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Terse, Trupti Madhukar

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UNIVERSITY OF CALIFORNIA
RIVERSIDE

Advanced Graphene Nanostructures for Biosensing Applications

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Bioengineering

by

Trupti Madhukar Terse

December 2016

Dissertation Committee:

Dr. Ashok Mulchandani, Chairperson

Dr. Quan Jason Cheng

Dr. Jiayu Liao

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The Dissertation of Trupti Madhukar Terse is approved:

Committee Chairperson

University of California, Riverside

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Dedication

To My Beloved Family

ABSTRACT OF THE DISSERTATION

Advanced Graphene Nanostructures in Biosensing Applications

By

Trupti Madhukar Terse

Doctor of Philosophy, Graduate Program in Bioengineering

University of California, Riverside, December 2016

Dr. Ashok Mulchandani, Chairperson

Graphene is a sp^2 hybridized carbon, arranged perfectly in a honeycomb structure to form a 2D monolayer of graphitic structure. It has become a popular choice in the development of electrochemical/electrical biosensor devices due to its large surface area, faster electron transfer kinetics, tunable band gap and ultrahigh charge carrier mobility with ballistic electron transport. The goal of this work is to synthesize advanced graphene nanostructures with improved electrical and physiochemical properties suitable for the development of ultrasensitive electrical/electrochemical biosensors. In the first project, seamless graphene-carbon nanotubes (G-CNT) hybrid film was synthesized using a two-step chemical vapor deposition (CVD) method where carbon nanotubes (CNTs) are grown on already grown graphene film on copper foil using iron as a catalyst. This 3D G-CNT hybrid film has been studied for its potential in achieving direct electron transfer (DET) of glucose oxidase (GOx) and its bioelectrocatalytic activity in glucose detection. The constructed electrode detected glucose concentration over the clinically relevant range with the highest sensitivity compared to reported composite hybrid electrodes of graphene oxide and CNTs.

G-CNT structure used in this work has potential to be used for development of artificial mediatorless redox enzymatic biosensors and biofuel cell. In the second project, an electrical transduction based biosensor platform for detection of biomolecular interaction using a graphene nanogap electrode has been developed. Different nanofabrication methods including focused ion beam milling (FIB), nanoidentation and e-beam lithography (EBL) were studied in achieving reproducible planar nanogap electrodes of width less than 100 nm. Electrical biosensing concept was tested on nanogap electrodes using a high affinity interaction of streptavidin- biotin. Sensor performance was further optimized for achieving the high sensitive detection of streptavidin. The detection capability of this biosensor can be tuned down to a single to few molecules. Proposed biosensor platform can be used for any detection based on biomolecules affinity interaction such as for antigen-antibody or chemo-selective interaction with full potential to be used as a portable point-of-use biosensor.

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Chapter 1

Introduction

1.1 INTRODUCTION

Biosensor technology has truly revolutionized the quality of human life by providing selective, sensitive, and rapid (point-of-care) detection tools for disease diagnosis and monitoring. Whether it be detection of disease specific small molecule metabolites like glucose, protein biomarkers, DNA or pathogenic microorganisms, biosensors are being increasingly used in developing advanced detection techniques in healthcare and medicine.

A biosensor can be broadly defined as a device which can detect presence of chemical or biological molecules like enzymes, proteins, nucleic acid or microorganisms, using specific biorecognition elements like receptors, antibodies, enzymes, substrate etc. The information about the specific binding of analyte to biorecognition element is converted into a measurable output as a signal using a transducer. Depending upon the type of transducer, biosensors can be categorized as electrochemical, optical, thermal, piezoelectric etc.

Amongst these, electrochemical/electrical techniques are promising technologies due to their simplicity, high sensitivity, specificity and potential to integrate into miniaturized devices for point-of care applications. In such biosensors, transducer element converts the biological event such as affinity capture or biocatalytical conversion corresponding to concentration of analyte into an electrical signal such as current or voltage, based on either amperometric, potentiometric, conductometric or field effect

transistor principles. Figure 1 shows the basic design of electrochemical/electrical biosensor.

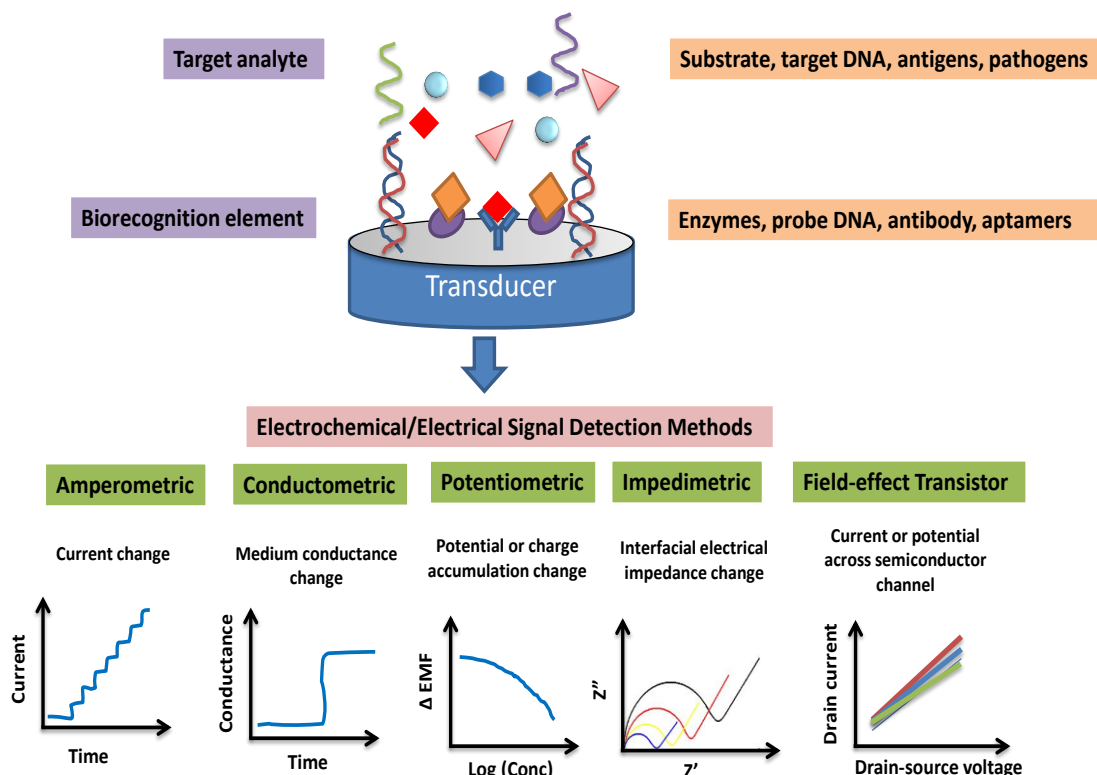


Figure 1.1: Schematic of electrochemical/electrical biosensor design

1.1.1 Graphene and advanced graphene nanostructures in electrochemical biosensing

With the discovery of carbon nanomaterials and advancement in nanofabrication methods and tools, carbon nanotubes (CNTs) and graphene have been extensively explored as transducer elements in the development of electrochemical biosensor devices. Graphene, a two dimensional flat monolayer of sp^2 hybrid carbon atoms arranged in honeycomb lattices, in particular has become a popular choice in the development of electrochemical biosensor devices due to its excellent electrical, chemical and material properties which include large surface area, charge carrier concentrations and exceptional electron mobility

in excess of $100,000 \text{ cm}^2/\text{V}\cdot\text{s}$) (Bolotin et al., 2008; Novoselov et al., 2005; Novoselov et al., 2004; Zhang et al., 2005) with ballistic electron transport. Moreover, the reactivity of graphene edges and functional groups introduced in the basal plane due to its synthesis procedure for e.g. oxygen containing groups introduced during graphene oxide synthesis by Hummer's method are crucial in electrochemical reactions and its functionalization with biorecognition elements.

However, the superior properties of the graphene structure only emerge in the 2D planar direction, limiting its performance in biosensing application. New efforts in graphene research have attempted to address this weakness by developing structures wherein graphene acts as a platform for support, scaffold, or a 2D planar substrate for anchoring other nanomaterials. For example, carbon nanotubes (CNTs), whose properties emerge in the axial direction, can be functionalized onto the surface of graphene, combining the properties of the two carbon allotropes in all directions while allowing for an increased active surface area and faster electron transfer kinetics. Similarly, combining metal/metal oxide nanoparticles (NPs) with graphene overcomes NP shortcomings of low stability and tendency to aggregate resulting in highly stable electrochemical sensing platforms for anchoring with good dispersion, excellent conductivity and catalytic properties of NPs. This transformation of 2D graphene into three-dimensional (3D) architectures expands the functionality of nanomaterials and serves as novel hybrid electrode material for applications in sensing (Dong et al., 2012b; Hwa and Subramani, 2014) as well as in electronics (Kim et al., 2014a), energy storage (Prasad et al., 2014).

Along with synthesizing graphene hybrid structures, synthesizing and patterning of graphene into advanced geometries can tune the properties of graphene opening new avenues for electrical biosensor development. **Graphene nanoribbons (GNRs)**, which are thin strips of graphene of width of 100 nm or less, can be simply visualized as single or multiwall CNTs cut along its parallel axis and unfolded to form planar structure of width corresponding to diameter of the CNTs. GNRs can be synthesized by chemical (Yang et al., 2008) or lithographical methods (Cai et al., 2010; Han et al., 2007). GNRs produced through lithographic methods have oxidized/rough edges due to oxygen plasma etching used in their fabrication. Compared to graphene and CNTs, high surface area, edge to basal plane ratio, electroactive defects and edge chemistry/functional groups of GNRs are advantageous in electrochemical biosensing.

GNRs are also promising structures as a semiconductor gate/channel material in field effect transistor (FET) based biosensors. The graphene has a zero band gap which limits their sensitivity and limit of detection in FET biosensing (Sarkar et al., 2014). In GNRs, the band gap opening is possible by the electronic confinement of the charge carriers in quasi-one dimension due to their narrower widths. Another advanced graphene nanostructure, **graphene nanowalls**, which are also called as carbon nanowalls, are mainly multiple graphene sheets assembled or *in situ* grown in vertical or parallel direction with exposed sharp edges. The vertical graphene nanowalls structures are grown *in situ* by various PECVD methods using microwave plasma (Shang et al., 2008; Suzuki et al., 2011; Tanaka et al., 2005; Wu et al., 2002), radio frequency (rF) (Davami et al., 2014), inductively coupled plasma (ICP) (Jain et al., 2011), radical injection (Kondo et al., 2008),

or e-beam excited, conductively coupled (Hiramatsu et al., 2004), on different substrates such as glass (Kim et al., 2014b), SiO₂ (Tanaka et al., 2005), sapphire, Al₂O₃ (Fang et al., 2014) , NiFe (Wu et al., 2002) ,epitaxial growth on SiC (Kumar Roy et al., 2015) etc., without need of additional catalyst. Whereas parallel graphene nanowalls or layered GO sheets can be synthesized by electrophoretic deposition (EPD) of GO from an electrolyte solution on graphite electrode (Akhavan et al., 2014; Akhavan et al., 2012) or by simple ultrasonication assisted intercalation methods (Yang et al., 2013). These graphene nanowalls have a large specific area with higher conductivity and electroactivity, due to its increased edge plane sites, are suitable for development of electrochemical biosensors. **Graphene nanogaps and nanopore structures** have been theoretically proposed for individual DNA sequencing due to graphene's thinnest and ion permeable structure.

1.2 OBJECTIVE OF THE WORK

Based on aforementioned discussion on important properties of graphene advanced structures in the form of hybrid as well as patterned graphene in biosensing, the objective of this dissertation is divided in two major goals.

1. Synthesis of seamless three-dimensional graphene-carbon nanotube hybrid and its application in direct electron transfer based electrochemical sensors

Enzyme based electrochemical biosensors have been widely used in the areas of health care mainly in disease diagnosis and monitoring. Among them, glucose biosensors dominate by accounting for approximately 85% of the world biosensor market. Extensive research is still ongoing in this area for the development of advance generation of electrochemical biosensors with simplicity, portability, higher sensitivity and selectivity.

Direct electron transfer (DET) is one of the promising approach mainly in the development of electrochemical enzyme based biosensors where there is direct electrical communication between the enzyme and the electrode surface. No chemical mediator is required for the electron transfer therefore there is no mediator associated toxicity. Biosensor based on DET will likely to have higher sensitivity and selectivity. However, the achievement of DET is challenging due to the buried location of redox center in the enzyme. Many researchers are trying to genetically engineer the location of the redox center to facilitate DET and its effect on the activity of the enzymes. There has been a parallel research in the development of nanostructure modified electrode in achieving DET. Different carbon nanostructures like graphene (Kang et al., 2009; Liang et al., 2013; Shan et al., 2009; Wu et al., 2010), graphite nanosheets (Fu et al., 2009), graphene oxide, multiwalled (Deng et al., 2010; Janegitz et al., 2011) and single walled carbon nanotubes (Guisseppi-Elie et al., 2002), as such or modified with metal nanoparticles have been explored in achieving DET based glucose biosensor. Graphene and CNT based electrodes are mostly planar in nature with electrical properties emerging only in 2D and 1D directions, respectively, only. Also they have poor electrical contacts and the aggregation of individual components on the electrode affect the electrical conductivity and overall effective surface area.

Our goal for this work is to synthesize a three dimensional carbon nanostructure of graphene and carbon nanotube using chemical vapor deposition (CVD) method. The hypothesis is that such a tructure will have improved electrochemically active surface area, combined properties of graphene and CNTs, ideal for efficient enzyme immobilization and can form conductive network around an enzyme molecule which can result in direct

electron transfer across enzyme redox center and electrode. Currently used three dimensional carbon nanostructures are mainly composite films of graphene oxide and carbon nanotubes in which graphene oxide has a high tendency to form aggregates and also there is no direct electrical contact of CNTs to the graphene.

We would study our G-CNT structures in DET of glucose oxidase (a widely used enzyme in the development of glucose biosensor) as well as heme peptide and horseradish peroxidase in amperometric sensing.

1) Fabrication of graphene nanogap electrode and development of electric transduction based biosensor

Nanogap electrodes have emerged as a powerful method in the development of biosensors for detecting biomolecular interactions occurring in the nanometer dimensions. This indicates their ability to measure very small quantities of biomolecules. Nano sized biomolecules can be trapped into a gap between two electrodes and connecting the electrodes. In this sensor, detection is based on their electrical behavior (resistance/impedance, capacitance/dielectric, or field-effect). Electrical biosensors are capable of behaving at high performance with a simple miniaturized readout due to excellent compatibility with advanced semiconductor technology, miniaturization, and low cost.

Gap based biosensing using gold nanoparticle (AuNP) labelled analyte has been reported in 2002 (Park et al., 2002b) where capture of AuNP in the micron size gap due to target probe binding of DNA, does not show significant change in conductance without silver enhancement step. Nanogap electrodes (gold electrode with nanogap) of gap size of 30-90 nm avoids the silver enhancement step in the biosensing, but does not report

sensitivity and LOD data and also do not establish co-relation between gap size ,gold nanoparticle diameter and protein/analyte coverage on the AuNP. We hypothesized that nanogap fabrication in the structure such as graphene, an atomically thin material, would be easier compared to CNTs or metal. Also monolayer graphene electrode thickness is around 0.33 nm compared to metal electrode (Au/Cr or Au/Ti) whose minimum thickness reported is about 20 to 120 nm (Blom et al., 2007; Haguët et al., 2004; Marcon et al., 2008a; Marcon et al., 2008b). Atomically thin electrode material provides more flexibility with use of gold nanoparticle of larger diameter and its bispecific capture in the gap, in turn improving the LOD of the sensing.

The major goal of this work is to fabricate the planar nanogap electrode of gap size less than 100 nm using a reproducible and reliable method and a highly conductive graphene as an electrode material. Also to develop and perform analytical characterization of highly sensitive biosensor based on simple electrical conductivity transduction using novel graphene nanogap electrodes.

1.3 ORGANIZATION OF THE DISSERTATION

Chapter 2 describes the synthesis and characterization of graphene-carbon nanotube hybrid. Chapter 3 presents the studies of direct electron transfer (DET) of glucose oxidase at graphene-carbon nanotube hybrid and its bioelectrocatalysis. Chapter 4 reports the nanofabrication methods used in graphene nanogap electrodes fabrication. Chapter 5 describes the development and analytical characterization of an electrical biosensor using graphene nanogap electrodes optimization of biosensor performance. Chapter 6 gives the concluding summary of the work done. Appendix section includes the DET studies done

on heme peptide and horseradish peroxidases immobilized on graphene-carbon nanotube hybrid and hydrogen peroxide sensing.

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Chapter 2

Synthesis of Graphene-Carbon Nanotube Hybrid by Two Step Chemical Vapor Deposition

Three-dimensional seamless graphene-carbon nanotubes (G-CNT) hybrid film was synthesized by using a two-step chemical vapor deposition (CVD) method. First, a graphene film was grown on copper foil followed by CNT on the surface of graphene using iron as catalyst. Compared to assembled G-CNT hybrids, CVD growth of hybrid ensures ohmic contact between graphene and CNTs also it involves less processing steps and guarantee more uniform distributed growth of CNTs on the graphene. Two-step method of growing G-CNT was found to be more reproducible with smaller diameter MWCNTs than one-step method where graphene and CNT are grown together in one CVD step. Material characterization of G-CNT film was carried out using scanning electron microscope (SEM), transmission electron microscope (TEM), X-ray photoelectron spectroscopy (XPS) and Raman spectroscopy. Microscopic characterization shows uniform dense coverage of multiwall carbon nanotubes (MWCNT) grown directly on graphene with seamless contacts. Electrochemical characterization of G-CNT was carried out using electrochemical impedance spectroscopy (EIS), cyclic voltammetry. This hybrid film is promising nanomaterial in the areas of electrochemical electrodes in biosensing, flexible electronics, and energy storage/conversion.

2.1. INTRODUCTION

Graphene is a two dimensional flat monolayer of sp^2 hybrid carbon atoms in a honeycomb lattice. It has gained a wide attention in the development of enzymatic electrochemical biosensors due to its high surface area, superior electron mobility, high thermal conductivity, mechanical stability, and biocompatibility. Since its discovery in 2004 by Geim and Novoselov, graphene has been synthesized by various methods, e.g. starting from mechanical exfoliation of graphite, thermal decomposition of silicon carbide wafers under ultrahigh vacuum, chemical oxidation of graphite to form graphite oxide (GO) and subsequent chemical or thermal reduction, and chemical vapor deposition (CVD) method using metal substrates. Among these, CVD has been recognized as one of the promising methods for manufacturing of large area single layer or multilayer graphene (Miao et al., 2011).

Similarly, carbon nanotubes, both single walled and multi-walled, have been extensively studied and proven to be promising material in enzyme based electrochemical biosensing. These rolled sheets of graphene, due to their large length to diameter aspect ratio, provide high surface to volume ratio. CNTs also possess superior electronic, physicochemical, and electrochemical properties. Functionalized CNTs can be used for immobilization of various bio recognition element like enzymes, antibodies etc.

However, these properties only emerge in the 2D planar direction of the graphene structure, limiting its scope and application. New efforts in graphene research have attempted in developing structures wherein graphene acts as a platform for anchoring other nanomaterials. For example, CNTs whose properties emerge in 1D direction, can be

functionalized onto the surface of graphene resulting in the hybrid structures, combining the properties of the both the carbon allotropes in all directions while allowing for an increased active surface area and faster electron transfer kinetics. Similarly, combining metal/metal oxide nanoparticles (NPs) with graphene improves electroactive surface areas also improving the stability of NPs and resulting in highly stable electrochemical sensing platforms. This three-dimensional (3D) architectures expands the functionality of nanomaterials and serves as novel hybrid electrode material for applications in electronics (Kim et al., 2014a), energy storage (Prasad et al., 2014), and sensing (Dong et al., 2012b; Hwa and Subramani, 2014).

These hybrid structures of graphene and CNTs can be grown by either assembly method or in-situ growth method (Badhulika et al., 2015). In assembly method, graphene/graphene oxide and CNTs are physically mixed/dispersed or chemically or layer by layer assembled structures. Assembly processes are generally simpler than in situ fabrication, but produce lower quality materials with undesired phenomenon like aggregation, poor contact, residues, and non-uniformity of the films. On the other hand in situ growth of graphene hybrids provides better fabrication control in terms of morphology, density, and orientation of the hybrid structures with Ohmic contact of CNTs or SNMs with graphene resulting in high conductivity architectures. When in in-situ methods, growth of graphene and CNTs is carried out simultaneously in a single CVD step, these hybrids are termed here as one-step G-CNT hybrids (OS hybrids). In this method, catalysts for CNTs growth, such as Fe and Ni, are deposited onto the growth substrate of graphene such as copper foil (Ghazinejad et al., 2013; Paul et al., 2010), nickel foam (Xiaochen et

al., 2012) and MgO (Zhu et al., 2012a) by thin film evaporation methods or by immersing in metal salt solution. The catalyst coated substrate is subjected to CVD growth using a carbon source gas for simultaneous growth of graphene and CNTs. Lamella-like mixed catalyst, Fe/MgO and MgO were prepared using the hydrothermal method and subjected to CVD for the growth of G-CNT hybrid (Zhu et al., 2012a). MgO catalyzed the growth of graphene and the Fe/MgO layer catalyzed growth of CNTs.

Depending on the metal catalyst (CNT growth) and metal substrate (graphene growth) interaction, it may be necessary to use a two-step growth method. Specifically in the case of iron catalyst on copper foil it has been reported that CNT growth was difficult due to hindrance of the active catalyst surface by possible formation of Fe/Cu alloy (Duc Dung et al., 2012). Two step hybrids with single to few walled CNTs have been shown to seamlessly connect with graphene (Zhu et al., 2012b). In this method first Fe film catalyst (1 nm) and then alumina layer (3 nm) was deposited on the CVD grown graphene/copper foil. The metal coated graphene/copper foil was again subjected to CVD for CNT growth. Alumina layer acted as the floating buffer layer which helped in covalent binding of CNTs to graphene as well as in controlling the diameter of CNTs.

In this study, we report optimization of synthesis of G-CNT hybrid using a two-step CVD method on copper substrate. Detailed material and electrochemical characterization of the material was carried out and compared with one-step G-CNT hybrid. We also report G-CNT hybrid synthesis on substrates like Indium tin oxide (ITO) and Silicon dioxide (SiO_2). Further we also studied the metal decoration on the G-CNT structures in achieving different geometries.

2.2. EXPERIMENTAL

2.2.1 Instruments

All electrochemical experiments were performed with a CHI 750C (CH instruments Inc., USA) using a three electrode system. Bare or modified glassy carbon electrode (GCE, area 0.07 cm^2) was used as a working electrode. Ag/AgCl with 3 M KCl solution and platinum wire were used as the reference and counter electrode, respectively. Electrochemical impedance spectroscopy (EIS) measurements were performed in 0.05 M $\text{K}_3\text{Fe}(\text{CN})_6$ containing 0.1 M KCl with a frequency ranging from 100 kHz to 0.1 Hz. Scanning electron microscopy (SEM) images were obtained on a XL30 FEG system after transferring G-CNT film to the SiO_2/Si substrate. Transmission Electron Microscopy (TEM) images were taken using PHILIPS TECNAI 12 Transmission Electron Microscope after transferring the G-CNT film on the 400 nm lacey carbon TEM grid. Raman spectra were recorded using Horiba LabRam/ AIST-NT AFM microscope with laser of $\lambda_{\text{EX}} = 532\text{ nm}$ and power of 5mW. X-ray photoelectron spectroscopy (XPS) characterization was performed by using a Kratos AXIS ULTRADLD XPS system equipped with an Al $\text{K}\alpha$ monochromated X-ray source and a 165-mm mean radius electron energy hemispherical analyzer. The binding energy was calibrated by referencing the Au 4f_{7/2} peak to 84.0 eV. XPS samples were prepared by transferring G-CNT film on gold (180 nm) coated SiO_2/Si chips. E-beam evaporator (Temescal, BJD-1800) was used for deposition of iron on the copper foil/graphene. Tube furnace (Linderberg/Blue, Mini-Mite TM by Thermo scientific) with mass flow controller (MFC) was used for chemical vapor deposition growth of carbon nanostructures.

2.2.2 Synthesis of graphene-carbon nanotube hybrid film

Graphene-carbon nanotubes hybrid film was synthesized by the method reported previously. In brief, first a graphene film was grown by chemical vapor deposition (CVD) method using a copper foil (99.99% purity, 25 μm thickness and 2.54 cm^2) as a growth substrate. Initially, the copper foil was cleaned in 5% acetic acid, deionized water, acetic acid and isopropyl alcohol respectively for 10 min. each to remove oxide and other impurities. The copper foil was dried by blowing nitrogen gas, placed inside a fused silica tube (5 cm inside diameter $\times$ 100 cm long) and the temperature of the tube furnace was raised to 1030 $^{\circ}\text{C}$, under flowing mixture of Ar (190 sccm) and H_2 (5 sccm) at the atmospheric pressure. After annealing at 1030 $^{\circ}\text{C}$ for 30 min, CH_4 (5 sccm) was supplied for 7-10 min. Once the growth process was complete, CH_4 flow was stopped and the furnace cooled to room temperature under the flow of Ar/ H_2 .

