Nanocarbon based field effect transistors for biosensing applications

Herry Gunadi Sudibya

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**NANOCARBON BASED FIELD EFFECT TRANSISTORS
FOR BIOSENSING APPLICATIONS**

**HERRY GUNADI SUDIBYA
SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING**

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FOR BIOSENSING APPLICATIONS**

HERRY GUNADI SUDIBYA

**SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING**

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Doctor of Philosophy

2011

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Everybody in this world could learn from every phenomenon they experience in their life. Some of them may learn from amazing and great experiences, some other learn from difficulties and tribulations, and the rest may learn from their mistakes and bad decisions they made. Three years of journey in my postgraduate has finally finished; with the mixture of those three components. Many good experiences have been obtained; my class teaching experiences, great sharing moment with many undergraduate students, successful paper publications, good teamwork with colleague and collaborators, and last but not least the increment after Qualification Examination (QE); while several bad moments also left behind, such as some paper rejections, experiment failures, machine breakdown, and also other imperfections.

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Abstract

Nanocarbon materials, including Single Walled Carbon Nanotubes (SWNTs) and graphene, with their unique combination of electrical, physiochemical, and structural properties, has recently emerged as a novel nanoelectronic biosensor for chemical and biomolecular detection. Their major advantages; including the simple detection scheme, high sensitivity and selectivity, and capability to show real time monitoring; make those materials to receive increasing attention in the last few years.

In the synthesis process of carbon nanotubes (CNTs), the product obtained is always mixture of metallic and semiconducting; an electrical property which is determined by their chiralities. Effective separation technique, which mainly aims for high purity semiconducting tubes, should be achieved to develop high-quality sensor. Among various techniques for chirality characterization, our results demonstrate that the electrical transport measurement (through I_{on}/I_{off} ratio) of thin film Field Effect Transistors (FETs) is the most sensitive technique; compare to photoluminescence excitation spectroscopy, optical absorption spectroscopy, or Raman spectroscopy; in order to evaluate the purity of semiconducting CNTs.

Current exploitations of nanocarbon materials for biosensor devices, so far, are still dominated by the design of static in-vitro molecular or chemical detections. Various biomolecules, including protein, DNA, amino acid, and so on, have been detected with high sensitivity through carbon nanotubes. Besides, the use of graphene sheet has been well established for gas monitoring and a few molecular detection. Applications of *in vivo* biosensing, which addresses dynamic systems in living organisms, unfortunately, are

still very limited. This is mainly due to the difficulties of interfacing the living cells while preserving the sensor functionalities at the same time.

In our research, we mainly focus to overcome this challenge and explore the possible solutions for conducting a simple, non-invasive, sensitive, selective, and high-throughput detection of dynamic cellular activities; without neglecting the typical biomolecular detection explored and presented extensively in the recent publications. We developed nanoelectronic biosensors based on nanocarbon material, configured as field-effect transistors, which can interface with living cells and subsequently detect various cellular activities such as biomolecule release (exocytosis), glucose metabolism, and ion channel activities. In particular, we have successfully demonstrated a real time Adenosine Tri Phosphate (ATP), catecholamine molecule secretion from living astrocytes and PC12 cells, and glucose metabolism processes from bacteria, with high sensitivity and high temporal resolution. An in-depth investigation of E.coli metabolism activity, together with the sensor utilization as a drug screening tool, has also been established.

Besides all these unique cell activity demonstrations, it is also shown that our nanocarbon sensor can perform physiological and environmental diagnosis with excellent results. A modification/functionalization of our nanocarbon sensor was achieved to create a platform for detection of specific antibody-antigen interactions, physiological calcium ion release, and the presence of toxic heavy metal; inside aqueous solution. As the final word, we do expect all of these realizations can make great impact in the development of biosensor based on nanocarbon materials.

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Chapter1

Introduction

1.1. Research Background

Carbon nanotubes (CNTs), which equipped with remarkable electrical, structural, and physiochemical properties, have been employed as nanoelectronic biosensors for rapid detection of biomolecules¹⁻². They are known to be extraordinarily sensitive to the electrochemical perturbations, attributable to the fact that they have very large surface to volume ratio and most of electrical carriers flowing on its sidewall. In addition, their nanoscale dimensions allow them to interact intimately and to be used as an investigating tool for the nanoscale world of biomolecules and molecular machineries. More recently, a single layer graphene has also been employed as chemical and biomolecular sensor as the alternative of CNTs³⁻⁴. Its excellent sensing performance, relatively low 1/f noise, and cost-effective compared to other nanomaterials, promote this material to be scientifically and commercially attractive in biosensing application. Its single layer structure, in addition, could provide potential of better interface to the cell membrane compare to nanotubes network.

Despite their tremendous potential and well-established demonstrations in chemical and biomolecules monitoring, the role of nanocarbon materials in analyzing dynamic cellular activities is still very limited. The cytotoxic interface between nanocarbon and the living cells largely limits its applications in biological system⁵⁻⁶. Some covalent chemical functionalization to improve cells adhesion, on the other hand, might affect the electrical properties of the nanocarbons, as well as their electrical

recording when they are utilized as a sensor. A breakthrough technique, therefore, must be designed properly thereby can ensure good interface between cells and nanocarbon and still preserving the sensor functionalities at the same time.

Why do we, in the beginning, focus our interest in the study of the dynamic cellular signal? The answer for this question is this: in high-level living organism, some excitable cells (such as myocytes, neurons, endocrine cells, and sensory cells) are able to generate bioelectrical signals resulting from specific signaling molecules, endo/exocytosis process, or dynamic ion flow activities through ion channels embedded within the cell membrane. Information about these bioelectrical signals are indeed essential to many fundamental biological processes, such as, communication between neurons, contraction of heart or other muscles, vision, hearing, and so on. Else, since most biological processes involve this typical electrostatic interactions and charge transfer, it opens a great opportunity to realize the electronic detection of biological molecules as an alternative to optical characterization.

In the long journey of obtaining and investigating bioelectricity signals from living cells, several state-of-the-art electrophysiological methods, such as microfiber glass capillaries and patch-clamp electrodes ⁷ have been established and enable a good signal-to-noise ratio with high sensitivity. Their applications, unfortunately, are very invasive and damage the cell just in a few hours. An alternative method such as fluorescence with voltage sensitive dyes ⁸, meanwhile, cause a toxic effect to the cells upon illumination makes it unsuitable for long-term recordings. Those typical bottlenecks have been overcome by applying a non-invasive extracellular recording: via microelectronic devices like microelectrode arrays (MEAs) ⁹⁻¹¹ or field-effect transistors

(FETs)¹²⁻¹⁵; a platform which is found to be sensitive enough to detect changes of the extracellular electrochemical perturbation, can be reused for many times, and is capable of recording for long periods of time in the absence of an operator (as long as the sterility is maintained)¹⁶.

In FET configuration, a small potential change from electrical active cells (myocytes) cultured on planar array devices can be detected. During an action potential, capacitive and ionic currents from the cell flow through the attached membrane and along the cleft between the cell and the sensor. This current signal is then transferred to the gate of FET through the electrolyte, giving rise to an extracellular voltage, which then modulates the current between the source and drain electrode¹⁷. Explanation of extracellular recorded signal shapes was first attempted by *Regehr et al*¹⁸ using a simple equivalent electrical circuit (point contact model). In this model, it is assumed that all currents are flowing through one common point in the cell-transistor coupling. *Ingebrandt et al*¹⁹ suggest that additional effects like electrodiffusion, ion sensitivity of the sensors and more detailed electronic models should also be included.

In our research work, we exploited the potential of nanocarbon materials (including carbon nanotubes and graphene), which are configured as a field-effect transistor (FET) to obtain electrophysiological recordings from living cells, including action potentials and biomolecule secretion. Initialized by an effort to improve sensor device properties (sensitivity, specificity, low leakage current, low noise level) with high throughput/ reproducibility, we focused on how to overcome the cytotoxic interaction between living cells and nanocarbon materials using proper device modification/ functionalization. As the final step, we achieved extracellular signal detection in real time

with simple device scheme and high temporal resolution. In addition to the successful demonstration of dynamic bioelectricity signals, we further exploited our sensor for detection of specific antibody-antigen binding, physiological calcium ion detection, as well as heavy toxic metal detection.

1.2. Significance of Research

This research work investigates deeply the application of carbon nanotubes and graphene -- materials which have received high interest in biological research-- as novel *in vivo* biosensor tool. By utilizing the concept of field-effect transistors, followed by developing a biocompatible interaction between cell and the device, we achieved a non-invasive, real time, and sensitive detection of cell activity with high temporal resolution. Besides, the fabrication of ultrasensitive sensors for physiological calcium and toxic metal ion detection has been established. We believe that all this realizations are significant contributions towards further developments in the field of biosensor research.

In a larger point of view, our bioengineering laboratory studies at the interface of nanotechnology, biophysics, and biology with the aim to develop novel biosensors and study cell physiology at the same time (particularly, the biological functions mediated by or occur at the cell membrane). We believed that fusion of those interdisciplinary researches brings unprecedented opportunities and new tools to investigate on the complex biological system at the single cell or even at the single molecule level.

1.3. Research Objectives

In this research, our main objectives is to develop sensing technology using nanocarbon materials, including Single Walled Carbon Nanotubes (SWNTs) and

graphene, based field-effect transistors (FET) to detect cell activities with high sensitivity and selectivity, simplicity, low cost, and high throughput.

In order to achieve that overall objective, we divide some specific aims in our research progress including:

- Fabrication of low noise and high dynamic range nanocarbon sensors with sensitive electrical response.
- Initial investigation of cell adhesion characteristic on various substrates (aimed for further research work).
- Method development for interfacing living cells with nanocarbon sensors with full retention of functionalities of both membranes and sensors.
- Device functionalization to obtain specific biological binding/interaction to be investigated.
- Detection/sensing of specific biomolecules or metal ions as well as certain cell activities (with non-invasive method to obtain clear signal from individual or multiple cells directly cultured on nanocarbon materials).

1.4. Organization of the thesis

This thesis is organized as following:

Chapter 1 Introduction describes the overall and specific objectives of this research work and background motivation about the aims of this research.

Chapter 2 Literature Review provides the review of current literature related to our research work. It starts with the general overview of biosensor, followed by a description

of carbon nanotubes and graphene; materials which are applied as sensing materials. Then the concept of a field-effect transistor is described followed by recently proposed biomolecule detection mechanisms. At the end of this chapter, some recent research works related to nanocarbon-based biosensor are reviewed.

Chapter 3 Research Methodology and Preliminary Results describes the methodologies we used in our research. Preliminary results regarding the electrical characterization of the device are also described in this chapter.

Chapter 4 Label-free Detection of ATP Release from Living Astrocytes with High Temporal Resolution Using Carbon Nanotube Network describes a successful demonstration to detect dynamic cellular secretion from living cell. Living astrocytes were cultured on the CNT-net devices. After stimulation with glutamate, the Adenosine Tri Phosphate (ATP) release from the cells was detected in real time.

Chapter 5 Interfacing Glycosylated Carbon Nanotube Network Devices with Living Cells to Detect Dynamic Secretion of Biomolecules. This chapter demonstrates our successful strategy in interfacing nanotubes with PC12 living cells through carbohydrate-carbohydrate interaction. Dynamic catecholamine release from these cells was successfully detected.

Chapter 6. Real Time Detection of Cardiac Muscle Action Potentials and the Escherichia coli Glucose Metabolism Process based on Carbon Nanotube Network Field-Effect Transistors. After two demonstrations of mammalian cells activities in the

two previous chapters, we utilize our CNT devices as a platform to detect glucose metabolism activity in bacteria. Effect of various antibiotic drugs on the bacteria metabolism was also investigated.

Chapter 7. Centimeter-Long and Large-Scale Micropatterns of Reduced Graphene Oxide Films: Fabrication and Sensing Applications. In this chapter, experiment with the graphene FET sensors (as an alternative to the carbon nanotubes) are discussed. It is interesting to note that graphene performs ambipolar characteristic (both p-type and n-type), properties that enable us to perform ‘inversed’ electrical response just by varying the gate voltage bias. Subsequently, both in vitro and in vivo catecholamine detections, similar to those shown in chapter 5, were demonstrated using the graphene sensor.

Chapter 8. Application of Graphene-Field Effect Transistors for the Detection of Metal Ions and Specific Biomolecular Interactions. This chapter further elaborates the application of the graphene devices as sensitive and specific biomolecule sensor. In particular, the detection of calcium and magnesium ions in a dynamic physiological (micromolar) range is demonstrated; in addition to the preliminary sensing of pH variations and antigen-antibody interactions. Besides that, nanomolar concentration of heavy toxic metal (including mercury and cadmium) ions, which is known to have lethal effects on environmental and living organisms, has also been detected with acceptable selectivity. It was found that the limit of detection (LOD) was comparable to other methods, which are currently available.

Chapter 9 Conclusion and Future Directions. This chapter wraps up all the idea and demonstrations of various biosensing techniques (via nanocarbon materials), that were investigated in the past few years. Some future directions of this project are discussed and elaborated.

Chapter 2

Literature Review

2.1. Nanoscale Electronic Biosensor

A biosensor is defined as a self-contained integrated device that is capable to detect biological components; such as proteins, polynucleotides, carbohydrates, enzymes, and the whole biological tissues, providing either quantitative or semiquantitative analytical information ²⁰. It is distinguished from chemical sensor in that it comprises of an analyte combining the biological recognition element with a physicochemical detector.

In general, there are two types of detection in biosensors: *in vitro* and *in vivo* ²¹. During *in vitro* biosensing, the sample solution is dropped at the top of the sensing element and subsequently the output signal is obtained and investigated to provide the desired information of the solution. On the other hand, *in vivo* biosensing is a detection scheme, which addresses the dynamic systems in a living organism, such as measuring rate of uptake or efflux in of a certain material through a particular cell. Current applications of biosensors are limited to static *in vitro* measurement, either to monitor biological process or to recognize single specific biomolecules. Not many applications of *in vivo* biosensing are demonstrated up to date due to the difficulties in interfacing the sensor components with living cells while preserving proper functionalities of both systems at the same time.

In classical approaches, optical techniques (such as mass spectrometry and biolabeled fluorescence) play a significant role in biosensing research because of their high sensitivity. However, one of the key disadvantages is: they require numbers of steps

before they can be used for detection. Besides, such methods are very difficult to be miniaturized ². In order to tackle those issues, some conventional transistors have been used for bimolecular sensing. Unfortunately, they fail to exhibit sensitivity comparable to previous methods. A promising result has subsequently been achieved when molecular nanoscale devices were developed ²²⁻²⁵. Hence, nanoscale devices (using carbon nanotubes and graphene) started to receive increasing attention in biosensing research from that point of time. Attributable to the fact that most biological processes involve ion movement, electrostatic interactions and charge transfer, this opens a great opportunity for the direct electronic detection of biological molecules as an alternative to the optical characterization.

In general, carbon nanomaterials electronic devices have shown several advantages compared to macroscopic materials. The first advantage, of course, is related to its intrinsic nanoscale size. The size compatibility between carbon nanomaterials and biological entities is essential for practical applications; single walled carbon nanotubes, for instance, have diameters about 1-2 nm, which is comparable to the size of the DNA duplex ². The second advantage is related to the outstanding electrical properties of these materials. Carbon nanotubes and graphene were found to perform ballistic transport with very high electron mobilities. Furthermore, they have atoms on their structures exposed to the surface, such that any small change in the environment is generating measurable response in the electrical properties. In addition, the unique electrochemical properties possessed by this typical materials enable them to identify biomolecules electrochemically and even to quantify the concentration of mixed biomolecules ²⁶⁻²⁷. Their ability to act as an efficient ion-to-electron transducer in potentiometric analysis ²⁸⁻²⁹ demonstrates

another unique strength. One disadvantage, however, is that carbon nanomaterials are not easy to handle and are rather heterogenous in nature, at least at present.

After reviewing briefly about the fundamentals and history of the carbon nanoscale device biosensors, the next discussion will focus on the use of carbon nanotubes (CNTs) and graphene as a potential material for biosensors. Some questions related on how those nanocarbons are fabricated into biosensor devices, how they are used to interact with the biological components, or what kind of interaction mechanism can cause the effect in their electrical properties are discussed in the following section.

2.2. Carbon Nanotubes

2.2.1 General Information about Carbon Nanotubes

Carbon nanotubes (CNTs), which were first accidentally discovered by Iijima in 1991³⁰, are considered as almost ideal 1-D systems. Carbon nanotubes can be formed by rolling hollow cylindrical tubes with diameter ranging between 1 and 100 nm. Compare to their relatively long length (μm range), CNTs possess very large aspect ratios (around 1:10,000). CNTs have various unique properties, including high tensile strength and elastic modulus, high thermal conductivity, high ductility, high resistance to heat, relative chemical inactivity, and some unique electrical properties³¹; characteristics that bring this nanomaterial to intense investigation since their first discovery. All of those properties place CNTs as a very attractive candidate in diverse nanotechnological applications till nowadays.

Baughman et al.³² have reviewed some of the current applications of CNTs including the conductive and high-strength composites, energy storage, energy

conversion devices, biosensors, field emission displays, radiation sources, hydrogen storage media, nanometer-sized semiconductor devices, probes, and also interconnects. Besides that, there are actually a lot of other applications can be achieved using this material.

Chemical bonds inside CNTs are very strong, which leads to extremely high mechanical, thermal, and chemical stability. The lack of interface states as in the silicon/silicon dioxide interface even provides a greater flexibility to the fabrication process.

In general, carbon nanotubes can be categorized into two types: Single Walled Carbon Nanotubes (SWCNTs) and Multiwalled Carbon Nanotubes (MWCNTs). SWCNTs consist of a single graphite sheet wrapped into a cylindrical tube. On the other hand, MWCNTs consist of an array of such nanotubes that are concentrically nested like rings with an interlayer spacing of 3.4 Å. As the consequences, they also have a larger diameter compare to SWCNTs.

2.2.2. Synthesis and Purification of CNTs

There are three common methods for synthesizing CNTs: electric arc discharge³³, laser ablation³⁴, and catalytic decomposition of hydrocarbon described as chemical vapor deposition³⁵⁻³⁶.

The arc discharge method produces CNTs through arc-vaporization of two carbon rods placed end to end (with 1 mm gap) in a low-pressure container that is usually filled with inert gas. A potential difference (~ 20 V) was applied to drive a direct current (~ 50-100 A), which in turn creates a high temperature discharge between the two electrodes.

The discharge vaporizes the surface of one of the carbon electrodes, and produces a small rod-shaped deposit on the other electrode.

Laser ablation methods, on the other hand, prepared the sample by laser vaporization of graphite rods with a 1:1 catalyst mixture of Cobalt and Nickel at 1200°C in flowing argon, followed by vacuum treatment at 1000°C to remove the C₆₀ and other fullerenes impurities. Two consecutive laser vaporization pulses were exposed to vaporize the target more uniformly and to minimize the amount of carbon deposited as soot. The average nanotube diameter and size distribution can be controlled by varying the growth temperature, the catalyst composition, and other process parameters. The nanotube formed by this method appears as a mat of "ropes", 10-20nm in diameter and up to 100µm or more in length. Each rope is found to consist primarily of a bundle of single walled nanotubes, aligned along a common axis.

Arc discharge and laser ablation methods, though they are commonly used recently, actually still suffer two major drawbacks. The first issue is related to its low yield result, which is unsuitable for large industrial application, and the second issue is the problem in purifying and manipulating the final product, which certainly becomes an obstacle for further practical applications.

Currently, chemical vapor deposition (CVD) method is commonly preferred since it requires much lower temperatures compare to the previous two methods. In CVD method, instant methane, alcohol vapor, or other hydrocarbon gases were used as the carbon source. Some catalyst particles (Fe or Co) are deposited onto the substrate. The catalyst will then act as a template to decompose the hydrocarbon (to produce carbon and hydrogen) and start to precipitate the nanotubes.

In theory, CNTs are pure allotrope of carbon. However in reality, they almost always contain some impurities, such as metallic nanoparticles or compounds derived from the catalysts used in nanotube growth, which remain trapped between the nanotubes even after extensive acid washing³⁷⁻³⁹. Typical purification methods of CNTs consist of thermal oxidation (at around 350 °C) to remove amorphous carbon, heating in non-oxidizing acid solution to remove the free metal particles, and finally annealing of the samples to remove the defects created in the earlier steps⁴⁰. Unfortunately, oxygen-containing moieties were usually created during the washing steps. All of these impurities, particularly the metallic compounds, are responsible for the electrocatalysis and electrical performance seen at the nanotube-modified electrodes and devices.

2.2.3. Electrical Properties of CNTs

In the perspective of molecular electronics with carbon nanotubes, SWNTs are more commonly used. It is due to its superb electrical properties which can not be exhibited by MWNTs. MWNTs are considered as metallic conductors, whereas SWNTs can be metallic or semiconductors, depending on their diameter and chirality (arrangement of the hexagon rings) along the nanotube structure. The chirality concept is illustrated in the figure below.

Figure 1. Map of chiral vectors that determine the chirality of SWCNTs. Each vector is specified by (n,m) indices. From reference ⁴¹

Chirality can be obtained from a pair of integers (n,m) called chiral vectors. If $m=0$, the nanotubes are called "zigzag". If $n=m$, the nanotubes are called "armchair". For the rest, they are called "chiral". All nanotubes which the $n-m = 3i$ (i are zero or positive integer) are metallic. Otherwise they are considered to be semiconducting. For small-diameter SWNTs, approximately two-thirds are semiconductors and one-third is metallic ⁴².

For the semiconducting nanotubes, the band gap also depends on nanotube diameter. Since SWNTs with almost identical diameters might have different chiral vectors and hence different electronic properties, SWNTs have to be monodisperse in diameter and in chirality such that uniform electrical (and optical) properties can be

achieved. Even then, SWNTs with identical chiral vectors can possess different chiral handedness, which will influence the interaction of SWNTs with circularly polarized light. A lot of research effort in recent years has been seeking large-scale, economical production of monodisperse SWNTs, with control of electronic type, diameter, length, and chiral handedness. However, of the many techniques developed, none has overcome all the obstacles to date.

2.2.4. Bioapplications of CNTs

While fundamental research and science of nanotubes focuses on the properties of isolated carbon nanotubes, applications heavily rely on interactions of nanotubes with their environment. In biological research, two major limitations of CNTs are their low solubility in aqueous media and their cytotoxic interface to living systems⁵⁻⁶. To solve those issues, various covalent and non-covalent modifications of CNTs have been attempted over the past decade by incorporating chemical and biological functional groups^{40,43-44}.

Covalent modifications, either applied to the end-group or to the sidewall of the tubes⁴³, were initially developed and were known to have a remarkable potential to provide high degree of tunability of the SWNT properties. Those methods, nevertheless, have the disadvantage that they impair the tubes physical properties and consequently to their electrical properties as well. For this reason, people started to explore an alternative in supramolecular approach to the solubilization problem – namely, the noncovalent functionalization. This is done by wrapping polymers such as poly(vinyl pyrrolidone) and poly(styrene sulfonate) around the tubes. This novel method can preserve the intrinsic sp^2

structure and conjugation of the tubes, which yields in the conservation of the SWNTs electrical properties ^{5,43}. Various biological molecules have also been immobilized onto SWNTs sidewalls through π - π interactions involving the mediation of 1-pyrenebutanoic acid and succinimidyl ester linkers ⁴⁴⁻⁴⁵. This typical approach tends to retain biological activity and functionality, properties which are very important in biosensing application.

