RC before and after digestion by MMP-7. The electrostatic potential of polypeptide was calculated at pH = 7 rendered using the software spdbv (version 4.10). It is obvious that the negative potential (red color) decreases significantly after the digestion.

Proteases (see Figure 6.3) are enzymes involved in the digestion of long protein chains into shorter fragments (a process known as proteolysis) by splitting the corresponding peptide bonds. Due to their significant roles in numerous physiological processes and involvement in several pathological conditions, proteases have attracted considerable attention recently. In particular, matrix metalloproteinases have been intensively studied over past decades as their dysregulations can be related to cancers [7, 8], AIDS [9], inflammation [10], *etc.* Discovered in 1988 [11], MMP-7 was found to be involved in the degradation of various macromolecules [12]. This molecule plays a key role in the regulation of lung defence [13] and acts as an indicator of certain cancers (e.g., lung cancer and salivary gland cancer) [14]. Also, it is believed that MMP-7 contributes to invasive growth and metastasis of tumors and carcinomas [15, 16]. Due to its various roles in physiological processes, MMP-7 has attracted intensive attention and various sensing approaches have been developed. Currently, sensing approaches for MMP-7

include western blot analysis [17], ELISA [18] and Förster resonance energy transfer (FRET) analysis [19]. Nevertheless, these approaches have their respective limiting factors: ELISA suffers from limited throughput due to its resource intensive nature [20]; western blot analysis is a non-quantitative method which provides limited information on analyte concentration; FRET are limited by concerns such as photo-bleaching of probes and toxicity of quantum dots [3]. Chen *et al.* described a colorimetric detection of MMP-7 based on polypeptide functionalized gold nanoparticles [3]. In this assay, AuNPs were functionalized with synthetic polypeptides containing recognition/cleavage sites for MMP-7. Cleavage of the polypeptides induced nanoparticle aggregation and a concomitant color shift that enabled a direct and quantitative measurement of the concentration of MMP-7. However, the LOD achieved by this assay was 5 nM, approximately equivalent to the critical level of MMP-7 related to salivary gland cancer [14].

Figure 0.3 Schematic structure of MMPs family [21].

6.2 Experimental Methods

6.2.1 Synthesis

MMP-7 was purchased from Merck Pte Ltd, Singapore and used without further purification. Pyrene maleimide and all chemicals used for rGO preparation were obtained from Sigma Aldrich Inc.. Human plasma (GTX73265, normal human plasma) was purchased from Genetex Inc., USA.

The polypeptides were synthesized as reported previously [22, 23] and the electrostatic potential of the polypeptides was calculated at $\text{pH} = 7$. More specifically, the polypeptides were synthesized on an automated peptide synthesizer using standard fluorenylmethoxycarbonyl (Fmoc) chemistry with O-(7-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU, Alexis Biochemicals) as the activating reagent. The synthesis was performed on a 100 μmol scale with an Fmoc-Gly-PEG-PS resin, and a four-fold excess of amino acid was used in each coupling. The resin was washed with 20 mM diethyldithiocarbamic acid in dimethylformamide (DMF), 30 mM diisopropylethylamine (DIPEA) in DMF, DMF and dichloromethane (DCM), respectively, followed by desiccation. Peptides were cleaved from the resin by incubation with a mixture of trifluoroacetic acid (TFA), ethanedithiol, water, and triisopropylsilane (94: 2.5: 2.5: 1, v/v/v/v) for 2 h at room temperature. After filtration and evaporation of TFA, polypeptides were precipitated (upon addition of cold diethyl ether), centrifuged, re-suspended in diethyl ether, and lyophilized. The products were then purified by reversed-phase high performance liquid chromatography (HPLC) [24] and peptides were eluted with 0.1% TFA and 30% – 50% aqueous 2-propanol.

6.2.2 Characterization

rGO samples prepared by the method proposed were examined by FESEM and AFM. Figure 6.4 shows the FESEM image of the rGO flakes deposited on the Si/SiO₂ substrate. The bright regions are the substrate, while the dark regions are deposited rGO flakes. Figure 6.5 shows the AFM image of the GO flakes and the corresponding section

analysis. As observed, the thickness of GO flakes on the substrate was ~ 1.2 nm, which is consistent with the thickness of GO monolayer reported elsewhere [25].

The immobilization of JR2EC with pyrene maleimide as linker molecule was investigated using two control groups. For Group I (red), the rGO-FET device was passivated with PEG and incubated with JR2EC directly. For Group II (blue), the rGO-FET device was incubated with pyrene maleimide, passivated with PEG and then incubated with JR2EC. As shown in Figure 6.6a, the device in Group II exhibited a significant I_d response as compared to that of device in Group I, demonstrating successful immobilization of JR2EC with pyrene maleimide as linker molecule. Figure 6.6b shows the transfer characteristics (I_d vs. V_g) of the rGO-FET devices before and after polypeptide functionalization. As observed, device before polypeptide functionalization exhibited a typical semiconductor behavior and polypeptide-functionalized device generated an I_d - V_g curve similar to that of non-functionalized device, demonstrating that both polypeptide-functionalized and non-functionalized devices showed typical FET responses.