A 1.5 - 2.0 nm thick iron film was deposited using E-beam evaporator on the graphene film grown on the copper foil for growing the carbon nanotubes. A similar setup was used as described for the graphene growth. To grow carbon nanotubes, the substrate (Cu-graphene-Fe) was placed in the tube furnace, heated to 750 $^{\circ}\text{C}$ under the flow of Ar (100 sccm) and H_2 (50 sccm) and annealed for 3 min. C_2H_2 (15 sccm) was supplied for 5 - 10 min together with Ar/ H_2 . Once the growth process was complete, C_2H_2 flow was stopped by turning off the respective MFC, followed by cooling to room temperature under the flow of Ar/ H_2 (Figure 2.1).

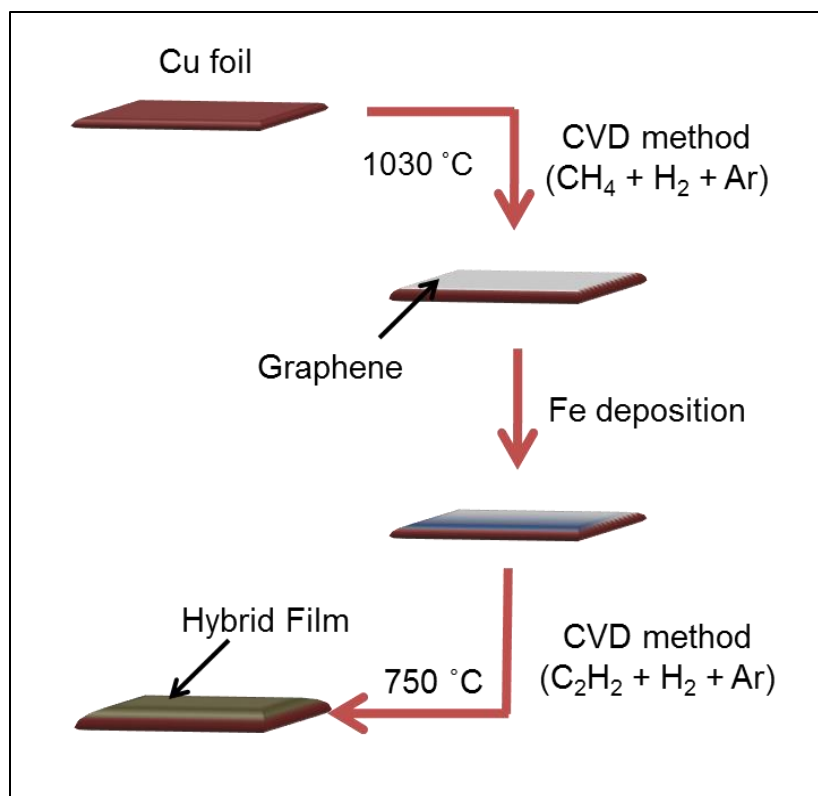


Figure 2.1 Schematic of two-step CVD growth of G-CNT hybrid

The backside of the cooled substrate with only the graphene layer was etched with reactive ion etching (RIE) using oxygen plasma, followed by etching of copper by a 0.3 M FeCl₃ solution. The graphene-carbon nanotube (G-CNT) hybrid film floating on the ferric chloride solution was further cleaned with aqueous HCl (5%) and deionized water to make sure no copper or ferric chloride traces were left (Figure 2.2B). For one-step G-CNT hybrid, iron film was directly deposited on the copper foil and 2nd CVD step is directly carried out. Post-processing steps are same as that of two-step hybrid.

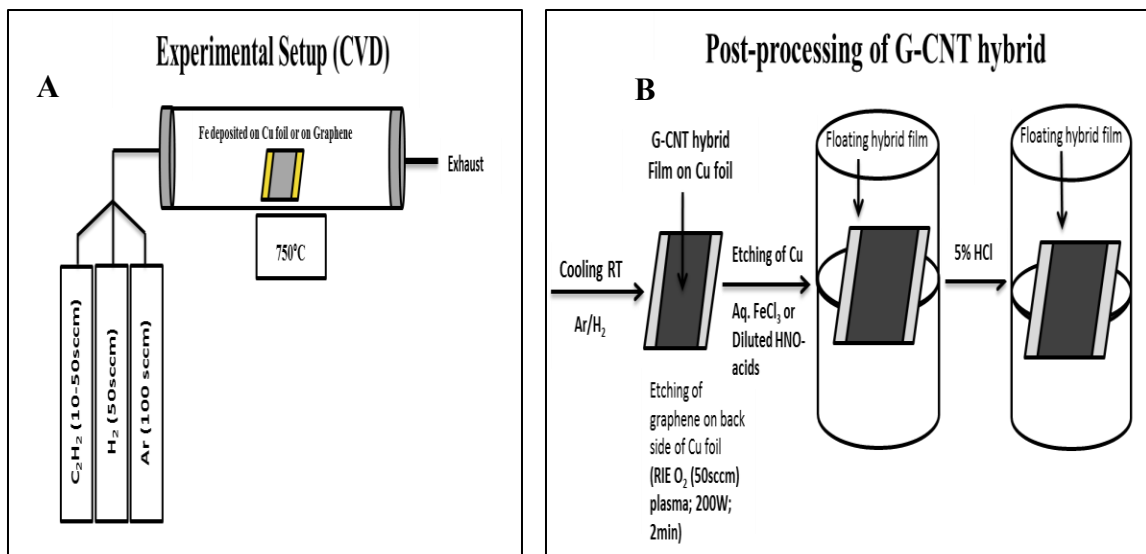


Figure 2.2 A) Schematic of experimental CVD set up and B) post processing of G-CNT hybrid

2.2.3 Preparation of the electrode

A glassy carbon electrode (GCE) of 3 mm diameter was polished first with an emery paper then with aqueous slurries of alumina solution of $0.3\ \mu m$ and $0.05\ \mu m$, respectively. The electrodes were then rinsed with water followed by sonication in deionized water and isopropyl alcohol respectively for 1 min each and was dried by a nitrogen stream.

The G-CNT film was transferred to the GCE by simple contact lifting and allowed to air dry followed by oven drying at $50\ ^\circ C$ for 1 h. The G-CNT/GCE was then incubated in 10% v/v aqueous hydrochloric acid for 30 min to remove iron nanoparticles followed by thorough washing with D.I. water.

2.3. RESULTS & DISCUSSION

2.3.1 Characterization of two-step G-CNT hybrid film (TS hybrid)

SEM and TEM were used to probe the CVD grown G-CNT hybrid film. Similar to previous study, the SEM image (Figure 2.3A) showed a uniformly distributed dense coverage of carbon nanotubes in network-like fashion on the surface of graphene with an average

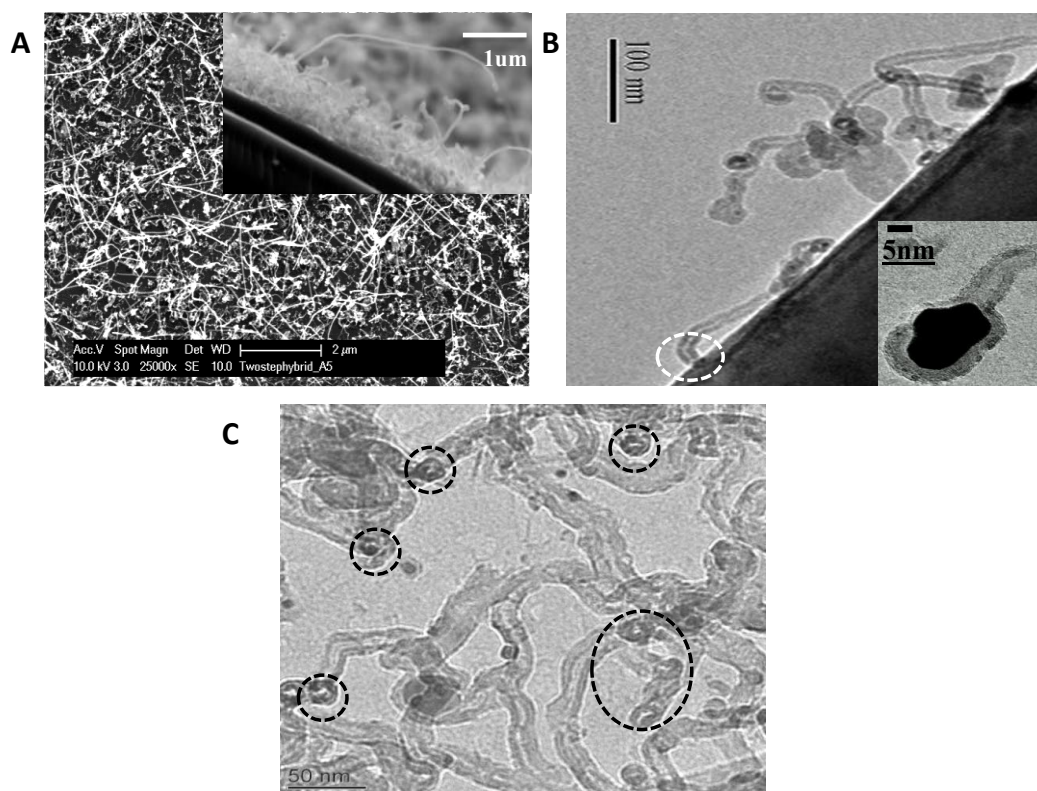


Figure 2.3. (A) SEM images of G-CNT film. Inset shows the cross-section of G-CNT film. (B) TEM image of G-CNT film showing a folded edge with CNTs growing directly on graphene floor. Inset shows TEM image of G-CNT film showing the tip of the multiwall carbon nanotube with iron catalyst particle. (C) TEM image of bottom view of G-CNTs hybrid film showing contact between individual CNTs root (circles) and the graphene surface.

diameter of ~ 30 nm. Cross-section SEM image of the G-CNT film (Inset of Figure 2.3A) showed CNTs growing out of the graphene plane and as they grew longer falling on neighboring CNTs to form the network-like morphology. TEM images of the cross-section

(Figure 2.3B) and graphene side (Figure 2.3C) illustrated the CNTs to be multiwalled (see inset in Figure 2.3B) and growing seamlessly on the still intact graphene floor (the contact points are circled). This differentiated the structure of G-CNT hybrid from composite films of GO and CNTs where CNTs are instead lying on the graphene surface. Diameter of CNTs in TS hybrid is much smaller than one step hybrid. It could be because of iron-copper interaction. Contact angle of iron nanoparticles on the surface of graphene coated copper foil should be much smaller than iron nanoparticles directly on copper foil.

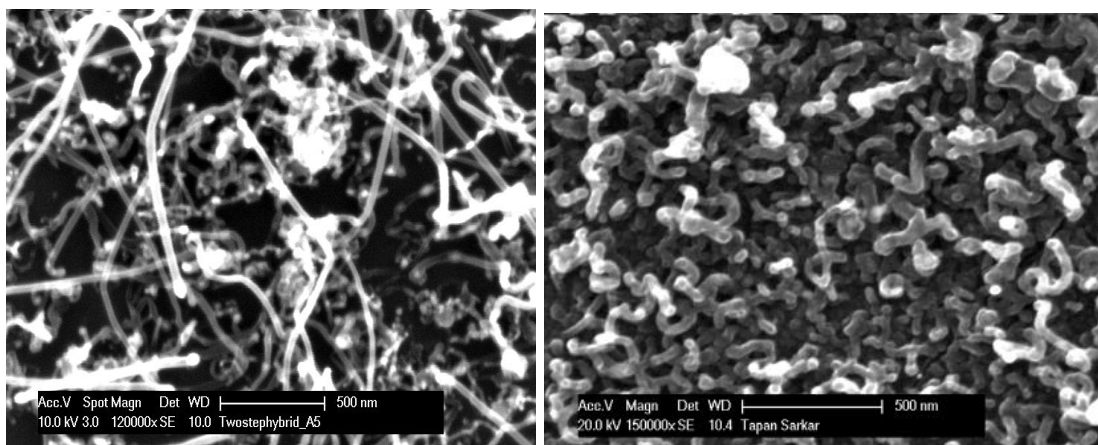


Figure 2.4 SEM images of A) Two-step hybrid B) one step hybrid

The Raman spectra of various carbon nanostructures synthesized in this work - graphene synthesized after the first CVD, G-CNT synthesized after the second CVD and graphene surrounding the CNTs after the second CVD- are exhibited in Figure 2.5. An analysis of the spectra showed all the materials displayed the three peaks - namely D, G and 2D at Raman shifts of 1346 cm^{-1} , $\sim 1578\text{ cm}^{-1}$, and $\sim 2695\text{ cm}^{-1}$, respectively - characteristic of graphitic material. What differentiated them were the intensity of these peaks. As expected, the graphene synthesized after the first CVD had a negligible D peak and the 2D to G peak intensity ratio was ~ 1.7 , confirming it to be monolayer (Graf et al.,

2007). On the other hand, both G-CNT and OG-CNT, which has G-CNT as foundation, exhibited a high intensity D peak and sharp intense G peak, attributed to defects and higher sp^2 content, respectively, typically observed in MWCNTs Raman spectrum (Datsyuk et al., 2008; Eswaraiah et al., 2011).

To ascertain that changes in the Raman spectrum peaks of G-CNT were not emanating from erosion in the quality of the underlying graphene grown in the first CVD, i.e., the graphene layer was still intact, a control experiment where a sub-section of the graphene on copper foil synthesized in the first CVD was patterned with iron catalyst using photolithography and the CVD was performed to grow CNTs. SEM image (Figure 2.5B) of the graphene in the region next to patterned G-CNT clearly shows it to be fully intact, i.e. undamaged. Further, the Raman spectrum (Figure 2.5C) on the graphene surrounding G-CNT showed the three characteristic peaks corresponding to graphitic structure, but with 1) a lower ratio of intensity of 2D to G ($I_{2D/G}$) peaks, an indicator of few layer graphene possibly due to adlayer formation (Han et al., 2014; Li et al., 2013), and 2) a broader D peak surrounding graphene suggesting the presence of some amorphous carbon possibly due to absence of catalytic sites on graphene/copper surface. It should be noted that the D peak in the spectrum taken in G-CNT hybrid region (Figure 2.5A) did not broaden, signifying an absence of amorphous carbon and full utilization of the carbon gas for CNT growth at iron catalytic sites.

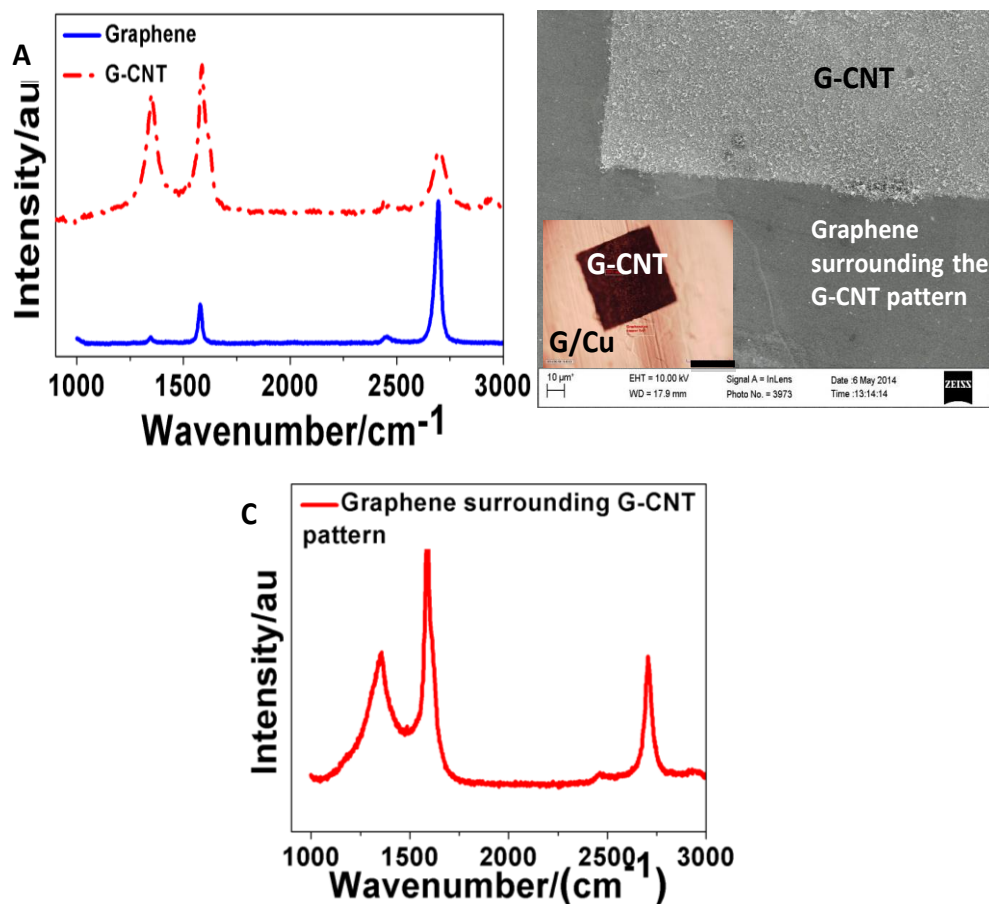


Figure 2.5 A) Raman spectra of graphene and G-CNT hybrid. B) SEM image of patterned G-CNT film. Inset shows the optical image of G-CNT pattern on graphene/copper foil. C) Raman spectra of graphene surrounding the G-CNT pattern

2.3.2 Electrochemical Properties of the G-CNT Film

We next performed cyclic voltammetry (CV) of $K_3[Fe(CN)_6]$, a redox molecule the electron transfer of which is known to depend on the graphene edge and oxygen-containing functional groups at the carbon surface (Chen and McCreery, 1996),(Ji et al., 2006),(Ko et al., 2014) , at the G-CNT/GCE, G/GCE and GCE (Figure 2.5). As shown in the figure 2.6,

based on the peak current, electroactive surface area of the G-CNT/GCE is almost 3 times higher than that of G/GCE.

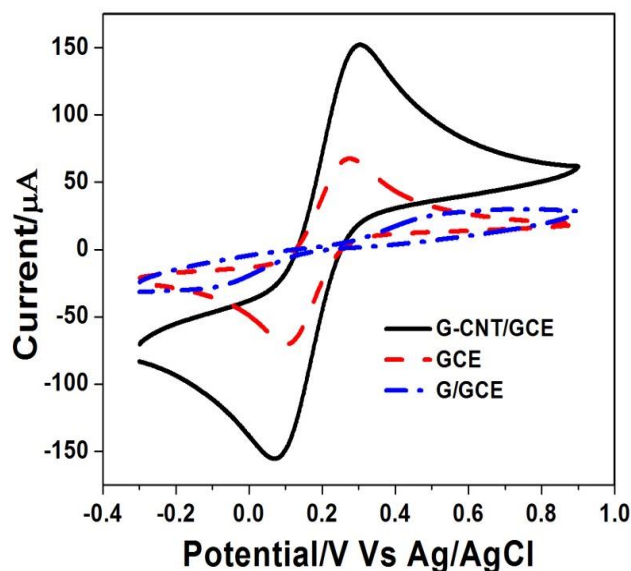


Figure 2.6 Cyclic voltammograms in 0.05 M $K_3Fe(CN)_6$ containing 0.1 M KCl at 50 mV/s for GCE, G/GCE and G-CNT/GCE, OG-/GCE.

2.4 CONCLUSION

Two-step chemical vapor deposition method has been used in the synthesis of graphene-carbon nanotube hybrid. Morphological characterization showed growth multiwalled CNTs on the graphene. Direct growth of CNTs on graphene surface resulted in seamless contact points which has led to lower contact resistance at the junction. Improved electroactive surface area with 3D conductive nanostructures has tremendous potential to be used in electrochemical biosensors.

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Chapter 3

Electrochemically Functionalized Seamless Three-Dimensional Graphene-Carbon Nanotube Hybrid for Direct Electron Transfer of Glucose Oxidase and Bioelectrocatalysis

Seamless chemical vapor deposition (CVD) grown graphene-carbon nanotubes (G-CNT) hybrid film has been studied for its potential in achieving direct electron transfer (DET) of glucose oxidase (GOx) and its bioelectrocatalytic activity in glucose detection. The G-CNT hybrid film was electrochemically modified to introduce oxygenated functional groups for DET favorable immobilization of GOx. Pristine and electrochemically functionalized G-CNT film was characterized by electrochemical impedance spectroscopy (EIS), cyclic voltammetry, XPS and Raman spectroscopy. The DET between GOx and electrochemically oxidized G-CNT electrode was studied using cyclic voltammetry which showed a pair of well-defined and quasi-reversible redox peaks with a formal potential of -459 mV at pH 7 corresponding to redox site of GOx. The constructed electrode detected glucose concentration over the clinically relevant range of 2 mM to 8 mM with the highest sensitivity of $19.31\text{ }\mu\text{A/mM/cm}^2$ compared to reported composite hybrid electrodes of graphene oxide and CNTs. Electrochemically functionalized CVD grown seamless G-CNT structure used in this work has potential to be used for development of artificial mediatorless redox enzyme based biosensors and biofuel cell.

3.1 INTRODUCTION

Carbon-based nanomaterials, including graphene, graphene oxide (GO), reduced graphene oxide (rGO) and carbon nanotubes (CNTs) have attracted a lot of research interest over the past two decades. These sp^2 hybridized carbon allotropes, with superior electrical, physicochemical, and electrochemical properties have been extensively studied and proven to be promising carbon nanomaterials in development of electrochemical electrodes for biosensing (Jacobs et al., 2010; Pumera et al., 2010), energy storage (Frackowiak and Beguin, 2001) and conversion (Arico et al., 2005). Recently, in the area of enzyme based electrochemical biosensing, graphene oxide (GO) (Kang et al., 2009; Liang et al., 2013; Shan et al., 2009; Wang et al., 2009; Wu et al., 2010), graphite nanosheets (Alwarappan et al., 2012; Fu et al., 2009) and CNTs (Cai and Chen, 2004), both multi-walled (Deng et al., 2010) and single-walled (Guisseppi-Elie et al., 2002), are being extensively studied for achieving the direct electron transfer of redox enzymes mainly glucose oxidase (GOx) and their application in glucose biosensing and biofuel cells. However, the electrodes prepared with graphene or CNTs are mostly planar in nature with electrical properties emerging only in 2D and 1D direction respectively. In addition, poor electrical contacts and aggregation of the individual components on the electrode affects the electrical conductivity and overall effective surface area of the electrode. To address this issue, the synthesis of three-dimensional (3D) hybrid structures using graphene and CNTs has been demonstrated to utilize the unique properties of both the carbon allotropes. (Badhulika et al., 2015) The enhanced specific surface area and high electrochemical activity of this graphene-carbon nanotube (G-CNT) hybrid with 3D conductive network is expected to be a superior

material in the achieving DET of redox enzymes which is otherwise difficult due to deeply embedded redox factor of the enzyme.

Recently, composite G-CNT films prepared by physical mixing/dispersion of reduced GO with single-walled as well as multi-walled carbon nanotubes (Chen et al., 2012; Mani et al., 2013; Palanisamy et al., 2014) have been reported for the DET of GOx. However, these composite films exhibit low electrical conductivity due to structural defects, non-ohmic contact between the CNT and graphene and tendency to form aggregates. On other hand, the *in-situ* chemical vapor deposition (CVD) grown (Badhulika et al., 2014; Dong et al., 2012a; Due Dung et al., 2012; Paul et al., 2010; Rajesh et al., 2013; Zhu et al., 2012b) G-CNT hybrids are seamless in nature with better electrical conductivity and low contact resistance at the graphene-CNT junction compared to such composites films (Badhulika et al., 2015). However, to the best of our knowledge the seamless G-CNT film has not been studied in direct electron transfer (DET) of GOx and its application in electrochemical glucose sensing. In the present work, we investigate the performance of CVD grown seamless G-CNT hybrid film for achieving DET between GOx and the G-CNT modified electrode surface. We electrochemically oxidized G-CNT hybrid film for DET favorable immobilization of GOx (Moumene et al., 2013). We also studied bioelectrocatalytic activity of constructed electrode for its potential application in glucose detection. Based on which we propose that the oxidized hybrid film has potential to be used as promising material in developing artificial mediatorless electrochemical biosensors using redox enzymes and also can be exploited in mediatorless biofuel cell.