Besides the solubilization issue, functionalization also aims to improve biocompatible interactions between carbon nanotubes and living cells. In the journey of obtaining this goal, nanotubes have been functionalized with positively charged polyelectrolytes, such as poly(ethylene)imine (PEI) ⁴⁶ and poly-L-lysine (PLL) ⁴⁷, in order to promote adhesion of the cell membrane. Charged groups can also be imparted to nanotube surface via chemical functionalization ⁴⁸. Alternatively, CNTs have been coated with adhesion proteins found in the extracellular matrix ⁴⁹. All of those functionalization methods, however, are also possible to cause an adverse effect to the nanotubes properties.

The issue regarding interaction between nanotubes and mammalian cells has received increasing attention nowadays. Their application to excitable neuronal cells, in particular, has provided significant impacts in the development of neuroscience nanotechnology, as demonstrated by several research groups. *Lovat et al.* ⁵⁰, for example, have demonstrated the possibility of using carbon nanotubes (CNTs) as potential devices able to improve neural signal transfer, while supporting dendrite elongation and cell adhesion. Besides, *Geith et al.* ⁵¹ have demonstrated the use of SWNT layer-by-layer (LBL) films to support the growth, viability, and differentiation of neuronal NG108-15 neuroblastoma/glioma hybrid cells. They also successfully demonstrated the possibility

of utilizing the electrical conductivity of LBL-assembled, modified SWNT films to stimulate the neurophysiological activity of NG108-15 cells. An interesting example of stimulating the neuronal circuits via the SWNT layers has been effectively done by *Mazzatenta et al.*⁵². Meanwhile, *Wang et al.*⁵³ have demonstrated the first electrical stimulation of primary neurons via nanotube electrodes.

In our approaches (which is described in more details in chapter 4 to 6), we tried to develop a non-covalent functionalization method for the carbon nanotubes network to interface the cell membrane. Quick detection of various types of cell activity was then achieved in real time electrical recordings.

2.3.Graphene

Compared to other forms of sp^2 carbon such as carbon nanotubes or fullerenes; which have been synthesized, isolated, and deeply investigated during the past two decades, the development of single-layer graphene was lagging behind. Its structure and properties has just recently examined⁵⁴.

Graphene is a 2-dimensional semi-metallic material consists of individual layers of sp^2 - hybridized carbon atoms. It has the π -stacked hexagonal structure of graphite with an interlayer spacing of 3.34 Å, which is the van der Waals distance for sp^2 -bonded carbon⁵⁵ and its carbon-carbon bond length is about 0.142 nm. Graphene is known as the strongest material, having elastic stiffness of 340 N/m and Young Modulus of about 1 TPa⁵⁶. Besides, it has very high thermal conductivity and mechanical stiffness (~3,000 Wm⁻¹K⁻¹ and 1,060 GPa, respectively).⁵⁷

Graphene sheets can be produced using several methods: mechanical exfoliation via repeated peeling of highly ordered pyrolytic graphite (HOPG)^{54,58}, epitaxial growth by using SiC substrate⁵⁹⁻⁶⁰, and by chemical conversion from graphite⁶¹⁻⁶⁴. The method of mechanical exfoliation was the first invented, which led to the invention of graphene sheets⁵⁴. Although it is quite simple, this method is unfortunately not controllable, which results in low productivity and doesn't permit a large-scale use. More recently, an epitaxial growth method using SiC substrate was introduced⁶⁰. In this technique, the graphite is heated to ultrahigh temperature (> 1,000 C) under vacuum conditions. This results in higher yield with less defect compare to mechanical exfoliation. Nevertheless, it is still difficult to achieve larger graphene areas. Due to the very high temperature, moreover, the application of SiC method is very costly and is impossible to be used in combination with electronic circuits.

The newest method in graphene production is using graphene oxide (GO) as a precursor^{61,64}. Graphene Oxide (GO) is produced by oxidative treatment of graphite via one of three methods developed by Hummers⁶² (the most common one), Broide, or Staudenmeier. The oxygen containing group (carboxyl and hydroxyl) existing in GO makes it dispersible in water and provides reactive sites for chemical modification using carbon surface chemistry. Subsequently, GO is chemically reduced by dissolving them in hydrazine followed by deposition on various substrates to form single or few graphene layers. Deposition of graphene films to various substrates has been demonstrated via dropcasting, dip-casting, spraying, electrostatic absorption, spin coating, as well as filtration^{63,65-72}.

Owing to its unique electrical, optical, and structural properties, graphene has been used in various applications including individual gas sensors ⁷³, integrated circuits ⁷⁴, transparent conducting electrodes ⁷¹, and ultracapacitor ⁷⁵. Its one-layer structure also provides potential for better interface to the cell rather than carbon nanotube networks. This property is significant for successful detection of cell activities. Due to its only one single-atom thick, graphene can perform very high sensitivity towards a local environment and electrochemical perturbations ⁷⁶. Besides, it has extremely high charge mobility ($\sim 15,000 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$), large carrier capacities ($\sim 10^{12} \text{ cm}^{-2}$), and relatively low $1/f$ noise ^{54,58,77}; properties which altogether promoted this material to be particularly attractive for sensor applications ^{54,77}.

Graphene sensor is typically operated in electrochemistry or field-effect transistor scheme. The facile solution process together with its residual and chemically active defect sites renders rGO a promising material in electronic sensors, which have shown promising results in vapor and biomolecule sensing by using either single layered ⁷⁸⁻⁷⁹ or few-layered rGO films ⁸⁰⁻⁸¹. So far, it remains a great challenge to obtain large-scale continuous rGO thin films with only single to a few layers ⁸²⁻⁸³. It is also noted also that most of the reported rGO thin-film devices rely on microfabrication by lithography methods ⁸⁰⁻⁸¹.

2.4. Recent Development of the Nanocarbon Material in Biosensors

The ultimate aim of biosensor in the modern science is to achieve simple monitoring of biomolecular interactions with high sensitivity in real time. In general, this development can be categorized into three main approaches, including: optical biosensor,

electrochemical biosensor, and field effect transistor based biosensor. Review of these three techniques to will be described in the following sections.

2.4.1. Optical Biosensor

In general, the optical methods for biosensor typically rely on two different modes: the fluorescence quenching ⁸⁴ and the NIR photoluminescence exhibited by semiconducting nanotubes ⁸⁵.

The ability of CNTs to quench fluorescence has been explored by several research groups and such strategy has been proven to result in nanomolar sensitivity. For instance, *Yang et al.* ⁸⁶ used the preference for single-stranded oligonucleotides to wrap around SWNTs compared with the related duplexes and *Doornet et al.* ⁸⁷ utilized a dye–ligand conjugate in which the dye complexed with the SWNTs, thus causing its fluorescence to be quenched. The interaction between analyte and nanotube-bound receptor ligand displaced the dye–ligand conjugate from the nanotubes and hence the fluorescence tag can be recovered.

On the other hand, the NIR luminescence of semiconducting SWNTs provides particularly interesting advantage because NIR radiation is not absorbed by biological tissue, and therefore can be used for biosensing within biological samples or organisms. The use of NIR luminescence for DNA–SWNTs sensor system has been reported to be able to detect single-molecule interactions when wrapped either in DNA ⁸⁸ and collagen ⁸⁹. Wrapping the nanotubes with single-stranded DNA (ssDNA) has been explored for monitoring DNA hybridization and for monitoring small-molecule interactions with the

DNA. Furthermore, in the research conducted by *Strano et al.*⁹⁰, infrared luminescence was used in which semiconducting SWNTs are wrapped in double-stranded DNA (dsDNA). The shift in optical properties upon the change in dsDNA structure was subsequently applied to detect metal ions that induced such changes in DNA structure. This DNA-wrapped SWNT biosensors were shown to be able to detect all these metals with the sensitivity decreasing in the order $\text{Hg}^{2+} > \text{Co}^{2+} > \text{Ca}^{2+} > \text{Mg}^{2+}$. Recently, an interesting development has been achieved by *Heller et al.*⁹¹. They reported the ability to detect multiple different analytes simultaneously on the same sample of ssDNA-wrapped SWNTs is due to the differing optical responses of (6,5) and (7,5) SWNTs.

Similar approach of fluorescence-quenching involving CNTs have already been have developed in graphene oxide, as reported by *Lu et al.*⁷⁸. DNA fragments and proteins in solution by exploiting has been detected by quenching of fluorescent tags during adsorption of biomolecules. It was mentioned that the addition of the complementary strands of DNA caused hybridization, displacing the tagged DNA from the graphene oxide surfaces and restoring approximately 77 % of the original fluorescence signal.

In general, the number of research studies that exploit the optical properties of nanocarbon material for biosensing is relatively small compared with studies that use their electrochemical or electrical properties, which would be described on the following sections. Nevertheless, this optical-based system has great potential to be developed as the future sensor since it might be the only way to develop entirely nanoscale biosensors which could operate inside the living cells. This particular advantage has been illustrated

very well by *Jin et al.*⁸⁹ In their study, the ability of the DNA–SWNTs to detect drugs and reactive oxygen species inside living cells has been demonstrated. The DNA–SWNTs had been shown to be able to enter 3T3 fibroblasts by endocytosis without being genotoxic, and retain their photoluminescence.

2.4.2. Electrochemical Biosensor

Electrochemical biosensors are developed based on enzymatic catalysis of a reaction that generates or loses electrons in redox enzymes. The substrate of the sensor usually contains three different electrodes, including a reference electrode, a working electrode and a sink electrode. Besides that, an additional counter electrode may also be present as an ion source. Analyte in the sensor is involved in the redox reaction that takes place on the active electrode surface, and subsequently the ions produced create a potential which is subtracted from that of the reference electrode and recorded as a signal. The success of an electrochemical biosensor is based on their ability to promote electron transfer in electrochemical reaction. Therefore, the main challenge for the design of electrochemical biosensors with nanocarbon materials is how to incorporate these nanomaterials into bulk electrodes to obtain optimum result. In general, there are two different recording modes. Firstly, the current is measured at fixed potential (rate of flow of electrons is directly proportional to the analyte concentration) and secondly the potential is measured at zero current which gives a logarithmic response.

This typical detection scheme offers several advantages over conventional fluorescence measurement methods: better performance with less background noise, low-

cost components, portability, and the ability to carry out measurements in turbid samples. During the past few years, there have been many reports of nanocarbon-based electrochemical biosensors for the detection of diverse biological structures such as DNA, viruses, antigens, disease markers, and whole cells.

One of the most popular examples of electrochemical biosensor is the detection of glucose molecules by using enzymatic reaction of glucose oxidase followed by the signal measurement of the resulting H_2O_2 concentration. This detection approach was successfully demonstrated by Wang *et al.*⁹² by using Nafion, a sulfonated tetrafluoroethylene-based polymer, to incorporate MWNTs into composite electrodes. This typical technique has also been studied extensively by several other research groups⁹³⁻⁹⁵, attributable to the fact that the nanotubes can similarly provide a high surface area for growth of catalytic nanoparticles. The electrochemical properties of CNTs can also be improved on by combining them with other functional materials, such as conducting polymers, metal nanoparticles, or ionic liquids, to enhance their sensing performance, as shown in several reports, such as Gao *et al.*⁹⁶ who have reported glucose biosensors based on aligned CNTs coated with a conducting polymer, Dai *et al.*⁹⁷⁻⁹⁸ who developed a versatile and effective approach for decorating CNTs with metallic nanoparticles, and Fisher *et al.*⁹⁹ who developed a SWNT-based electrochemical biosensor that used Au-coated Pd (Au/Pd) nanocubes.

More recently, demonstration of electrochemical approach in graphite nanoplatelets has been demonstrated by Lu *et al.*³⁻⁴. The nanocomposites of graphite nanoplatelets and Nafion exhibited significant electrochemical response (oxidation and

reduction currents in H_2O_2 solution). The composite electrodes made from the nanoparticle-decorated nanoplatelets and Nafion had exceptionally high surface-area-to-volume ratio, thus offering outstanding performance, with a marked decreased in the overpotential needed to detect H_2O_2 , at low cost. A different approach to glucose detection was attempted by *Shan et al.*¹⁰⁰, who made electrodes from reduced graphene oxide covered with polyvinylpyrrolidone, from a polyethylenimine-functionalized ionic liquid and glucose oxidase. This approach was intended to exploit the high surface area and moderate electrical conductivity of reduced graphene oxide and attempt direct electron transfer between the glucose oxidase and the electrode. It is interesting to note that the use of graphene materials can respond to glucose up to three times better than most sensors made with CNTs⁴.

Another interesting development in electrochemical biosensors is the use of aptamers, an oligonucleotide sequences that can be generated to have affinity for a variety of specific biomolecular targets such as drugs, proteins, and even bacteria. Furthermore, aptamers can self-assemble on CNTs through π stacking between the nucleic acid bases and the nanotube walls. Consequently, considerable efforts have been directed towards incorporating aptamers and CNTs into the design of biosensors¹⁰¹. One significant study has been demonstrated by *Rius et al.*¹⁰² They reported a novel potentiometric biosensor made with aptamer-modified SWNTs that allowed specific real-time detection of one single colony-forming unit (CFU), effectively a single bacterium, of *Salmonella typhi*.

In the area of neuroscience, the application of electrochemical biosensor has also received increasing attention in the past few years ⁴⁶. The high stiffness of nanotubes makes them potential material for the electrodes to penetrate tissue and operate such that it does not significantly slow down the flow of electrons. This would aid in decreasing impedance and thus promoting the charge transfer.

Keefer et al. ¹⁰³ have investigated the use of nanotube-coated electrodes in preparing brain–device interfaces and showing that these combined materials also appears to be biocompatible. Furthermore, *Kotov et al.* ¹⁰⁴ fabricated layer-by-layer-assembled composites from SWNTs and laminin which is a significant part of human extracellular matrix. These thin films were found to be able to stimulate neural stem cells differentiation and subsequently promote further neuronal excitation. Another significant research is the work of *Lin et al.* ¹⁰⁵, in which a sensor was developed for a continuous and simultaneous monitoring of glucose and lactate in rat brain tissue. Real-time monitoring of the metabolic intermediate glucose or the circulatory impairment molecule lactate has been realized by using a complex electroanalytical platfor. Prior to the recording, the SWNTs were initially functionalized with glucose dehydrogenase or lactate dehydrogenase. More recently, the edges of multilayer graphene nanoflakes or the edges and functional groups of reduced graphene oxide has also been explored to perform at electrochemically detecting important neurotransmitters like dopamine and serotonin

²⁶.

2.4.3. Field-effect transistor based biosensor

One of the significant applications of SWCNTs in molecular electronics is for developing intramolecular field-effect transistors¹⁰⁶⁻¹⁰⁷. The intramolecular FET-CNT is then further developed as a basis of a biosensor; things that we will investigate in this thesis. In prior to that, a brief introduction about field-effect transistor, electrical transport phenomenon, and electrical transfer characteristic is described in the following sections.

a. Field Effect Transistor

Among various electronic devices, a transistor is suitable for sensing purposes. For this fact, one should start from the fundamental concept of conventional field-effect transistors (FET), where the doping agent material plays a significant role.

A field-effect transistor (FET) is a type of transistor that contains semiconducting material in the channel for its conductivity and relies on an electric field to control the channel conductivity. In the FET concept, electrons flow from the source terminal towards the drain terminal, if influenced by an applied voltage (both from source or gate). A conventional FET is constructed from different types of semiconductors in which Si became the most common. Conventional FET is normally categorized into two different types: Metal Oxide Semiconductor FETs (MOSFET) or Ion Sensitive Field Effect Transistor (ISFET). Although there was vibrant development of chemically sensitive FETs (CHEMFETs)¹⁰⁸, which incorporate a chemically selective layer or membrane into the MOSFET or ISFET device architecture, the commercial success of this new approach, was found to be very limited.

There are two different types of doping agents used in a conventional FET configuration: negative and positive doping agents. Impurity of valence five elements (such as N, P, or As) will act as a negative doping agent. It donates its electron to a valence four semiconductor (such as Si) so that the number of its electron charge carrier increases. It then generates n-type semiconductor in which the current between source and drain is increasing with the increment gate voltage. In contrast, valence-three doping agents (B or Al) will act as an electron acceptor. They will create extra hole (positive) charge carriers by taking away some electrons from the semiconductor lattice. This is called p-type semiconductor. In p-type semiconductors, the current is decreasing with the increment gate voltage.

FET developed based on nanocarbon materials is different from the conventional FET. In nanocarbon-FET device, the conductance is dominantly modulated through the Schottky Barrier at the nanocarbon-metal interface (though some other mechanism also contribute significant role, as described in section 2.4.3c). In a MOSFET, the conductance of the device is controlled by applying a V_g to the surface of the SiO_2 insulation layer separating the gate and Si conduction channel (which is called inversion layer). Once sufficiently positive V_g is applied a region of mobile charges in the underlying Si layer forms between the source and drain electrodes, the initially p-type Si will be turned into an n-type conductor, and then the device will turn on and current will flow. On the other hand, the ISFET was developed in the early 1970s for use in liquid environments and is illustrated in Figure 2. Operation of an ISFET is similar to a typical MOSFET except the device conductance is modulated through the electrochemical potential of a liquid environment using a reference electrode instead of an insulated gate

electrode. In this configuration the ISFET performance is then controlled by the chemical environment, and shows sensitivity to pH and metal ions in solution. In the current sensor, it is typically called as liquid gate or front gate voltage.

A conventional example of a gas-sensitive MOSFET is the H_2 sensitive FET constructed with a Pd gate, which was first reported by Lundström et al in 1974¹⁰⁹. In this sensor device, the response relied on gas interaction with the device gate electrode, which was located on top of the semiconducting SiO_2 conduction channel. The dissolved hydrogen atoms subsequently diffused into the Pd- SiO_2 interface, which in turn changed in the Pd work function and shifted in the FET transfer characteristic.

A schematic of a typical top-gated MOSFET device is illustrated in

Figure 2. Schematic diagram of: a.) a metal oxide semiconductor FET (MOSFET) and b.) an ion selective FET (ISFET).¹⁰⁸.

On the subsequent development, nanotube based FET devices was found to perform several intrinsic properties that make them an attractive alternative to more established semiconductor FET architectures. Nanotubes constructed in a top-gate

configuration was found to perform a nearly four-fold increase in the I_{ON} , more than a two-fold increase in the transconductance, and an I_{OFF} approximately half that of the Si-MOSFET device¹¹⁰. Though the device performance of MOSFET has not yet been optimized, this finding clearly shows that the carbon nanomaterial has great potential for success in applications previously dominated by Si-technologies. In sensor applications point of view, this tremendous breakthrough will result in a larger response for any given shift in the transfer characteristic, thus result in much more sensitive sensor device.

In addition to enhanced electrical transport properties, the unique structural and physiochemical characteristics of nanocarbon materials, as described earlier, has set them apart as an excellent choice for sensor applications.

b. Typical Device Characteristic in CNTs-FET and Graphene-FET

Most of the time, nanotube transistors show a p-type characteristics (as shown in figure 3 on the next page); unless it is subjected to further treatment¹¹¹. Graphene, on the contrary, show a typical ambipolar V-shape characteristic with shifted dirac delta point upon modulation of the gate condition^{73,76,112} (figure 4).

The reason why CNTs can show this p-type characteristic remains under debate. A possible mechanism to describe this phenomenon is the fact that nanotubes channel is attributed to oxygen adsorption. If we compare this to what occurs in a conventional transistor, we can suggest that the presence of oxygen will take away electrons from the semiconductor substrate. Extra hole generated by that atom transforms the device to become p-type. In an experiment about bipolar p-n diode or logic-gate circuit application, it was reported that potassium doping or simple spin casting polyethylenimine (PEI) can

change p-type to n-type characteristic ¹¹³. This can support the idea of the doping mechanism by the oxygen adsorption.

On the contrary, a report by *Heinze et al.* ¹¹⁴ has assigned effects of oxygen exposure is different to what happens in an conventional transistor. They reported that oxygen adsorption will change the work function of the exposed portion of the metal electrodes instead of acting as a doping material. It also fits with the other reports on logic circuits, in which metals with different work functions were used to control the n-p polarities of the SWCNTs transistors.

Figure 3. a.) Transistors transfer characteristic I_d - V_g of a CNTFET at different source-drain voltages and b.) transistors output characteristic I_d - V_{ds} for different gate voltages ¹¹⁵

A typical device characteristic for a nanotube transistor is shown in figure 3 above. The p-type characteristic is shifted with various applied bias voltages (V_{ds}) as shown in figure 3a. The plot of the I_{ds} vs drain voltage is also shifting with various applied gate voltages (figure 3b). In the lower gate voltage, the device conductance decreases. As a consequence, the I_d - V_d curve looks flatter. As discussed above, a semiconducting nanotube is required in order to achieve this transistor characteristic. Metallic tube should be removed from the mixture after the synthesis.

Figure 4. Transistors transfer characteristic I_d - V_g of a graphene-FET at different modulation of chemical doping⁷³

Different from CNTs, ambipolar V-shape characteristic is typically observed on graphene-FET (figure 4). The parallel-shift in the figure above corresponds to the chemical doping and possible inducement of charge impurities at the same time. This

typical shift is often utilized for the detection of individual gas molecules^{73,80} and pH of the solution^{76,79,112}. As illustrated on the figure 4, doping increased from zero (black curve) to $\sim 1.5 \times 10^{12} \text{ cm}^{-2}$ (red curve) due to increasing exposure to NO_2 . The gradient of the $\sigma(V_g)$ curves (away from the Dirac point) characterizes the charge carrier mobility, μ . Conductivity (σ) of single-layer graphene away from the dirac point changes was almost linear with increasing V_g .

Doping with NO_2 adds holes and induces charged impurities. The latter apparently do not affect the mobility of either electrons or holes. This parallel shift implies that a negligible potential scattering effect of the charged impurities induced by chemical doping. The practically constant σ for the same concentration of holes (n_t) yields that the absolute mobility, $\mu = \sigma/n_t e$, as well as the Hall mobility are unaffected by chemical doping.

c. Detection mechanism of nanocarbon based biosensor

Although an appreciable amount of biosensing studies has been conducted using carbon nanotube transistors, the physical mechanism that underlies sensing is still under debate. Various mechanisms have been proposed to describe the interaction between biomolecules and nanocarbon materials. As reviewed in many articles, biomolecules may influence the nanotube conductance via several ways including oxidation induced electron transfer/electrostatic gating¹¹⁶⁻¹¹⁷, change in the gate-coupling efficiency⁴⁴, reducing carrier mobility², and also causing Schottky barrier effects¹¹⁸. The lack of a good understanding of the sensing mechanism hampers the further exploitation of these promising nanosensors.