Figure 0.4 FESEM image of the rGO flakes deposited on the substrate. The bright regions are the substrate, while the dark regions are the deposited rGO flakes.

Figure 0.5 AFM image of GO flakes deposited on the substrate and the corresponding section analysis. The thickness was ~ 1.2 nm, which is consistent with the thickness of GO monolayer reported previously [25].

Figure 0.6 (a) I_d responses of the proposed rGO-FET in two control groups. In Group I (red), the PEG-passivated rGO-FET device was incubated with JR2EC; in Group II (blue), the rGO-FET device was incubated with pyrene maleimide, PEG and JR2EC consecutively (Error bars refer to the standard deviations of data collected on n devices, $n > 3$); (b) transfer characteristics (I_d vs. V_g) of the rGO-FET devices before and after polypeptide functionalization.

6.3 Principal Outcomes

6.3.1 Kinetic Measurements

For kinetic measurements in buffer, solutions with MMP-7 at different concentrations were measured using the method described previously., I_d decreased as a result of

incubation with MMP-7 (see Figure 6.7a) and the decreasing magnitude increased exponentially with the concentration of MMP-7 (see Figure 6.7b).

Figure 0.7 (a) Kinetic measurements of MMP-7 at different concentrations (0.01 $\mu\text{g/mL}$, 0.05 $\mu\text{g/mL}$, 0.1 $\mu\text{g/mL}$, 0.5 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$) in buffer; (b) I_d response of rGO device vs. MMP-7 concentration in buffer tests using devices from different batches ($n>3$). Error bars are the standard deviation of at least three repetitive experiments.

The results indicated that MMP-7 could be quantitatively measured by the proposed methodology. The LOD achieved was 10 ng/mL (400 pM), which is one order of magnitude higher than the LOD achieved by the colorimetric approach reported [3]. The excellent sensitivity could be attributed to the significantly reduced net charge carried by the immobilized polypeptides (from 5 negative charges per peptide to 1 negative charge per polypeptide, at pH 7), as well as reduction of polypeptide size. Indeed, the reduction of charges leads to a reduction of electrostatic potential/gate voltage in the FET device.

6.3.2 Serum Test

Besides kinetic measurements in buffer, detections of MMP-7 of different concentrations in human plasma were also demonstrated using the proposed assay. The results indicated that I_d decreased as a result of incubation with MMP-7 (Figure 6.8a) and the response increased exponentially with the concentration of MMP-7 (Figure 6.8b). The LOD of plasma test was 40 ng/mL. Sensitivities of the proposed assay for detection of MMP-7 in both buffer and human plasma are higher than that of the colorimetric approach reported

previously [3] and comparable to ELISA and optical assays [4-6]. Additionally, LODs achieved by the assay proposed are within clinically relevant ranges (e.g., 0.1~5 $\mu\text{g/mL}$ for salivary gland cancer and 1~10 ng/mL for bladder cancer patients). Therefore, the proposed assay shows a great potential for applications in tumor detection and carcinoma diagnostic.

Figure 0.8 (a) Kinetic measurements of MMP-7 at different concentrations (0.01 $\mu\text{g/mL}$, 0.05 $\mu\text{g/mL}$, 0.1 $\mu\text{g/mL}$, 0.5 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$) in human plasma; (b) I_d response of rGO device vs. MMP-7 concentration in plasma tests using devices from different batches ($n>3$). Error bars are the standard deviation of at least three repetitive experiments.

6.3.3 Control Experiments

Three control experiments were also designed to validate the mechanism of the assay proposed. The results are shown in Figure 6.9. In the first experiment (control group A), no polypeptides were immobilized on rGO prior to device incubation with MMP-7 in order to investigate the non-specific binding of MMP-7 on rGO films. No significant I_d responses were triggered by MMP-7 in the absence of polypeptides, demonstrating that non-specific binding is minimized by surface passivation using PEG. In the second experiment (control group B), the polypeptide-functionalized rGO devices were incubated with deactivated (realized by heating at 100 $^{\circ}\text{C}$ for 10 min) MMP-7. The device showed negligible responses to deactivated MMP-7, indicating that the proposed assay is capable of providing essential information on the activity of MMP-7. In the third experiment (control group C), the polypeptide-functionalized rGO devices were

incubated with MMP-1 to investigate the specificity of the polypeptide proposed. Negligible I_d responses as compared to the responses in kinetic measurements were observed, demonstrating that the polypeptide is specifically digested by MMP-7.

In virtue of the robustness of the synthetic polypeptides used and possible miniaturized structure (a portable electronic circuitry) of the FET platforms, the proposed assay could be employed for point-of-care diagnostic applications. It is also envisioned that the proposed approach could be extended for detections of other polypeptide-digesting enzymes upon appropriate selection or modification of polypeptides.

Figure 0.9 I_d responses to three control groups as compared to that of kinetic measurements: Control group A = non-functionalized rGO device incubated with active MMP-7; Control group B = JR2EC functionalized rGO device incubated with deactivated MMP-7; Control group C = JR2EC functionalized rGO device incubated with MMP-1. (Error bars are the standard deviation of at least three repetitive experiments).