3.2 EXPERIMENTAL

3.2.1 Materials

Glucose oxidase (GOx) from *Aspergillus niger* Type X was purchased from MP Biomedicals, USA. One M stock solution of glucose was prepared using deionized water and allowed to mutarotate at room temperature for 24 h. Unless otherwise specified, 0.033 M, pH 7 phosphate buffer containing 0.1 M KCl (PB/KCl) was used as a supporting electrolyte. All other reagents used were obtained from Fisher Scientific and were of analytical grade. Deaeration of solution was performed by purging pure nitrogen for at least 20 min.

3.2.2 Instruments

All electrochemical experiments were performed with a CHI 750C (CH instruments Inc., USA) using a three electrode system. Bare or modified glassy carbon electrode (GCE, area 0.07 cm^2) was used as a working electrode. Ag/AgCl with 3 M KCl solution and platinum wire were used as the reference and counter electrode, respectively. Electrochemical impedance spectroscopy (EIS) measurements were performed in 0.05 M $\text{K}_3\text{Fe}(\text{CN})_6$ containing 0.1 M KCl with a frequency ranging from 100 kHz to 0.1 Hz. Scanning electron microscopy (SEM) images were obtained on a XL30 FEG system after transferring G-CNT film to the SiO_2/Si substrate. Transmission Electron Microscopy (TEM) images were taken using PHILIPS TECNAI 12 Transmission Electron Microscope after transferring the G-CNT film on the 400 nm lacey carbon TEM grid. Raman spectra were recorded using Horiba LabRam/ AIST-NT AFM microscope with laser of $\lambda_{\text{EX}} = 532\text{ nm}$ and power of 5mW. X-ray photoelectron spectroscopy (XPS) characterization was performed by using a

Kratos AXIS ULTRADLD XPS system equipped with an Al K α monochromated X-ray source and a 165-mm mean radius electron energy hemispherical analyzer. The binding energy was calibrated by referencing the Au 4f $_{7/2}$ peak to 84.0 eV. XPS samples were prepared by transferring G-CNT film on gold (180 nm) coated SiO $_2$ /Si chips. E-beam evaporator (Temescal, BJD-1800) was used for deposition of iron on the copper foil/graphene. Tube furnace (Linderberg/Blue, Mini-Mite TM by Thermo scientific) with mass flow controller (MFC) was used for chemical vapor deposition growth of carbon nanostructures.

3.2.3 Preparation of the graphene-carbon nanotube hybrid film electrode

Graphene-carbon nanotubes hybrid film was synthesized by the method reported previously in chapter 2. A glassy carbon electrode (GCE) of 3 mm diameter was polished first with an emery paper then with aqueous slurries of alumina solution of 0.3 μ m and 0.05 μ m, respectively. The electrodes were then rinsed with water followed by sonication in deionized water and isopropyl alcohol respectively for 1 min each and was dried by a nitrogen stream.

The G-CNT film was transferred to the GCE by simple contact lifting and allowed to air dry followed by oven drying at 50 °C for 1 h. The G-CNT/GCE was then incubated in 10% v/v aqueous hydrochloric acid for 30 min to remove iron nanoparticles. The electrochemical oxidation of pristine G-CNT/GCE was carried out at a constant potential of 1.8 V (vs. Ag/AgCl) for 10 - 12 s in a 2.5% w/v potassium dichromate solution in 10% v/v aqueous nitric acid. The resulting electrode (OG-CNT/GCE) was thoroughly rinsed with deionized water followed by PB/KCl and incubated with GOx solution (2 mg/100 μ l)

in PB/KCl at 4 °C for 24 h. Afterwards, the electrode (GOx/OG-CNT/GCE) was rinsed with PB buffer/0.1 M KCl and stored at 4 °C when not in use.

3.3. RESULTS & DISCUSSION

3.3.1 Characterization of electrochemically functionalized G-CNT hybrid film

The Raman spectra of various carbon nanostructures synthesized in this work - graphene synthesized after the first CVD, G-CNT synthesized after the second CVD, and OG-CNT – are exhibited in Figure 3.1. An analysis of the spectra showed all the materials displayed the three peaks - namely D, G and 2D at Raman shifts of 1346 cm^{-1} , $\sim 1578\text{ cm}^{-1}$, and $\sim 2695\text{ cm}^{-1}$, respectively - characteristic of graphitic material. What differentiated them were the intensity of these peaks. As expected, the graphene synthesized after the first CVD had a negligent D peak and the 2D to G peak intensity ratio was ~ 1.7 , confirming it to be monolayer (Graf et al., 2007). On the other hand, both G-CNT and OG-CNT, which has G-CNT as foundation, exhibited a high intensity D peak and sharp intense G peak, attributed to defects and higher sp^2 content, respectively, typically observed in MWCNTs spectrum (Datsyuk et al., 2008; Eswaraiah et al., 2011). A further probing of the Raman spectra revealed an increase of the intensity ratio of D to G peak from 0.83 for G-CNT to 1.03 for OG-CNT (Figure 3.1B). This increase is ascribed to reduction of sp^2 carbon (G peak) and subsequent increase in defects (D peak) resulting from the introduction of oxygenated-functional groups in the graphitic structure by electrochemical oxidation step without significantly destroying the sp^2 structure.

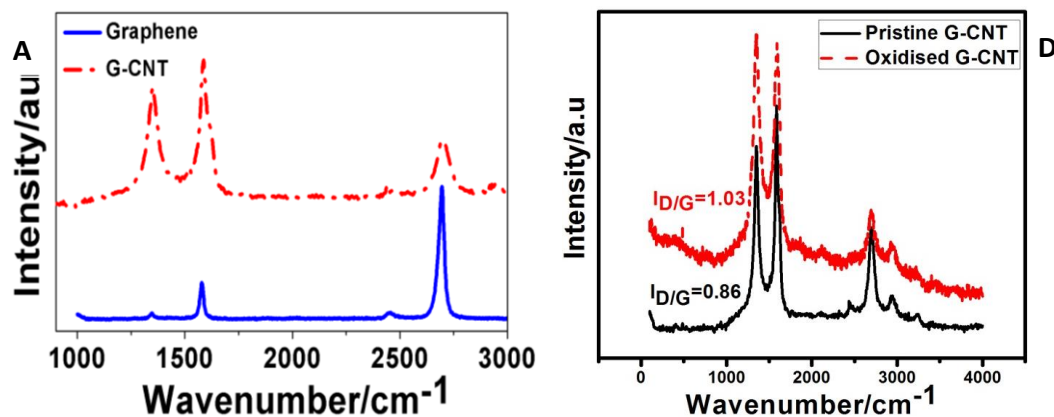


Figure 3.1. A) Raman spectra of graphene and G-CNT hybrid. B) Raman spectra of pristine G-CNT and OG-CNT film

XPS measurements were performed to identify and quantify the functional groups introduced in G-CNT by electrochemical oxidation. Figure 3.2A displays XPS survey spectra of G-CNT before and after electrochemical oxidation. Pristine G-CNT showed a strong C 1s peak (284.2 eV) indicating carbon rich material and weaker oxygen peaks ascribed to impurities from ambient air oxidation and/or acid treatment during catalyst impurities removal. In comparison, in OG-CNT O 1s peak (532.3 eV) was higher than C 1s peak due to oxidation process. The C 1s spectra for both G-CNT and OG-CNT upon deconvolution exhibited 6 components, which are attributed to major graphitic structure with sp² carbon C=C at ~284.2 eV, sp³ carbon or defects at ~285.3 eV, and most significantly oxygen containing functional groups, C-O at ~286.2 eV, C=O at ~288.0 eV, O-C=O (COO) at ~289.3 eV and π - π interaction carbon at ~291.5 eV based on the binding energies reported in previous literatures (Liang et al., 2013),(Doepke et al., 2012),(Datsyuk et al., 2008),(Okpalugo et al., 2005). Comparing the C 1s XPS spectra of pristine and OG-

CNT, we can clearly see that percentages of the components containing oxygen have increased after oxidation. In particular, peaks at C-O and C=O for OG-CNT significantly increased in comparison with G-CNT. In pristine sample, atomic concentrations of C and O were found to be 86.6% and 13.4%, respectively, whereas in OG-CNT sample they are 75.2% (C) and 24.8% (O). Therefore, the relative ratio of oxygen to carbon (O/C) in OG-CNT is 0.33, which is 2.2 times higher than the ratio (0.15) of pristine G-CNT. This clearly indicates introduction of oxygen containing groups by electrochemical oxidation.

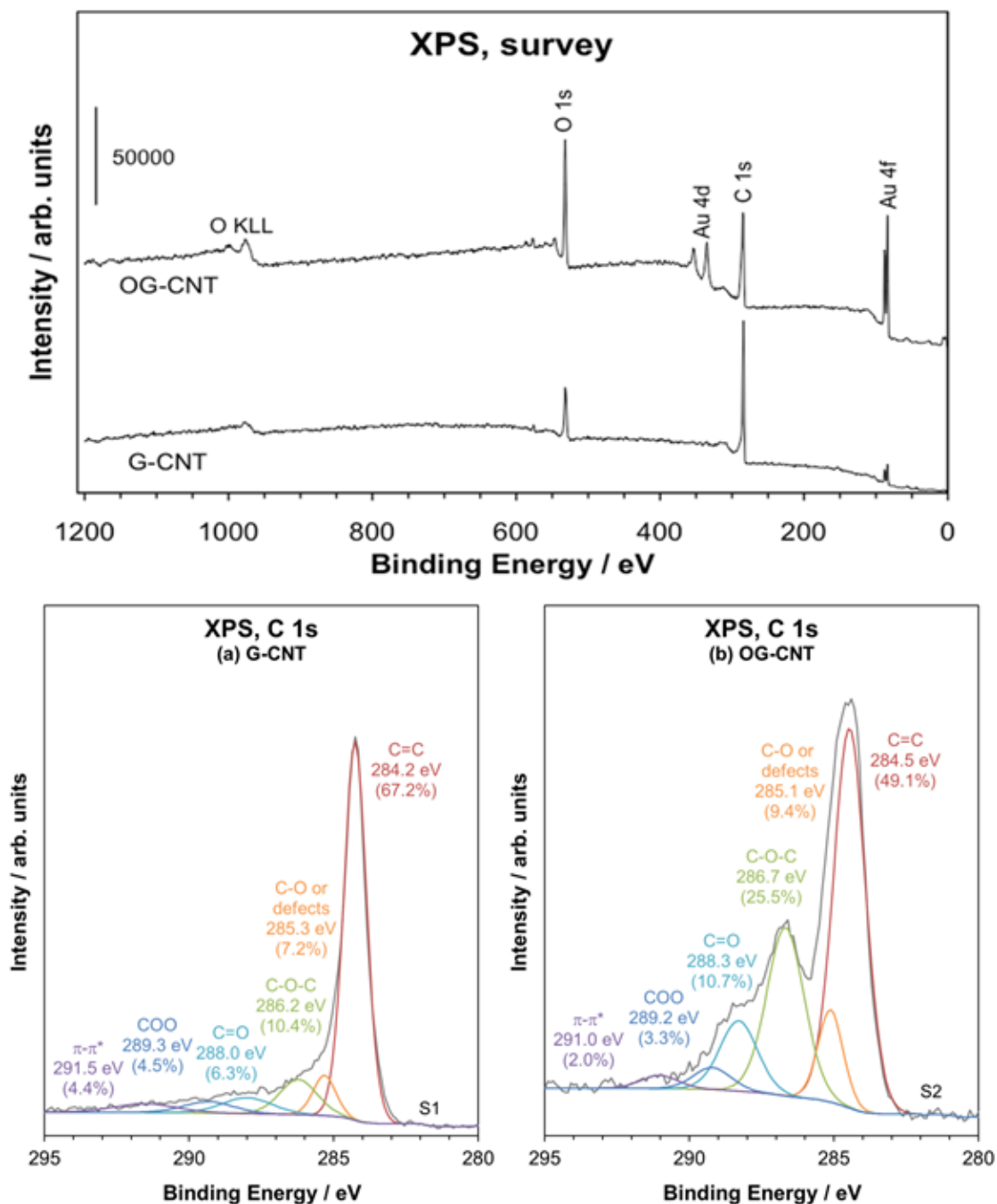


Figure 3.2 A) Survey XPS spectra of pristine G-CNT and OG-CNT. B) Deconvoluted C 1s XPS spectra of pristine a) G-CNT and b) OG-CNT.

3.3.2 Electrochemical Properties of the OG-CNT Film

We next performed cyclic voltammetry (CV) of $K_3[Fe(CN)_6]$, a redox molecule the electron transfer of which is known to depend on the graphene edge and oxygen-containing functional groups at the carbon surface (Chen and McCreery, 1996),(Ji et al., 2006),(Ko et al., 2014), at the G-CNT/GCE, and OG-CNT/GCE (Figure 3.3A). As shown in the figure, there was a very small increase in the peak-to-peak potential separation (ΔE_p) and decrease in the peak currents (anodic and cathodic) at OG-CNT/GCE compared to G-CNT/GCE. This is attributed to presence of negatively charged groups in the former electrode. Additionally, the very small differences in ΔE_p and peak currents between the G-CNT/GCE and OG-CNT/GCE affirmed that electron transfer in latter was not significantly affected because G-CNT was being oxidized only mildly. The above findings were also confirmed from the electrochemical impedance spectroscopy (EIS). Figure 3.3B shows the Nyquist plots, in which the diameter of the semicircle represents the charge transfer resistance (R_{ct}). As shown in the figure, the R_{ct} value of OG-CNT/GCE was slightly larger than of G-CNT/GCE. A very small increase of R_{ct} of G-CNT/GCE upon electrochemical oxidation is ascribed to the presence of negatively charged functional groups at the OG-CNT hybrid surface.

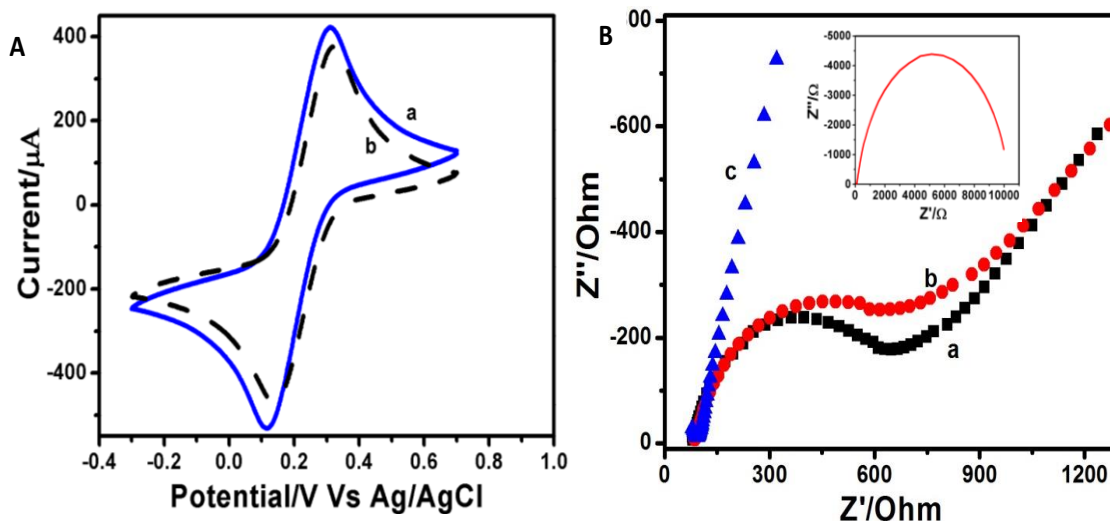


Figure 3.3. A) Cyclic voltammograms in 0.05 M $K_3Fe(CN)_6$ containing 0.1 M KCl at 50 mV/s for a) G-CNT/GCE and b) OG-CNT/GCE. B) EIS spectra of a) G-CNT/GCE, b) OG-CNT/GCE and c) GOx/OG-CNT/GCE in 0.1 M KCl aqueous solution containing 0.05 M $K_3Fe(CN)_6$. Inset shows the zoomed-in EIS spectra for GOx/OG-CNT/GCE

3.3.3. Direct electrochemistry of GOx/OG-CNT/GCE electrodes

To investigate the DET between the GOx redox center and OG-CNT/GCE, we performed cyclic voltammetry in deaerated PB/KCl (pH 7.0). As illustrated in Figure 3.4, while no redox peaks were observed at, GOx/G-CNT/GCE, G-CNT/GCE and OG-CNT/GCE, there was a pair of well-defined reversible redox peaks at the GOx/OG-CNT/GCE with anodic peak potential (E_{pa}) at about -0.451V and cathodic peak potential (E_{pc}) at -0.468 V. The obtained formal potential for GOx/OG-CNT/GCE of -0.459 V was close to the standard electrode potential of GOx (Kang et al., 2009). This result indicates that the surface of OG-CNT has a great advantage in the DET with GOx compared to G-CNT. In particular, both defects/edge sites and oxygen-containing functional groups at the OG-CNT surface might play an important role in the GOx adsorption and/or its orientation. Thus, OG-CNT with

rough face consisting of electrochemically oxidized CNTs-grown graphene might be further important for the DET with GOx.

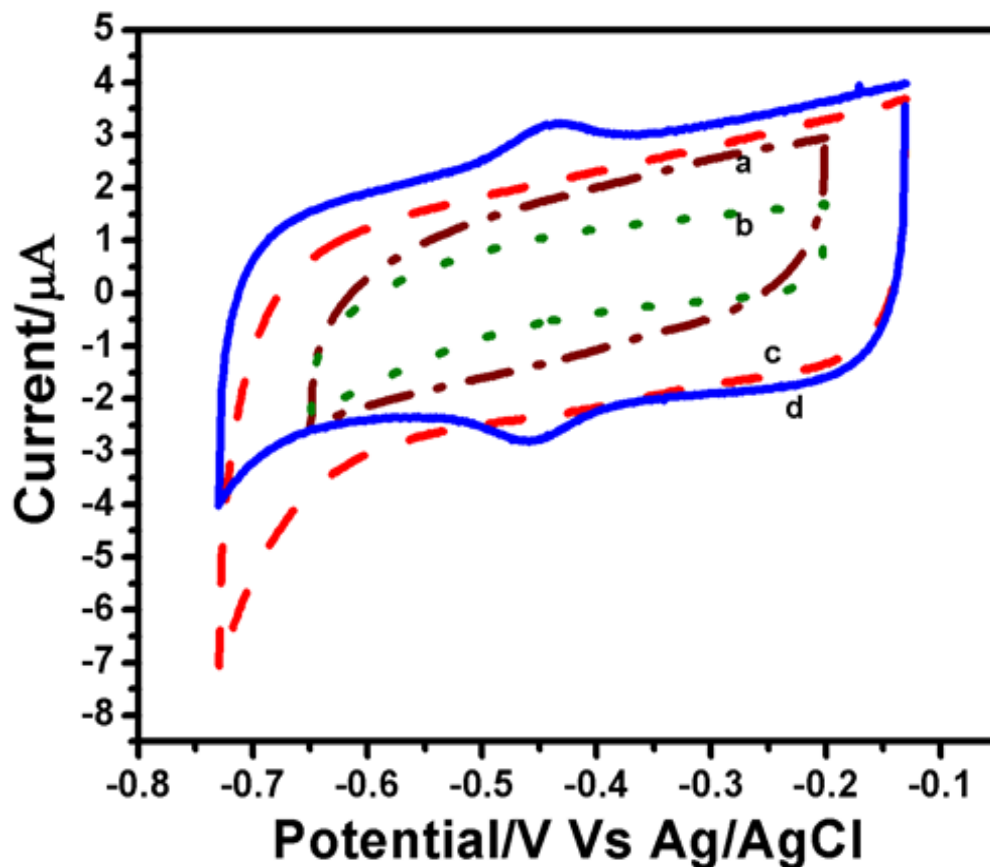


Figure 3.4. Cyclic voltammograms of a) G-CNT/GCE, b) GOx/G-CNT/GCE, c) OG-CNT/GCE and d) GOx/OG-CNT/GCE in deaerated PB/0.1 M KCl at 50 mV/s.

As the direct electrochemistry of GOx is a two electron and two proton coupled reaction (eqn. 1), the effect of different pH environments on peak potentials was studied for GOx/OG-CNT/GCE.



Figure 3.5A shows CVs of GOx/OG-CNT/GCE in PB/0.1 M KCl of different pH values.

As expected, the observed redox peaks showed a negative shift with increasing pH values

from 5.5 to 8.5. The formal potential exhibits linear relationship with solution pH with a slope of -66.9 mV/pH (Figure 7A (inset), $R^2 = 0.9903$) which is close to theoretical value of -58.6 mV/pH for a reversible two proton and two electron transfer reaction. These confirmed the DET of GOx at the OG-CNT/GCE.

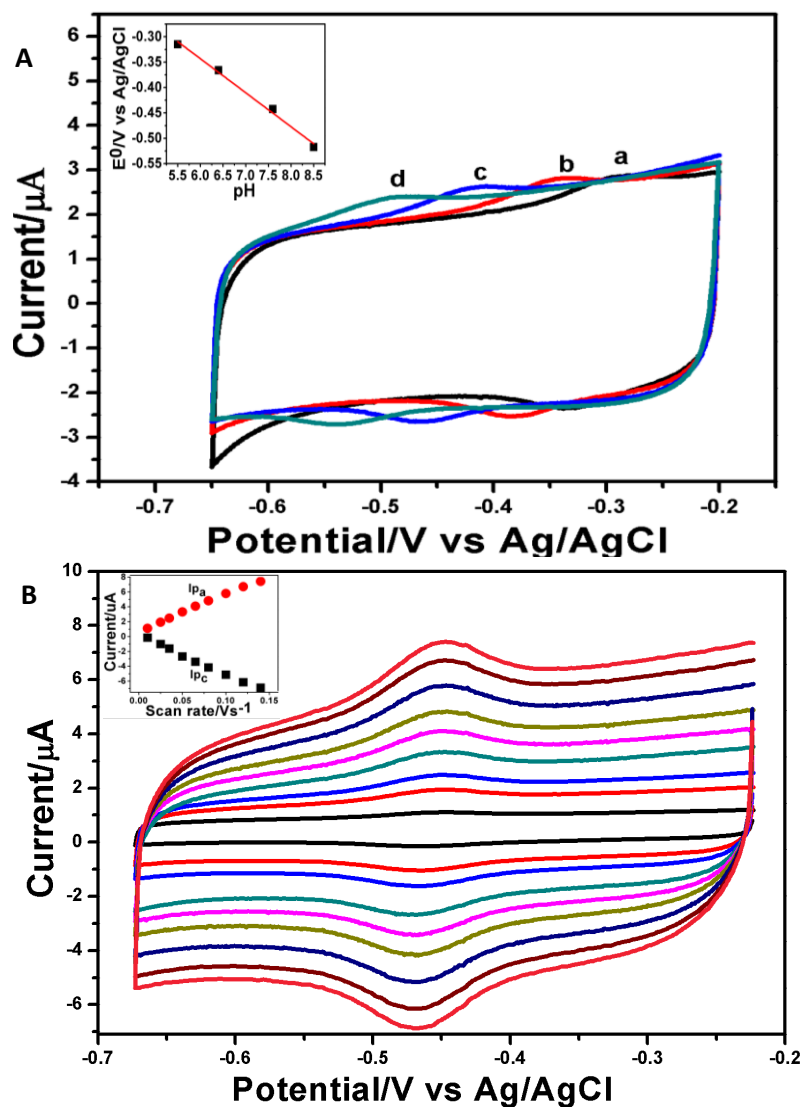


Figure 3.5. A) Cyclic voltammograms of GOx/OG-CNT/GCE in deaerated PB/0.1 M KCl at pH of (a) 5.5, (b) 6.4, (c) 7.6 and (d) 8.5. B) Cyclic voltammograms of GOx/OG-CNT/GCE in deaerated PB/0.1 M KCl pH 7 at scan rates of 10, 25, 35, 50, 65, 80, 100, 120 & 140 mV/s.

Figure 3.5B illustrates the effect of scan rate on cyclic voltammetry characteristics of the DET of GOx/OG-CNT/GCE. As the scan rate increased from 0.01 V/s to 0.14 V/s, both anodic (I_{pa}) and cathodic (I_{pc}) peak currents increased linearly with scan rate, indicating that the redox reaction is a surface controlled process.

The amount of glucose oxidase enzyme immobilized on the electrode surface (Γ mol/cm²) can be evaluated using the formula,

$$Q = nFA\Gamma \dots\dots\dots(2)$$

where, Q is the charge (C) calculated by peak integration, A (cm²) is the electrode area, F is Faraday constant (=96493 C/mol) and n is the number of electrons (assumed to be 2). The amount of electroactive GOx was calculated to be 1.24×10^{-10} mol/cm², which is about 40 times higher than the theoretical value of 2.86×10^{-12} mol/cm² for monolayer GOx on bare GCE⁴¹ and comparable to other reported values (Table S1). This demonstrates that the OG-CNT film has provided high surface area for GOx loading.