Figure 5. Calculated I - V_g -curves before (black) and after (red) protein adsorption for four different sensing mechanisms. The bias voltage is 10 mV. (a) Electrostatic gating effect corresponding to a 50 meV shift of the semiconducting bands downward. (b) Schottky barrier effect that corresponds to a change of the difference between metal and SWNT workfunctions of 30 meV. In panels a and b, left and right insets illustrate the corresponding changes in the band diagrams for hole and electron doping respectively. (c) Capacitance mechanism for a 90% coverage of SWNT with protein ($\epsilon = 10$, diameter = 6 nm). In panels c and d, the insets illustrate the corresponding changes in the band diagrams. (d) Mobility mechanism that corresponds to a mobility reduction to a mere 2% of the initial value.

The four general detection mechanism in CNTs device has investigated by *Heller et. al.* as illustrated in the figure 5 above. Charge transfer/electrostatic gating (figure 5a) is an effect caused by adsorption of charged species that induce a screening mechanism in the SWNTs and results in I_d - V_g curve shifting along the voltage axis. Donor of electron

charge will shift the curve to more negative voltage. On the other hand, withdrawal of electron will cause adverse effect.

Adsorbed biomolecules may also affect the metal contact and modulate local work function of the device. As the consequences, the device characteristic is affected. This phenomenon is described as schottky barrier mechanism, as shown in the figure 5b. This mechanism was initially suggested by *Chen et al.*⁴⁴, where adsorbed biomolecules at the metal contact modulate the local workfunction and thus the band alignment. Since the Schottky barrier height changes in opposite directions for hole (p) and electron (n) transport, a characteristic asymmetric conductance change for p- and n-branches of $I-V_{lg}$ curves is obtained.

Change in gate efficiency coupling, as another mechanism (shown in figure 5c), could occur when the capacitance of the gate is reduced because of the low permittivity of the biomolecules relative to the electrolyte. Since the nanotube electrolyte interfacial capacitance is normally dominated by the SWNT quantum capacitance, the electrostatic capacitance reduction due to adsorbed biomolecules can only become apparent in case of near-full coverage under the disputable assumption that ions cannot permeate through this protein layer¹¹⁹. In this mechanism, biomolecules may inhibit the binding of ions out of the electrolyte to the nanotube network, thereby reducing the electrical conductance.

The last proposed mechanism is the reduction of the carrier mobility (potential scattering) as illustrated in figure 5d. This mechanism explains that addition of molecules could suppress the conductance on both p- or n-type device. However, this typical mechanism is unlikely to dominate the sensing mechanism.

Among four different mechanisms described above, the interaction between biomolecules and nanotubes in field effect configuration is dominated with the electron transfer or schottky barrier modulation.

Interaction of streptavidin and SWNT in an experiment carried out by *Bradley et al.*¹²⁰, was described as an example of charge transfer mechanism. Using NH_3 as a model, it was assumed that each of the approximately 4000 amine groups per streptavidin molecule in contact with the SWNT donated 0.04 electrons into the SWNT valence band.

One well-known experiment by *Star et al.*¹²¹ described an interesting biotin-streptavidin binding in SWNT device. They mentioned that there were two different mechanisms between the bare and polymer-biotin functionalized NTFET in response to streptavidin binding. The negative shift in the $I-V_G$ transfer characteristic and decreased on-state current of the bare NTFET device suggests streptavidin adsorption resulted in a direct electron donation into the SWNT. On the other hand, the decreased slope in the $I-V_G$ transfer characteristic of the biotin-polymer functionalized NTFET suggests streptavidin binding resulted in a reduction in charge mobility.

On the BSA detection experiment carried out by *Minot et al.*, it was reported that BSA interaction with SWNTs was also determined by charge transfer mechanism. In this report the influence of the physical structure of the gate electrode on the behavior of liquid gated NTFET devices during protein adsorption was investigated.

On contract to the reports mentioned above, an interesting research was reported by Byon and Choi¹¹⁸ who measured the response of NTFET devices with extended Schottky contact areas to proteins such as SpA (derived from *Staphylococcus aureus*), streptavidin, mouse antibody β -hCG, human chorionic gonadotropin (hCG), and rabbit

immunoglobulin G (IgG). This report is a smart demonstration of how identification and exploitation of the dominant response mechanism can be used to maximize the device sensitivity.

On another experiment result, *Dai et al.*¹²² demonstrated that protein and SWNT interaction activity actually rely on the effect Schottky barriers modulation rather than direct interaction between SWNT and protein. This is confirmed by their results which showed that unpassivated NTFET devices showed significant response to several proteins including human chorionic gonadotropin (hCG), polyclonal human IgG (hIgG) and bovine serum albumin (BSA). In contrast, devices with passivated electrodes did not show response to protein exposure.

SWNT response to DNA hybridization is a direct result of modification of the metal work function of the Au device electrodes and did not originate from a change in the electronic density of the SWNT valence band, as reported by *Tang et al.*¹²³. In their experiment, SWNT devices with Au electrodes covered with a self-assembled monolayer (SAM) of mercaptohexanol (MCH) were used to study the hybridization of complementary strands of thiolated DNA where a Pt wire acted as a liquid gate electrode. They found that DNA hybridization did not occur on the SWNTs, but rather it occurred only on the MCH covered electrodes. In contrast to that, *Gui et al.*¹²⁴ have recently reported that SWNT response to DNA hybridization can be attributed to a combination of charge transfer with the SWNT and Schottky Barrier modification. The Schottky barrier modification, however, is still considered as the dominant mechanism driving the device response.

Different from SWNT device, the interaction mechanism between biomolecule and graphene, so far, is not verified yet. To date, it is dominantly described as the effect of polarity specific gating upon the binding of negatively or positively charged species (which causes an increase or decrease in device conductivity, respectively) ¹²⁵ or as an effect on electrical carrier (both hole or electron) doping ^{73,112}. It has also been reported that direct molecule absorption would possibly cause small conductance changes ⁷⁹.

d. Current application of FET sensor based on nanocarbon materials

The nanocarbon FET has shown great promise in becoming a platform technology for studying a vast array of biological systems. The nano-scale dimensions of the conduction channel allow the design and fabrication of novel electronic architectures that are inherently sensitive to their local molecular environment. More recently, interaction with living system has also started to receive more attention.

CNTs can act as a metallic or semiconducting material simply based on their chirality (as described in section 2.2.3), while on the other hand graphene typically shows a V-shape characteristic corresponding to their ambipolar (both p- and n- type) performance. For its application as transistor, semiconducting CNTs and graphene are used as the conducting channel between the source and drain electrode (shown in the figure 6 on the next page) as the replacement of Si in conventional FETs. The gate voltage can be applied from the backside of the substrate and this configuration is named backgate voltage (similar to MOSFET application). On the other hand, one may also apply the gate voltage from the top of the source and drain voltage using the electrolyte solution, which is called front-gate voltage or liquid-gate voltage (similar to ISFET

application). It is critical to study the performance of nanocarbon materials in an aqueous solution, especially when it is exploited for biological sensing applications. Interestingly it has been shown that graphene immersed in an electrolyte shows better transfer characteristics (almost ~30 times) compared with their characteristic in vacuum ⁷⁹, and the same phenomenon also happens with carbon nanotubes. This occurs because the formation of a very thin top-gate double layer in the electrolyte. The application of the gate voltage, meanwhile, falls across at this solution-nanocarbon interface.

Figure 6. Schematic diagram of a field-effect transistor based on carbon nanotube (CNTs-FET) ²

Transistor device developed based on nanocarbon materials can offer several advantages. Firstly, they act as the conducting channel having direct contact to the environment. Since all the current carriers flow at the surface, the conductance of the nanocarbon is highly sensitive to the electrochemical perturbations at their surface or in their immediate vicinity ¹. Secondly, this typical electrical measurement can provide very simple and real-time detection of the biomolecules.

In general, there are two types of nanotube transistor device. The first one is a device that is fabricated based on a single nanotube. The other type is based on the carbon nanotube network. Single-channel nanotube devices were found to have superior electronic aspects. However, major problems are the reproducibility and the efficiency. The device performance varies a lot from tube to tube. Therefore, carbon nanotube networks which are reproducible low cost and highly efficient are better suited for applications. In addition, the network devices typically have lower $1/f$ noise and provide a larger area for cell interfacing. As reported in recent reviews, nanotube network has extensively studied as thin-film transistor, diodes, field-activated optical modulators, strain and chemical sensors, field emission devices, and transparent conductive coatings¹²⁶. On the other hand, reduced graphene oxide (rGO),^{69,127} prepared from the chemical⁶⁸ or thermal reduction^{71,128} of graphene oxide, has emerged recently as a competitive alternative material. Though the development of this material as sensor is still relatively new, it can show potential advantages compared to CNTs.

In the next section, we categorized the application of field-effect transistor based nanocarbon materials into three different groups: detection of environmental changes, detection, of specific biomolecules, and detection of living cells.

Detection of Environmental Changes

The effect of environmental change on the nanocarbon-transistor surface is similar to an impurity material in a conventional transistor, which may results in two different mechanisms: firstly, charge transfer from molecule to the nanocarbons and secondly, reduction of the carrier mobility (potential scattering).

The charge transfer mechanism can shift the device characteristic towards more negative voltages (corresponding to an electron donation from molecule to nanocarbon) or positive voltages (corresponding to a hole donation) as shown in figure 7a. On the other hand, molecules to which act as a scattering center, can reduce the carrier mobility, which causes the current to drop without shifting the p-type characteristic (as shown in the figure 7b).

Figure 7. Change in the transistor electrical characterization in the presence of an adsorbed species. a.) Electron-transfer effect from absorbed analyte to the sensing area. b.) potential scattering (reducing the carrier mobility) of charge carriers by the scattering potential, because of the analyte absorbed ².

Interaction of devices with nitrogen gas such as NH_3 and NO_2 has been investigated by *Chang et al.* ¹²⁹ and *Schedin et al.* ⁷³. As a result, the shifting phenomena in the p-type or ambipolar characteristic can be observed in CNTs and graphene devices. It also strongly suggests that the NH_3 and NO_2 gasses will cause charge transfer effects.

NH_3 will act as an electron donating species and NO_2 will act as electron withdrawing species (figure 8). In estimation, each adsorbed NH_3 molecule donates 0.04 electrons to the nanotubes conducting channel. In more recent research by *Bondavalli et al.*¹³⁰, however, the effect of those amine gasses are suggested to originate from modulation of the Schottky barrier instead of this typical electron transfer.

Figure 8. Charge transfer effect in nanotube network device by nitrogen gas²

Besides the influence from gas molecules, effect caused by the coating of polymers, such as poly(ethylene) glycol (PEG), poly L-lysine (PLL), and poly(ethylene) imine have also been explored^{129,131}. The PEG layer was found to have a little influence to the device characteristic, while PEI in contrast caused a dramatic change. Effect of this polymer coating is similar to an electron donation by the NH_3 groups (electron donating). PLL coating would also cause an electron donation based on its positive charge nature.

Detection of specific biomolecules and biological processes

To date, interactions between nanocarbon sensors and specific biomolecules has been extensively studied with electron transfer or Schottky barrier modulation were found to be the dominant mechanism (described in section 2.4.3c).

Various types of biomolecules, including proteins^{79,132}, DNA¹²⁴⁻¹²⁵, ion molecules¹³³, dopamine¹³⁴, organophosphorus compounds¹³⁵, cholesterol¹³⁶, NAD⁺⁹⁴, nitric oxide¹³⁷, cytochrome c¹³⁸, and morphine¹³⁹, have been successfully detected using nanocarbon biosensors. Specific binding of the molecules to the sensors were characterized by either a conductance changes in real time measurements or shifts in the I_d - V_g device characteristic. It was suggested that most of the interactions to nanocarbon are based on the noncovalent π - π stacking, direct absorption, or the hydrophobic interaction on the carbon surface.

The detection mechanism of nanocarbon biosensor typically relies on the use specific antigen-antibody interactions^{121,140}, synthetic oligonucleotides¹⁴¹, or enzymatic reactions¹¹⁶⁻¹¹⁷, whereas a nonspecific adsorption should be avoided. A layer consisting of poly(ethylene)imine (PEI), poly(ethylene) glycol (PEG), or Tween 20, were typically used for blocking the non-specific binding to the sensor.

The first graphene-based electrical biosensors were demonstrated by *Mohanty and Berry*, who produced sensors from graphene oxide or from graphene amines made by treating graphene oxide with nitrogenous plasmas or ethylenediamine¹²⁵. Electrical measurements on this device showed that the initial attachment of single DNA strands more than doubled the conductivity of the graphene oxide, which was plausibly attributed to the negatively charged molecules providing negative gating, thus increased the hole

density. Interestingly, DNA hybridization caused a further increase in conductivity, which was completely reversible during cycles of denaturing and rehybridization. They also demonstrated that adsorption of polyelectrolyte molecules on the modified graphenes can generate polarity-dependent changes in the device electrical conductivity.

A very recent study by *Ohno et al*⁷⁹. demonstrated electrical detection of dissolved bovine serum albumin (BSA), at a level as low as 3 nM, by means of an electrolyte-gated graphene FET. Besides, they were also able to explore the changes in conductance of the device with different top- or bottom-gate voltages, as well as with different pH values. It was also shown that the position of the Dirac point increased linearly over the range of pH values examined, from 4.0 to 8.2.

Detection of Living Cells

Published research works related to the detection of living cells are relatively rare compare to the last two sections discussed before. One of the main reasons is the difficulty to achieve a good interface between the nanocarbon material and the cell membranes, whereas functionalities of both sensors and cells should properly be maintained.

This typical detection scheme has significant impacts in many areas. One example is recognition of pathogenic organisms, such as yeast and bacteria. This is very important for the quality control processes in the food industry or for water purity controls. Current methods that are clinically used, however, only rely on selective growth of that pathogenic organism from a contaminated sample, which can take several days. Although recently, some faster techniques, including fluorescently labeled antibodies¹⁴², DNA

probes ¹⁴³, and bacteriophages ¹⁴⁴ have been developed. Electrical characterization by applying nanocarbon sensors is a still very promising alternative, since it can provide very sensitive detection in real time and with a very high temporal resolution.

So far, detections of pathogenic materials by applying nanocarbon materials have been successfully demonstrated by several research groups. *Elkin et al.* ¹⁴⁵ have successfully demonstrated the recognition of specific target *E.coli* O157 via antibody–antigen interactions by using immuno-carbon nanotubes. These nanotubes were prepared by conjugating BSA functionalized SWNTs with specific antibodies. Similar strategies of antigen-antibody reactions have also been used for the detection of *Candida albicans* ¹⁴⁶ yeast and *Salmonella infantis* ¹⁴⁷ bacteria. In this case Tween 20 at 0.5% was applied to block non-specific binding. Slightly different strategies were performed by *Odaci et al.* ¹⁴⁸. Based on the respiratory activity of the cells, they developed sensors for two types of bacteria: *Pseudomonas fluorescens* and *Pseudomonas putida*. For this experiments they used chitosan modified nanotubes. In a recent experiment using chemically modified graphene device, as reported by Mohanty and Berry ¹²⁵, an electrostatic adsorption were achieved between negatively charged bacteria on the positively charged graphene amine, where they survived for up to 4 h. Adsorption of a single bacterium on a graphene–amine device was found to cause a 42% increase in conductivity, thus demonstrating single-cell sensitivity.

The interaction between nanocarbon and virus has also been investigated ¹. However, it was found that the shifting of the device characteristic, when that material interacts with viruses, is not significant. It was found to be about an order of magnitude smaller than in the case of streptavidin. This suggests that the binding of the surface

proteins to the nanocarbon is weak. In addition, binding between them is also determined by the arrangement of the viral surface proteins.

Investigation of various dynamic cell activities has also received increasing attention nowadays. Extracellular recording of dynamic cardiomyocytes beating ^{12,149-151} and action potentials of excitable neurons ^{17,19,152} using FET arrays has been extensively investigated in the past few years. Their sensitive, fast, and invasive detection scheme is really a clear advantage compared to classical electrophysiological methods, such as microfiber glass capillaries and patch-clamp electrodes ⁷ (which are very invasive to the cell) or fluorescence methods with voltage sensitive dyes ⁸ (which are found to be toxic upon illumination). In addition to those research works, detection of some dynamic cellular secretions has been successfully demonstrated in our lab, including ATP released from astrocytes ¹⁵³ and catecholamine release from PC12 cells ¹⁵⁴.

By reviewing to another research direction, interaction between a cell and a sensor has also drawn interest on the application of cell-based biosensors (CBBs), which treat living cells as sensing elements, to detect the functional information of biologically active analytes ¹⁵⁵. To the best of our knowledge, the application of nanocarbon materials in this research topic, nevertheless, has not been extensively studied. This is limited by two major issues: the variability of the cell response in every experiment (which causes this system to be less reliable) and the contamination issue (devices must be maintained sterile during the whole cell culture over several days).

Chapter 3

Research Methodology and Preliminary Result

3.1. Cell Culture Work

In our research, several cell lines were used, including PC12 cell, smooth muscle cell, and HL-1 cardiomyocytes. On the other hand, astrocytes and hippocampal neuron cells were extracted directly from rats as primary cells. All cell culture protocols were described in detail in appendix C.

3.2 CNTs and Graphene Device Fabrication Methods

The CNT-net fabrication was done using three different techniques: dropcasting, film transfer, and evaporation methods. In dropcasting method, small drop of CNTs solution was cast directly on the substrate and evaporated. In film transfer method, CNTs thin film was initially prepared on the top of pentane layer before it was transfer-printed onto the substrate. CNTs pattern could be formed by using evaporation method. In this technique, substrate was located on the CNTs solution and then the solution was evaporated overnight. Band pattern of CNTs were formed directly on the substrate. The detail procedures for those three CNTs device fabrication methods were described in appendix E. On the other hand, reduced graphene oxide (rGO) device were prepared as rGO pattern (using hard PDMS stamp) and rGO sheet form (using chemical vapor deposition). Experiment protocols for those two methods were described in appendix F.

3.3. CNTs and Graphene Device Preliminary Result

The different methods of CNTs device fabrication (dropcasting, film transfer, and evaporation) and reduced graphene oxide production (on various substrates) were examined. The source-drain current under a voltage bias of 400 mV was measured while varying the solution gate voltage (-1 V to 1 V). Leakage current was measured by detaching the source probe from the system. The real current is assumed to be the initial front-gate measurement deducted by the leakage current.

Figure 9. Typical device characterization with front gate operation measured in a bath solution. Bias voltage $V_{ds} = 400\text{mV}$. Red line: front gate measurement. Yellow line: leakage current. Blue line: front gate current after deducted with leakage.

This graph is an example of a good device with a high on-off ratio. The SWNT-net manifests a p-type field-effect characteristics and its conductance is very sensitive to voltage gating. Specifically, the current decreased more than 10 times as the gate voltage varied from -1 V to 1 V. It is noted that besides the device characterization, film uniformity is another important issue for the optical imaging.

Summary of the device fabrication results are mentioned below:

3.3.1. Dropcasting method

Figure 10 . SEM image picture of a CNTs film (concentration 0.01 mg/mL) on different area fabricated using dropcasting method

The sample fabricated with the dropcasting method provided promising results in term of sensitivity and on-off ratio. However, the conductivity of those samples also tend to be high, because thick films were obtained using this method and the uniformity of the films can only be found in the side part (Figure 10). The size of the area network can be obtained using this method and can be easily controlled by adjusting the amount of solution deposited. The dropcasting method with concentration 0.01-0.02 mg/mL was

found to be appropriate enough for device fabrication. This method was also the simplest compared to the other two methods mentioned afterwards. This method, however, results in non-uniformity of CNTs network on the whole drop area.

Intermolecular attraction force of the CNTs solution particle near the edge of the solution droplet is weaker compare to the CNTs particle in the center of the droplet. The water molecules, therefore, can escape easily through evaporation process becoming water vapor. The position of evaporated water molecules were replaced by the water molecule from the inside, which also caused the shifting of CNTs particle to move to the edge of the solution drop. This phenomenon keeps going on until the whole water molecules were evaporated. A ring of SWNT-net was formed with decreasing density from the edge till disappearance at $\sim 140\text{ }\mu\text{m}$ away from the edge as shown on the SEM image on figure 10.

3.3.2. Film transfer method

After centrifugation of CNTs and removal of the impurities, uniform films were obtained as long as concentration was high enough (0.05 mg/mL) as presented in figure 11. However, the device fabricated with this method typically resulted in a lower on-off ratio of the devices compared to the dropcasting method due to significant leakage currents. Variations in the electrical characterization results were larger when this method was applied.

Figure 11. SEM image picture of CNTs film (concentration 0.05 mg/mL) fabricated using film transfer method.

It is interesting to note that we used both film transfer method and dropcasting method for different biomolecule detections. Devices fabricated using film transfer method was used in the detection of ATP released and dynamic secretion of biomolecules from living cells (chapter 4 and 5). The reason for choosing this method is the film uniformity of the CNT network, which is expected to result in better interface between the living cells and carbon materials.

The film-transfer devices, however, fail to function well when they are used in the detection of cardiac muscle action potential and *Escherichia coli* glucose metabolism process (chapter 6). Interestingly, devices fabricated using drop casting method can give good and consistent experiment results. The presence of non-uniform network made the device sensitivity to be concentrated at limited centralized area (at the CNT ‘ring’), hence, enhance the sensing capability at that particular location.

3.3.3. Evaporation method

This method had advantages in generating aligned CNTs, since cells can grow smoothly according to that line (figure 12). The higher surface roughness, which located on that line, possibly attracts the cells, inducing migration, and enhance adhesion.

Figure 12. PC12 cells can attach to the substrate according to the CNTs line

However, it was unfortunate that the on-off ratio of devices fabricated using this method was found to be very low. This was possibly due to the massive mixture of metallic and semiconducting CNTs in every line generated. The effort to scratch out some strips from the device was quite risky in losing the device conductance and did not improve the on-off ratio significantly. This method, in addition, was difficult to be controlled, which resulted in high variability. So far, no significant improvements were achieved to obtain good devices using this fabrication method.

3.3.4. Graphene Pattern and Graphene Sheet Results

Figure 13 a.) Typical device characterization of a rGO pattern device measured with front gate operation in a bath solution. Bias voltage $V_{ds} = 400$ mV. b.) Typical device characterization of rGO sheet device measured with front gate operation in bath solution. Bias voltage $V_{ds} = 400$ mV.

A typical device characterization of on both graphene pattern device and a graphene sheet device is presented in figure 13. Although the graphene sheet presents a much larger conductance (about 2 orders of magnitude difference) compared to the graphene pattern device, a similar ambipolar characteristics with on-off ratios $\sim 1.5 - 4.5$ was obtained with both types of devices. Specifically, the device conductance of the graphene pattern device decreased from 965.15 nA to 223.35 nA and from 231.1 μA to 150.121 μA on the graphene sheet device. Besides, this is noted that the devices' dirac delta point of graphene was typically slightly positive (0.2 to 0.4 V). This dirac point location would be important in determining the p- and n- type region of the devices and consequently the response to biomolecules.