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Chapter 7

Bio-Sensing Using D-AptaTRPS

This chapter proposes an aptamer sensor using a Dipstick and Tunable Resistive Pulse Sensing (D-AptaTRPS) for quantitative protein detection. Detection of cTnI, which is a key myocardial infarction biomarker, in buffer and human serum were demonstrated. The format of the stick allows rapid and affordable replicate measurements on the same sample to enhance assay reliability. The presence of the target protein caused a conformational change to the aptamer and the release of immobilized nanoparticles into solution, and their concentration, counted by TRPS, was proportional to the concentration of the target protein. The LOD achieved was 6.7 ng/mL in buffer and 16.7 ng/mL in serum. Also, the proposed assay can be easily extended to multiplexed biomarker detection by involving beads of different sizes, with each targeting one specific protein..

7.1 Introduction

An aptamer sensor using a Dipstick and Tunable Resistive Pulse Sensing (D-AptaTRPS) for rapid (≤ 10 min, see Table 7.1) and label free detection of cTnI, a myocardial infarction biomarker, is proposed (see Figure 7.1). The cTnI aptamers were immobilized on a slide (dipstick) which enabled the immobilization of nanoparticles (SPBs) modified with partial complementarity sequences. The presence of the target protein caused a conformational change to the aptamer and the release of the immobilized nanoparticles into solution, and their concentration, counted by TRPS, was proportional to the concentration of the target protein. Optimization of the partial complementary sequence produces an assay that was reliable and easy to setup with a LOD of 6.7 ng/mL (in buffer), which is comparable to assays previously reported. More importantly, quantitative detection of cTnI in human serum using the proposed assay has also been demonstrated and the sensitivity (LOD = 16.7 ng/mL) indicates great potential of the proposed assay for clinical applications. The format of the stick allows rapid and affordable replicate measurements on the same sample to enhance assay reliability. In addition, the proposed assay can be easily extended to multiplexed biomarker detection by involving beads of different sizes, with each targeting one specific protein.

Table 0.1 Detection time of different cTnI assays.

Detection method	Detection time	References
Fluorescence microscopy	120 min	[1]
Electrochemical impedance spectroscopy	30 min	[2]
Colorimetric assay	30 min	[3]
Microfluidic immunoassay	15 min	[4]
Förster resonance energy transfer	10 min	[5]
AptaTRPS	≤ 10 min	Present work

Figure 0.1 Schematic illustration of D-AptaTRPS proposed. Biotin-modified aptamers were immobilized on a glass slide using avidin as the linker and SPBs functionalized with DNA sequences that are complementary to the aptamers were then attached to slide. In the presence of target protein molecules, SPBs immobilized on the glass slide were released into the solution due to the higher binding affinity of cTnI towards the aptamers. Afterwards, the solution containing free SPBs was collected and measured using TRPS.

Nanopore systems based upon the Coulter technology have seen resurgence over the past decades [6, 7]. Denoted as resistive pulse sensing (RPS), these sensing platforms are advantageous as information on individual particles within their natural environment can be provided [8]. Indeed, RPS has exhibited high resolution for the monitoring numerous biological particles, protein molecules [9] and metallic nanoparticles [10]. TRPS, a recent adaptation to RPS, has attracted increasing attention due to a further versatility [11]. Herein, the conical tunable elastomeric pore on a polyurethane elastomeric membrane

used in this system can be mechanically stretched in real time according to conditions of different samples [12]. Determination of the concentration, size, and surface charge of dispersed inorganic particles using TRPS has been demonstrated [6, 10, 13]. As powerful reagents that bind target ligands with affinities comparable to antibodies [14, 15], aptamers have been used to modify particle surface in TRPS assays. Information on size and surface charge of a particle can be obtained simultaneously by analyzing the pulse in response to its movement through the pore [16-19]. Myocardial infarction (commonly known as heart attack) has been regarded as one of the top killing diseases [20] as it may partially or completely disable the pumping function of the heart, resulting in death or physical disability [21]. Therefore, early diagnosis of myocardial damages is of great significance and has attracted increasing attentions [22]. Among various cardiac biomarkers reported, cTnI has been intensively studied [23-25]. This protein is a cardiac muscle one that can form a heteromeric protein complex (known as troponin, which plays a key role in contraction of skeletal and cardiac muscles) together with troponin T and troponin C [26]. In cases of myocardial damages, the troponin proteins are released into the bloodstream and a significant increase of the cTnI circulating level in blood can be detected in the blood after 4-8 h [27]. To date, various cTnI detection assays, including ELISA [28], colorimetric assays [29], and luminescence assays [30], have been reported. Nevertheless, these assays exhibit different limiting factors: ELISA suffers from limited throughput due to its resource intensive nature (sophisticated instrument and well-trained personnel are typically required), colorimetric assays are not highly sensitive in some cases (compared with other sensing assays) [31], and optical assays are usually limited by poor specificity due to non-specific binding issues (thus not an ideal candidate for real sample applications) [32].