3.3.4. Electrocatalytic activity of GOx/OG-CNT electrode and glucose detection

Electrocatalytic activity of GOx/OG-CNT/GCE was studied using cyclic voltammetry in PB/KCl (pH 7) saturated with nitrogen and air (Figure 3.6A). As expected, reversible redox peaks appeared in deaerated buffer (Figure 3.6A, curve a). In air saturated buffer, the electrode showed a sharp increase in the cathodic current (Figure 3.6A, curve b) compared to nitrogen saturated buffer. To determine a reason, we performed CVs at OG-CNT/GCE in aerated and deaerated buffers and in 5 mM H₂O₂ (Inset Figure 3.6A). The results showed that for OG-CNT/GCE in air saturated and H₂O₂ buffer, onset of reduction potential for O₂ and H₂O₂ is in the examined potential though magnitude of cathodic current increase was

lower than that of GOx/OG-CNT/GCE in aerated buffer. Therefore, the cathodic current increase at GOx/OG-CNT/GCE in aerated buffer when compared with deaerated buffer is due to the overlapping electrocatalytic reduction of O₂ and H₂O₂ (eqn. 3) at the electrode and electrochemical reaction (eqn. 1).^{7,9}



In aerated buffer when glucose is added, the cathodic current decreased (Figure 7A, curve c) indicating the increased consumption of oxygen and restriction of electrochemical reaction (eqn. 1) due to enzymatic reactions (eqns. 3 and 4). This cathodic current decrease was proportional to glucose concentration. Figure 7B shows CVs at the GOx/OG-CNT/GCE and plots of peak cathodic (at - 485 mV) and anodic (at - 444 mV) currents as a function of glucose concentration. As illustrated, both cathodic ($R^2 = 0.9485$) and anodic ($R^2 = 0.9884$) peak current are a linear function of glucose concentration in the range of 2 mM to 8 mM with sensitivity (slope/electrode area) of 19.31 $\mu\text{A mM}^{-1} \text{cm}^{-2}$ and 3.51 $\mu\text{A mM}^{-1} \text{cm}^{-2}$, respectively (Inset Figure 7B). This finding demonstrates that the GOx/OG-CNT/GCE can directly evaluate the glucose concentration without an artificial mediator. Compared to other reported GO/CNT composite hybrids, including the one using mediator¹⁶ (Table S2), the seamless OG-CNT electrode in this work has the highest sensitivity for glucose detection. Since the normal blood glucose concentration is 4.4 - 6.6 mM and diabetic patients typically show blood glucose levels more than 7 mM, the present glucose biosensor based on OG-CNT will find application in diagnosis as well as monitoring the blood glucose levels of diabetic patients. Furthermore, the negative

operating potential of the biosensor alleviates the interference from electroactive compounds like ascorbic acid, acetaminophen, dopamine hydrochloride and uric acid present in the blood which are oxidized at much higher potential (Figure S1).

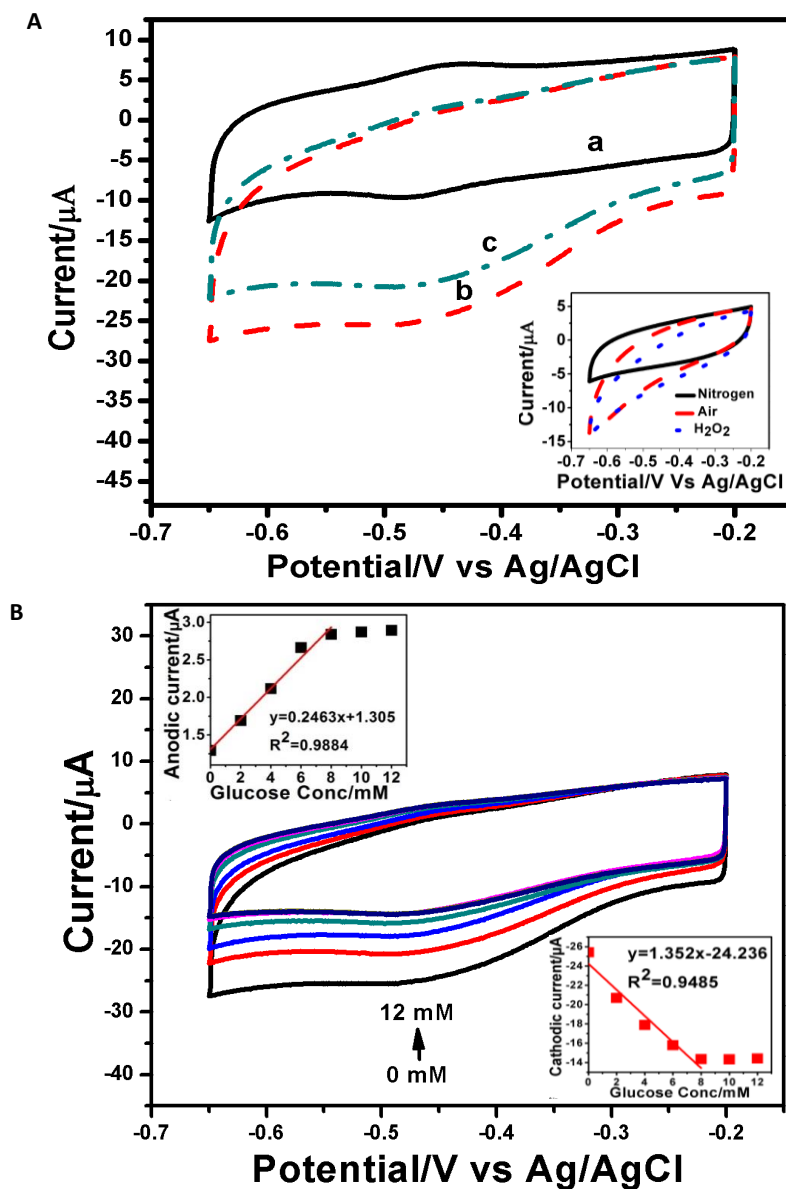


Figure 3.6. A) Cyclic voltammograms of GOx/OG-CNT/GCE in PB/0.1 M KCl pH 7 (a) deaerated, (b) aerated and (c) aerated with 2 mM glucose. Inset shows cyclic voltammograms of OG-CNT/GCE in PB/0.1 M KCl pH 7 deaerated, aerated, aerated with 5 mM H₂O₂. B) Cyclic voltammograms of GOx/OG-CNT/GCE in aerated PB/0.1 M KCl pH 7 with increasing glucose concentration of 0, 2, 4, 6, 8, 10, 12 mM. Insets are the calibration plots corresponding to anodic and cathodic current responses at - 485 mV and - 444 mV respectively. Scan rate: 100 mV/s.

The biosensor had good precision, as evident by the residual standard deviation (RSD) of 2.83% for 5 replicate measurements of 6 mM glucose. Furthermore, the biosensor retained more than 90% of its original response even after 30 days of storage at 4°C.

3.4 CONCLUSIONS

A seamless G-CNT hybrid film with CNTs fused to the graphene surface was synthesized using a two-step CVD method. Electrochemical oxidation of “high surface area G-CNT film” resulted in favorable immobilization/orientation of GOx resulting in DET of GOx at the electrode. We have successfully demonstrated the potential of the GOx functionalized electrochemically oxidized CVD grown seamless G-CNT film in achieving improved glucose biosensing response compared to reported physically dispersed/assembled hybrids of graphene oxide and CNTs. GOx/OG-CNT showed a linear response to glucose in clinically relevant range with highest sensitivity ($19.31 \mu\text{A}/\text{mM}/\text{cm}^2$) compared to reported composite hybrid electrodes of graphene oxide and CNTs which proves the advantage of CVD grown seamless G-CNT hybrid in glucose bioelectrocatalysis performance. The electrochemically oxidized hybrid structure of carbon nanotube-graphene can also be studied for development of electrochemical biosensors based on other redox enzymes as well as in the development of mediatorless biofuel cells.

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Chapter 4

Advanced Methods in Fabrication of Novel Graphene Nanogap Electrode

Planar graphene nanogap electrode were fabricated using combination of photolithography and advanced nanofabrication method. We studied and compared three different methods including, focused ion beam milling (FIB), nanoindentation and e-beam lithography (EBL) in fabricating the reproducible nanogap of gap width less than 100 nm. Current-voltage (I-V) curves of graphene electrodes before and after nanogap fabrication was measured to confirm the successful fabrication of high resistance nanogaps. Based on the SEM studies, the lowest width of nanogap obtained using these three methods was between 30 to 40 nm. Based on the advantages and disadvantages of each method, EBL was chosen as the method of nanogap fabrication. This graphene nanogap electrodes will be used in developing an electrical biosensor based on conductance change

4.1 INTRODUCTION

Nanogap electrodes gained a lot of attention due to their potential of integrating into next generation molecular electronics with high efficiency and low power dissipation. These nanogap electrodes are fabricated using methods such as mechanical break junctions (Reed et al., 1997), surface catalyzed chemical deposition (Park et al., 2011), electromigration (Park et al., 2002a), and direct nanotransfer printing (Strobel et al., 2009) etc.

Graphene, an atomically thin material, has attracted world attention due to its superior conductivity, stability and tunable band gap with highest electron mobility. Therefore, patterning of graphene is important to be able expand the scope of its use in electronics and sensing. Graphene nanogaps have been fabricated using E-Beam lithography, chemical etching or using iron or nickel catalyst (Ci et al., 2008; Datta et al., 2008; Wang et al., 2010), atomic force microscopy lithography (AFM) lithography (He et al., 2010) etc. We study nanoindentation method, for the first time, in fabricating the graphene nanogap electrode along with EBL and FIB. Nanoindentation is a common technique used in the testing the mechanical properties of the material such as modulus of elasticity, hardness, yield strength, fracture toughness, scratch hardness and wear properties. We compared these three methods in fabricating nanogap width of less than 100 nm reproducibly.

4.2 EXPERIMENTAL

4.2.1 Instruments

Semiconductor parameter analyzer (HP Agilent 4156B) was used in the electrical measurements. E-beam evaporator (Temescal BJD-1800) was used in depositing metal contact pads. E-beam lithography system used is a Leo SUPRA 55 with nanometer pattern generation system (NPGS) capabilities. The Focused Ion Beam Milling system (FIB) used is a Leo XB1540. Scanning electron microscopy (SEM) images were obtained on a XL30 FEG system. Hysitron Inc. TI-950, triboindenter was used in nanoindentation of graphene. An atomic force microscope (AFM) (Veeco Innova, Santa Barbara, CA) was used for charactering the nanogap structure. The SpmLabAnalysis software was used for analyzing the AFM images.

4.2.2 Methods

4.2.2.1 Graphene synthesis and electrode preparation

Graphene (Gr) was synthesized by chemical vapor deposition method using copper (Cu) foil (99.99% purity, 25 μm thickness) as a substrate for growth. Initially Cu foil was cleaned in 10% acetic acid, deionized water, acetone and 2-propanol respectively for 10 min. each to remove any oxide and other impurities. The Cu foil was dried by stream of nitrogen gas, placed inside a fused silica tube (5 cm inside diameter $\times$ 100 cm long) and the temperature of the tube furnace was raised to 1030 $^{\circ}\text{C}$, under flowing mixture of Ar (190 sccm) and H_2 (8 sccm) at the atmospheric pressure. After annealing at 1030 $^{\circ}\text{C}$ for 30 min, CH_4 (10 sccm) was supplied for 10-12 min. Once the growth process was complete,

CH₄ flow was stopped and the furnace cooled to room temperature under the flow of Ar/H₂. To transfer the graphene from the Cu foil to the SiO₂/Si substrate, a PMMA solution was spin coated onto the Gr/Cu foil and baked at 60 °C for 10 min. The PMMA/Gr/Cu foil was then cut into desired size pieces. The Cu was then etched with 0.3 M FeCl₃ solution and the PMMA/Gr film floating on the ferric chloride solution was further cleaned with aqueous HCl (5%) and deionized water to make sure no copper or ferric chloride traces were left.

4.2.2.2 Fabrication of graphene microelectrode

The process starts with transferring of PMMA/Gr film to the silicon substrate with 300 nm silicon oxide. The film is then air dried followed by oven drying at 50°C for 1 hr. To remove the PMMA from the graphene a drop of fresh PMMA solution is placed on the PMMA/Gr film for 1 min, thinned out by spinning at 3000 rpm for 30 sec and finally dipped in the acetone bath at 60°C. The fabrication process for planar graphene electrodes is shown in Figure 4.1. The graphene was patterned using standard photolithography method using AZ5214 photoresist and contact pads of Cr/Au (20/180 nm) for electrical measurements. The chip patterned is shown in the Figure 4.1.

4.2.2.3 Nanogap fabrication by E-beam Lithography method:

Graphene microelectrodes were first cleaned with acetone and IPA followed by drying with the stream of nitrogen gas. The EBL resist, i.e. PMMA was spin coated onto the chips in two steps, step I: 1000 rpm at 300 rpm/s for 3 s, step II: 5000 rpm at 1000 rpm/s for 45 s. The chips were then baked at 180 °C for 30 min. The gold pads on these chips were used

as alignment marker and a line pattern was fabricated in the center of the graphene electrode by EBL writing (20keV, 20pA and varied line dose). The line pattern transferred on the PMMA was developed using MIBK:IPA (1:3) solvent for 70-90 secs. The exposed graphene in the line pattern was subjected to oxygen RIE. Finally, PMMA was removed by washing with the acetone.

4.2.2.4 Nanogap fabrication by Focused ion beam milling (FIB)

Graphene micorelectrodes were also used as a substrate in this method. Ga^+ ions with an accelerating voltage of 30 keV was used as the ion source. Focused beam introduced the trench profile in a single line pattern across the graphene micorelectrodes. Different combinations of ion beam currents and milling time or depth were used to achieve the nanogap width of lower than 100 nm.

4.2.2.5 Nanogap fabrication using Nanoindenter

Nanogap fabrication by nanoindentation was performed using a displacement- controlled indentation tool with an ultralow noise capacitive force sensor. The diamond tip of 80 nm was approached the surface of the graphene at predetermined rate and vertical depth was varied.

4.3 RESULTS AND DISCUSSION

4.3.1 Fabrication of graphene nanogap electrode

Figure 4.1 shows the schematic for fabrication of graphene microelectrode design with 5 pairs of electrodes.

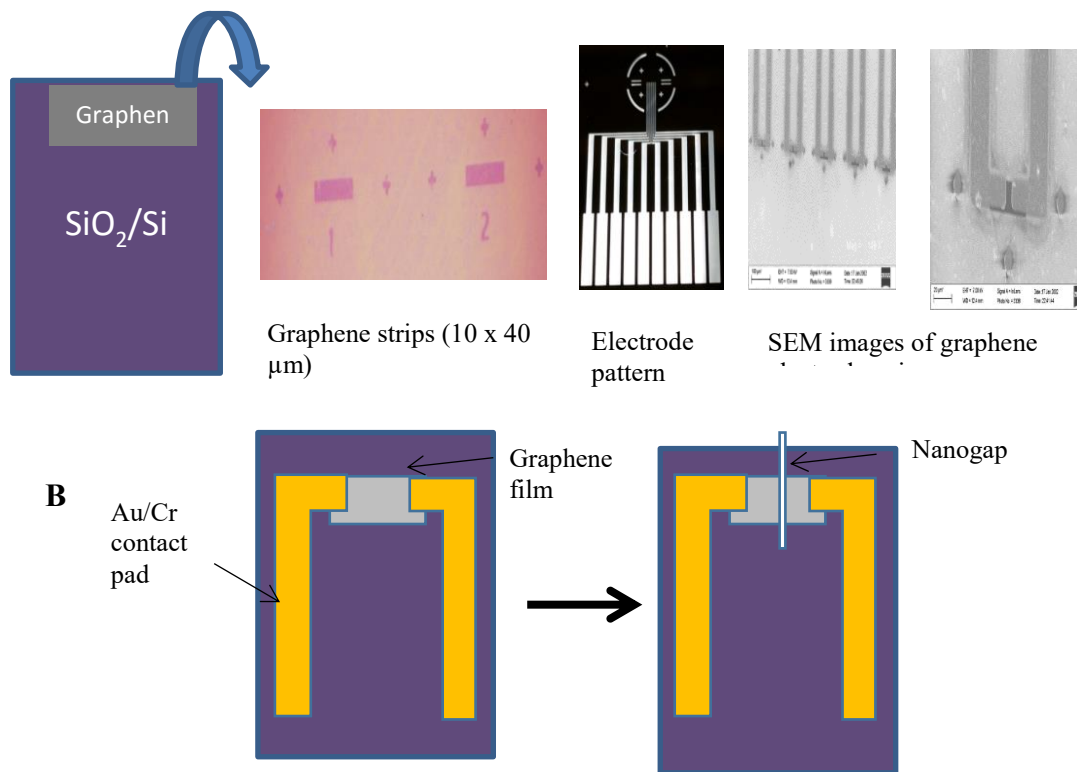


Figure 4.1 A): Photolithography for writing the planar micro patterned graphene electrodes.

B) Schematic for planar nanogap electrode fabrication

We investigated three different methods of fabricating the planar gap of less than 100 nm in the graphene. These methods were namely focused Ga⁺-ion beam milling, nanoindentation and e-beam lithography. Our goal was to find out the method which can reproducibly make nanogaps without affecting the graphene properties and would result into a device suitable for detecting the biomolecular interaction at the nanoscale.

Focused ion beam (FIB) milling using gallium ions was the first method used in making nanogaps as it's a direct method without any lithography steps. In this method, highly accelerated Ga^+ ions knock off the carbon atoms from the graphene in the desired pattern (Figure 4.2A) as a single line pattern for planar nanogap. The nanogap electrodes of width around 30-80 nm were fabricated using FIB milling using varied milling current, time or depth. Figure 4.2C and 4.2D shows SEM image of a 50-60 nm nanogap. Current voltage curve (I-V) before and after the FIB milling shows that milling has resulted in the high resistance graphene nanogap (Figure 4.2 B).

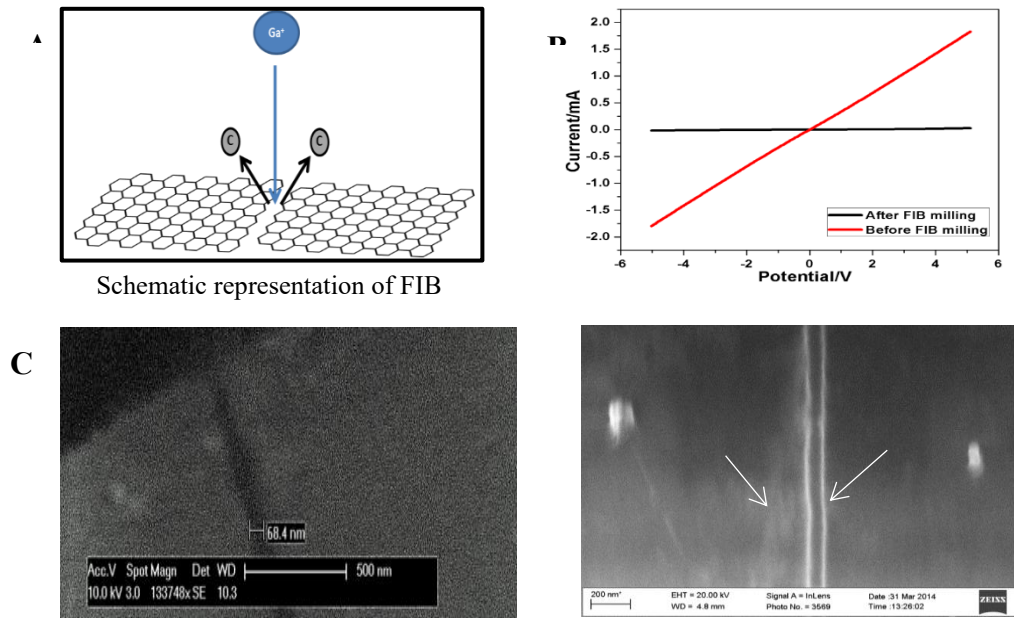


Figure 4.2 A): Schematic representation of FIB milling B) I-V curve of graphene electrode before and after nanogap by FIB C) & D) SEM images of graphene nanogap

Second method used was Nanoindentation. It is a common technique used in the testing the mechanical properties of the material such as modulus of elasticity, hardness, yield strength, fracture toughness, scratch hardness and wear properties. To our knowledge, this method has been used for measuring the mechanical properties of graphene but has not been studied in nanogap fabrication. In this method nanoindenter with a cube corner diamond tip of radius of 80 nm was used to physically etch the graphene in making the nanogap. The vertical displacement of the tip on the graphene substrate is directly proportional to the final width of the gap. Table 4.1 shows the different gap widths obtained by varying the depth of the tip.

Selected depth/ vertical displacement (nm)	Width of the gap (nm) across trench
50	132
20	90-95
15	80-85
10	45-50

Table 4.1 Gap widths obtained varying the vertical displacement of nanoindenter tip

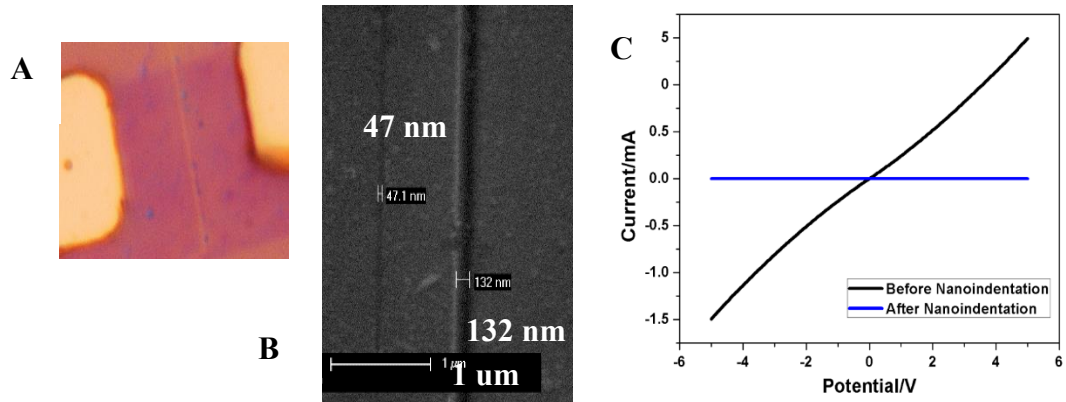


Figure 4.3 A): Optical image of nanogap by indenter B) SEM image of nanogap by indenter before and after nanogap C) I-V curve of graphene electrode before and after nanogap by indenter

Figure 4.3 shows the optical and SEM image of nanogaps on graphene electrode using nanoindenter. Electrical measurements (I-V curve) proves the successful formation of graphene nanogap.

Different width of nanogap were also fabricated using EBL. Optimized aperture size (Figure 4.4), line dose and beam current (Figure 4.5) resulted in nanogap widths varying from 30-300 nm. After EBL writing and development, RIE step is performed to etch the graphene exposed in the line pattern, rest of the area is still covered with PMMA resist. Au/Cr deposited to better visualize the gap widths and features. I-V curve after RIE etching of graphene in the developed line pattern shows the high resistance gap formation (Figure 4.6).