Figure 14. Contact resistance measurement with various lengths of the rGO patterns. It is shown that the conductance measured was achieved dominantly from the graphene and not from the contact junction.

Besides, the measurement of the contact resistance of the devices (figure 14) presents a good linear relationship with a y-intercept very close to 0. This suggests that the conductance measured was dominantly obtained from the graphene itself and not from the metal-graphene contact junction.

3.4. Assessment of (n,m) Selectively Enriched Small Diameter Single-Walled Carbon Nanotubes¹⁵⁶

Synthetic processes for carbon nanotube fabrication, either via arc discharge, laser ablation, or chemical vapor deposition, always produce mixtures of metallic and semiconducting tubes. In order to develop sensitive sensors, a good separation technique, which mainly aim at pure semiconducting tubes, should be achieved.

In one of the approaches, we selectively enriched SWNTs, which possessed small diameters (<0.9 nm) and a narrow (n,m) distribution, synthesized on cobalt-incorporated MCM-41 catalysts. The (7,5) enriched SWNTs were obtained from sodium cholate (SC) dispersion, whereas (6,5) were from cosurfactant mixtures of sodium dodecyl sulfate (SDS):SC at a ratio of 1: 4. A density gradient ultracentrifugation was applied to further improve the separation.

The characterization of the nanotube chiralities was then achieved via photoluminescence excitation spectroscopy, optical absorption spectroscopy, Raman spectroscopy, and electrical transport measurements. Among these techniques, the results demonstrated that the electrical transport measurements (via $I_{\text{on}}/I_{\text{off}}$ ratio measurement) of thin-film FETs was the most sensitive technique to evaluate the purity of the semiconducting SWNTs. Besides, we found that devices fabricated from CNTs separated by SC alone had better electrical properties compared to those separated using SDS/SC.

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Chapter 4

Label-free Detection of ATP Release from Living Astrocytes with High Temporal Resolution Using Carbon Nanotubes Network¹⁵³

4.1. Abstract

Single-walled carbon nanotubes (SWNTs), with their remarkable electrical, physiochemical, and one-dimension structural properties, were recently employed as a nanoelectronic biosensor for chemical and biomolecule detection with extraordinary sensitivity and specificity. Nevertheless, current applications are limited to static in-vitro measurement. Herein, we demonstrate that SWNT network can directly interface with living neuroglial astrocytes and label-freely detect local ATP secretion from these cells upon stimulation in real-time with a millisecond resolution.

4.2. Background of the study

As described in the literature review, interaction of nanodevices and nanomaterials with biological systems has drawn increasing interest in various research fields with high significance. This is supported by their substantial advantages, including size compatibility and unique molecular specificity of nanomaterials to biomolecules.

In our research work, we use SWNT network to achieve localized real-time detection of ATP release from living astrocytes directly cultured on top of the SWNTs. In comparison to single SWNT based device¹²⁶; SWNT network, with compromise in sensitivity and some other electrical properties, can provide higher reproducibility, larger detection area, lower 1/f noise, and relative simplicity in the fabrication process. In

addition, the size dimension of the network would be more appropriate to interface mammalian living cells (which are about ~10 μm in diameter).

4.3. ATP Review

Adenosine Triphosphate (ATP) - which is commonly known as a universal energy source of cells- is also an important signaling molecule, which regulates many biological functions¹⁵⁷. Particularly, it plays a role as a neurotransmitter or neuromodulator in both central and peripheral nervous systems¹⁵⁸. ATP signaling, mediated by inotropic or metatropic purinergic receptors expressed on the cell membrane, is implicated in cognitive functions such as learning and memory formation. It has been recently known that ATP release from astrocytes has a significant contribution in astrocyte-neuron communication, hence, consequently shaping synaptic neurotransmission¹⁵⁹. This is adding a new piece of evidence that glial cells, which were merely regarded as neuronal supporting cells, actually take active roles in the central nervous system¹⁶⁰. In the astrocyte-neuron communication, glutamate neurotransmitter secreted by neurons can trigger glutamate receptors on the membranes of the adjacent astrocytes to stimulate Ca^{2+} release from endoplasmic reticulum (ER), and subsequently increase cytosolic Ca^{2+} and trigger ATP release via secretory vesicles¹⁶¹. After binding with the receptors on the nearby astrocytes and neurons, the released ATPs cause a propagation effect of Ca^{2+} wave throughout the astrocyte-neuron network¹⁶². Such mechanism contribute further glutamate release from neurons, working as a positive feedback in a form of activity-dependent mechanism of the neuronal network¹⁶³.

Methods development to detect ATP signaling is indeed significant due to the important physiological role of this molecule. However, due to the lack of methods in monitoring the dynamic process in real time, interaction and mechanisms of ATP release from astrocytes are not yet fully verified up to this moment. ATP detection currently depends on bioluminescence assays¹⁶⁴, represented by a widely adopted methods named firefly luciferase assay¹⁶⁵. Despite its sensitivity, this method can only report the number of diffused ATP in the bulk extracellular medium. The measurement does not reflect the true local concentration of released ATP at the same time, because of medium dilution and high ecto-ATPase activities. It cannot be determined whether the extracellular ATP is actually obtained from nonspecific cytolysis or from a particular release mechanism which is difficult to be recognized.

Contrary to the release of ATP, which is expected to be quick and highly regulated, current off-time measurements, unfortunately, are not able to report the dynamic ATP flux at the cell surface. Therefore, firefly luciferase is adhered to the surface of the cell to detect the local ATP flux in order to solve this issue¹⁶⁶. This method, however, is not a real-time measurement and needs a complicated molecular engineering in the process.

In an approach named sniffer cell, a cell which is transfected with ATP receptors is used as the ATP sensor, and ATP excited from the target cell is relayed to the responding transmembrane current and continuously analyzed via patch-clamp recordings.¹⁶⁷ Such indirect detection requires tedious techniques and produce low throughput. Electrochemical approaches have been established as an alternative. Nevertheless, those

techniques often depend on enzymes mediation ¹⁶⁸ or ATP binding molecules ³⁷, and demonstrate low sensitivity and temporal resolution.

Considering all limitations of the current methods, here we proposed an innovative idea by employing a nanotechnology approach, particularly carbon nanotube networks, to engage the biological system and to investigate the ATP release phenomenon.

4.4. CNTs-FET Device Fabrication

Figure 15. (a) Representation of SWNT-net device fabrication (b) AFM image of a SWNT-net. A representative cross-section profile is shown as inset. (c) Conductance of SWNT-net device is sensitive to the solution gate voltage (V_g). V_{ds} is 400mV. V_g is applied as shown in the insert, to the bath solution via an Ag/AgCl electrode.

The CNT-FET devices were fabricated using the film-transfer technique (as described in Appendix A section E2). An ultra-thin network consisting of small SWNT bundles (10-20 nm) was generated on the coverslip substrate, as revealed by atomic force microscopy (figure 15b). Electrical properties of a SWNT-net device are characterized as shown in figure 15c. The source-drain current under a voltage bias of 400 mV was measured while varying the solution-gate voltage. The gating voltage was applied via an Ag/AgCl electrode immersed in the physiological bath solution. It is noted that SWNT-net manifests p-type field-effect characteristics and its conductance is very sensitive to voltage gating. The source drain current specifically decreased from 9.49 μ A to 0.12 μ A as the gate voltage varied from -1 V to 1 V. This data suggests that our SWNT-net device is highly responsive to the electrical perturbation in the solution, hence desirable for electronic biodetection.

4.5. Astrocytes Isolation, Cultures, and Staining

Astrocytes were isolated from the brains of neonatal rats (as previously described in Appendix A section C3). A bright-field image of astrocyte cells on SWNT-net is presented in figure 16. Immunostaining using a specific marker of astrocytes in the central nervous system (type III intermediate filament protein glial fibrillary acidic protein -- GFAP) was conducted in order to confirm the cell type. The cells were initially fixed by 4% formalin at 37 °C for 30 minutes and subsequently treated with permeabilization buffer (0.15% Triton X-100 in PBS, 10% goat serum) for 1 hour followed by the addition of 10 μ g/mL anti-GFAP primary IgG antibody (Promega). After 18 hours incubation at room temperature and dialysed with PBS, cells were treated with

permeabilization buffer containing 10 $\mu\text{g/mL}$ Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch), and incubated in the dark for one hour. After rinsing with PBS, confocal fluorescence images was taken (excitation: 550 nm; emission: 570 nm) to verify the astrocyte cell type (figure 16b).

Figure 16. (a) Bright-field image of astrocytes cultured on a SWNT-net. (b) Confocal fluorescent image of immunostained astrocytes on a SWNT-net. (c) Illustration of how the ATP-release from an astrocyte is triggered and detected.

A schematic illustration on the process of stimulating ATP release from astrocytes and detection via SWNT-net device is shown in figure 16c. Glutamate, one of the most common neurotransmitters in the central nervous system, was applied to trigger the secretion of ATP from the cells¹⁶⁷, through a series of molecular events. Activation of phospholipase C (PLC) occurs initially after the binding of glutamate to the metabotropic glutamate receptor (mGluR); followed by hydrolysis of membrane lipid phosphatidylinositol biphosphate (PIP₂) to generate inositol triphosphate (IP₃). IP₃ subsequently opens IP₃ sensitivity Ca²⁺ channels on the internal Ca²⁺ stores inside the endoplasmic reticulum, trigger Ca²⁺ release into the cytoplasm and consequently elevate cytosolic Ca²⁺ concentration. Finally, Ca²⁺ ions stimulate cascade reactions of a set of secretory proteins, and induce fusion of secretory vesicles with the plasma membrane to release ATP molecules inside to the extracellular space. The released ATP molecules then diffuse into the interface gap between the SWNT-net and the cells, and interact with SWNT-net via non covalent π - π stacking interaction¹⁶⁹. The ATP molecules, which are in close contact to SWNT by such interaction, exert strong influences on conductance and thus trigger measurable current response.

4.6. Cells Electrical Recording

The astrocyte cell activity was continuously monitored as a function of source drain current (I_{ds}) by using a semiconductor device analyzer (Agilent Technologies, B1500A), while biasing the source and drain electrodes at 400 mV. A typical experiment is presented in figure 17a on the next page.

Figure 17. (a) Real-time current response of a SWNT-net device, while astrocytes were stimulated by the addition of 300 μ M glutamate to the bath solution. V_{ds} was 400mV (same in b, c). Two representative spikes are depicted with an expanded time scale. (b) Responses of cell-free SWNT-net to locally delivered bath solution containing ATP (1 mM) or glutamate (1 mM), or a high concentration of protons (pH 5.0) using a puffing quartz nanopipette (100~200 nm in diameter) positioned $\sim$ 1 μ m above the SWNT-net in the bath. (c) SWNT-net current recorded in the bulk bath solution containing ATP (0.5 – 10 mM) or glutamate (1 mM) or of low pH (pH 5.0).

Initially the cell medium was replaced by the physiological bath solution to incubate living astrocytes. After introduction of 300 μ M glutamate into the recording chamber, many upward current spikes were observed (with about 100-second delay). In a

closer investigation, we found that single spike indeed exhibits two biphasic kinetic changes, including an initial rapid rising phase (a few ms scales) followed by a long-lasting decaying phase (a few hundred milliseconds to few seconds). This phenomenon is plausibly corresponds to the burst discharge of ATP molecules from each single astrocyte cell.

Interestingly, this similar response from a single astrocyte was observed in a previous study using the sniffer cell approach¹⁶⁷. Compared to Ca^{2+} dependent secretion of neurotransmitters from several neuroendocrine cells, which often requires time within a few milliseconds after triggering, the delay following the stimulation is rather long. This is possibly due to complicated molecular reactions (as described earlier) involved in the ATP secretion process. In addition, it is also conceivable that secretory vesicles in astrocytes may need to go through a series of steps to be readily discharged including, transport to the plasma membrane, docking, and priming. This is different from neurons and neuroendocrine cells, which contain a lot of readily releasable vesicles waiting for final Ca^{2+} triggering. The ATP molecules discharge seems to be very rapid and highly synchronized and reaches a peak value in a few milliseconds. Such burst release may be necessary for synergetic reactions of the downstream molecular events. The decay in the current signal presumably is due to diffuse-away of ATP molecules and ATP break-down by ecto-ATPases, which are believed to be co-localized with in the vesicle fusion sites¹⁷⁰.

The application of calcimycin, a calcium ionophore that inserts into cell membrane and selectively transports Ca^{2+} ions from the extracellular to the intracellular space, produced similar responses as glutamate. This suggests that the stimulated ATP release from astrocytes is indeed Ca^{2+} -dependent. On the contrary, voltage pulse

stimulation (400 – 800 mV in amplitude on top of the biasing voltage, 5 -100 ms in duration) to the SWNT-net, which is expected to depolarize the cell membrane and open the voltage-dependent Ca^{2+} channels ⁵¹ fails to elicit any responses, implying that our observations are not derived from the Ca^{2+} influx through these Ca^{2+} channels.

After experiment, the same device used was treated with 0.125% trypsin followed by repeated dialysis with bath solution to completely remove the cells. Subsequently, a quartz nanopipette (diameter of ~100 – 200 nm) positioned ~1 μm above SWNT-net was used to locally puff in pulses of small amount of 1 mM ATP containing bath solution onto the SWNT-net sensor. We found that each puff gave rise to a spike having amplitudes comparable to the recordings from the astrocytes (figure 17b). This was a good control simulation, which demonstrated that small ATP flux locally discharged onto SWNT-net was actually a derived signal from the astrocytes. Other possibilities that acidic vesicular fluids (~pH 5.0) or glutamate molecules may also be contained in the vesicles responsible for the recorded SWNTnet responses are ruled out by the observations that nanopipette administration of bath solution of pH 5.0 or with 1 mM glutamate did not elicit any appreciable responses from the SWNTs.

In another control experiment (figure 17c), the SWNT-net was pre-incubated with the culture medium for 2 days and immersed into the bath solution prior to the testing. This cell free device showed an increase in conductance (with duration and amplitude similar to the cell recording results) to the bulk bath solution containing ATP (0.5 – 10 mM), but elicited no response to administration of acidic (pH 5.0) or glutamate containing (1 mM) bath solution.

These control experiments together with the condition that SWNT-net can respond only after application of the glutamate neurotransmitter, and demonstration that not all biomolecules are able to directly interact with the nanotubes (good specificity), establish a direct conclusion that our observations are resulted from triggered ATP release of the cells.

The interaction mechanism between the nanotubes and ATP, which cause the conductance increase, may be originated from a Schottky energy barrier decrease at the nanotube-electrode contacts or a direct charge transfer into the nanotube or electrostatic gating. The first two mechanisms, however, can be excluded because the nanotube-electrode contacts are insulated and the aromatic adenine base is unlikely to be hole-dopant. It is more plausible that highly negatively charged ATP molecules may generate an increase of the concentration of the major current carrier (holes) in the nanotube and consequently elevate its conductance.

4.7. Conclusions

In conclusion, we have successfully demonstrated that a SWNT-net can be employed as a biocompatible substrate for astrocytes and subsequently used as a platform for label-freely detection of local ATP release immediately upon stimulation. This realization provides a novel technique to investigate ATP release and signaling with high time resolution and simplicity. Finally, in a large point of view, SWNT-net devices can contribute as a novel nanotechnology application in biological research¹⁷¹⁻¹⁷².

4.8. Acknowledgement

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- Huang Y, Sudibya HG, Fu D, Xue R, Dong X, Li LJ, and Chen P. *Label-free detection of ATP release from living astrocytes with high temporal resolution using carbon nanotube network. **Biosensors and Bioelectronics**, 24(8):2716-2720, 2009. (Copyright © 2008 Elsevier B.V. Reproduced with permission).*

Chapter 5

Interfacing Glycosylated Carbon Nanotube Network Devices with Living Cells to Detect Dynamic Secretion of Biomolecules¹⁵⁴

5.1. Abstract

In our research work, fabricated thin-film networks of single-walled carbon nanotubes (SWNTs) were glycosylated with monosaccharides abundantly found in living systems, specifically, N-acetyl-D-glucosamine (GlcNAc), D-glucose, or D-mannose. These sugar moieties are anchored onto nanotubes via either a pyrene or lipid tail. We subsequently demonstrated that this glycosylated CNTs network can be applied to detect dynamic catecholamine secretion from PC12 cells.

5.2. Background of the study

Carbon nanotubes (CNTs) have stimulated much interest in their biological applications ever since their initial discovery, because of their remarkable electrical, structural, and physiochemical properties^{6,173}. For instance, they have been employed as nanoelectronic biosensors for rapid detection of biomolecules with extraordinary sensitivity^{1-2,40}, as nanovectors to deliver macromolecules into cells¹⁷⁴⁻¹⁷⁵, and as nanofibrous scaffolds for tissue engineering¹⁷⁶⁻¹⁷⁷. Despite all these promising potentials, the non-ideal, actually in many cases cytotoxic, interface between CNTs and the living cells, however, largely limits the nanotube applications in biology¹⁷⁸⁻¹⁷⁹, for example, in developing CNT based biosensors to detect biological activities from living cells. Herein, we devised a strategy to functionalize carbon nanotubes with bioactive monosaccharides

and demonstrated that glycosylated CNT network devices are able to biocompatibly interface with living cells and detect dynamic secretion of biomolecules from them.

To achieve biocompatible interactions between carbon nanotubes and living cells, hydrophobic nanotubes have been coated with positively charged polyelectrolytes, such as poly(ethylene)imine (PEI) ⁴⁶ and poly-L-lysine (PLL) ⁴⁷, in order to promote adhesion of the negatively charged cell membrane. Charge groups can also be imparted to nanotube surface via chemical functionalization ^{48,50,52}. Non-physiological strong electrostatic interactions between the highly charged surface and the cell membrane, nevertheless, may lead to cell death or cytotoxic effects ¹⁸⁰. Moreover, the introduced surface charges and chemical modifications often significantly alter the electrical properties of nanotubes, which is undesirable when nanotubes are used as electrical sensing elements. Alternatively, CNTs have been coated with adhesion proteins found in the extracellular matrix ⁴⁹. However, the thick gel-like layer formed by the adhesion proteins precludes the intimate contact between the nanotubes and the cells. In addition, the charged groups of these macromolecules also may adversely influence electrical properties of the nanotubes. Therefore, new strategies are needed to modify CNTs, thereby ensuring biocompatible interactions between CNTs and living systems and the preservation of the functionalities of both.

Carbohydrates, a major component of the cell membrane, have recently been used to decorate CNTs in order to establish natural contacts with living cells ¹⁸¹⁻¹⁸². Carbohydrates, in the form of glycans, proteoglycans, glycoproteins, or glycolipids, populate the surfaces of both eukaryotic and bacterial cells and are critically involved in a wide spectrum of biological processes such as cell-cell communication and interaction,

cell growth and development, immune recognition and responses, and signal transduction¹⁸³⁻¹⁸⁴. In particular, carbohydrates are important in mediating cell adhesion process through carbohydrate-carbohydrate or carbohydrate-receptor (lectin) interactions¹⁸⁴⁻¹⁸⁵. In the present work, fabricated thin-film networks of single-walled carbon nanotubes (SWNTs) were glycosylated with monosaccharides abundantly found in living systems, specifically, N-acetyl-D-glucosamine (GlcNAc), D-glucose, or D-mannose. These sugar moieties are anchored onto nanotubes via either a pyrene or a lipid tail. We demonstrate that the GlcNAc-modified SWNT-net can biocompatibly interface with living neuroendocrine PC12 cells, and support their adhesion and proliferation. In addition, we show that the glycosylated SWNT-net devices are able to detect triggered secretion of catecholamines from individual PC12 cells with a high temporal resolution.

5.3. Synthesis of the carbohydrate compound

The synthetic routes of (16-allyloxy) hexadecyl-2-acetamido-3,4,6-tri-O-acetyl-2-deoxy- β -D-glucopyranoside (Nacetyl-D-glucosamine with lipid tail or GlcNAc-lipid in short) and 4-(1-pyrenyl)butyl 2-acetamido-2-deoxy- β -D-glucopyranoside (N-acetyl-D-glucosamine with pyrene tail, or GlcNAc-pyrene) are depicted in figure 18.

In brief, N-acetyl-D-glucosamine was acetylated by acetic anhydride in pyridine to produce pentacetyl carbohydrate. The anomeric acetyl group was then cleaved by methyl amine and subsequently transformed to trichloroacetimidate. Glycosylation reactions were performed in the presence of trimethylsilyl triflate, followed by deacetylation to generate GlcNAc-lipid or GlcNAc-pyrene. 4-(1-pyrenyl)butyl β -D-

glucopyranoside (D-glucose with pyrene, or glc-pyrene) and 4-(1-pyrenyl)butyl α -D-mannopyranoside (mannose with pyrene, or man-pyrene) were similarly synthesized.

Figure 18. Synthetic routes to GlcNAc-lipid and GlcNAc-pyrene. Ultra-thin networks comprising of small SWNT bundles (10-30 nm) were prepared through phase separation facilitated self-assembly and drop-casted onto glass coverslip as previously described¹⁸⁶. An atomic force microscopy (AFM) image of such a network is presented in figure 19a.

5.4. CNTs devices fabrication and functionalization

CNT-FET devices were fabricated using film-transfer technique (as described in Appendix A section E2). The SWNT-nets were functionalized by incubation with 5 mM glyco-conjugates equipped with either a lipid or pyrene tail for 24 hours, followed by DI water rinsing to remove unbound molecules. The hydrophobic lipid tail anchors onto the nanotubes via hydrophobic interaction, while aromatic pyrene tail firmly attaches to the nanotube sidewall via a π - π stacking interaction (Figure 19b). The effectiveness of the glycosylation was visualized using fluorescein isothiocyanate (FITC) conjugated sugar

binding proteins (lectins), specifically, FITC conjugated Helix Pomatia Agglutinin (HPA) that selectively binds to GlcNAc and FITC conjugated Concanavalin A (conA) that selectively binds to glucose and mannose. The functionalized SWNT-net was incubated with 5 mM FITC-conjugated lectin for 24 hours, followed by rinsing to remove unattached protein and subsequent fluorescence imaging. As revealed by the fluorescence labeling, GlcNAc-lipid formed inhomogeneous patches on the SWNT-net (not shown) whereas GlcNAc-pyrene uniformly covered the SWNT-net (Figure 19c, left), indicating that pyrene is a better linker. The bare SWNT-net, by contrast, only showed background fluorescence (Figure 19c, right), because FITC-HPA was not able to bind onto the nanotube substrate without the GlcNAc bait.

Figure 19. (a) AFM image of a SWNT-net with a representative cross-section profile. (b) Illustration of the GlcNAc-pyrene functionalization onto SWNTs. (c) Fluorescence staining of GlcNAc functionalized SWNT-net (left) and bare SWNT-net (right) by helix pomatia agglutinin (HPA) lectin. The scale bars = 500 μm . (d) Electrical characterization of SWNT-net before (red circles and polynomial fitting) and after (blue triangles and polynomial fitting) glycosylation of GlcNAc. Biasing at 400 mV, the source-drain current (I_{ds}) was measured while varying the gate voltage (V_g) applied to the bath solution via an Ag/AgCl electrode.