7.2 Experimental Methods

DNA aptamers against cTnI [33] and their complementary sequences (full sequence shown in Table 3.1) were purchased from Thermo Fisher Scientific UK and Sigma Aldrich UK, respectively; their full sequences are listed in Table S1. cTnI was purchased from Abcam Ltd. (Cambridge, UK). 125 nm streptavidin-modified SPBs were purchased

from Ademtech (Pessac, France) and used without further purification. The qNano system for TRPS measurements and SPBs of known concentration and size were sourced from Izon Science. Avidin (A9275) and BSA (A2153) were obtained from Sigma Aldrich UK. Appropriate Ethical and Trust approvals for carrying out research on human biological samples were sought from Peterborough & Stamford Hospitals NHS Foundation Trust. Human serum samples were collected and prepared at Peterborough City Hospital Pathology Laboratory, U.K.. Briefly, serum samples gathered using blood from a healthy volunteer donor were collected into Sarstedt monovette/collection tubes, followed by centrifugation (3000 rpm, 6 min). The supernatants from each sample were transferred into separate vials and stored at - 80 °C.

7.2.1 DNA Modification of SPB

A solution containing streptavidin-modified SPBs (150 µL, concentration = 1×10^{10} particles/mL) were mixed with solutions containing biotin-modified DNA sequences that are complementary to aptamers (50 µL in buffer, concentration = 10 µM) and left for 30 min. DNA-functionalized SPBs were then isolated using a magnet and re-dissolved in buffer. The SPBs were examined using TRPS before and after DNA functionalization (complementary sequences A and B, as a demonstration) to determine changes in their diameters and surface charges.

7.2.2 Preparation of the Dipstick and Assay

Aptamer-immobilized slides were incubated with DNA-functionalized SPBs for overnight (> 12 h) followed by rinsing with buffer for three times. Afterwards, SPB-immobilized slides were incubated with 50 µL of cTnI at different concentrations (100 ng/mL, 1 µg/mL and 10 µg/mL) for 6 h. The initial solution of equal volume (50 µL) was collected in a centrifuge tube and the chamber was washed with buffer (40 µL), which was added into the tube. A 90 µL solution was obtained. For detections of cTnI in human serum, free SPBs collected after protein incubation were isolated and re-dissolved in

buffer of equal volume. As a control group, SPB-immobilized slides were incubated with 50 μ l of BSA solution at 100 μ g/ml for a comparable time period.

7.3 Principal Outcomes

7.3.1 Verification of SPB Modification

The first step required surface of SPBs to be functionalized with complementary DNA. The velocities of SPBs were measured before and after incubation with complementary sequences to validate DNA functionalization. Figure 7.2 shows particle velocities of SPBs, which have been demonstrated to be proportional to their surface charges, before and after DNA functionalization. As can be seen, the velocities of SPBs increased in response to functionalization by both complementary sequence A and complementary sequence B, indicating effective DNA immobilization on SPB surface. Notably, the velocity of SPBs functionalized with complementary sequence A were higher than that of SPBs functionalized with complementary sequence B. This can be attributed to the fact that complementary sequence A consists of 19 bases, while complementary sequence B consists of 15 bases. This is consistent with previous studies and the increase in velocity is attributed to an increase in zeta potential of the beads.

Figure 0.2 Relative particle velocities at different blockage reference points for SPBs functionalized with complementary sequence A (half complementary) and complementary sequence B (first 15 bases complementary).

7.3.2 Selection of DNA Sequence

Figure 0.3 Predicted structure of cTnI aptamer (determined using mfold) with complementary sequences adjacent (red). Highlighted in blue are the primer regions, which were used in the development of the aptamer sequence.

Four DNA sequences with partial complementarity to the troponin aptamer used in this study: half-complementary sequences, labelled as complementary sequence A; first 15 bases complementary sequences, labelled as complementary sequence B; first 10 bases

complementary sequences, labelled as complementary sequence C; middle 10 bases complementary sequences, labelled as complementary sequence D.

Figure 7.3 depicts the predicted structure of the cTnI aptamer used and its interaction with different complementary sequences. The primer regions in the aptamer structure stayed invariant in the original SELEX process [34] and have not been optimized to interact with the target protein, despite their potential role in aptamer structure. Therefore, the primer regions were excluded in the design of complementary sequences. These sequences were selected due to their appropriate affinity to the aptamer. More specifically, complementary sequences in this case must be designed in a way that their affinity to the aptamer is slightly lower than the affinity of cTnI to the aptamer [35], but not so low that other proteins/particles may also displace them [36]. Therefore, sequences with less than ten complementary bases were excluded. On the other hand, sequences with over-high affinity to the aptamer should also be avoided as these aptamers might have higher affinity to the aptamer than cTnI does, which would lead to limited sensitivity of the assay proposed. Hence, sequences in which more than half of all bases are complementary to the aptamer were excluded.