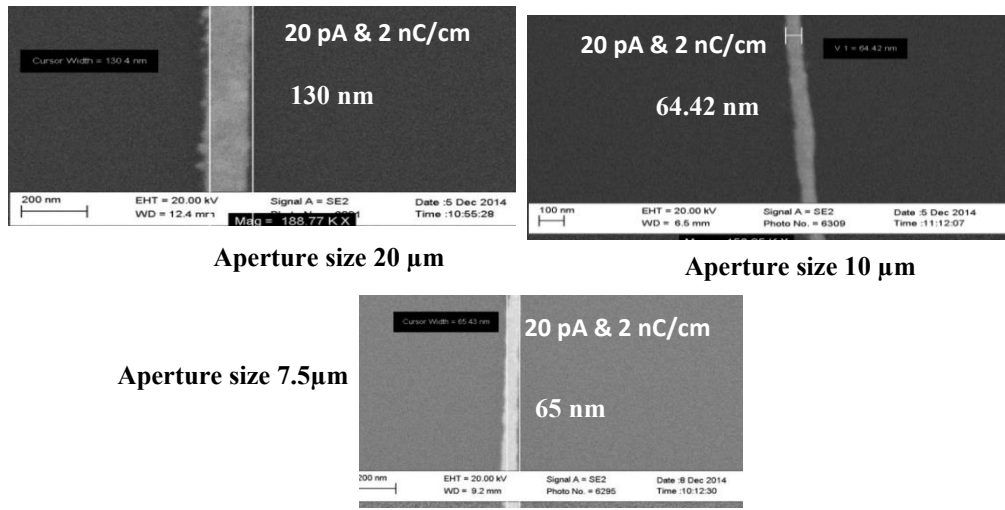


Figure 4.4 Effect of aperture size on gap width

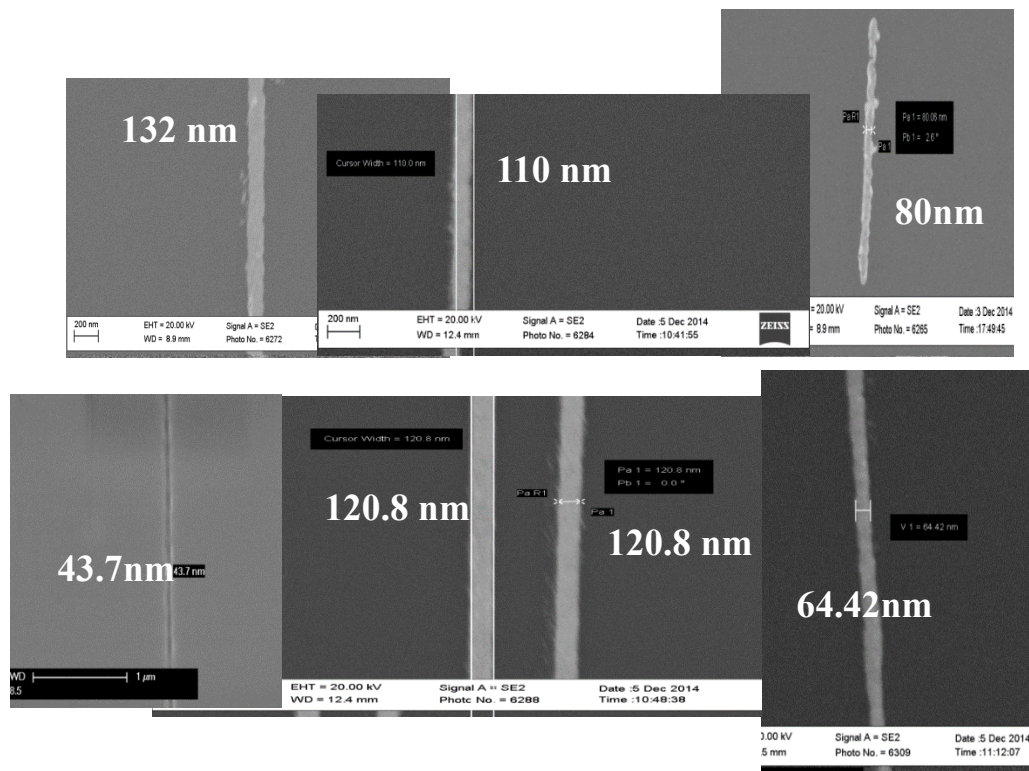


Figure 4.5 Effect of beam current and line dose on gap width

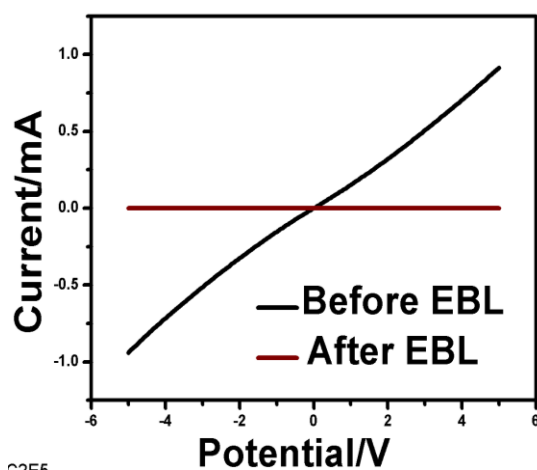


Figure 4.6 I-V curve of graphene electrode before and after nanogap by EBL.

4.3.2 Comparison of the nanofabrication methods: We studied pros and cons of each method to decide the most reproducible method without affecting conductivity of graphene. Table 4.2 lists the pros and cons of each method. Based on which EBL was chosen as method for further experiments of nanogap fabrication.

4.4 CONCLUSION

Based on our experiments, E-beam lithography has proven to be a reproducible method in fabricating graphene nanogap of less 100 nm. These nanogap electrodes will be further studied in the development of highly sensitive electrical biosensor.

Method	Pros	Cons
FIB	1. Direct method	1. Gallium ion implantation generates defect in the graphene, affecting the electron conductivity of graphene
Nanoindentation	1. Direct method 2. No ion implantation	1. Wear and tear of the diamond tip affects the gap size 2. Surface contaminants or roughness due to polymer/resist residues or any solid particles affect the tip movement and therefore the gap size
One step EBL	1. No ion implantation	1. Lithography step is involved 2. RIE use

Table 4.2 Comparison of nanofabrication methods in graphene nanogap fabrication

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Chapter 5

Development of Electrical Biosensor Using Graphene Nanogap

Electrode: A Proof of Concept Study

We report an electrical biosensor platform for detection of biomolecular interaction using a graphene nanogap electrode fabricated using E-beam lithography (EBL). High affinity interaction of streptavidin-biotin was chosen as a model system in the testing the electrical biosensing concept. Nanogap functionalized with biotin possessing high affinity for target analyte, i.e. streptavidin. Streptavidin are coated on the gold nanoparticles (AuNP). Affinity interaction of streptavidin-AuNP to the biotin in the gap, result in localization of AuNP and was detected based on the change in the conductance. Significant change in conductance was observed due to a narrower gap size and superior conductivity of the electrode material. The biosensing performance was optimized by varying the gap size, AuNP diameter and streptavidin coverage on the AuNP. The Sensitivity and LOD of streptavidin detection with optimized parameters was found to be 0.3 $\mu\text{A}/\mu\text{M}$ of streptavidin and 0.24 pM, respectively. The detection capability of this biosensor can be tuned down to the single to few molecules. Proposed biosensor platform can be used for any detection based on biomolecules affinity interaction such as for antigen-antibody or chemo-selective interaction with full potential to be used as a portable point of use biosensor.

5.1 INTRODUCTION

Nanogap electrodes have emerged as a powerful method in the development of ultrasensitive electrical/electrochemical biosensors. The biospecific or chemospecific binding events are directly transduced to produce useful signal in the form of electrical resistance, electrochemical impedance (Lu et al., 2016; Singh et al., 2010), capacitance (Hsueh and Lin, 2016; Mannoor et al., 2010; Yi et al., 2005) or field effect (Gu et al., 2009; Kim et al., 2009). Planar (single or interdigitated (Singh et al., 2010)) and vertical nanogap electrodes (Jang et al., 2007; Strobel et al., 2007) for biosensing have been fabricated.

Earlier in 2002, an electrical detection of DNA using gold nanoparticle probes on micron size gap electrodes was reported (Park et al., 2002b). In which binding of oligonucleotide conjugated gold nanoparticles to its target located in the gap electrode, resulted in the conductivity changes. Owing to the wider gap size, silver enhancement/precipitation step was necessary to enhance the size of the gold nanoparticles for complete bridging of the gap. To avoid this additional step and background current from non-target related silver deposition, gap size between planar electrodes was reduced to less than 100 nm (Haguet et al., 2004). Significant conductance variation was still observed even without silver precipitation step in detecting interaction between biotin/streptavidin and antibiotin/biotin. Similar studies were also reported for electrical detection of antibodies from human serum (Marcon et al., 2008a) and for characterization of nanogap chemical reactivity (Marcon et al., 2008b). However, to best of our knowledge, the electrical biosensor performance data of gold nanoparticles- labeled nanogap electrodes in terms of sensitivity and limit of detection has not been reported. There is also need for

investigating correlation of gap size and diameter of the gold nanoparticles in further optimizing the limit of detection making it an ideal platform for high sensitive detection for early diagnosis.

Graphene is a sp^2 hybridized carbon, arranged perfectly in a honeycomb structure to form a 2D monolayer of graphitic structure analogous to a polycyclic aromatic hydrocarbon of quasi infinite size (Fitzer et al., 1995). It has become a popular choice in the development of electrochemical biosensor devices due to its excellent electrical, chemical and material properties which include large surface area, charge carrier concentrations and exceptional electron mobility with ballistic electron transport. CVD produces large area graphene which can be easily transferred to any substrate in fabricating miniaturized biosensor devices. We believe that being an atomically thin (0.33 nm) material, fabrication of nanogap would be much easier. Also large size gold nanoparticle conjugated ligand binding across the narrower nanogap would not hindered due to thickness of the electrode materials. The use of highly conductive graphene as a nanogap electrode for electrical biosensing is being reported for the first time in this work.

We will be using graphene nanogap electrode fabricated using E-beam lithography (EBL) in developing an electrical biosensor. We have chosen streptavidin –biotin interaction as a model system in testing on graphene nanogap electrode. We also study the effect of the gap size, gold nanoparticle diameter and protein coverage on the gold nanoparticles in optimizing the biosensor performance. The overall goal of this project is to develop a label-free, highly sensitive, selective, rapid point-of-care bisensor using a novel graphene nanogap electrode. This simple electrical transduction based biosensor

platform has great potential to be designed as a portable device with a battery operated handheld multimeter.

5.2 EXPERIMENTAL

5.2.1 Materials

Streptavidin labelled gold nanoparticles (Strep-AuNP) solution of 10 and 60 nm diameter was purchased from Nanocs Inc. 10 nm and 60 nm strep-AuNP solution contains 25 ug/ml of streptavidin, 2.8×10^{13} nanoparticles/ml and 2.3×10^{11} nanoparticles/ml respectively. Biotinamidohexanoic acid N-hydroxysuccinimide ester (Biotin-LC NHS) was purchased from Sigma Aldrich. Phosphate buffer 50 mM of pH 7.4 was used as incubation and blocking buffer (with 0.5% Tween 20). All organic solvents were of analytical grades and purchased from Fischer scientific. Water used in the sensing experiments is purified using Mili-Q purification system (Millipore).

5.2.2 Instruments

Tube furnace (Linderberg/Blue, Mini-Mite TM by Thermo scientific) with mass flow controller (MFC) was used for chemical vapor deposition (CVD) growth of graphene. Si (100) wafer with a 300 nm thermally grown oxide was used as a substrate for electrical device fabrication. Semiconductor parameter analyzer (HP Agilent 4156B) was used in the electrical measurements. E-beam evaporator (Temescal BJD-1800) was used in depositing metal contact pads. E-beam lithography system used is a Leo SUPRA 55 with nanometer pattern generation system (NPGS) capabilities. The Focused Ion Beam Milling system (FIB) used is a Leo XB1540. Scanning electron microscopy (SEM) images were obtained on a XL30 FEG system. Hysitron Inc. TI-950, triboindenter was used in nanoindentation

of graphene. An atomic force microscope (AFM) (Veeco Innova, Santa Barbara, CA) was used for charactering the nanogap structure. The SpmLabAnalysis software was used for analyzing the AFM images.

5.2.3 Methods

5.2.3.1 Fabrication of graphene nanogap electrode: Synthesis of graphene and graphene nanogap fabrication using E-beam lithography (EBL) on photolithographically patterned graphene chip as described previously in method section of chapter 4.

5.2.3.2 Functionalization of nanogap with biotin: The nanogap space of SiO_2 substrate between the pair of graphene electrodes is treated with piranha solution (sulfuric acid: hydrogen peroxide, 5:1) for 30 mins and washed thoroughly with water. The hydrophilic SiO_2 surface is then functionalized with a self-assembled (SAM) monolayer of 3-aminopropyl triethoxysilane (APTES) by incubating the chips in APTES solution for 1 hr. Chips are washed with water, dried, 10 mM Biotin LC NHS solution in DMF is drop casted (5 μl) to cover the electrode area and kept for incubation. After an hour, chips were washed with water and dried gently with nitrogen blow dry.

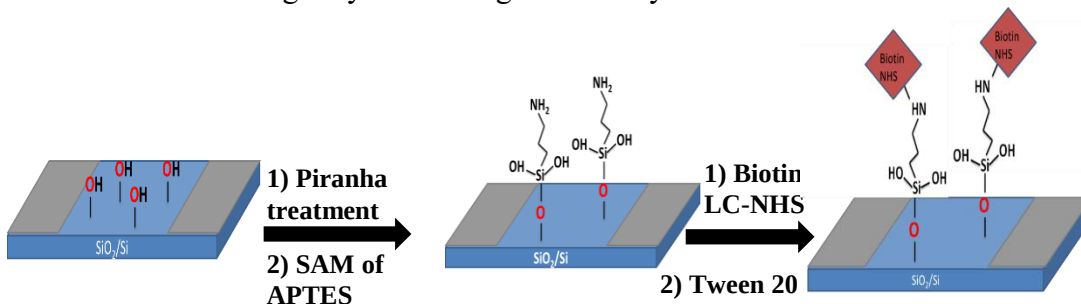


Figure 5.1: Schematic of steps in functionalization of nanogap with biotin

5.2.3.3 Incubation with streptavidin and electrical measurements

To prevent the nonspecific adsorption of streptavidin on the graphene surface, chips were incubated with aqueous 0.5% Tween 20 and 1 % BSA for 30 min. Chips were then incubated for 1 hour with 5 ul each of different dilutions of strep-AuNP. Chips washed with PB buffer, water and dried gently with N₂. Electrical measurements were performed before and after strep-AuNP incubation using semiconductor parameter analyzer. The applied voltage ranged from -5 to +5 V with 50 mV steps.

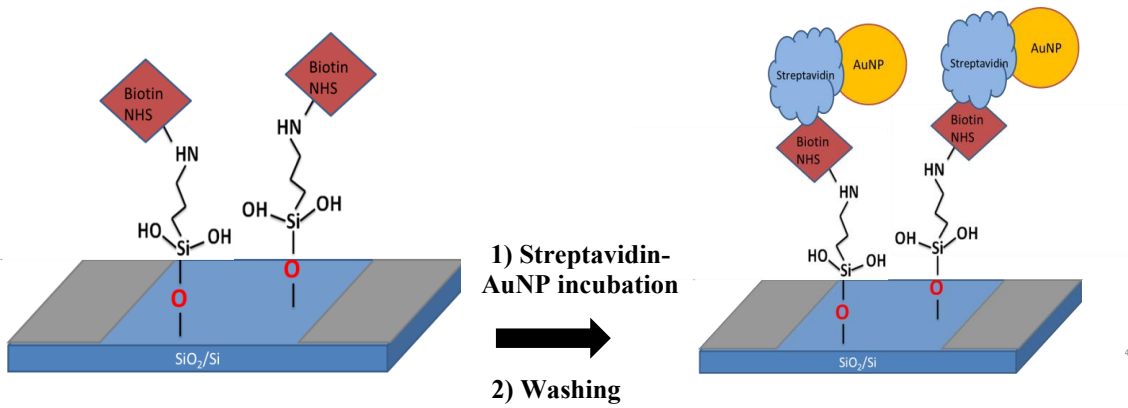


Figure 5.2: Schematic of strep-AuNP incubation and capture in the nanogap

5.2.3.4 Optimization of biosensor performance

To avoid the harsh treatment of piranha on the graphene electrode, modification in the functionalization procedure was carried out. In which after EBL development and O₂ RIE step, additional step of plasma ashing for 30sec at 100W was performed. Similar to piranha, plasma ashing can also activates the hydroxyl groups on the silicon dioxide in the nanogap

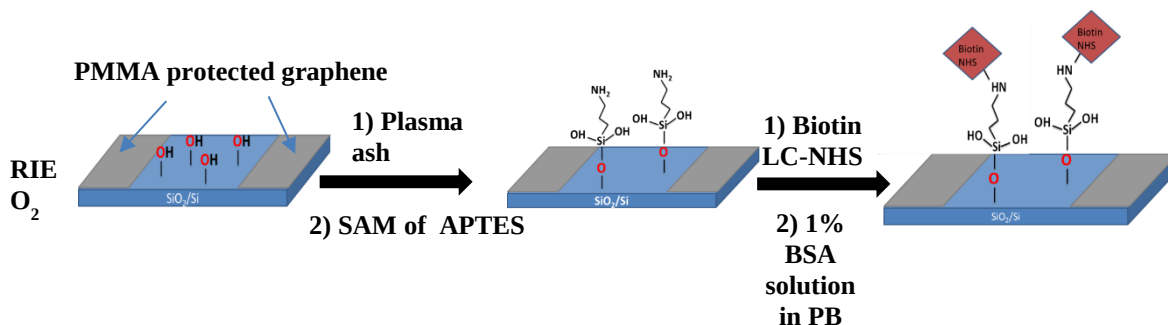


Figure 5.3: Schematic of modified protocol of functionalization of nanogap

for efficient functionalization with APTES. After APTES treatment is done then the chip is washed with acetone to remove the PMMA resist protecting the graphene electrode. The sensing performance in terms of lowest concentration of streptavidin on the AuNP that first bridge the gap (LCFB), resulting in conductance change, was measured by varying the gap size, AuNP diameter and protein coverage on the AuNP. Gap size of 30 and 60 nm with strep-AuNP of 10 and 60 nm diameter were used in the testing. To vary the protein coverage on the AuNP, streptavidin conjugation was carried out using passive adsorption method. Streptavidin needed to 100% coverage for each size of AuNP was first calculated using sodium chloride titration method (Thobhani et al., 2010). Based on which amount of protein varied for conjugation and rest of the surface on the AuNP was blocked with bovine serum albumin (1%) solution.

5.3 RESULTS AND DISCUSSION

The principle of electrical detection of streptavidin in the proposed biosensor is based on the electrical current/conductivity change associated with the bridging of the nanogap between two planar electrodes by metallic nanoparticle(s) (Figure 5.4). This bridging event

occurs as a result of biospecific binding of streptavidin captured on AuNP to the biotin immobilized within the nanogap. In principle, the electrical current/conductivity change is a function of the number of nanoparticles that fill the gap, which in turn is a function of number of target analyte molecules.

Graphene nanogap with 65 nm gap width and strep –AuNP of 10 nm was first tested. Five electrodes each with 65 nm gap were incubated with ten times diluted strep –AuNP of 10

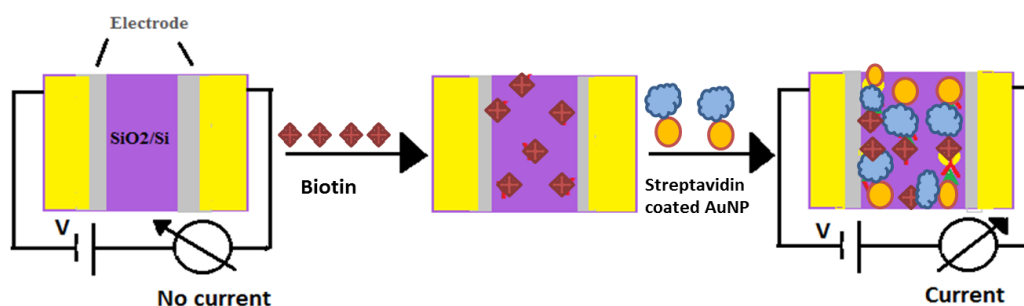


Figure 5.4: Operating principle of nanogap biosensor for detecting streptavidin-biotin

nm followed by I-V current measurements and SEM observation. Figure 5.5a shows the AuNP captured in the nanogap as well as outside. In contrast, no AuNP were seen in the nanogap when just PBS was incubated as well as when nanogap was not functionalized with biotin. This proves the bispecific capture of strep-AuNP in the nanogap area.

I-V curve was measured first after biotin functionalization with blocking step then after incubation with strep-AuNP (Figure 5.5b). Current measured after blocking step at $\pm 5V$ is below $10^{-9} A$ associated with background current. Subsequent incubation with strep-AuNP increased the current

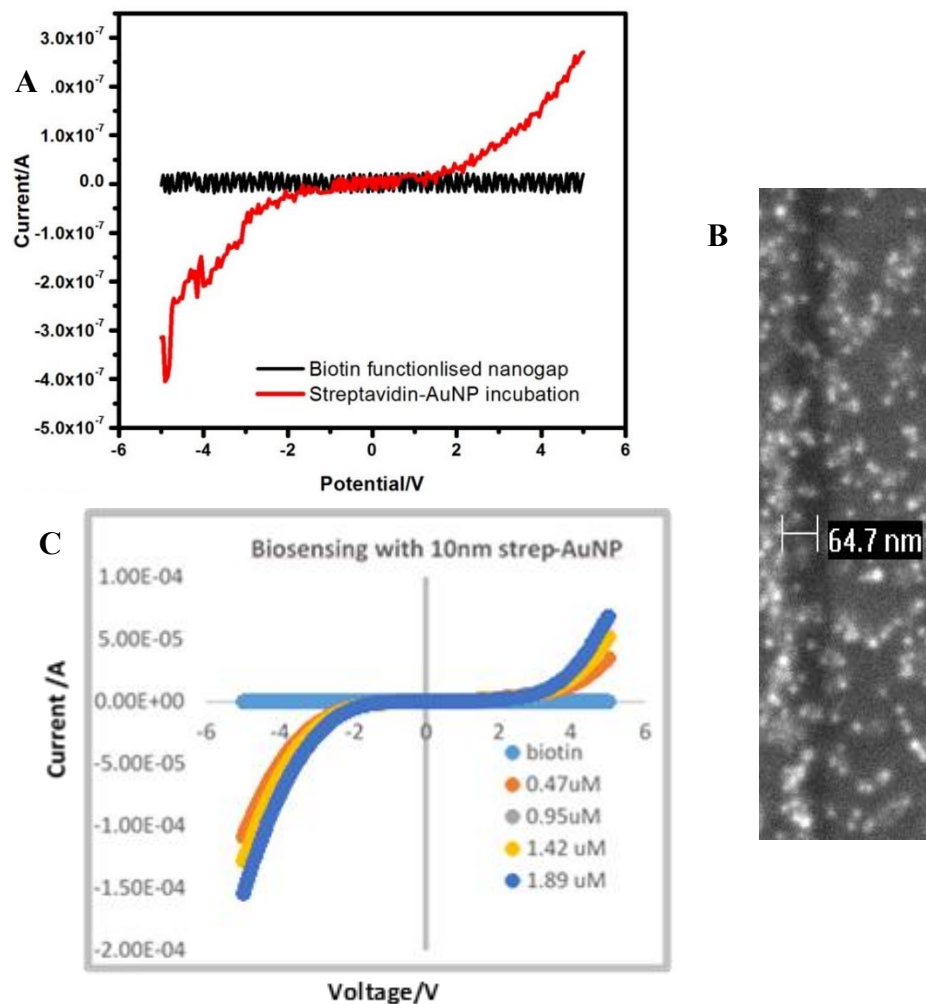


Figure 5.5: A) I- V curve with biotin functionalized and after detection of strep-AuNP B) SEM image of nanogap with captured strep-AuNP C) I-V curves with different concentration of streptavidin

from 10^{-9} to 10^{-7} A resulted from capture of strep-AuNP with threshold voltage of ± 1 V. A non-linear I(V) (s-shaped) curve attributes the presence of AuNP in the nanogap. Different concentration of strep-AuNP was then applied to the nanogap electrode and current

response was measured at $\pm 5V$. LCFB was found to be 25.77 ng. Figure 5.6 shows the I-V curves at different concentration of strep-AuNP.

5.3.1 Optimization of biosensor performance

To study the effect of AuNP diameter on LCFB of the 65 nm nanogap, AuNP size of larger diameter (60 nm) was used. Different concentrations of strep-AuNP was applied on the nanogap electrodes. Figure 5.6 shows the I-V curves for different concentration of strep-AuNP of 60 nm on nanogap of 65 nm. Change in the current was first observed at incubation of streptavidin concentration of 16.879 ng (LCFB). To further investigate the effect of gap size, nanogap of ~ 30 nm gap width was used for sensing experiment with strep-AuNP of 10 and 60 nm. LCFB for 30 nm gap and 10 nm Strep-AuNP was found to be 5 ng whereas LCOB for 30 nm gap and 60 nm strep-AuNP was found to be 0.2978 ng.