The conductance of the SWNT-net can be sensitivity-modulated by the gate voltage applied to the solution that baths the SWNTs. In a typical example is shown in figure 19d. The nanotube current dropped from 6.3 μA to 0.1 μA as the gate voltage varied from -1 to 1 V (circles), manifesting a p-type field-effect characteristics. This demonstrates that our SWNT-net device was highly responsive to the electrochemical perturbation in the solution, therefore desirable for electrical biosensing. Glycosylation did not compromise the nanotube properties (figure 19d, triangles). In comparison to single SWNT based devices which are usually employed for biosensing with higher sensitivity, the SWNT-net devices offer several advantages. They can be more readily fabricated and have a lower 1/f noise than commonly fabricated single nanotube devices¹⁸⁷. The SWNT-net is especially advantageous to probe cellular activities, because it could serve as cell growth substrate and interface with cells with a large contact to the sensing area.

5.5. Cells growth and proliferation on various sugar compound

Bare nanotube substrates, however, are usually not suitable for the cell growth. When PC12 cells were cultured on bare SWNT-nets, they abnormally aggregated into sparsely dispersed clumps (figure 20a, left) and their growth was apparently impaired. For comparison, we examined growth of PC12 cells on SWNT-nets noncovalently functionalized with glucosamine, glucose or mannose, which are sugar terminal residues of many membrane glyco-conjugates relevant to various biological processes. In particular, glucosamine is a major constituent of many proteoglycans, which are important cell adhesion molecules found on both cell membrane and extracellular matrix¹⁸⁸.

Figure 20. (a) Phase-contrast images of PC12 cells cultured on bare SWNT-net (left) and GlcNAc-SWNTs (right). Scale bars = 100 μm. (b) Proliferation curves of PC12 cells grown bare SWNT-net (circles), or SWNT-net modified with GlcNAc (squares), or glucose (triangles), or mannose (diamonds). Each data points are the average from 40 different areas (3 mm²) in 3 different substrates. The error bars indicate the standard deviations.

PC12 cells possess abundant proteoglycans, especially heparin sulfate that consists of a core protein with one or more covalently attached glycosaminoglycan polymerized from *N*-acetylglucosamine and glucuronic acid¹⁸⁹⁻¹⁹⁰. As anticipated, GlcNAc-pyrene coated SWNT-nets, in contrast to bare SWNT-nets, supported normal confluent growth of PC12 cells (figure 20a, right), suggesting the facilitating role of glucosamine on adhesion of PC12 cells. It has been shown that noncovalent interactions between similar glycans can provide strong adhesion forces (~250 pN) comparable to that of protein-protein interaction¹⁹¹. Therefore, it is plausible that a nanotube displayed with many glucosamine residues can strongly interact with glycosaminoglycans on the PC12 cell membrane. In addition, glucosamine specific lectins on the cell membrane can bind with glucosamine on the nanotube and promote cell adhesion as well¹⁹². In comparison

to GlcNAc-pyrene, glc-pyrene and man-pyrene appear to seriously inhibited cell adhesion therefore cell growth. Proliferation curves of cells cultured on bare SWNT-net or SWNT-net functionalized with different sugar moieties are shown in figure 20b.

These experiments were conducted in parallel and PC12 cells of the same initial density were introduced onto the respective substrates. As seen, PC12 cells grown on GlcNAc functionalized SWNT-nets can proliferate well, evident by 160.0% increase in cell density after 3 days culturing. On the other hand, proliferation of PC12 cells was largely inhibited on bare SWNT-nets. In this case, the cell density only increased by 60.9% after 3 days. On both glucose and mannose coated nanotubes, PC12 cells cannot adhere well and their density decreased by 57.5 % and 56.1 % in 3 days, respectively.

As demonstrated above, appropriate glycosylation enables biocompatible interfacing between SWNT-net and living cells without sacrificing SWNT functionalities. Hence, glycosylated SWNT-net provides a novel platform to probe dynamic cellular activities, for example, to continuously monitor biomolecular release from living cells with a high temporal resolution and a high sensitivity. Carbon nanotube devices have already been utilized as nanoelectronic biosensors to detect various biomolecules^{116,138,193}. However, these realizations are essentially limited to static in vitro measurements.

5.6. Electrical recording of catecholamines secretion

PC12 cells are a widely used cell model to study regulated secretion (or exocytosis), a fundamental and dynamic biological process in many cell types underlying, for instances, hormone secretion from endocrine cells and neurotransmitter secretion

from neurons. As illustrated in figure 21a, membrane depolarization (i.e., increase in intracellular potential) resulting from certain physiological stimulation opens voltage-gated Ca^{2+} channels. Ca^{2+} influx through Ca^{2+} -channels, in turn, triggers actions of several sets of protein machineries, which drive immobilization and final fusion of large dense core secretory vesicles. Upon vesicle fusion with the plasma membrane, catecholamine molecules (including dopamine, norepinephrine and epinephrine) inside the vesicle are discharged into the extracellular space. Once catecholamines are released into the narrow interface gap between the cell and the SWNT-net, they quickly diffuse onto the nanotubes and intimately interact with them via π - π stacking between the aromatic ring of the catecholamine molecule and the sidewall of the nanotubes. As the current flows solely at the surface, the conductance of nanotube is highly sensitive to the electrochemical perturbations at the surface induced by the interacting molecules. So, it is possible that triggered catecholamine release from PC12 cells can be reported by changes in nanotube current.

Indeed, when the glycosylated SWNT-net was biased at 400 mV, and high potassium (K^+) solution, which depolarizes the cell membrane and is commonly used to trigger catecholamine release from PC12 cells, was introduced to PC12 cells cultured on top of SWNT-net, a train of current spikes was detected (Figure 21b). When another common secretagogue, calcymycin (1 mM), which inserts into cell membrane and forms Ca^{2+} selective ion channel, was applied, similar current spikes were elicited.

Figure 21. (a) Schematic illustration of triggered exocytosis of catecholamins from PC12 cells and subsequent detection by a SWNT-net device. (b) Nanotube responses to exocytosis of PC12 cells triggered by high K^+ stimulation. The SWNT-net was biased at $V_{ds}=0.4$ V. (c) Stimulation of single PC12 cell through micropipette perfusion of high K^+ solution. (d) Transient perfusion of 1 mM dopamine or norepinephrine on glycosylated SWNTs-net results in current spikes. In contrast, perfusion of acetylcholine, acidic solution (pH 5.0), and high K^+ did not cause any appreciable responses. In (c) and (d), the arrows only roughly indicate, where the stimulations were applied.

We hypothesize that each spike corresponds to Ca^{2+} -dependent exocytosis of catecholamines from single PC12 cell. To test this hypothesis, we used an application pipette (with a tip size of 1-2 μm) positioned 1-2 μm away from the target cell to locally deliver high K^+ solution or calcymycin, which exclusively stimulate this one cell. Single current spikes, with similar amplitude and time scale as observed in figure 21b, was resulting from such local stimulation (figure 21c), suggesting that each recorded spike in figure 21b is due to a single cell activity.

Furthermore, when a puff of bath solution contained 1 mM dopamine or norepinephrine was delivered onto a cell-free and 400 mV-biased glycosylated SWNT-net via an application micropipette, a current spike was also induced (figure 21d), indicating that local discharge of catecholamines can cause transient increase in nanotube current as observed in the experiment depicted in Figure 21b. High K^+ solution and calcymycin failed to produce any signals in the absence of cells (figure 21d). It is arguable that the observed spike may be due to the acidic vesicular fluids ($\sim\text{pH}$ 5.0-6.0).

This possibility was ruled out by the observation that nanotubes were not responsive to the micropipette application of bath solution of low pH (5.0) (figure 21d). Upon Ca^{2+} triggering, PC12 cells also release acetylcholine from small synaptic-like vesicles. But as shown in figure 21d, acetylcholine was not able to modulate the nanotube current. Moreover, it is not likely that the recorded transient signals are originated from constitutively secreted molecules (e.g., metabolites) of the cells, because in comparison to triggered rapid exocytosis, the constitutive secretion is a slow and constant process. The spiky nanotube responses were only observed after application of the secretagogues, which lead to Ca^{2+} dependent exocytosis.

Exocytosis of biomolecules are conventionally analyzed by biochemical assays which, however, only offer averaged ensemble measurements from a population of cells with low temporal resolution and sensitivity. Although the state-of-the-art electrophysiological methods, such as membrane capacitance measurement and carbon micro-fiber amperometry, provide s high sensitivity and temporal resolution, they require high skills and specialized equipments. These methods involve tedious procedures and only record one cell at a time, therefore are of very low-throughput. In addition, membrane capacitance measurement is invasive and carbon fiber microelectrode (typically 5 μm in size) can only electrochemically detect the release of electroactive molecules from $\sim 5\%$ of the total area of the cell surface. Comparing to these current methods, our nanotube approach provides a novel alternative to detect exocytosis from individual cells.

5.7. Conclusions

In summary, we demonstrate that non-covalent functionalization with bioactive sugar moieties confers biocompatibility to carbon nanotubes without compromising its sensing capabilities. Glycosylated SWNT-net can directly interface with living cells by supporting their adhesion and growth, and detect dynamic biomolecular release from these cells.

Comparing to current methods to detect exocytosis, our nanotube approach provides real-time and noninvasive measurements from living cells with high sensitivity, high temporal resolution, high throughput and ease of detection. The study represents

another example of how nanotechnologies and nanomaterials can bring new opportunities to biology^{172,194}.

5.8. Acknowledgements

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Chapter 6

Real Time Detection of Cardiac Muscle Action Potential and Escherichia coli Glucose Metabolism Process Based on Carbon Nanotube Network Field-Effect Transistor¹⁹⁵

6.1. Abstract

In this research work, we advanced the application of our carbon nanotube network devices not only as a sensitive platform for dynamic biomolecular detection but also as a possible high-throughput drug screening tool. Real time electrical monitoring of cardiomyocytes action potential and bacterial glucose metabolism processes (of secreted organic acid molecules) were demonstrated after interfacing those living systems to the nanotube sensors. Subsequently, effects of various drugs influencing the living cells were carefully analyzed and investigated. This realization really opens up an alternative avenue to study of cellular or bacteria dynamic systems in drug screening with enhanced efficiency compared to the currently established methods.

6.2. Background of the study

Excitable cells (such as myocytes, neurons, endocrine cells, and sensory cells) are able to generate bioelectrical signals resulting from the orchestrated activities of ion channels embedded within the cell membrane. Such bioelectrical signals are essential to many fundamental biological processes, including communication between neurons, contraction of heart or other muscles, vision, hearing, and so on. Hence, drug targeting to various ion channels have been developed for critical diseases such as cardiac arrhythmia,

hypertension, Parkinson's disease, diabetes, depression, and neuropathic pain. Despite longstanding recognition that these are major therapeutic targets and billions of dollars spent on research, ion channels are still largely under-exploited for drug development. The primary bottleneck in such efforts is the lack of high-throughput electrophysiological screening technologies.

On the other hand, recognition of pathogenic organisms, such as yeast and bacteria, is very important in the practice to ensure safety and high quality in the food ¹⁹⁶ and water-treatment industry ¹⁹⁷⁻¹⁹⁸ as well as to perform clinical diagnosis ¹⁹⁹. Some conventional methods have been established in this area including culturing and colony counting methods ²⁰⁰⁻²⁰², polymerase chain reaction (PCR) methods ²⁰³⁻²⁰⁶, and immunology based methods (enzyme immunoassay (EIA) ²⁰⁷, immunomagnetic separation (IMS) ²⁰⁸⁻²⁰⁹, enzyme-linked immunosorbent assay (ELISA) ²¹⁰, flow-injection immuno assay ²¹¹, and so on). Despite their excellent sensitivity and accessibility of sufficient qualitative and quantitative information, those techniques unfortunately involve multistep procedures and long analysis times, which are obviously inconvenient for industrial applications. The use of more rapid and effective techniques such as electrochemical and optical detection, therefore, started to emerge to overcome this limitation. On the next progress, however, all of those rapid methods are found to be “*not so perfect*” either. Electrochemical techniques (including amperometric, potentiometric, and impedance measurement) ²¹², though are rapid and much easier to use, still show inadequate performance compared to traditional methods, mostly due to presence of high background noise. On the other hand, the optical detection (including fluorescently labeled antibodies ²¹³⁻²¹⁴, Raman spectra ²¹⁵⁻²¹⁶, fourier transform infrared spectroscopy

(FTIR)²¹⁷, and surface plasmon resonance (SPR)²¹⁸⁻²²⁰) despite their popular selectivity and sensitivity, require high equipment cost and complex operation procedures. These are major obstacles for most of the end users.

Therefore, alternative detection methods are still desired to provide a simple, reliable, high throughput, and low-cost platform, which is able to recognize the microorganism presence as well as to perform electrophysiological drug screening with high sensitivity, selectivity, real time configuration, and high temporal resolution. Electrical characterization technique seems to promise a good solution to address this challenge.

Carbon nanotubes (CNTs), owing to its unique electrical, structural, and physiochemical properties, have been commonly configured as FETs and utilized as nanoelectronic biosensors to detect various biomolecules^{116-117,121,221} and dynamic activity of living cells^{19,53,153-154}. In addition, detections of various pathogenic materials by employing carbon nanotubes have also been successfully demonstrated with a good selectivity (as reviewed in the literature review chapter section 2.4). *So et al.* have demonstrated a FET device with single- or a few SWCNTs as the conducting (sensing) channel to detect *E. coli* with a detection limit of 10^3 cfu/mL²²². In the work of Vallamizar and co-workers, FETs based on a network of SWCNTs were developed to detect *Salmonella Infantis* with a detection limit of 100 cfu/mL¹⁴⁷. Herein, we demonstrate the detection of action potential activity from cardiomyocytes as well as organic acid release from bacteria during their physiological glucose metabolism process (which causes an acidification in the extracellular environment) by using CNTs network

device. Furthermore, their activities under treatment of various drugs is also carefully investigated.

6.3. SWNTs-FET device fabrication and functionalization

Figure 22. a) Schematic diagram of SWNTs-net device fabrication by the dropcasting method. b) Scanning Electron Microscopy (SEM) images of carbon nanotubes network fabricated via dropcasting method. Figure c, d, and e present the density differences in different area region.

Single-walled carbon nanotube networks (SWNT-net) were simply fabricated by drop-casting aqueous dispersion of carboxylated SWNTs onto a glass coverslip (as described in Appendix A the section E1). Due to the interstitial capillary force during water evaporation (inside an oven at 70 °C), SWNTs moved to the edge of the solution drop and formed a ring of SWNT-net (~ 3-4 mm in diameter) with decreasing density from the edge till disappearance at ~140 μm away from the edge (Figure 22). Two electrodes, representing the source and drain, were subsequently prepared using silver conductive paint. Silicone rubber was used to insulate the electrodes and to form a

chamber for bacteria culturing and recording. SWNT-net conductance can be sensitively modulated by the gate voltage (V_g) applied to the phosphate buffer saline (PBS, pH 7.2) on the SWNT-net, manifesting a prominent *p*-type field-effect characteristics. This suggests that our SWNT-net is able to detect electrical perturbations in solution occurring in the vicinity of SWNTs. Scanning Electron Microscopy (SEM) images of our SWNTs network in some different regions are presented in figure 22. Before applying cardiac cells or E.coli bacteria on the devices, the SWNT-net device was sterilized by immersion in 70% alcohol for 2 minutes following by DI water rinsing, and subsequently coated with fibronectin/gelatin and Poly L-Lysine (PLL), respectively.

6.4. Cardiac cells and *Escherichia coli* addition onto the SWNT-net device

HL-1 cells, a cardiac muscle cell line (HL-1), was a kind gift from Prof. William C. Claycomb (Louisiana State University). They were planted on the pre-sterilized and pre-coated SWNT-net devices with a density of 2.0×10^5 cells/mL, and cultured till confluence (after 4 or 5 days) in Claycomb medium supplemented with 100 μ M norepinephrine, 2 mM L-glutamine, 10% fetal bovine serum, and 100 U/mL penicillin-streptomycin (as described in Appendix A section C4).

Escherichia coli bacteria (strain DH5 α) stock in LB medium were prepared and employed for our bio-detection experiment (as described in Appendix A section D). This bacteria stock solution were diluted to reach small concentration (~50 CFU) when it is added to the SWNTs devices.

6.5. Electrical recording of cardiac action potential

After sterilizing the SWNT-net and subsequent coating with adhesion protein (fibronectin and gelatin), cardiac myocytes (HL-1 cell line) were planted onto the SWNT-net device and grew for 4 days to reach confluence before the experiments (Figure 23a).

Figure 23 (a) Phase-contrast image of cardiomyocytes grown on the SWNT-net to confluence after 4-days culturing. (b) Illustration of simultaneous extracellular recording using a SWNT-net device and intracellular recording using an impaling nanopipette of bioelectrical signals (action potentials) from cardiomyocytes grown on top of the SWNT-net. (c) Spontaneous and periodic action potentials recorded intracellularly by the nanopipette (top) and simultaneous current responses of the SWNT-net (bottom).

Cardiomyocytes periodically produce bioelectrical signals called action potentials (APs), which are a transient increase in intracellular electrical potential due to orchestrated transmembrane ionic currents flowing through voltage-gated ion channels in

the cell membrane. The periodic APs underlie the spontaneous contraction of cardiomyocytes and thus heart beating. It is conceivable that when the transmembrane ionic current flows from the intracellular space into the extracellular narrow gap at the interface between the cardiomyocytes and the SWNT-net or vice versa, the electrical potential at the interface (V_i) is altered. Changes of the electrical potential at the vicinity of SWNTs, in turn, influence the SWNT-net conductance, leading to measurable current signals from the voltage biased SWNT-net. This view is in line with the Fromherz model that explains well the coupling between excitable cells and conventional planar FETs²²³. Therefore, it is anticipated that when electrogenic cardiomyocytes are cultured on top of a SWNT-net, the transmembrane ionic currents accompanying the periodic action potentials would cause periodic SWNT-net responses. We monitored the SWNT-net current and, at the same time, recorded action potentials from a cardiomyocyte on top of the SWNT-net using a quartz nanopipette with a tip size of ~100 nm impaled inside the cell (as illustrated in Figure 23b). As expected, periodic SWNT-net current signals were observed (Figure 23c), and they synchronized with the contraction of cardiomyocytes. All cardiomyocytes in the confluent sheet contract as a whole due to the synchronization of their action potentials through gap junctions between cells. As shown in Figure 23c, the SWNT-net signals are in concert with the spontaneous action potentials recorded intracellularly from a myocyte on the SWNT-net. This unambiguously indicates that each episode of the SWNT-net response corresponds to an action potential generated in cardiomyocytes by sequential activation of sodium, calcium and potassium currents. The action potential of the HL-1 cells proceeds with a rapid depolarization phase (i.e., the intracellular potential increases from the resting level ~ -60 mV) due to activation of the

inward Na^+ current through the fast voltage-gated Na^+ channels, followed by a slow repolarization phase (i.e., the intracellular potential returns back to the resting level) resulting from the net action of the inward Ca^{2+} current through L-type Ca^{2+} channels and delayed outward K^+ current through delayed-rectifier K^+ channels.

6.6. Detection of Bacterial Incubation on SWNT-net Device

During the bacteria detection experiments, PBS solution (300 μL) was added in the recording chamber and an Ag/AgCl electrode was immersed to apply gate voltage (V_g). While biasing the device at $V_{ds} = 400$ mV and the liquid-gate electrode at $V_g = 0$ mV, introduction of *E. coli* to the final concentration of 100 cfu/mL caused obvious decrease of the device current over time (figure 24a). The current (or conductance) decrease reached a plateau in ~ 2 hours as the bacteria gradually settled down onto the SWCNT network. Appreciable response could be noticed at 20th minute with a signal-to-noise ratio of ~ 10 (~ 32 pA current decrease vs. ~ 3 pA current noise). In contrast, the SWCNT conductance remained stable in the absence of bacteria. Figure 24b depicts a SEM image of an *E. coli* on SWCNT network. The rate of bacteria attachment at the concentration of 10^5 cfu/mL was shown in figure 24c as quantified under the phase-contrast optical microscope. As shown in figure 24d, the electrical characteristics (conductance vs. V_g) was significantly altered by the attachment of bacteria in a incubation-time (bacteria-density) dependent manner.

Figure 24. a.) Percentage change of SWCNT-network conductance over time without (circles) or with (squares) addition of *E. coli* (100 cfu/mL). Each data point is the average value from 3 devices. The error bars indicate the standard errors. b.) SEM image of an *E. coli* on a poly-L-lysine (PLL) coated SWCNT-network. c) Number of *E. coli* per mm^2 attached on SWCNT-network, when SWCNT-network was incubated with *E. coli* (10^5 cfu/mL) for 15 min or 1 h or 3 h. d.) Conductance vs. gate voltage of bare PLL-coated SWCNT devices (circles) pre-incubated with *E. coli* (10^5 cfu/mL) for 1 h (squares) or 3 h (triangles). The conductance is normalized to the maximum conductance of the bare devices. Each data point is the average value from 6 devices. The error bars indicate the standard errors.

Our experiments corroborated the ability of SWCNT sensors to detect bacteria. The achieved detection limit (< 100 cfu/mL) is comparable to the state-of-the-art ELISA analysis²²⁴. The observed conductance decrease induced by bacteria is consistent with the previously reported SWCNT sensors²²². However, the observed field-effect due to the negatively charged bacterial surface would predict an increase in conductance because the *p*-type characteristics of the SWCNT FET (figure 24d). Therefore another mechanism should cause the observed recordings. It is conceivable that the conductance

decrease may be attributable to doping effects imposed by the interacting biomolecules on the bacterial surface or scattering effects imposed by the charged molecules on the bacteria. The underlying mechanisms deserve further investigations.

6.7. Detection of bacteria glucose metabolism process

Figure 25. a) Illustration of SWCNT-FET and bacteria interface for detection of bacterial metabolic activities as an indicator of susceptibility to antibacterial drugs. b.) Real-time current recordings ($V_{ds} = 400$ mV and $V_g = 0$ mV) of a SWCNT device pre-incubated with *E. coli* (10^5 cfu/mL) for 3 h, a device pre-incubated with *E. coli* for 1 h, and a bare SWCNT device, with application of glucose to the PBS recording buffer at indicated time points to reach the concentrations of 1, 3, 6, 10, and 15 mM. c.) Percentage change in conductance of bare SWCNT devices (circle), devices pre-incubated with *E. coli* for 1 h (squares), and devices pre-incubated with *E. coli* for 3 h (triangles), upon application of different concentrations of glucose. Each data point is the average from 6 devices. The error bars indicate the standard errors. d.) Percentage change in conductance of SWCNT devices pre-incubated with *E. coli* for 3 h without (squares) or with antibacterial drugs (10 μ g/mL), upon application of different concentrations of glucose. Each data point is the average from 6 devices. The error bars indicate the standard errors.