7.3.3 Immobilization of SPBs on Aptamer Surface

The concentrations of DNA-modified SPBs before and after incubation with the aptamer modified slide were measured and the results are shown in Figure 7.4. The concentration of particles in solution was reduced from 1×10^{10} particles/mL to 1.5×10^9 particles/mL following this step, demonstrating successful SPB immobilization on aptamer-modified glass slides via the approach proposed. The mechanism of the proposed assay was validated by two control experiments. In the first experiment, SPB-immobilized slides were incubated with BSA (100 μ g/mL); no free SPBs were detected in the solution collected, demonstrating excellent specificity of the assay proposed. In the second experiment, SPBs were functionalized with DNA sequences with no complementarity to the aptamer used; no free SPBs were detected in the solution collected, indicating negligible non-specific binding of DNA-functionalized SPBs on glass slides.

Figure 0.4 Concentration of superparamagnetic beads modified with complementary sequence A and B before and after glass incubation.

7.3.4 Assay Measurement

The reaction chambers were incubated with different concentrations of cTnI. Figure 7.5 depicts real time SPB measurements using TRPS for different complementary sequences. Herein, SPB concentration in the solution is proportional to the quantity of particles detected in unit time. As we can see, all measurements showed good linear regression. Figure 7.6 shows the concentrations of free SPBs corresponding to different cTnI concentrations (100 ng/mL, 1 μ g/mL, and 10 μ g/mL, in buffer). The particle concentrations are calculated from the particle count rate, and calibrated against a particle of comparable size and known concentration [18].

Figure 0.5 Real time free SPB measurements using TRPS corresponding to different complementary DNA sequences: (a) half complementary sequences, (b) first 15 bases complementary sequences, (c) first 10 bases complementary sequences, and (d) middle 10 bases complementary sequences. All measurements showed good linear regression.

Detectable quantities of free SPBs were observed in all measurements, suggesting that SPBs immobilized on glass slides were released in the presence of cTnI. More importantly, the concentration of free SPBs in the solution collected was proportional to cTnI concentration, indicating that cTnI detections by the assay proposed were quantitative. The LOD calculated using the $3\sigma/S$ approach [37] was 6.7 mg/mL, which is comparable to previous studies [38]. Moreover, assays using all complementary sequences mentioned above showed detectable concentrations of free SPBs. Nevertheless, assays using complementary sequence A exhibited relatively low efficacy (especially for cTnI at high concentrations) compared with other complementary sequences used in this study. This can be attributed to the high affinity of complementary sequence A induced by its long sequence (19 bases), which makes it difficult for cTnI to displace its

interaction with the aptamer. Notably, negligible differences in the efficacy of assays using complementary sequence B, C and D were observed, despite their varying sequence lengths and regions of complementary.

Figure 0.6 Concentrations of free SPBs corresponding to different cTnI concentrations. The concentration of free SPBs in the solution collected was proportional to cTnI concentration.

Quantitative detection of cTnI in human serum has also been achieved. Figure 7.7 shows the concentrations of free SPBs detected corresponding to cTnI in human serum at different concentrations. As observed, the concentration of free SPBs collected increased with the concentration of cTnI and the LOD achieved (16.7 ng/mL) was comparable to that of cTnI in buffer (6.7 ng/mL as mentioned above). The specificity of the assay proposed was demonstrated by the fact that no free SPBs were detected in the control group where the slide was incubated with serum containing no target protein. Besides the excellent specificity of DNA interactions, the promising sensitivity achieved can be attributed to the utilization of SPBs, which can be easily separated from unwanted constituents in serum using a magnetic separation step [39].

Figure 0.7 Concentrations of free SPBs corresponding to different cTnI concentrations in human serum and buffer using complementary sequence B.

Figure 7.8 illustrates corresponding real time SPB measurements, all of which exhibited good linear regression.

Figure 0.8 Corresponding real time free SPB measurements of cTnI in human serum.

In conclusion, the concentration of free SPBs was proportional to cTnI concentration and the LOD achieved was 6.7 ng/mL (in buffer), which is comparable to assays previously reported. More importantly, real sample detection of cTnI using the proposed assay has been demonstrated and the sensitivity (LOD = 16.7 ng/mL) was consistent with detections of cTnI in buffer, indicating great potential for clinical applications. The format of the stick allows rapid and affordable replicate measurements on the same sample to enhance assay reliability. Moreover, the proposed assay can be easily extended to multiplexed biomarker detection by involving beads of different sizes, with each targeting one specific protein.

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Chapter 8

Discussion and Future Work

This chapter summarizes projects discussed in this thesis in terms of key findings and contributions to the study. Statements of hypotheses are clarified based on the findings and conclusions obtained in projects mentioned above, with all hypotheses demonstrated to be valid. Renaissance works that have not been completed are also described. Additionally, outstanding questions (design of synthetic receptors that are specific to sports fatigue related biomarkers, demonstration of aptamer based FET sensor for monitoring of sports fatigue related biomarkers, and comparison of FET sensors using synthetic molecules as recognition element and peers using natural molecules as recognition element) and future outlook (suggestions on future studies in this field) are presented for the reference of future studies in this field.

8.1 General Discussion

This thesis discussed the application of carbon nanomaterial based FET sensors in detection of sports fatigue related biomarkers and the viability of synthetic molecules as recognition elements in biosensors. This section summarizes the outcomes of the hypotheses proposed in Chapter 1.