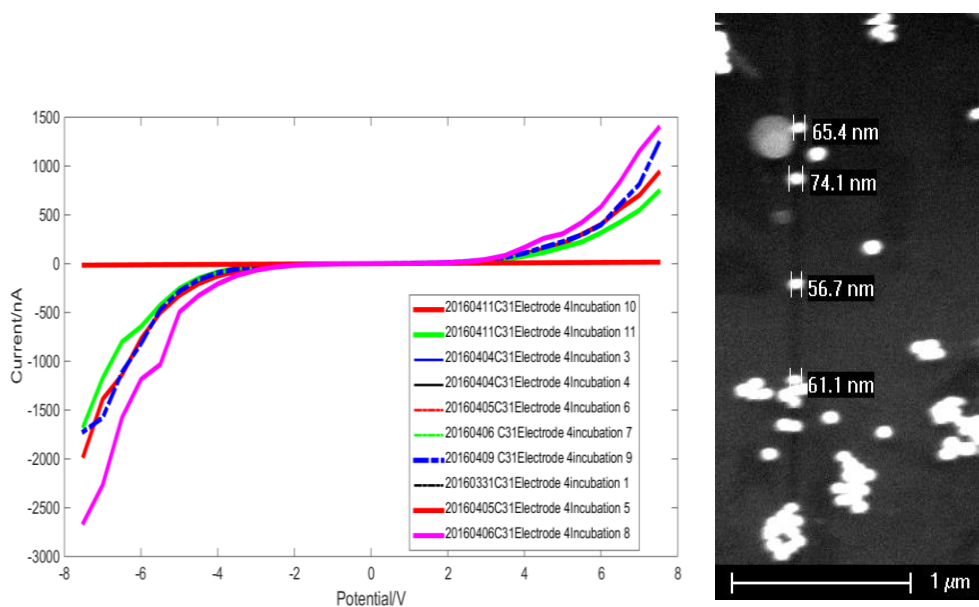


Figure 5.6: I-V curve response with increasing concentration of strep-AuNP of 60 nm

Based on our hypothesis that streptavidin coverage (number of streptavidin) on the AuNP should play an important role as it will decide how efficiently the streptavidin comes in vicinity of the biotin for the capture at the same time it will also be decisive factor for LCFB. Therefore, we increased the surface coverage of the streptavidin on AuNP compared to the commercial strep-AuNP. We observed that increasing the coverage on the streptavidin resulted in higher LCFB. LCFB for 15 nm strep-AuNP with coverage of 4 % on 65 nm and 30 nm gap is 112 ng and 11.2 ng respectively.

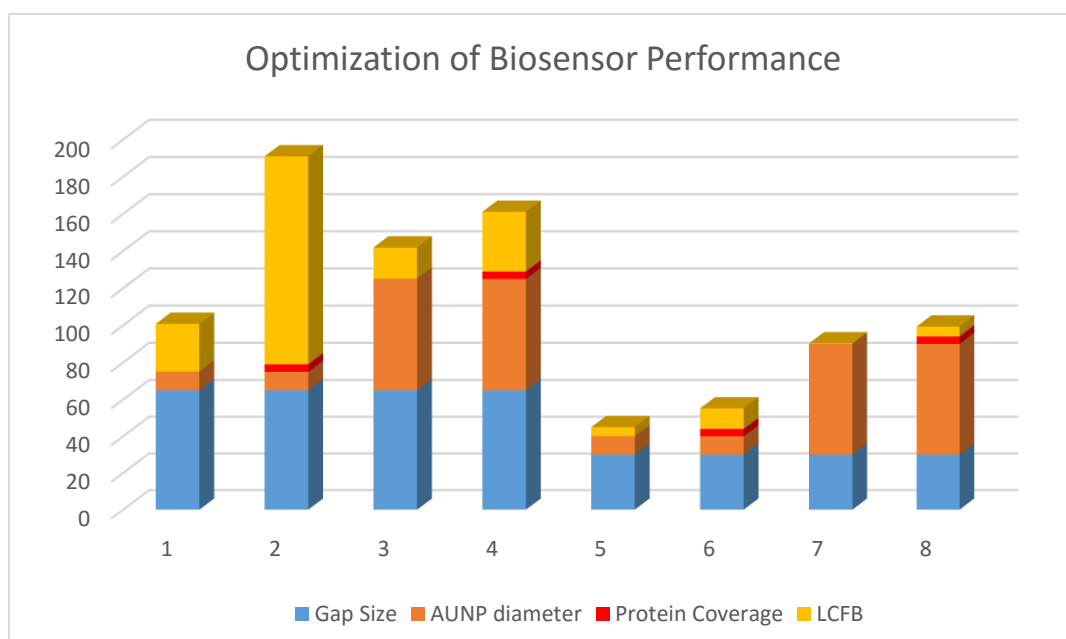


Figure 5.7: Column chart compiling all the nanogap sensor parameters and their LCFB.

Whereas testing of 40 nm strep-AuNP with coverage of 4 % on 65 nm and 30 nm gap resulted in LCFB of 32.1 and 5.35 ng respectively. In case of too low streptavidin coverage (less than 1 %) probability for streptavidin coming in vicinity of biotin goes down (data not shown). Therefore, the streptavidin coverage of 2% was found to be optimum with gap size of 30 nm and 60 nm strep-AuNP. Figure 5.7 shows the column chart compiling all the parameters and their LCFB.

Analytical characterization was carried out for the optimized combination of gap size, strep-AuNP and protein coverage. Calibration response was measured with increasing concentration of strep-AuNP (Figure 5.8). Sensitivity and LOD of streptavidin detection with optimized parameters was found to be 0.3 $\mu\text{A}/\mu\text{M}$ of streptavidin and 0.24 pM respectively.

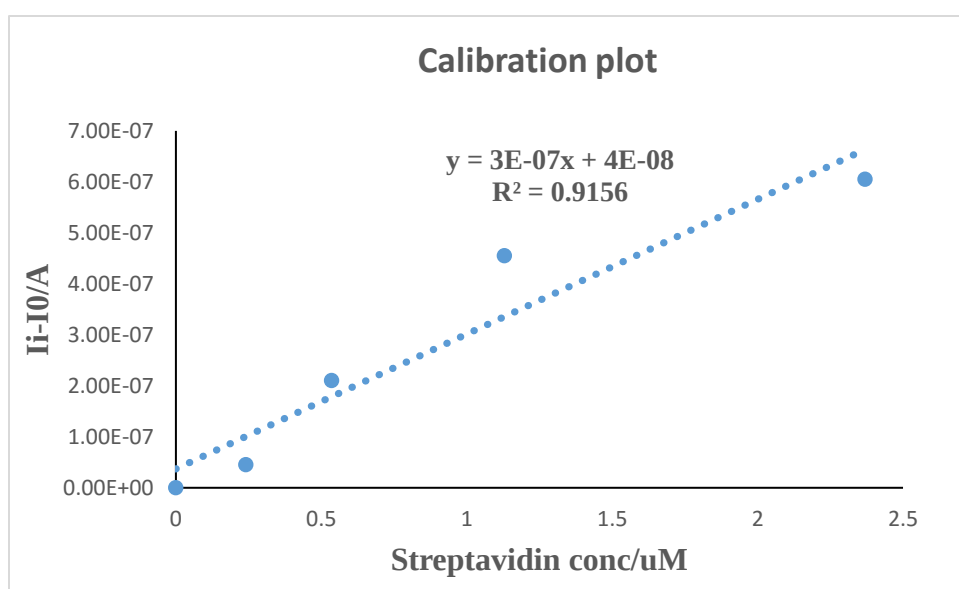


Figure 5.8: Calibration plot for optimized combination i.e.30 nm gaps width, 60 nm Strep-AuNP with 2% coverage.

5.4 CONCLUSION

A proof-concept study for graphene based electrical biosensing has been successfully carried out. For the first time graphene nanogap electrodes are used for electrical biosensing with their analytical characterization in terms of sensitivity and LOD. Detailed studies were carried out to establish the co-relation between gap size, AuNP and protein coverage, in developing high sensitive electrical biosensor platform. This high sensitive

biosensor platform has tremendous potential to be used as a point-of-care device in clinical diagnosis mainly in early disease diagnosis

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Chapter 6

Conclusions

Graphene advance nanostructures, graphene-carbon nanotube (G-CNT) hybrid and graphene nanogap electrode have shown great promise in the development of highly sensitive biosensor development. The G-CNT hybrid was grown by two-step CVD method with seamless nanostructure resulting in faster electron transfer kinetic. The 3D conductive network of electrochemically oxidized G-CNT resulted in DET favorable orientation of glucose oxidase on the electrode. The sensitivity of G-CNT based glucose biosensor was found to be higher than composite hybrid of G-CNT where graphene and CNTs are physically mixed together. Electrochemically functionalized CVD grown seamless G-CNT structure used in this work has potential to be used for development of artificial mediatorless redox enzyme based biosensors and biofuel cell.

A proof-of-concept for novel graphene nanogap based electrical biosensor was presented using streptavidin-biotin model system. Three different nanofabrication methods i.e. FIB, EBL and nanoindentation, were studied in achieving reproducible nanogap of less than 100 nm. EBL was found to be reproducible method over other where graphene structure was damaged due to gallium ions in FIB or non-reproducibility due to wear and tear of the nanoindenter tip. Reproducible planar graphene nanogap electrodes were fabricated using combination of photolithography and EBL. Affinity interaction of streptavidin from the AuNP across to the biotin in the gap, result in localization of AuNP and is detected based on the change in the conductance. Significant change in conductance was observed due to a narrower gap size and superior conductivity of the electrode

material. Biosensor performance was optimized in terms of gap size, AuNP diameter and streptavidin coverage on the AUNP. Optimized combination based on the lowest concentration for gap bridging was found to be 30 nm nanogap width, 60 nm Strep-AuNP diameter with 4% coverage on the AuNP. The Sensitivity and LOD of streptavidin detection with optimized parameters was found to be 0.3 $\mu\text{A}/\mu\text{M}$ of streptavidin and 0.24 pM respectively. The detection capability of this biosensor can be tuned down to the single to few molecules. Proposed biosensor platform can be used for any detection based on biomolecules affinity interaction such as for antigen-antibody or chemo selective interaction with full potential to be used as a portable point of use biosensor.

Appendix #1

Electrochemical Properties of Seamless Three-Dimensional Carbon Nanotubes-Grown Graphene Modified with Horseradish Peroxidase

Horseradish peroxidase (HRP) was immobilized through sodium dodecyl sulfate (SDS) on the surface of a seamless three-dimensional hybrid of carbon nanotubes grown at the graphene surface (HRP-SDS/CNTs/G) and its electrochemical properties were investigated. Compared with graphene alone electrode modified with HRP via SDS (HRP-SDS/G electrode), the surface coverage of electroactive HRP at the CNTs/G electrode surface was approximately 2-fold greater because of CNTs grown at the graphene surface. Based on the increase in the surface coverage of electroactive HRP, the sensitivity to H_2O_2 at the HRP-SDS/CNTs/G electrode was higher than that at the HRP-SDS/G electrode. The kinetics of the direct electron transfer from the CNTs/G electrode to compound I and II of modified HRP was also analyzed.

1. INTRODUCTION

Seamless three-dimensional (3D) sp^2 carbon-based nanomaterials, such as foam-like graphene [1, 2] and carbon nanotubes-grown graphene (CNTs/G) hybrid materials [3, 4], have recently attracted great attention not only because of their high electrical conductivity but also their high surface area per unit planar/footprint area. Additionally, since charge carriers are movable in all three dimensions without significant contact resistance, such seamless 3D sp^2 carbon-based nanomaterials are expected to provide electrode materials for high power fuel cells as well as highly sensitive sensors, compared with seamless 2D sp^2 carbon-based nanomaterials, such as graphene. Also, because of the flat surface of its backside, the CNTs/G hybrid has the potential for the development of flexible and wearable biosensors for physiological monitoring.

Redox enzymes, such as horseradish peroxidase (HRP) and glucose oxidase (GOx), are widely used for electrochemical biosensors. However, they are generally difficult to directly communicate with flat metal electrodes due to the deeply embedded active site in the thick insulating polypeptide layer. Meanwhile, it is known that CNTs and graphene flakes can communicate electrochemically with redox enzymes directly, because of their small nanostructure with high conductivity [5–11]. Such carbon nanomaterials have therefore been expected to be utilized for the development of enzyme-based electrochemical biosensors. However, properties of CNTs and graphene emerge only in one direction for CNTs and planar direction for graphene due to their one- and two-dimensional structures, respectively [12–14]. Therefore, controlling the orientation of those carbon nanomaterials is necessary. Additionally, there are also some non-trivial

problems, such as the aggregate formation and significant contact resistance among CNTs and graphene flakes.

Toward the development of highly sensitive enzyme-based electrochemical biosensors without the above problems, we previously constructed CNTs/G hybrid electrodes modified with GOx [15] or heme peptide (HP) [16], which has a similar structure to the active center of peroxidase and has often been used for H₂O₂ sensing [17–19]. However, the catalytic activity of HP is lower than that of HRP and therefore an HRP-based biosensor would be more sensitive compared to one based on HP. Thus, there is an interest in using the full HRP for H₂O₂ sensing. In the present work, we constructed HRP-modified CNTs/G electrodes and studied the kinetics of electron transfer from the CNTs/G hybrid to peroxidase. Based on the previous report by Yan et al., we used surfactant sodium dodecyl sulfate (SDS) to immobilize HRP at the CNTs/G [6]. Furthermore, based on the direct reduction of HRP(P)Fe⁴⁺-oxygen complexes (P: porphyrin ring), such as compound I and II, in the presence of H₂O₂ at sufficiently higher potential relative to the formal potential of the HRP(P)Fe^{2+/3+} couple [20, 21], we compared catalytic reduction currents of H₂O₂ at the HRP-modified CNTs/G electrode with those at HRP-modified graphene electrode (Figure 1). Thus, we confirmed the superiority of CNTs/G hybrid films in electrochemical biosensing, compared with graphene films.

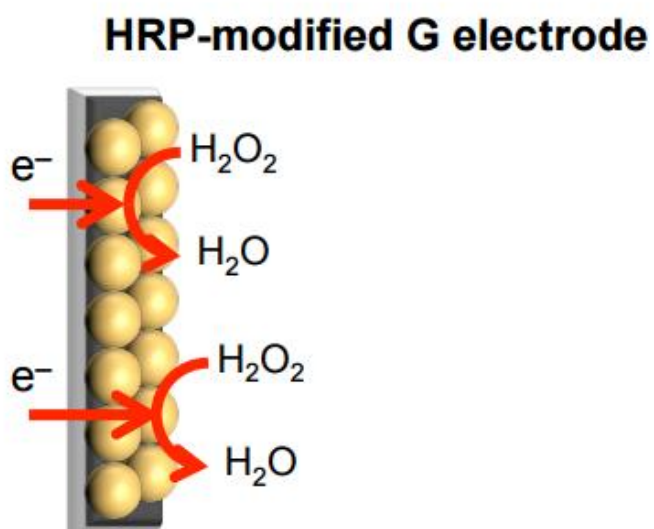
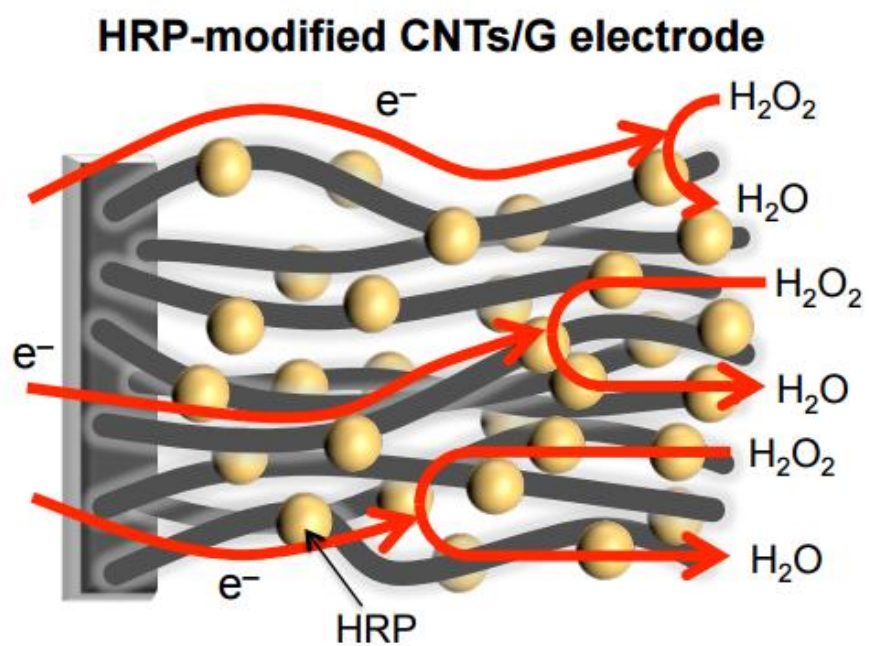


Figure 1. Schematic illustration of a CNTs/G and G electrodes modified with HRP.

2. EXPERIMENTAL

2.1. Preparation of CNTs/G Films

CNTs/G hybrid was prepared according to the previous report [16]. Briefly, a copper (Cu) foil ($\sim 6.5 \text{ cm}^2$ and $25 \text{ }\mu\text{m}$ thick), which is a catalyst and substrate for graphene growth, was placed inside a quartz tube (ca. 3 cm in inner diameter and ca. 75 cm long) and annealed at $1030 \text{ }^\circ\text{C}$ under flowing Ar (190 sccm) and H_2 (10 sccm) at atmospheric pressure in an electric furnace (Lindberg/Blue MTM Mini-MiteTM Tube Furnace, Thermo Scientific). At this time CH_4 (5 sccm) was introduced in the tube for 9 min to grow graphene followed by cooling of the furnace to room temperature in flowing Ar/ H_2 atmosphere. Subsequently, the graphene-formed Cu foil was decorated with 1.5 nm thick iron film, which acted as catalysts for CNTs growth, using an e-beam evaporator (Temescal BJD-1800, Technical Engineering Services). The graphene/Cu foil with the iron film was placed back in the quartz tube and heated to $750 \text{ }^\circ\text{C}$ in flowing Ar (100 sccm) and H_2 (50 sccm) atmosphere. After the temperature was stabilized at $750 \text{ }^\circ\text{C}$, a 15 sccm flow of C_2H_2 was supplied in the tube for 5 min, followed by cooling of the furnace to room temperature in flowing Ar/ H_2 atmosphere. The graphene film formed on the backside of CNTs/G/Cu foil was removed by O_2 plasma, followed by etching of the Cu foil in a 1 M FeCl_3 aqueous solution to obtain the CNTs/G hybrid. The CNTs/G hybrid was cleaned with 5% HCl solution to completely remove the Cu foil and the iron nanoparticles. Note that we also obtained the graphene film using the same procedure. Fundamental properties of CNTs/G and graphene films were reported previously [15, 16].

2.2. Preparation of HRP-Modified Electrodes

A glassy carbon (GC) electrode (3 mm in diameter, ALS Co., Ltd) was polished with 1 and 0.05 μm alumina slurries on a polishing cloth and then thoroughly rinsed with distilled water, followed by sonication in 2-propanol and distilled water, respectively. After drying with a high-purity nitrogen stream, the CNTs/G or graphene was transferred to the GC electrode surface and then kept at 50 °C in an electric oven for 1 h to be firmly attached on the GC surface. Subsequently, the CNTs/G and graphene electrodes were immersed in either A) pH 7, 67 mM phosphate buffer (PB) containing 0.5 mM HRP (Sigma-Aldrich) with 20 mM SDS for 12 h at 4 °C to obtain the HRP-SDS/CNTs/G and HRP-SDS/G electrode, respectively, or B) pH 7, 67 mM phosphate buffer (PB) containing 0.5 mM HRP (Sigma-Aldrich) for 12 h at 4 °C to obtain the HRP/CNTs/G and HRP/G electrode, respectively.

2.3. Electrochemical Measurements

Electrochemical measurements were performed with a potentiostat Versa STAT (Princeton Applied Research, USA) in 67 mM phosphate buffer (pH 7.4) in a batch system. A Ag|AgCl|KCl (sat.) and a coiled platinum wire were used as reference and counter electrodes, respectively. The catalytic activity of HRP-SDS/CNTs/G and HRP-SDS/G electrodes toward H_2O_2 reduction was evaluated by amperometric measurements. After the working electrode was polarized at +150 mV and a steady-state current was obtained, a H_2O_2 solution was added into the electrolyte solution. From the steady-state current obtained here, the catalytic activity of HRP at the CNTs/G and graphene electrodes was determined.

3. RESULTS AND DISCUSSION

3.1. Electrochemical Characterization

Cyclic voltammetry (CV) were performed at the G, CNT/G, HRP/G, HRP/CNTs/G, HRP-SDS/G and HRP-SDS/CNTs/G electrodes in deaerated PB. As shown in Figure 2A, there was no apparent background redox peak at both the CNT/G and graphene electrodes. Similarly, the HRP/CNTs/G and HRP/G electrodes also did not show redox peak (data not shown). On the other hand, redox peaks appeared at both the HRP-SDS/G and HRP-SDS/CNTs/G at approximately -320 mV (vs. Ag|AgCl), corresponding to $(P)Fe^{2+/3+}$. These findings are similar to reported by Yan et al. [6] and as in their case can be ascribed to properties of the SDS for facilitating protein electrochemistry and the structural properties of CNT and the 3D morphology of the CNT/G electrode. We also evaluated cetyltrimethylammonium bromide (CTAB) was for the immobilization of HRP in the phosphate buffer (pH 7.4). However, no redox peaks of $(P)Fe^{2+/3+}$ were clearly observed. Since CTAB is a cationic surfactant and the isoelectric point of HRP is 8.9, HRP might not be immobilized through CTAB probably due to the electrostatic repulsion between CTAB and HRP. We therefore used only SDS modification for HRP immobilization for further studies.

The redox peak current for both electrodes was proportional to the scan rate below at least 1.5 V s^{-1} (Figure 2B), indicating that the redox reaction was a surface controlled process. In addition, the redox peak current at the HRP-SDS/CNTs/G electrode was approximately 2-times larger than that at the HRP-SDS/G electrode. This is in accordance with the previously reported ~ 2.5 -fold larger apparent electroactive surface area for the

CNTs/G film compared to graphene because of CNTs grown at the graphene surface [16]. Since the peak current was proportional to the scan rate below at least 1.5 V s^{-1} (Figure 2B), the surface coverage Γ of electroactive HRP at both CNTs/G and graphene electrodes was determined from the equation,

$$I_p = n^2 F^2 \Gamma \nu / 4RT \quad (1)$$

where n is the number of electrons transferred ($n = 1$), F is the Faraday constant, ν is the scan rate, R is the gas constant, and T is the temperature. Based on equation 1, Γ was estimated to be $\sim 4.3 \times 10^{-11} \text{ mol cm}^{-2}$ for the HRP-SDS/CNTs/G electrode and $2.1 \times 10^{-11} \text{ mol cm}^{-2}$ for the HRP-SDS/G electrode. On the basis of the reported Γ value of $5 \times 10^{-11} \text{ mol cm}^{-2}$ for the HRP monolayer-modified basal plane pyrolytic graphite (BPPG) electrode through didodecyldimethylammonium bromide [22], we hypothesize that HRP molecules formed a monolayer at the graphene and CNTs/G surfaces.

As mentioned above, the present redox reaction of HRP is a surface controlled process. In addition, as the scan rate increased, the peak-to-peak separation (ΔE_p) also gradually increased. To obtain the kinetic parameter for HRP redox, the apparent

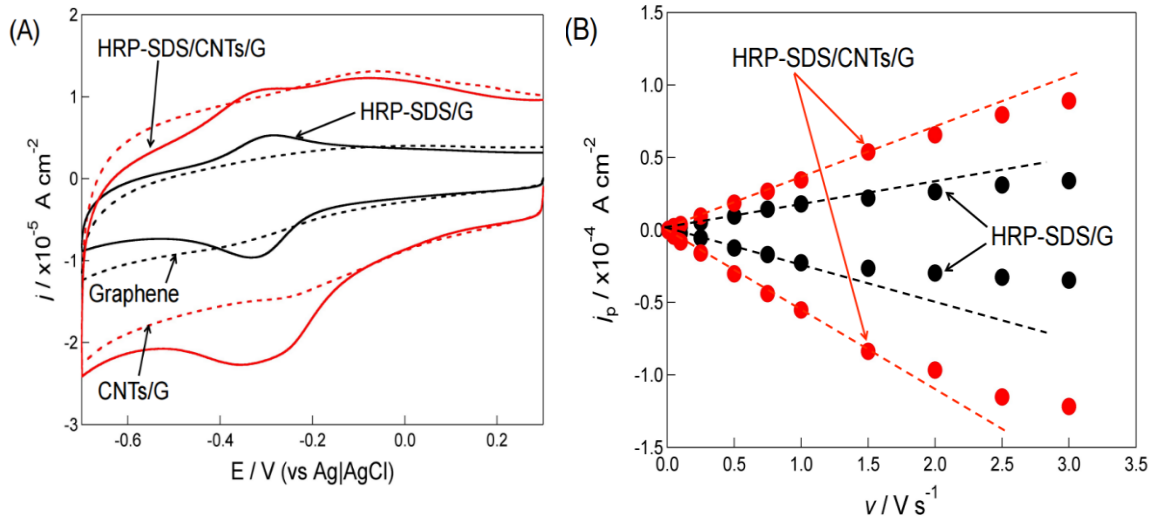


Figure 2. (A) CVs of graphene, CNTs/G, HRP/G, and HRP/CNTs/G electrodes in a 0.067 M phosphate buffer solution (pH 7.4) at scan rate of 100 mV s⁻¹. (B) Relationship between anodic and cathodic peak currents of HRP/G and HRP/CNTs/G electrodes and scan rate.

heterogeneous electron transfer rate constant k_{app} was therefore calculated according to the Laviron's theory [23]. The anodic and cathodic peak potential E_{pa} and E_{pc} can be represented as follows:

$$E_{pa} = E^0 - (2.3RT/anF)\log(\alpha/m) \quad (2)$$

$$E_{pc} = E^0 + \{2.3RT/(1-\alpha)nF\}\log\{(1-\alpha)/m\} \quad (3)$$

$$m = RTk_{app}/F\eta v \quad (4)$$

where α is the transfer coefficient. Based on the plots of E_{pa} or $E_{pc} - E^0$ versus $\log v$, α can be determined from the slopes of the anodic and cathodic processes (Figure 3) to be about 0.50 ± 0.02 and 0.41 ± 0.04 for HRP-SDS/CNTs/G and HRP-SDS/G, respectively.