It is known that glucose metabolism in bacteria leads to release of organic acids (e.g., pyruvic, citric, and lactic acids), and consequently, extracellular acidification. We hypothesized that discharge of organic acids into the nano-gap between the SWCNTs and the interfacing bacterial surface would significantly lower the local pH (the local electrochemical environment) at the vicinity of SWCNTs, thus possibly change the SWCNT conductance (figure 25a). We first verified that the conductance of our carboxylated SWCNTs is actually highly responsive to pH change as shown in figure 26.

To test our hypothesis, the SWCNT-network FETs were firstly incubated with *E. coli* suspension in PBS (10^5 cfu/mL) for 1 or 3 h, followed by gentle rinse and replacement with fresh PBS. While the SWCNT current was continuously monitored, glucose was added into the recording chamber to reach different final concentrations. Indeed, as expected, when glucose (1 mM) was applied to a SWCNT device pre-incubated with *E. coli* for 1 hour, an immediate increase of the SWCNT current was observed (Figure 25b, middle trace), and the current further increased upon addition of higher doses of glucose. A similar recording from a SWCNT device pre-incubated with *E. coli* for a longer time (3 hour) is shown in parallel (Figure 25b, top trace). With a higher density of deposited bacteria, this device produced a larger response at a given glucose concentration. In comparison, glucose was not able to trigger any response from a device without bacteria (figure 25b, bottom trace). The statistics of these experiments is summarized in figure 25c. As it can be seen, the magnitude of the SWCNT response is positively correlated with the concentration of glucose and the bacteria density (the pre-incubation time). These observations clearly suggest that the SWCNT responses were resulted from the glucose-induced bacterial metabolism.

Figure 26. a) Conductance vs. gate voltage of SWCNT devices in buffer solutions with different pH values (pH 2, 5, 7, 9 and 12). $V_{ds}=400mV$; $V_g=0V$. b.) Real-time current of the SWCNT device while changing solutions with different pH values. c.) Percentage change of SWCNT-network conductance over pH values. Each data point is the average value from 6 devices. The error bars indicate the standard errors.

Current detection methods, such as PCR, are not able to distinguish dead cells from live ones. Our detection of glucose metabolism can provide unambiguous evidence of the existence of live bacteria, with a signal strength reflecting their number. The metabolic activities of bacteria can serve as an indicator of their susceptibility to drugs. Antibacterial drugs attack bacteria by inhibiting protein or DNA synthesis, damaging the outer bacterial wall, inhibiting synthesis of outer wall or inner membrane, or combination of the above. These actions directly or indirectly impair the metabolic activities (thus survival) of such simple life forms.

6.8. Antibiotic drug effect on bacterial metabolism

As a proof-of-concept demonstration, we examined the effects of several common antibacterial drugs (ampicillin, kanamycin, chloramphenicol, mixture of penicillin and streptomycin) on glucose metabolism using our SWCNT-network FETs. The devices were pre-incubated with 10^5 cfu/mL *E. coli* for 3 h in the presence of the testing antibacterial drug (at a dose of 10 μ g/mL), followed by replacement with fresh PBS. The drugs did not affect the bacteria attachment onto SWCNTs. The inhibitory effects of these drugs on the induced metabolic activities of *E. coli* (indicated by increase of SWCNT conductance) at different concentrations of glucose are shown in figure 25d.

Ampicillin, which inhibits a critical enzyme (transpeptidase) for peptidoglycan synthesis of the bacterial cell wall, significantly reduced the SWCNT response to the application of 55 mM glucose by ~33.8 % as compared to the response without the drug. Kanamycin and chloramphenicol, which inhibit protein synthesis, gave more profound inhibition. The cocktail of penicillin and streptomycin, which is commonly used to prevent infection in animal cell cultures, because these two antibiotics can synergistically, thus more effectively, kill bacteria. Inhibition of peptidoglycan synthesis by penicillin facilitates penetration and disruption of bacterial wall by streptomycin. Therefore, not surprisingly, the mixture of penicillin and streptomycin (10 μ g/mL altogether) produced the most potent inhibition on bacterial metabolism. And as anticipated, reducing the dose of a drug reduces its inhibitory effect. Bacteria can grow confluent on our devices to form a biofilm. It is known that bacterial biofilm, whose formation involves in a variety of infectious diseases (e.g., the fatal cystic fibrosis), is more resistant to antibacterial

drugs. In agreement with this notion, we found that the inhibitory effects of all the tested drugs were less potent on *E. coli* biofilms (Figure 27).

Figure 27. Percentage change in conductance of SWCNT devices with *E. coli* biofilms (10^7 cfu/mL, incubated for 3h) without (squares) or with antibacterial drugs ($10 \mu\text{g/mL}$), upon application of different concentrations of glucose. Each data point is the average from 6 devices. The error bars indicate the standard errors.

6.9. Conclusions

In summary, we demonstrated a simple SWCNT device for high-throughput detection, functional studies, and drug screening of bacteria. Our devices, however, still have limitation in their detection specificity. In the future work, the recognition elements, such as antibodies²²⁵ or aptamers²²², could be functionalized onto SWCNTs for detection of specific bacterial strains.

Besides that, it should be noted that our approach still cannot compete compared to the state-of-the-art techniques in the aspect of sensitivity. Some established methods, such as colony culture counting or immunology based method, despite their tedious and time consuming pretreatment steps, has been proven to be able to for detecting

microorganisms enable the quantification of very low concentration of bacteria. With the rapid advance of SWCNT-nanoelectronics technologies ²²⁶, we envision that their sensitivity and capability would be greatly extended to reach the standard of conventional approaches.

Nanoelectronic biosensors have also been coupled with animal cells to detect their dynamic activities for high-throughput functional studies ^{151,227-228} and high-throughput drug screening. This herein demonstrated nanoelectronics-microorganism interface adds a new dimension to this emerging technology.

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Chapter 7

Centimeter-Long and Large-Scale Micropatterns of Reduced Graphene

*Oxide Films: Fabrication and Sensing Applications*²²⁹

7.1. Abstract

Recently, the field-effect transistors (FETs) with graphene as the conducting channels have been used as promising chemical and biological sensors. However, the lack of low-cost and reliable large-scale preparation methods of graphene films limit their applications. In this contribution, we report the fabrication of centimeter-long, ultrathin (1–3 nm), and electrically continuous micropatterns of highly uniform parallel arrays of reduced graphene oxide (rGO) films on various substrates including flexible polyethylene terephthalate (PET) films by using the micromolding in capillary method. Compared to other methods for the fabrication of graphene patterns, our method is fast, facile, and substrate independent. In addition, we demonstrate that the nanoelectronic FETs based on our rGO patterns are able to label-freely detect the hormonal catecholamine molecules and their dynamic secretion from living cells.

7.2. Background of the Study

Graphene, owing to its unique structure and properties²³⁰⁻²³¹, shows great potential in various applications including transparent electrodes^{67,71,232}, field-effect transistors (FETs)^{54,233-234}, and sensors^{73,79,81,112,125,235-240}. In particular, the graphene-based FETs used for chemical^{73,81,236,238} and biological sensors^{79,125,235,237} have recently

gained considerable interest, because the electrical property of graphene is highly sensitive to its local environment. Despite its potential, there are only a few reports about the graphene-based FETs being used for biosensing. For example, the electrolyte-gated FETs based on an individual pristine graphene sheet have been explored for pH and protein sensors⁷⁹. Chemical vapor deposition (CVD)-grown graphene films were used to detect DNA with single-base-mismatch sensitivity²³⁷. Recently, Lieber *et al.* reported the detection of bioelectrical signals at the graphene-living cell interface²³⁵. Most graphene-based devices mentioned above, however, rely on the single-layer graphene sheets produced by the low-yield or high-cost methods, such as mechanical exfoliation²³⁵⁻²³⁶ or thermal growth (CVD^{237,241-242} or epitaxial growth^{59,112}).

Reduced graphene oxide (rGO), prepared by the chemical^{68,243} or thermal reduction^{71,232} of graphene oxide (GO), emerges as a competitive alternative material of graphene. The facile and scalable solution process together with its residual chemically active defect sites renders rGO a promising material for functional electronic sensors. A single-layer rGO sheet^{78,125,240} or few-layer rGO films^{80-81,238} have been used to detect chemicals or biomolecules. Nevertheless, it remains a great challenge to obtain highly reproducible and large-scale continuous single or a few layer rGO films.^{67,83} In addition, the previously reported rGO thin-film devices have to rely on the microfabrication techniques owing to the short length of the conducting rGO films.⁸⁰⁻⁸¹

Fabrication of graphene-based electronics on various substrates, especially the flexible substrates, is highly desirable since it would greatly extend the applications of graphene in the plastic electronics field²⁴⁴. Although thermally grown graphene²⁴⁵⁻²⁴⁷ and rGO thin films⁶⁷ have been successfully coated on flexible substrates, these methods

usually incorporate a transfer step which is complicated and may induce chemical contaminations²⁴⁸. Therefore, the direct patterning of graphene on polymer substrates is desirable for flexible electronics, especially flexible biosensors which can conformally attach to tissue or organ surfaces²⁴⁹.

In this contribution, we present the fabrication of large-scale micropatterns of continuously conductive rGO films that are centimeters in length and micrometers in width on various substrates and then demonstrate their capabilities in sensing applications based on the front-gated (solution-gated) FETs. As compared to the back-gated FET on SiO₂ (typical 300 nm thickness), the solution-gate voltage falls across the thin electric double-layer capacitance (<1 nm in physiological ionic strength) formed at the rGO–solution interface, leading to superior transconductance and hence better field-effect characteristics in response to the perturbations introduced by the biomolecules in solution. The achieved parallel arrays of rGO films are highly uniform and can be easily observed by optical microscopy. We found that the randomly stacked networks of certain-sized rGO flakes (ca. 500 nm to 1.5 μ m) in our experiment exhibit the similar ambipolar FET characteristics as the individual single-layer graphene sheet^{76,233} and graphene thin films²³⁷ in the front-gate FETs. Finally, we used such patterned rGO film-based devices for detection of the catecholamine molecules in buffer solution, and label-free real-time monitoring of catecholamines secretion from living neuroendocrine PC12 cells. It was found that similar sensing capabilities were realized on both hard and flexible substrates.

7.3. Micropatterns of rGO Films

The patterns of continuous rGO films were achieved by the micromolding in capillary (MIMIC) method with a GO aqueous solution, followed by chemical reduction with hydrazine vapor, as described in Appendix A section F. Prior to micropatterning, all substrates (SiO₂ wafer, quartz, and PET film) were firstly modified with 3-aminopropyltriethoxysilane (APTES) SAMs to provide positively charged surfaces, which are crucial to ensure the uniform GO patterns. Without the modification of APTES, the patterned GO films will aggregate on all substrates. Although the normal PDMS stamp was able to generate continuous GO patterns on substrates, some PDMS residues would remain on the substrates due to the plasma treatment²⁵⁰. To avoid such contamination, a hard-PDMS stamp was used in our experiments.

The width of GO patterns (determined by the width of PDMS channels) was set at 10 μm for two reasons. First, too large ($>50\ \mu\text{m}$) or too small ($<2\ \mu\text{m}$) channels were not able to give continuous GO patterns since the large channels lead to the aggregation of rGO flakes while the small ones result in the discontinuous GO patterns.

Second, the 10 μm size is similar to the size of an animal cell, which is desirable to interface with living cells and detect their activities. In addition, only the GO flakes with sizes ranging from 500 nm to 1.5 μm (selected by centrifuge) could produce the centimeter-long continuous films. In our experiment, the achieved smallest width of rGO patterns is 2 μm . The length of the rGO patterns was only determined by the microfluidic channels in the PDMS stamp.

The previous study on rGO thin films fabricated by the filtration method suggested that the thickness of an rGO film is crucial for its field-effect property⁶⁷. In

our experiment, the thickness of the rGO patterns can be easily controlled by adjusting the concentration of the GO solution used in MIMIC. After the hydrazine vapor reduction of the GO patterns, the obtained rGO patterns show similarity in the film morphology and thickness, suggesting that the generated GO patterns are very stable.

Figure 28. a.) Optical image of patterned rGO films (dark lines) on the SiO₂ substrate. b.) AFM image of patterned rGO on a SiO₂ substrate. c.) Section analysis indicates rGO films are composed of one to a few-layer of randomly stacked rGO sheets.

The optical image (Figure 28) shows the obtained large-area, centimeter-long, perfectly aligned rGO micropatterns on a SiO₂ wafer. An AFM image and height profile (Figure 28b and c) confirmed that the patterned rGO films contained single or a few-layer rGO sheets, since the measured thickness was about 1–3 nm (average thickness around 2.3 nm)^{243,251}. Our method was also successfully used to fabricate the rGO patterns on other substrates, such as quartz and PET films, although the quartz (rms = 0.5 nm in 10 ×

10 μm^2) and PET film (rms = 2.5 nm in 10 $\times$ 10 μm^2) were quite rough. It is worth mentioning that such micropatterned arrays allow parallel and multiplexed measurements, and therefore possibilities to resolve the propagation of biosignals ²⁵².

7.4. Electrical characterization and sensing applications

Electrical properties of the resulting rGO devices were monitored using a semiconductor device analyzer (Agilent B1500A). Silver conducting paint was used to prepare electrodes at both ends of the parallel rGO pattern arrays, that is, the source and drain electrodes. The length of the rGO conducting channel was fixed at 1 cm, which is the distance between the drain and source electrodes. Subsequently, silicon rubber (Dow Corporation) was used to insulate the electrodes and to define the chamber containing the electrolyte saline bath solution (the mixture of 10 mM HEPES, 140 mM NaCl, 1 mM MgCl_2 , 5.5 mM KCl, and 2 mM CaCl_2 , pH 7.2) in the electrical recording. The source-drain current (I_{ds}) was biased at 400 mV, and measured while varying the gate voltage (V_{g}) applied on the top gate (Ag/AgCl electrode).

The source-drain current (I_{ds}) was continuously monitored (biased at $V_{\text{ds}} = 400$ mV), when dopamine was manually added to the recording chamber with a gradual increase in its concentration. To detect the dynamic secretion of dopamine from living cells, PC12 cells were cultured on the rGO patterned devices for 2 days in the cell culture media (a mixture of Dulbecco's Modified Eagle's Medium, 10% Horse Serum, 5% Fetal Bovine Serum, 1% Pencillin-Streptomycin, and 5 μM Dexamethasone) and maintained at 37 $^{\circ}\text{C}$ in a humid atmosphere with 5% CO_2 /95% air ¹⁵⁴. Prior to the cell culture, the rGO devices were coated with poly-l-lysine (PLL) overnight, in order to help cells to

adhere to the substrates. High potassium solution (the mixture of 40 mM NaCl, 105 mM KCl, 6 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES) was introduced into the recording chamber after the cell culture medium was initially replaced by the physiological bath solution (pH 7.2) to incubate PC12 cells. The current (I_{ds}) through the rGO patterns was continuously monitored ($V_{ds} = 100$ mV, $V_g = 0$ V) during the high K⁺ stimulation.

7.5. Electrical properties

A set of parallel Au electrodes with increasing gap distance, deposited on the rGO strips by the sputtering technique, were used to investigate the conductance of the resulting rGO patterns on quartz. As shown in Figure 29, the obtained centimeter-long rGO patterns are electrically continuous and exhibit ohmic behaviors. The inset in Figure 29 confirms that the resistance of rGO patterns increases linearly with an increase in the effective length of the rGO conducting channels, that is, the gap distance between the two Au electrodes. This indicates that the resistance mainly arises from the intrinsic resistance of rGO patterns rather than the contact resistance between the rGO patterns and the Au electrodes, which is in agreement with the previous report for the individual single-layer rGO sheet¹²⁷. These results also suggest that the high uniformity of rGO patterns is crucial to achieve the reliable device performance. The large resistance (>2 M Ω) is likely due to the remaining defects on the rGO sheets and the scattering effects arising from the rGO stacking⁶⁷.

A typical front gate setup is used to study the properties of patterned rGO in the saline bath solution (pH 7.2). Source and drain electrodes can be easily prepared on such an array of perfectly aligned rGO microline patterns using silver paint without

microfabrication or microscopy. The silicone rubber was applied to define the recording chamber and provide insulation on the Ag electrodes. Such insulation ensures the small leakage current (typically $<0.1\%$) and avoids the influence of the metal–rGO contact on sensings.

Figure 29. a.) Linear I – V curves at V_{ds} from -1 to 1 V on rGO–quartz device. Inset: the resistance increases linearly with the gap distance between Au electrodes. b.) Plots of I_{ds} vs front-gate voltage (V_g) at $V_{ds} = 400$ mV on (top) rGO–PET and (bottom) rGO–quartz devices. c.) Current noise on rGO–quartz and rGO–PET devices. The distance between the drain and source electrodes in the devices in panels B and C was fixed at 1 cm.

Figure 29 shows the typical current *versus* gate potential plots of two devices, that is, the rGO patterns on PET and rGO patterns on quartz (referred to as rGO–PET and rGO–quartz devices, respectively). V-shaped ambipolar field-effect characteristics with Dirac (minimum current) point at *ca.* $+0.2$ V was reliably observed on all the devices.

The on/off ratio of the resulting devices ranged from 2 to 20, mostly ~5. The sensitivity of the current to front-gate voltage was comparable to the FET devices based on the individual single-layer graphene sheet¹¹². This result is remarkable since our conducting channel is formed by the random stacking of many small rGO sheets.

The bending of the rGO–PET devices (bent radius $r = 3$ cm) did not impair its field-effect characteristic. The flexible nature of our rGO devices enables wide applications including nanoelectronic biosensing, for example, for interfacing with curved living organs or tissues. Interestingly, as compared to the rGO–quartz device, the rGO–PET device showed slightly lower conductance and much larger noise ratio (>2 order) (Figure 29c). Such a difference may be attributable to the rougher surface of a PET film, which could affect the stacking quality of the rGO flakes.

When a highly concentrated GO suspension was used in MIMIC, the obtained thick rGO patterns (>5 nm) gave a weak field-effect response to the front-gate modulation and hence a lower sensitivity in sensings. When spin-coating was used to coat GO films on the substrates, the obtained thick GO films (>10 layers, *i.e.*, > 10 nm) showed no field-effect response, resulting in no sensing ability.

7.6. Detection of catecholamines molecules

As a proof of concept for sensing applications, we demonstrate the capability of our micropatterned rGO films in the detection of dopamine, a major neurotransmitter secreted by neurons and hormone secreted by neuroendocrine cells, with both rGO–PET and rGO–quartz devices. The conductance of the rGO devices was continuously

monitored at $V_g = \pm 0.6$ V, while dopamine with various concentrations was introduced in the buffer solution.

Figure 30. Detection of dopamine on rGO–PET device at $V_g = \pm 0.6$ V. Each step represents the gradual addition of dopamine with concentration increasing from 1 to 8 mM. Inset: Change of conductance vs dopamine concentration. The distance between the drain and source electrodes in the rGO–PET device is fixed to 1 cm.

As shown in Figure 30, the conductance of the rGO–PET device increased at $V_g = -0.6$ V, while it decreased at $V_g = +0.6$ V, with increasing concentration of dopamine. This is expected because the rGO device operated at the p-type region when V_g was less than the Dirac voltage (+0.2 V), and operated at n-type region when V_g was greater than the Dirac voltage (Figure 29b). The behavior of the rGO–quartz devices was similarly to that of the rGO–PET devices. After removing dopamine by thorough perfusion, the current (I_{ds}) returned to its initial level, and the device could be reliably used for many

times. Other catecholamine molecules, such as epinephrine, also caused the similar increase of rGO conductance as shown in Figure 31.

Figure 31. Test of epinephrine on rGO-quartz device at $V_g = -0.6$ V. Each step represents the gradual addition of dopamine with the concentration from 1 to 10 mM. The distance between the drain and source electrodes in rGO-quartz device was fixed to 1 cm.

As anticipated, when $V_g = 0$ V, the devices also gave a p-type response but with a smaller amplitude (Figure 32). The low level V_g is advantageous in terms of minimizing possible disturbance to the biological samples (*e.g.*, living cells) and avoiding the instability of devices due to the induced electrochemical or charging effects. As evidenced from Figure 32, at $V_g = 0$ V, the rGO–PET device is more sensitive than the rGO–quartz device (note that the scale bars in Figure 32a and b are different). These results are reliably observed on all fabricated devices. In response to 10 mM dopamine, the rGO–PET FETs gave an increase in conductance of $31.3 \pm 4.1\%$ ($n = 6$ devices) at $V_g = -0.6$ V and $22.8 \pm 2.1\%$ ($n = 6$ devices) at $V_g = 0$ V.

Figure 32. Detection of dopamine on (a) rGO–quartz and (b) rGO–PET devices at $V_g = 0$ V. The distance between the drain and source electrodes in the devices in panels A and B was fixed to 1 cm. Insets: Change of conductance vs. dopamine concentration.

The response of the rGO FET device to dopamine is not due to the modulation of the energy barrier at the electrode–rGO contact, because the source and drain electrodes are well-insulated. It is neither due to electrostatic gating because, in this case, positively charged dopamine at pH 7.2 should cause current decrease when the rGO devices operate at the p-type region. Therefore, the rGO responses are likely due to the doping effect

since dopamine can strongly interact with rGO through the strong π - π interaction. In fact, as shown in figure 33, the addition of dopamine caused the right-shift of the Dirac point in the I_{ds} - V_g curve of the rGO-PET device, indicating that the aromatic catecholamine molecules induce p-doping (or increase of hole concentration) in the rGO film. This is in agreement with the previous study on the doping effects of aromatic molecules on graphene²⁵³.

Figure 33. The plot of I_{ds} vs. front-gate voltage (V_g) at $V_{ds} = 400$ mV of a rGO-PET device before (●) and after (●) addition of 10 mM dopamine. The distance between the drain and source electrodes is fixed at 1 cm.

7.7. Detection of dynamic vesicular secretion of catecholamines from PC12 cells

As compared to one-dimensional semiconducting nanomaterials (e.g., carbon nanotubes, silicon nanowires), the two-dimensional (2D) graphene offers several advantages in the sensing applications, including large sensing area, ease of

homogeneous functionalization ²⁵⁴, biocompatibility ²⁵⁵, and suitability to interface with 2D cell membranes. In our experiment, we coupled our rGO FETs with living neuroendocrine PC12 cells and detected their triggered vesicular secretion of catecholamine molecules (dopamine, epinephrine, and norepinephrine).

Figure 34. a) Optical image of PC12 cells grown confluent on a poly-l-lysine coated rGO–PET device. b.) Schematic illustration of the interface between a PC12 cell and a rGO FET. c.) Real-time response of rGO–PET FET to the vesicular secretion of catecholamines from PC12 cells stimulated by high K^+ solution. ($V_{ds} = 100$ mV, $V_g = 0$ V). The distance between the drain and source electrodes in the device was fixed at 1 cm.

The PC12 cells were directly cultured on top of poly-l-lysine-coated rGO FET devices. When a high K^+ solution, which depolarizes the cell membrane and elicits Ca^{2+} influx through the voltage-gated Ca^{2+} channels of the cell membrane, was introduced into the recording chamber, current responses (spikes) of rGO FET were observed (Figure 34).