8.1.1 Outcome of Hypothesis 1: Carbon nanomaterial based FET sensors can measure sports fatigue related biomarkers quantitatively and in real time.

The over-training issue, which has been demonstrated to be a key contributor to fatigue and injury of athletes [1], is targeted in this study. As a key part in athletes' daily life, training is regarded as the most effective and efficient way for athletes to keep their body conditions at a relatively high level [2] and make further performance improvements. Hence, athletes and coaches tend to maximize training loads whenever possible to seek for better performances in coming sports events. However, recent studies revealed that training loads over a threshold level had a negative effect on athlete performance (see Figure 8.1) due to physiological and psychological fatigue, as well as injury risks [3]. In most cases, the threshold level is expressed by levels of several biomarkers. To maximize the effectiveness of training and avoid over-training, real time and *in situ* monitoring of physiological conditions of athletes using a portable and sensitive biosensor must be achieved as coaches and consultants can never make appropriate/informed decisions on the duration, intensity, and frequency of training sessions without real time information about physiological and psychological conditions of athletes.

Aimed at portable biosensors for *in situ* monitoring of athletes, we propose that FET biosensors are promising sensing platforms owing to their miniaturization potential. As mentioned in Chapter 4, quantitative detection of IL-6, a key biomarker related to sports fatigue, has been achieved using an FET biosensor based on horizontally aligned CNT as a demonstration. The proposed assay shows excellent sensitivity (LOD = 1.37 pg/mL, which is comparable to that of ELISA and other assays) and good specificity.

Additionally, the developed biosensor exhibits great long-term robustness, thus are suitable for *in situ* measurements. Based on the results obtained in this project, it can be concluded that carbon nanomaterial based FET biosensor are applicable for real time detection of sports fatigue related biomarkers.

Figure 0.1 Sports performance vs. training load. The sports performance degrades drastically as the training load exceeds the training threshold.

8.1.2 Outcome of Hypothesis 2: Synthetic receptors such as liposomes and polypeptides can be used as recognition element in carbon nanomaterial based FET sensors for monitoring of sports fatigue related biomarkers.

Owing to their superior binding affinity and specificity, natural biomolecules such as antibodies have been widely used as recognition element in biosensors. However, biosensors using antibodies as recognition element exhibit several limitations. First, antibodies are limited by complicated and fragile functional structures. As a result, antibodies are readily denaturized in typical working environments of biosensors [4]. Second, antibodies are limited by steric hindrance effect caused by large molecular sizes [5, 6]. As a result, the quantity of receptors allowed per unit surface area is relatively low.

Third, antibodies are limited by tedious preparation procedures [7]. As a result, short shelf life, limited stability, and poor reproducibility have been observed in antibody based biosensors.

In this study, an rGO based FET sensor using reporter-encapsulated liposomes as the recognition element is proposed for enzymatic sensing. Detection of PLA₂ by the proposed sensor via real time monitoring of I_d responses of the rGO-FET upon rupture of reporter-encapsulated POPC liposomes triggered by the PLA₂ was demonstrated.

Besides the liposome based sensor, an rGO based FET sensor using *de novo* designed synthetic polypeptide as the recognition element is proposed for rapid and sensitive detection of matrilysin. The hydrolysis of JR2RC facilitated by MMP-7 leads to reduction of size and net charge (from -5 to -1, as shown in Figure 6.2) of the polypeptide. As a result, I_d of the rGO-FET device decreased drastically and the change in I_d could be readily detected in real time. It was observed that the change in I_d is dependent on the concentration of MMP-7, thus enabling a quantitative monitoring of the concentration of this protease. The outcomes of this part verify the hypothesis that synthetic receptors such as liposomes and polypeptides can be used in combination with carbon nanomaterial based FET sensors for bio-sensing applications. Nevertheless, proteins detected in this study are diagnostic related biomarkers instead of sports fatigue related biomarkers and further investigations are needed to fully verify this hypothesis.

8.1.3 Outcome of Hypothesis 3: Aptamers can be used in combination with tunable resistive pulse sensing assays for bio-sensing applications.

Owing to their folding capability upon binding [8], aptamers exhibit binding affinity and specificity that are comparable to that of antibodies and these nucleic acid ligands are termed as “chemical antibody” [9, 10]. More importantly, aptamers have shown unprecedented advantages over antibodies, including good reproducibility (thanks to well-established chemical synthesis routes) [11], low synthesis cost, and excellent stability in harsh environments. Additionally, the significant conformational changes upon target binding (see Figure 8.2) allow ultra-sensitive detections [12] and the

reversible interactions between aptamers and target molecules make it possible to develop non-disposable biosensors using aptamers as recognition element [13].

Figure 0.2 Schematic of target induced conformational changes of aptamers immobilized on electrode surface. (a) Binding of target leads to a conformational change and shields MB from electron-transfer communication with the electrode, resulting in a negative signal [13]. (b) Binding of targets blocked the binding of the cationic units, thus depleting the electrochemical response of the sensor [14].