According to the equations 2–4, ΔE_p can be obtained by the following equation, which is accepted above 200 mV:

$$\Delta E_p = \{2.3RT/(1-\alpha) \alpha nF\} \{ \alpha \log(1-\alpha) + (1-\alpha) \log \alpha - \log(RT/nF) - \log k_{app} \} + \{2.3RT/(1-\alpha) nF\} \log v \quad (5)$$

Based on this equation, k_{app} was determined to be $19.2 \pm 2.5 \text{ s}^{-1}$ for the HRP-SDS/CNTs/G electrode and $15.7 \pm 2.3 \text{ s}^{-1}$ for the HRP-SDS/G electrode, respectively. The difference in k_{app} can be ascribed to a small amount of defects and/or graphene edge sites in the CNTs/G hybrid. It is known that heterogeneous electron transfer reactions of redox species at edge-oriented pyrolytic graphite (EOPG) electrodes are generally faster than those at BPPG electrodes [24–27]. In addition, it is known that the heterogeneous electron transfer reaction rate is accelerated at carbon nanofibers with stacked graphene, which provided a high density of the edge site at their surfaces [28, 29]. As reported previously, the existence of defects and/or graphene edge sites in the CNTs/G hybrid were confirmed from Raman spectroscopy, whereas the graphene film formed a monolayer sheet with very few defects and edge sites [16]. Thus, the heterogeneous electron transfer rate of HRP might be facilitated at the CNTs/G in comparison with the graphene. Meanwhile, the k_{app} value obtained here was larger than that for previously reported HRP-SDS/CNTs (3.5 s^{-1}) [6] and HRP-Nafion/G (4.63 s^{-1}) [30] electrodes. The present CNTs/G and graphene electrodes were basically the seamless structure, whereas the previous CNTs and graphene electrodes consisted of their nanotubes and nanoflakes assembled on the GC electrode. Compared

with the assembled structure, the seamless structure might facilitate the electron transfer to some extent.

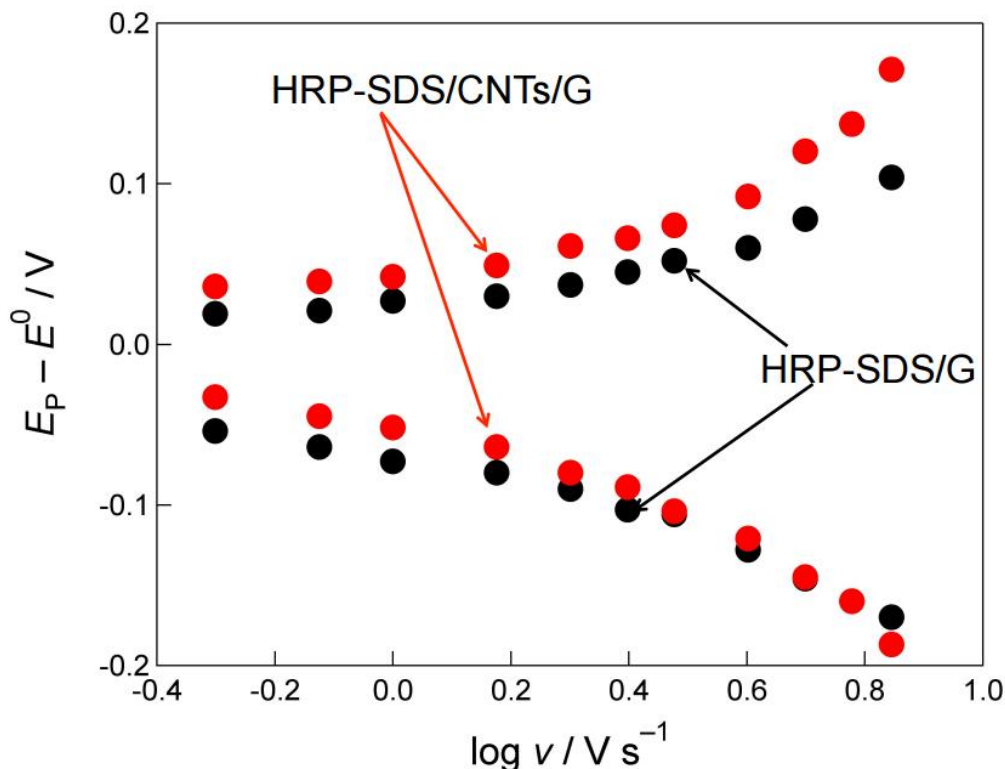
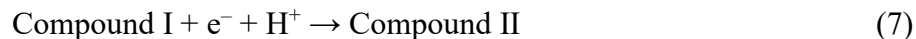


Figure 3. Dependences of $E_P - E^0$ of redox couple for HRP/G and HRP/CNTs/G electrodes on the logarithm of scan rate (Laviron plots) in the 0.067 M phosphate buffer solution (pH 7.4).

3.2. Catalytic Reduction of H₂O₂

Figure 4 shows typical cathodic current responses to H₂O₂ at CNTs/G and graphene electrodes, on which HRP was immobilized via SDS. The cathodic current at the HRP-SDS/CNTs/G and HRP-SDS/G electrodes was significantly larger than that at CNTs/G and graphene electrodes without HRP. This considerably enhanced current responses at enzyme modified electrodes can be credited to electron transfer from CNTs/G and graphene electrodes to HRP. The possible reaction mechanisms are proposed as follows [20, 21].



where compound I and II are oxidized complexes of HRP, such as $[(\text{HRP})\text{Fe}^{4+}=\text{O}]^{+\bullet}$ and $[(\text{HRP})\text{Fe}^{4+}-\text{OH}]^+$, respectively. Further, as shown in Fig. 4, the cathodic current at the HRP-SDS/CNTs/G and HRP-SDS/G electrodes increased and then leveled off, as the concentration of H_2O_2 increased. The initial linear increase of current as a function of H_2O_2 concentration suggests the reaction 6 to be rate determining. On the other hand, the subsequent plateauing of current points to reaction 7 and/or 8 to be rate determining. It is to be noted, here the current response was not influenced when the electrolyte solution was stirred, an indication the catalytic cathodic currents obtained at both HRP-SDS/CNTs/G and HRP-SDS/G electrodes were kinetically controlled. Therefore, all electroactive HRP molecules should contribute to the current. The cathodic current response at the HRP-SDS/CNTs/G electrode was about 2-fold larger than that at the HRP-SDS/G electrode, reflecting that the surface coverage of electroactive HRP at the former is about 2 times larger than that at the latter, as mentioned above.

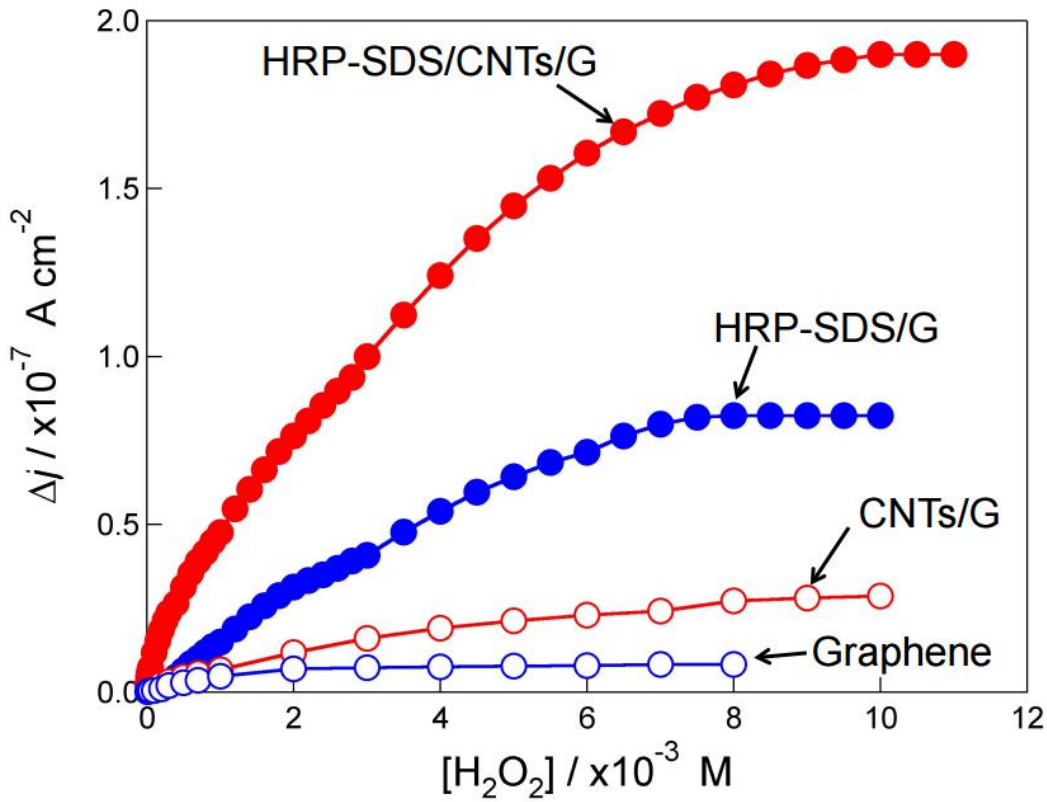


Figure 4. Cathodic current densities of H₂O₂ reduction at the graphene, CNTs/G, HRP/G, and HRP/CNTs/G electrodes in the 0.067 M phosphate buffer solution (pH 7.4) at +150 mV vs. Ag|AgCl

3.3. Analysis of Reaction Kinetics

Based on the above findings that cathodic current was not diffusion controlled and was proportional to the surface coverage of electrocative HRP molecules, the rate constants for the three reactions (equations 6 to 8) can be calculated using the equation 9 [20, 21, 31],

$$i = 2F\Gamma/[1/k_1C_S + (k_2 + k_3)/k_2k_3C_H] \quad (9)$$

where k_1 , k_2 , and k_3 are the rate constant of reactions 6-8, C_S and C_H are the H₂O₂ and proton concentration in the bulk solution, respectively. At low H₂O₂ concentrations (linear

range of current vs. H_2O_2 concentration plot in Fig. 4), when almost all HRP is in ferric state, the current response is determined by the rate of reaction 6 and therefore given by the equation [20, 21]

$$i_1 = 2F\Gamma k_1 C_S \quad (10)$$

Based on this equation, the rate constant k_1 for HRP-SDS/CNTs/G and HRP-SDS/G electrodes were estimated to be approximately 370 and 150 $\text{M}^{-1} \text{s}^{-1}$, respectively. Based on the fact that HRP was immobilized through SDS at essentially the same geometrical structure of sp^2 bonded carbon, we predicted the k_1 value at the HRP-SDS/CNTs/G and HRP-SDS/G electrodes to be almost the same. The reason for a larger k_1 at the HRP-SDS/CNTs/G is unclear. A plausible explanation could be an enhanced conformational stability of HRP on the nanofiber structure of CNTs grown at the graphene compared with the flat graphene. Meanwhile, k_1 for the HRP-SDS/CNTs/G electrode was larger than that for CNTs/G electrode modified with HP (ca. 120 $\text{M}^{-1} \text{s}^{-1}$) reported previously [16] because of the difference in molecular complexity. However, the k_1 values obtained here were much smaller than the value for dissolved HRP ($1.8 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$) [32]. A reason is the likely denaturation of HRP at CNTs/G and graphene due to its strong adsorption via SDS.

At higher H_2O_2 concentrations (plateau region of current vs. H_2O_2 concentration plot in Fig. 4), assuming that all the HRP molecules are existing as compound I or II and the reaction is not controlled by proton diffusion but the enzymatic reaction, the cathodic current i_{23} is given by the equation [20, 21]

$$i_{23} = 2F\Gamma k_2 k_3 C_H / (k_2 + k_3) \quad (11)$$

Based on the equation 11, the $k_2k_3/(k_2 + k_3)$ value at the HRP-SDS/CNTs/G electrode was calculated to be about $7.6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ which was slightly larger than that at the HRP-SDS/G electrode (ca. $6.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$). In addition, the values of the apparent heterogeneous rate constant, $C_H k_2k_3/(k_2 + k_3)$, at pH 7.4 were estimated to be $\sim 3.0 \times 10^{-2}$ and $2.5 \times 10^{-2} \text{ s}^{-1}$ for HRP-SDS/CNTs/G and HRP-SDS/G electrodes, respectively. This difference is likely due to the existence of the small amount of defects and/or edge sites in the CNTs/G, as mentioned above. Actually, the $k_2k_3/(k_2 + k_3)$ values obtained here were one order of magnitude smaller than that obtained at pH 7.0 for the HRP-adsorbed pyrolytic graphite electrode reported by Razgus et al. ($6.6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) [33]. In contrast, the obtained values were larger than that at a HRP-adsorbed graphite powder-coated electrode ($5.6 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$) [20]. For the graphite powder coating, a binder is often required for the fabrication of such graphite electrodes. It is known that the binder sometime interferes with the direct electron transfer between enzymes and graphite powder [20]. In the present work, no binder was required for the preparation of CNTs/G modified electrodes. Thus, the direct electron transfer from HRP to the CNTs/G might be accelerated, though the edge sites of graphene were virtually absent. However, the k_1 value for CNTs/G was significantly small, as mentioned above. Due to this, the lower detection limit of H_2O_2 for the HRP-SDS/CNTs/G electrode ($\sim 10^{-5} \text{ M}$) was five orders of magnitude larger than that for the HRP-adsorbed graphite powder-coated electrode ($\sim 2 \times 10^{-10} \text{ M}$) [20]. By improving this parameter, we strongly believe that CNTs/G could be applied for the development of a highly sensitive electrochemical H_2O_2 biosensor. For example, the surface modification of

the CNTs/G with metal nanoparticles [34, 35] and metal nanosheets [36] might help retain enzymatic activity.

4. CONCLUSION

The electrochemical properties of the CNTs/G electrode modified with HRP through SDS were investigated to explore the enhancement of the sensitivity to analyte H_2O_2 because of the increase in the surface coverage of electroactive HRP at the CNTs-grown on graphene surface. Compared with the HRP/G electrode, the catalytic current for H_2O_2 reduction at the HRP/CNTs/G electrode increased by a factor of about 2. The surface coverage of HRP was about $\sim 4.3 \times 10^{-11} \text{ mol cm}^{-2}$ at the CNTs/G electrode. The rate constant k_1 was estimated to be $\sim 370 \text{ M}^{-1} \text{ s}^{-1}$. The overall rate constant of electron transfer reaction $k_2k_3/(k_2 + k_3)$ and the apparent heterogeneous rate constant $C_Hk_2k_3/(k_2 + k_3)$ at pH 7.4 were $\sim 7.6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $3.0 \times 10^{-2} \text{ s}^{-1}$, respectively. Thus, we hypothesize that the CNTs/G hybrid would be a good candidate as the conductive nanoscaffold for enzymes toward the development of highly sensitive electrochemical biosensors.

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Appendix #2

Bioelectrochemistry of Heme Peptide at Seamless Three-Dimensional Carbon Nanotubes/Graphene Hybrid Films for Highly Sensitive Electrochemical Biosensing

A seamless three-dimensional hybrid film consisting of carbon nanotubes grown at graphene surface (CNTs/G) is a promising material for the application to highly sensitive enzyme-based electrochemical biosensors. The CNTs/G film was used as conductive nanoscaffolds for enzymes. The heme peptide (HP) was immobilized on the surface of the CNTs/G film for amperometric sensing of H_2O_2 . Compared with flat graphene electrodes modified with HP, the catalytic current for H_2O_2 reduction at the HP-modified CNTs/G electrode increased due to the increase in the surface coverage of HP. Additionally, microvoids in the CNTs/G film contributed to diffusion of H_2O_2 to modified HP, resulting in the enhancement of the catalytic cathodic currents. The kinetics of the direct electron transfer from the CNTs/G electrode to compound I and II of modified HP was also analyzed.

1. INTRODUCTION

Nanomaterials based on sp^2 carbon, such as graphene and carbon nanotubes (CNTs), are one of the promising materials for the development of batteries, fuel cells, capacitors, electronics, and sensors because of their high electrical conductivity, high specific surface area, and good chemical stability.¹⁻³ In particular, graphene and CNTs have attracted attention for the development of enzyme-based electrochemical biosensors due to their ability to achieve direct electrochemical communication with redox enzymes,⁴⁻⁷ that is otherwise difficult because of the thick insulating polypeptide layer covering the active site. However, such superior properties emerge only in the planar direction for graphene or one direction for CNTs due to their two or one-dimensional structures, respectively. In addition, graphene and CNTs are widely used after their coating on a substrate, such as electrodes, but contact resistance among those sp^2 carbon-based materials is significant.

To overcome this limitation, seamless three-dimensional (3D) sp^2 carbon-based nanomaterials, such as foam-like graphene^{8, 9} and carbon nanotubes-grown graphene (CNTs/G) hybrid materials,^{10, 11} have recently been synthesized. Due to the seamless structure, these 3D carbon nanomaterials enable charge carriers to move in all the three dimensions without significant contact resistance. Furthermore, the 3D carbon nanomaterials also enable the enhancement of the total surface area per unit planar/footprint area on the substrate. Hence, it is expected to improve the sensitivity and/or expand the dynamic range due to the increase in the amount of enzymes per unit area. For the development of biosensors, compared with the foam-like graphene, the CNTs/G film is particularly a preferred structure to attach on the substrate because its

backside is flat surface. For instance, the CNTs/G film is expected to develop flexible and wearable biosensors for physiological monitoring. However, the electron transfer reaction at the interface of the CNTs/G film is not yet well elucidated.

Toward the development of highly sensitive enzyme-based electrochemical biosensors, there is a need for the elucidation of the electron transfer between the CNTs/G film and enzymes. In addition, the three-dimensional structure-based microvoid effects on the sensitivity and/or dynamic range for an analyte also need to be assessed. In the present work, we constructed a heme undecapeptide (HP)-modified CNTs/G film-coated (HP/CNTs/G) glassy carbon electrode and studied the kinetics of electron transfer from the CNTs/G film to HP, which has a similar structure to the active center of peroxidase and has been used for H_2O_2 sensing.¹²⁻¹⁴ By comparing with catalytic reduction currents of H_2O_2 at HP-modified graphene-coated (HP/G) electrodes, we showed advantages of the HP/CNTs/G electrode in the sensitive detection of H_2O_2 . Furthermore, since the active center of HP is not fully covered with the insulating polypeptide and electrochemical responses for H_2O_2 at HP multilayer-modified electrode enhanced through the electron self-exchange among HP molecules. The microvoid effect at the HP multilayer-modified CNTs/G (HP-ML/CNTs/G) electrode on diffusion of H_2O_2 was also examined from the catalytic currents of H_2O_2 reduction in comparison with the case in the HP multilayer-modified graphene (HP-ML/G) electrode.

2. EXPERIMENTAL

Preparation of CNTs/G Hybrid Films.

CNTs/G hybrid films were prepared by a two-step process (Figure 1). First, graphene film was grown on the surface of a copper (Cu) foil ($\sim 6.5 \text{ cm}^2$ and $25 \text{ }\mu\text{m}$ thick) by chemical vapor deposition (CVD) method. The Cu foil, which is a catalyst and substrate for graphene growth, was placed inside a quartz tube (ca. 3 cm in inner diameter and ca. 75 cm long) and annealed at $1030 \text{ }^\circ\text{C}$ under flowing Ar (190 sccm) and H_2 (10 sccm) at atmospheric pressure in an electric furnace (Lindberg/Blue MTM Mini-MiteTM Tube Furnace, Thermo Scientific). At this time CH_4 (5 sccm) was introduced in the tube for 9 min to grow graphene followed by cooling of the furnace to room temperature in flowing Ar/ H_2 atmosphere. Next, CNTs were grown on the surface of the graphene-formed Cu foil by a second CVD. To achieve this, the graphene-formed Cu foil was decorated with 1.5 nm thick iron film, which acts catalysts for CNTs growth, using an e-beam evaporator (Temescal BJD-1800, Technical Engineering Services). The graphene/Cu foil with the iron film was placed back in the quartz tube and heated to $750 \text{ }^\circ\text{C}$ in flowing Ar (100 sccm) and H_2 (50 sccm) atmosphere. After the temperature was stabilized at $750 \text{ }^\circ\text{C}$, a 15 sccm flow of C_2H_2 was supplied in the tube for 5 min followed by cooling of the furnace to room temperature in flowing Ar/ H_2 atmosphere. The CNTs/G film formed on the backside of Cu foil was removed by O_2 plasma, followed by etching of the Cu foil in a 1 M FeCl_3 aqueous solution to obtain the CNTs/G film. The CNTs/G film was cleaned with 5% HCl solution to completely remove the Cu foil and the iron nanoparticles. Note that we also obtained the graphene film using the same procedure. The morphology of the CNTs/G

film was observed by a field emission scanning electron microscope (FE-SEM, Philips XL30 FEG system). The CNTs/G film was also characterized using Raman spectrometer (LabRAM HR Evolution, HORIBA Ltd.) and XPS (PHI Quantera SXM™, Ulvac-Phi Inc.).

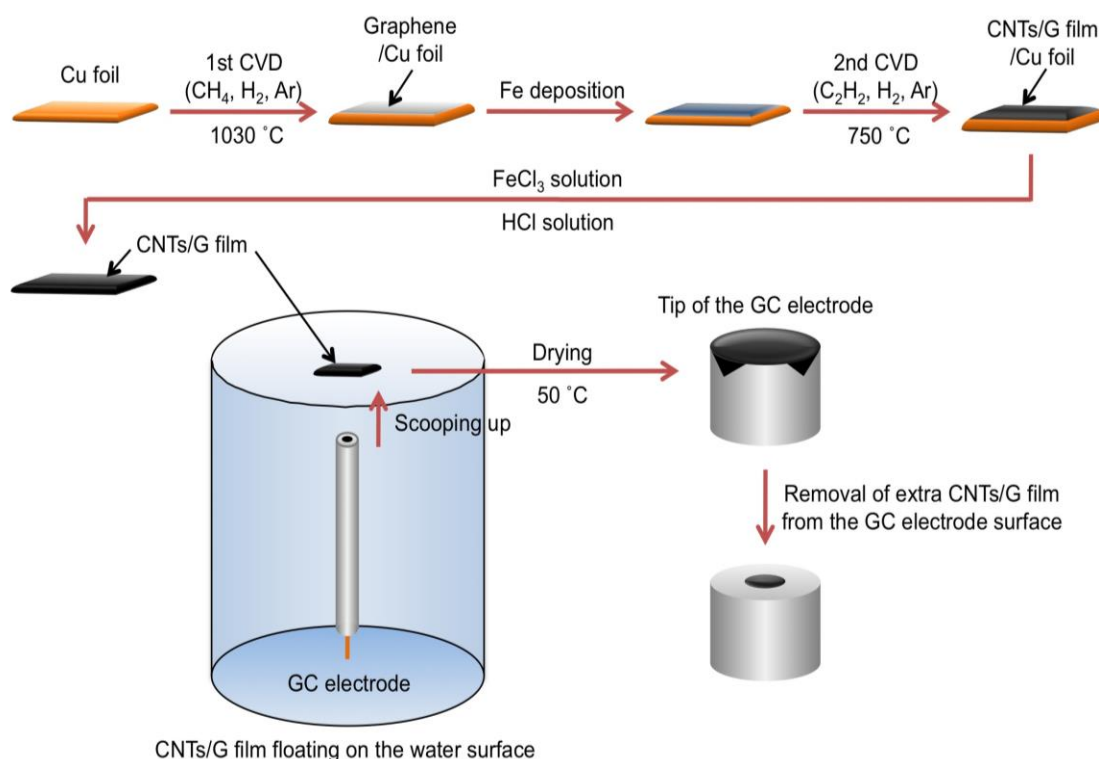


Figure 1. Schematic illustration of the preparation of CNTs/G electrode.