As demonstrated in our previous study, where the carbon nanotube network based FETs were interfaced with PC12 cells¹⁵⁴, each observed current spike likely corresponds to the vesicular release of catecholamines from a single PC12 cells. This experiment postulates the capability of graphene FETs for the nanoelectronic biosensing of dynamic cellular activities.

7.8. Conclusions

The ability to pattern large-area ultrathin rGO films with high yield and reproducibility will open up an avenue to widely incorporate graphene into electronics or bioelectronics. In this study, we demonstrated the patterning of centimeter-long, micrometer-wide, ultrathin (1–3 nm), continuous rGO–network films as the conducting channels of FETs on various substrates. Our rGO FETs exhibit remarkable and bending-insensitive field-effects, which are able to detect the presence and dynamic cellular secretion of biomolecules. The specificity of the demonstrated detection is realized in the defined biological context. In a parallel effort, we found that our rGO FETs can also specifically detect biomolecules with high sensitivity by functionalizing the rGO patterns with specific antibodies. This study highlights the promising potentials of graphene in flexible nanoelectronics and bioelectronics.

7.9. Acknowledgements

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Chapter 8

Application of Graphene-Field Effect Transistor for Detection of Metal

*Ions and Specific Biomolecular Interactions*²⁵⁶

8.1. Abstract

The unique electrical property of graphene, which is highly sensitive to the change of its local environment due to its unique structure of two dimensional and one-atom thick carbon sheets, makes graphene a promising channel material for electronic sensors. The material has shown promising results in the detection of chemical and biomolecule, such as various gases, protein and DNA, and the bioactivity of living cells. Solution-processed, reduced graphene oxide (rGO) is favored due to its low cost, potential mass production and simple surface functionalization protocols. In this chapter, initialized by performing typical pH sensing and detection of biotin-avidin interactions in the graphene platform, we successfully performed sensitive and selective detections of physiological calcium and magnesium ions as well as heavy metal pollutant in aqueous solution (e.g. 1 nM of Hg^{2+}) based on the bio-recognition between the metal ions and proteins on the rGO channel.

With the advantages of simplicity, real time detection, and efficiency; the electrical detection technique via rGO-FET devices exhibits asensitivity, selectivity, and detection limit comparable to the recent alternative methods. Different from all demonstrations in the previous chapter, in this part we focus on the real engineering application on the top of understanding fundamental principles of the devices.

8.2. Background of the study

Graphene, a two-dimensional layer of sp^2 -hybridized carbon atoms, has received increasing attention since its initial discovery. It has been used in various applications because of its remarkable electronic, mechanical, and optical properties. Attributable to the fact that graphene is only one single-atom thick, it can perform very high sensitivity towards a local environment and electrochemical perturbations⁷⁶. Furthermore, it has extremely high charge mobility ($\sim 10^4 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$), large carrier capacities ($\sim 10^{12} \text{ cm}^{-2}$), and relatively low $1/f$ noise; properties which promoted this material to be particularly attractive for sensor applications^{54,58,77}. The use of graphene in biological research, in particular, has recently been demonstrated including microbial diagnosis¹²⁵, label-free biomolecules sensor⁷⁸⁻⁷⁹, and pH detection^{76,112}.

It is critical to study the performance of graphene in aqueous solution especially when it is exploited for chemical or biological sensing applications. Interestingly it has been shown that graphene immersed in an electrolyte shows better transfer characteristics (almost ~ 30 times) compared with its characteristic in vacuum⁷⁹. The reason for this is occurs because the formation of a very thin top-gate double layer in the electrolyte. The application of the gate voltage, meanwhile, falls across this solution-graphene interface.

The interaction mechanism between biomolecules and graphene, so far, is dominantly described as the effect of polarity-specific gating upon the binding of negatively or positively charges species, which cause an increase or decrease in device conductivity respectively^{112,125}. The absorption of individual gas molecules, on the other hand, is reported to cause a chemical doping effect to graphene device⁷³. It has also been

reported that direct molecule absorption would possibly cause small conductance changes of graphene layer ⁷⁹.

To the best of our knowledge, demonstrations of the graphene material to detect a highly specific biological interaction are still very limited. One demonstration has been achieved recently by *Lu et al.* ⁷⁸ using fluorescently labeled aptamer GO to detect specific protein. Despite its good sensitivity and specificity, this typical detection scheme, nevertheless, was not used to achieve data in real time. Here, we demonstrate a real-time electrical detection of highly specific biomolecules binding by using rGO pattern devices and graphene sheets, including: biotin-avidin interaction and Ca^{2+} and Mg^{2+} ion binding to calmodulin. Besides, we also initially demonstrate that the graphene device is responsive to pH modulation. This is the first time a graphene-FET device can be used as a platform to detect a heavy metal pollutant with high sensitivity via an electrical transport characterization.

8.3. rGO-FET as pH sensor

Figure 35 presents the conductance plotted as a function of gate voltage (V_g) for a rGO-FET (which was fabricated by using the methods mentioned in Appendix A section F4) in phosphate buffer saline (PBS) at various pH values from 2 to 10. The dependence of the transfer characteristics and conductance of the rGO sheet on the pH value was evaluated. We observed that the conductance of the rGO-FET was right-shifted with increasing pH values. This behavior implies that the electrochemical double layer at the graphene / electrolyte interface is sensitive to a pH modulation.

Figure 35. Transfer gate characteristic (I_{ds} - V_g) of a rGO-PET device in pH 2 (red), 5 (yellow), 7 (green), and 10 (blue). The dirac delta point tends to shift to more positive value with increasing pH.

Similar changes in the gate transfer characteristics have been observed previously in rGO-FET devices exposed to NO_2 ⁷³. The graphene/electrolyte interface allows the capacitive charging of the surface by H_3O^+ or OH^- ions, which plays a role as a potential gating effect to modulate the Fermi energy level (in this case, with holes as majority electrical carrier). Interestingly, the phenomenon of a conductance increase has also been observed in carbon nanotube pH-sensors. It is described as the attachment of hydroxide ions, which act as electron scavengers, causes the conductance to increase²⁵⁷⁻²⁵⁸. In fact, this typical demonstration of pH sensitivity on graphene device is very similar to those previously presented in recent publications^{76,79,112}.

8.4. Detection of biotin-avidin specific binding

Biotin specific binding to avidin is known as one of the strongest protein-ligand interactions ($K_d \sim 10^{-15}$ mol/L), almost approaching the strength of covalent bonds²⁵⁹. In

our experiment, the rGO patterns (fabricated using the methods mentioned in 3.61-3.6.3) were initially submerged overnight in a 10 wt % solution of poly(ethylene imine) and 10% poly(ethylene glycol) in water to avoid non specific binding of proteins, followed by rinsing with distilled water. As presented in figure 36a, a typical V-shape ambipolar characteristic was observed for a modulation of the solution gate voltage from -1 V to 1 V for both before and after the polymer coating. It is noted that a drop in conductance were observed after the PEI-PEG coating, which can be caused either from the negative charge of the PEI groups or from scattering of the device potential. Subsequently, the polymer-coated devices were biotinylated by submerging them overnight in a 15 mM DMF solution of biotin-*N*-hydroxysuccinimide ester (Sigma) at room temperature. This compound readily reacts with the primary amines in the PEI under ambient conditions. It was shown that biotin functionalization would cause further a decrease in device conductance. The rGO devices were then rinsed with DMF and distilled water. An increasing concentration of avidin molecules (1 to 4 μ M) were tested afterward with those functionalized-rGO devices.

Prior to the biotin-avidin test, several control experiments were performed in order to investigate the existence of possible nonspecific binding (NSB) in the detection scheme. No significant conductance change was observed upon the addition of avidin on both bare device (Figure 36b upper trace) as well as polymer coated devices (figure 36b lower trace), suggesting that there was a relatively small NSB occurring in the recordings. On the contrary, significant responses were observed with the biotinylated polymer-coated rGO device. This is suggesting that the specific biotin-avidin interaction has successfully been detected (figure 36c and d). It is interesting to note that both p-type and

n-type recording can be achieved with the same device just by varying the gate-voltage offset. Besides, sensitivity of the FET can also be readily calculated from the slope of the specific gate voltage.

Figure 36. a) rGO-pattern device characteristic comparison of bare (red), after PEI-PEG coating (yellow), and biotin biotinylated polymer-coating (green) of the device. b.) Real-time avidin detection with a bare rGO device (upper trace) and a polymer-coated device (lower trace). (c and d.) Real-time avidin detection with a biotinylated polymer-coated rGO device. The offset voltage was set to -0.6V or +0.6V, respectively.

Avidin is positively charge in the used recording environment (pH 7.2), since it has an acidic isoelectric point (pI) of ~ 10.5 . As expected, the chemical doping of avidin would cause a conductance increase if the device was biased at a negative voltage (p-type region). On the contrary, the conductance was decreased if the device was biased at positive voltage (n-type region) during avidin addition. We also found that the sensitivity in the p-type region was more significantly quantitative (7.03%) compared to this in the

n-type region (4.28 %). This corresponds to the much larger slope in p-type (~300 nA/V) compared to n-type region (~ 80 nA/V) of this specific device.

8.5. Calcium Ion Detection via Calmodulin Conformational Change

Calmodulin (CaM), approximately 16,500-mol wt, is a popular and well-studied E-F- hand family of calcium sensor proteins. It is ubiquitous in eukaryotic cells and participates in various signaling pathways that regulate many crucial processes such as cell growth, proliferation, and movement. Different from those proteins, which function as Ca^{2+} buffer or Ca^{2+} transporter (eq: parvalbumin and calbindin) which only undergo minor structural alteration, the binding of Ca^{2+} ions to calmodulin causes a significant structural rearrangement followed by an exposure of hydrophobic domains on its surface. This platform, at the same time, serves as an interface for both CaM-CaM binding protein interactions and CaM-hydrophobic ligand interactions²⁶⁰⁻²⁶¹.

In order to demonstrate their possible applications, the fabricated rGO-FETs were used for detection of metal ions. As an example, we explored the detection of Ca^{2+} ions, an important secondary messenger for cells and the major determinant for water hardness. To achieve this specific detection, the patterned rGO films of the rGO-FET devices were functionalized with calmodulin (CaM), a Ca^{2+} binding protein ubiquitously expressed in eukaryotic cells, via pre-coated pyrene linker molecules, which covalently reacts with CaM in one end and firmly attaches to rGO in the other end (pyrene tail) via a strong π - π stacking interaction.

Figure 37. a.) Typical real-time recording of I_{ds} with addition of Ca^{2+} ions. (b, c) The change of I_{ds} measured with GO-FETs (tested sample number $n=6$) with addition of (b.) Ca^{2+} and (c.) Mg^{2+} ions at $V_g = -0.6\text{ V}$ (circle), 0 V (square) and $+0.6\text{ V}$ (triangle).

In a typical experiment, as shown in Figure 37a ($V_g = -0.6\text{ V}$), the introduction of Ca^{2+} ions caused a significant current (conductance) decrease in the CaM functionalized rGO-FETs. Both the p-type and n-type detection can be realized in the same rGO-FET by changing the solution-gate voltage (V_g). Figure 37b shows the statistics of device responses with different V_g values (-0.6 , 0 , and $+0.6\text{ V}$). In response to the addition of Ca^{2+} ions, the CaM-modified rGO-FETs exhibited a conductance decrease while operating at the p-type region ($V_g = 0$ or -0.6 V), and a conductance increase while operating at the n-type region ($V_g = +0.6\text{ V}$). At $V_g = -0.6\text{ V}$, a detection limit of $\sim 1\text{ }\mu\text{M}$ was achieved with a signal-to-noise ratio of ~ 20 - 30 . This detection limit was consistent with the binding affinity (K_d) between Ca^{2+} and CaM, which is ~ 0.1 to $1\text{ }\mu\text{M}$ ²⁶²⁻²⁶³. Importantly, the achieved detection limit in this experiment was lower than that obtained

with the silicon nanowire-based electronic sensors ²⁶². Moreover, we believe that the detection of lower concentrations of Ca^{2+} ions could be achieved if functionalization of the rGO-FETs was done with a recognition element of higher binding affinity with Ca^{2+} .

It is well known that CaM also binds to Mg^{2+} , but with a much lower affinity. Therefore, the rGO-FETs can also be used to detect Mg^{2+} (Figure 37c). As shown in Figure 37b and c, the change of conductance, caused by the binding of Ca^{2+} or Mg^{2+} onto the rGO layer, can be attributed to a field-effect modulation of the rGO-FETs introduced by the positively charged ions with the same sensing mechanism than for the silicon nanowire-based Ca^{2+} sensors. ²⁶² In contrast, the CaM-modified rGO-FETs did not show any response to the Na^+ or K^+ ions (figure 39), indicating the specificity of detection due to the CaM functionalization of rGO. Importantly, the rGO-FETs without functionalization of CaM are not sensitive to Ca^{2+} or Mg^{2+} (Figure 39), indicating that the CaM receptor is essential for the detection scheme mentioned above.

Figure 38. Control experiment of K^+ and Na^+ ions with CaM- functionalized rGO-FET devices.

Figure 39. Control experiments of Ca^{2+} , Mg^{2+} , Hg^{2+} , and Cd^{2+} ions with bare rGO-FET device.

8.6. Heavy Metal Ion Detection via Metallothionein II Functionalized Device

Metallothioneins (MTs) are very unique proteins, which have low molecular weights (6–10 kDa) and a high composition of cysteine residues (~30% of the total amino acid residues). Owing to their very high affinity to various heavy metals, such as copper, zinc, cadmium, selenium, and mercury, MTs were recently applied to sensor devices to detect metal analytes²⁶⁴⁻²⁶⁵ and were considered as a potential biomarker for environmental metal contamination.

Further, we explored the possibility for detection of heavy metal ions with our rGO-FETs. First, the metallothionein type II protein (MT-II), which binds with both physiological (e.g., zinc, copper, selenium) and xenobiotic (e.g., cadmium, mercury) heavy metals with high affinity, was used to functionalize the micropatterned rGO films with pre-adsorbed pyrene molecules in rGO-FETs. As shown in figure 40a, the addition of Hg^{2+} , even with a concentration as low as 1 nM, has caused an obvious current increase in the rGO-FET device, biased at $V_{ds} = 400\ \text{mV}$ and $V_g = -0.6\ \text{V}$.

Figure 40. a.) Typical real-time recording of I_{ds} with addition of Hg^{2+} ions. (b, c) The change of I_{ds} in the rGO-FETs (tested sample number $n=6$) with addition of (b) Hg^{2+} and (c) Cd^{2+} ions at $V_g = -0.6$ V (circle), 0 V (square) and +0.6 V (triangle).

Figure 40b shows the plot of the drain to source current change of the device with increasing Hg^{2+} concentration and its polarity dependence on the gate voltage (V_g). The detection limit for Hg^{2+} was about ~ 1 nM with a signal-to-noise ratio of 25-30. As compared to the detection of Ca^{2+} , the much higher sensitivity for Hg^{2+} detection arises from the high binding affinity of MT with heavy metals (K_d in 1 fM range)²⁶⁵. To the best of our knowledge, this is the first demonstration of the real-time electronic detection of heavy metal ions, which shows higher detection sensitivity than the previously reported fluorescence-based methods²⁶⁶⁻²⁶⁸. In addition, Cd^{2+} was also detected at a concentration of 1 nM with a slightly smaller change of current (signal-to-noise ratio of 15-20, Figure 40c).

It is worthy to mention that the polarity of the device response caused by Hg^{2+} or Cd^{2+} was opposite to that of Ca^{2+} and Mg^{2+} (Figure 40b), although all of them are positively charged ions. Therefore, it can be concluded that the sensing mechanism of heavy metals does not arise from the field effect. It has been previously reported that the binding of heavy metal ions causes the significant conformational change of MT²⁶⁵. Such a conformational change might alter the interaction between MT and rGO channel, and consequently might cause a conductance change in the rGO-FETs. In a control experiment, we found that the MT-II modified rGO-FETs were not responsive to K^+ , Na^+ , Ca^{2+} and Mg^{2+} (Figure 41), and the rGO-FETs without functionalization with MT-II were not sensitive to Hg^{2+} and Cd^{2+} (Figure 39). Importantly, the MT-II modified rGO-FETs can be re-used for 3-4 times without any significant loss in the sensitivity, after they were washed with acidic buffer (100 mM glycine, pH 2.3) to remove the bound metal ions from MT-II.

Figure 41. Control experiment of K^+ , Na^+ , Mg^{2+} , and Ca^{2+} ions on a MT-II functionalized rGO-FET device.

The performance of our rGO sensors is stable. As shown in figure 42, a steplike current increase in response to Hg^{2+} remained constant during the long recording time (>15 min). The MT-II modified rGO-FETs can be reused for 3-4 times without any significant loss in the sensing sensitivity, after they are washed with acidic buffer (100 mM glycine, pH 2.3) to remove the bound metal ions from MT-II. Degradation of the devices performance was observed after over-reuse likely due to denaturation of the receptor proteins. To further demonstrate the practical use of our sensors, we tested the natural water collected from Nanyang Lake at our campus.

Figure 42. Real-time recording of I_{ds} with addition of Hg^{2+} at $V_{ds} = 400 \text{ mV}$ and $V_g = -0.6 \text{ V}$.

As shown in the right inset of figure 43, the addition of lake water, which is a complex soup consisting of various ions, microorganisms, and impurities, could not elicit any appreciable response of our devices. It indicates the high specificity of our sensors and proves that this small and isolated lake is not contaminated by heavy metals. In comparison, lake water with the addition of Hg^{2+} (concentration as low as 1 nM) caused a significant increase of rGO current while V_g was biased at -0.6 V (Figure 43). The experiments showed the practical use of our sensors in environmental monitoring.

Figure 43. A typical real-time recording of I_{ds} with the addition of Hg^{2+} ions into 200 μ L natural lake water on the top of rGO-FET device, with $V_{ds}=0.4V$ and $V_g=-0.6 V$. Inset: Real-time recording of I_{ds} with addition of 100 μ L lake water into the existing 100 μ L of DI water on the device.

8.7. Conclusion

In summary, we have successfully demonstrated sensitive biomolecules detection via protein-modified graphene device as the platform. The results were confirmed and verified with preliminary measurements (pH detection and biotin-avidin binding which is commonly known). Calmodulin (CaM) functionalized graphene has shown a detection limit in micromolar range (which is similar to the physiological condition) and was selective only to calcium and magnesium ions.

It was also demonstrated that metallothionein-modified-graphene can be utilized as cadmium and mercury sensor with a detection limit in nanomolar range. It is interesting to note that both p-type and n-type recording can be achieved on the same device just by varying the gate voltage offset. Sensitivity of the FETs can also be readily calculated as the slope of the specific gate voltage. In addition, pattern shape fabricated on our device (size: 10 μ m), are indeed compatible to the size of mammalian cells,

realizing this platform for interfacing living cells and further investigation of dynamic cellular activity²²⁹.

It was demonstrated that a simple and easy to use reduced graphene oxide (rGO)-based FET sensor, readily fabricated on bench-top protocol, can successfully be utilized to rapidly and label-freely detect various metal ions in solution with a high sensitivity. To the best of our knowledge, this is the first report on the real-time electronic detection of heavy metal ions. This nanoelectronic approach is amenable for the development of lab-on-a-chip devices. We believe that the micropatterned rGO film array, in principle, enables the parallel recording and sensing of multiple targets.

8.8. Acknowledgement

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Chapter 9

Conclusions and Future Directions

9.1. Conclusions

Nanocarbon materials, particularly carbon nanotubes (CNT) and graphene, have a significant role in the new developments for the size reduction of biosensor devices. This significance has even received explosive attention due to their ability to address some key issues in biosensor development. These are including the increment of the sensor sensitivity and selectivity, improvement in the response time, better design of biosensing interface/modification, as well as the achievement of an efficient transduction at the recognition element.

All of those issues mentioned above have been considered very carefully in biosensor studies, especially when they are intended to be combined with biological living systems. This is attributable to the fact that those nanocarbon materials have been previously found to exhibit cytotoxicity effect to living cells. Strategy to non-invasively interface living cells while maintaining sensor properties, therefore, are essential to establish a stable and promising biodetection platform.

In our studies about these nanocarbon materials, several successful demonstrations of non-invasive interfacing between those materials and living systems have been established. We realized real time detection of dynamic cellular activities in a high temporal resolution and investigations of several drugs/stimulation effects in cells. Carbon nanotubes networks, configured as field-effect transistors (FET), have been utilized to detect the dynamic ATP secretion from astrocytes, catecholamine release from

neuroendocrine PC12 cells, dynamic action potentials (regular beating) from cardiomyocytes, as well as glucose metabolism mechanism detection from *Escherichia coli* bacteria. Graphene FET sensors, on the other hand, have been utilized as a platform to detect the exocytosis activity of PC12 cells. All of these realizations represent a significant development in the field of cell biology and open up an alternative way to advance our fundamental understanding of cell activities from single cells compared to several established methods. In addition, realization of our graphene FETs as metal ions molecular sensors, provide a significant impact in the study of cellular physiology and detection of environmental pollutant.

In general, limit of detection (LOD) of our developed devices is excellent for practical use in both scientific research and engineering application. ATP molecules, catecholamine molecules, calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions, for instance, can be detected in physiological (micromolar) range. On the other hand, heavy metal ions (pollutant) in the lake water sample can be detected until nanomolar range. All this results may not as sensitive as optical (fluorescence) method in the similar research field^{196,214,266,268}. Our nanocarbon-based sensors, nevertheless, provide many competitive advantages in the various aspects. With such simple detection scheme, our devices are easy to fabricate with a low cost, high throughput, stable, consistent (can be reused for several times), and can be utilized as a real time monitoring tool.

9.2. Mechanism of detection

In this section, the underlying mechanism for various detection in our CNTs and graphene sensors in this thesis is described and compared qualitatively. In this case, graphene sensors have advantage compared to CNTs, attributable to the fact that they have ambipolar (both p-and n-type) characteristic. This enables opposite responses to be observed on the same graphene device just by varying the gate voltage offset. CNTs, on the other hand, can only show p-type characteristic response.

In the study of detection mechanism applied in our devices, we assume that Schottky barrier modulation is always be excluded. The reason for this is because the nanocarbon-electrode contacts were already well insulated in the device fabrication process, prior to the recording experiment.

Various detection mechanisms in this thesis are described as following. In the study of Adenosine Tri Phosphate (ATP) molecules (Chapter 4), whether it was added directly onto the sensor or secreted from astrocyte cells, ATP consistently caused an elevation of CNTs conductance. This is plausibly contributed from the negative charge of the ATP molecule which increases the concentration of major current carrier (hole) of the nanotubes (under the category of electrostatic gating effect).

The similar electrostatic gating effect were also observed on positively charged Calcium (Ca^{2+}) and Magnesium (Mg^{2+}) ions detection by using calmodulin modified graphene platform (Chapter 8). It was shown that the positive charge on those ions caused decrease response in the p-type region and the opposite response on the n-type region.