In the third part of this study, aptamer is combined with TRPS for rapid and label free detection of cTnI. Firstly, aptamers were immobilized on a slide (used as a dipstick) to allow immobilization of nanoparticles modified with partially complementary sequences. The presence of the target protein caused a conformational change to the aptamer and the release of immobilized nanoparticles into solution. The concentration of nanoparticles released was proportional to the concentration of the target protein. Optimization of the partially complementary sequence produces an assay that was reliable and easy to setup with a LOD of 6.7 ng/mL in buffer. More importantly, quantitative detection of cTnI in serum using the proposed assay has also been demonstrated and the excellent sensitivity makes the proposed assay highly promising for clinical applications. The outcome of this part verifies the hypothesis that aptamers can be used in combination with tunable resistive pulse sensing for bio-sensing applications.

8.2 Reconnaissance Work

Despite the ultra-high sensitivities in virtue of the sensitive nature of the transducer layers used [15], electrical sensing platforms have exhibited several intrinsic drawbacks. First, electrical sensors tend to be vulnerable to external interferences. As a result, the signal-to-noise ratios of electrical sensors are relatively low [16]. Second, non-specific binding could be a severe issue for electrical sensors. Non-specific binding is regarded as a threat to device reliability as it may lead to false alarm (in cases molecules similar to the analyte are presented) and reduced sensitivity (in cases the analyte concentration is around the lower limit) [17]. In order to overcome these limitations, we have attempted to develop a platform combining electrical (FET) and optical (SPR) sensing mechanisms as optical sensing mechanisms (more specifically sensors based on nanoplasmonic mechanisms) are ideally complementary to electrical mechanisms. This can be attributed to the high resistances of optical mechanisms optical to interferences in bulk solutions owing to the localized working area [18]. As a preliminary study, PSA was detected using a customized sensing device by individual electrical and optical measurements.

Figure 0.3 Real time monitoring of the UV absorbance peak shifts caused by the presence of target molecule (red line = exact peak position, black line = centroid of the peak region). Analyte solution containing 1 μM PSA was injected and detectable signals in both data forms were obtained.

Firstly, detection of PSA using SPR-based biosensor has been achieved. As shown in Figure 8.3, the introduction of buffer solution led to a 10 nm peak shift as a result of refractive index change from 1.0 (air) to 1.33 (water) and the peak was stabilized after several rinsing cycles. Then, analyte solution containing 1 μM PSA was injected and detectable signals in both data forms were obtained. The results serve as an indicator that the designed device can be used for nanoplasmonic sensing of common protein molecules of reasonable concentration levels.

Figure 0.4 Real time monitoring of PSA using FET biosensor. The solution containing 10 nM analyte led to a 60 nA reduction of I_d .

Meanwhile, detection of PSA using FET biosensor has been achieved. As shown in Figure 8.4, the solution containing 10 nM analyte led to a 60 nA reduction of I_d , indicating that the sensitivity is at nano-Molar level. Nevertheless, no further shift was observed for solutions with higher analyte concentration (100 nM and 1 μM). This may be attributed to the limited amount of antibodies immobilized on the surface. In other

words, all antibodies immobilized on the surface were occupied in the incubation with the solution containing 10 nM analyte.

Nevertheless, no further investigations have been done to fully explore the possibility of combining electrical and optical sensing mechanisms in one device and this part has been regarded as reconnaissance work of this study.

8.3 Outstanding Questions

8.3.1 Design of Synthetic Receptors that are Specific to Sports Fatigue Related Biomarkers

In the second part of this study, it has been demonstrated that protein detections using FET sensors with liposomes and polypeptides as recognition element are viable and the sensitivity and specificity achieved are reasonable. Hence, it can be concluded that synthetic receptors can be used in combination with carbon nanomaterial based FET sensors for bio-sensing applications. Nevertheless, proteins detected in this study are not sports fatigue related biomarkers and it is essential to demonstrate detection of sports fatigue related biomarkers such as IL-6 using FET sensors with synthetic molecules as recognition element so that Hypothesis 2 proposed in this study can be fully verified. In order to achieve that, the first and key step is the design of synthetic receptors that are specific to these biomarkers.

Theoretically, it is absolutely viable to design or discover polypeptides that bind specifically to IL-6, which is a protein with similar structures and characteristics as proteins involved in our study. Therefore, great efforts have been made to design peptides against sports fatigue related biomarkers such as IL-6 over the past decades and several IL-6 binding peptides have been reported and/or isolated. In 1988, Yamasaki *et al.* reported the isolation of human IL-6 receptor peptide for the first time [19]. Among the 468 amino acids in the peptide, a section consisting of ~19 amino acids serves as the signal peptide. Later in 2000, Mizuguchi *et al.* developed a peptide against IL-6 by affinity panning from a random phage library to obtain oligopeptide ligands that interact

with IL-6 [20]. More recently, Ranganath *et al.* developed a disulfide-rich PEGylated peptide that binds specifically to IL-6 (see Figure 8.5a) based on approaches reported elsewhere [21]. A helix-loop-helix ZDC scaffold (see Figure 8.5b) was used as the starting scaffold for peptide synthesis and optimization.

Figure 0.5 (a) Schematic illustration of the disulfide-rich PEGylated peptide (cyan tube) binding to IL-6 (blue structure). (b) The helix-loop-helix ZDC scaffold used as the starting scaffold for peptide synthesis and optimization [21].

Nevertheless, the IL-6 binding polypeptides mentioned above have not been used in FET biosensors. The working environment of FET biosensors exhibit unique characteristics and further optimizations may be required for current peptides. Therefore, the design of synthetic receptors that are specific to sports fatigue related biomarkers and can work with FET biosensors should be achieved in the next stage.

8.3.2 Demonstration of Aptamer Based FET Sensor for Monitoring of Sports Fatigue Related Biomarkers

In the third part of this study, protein detection using aptamer as the recognition element has been demonstrated on the TRPS platform for rapid and label free detection of cTnI. TRPS sensing platforms are essentially based on electrochemical mechanisms [22], which shared considerable similarities with electrical mechanisms in terms of transducing mechanism, signal processing, etc.. Hence, it is reasonable to deduce that aptamers can

also be used as recognition element in electrical biosensors such as FET biosensors. As mentioned in Chapter 2, various assays combining aptamers and FET for bio-sensing applications have been reported. Zayats *et al.* monitored the interaction between adenosine and its aptamer. Herein, aptamers immobilized on the transducing layer were initially hybridized with complementary sequences [23]. Upon introduction of target molecules, the complementary sequences were displaced, resulting in variations in the gate potential. Maehashi *et al.* reported an aptamer-modified CNT-FET for the detection of IgE. Presence of IgE caused I_d reductions that were proportional to the concentration of target molecules. Moreover, this study demonstrated that the sensing performance of aptamer-modified devices was superior to that of antibody-modified ones under similar conditions. These studies demonstrated that aptamers can be used as recognition element in electrical biosensors such as FET biosensors.

The next step is the design of aptamers that bind specifically to IL-6. To date, several IL-6 binding aptamers have been discovered using the SELEX process. For instance, Gupta *et al.* reported two chemically modified DNA aptamers (Bn-dU and Nap-dU) for IL-6 [24]. The binding affinity of the proposed aptamers is so high that they can inhibit interactions between IL-6 molecules and their antibodies. Therefore, these aptamers are promising candidates for recognition element for detection of IL-6. Based on the creation of aptamers for other molecules using the SELEX process [25], Barton concluded that IL-6 binding aptamers can be easily obtained in a trial and error way [26]. These studies provide good references for aptamer design in our study.

Figure 0.6 Chemical structures of aptamers that specifically bind to IL-6 [24].

Nevertheless, the IL-6 binding aptamers previously reported have not been demonstrated on FET platforms. The unique working environment of FET biosensors may require further modifications of current aptamers to optimize performances of aptamer-based FET biosensors.

8.3.3 Comparison of FET Sensors Using Synthetic Molecules as Recognition Element and Peers Using Natural Molecules as Recognition Element

To advance studies of synthetic receptor based biosensors and promote their applications, comparisons of these devices with conventional ones based on natural molecule receptors are urgently needed. Over the past decades, great efforts have been made and considerable progresses have been achieved. For instance, Gaita *et al.* reported a comparison of antibody fragment-antigen binding (Fab', Figure 8.7a) units, antibody single-chain Fv fragments (scFv, Figure 8.7b), and aptamers (Figure 8.7c) applied in biosensors [27].

Figure 0.7 Schematic illustration of (a) antibody fragment-antigen binding units, (b) antibody single-chain Fv fragments, and (c) aptamers.

According to this study, antibody related receptors (Fab' and scFv) are limited by minimal regenerability, susceptibility to harsh working environments, presence of immunogenic regions, and absence of versatility for different analytes. Chen *et al.* summarized differences between aptamers and antibodies as recognition element in

biosensors [28]. It is claimed in this report that aptamers exhibit excellent chemical stability (nucleic acid nature), minimized batch variation, good device reusability (reversible conformational changes), extended detection range, and low cost synthesis (in vitro synthesis). However, it is mentioned in the same article that practical applications of aptamer-based biosensors are still lagging behind those of antibody-based ones, despite the remarkable advantages mentioned.

Nevertheless, current studies involving comparisons of synthetic receptors and antibodies are based on characteristics summarized from different assays that are not directly comparable to each other. In order to evaluate performances of FET sensors using synthetic molecules as recognition element, it is essential to make a direct comparison of these sensors and their peers using natural molecules as recognition element. More specifically, detection of the same target molecule by identical sensing platforms using different recognition elements should be executed and comparisons on various aspects (e.g., cost, sensitivity, specificity, assay duration, stability, and reproducibility) must be achieved to reach conclusions. Therefore, effects of synthetic molecule receptors and natural molecule receptors on sensing performance of a specific biosensor/assay need to be further investigated in the future.

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Appendix

Figure A. 1 (a) Molecular structure of MMP-7. (b) Degradation of extracellular matrix by MMP-7.