It is interesting to note that detection of heavy metal ions such as Hg^{2+} and Cd^{2+} in protein (MT-II) modified graphene, despite their positive charge, showed an opposite result compared to in Ca^{2+} and Mg^{2+} ions (Chapter 8). This suggested that it is the protein conformational change caused by those ions which subsequently alters the interaction between protein and graphene interface which provide more dominant effect compared to the charge.

Electrostatic gating mechanism also fails to explain catecholamine (dopamine, epinephrine, norepinephrine) detection response in CNTs devices (Chapter 5). In this case, the conductance increase is plausibly contributed from the π - π stacking between the aromatic ring and the CNTs side wall rather than gating effect. Similar response for catecholamine detection was observed on the p-type region of the graphene (Chapter 7). And as predicted, the opposite (decrease) response can be observed when the graphene was operated in n-type region.

Electron transfer mechanism can be used to describe the result of pH modulation on our graphene devices (Chapter 8), which is consistent compared to previous reports²⁵⁷⁻²⁵⁸. It was described that attachment of OH^- ions, which acts as electron withdrawer, contributed conductance increase (positive shifting) as pH value increase. Mechanism of detection of bacterial presence (Chapter 6), on the other hand, is most likely under doping effect imposed by the interacting biomolecules on bacterial surface or potential scattering effect imposed by the charged molecules on the bacteria, as previously reported²²².

9.3. Nanotubes or Graphene?

After all of the comparisons and summary described in previous sections, it still leaves us a big question: so, which one is better between carbon nanotube and graphene sensor? It is indeed a difficult question to answer because the application of graphene as electrical sensor has just started to receive attention in the past few years, when *Geim et al.* start to work intensively on this material ⁵⁴. Though recently there have been few head-to-head comparisons of the relative performance of CNTs and graphene in biosensing, none of them is in completely optimized systems.

Nevertheless, there are some important differences in properties that suggest that graphene might have some competitive advantages over CNTs for the sensors applications. Firstly, graphene have addressed some of the problems that limit the application of CNTs. For example, we found the 2-D “flat” nature of graphene platform enables them to interface better to the living system compare to the 1-D CNTs. In addition, the high-aspect-ratio of CNTs, which is so useful in many ways, makes it difficult to controllably assemble nanotubes to make complex sensors or other devices.

Second advantage of graphene involves aspects of its performance which are superior to CNTs sensor in general. Graphene is a strictly two-dimensional material and, as such, has its whole volume exposed to surface adsorbates, which maximizes their effect. Besides, it is highly conductive, exhibiting metallic conductivity and, hence, low $1/f$ noise even in the limit of no charge carriers, where a few extra electrons can cause notable relative changes in carrier concentration. Our experiment results presented in this thesis also support the idea that graphene material has great potential as a choice for future electrical sensor. Further reductions in noise (i.e by adjusting the number of

graphene layer), and therefore improvements in sensitivity, might also be possible through minimizing substrate effects and improving the quality of electrical contacts to the graphene.

Other outstanding properties of graphene include its high flexibility, which is required for mechanically robust sensors, and its high transparency^{246,269}. All of these properties may contribute to future biosensor based on optical principles. Unfortunately, graphene lacks the diversity of optical response shown by different chiral forms of CNTs as well as the structural rigidity of CNTs, so that nanotubes might prove best for developing intracellular optical sensors in the long term.

Besides that, graphene is still not fully developed in terms of the production methods. Indeed, ever since the first experimental study on graphene⁵⁴, researchers have used traditional microfabrication approaches such as masking, electron-beam lithography, oxygen-plasma dry etching, and metal deposition to fabricate the devices. This can be observed in all graphene fabrication techniques such as mechanically cleaved graphene⁵⁴, epitaxially grown multilayer graphene⁶⁹, and chemical vapor-deposited graphene⁵⁹. This fabrication issue would determine whether it produces single or multiple layers of sheet in this end and contributes significantly to the performance of graphene devices.

9.4. Future Directions

At the end of the day, despite all the promises and potential, both graphene and CNTs actually still face considerable challenges. The most severe drawback for CNTs application is possibly its heterogeneity (about how to separate the semiconducting and metallic tubes). Meanwhile, the production of the two-dimensional nature of graphene

also faces some challenges such as: layer separation method, control of the number of layers, minimization of folding and bending during the process. All of these effects need to be carefully investigated. Although nanocarbon synthesis was not a subject of extensive studies in our project, we suggest that improvements in this area would be a significant issue to be improved in this field of research.

As the next step, a proper functionalization method for the carbon materials is another challenging milestone to be solved. Since manipulation through chemical functionalization would disrupt bonding by converting some atoms into sp^3 -hybridized carbon atoms, typically non covalent functionalization is preferred. One of the methods presented in this thesis was using carbohydrate compounds (chapter 5) to interface cells through carbohydrate-carbohydrate interactions. Indeed, similar alternatives can be applied to interface various types of living cells, making use of various possible carbohydrate-protein or antigen-antibody interaction. By this approach, our simple detection platform is very promising for universal single cells detection techniques, as an alternative tool to several state-of-the-art instruments (including patch-clamp and microfiber electrodes), with great potential for further improvements. For instance, the presented methods for patterning devices (for CNT or graphene) of a controllable size, can potentially be used for cross functionalization with several proteins and interfacing several types of cells at the same time, which would offer a parallel detection of different cell activities simultaneously. The application of molecular imprinting techniques could also be possible to be applied to improve selectivity of the devices.

Despite the promising result demonstrated in this thesis, one of the missing links is the full understanding of the signal transduction mechanisms between the cellular

activities and the nanocarbon material responses, which are actually critical to optimize the sensing capabilities of these devices. Assistance of computer modelling and simulations could be imparted in order to achieve this goal.

As a final remark, although the work presented in this thesis is still more related to demonstration of fundamental research, we find that there is still very spacious room for improvements in this emerging field of nanocarbon based biosensors. We could envision this rapidly evolving technology would bring great contributions and impacts to real industrial applications in various aspects including cell and molecular biology, biomedicine, and environmental studies in the near future.

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Appendix A

Experiment Protocol

A. Substrate cleaning and sterilization

Glass coverslips (12 mm or 25 mm) or silicon wafers were cleaned before they were used for device fabrication or cell related work. Piranha solution, consisting of sulfuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2) in 7:3 composition, was used. Since the piranha cleaning would provide -OH (hydroxyl) functionalization, which might affect the results of cell adhesion on the following experiment, alternatively we could also apply DI water rinsing, followed by ethanol wash and blow dry using nitrogen gas on the purpose of sterilizing the coverslip.

B. Physiological bath solution preparation

The bath solution is a liquid buffer containing several ion ingredients, which can support cell viability during electrical recordings. For the purpose of obtaining better cell images, cell medium (which typically contain phenol red) could be changed to a bath solution, which is transparent. Its composition includes: 10 mM HEPES, 140 mM NaCl, 1 mM MgCl_2 , 5.5 mM KCl, 2 mM CaCl_2 , adjusted to pH 7.2.

C. Cell culture work

In our work, several types of cell lines were cultured. They are maintained at 37 °C in humid atmosphere with 5% CO_2 /95% air.

C1. PC12 cell

PC12 is a cell line derived from a pheochromocytoma of the rat adrenal medulla. It is a widely used cell model to study regulated secretion, a fundamental and dynamic biological process in many cell types underlying, for instances, hormone secretion from endocrine cells and neurotransmitter secretion from neurons. Their medium composition including: advanced RPMI 1640, 10% Fetal Bovine Serum (FBS), 5 % Horse Serum (HS), and 1% Pencillin-Streptomycin.

C2. Smooth Muscle Cell

Smooth muscle cell is an involuntary non-striated muscle, found in internal organ of organism such as bladder, uterus, male and female reproductive tracts, gastrointestinal tract, respiratory tract, large and small arteries and veins (the tunica layer), ciliary muscle, and iris of the eye. This type of cell line were treated in medium composed of Dulbecco's Modified Eagle's Medium (DMEM), 10% Fetal Bovine Serum (FBS), and 1% Pencillin-Streptomycin (PS).

C3. Astrocytes and hippocampal neuron

A neuron is an excitable cell in the nervous system that is responsible in the processes of transmitting information, a phenomenon which is typically triggered by electrochemical signalling. On the other hand, astrocytes are a sub-type of the glial cells in the central nervous system, having a star-shaped form, working as a biochemical and nutrient support for the neurons.

In this work, primary astrocytes and hippocampal neuron cell lines were extracted directly from the brain of neonatal rats by following the protocol described before²⁷⁰⁻²⁷². Neonatal rats of 1~4 days were exposed to ethanol for anesthesia and sterilized. Rats were then decapitated and the brains were removed with a sterile technique. After elimination of all blood vessels and meninges, the brains (cortex part) were fragmented into small pieces and washed three times in $\text{Ca}^{2+}/\text{Mg}^{2+}$ - free Hanks' balanced salt solution (HBSS) (Gibco) with antibiotics. The brain tissues were then resuspended in HBSS mixed with 0.125% trypsin for digestion, and shaken for 15 minutes at 37°C. The undigested brain tissues were filtered out. After 5 min centrifugation (1200 rpm), the cells were resuspended in the culture medium (Dulbecco's Modified Eagle's Medium + 10% Fetal Bovine Serum + 1% Pencillin-Streptomycin) to a density of $\sim 1.0 \times 10^6$ cells/ml. Cells were subsequently plated in petri dishes for 1.5 hour to allow settle-down of the fibroblast cells. The suspension was transferred to 25 cm² flasks and maintained at 37°C in a humid atmosphere with 5% CO₂ / 95% air. On the 14th day, when cells reach 80~90% confluence, the flask was shaken for 2 hours to remove the microglia. After one hour incubation in the refreshed culture medium, the flask was shaken for 18 hours in 37°C at 260rpm to get rid of the oligodendroglia. Finally, the remaining astrocytes in the flask were trypsinized and replanted on the substrate for experiments.

Extraction of hippocampal neuron is the same with astrocytes (as described above) with little modifications; including: 1.) small portion of the brain (slightly behind the cortex) with grey colour was extracted instead of the cortex. 2.) During the wash and resuspension process after centrifugation: MEM⁺ solution (MEM (Gibco 11095-080), 10% FBS, 1.5 mM HEPES, 1 mM Pyruvate, 1% Penicillin-Streptomycin, and 20 mM

KCl) were used instead of the astrocytes culture medium. 3.) Four hours after plating, media MEM⁺ was removed, and replaced by a medium containing 70% MEM⁺ and 30% NB-B27 (NB (Gibco 21103-049), 2% B27 (Gibco 17504-044), 0.5 mM L-Glutamine, 1% Penicillin-Streptomycin). The combination of both NB and MEM⁺ resulted in high neuron survival rate, low glial cell proliferation, reduced antioxidant level, and also provided relatively pure cultures of hippocampal neurons²⁷¹. 5 μ M Cytosine arabinoside (AraC) (Sigma C1768) was used to arrest proliferation of nonneuronal cells²⁷².

C4. HL-1 cardiomyocytes

Cardiac muscle cell is an involuntary striated muscle found in heart wall, which is also known as cardiac myocytes (or cardiomyocytes). It has structure similar to skeletal muscles. However, it works involuntarily just like the smooth muscle cells. This is the reason that cardiac cells are often considered as an intermediate type between smooth muscle and skeletal muscle cells. HL-1 cardiomyocytes were treated with medium consist of Claycomb medium (Sigma 51800C), 10 % Fetal bovine serum, Penicillin/Streptomycin (100U/mL: 100 g/mL), 0.1 mM Norepinephrine, and 2mM L-Glutamine. This medium bottle should be wrapped in aluminum foil, since the medium is extremely light sensitive.

D. Escherichia coli Bacteria Preparation and Counting

Escherichia coli bacteria (strain DH5 α) stock were prepared and employed for the bio-detection experiments. Initially, bacteria were cultured inside the LB medium and shaken overnight to enhance growth and proliferation to reach confluence. The number of

bacteria inside the stock solution was then counted by growing on an LB agar medium plate. The agar was prepared by mixing 800ml H₂O, 10g Bacto-tryptone, 5 grams yeast extract, and 10 grams NaCl followed by pH adjustment to 7.5. Subsequently, 15 grams of agar solution was added and it was melted into solution in the microwave. Volume of the LB agar medium solution was added to 1 L with distilled water, followed by autoclave sterilizing. The bacteria stock solution was added (with a series of dilution factors) to several agar plates and incubated at 37 °C overnight. Finally, the number of bacteria colonies formed on the next day was counted.

E. CNT device fabrication

The CNT-net fabrication was done using three different techniques: dropcasting, film transfer, and evaporation methods. Before the device fabrication, carboxylated SWNTs (Carbon Solutions) were sonicated and dispersed in DI water (concentration 0.1 or 0.2 mg/mL). All samples were cleaned with piranha or ethanol (as described in section A). After film deposition, two electrodes (source and drain) were then prepared across the SWNT-net using silver conductive paint (RS Component). Finally, silicon rubber (Dow Corning) was used to insulate the electrodes and define the chamber for cell culturing and recording.

E1. Dropcasting Method

Figure 44. Device fabrication via dropcasting method

The CNT solution (concentration 0.01 - 0.02 mg/mL dispersed in DI water) was centrifuged at a speed of 18,000 rpm for 30 minutes and the sediments were removed. The solution was filtered afterwards. A small drop of 5 or 10 μL was cast on the cleaned glass sample and evaporated in the oven at 60 $^{\circ}\text{C}$.

E2. Film Transfer Method

Figure 45 . Device fabrication via film transfer method¹⁵³.

The CNT solution was centrifuged at 18,000 rpm for 30 min and sediments were removed. The solution was filtered afterwards. This solution (diluted to concentration 0.05 mg/mL) was taken in a petri dish and pentane was added to form an organic layer above the SWNT suspension, followed by injection of ethanol into the aqueous layer. The top layer of pentane was allowed to evaporate overnight at room temperature and a uniform SWNT film was found floating on the aqueous layer after evaporation. The CNT film was then transfer-printed onto a glass cover slip using a wire probe followed by rinsing with DI water. Finally, the samples were hard baked in a vacuum oven at 130 °C for 2 hours.

E3. Evaporation Method

Figure 46. Device fabrication via evaporation method

The CNT solution was centrifuged at 18,000 rpm for 30 minutes. Sediments were removed followed by further filtration. The CNT (concentration 0.001 mg/ml) solution was added to multi well plate. Cleaned and square 4 glass cover slips were carefully

placed in the well perpendicular to the plate with the sidewall support (Figure 46). The solution was allowed to evaporate in an oven at 60 °C overnight. Patterns like bands of CNT were formed on the cover slips due to evaporation of solution and change in liquid meniscus. Sample were removed the next day, washed with DI water and dried at 60 °C in oven. Patterns of CNT can be created on the substrate and some alignment of CNT is demanded in this case.

F. Reduced graphene oxide (rGO) device preparation ²²⁹

F1. Preparation of Graphene Oxide Aqueous Solution

Graphene oxide (GO) sheets were synthesized by a modified Hummer method using natural graphite ²⁵¹. The GO aqueous solution was obtained by dispersing as-prepared GO sheets in water followed by a two-step sonication. Briefly, GO was first sonicated in mild sonication (Branson model 2510, Branson Ultrasonic Corporation, USA) for 60 minutes to get a stable aqueous suspension. Subsequently, a strong sonication (Vibra cell of Sonics & Materials, Inc., USA) was applied to the presonicated GO solution for 10 min (amplitude 50%, 1 s pulse, 1 s stop) to break the big GO sheets. The resulting GO solution was then centrifuged (Kubota Co. 6200) at 1500 rpm for 2 h. The top layer solution was collected in order to remove most of the big GO sheets (>3 µm). Small GO sheets with diameter less than 300 nm were also removed after centrifuging the collected GO solution at 10000 rpm for 2 h. The bottom part of the GO solution was collected and used for GO patterning.

F2. Hard-PDMS Stamps

Hard polydimethylsiloxane (PDMS) stamps were fabricated according to the literature method²⁷³ using a negative silicon master (line patterns with 10 μm width and 5 μm height). Normal PDMS (Sylgard 184, A:B = 1:10, Dow Company) stamps²⁷⁴⁻²⁷⁵ were fabricated using the same silicon master.

F3. Patterning GO by Micromolding in Capillary

Figure 47. Schematic illustration of patterning GO films by MIMIC and reduction of the patterned GO films²²⁹.

The schematic illustration of the patterning method for graphene oxide on a flat substrate by using micromolding in capillary (MIMIC) is shown in figure 47²⁷⁶. The substrates, for example, SiO_2 wafer (Bonda Tech, Singapore) and quartz (SYST

Integration Pte Ltd., Singapore), were first coated with 3-aminopropyltriethoxysilane (APTES, Sigma-Aldrich)^{243,251,275,277}. After the polyethylene terephthalate (PET) film (3M, USA) was immersed into an aqueous solution of 3% APTES for 30 min, followed by an ample rinse with DI water, the APTES self-assembled monolayers (SAMs) were formed on the PET surface. The fabricated PDMS stamp with arrays of line channels (10 μm width, 5 μm height, and 1.5 cm length) was cut at both ends with a sharp knife to generate the open channels. The thus-cut PDMS stamp was treated with O_2 plasma to make the surface hydrophilic before it was placed on the APTES-modified flat substrates (*e.g.*, SiO_2 wafer, quartz, PET film) to form the conformal contact (Figure 47). A minitool (FX-117, minitool. Inc.) was used to apply force on the top of the PDMS stamp to ensure a close contact between the PDMS stamp and the substrate²⁷⁸. A drop of GO aqueous solution (*ca.* 1.5 mg/mL) was then dropped at one end of the open channels of the PDMS stamp (Figure 47). After the whole system was placed in a vacuum oven and degassed for 30 min to ensure the solution dried up²⁷⁸, the force on the PDMS stamp applied by the minitool was released. Then the PDMS stamp was carefully peeled off and the GO patterns were formed on the substrate, which were reduced in hydrazine vapor at 60 $^{\circ}\text{C}$ for 12 h to obtain the reduced GO (rGO) patterns (Figure 47)^{232,243}.

The resulting GO and rGO patterns on substrates were characterized by optical microscopy and atomic force microscopy (AFM). Optical images were recorded by Nikon Eclipse LV100 optical microscopy. AFM images were obtained by using Dimension 3100 (Veeco, CA) in tapping mode with a Si tip (Veeco, resonant frequency, 320 kHz; spring constant, 42 N m^{-1}) under ambient conditions with a scanning rate of 1 Hz and scanning line of 512.

F4. Fabrication of Graphene Sheet Device

Graphene films were grown on a Ni substrate at temperatures up to 1000 °C by chemical vapor deposition of carbon using a mixture of methane and hydrogen ²⁴¹. Graphene films were removed from the Ni substrate by etching in an aqueous solution of hydrochloric acid. First of all, the surface of the graphene on Ni substrate is coated with poly-methyl methacrylate (PMMA) and annealed at 120°C for 20 minutes. Subsequently, the graphene films on Ni substrate were immersed in HCl solution (HCl:H₂O=1:10). After the Ni was etched, the PMMA/Graphene peels off and can float on the surface of water. The film was then picked up, put on quartz substrate, and immersed in acetone in order to remove the PMMA. As the final step, the samples were annealed at 450 °C for 20 minutes (hydrogen/argon atmosphere) to remove the PMMA further. Silver electrodes were patterned on top of the graphene films and the silver/graphene contact was insulated with polydimethylsiloxane (PDMS).

G. CNTs and Graphene-FET Device Characterization

The source-drain current under a voltage bias of 400 mV was measured while varying the solution-gate voltage (typically we measure from -1 V to 1 V). The gating voltage was applied via a Ag/AgCl electrode immersed in the physiological bath solution which incubated the SWNTs and contained (in mM, titrated to pH 7.2): 10 HEPES, 140 NaCl, 1 MgCl₂, 5.5 KCl, and 2 CaCl₂ (as described in the section B).

The gate voltage can also be applied as backgate voltage, when silicon substrate is used instead of glass coverslips in the chip fabrication. The gate voltage was typically swept in a higher voltage range (from -10 to 10 V) in this case.

H. CNTs and Graphene-FET Device Sterilization and Electrical Recording

Before cell culturing on the nanocarbon sensor surfaces, the substrates have to be cleaned and coated with biocompatible layers. For sterilization, the sensor networks were immersed in 70% alcohol for 10 minutes. This was then followed by intensive rinsing with double distilled water. An intensive rinsing step is significant for the sensor to be maintained in optimal culture conditions.

The electrical recording of cells was conducted after cells were cultured for several days in a humid atmosphere with 5% CO₂/95% air. The cell medium was replaced by physiological bath solution (exactly the same to those used during device characterization), before electrical recordings were started. Micropipette used in the experiment can be produced using Narshige PC-10 glass cappilaries puller or Sutter instrument CO Model P-2000.

Electrical recording for device characterization and cell signal were done using Semiconductor Device Analyzer (Agilent Technologies B1500A and Axon Instruments Multiclamp 700B Digidata 1440A).

Appendix B

List of Publications

1. **Sudibya HG**, He Q, Zhang H, Chen P, *Electrical Detection of Metal Ions Using Field-Effect Transistors Based on Micropatterned Reduced Graphene Oxide Films*, **ACS Nano**, 5(3):1990-1994, 2011.
2. He Q, **Sudibya HG**, Yin Z, Wu S, Li H, Boey F, Huang W, Chen P, Zhang H, *Centimeter long and large-scale micropatterns of reduced graphene oxide films: fabrication and application in biosensing*, **ACS Nano**, 4(6):3201-3208, 2010
3. Pui TS, **Sudibya HG**, Luan X, Zhang Q, Ye F, Huang Y, Chen P, *Non-invasive detection of cellular bioelectricity based on carbon nanotube devices for high-throughput drug screening*, **Advanced Materials**, in press
4. **Sudibya HG**, Ma J, Dong X, Ng S, Li LJ, Liu X, Chen P, *Interfacing Glycosylated Carbon Nanotube Network Devices with Living Cells to Detect Dynamic Secretion of Biomolecules*, **Angewandte Chemie International Edition**, 48:2723-2726, 2009
5. Huang Y, **Sudibya HG**, Fu D, Xue R, Dong X, Li LJ, and Chen P. *Label-free detection of ATP release from living astrocytes with high temporal resolution using carbon nanotube network*. **Biosensors and Bioelectronics**, 24(8):2716-2720, 2009

6. Li W, Lee CW, Li LJ, **Sudibya HG**, Wang B, Chen LQ, Chen P, Yang YH, Chan-Park MB, Chen Y. *Assessment of (n,m) Selectively Enriched Small Diameter Single-Walled Carbon Nanotubes by Density Differentiation from Cobalt-Incorporated MCM-41 for Macroelectronics. **Chemistry of Material**, 20:7417-7424, 2008*
7. **Sudibya HG**, Huang Y, Chen P, *Detecting Metabolic Activities of Bacteria Using a Simple Carbon Nanotube Device for High-throughput Screening of Anti-bacterial Drugs. (in press)*