Preparation of HP-Modified CNTs/G Hybrid Electrodes.

A glassy carbon (GC) electrode (3 mm in diameter, ALS Co., Ltd) was polished with 1 and 0.05 μm alumina slurries on a polishing cloth and then thoroughly rinsed with distilled water. The GC electrode was sonicated in 2-propanol and distilled water, respectively, and then dried with a high-purity nitrogen stream. The CNTs/G or graphene film was

transferred to the GC electrode surface, followed by drying at 50 °C in an electric oven for 1 h to obtain CNTs/G and graphene electrodes (Figure 1). The CNTs/G and graphene electrodes were subsequently immersed in *N,N*-dimethylformamide (DMF) containing 6.0 mM 1-pyrenebutyric acid *N*-hydroxysuccinimide ester (PBASE) for 30 min. After thoroughly rinsing with DMF and 67 mM phosphate buffer (pH 7.4), the electrodes were immersed in the 67 mM phosphate buffer (pH 7.4) containing 1.0 mM HP (Sigma-Aldrich) for 12 h at 4 °C to obtain HP/CNTs/G and HP/G electrodes.

To fabricate HP-ML/CNTs/G and HP-ML/G electrodes, 12 μ L of 0.1 M 2-morpholinoethanesulfonic (MES) aqueous solution (pH 4.6) containing 1 mM HP and 0.3 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was cast on the surfaces of HP/CNTs/G and HP/G electrodes. Afterward, these electrodes were stored at 4 °C for 12 h and thoroughly rinsed with distilled water to remove excess casting solution.

Electrochemical Measurements. Electrochemical measurements were performed in 67 mM phosphate buffer (pH 7.4) in a batch system. A Ag|AgCl|KCl (sat.) and a coiled platinum wire were used as reference and counter electrodes, respectively.

The CNTs/G electrode was evaluated in a 0.1 M KCl aqueous solution containing 1.0 mM $[\text{Ru}(\text{NH}_3)_6]\text{Cl}_3$ by cyclic voltammetry (CV) using a potentiostat (CHI750c, CH Instruments Inc., USA). Direct electron transfer reaction between HP and electrode through CNTs/G or graphene film was evaluated by CV using a HSV-110 (Hokuto Denko) potentiostat. The catalytic activity of HP/CNTs/G, HP/G, HP-ML/CNTs/G, and HP-ML/G electrodes toward H_2O_2 reduction was evaluated by amperometry with the potentiostat LC-4C (BAS). After the working electrode was polarized at +150 mV and a steady-state

current was obtained, a H_2O_2 solution was added into the electrolyte solution. From the steady-state current obtained, the catalytic activity of HP at the CNTs/G and graphene electrodes was determined.

3. RESULTS AND DISCUSSION

Characterization of CNTs/G Hybrid Films. Figure 2A shows a photograph of CNTs/G hybrid films floating on water after chemical etching of Cu foil, indicating the formation of a large area film on Cu foil. The CNTs/G hybrid film can therefore be transferred to flat substrates because carbon nanotubes basically grow at the flat graphene surface. Figure 2B and C shows a FE-SEM image of the surface and cross-section of the CNTs/G hybrid film, respectively. We presume that the CNTs grew perpendicularly at the graphene surface during the early stage and then the tip of CNT fell down on neighboring CNTs during the later stage, resulting in the formation of a chain conformation and/or a network-like structure. This indicates that iron nanoparticles formed on the graphene surface for CNTs growth were randomly distributed on the graphene surface. Such structure will allow to increase surface coverage of chemicals, such as enzymes, due to the suppression of carbon nanotube bundle effects.

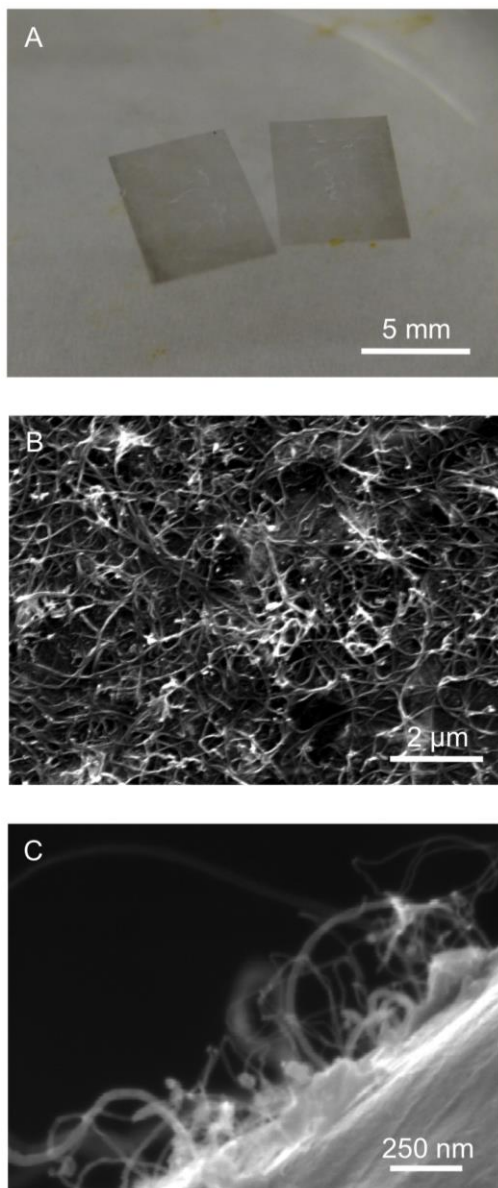


Figure 2. (A) Photograph of CNTs/G films floating on distilled water and FE-SEM images of (B) the surface and (C) cross-section of the CNTs/G film.

Figure 3A shows Raman spectra before and after growth of CNTs on graphene. Sp^2 carbon atoms, such as graphene, generally give a G band signal at around 1580 cm^{-1} and a G' band signal at around 2700 cm^{-1} .¹⁵ Meanwhile, carbon atoms adjacent to a defect or a graphene edge give a D band signal at around 1350 cm^{-1} .¹⁶ As shown (spectrum a), the G and G'

bands clearly appeared at 1584 cm^{-1} and 2678 cm^{-1} , respectively. The G/G' band intensity ratio was about 0.25. It is known that the G band and the G/G' ratio for monolayer graphene are about 1584 cm^{-1} and 0.25, respectively,¹⁷ which confirms the formation of a monolayer graphene. After growth of CNTs on the graphene surface, the D band also appeared at about 1350 cm^{-1} in addition to the G band (spectrum b in Figure 3A). The D/G band intensity ratio was calculated to be about 0.86. Unlike in the case of graphene, the G band intensity was much larger than the G' band. The G' band for the CNTs/G film was shifted by 22 cm^{-1} higher than that for graphene. The G/G' band intensity ratio was estimated to be about 2.72. These results fitted the characteristics of few-layer graphene¹⁷ and is attributed to the three-dimensional structure consisting of multi-walled carbon nanotubes grown on the monolayer graphene.

We further characterized the CNTs/G film by XPS. In the deconvoluted C1 spectrum (Figure 3B), a main peak of the graphitic structure appeared at about 284.3 eV. Moreover, five peaks attributed to defects on the nanotube structure (ca. 285.3 eV), oxygen-containing functional groups (ca. 286.7, 288.6, and 290.3 eV), and π - π^* transition (ca. 291.6 eV) were obtained.¹⁸ In the deconvoluted O1 spectrum (Figure 3C), two peaks attributed to oxygen-containing functional groups (ca. 531.7 and 533.3 eV) were also observed.¹⁸ Based on these spectra, the atomic oxygen/carbon (O/C) ratio of the CNTs/G film was determined to be about 12.3%. The reason that the oxygen-containing functional groups were existent at the CNTs/G film might be due to the acid treatment for the removal of Cu foil and Fe nanoparticles (see experimental section). Additionally, the CNTs/G film might be oxidized by atmospheric oxygen. Although the peaks for defects on the nanotube

structure was obtained here, there was no obvious D' band (ca. 1620 cm^{-1}), which is an index of the presence of graphene edges, in the Raman spectrum (Figure 3A).¹⁹ We supposed that the CNTs/G film wasn't heavily oxidized by the acid treatment.

We next examined electrochemical behavior of $[\text{Ru}(\text{NH}_3)_6]\text{Cl}_3$, the electron transfer kinetics of which is generally known to be an outer-sphere process and independent of surface sites and/or functional groups,²⁰ at the CNTs/G electrode. Figure 3D shows CVs recorded in a 0.1 M KCl aqueous solution containing 1 mM $[\text{Ru}(\text{NH}_3)_6]\text{Cl}_3$, where the scan rate was 25 mV s^{-1} . The reversible redox peak current at the CNTs/G electrode was about 2.5 times larger than that at the graphene electrode, indicating an increased apparent electroactive surface area, A_{app} , due to CNTs grown at the graphene surface. The A_{app} value for the CNTs/G film was compared with that for the graphene film using the Randles-Sevcik equation,²¹

$$I_p = (2.69 \times 10^5) n^{3/2} A_{\text{app}} D^{1/2} C \nu^{1/2} \quad (1)$$

where n ($= 1$) is the number of electrons transferred, D is the diffusion coefficient of $[\text{Ru}(\text{NH}_3)_6]^{2+/3+}$ ($= 9.1 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$),²² C is the concentration of $[\text{Ru}(\text{NH}_3)_6]\text{Cl}_3$, ν is the scan rate. The A_{app} values for the CNTs/G and graphene films were approximately 0.141 and 0.059 cm^2 , respectively. We further examined the stability of the CNTs/G film on the GC surface using the multiple scan CV measurement. If fragile, the CNTs/G film would be detached from the GC surface and as a result the redox peak current of $[\text{Ru}(\text{NH}_3)_6]^{2+/3+}$ would decrease. However, the redox peak current was approximately constant for at least 20 repeated cyclic potential sweeps from -0.5 to 0.1 V at the scan rate of 100 mV s^{-1}

(Supporting Information, Figure S1), confirming that the CNTs/G film was tightly attached at the GC surface due to π - π and/or hydrophobic interactions.

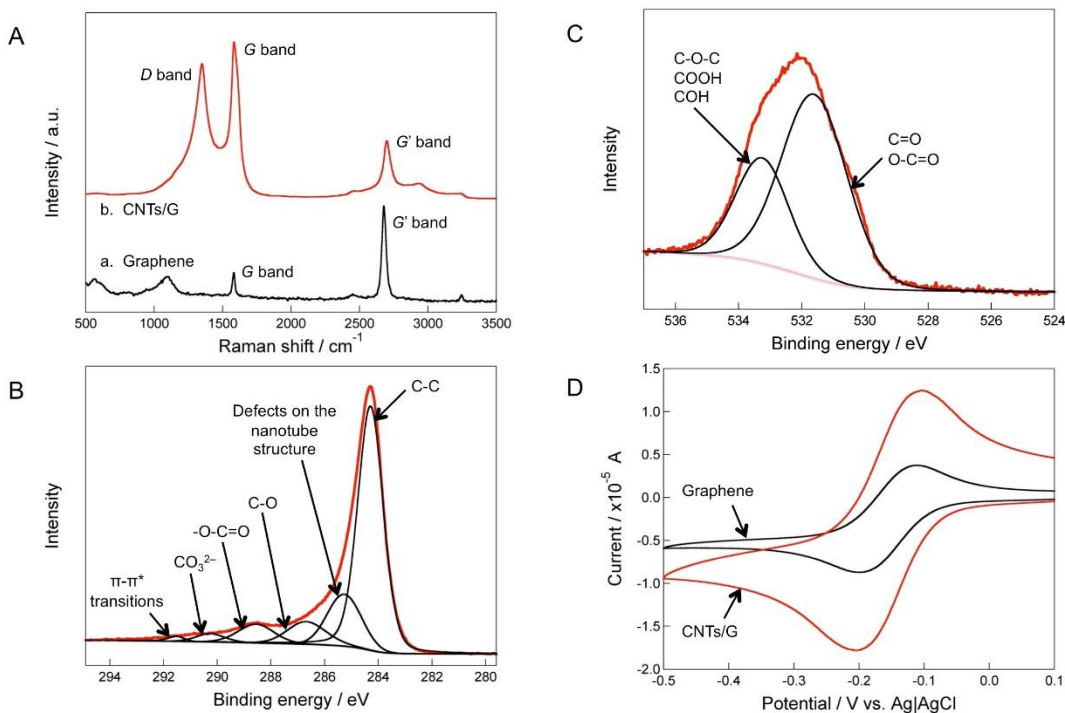


Figure 3. A) Raman spectra of (a) graphene and (b) the CNTs/G film. XPS B) C1 and C) O1 spectra of the CNTs/G film. D) CVs of 1.0 mM [Ru(NH₃)₆]Cl₃ in a 0.1 M KCl aqueous solution at the CNTs/G and graphene electrodes at scan rate of 25 mV s⁻¹.

Electrochemical Characterization of HP-Modified CNTs/G Hybrid Electrodes. CV measurements were carried out with the HP/G and HP/CNTs/G electrodes in 67 mM phosphate buffer (pH 7.4). As shown in Figure 4A, reversible redox peaks appeared at around -370 mV (vs. Ag|AgCl) in HP/G and HP/CNTs/G electrodes, corresponding to (P)Fe^{2+/3+} (P = porphyrin ring),^{23, 24}, whereas no redox peak was observed without HP (Figure 4B).). The redox peak currents per the GC surface area (ca. 7.1×10^{-2} cm²) at the HP/CNTs/G electrode were about one order of magnitude larger than those at the HP/G.

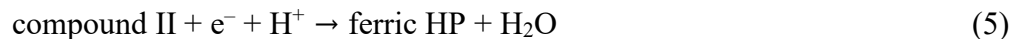
Since the peak currents for both electrodes were proportional to the scan rate below at least 100 mV s⁻¹ (Figure 4C), the redox peak currents were used to estimate the surface coverage Γ of electroactive HP at the CNTs/G film and graphene using the equation

$$i_p = n^2 F^2 \Gamma v / 4RT, \quad (2)$$

where n ($= 1$) is the number of electrons transferred, F is the Faraday constant, R is the gas constant, and T is the temperature. Based on the equation, the Γ values were estimated to be 1.3×10^{-11} and 1.1×10^{-10} mol cm⁻² for the graphene ($i_p/v = 1.2 \times 10^{-5}$ A cm⁻² V⁻¹ s) and CNTs/G electrodes ($i_p/v = 1.0 \times 10^{-4}$ A cm⁻² V⁻¹ s), respectively. The one order of magnitude higher value of the coverage for the CNTs/G is attributed to the larger electroactive surface area as a result of the 3-dimensional structure. Further, based on the reported Γ value for the HP monolayer-modified gold electrode to be 3.5×10^{-11} mol cm⁻², we hypothesize that there was a monolayer coverage of HP at both graphene and CNTs/G electrodes.²⁵

Catalytic Reduction of H₂O₂. Cathodic current responses of the HP/CNTs/G electrode to H₂O₂ were measured at +150 mV (vs. Ag|AgCl) in an air saturated 67 mM phosphate buffer solution (pH 7.4). After the background current stabilized, aliquots of H₂O₂ were added to the electrolyte solution and a steady-state cathodic current was observed after each addition. This current is attributed to electron transfer from the electrode to HP *via* the CNTs/G hybrid film. The possible reaction mechanisms are





where compound I and II are oxidized complexes of HP, in which the Fe(IV) of heme is coordinated to oxygen.^{12, 13} As shown in Figure 5A, the steady-state cathodic current densities for H₂O₂ reduction at the HP/CNTs/G electrode were clearly much larger than those at the bare CNTs/G electrodes. In addition, the cathodic currents at the HP/CNTs/G electrode were larger than those at the HP/G electrode. The cathodic current at the HP/CNTs/G and HP/G electrodes linearly increased for H₂O₂ concentration between 0.1×10^{-6} and 3.0×10^{-5} M, indicating that the equation 3 was the rate-determining step of the reaction. However, for H₂O₂ concentration of 3.0×10^{-4} M or higher, the cathodic current was independent of the H₂O₂ concentration, indicating that equation 4 and/or 5 were the rate-determining steps. In addition, the catalytic cathodic currents obtained at both HP/CNTs/G and HP/G electrodes were found to be kinetically controlled because the current response was not influenced when the electrolyte solution was stirred.

The kinetics of the reactions were analyzed on the basis of the observed currents (Figure 5A) and the HP coverage. As mentioned above, since the catalytic cathodic current for H₂O₂ reduction was kinetically controlled, all the HP molecules should contribute to the current and the current should be proportional to the surface coverage of HP. The cathodic current i is therefore given by equation 6.^{12, 13, 26, 27}

$$i = 2FI / (1/k_1 C_S + (k_2 + k_3)/k_2 k_3 C_H) \quad (6)$$

where k_1 , k_2 , and k_3 are the rate constant of equation 3-5, and C_S and C_H are the H₂O₂ and proton concentration in the bulk solution, respectively. According to equation 6, based on the I the catalytic current at the HP/CNTs/G electrode should be approximately an order

of magnitude larger than at the HP/G electrode. This was indeed observed experimentally (Figure 5A).

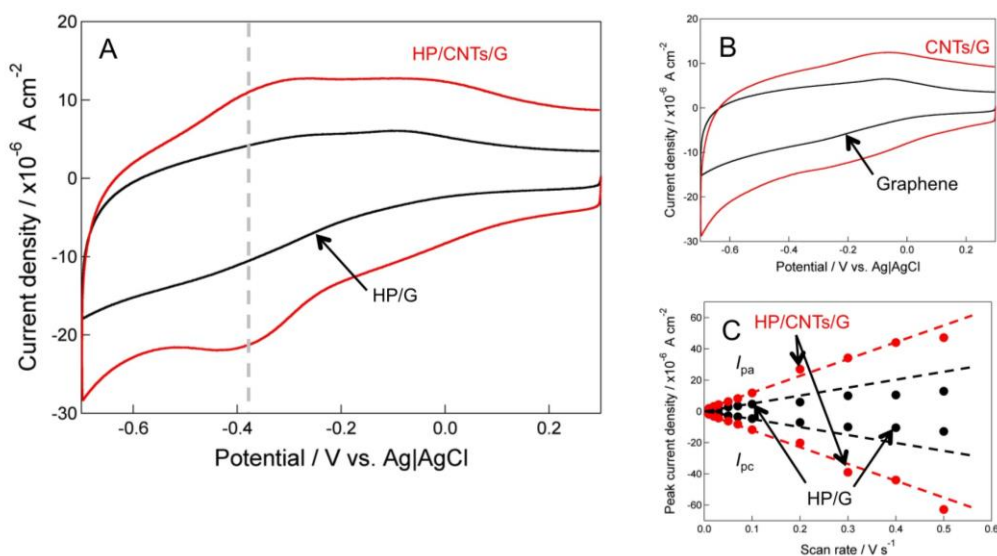


Figure 4. CVs of (A) HP/CNTs/G and HP/G and (B) CNTs/G and graphene electrodes in a 67 mM phosphate buffer solution (pH 7.4) at scan rate of 100 mV s⁻¹. (C) Relationship between anodic and cathodic peak currents of HP/CNTs/G and HP/G electrodes and scan rate.

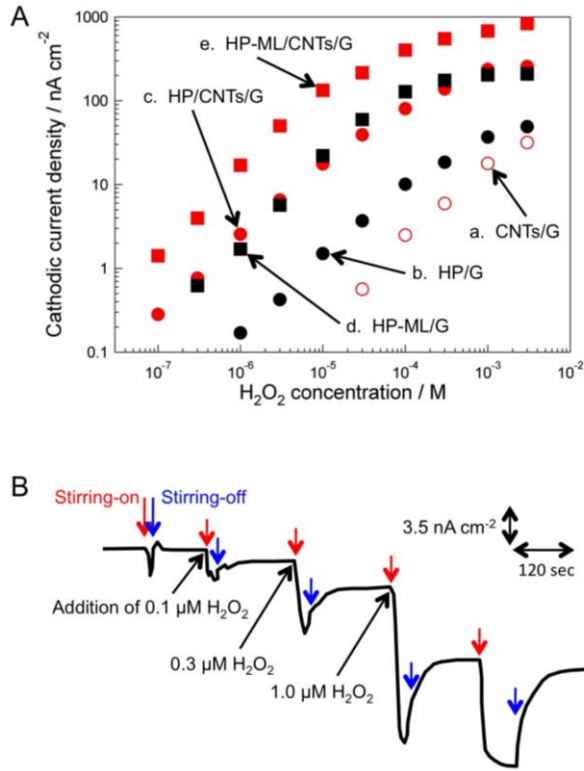


Figure 5. (A) Cathodic current densities of H_2O_2 reduction at (a) the CNTs/G, (b) the HP/G ($\Gamma = 1.3 \times 10^{-11} \text{ mol cm}^{-2}$), (c) the HP/CNTs/G ($\Gamma = 1.1 \times 10^{-10} \text{ mol cm}^{-2}$), (d) the HP-ML/G ($\Gamma = 5.5 \times 10^{-10} \text{ mol cm}^{-2}$), and (e) the HP-ML/CNTs/G ($\Gamma = 5.5 \times 10^{-10} \text{ mol cm}^{-2}$) electrodes and (B) chronoamperometric response of the HP-ML/CNTs/G electrode to H_2O_2 in an air-saturated 67 mM phosphate buffer solution (pH 7.4) at +150 mV vs. Ag|AgCl.

Using the equation 6, we calculated the rate constants for the three reactions. In the linear response region ($[\text{H}_2\text{O}_2] \leq 3.0 \times 10^{-5} \text{ M}$), the current response is determined by the rate of equation 3. Since the equation 3 is kinetically controlled, the current is given by the equation.

$$i_1 = 2F\Gamma k_1 C_S \dots \quad (7)$$

The k_1 values were estimated from the equation 7 to be about $120 \text{ M}^{-1} \text{ s}^{-1}$ for the HP/CNTs/G electrode ($i/C_S = 2.5 \times 10^{-3} \text{ A cm}^{-2} \text{ M}^{-1}$ at 0.1×10^{-6} to $1.0 \times 10^{-6} \text{ M H}_2\text{O}_2$) and

40 M⁻¹ s⁻¹ for the HP/G electrode ($i/C_S = 1.0 \times 10^{-4}$ A cm⁻² M⁻¹ at 1.0×10^{-6} to 1.0×10^{-5} M H₂O₂), respectively. It is known that the k_1 value at a heme nonapeptide-modified SnO₂-coated glass plate was 220 M⁻¹ s⁻¹¹² and was much smaller than those obtained for HRP-adsorbed graphite electrode by Ruzgas *et al.* (3.9×10^4 M⁻¹ s⁻¹)²⁸ and Tatsuma *et al.* (3.1×10^4 M⁻¹ s⁻¹)²⁹ due to the difference in the molecular complexity. We believe that the same tendency was observed in our present work. At the constant region ($\geq 3.0 \times 10^{-4}$ M), the current response is determined by the rate of equation 4 and 5. If those equations are not controlled by diffusion of protons but by the enzymatic reactions including electron transfer from CNTs/G or graphene to compound I and II, the current is given by the equation.

$$i_{23} = 2FI\Gamma k_2 k_3 C_H / (k_2 + k_3) \quad (8)$$

Substituting the i_{23} values of 2.6×10^{-7} or 4.9×10^{-8} A cm⁻² at 3.0×10^{-3} M H₂O₂ for the HP/CNTs/G or HP/G electrodes (see Figure 5A), respectively, the C_H value (= ca. 4.0×10^{-8} M), F , and Γ ($= 1.1 \times 10^{-10}$ mol cm⁻² for the HP/CNTs/G electrode or 1.3×10^{-11} mol cm⁻² for the HP/G electrode) into the equation 8, the $k_2 k_3 / (k_2 + k_3)$ value was calculated to be about 3.0×10^5 and 4.9×10^5 M⁻¹ s⁻¹ at the HP/CNTs/G and HP/G electrodes, respectively. The $k_2 k_3 / (k_2 + k_3)$ value at the HP/CNTs/G electrode is slightly smaller than that at the HP/G one. This might be comparably reasonable because the CNTs/G film consisted of the multilayered graphene. The $k_2 k_3 / (k_2 + k_3)$ values obtained here were one order of magnitude smaller than that obtained at pH 7.0 for the HRP-adsorbed graphite electrode by Ruzgas *et al.* (6.6×10^6 M⁻¹ s⁻¹)²⁸ although the molecular weight of HP is one order of magnitude smaller than that of HRP. This is presumably affected by the difference

in the plane site of carbon nanotmaterials. It has been known that the heterogeneous electron transfer rate constant at the edge plane site of graphite was about six orders of magnitude larger than that at the basal plane.³⁰ Therefore, such decline in the $k_2k_3/(k_2 + k_3)$ value was likely due to the fact that HRP was adsorbed at the edge plane site of graphite, whereas HP was immobilized at the basal plane of CNTs.

We initially expected to obtain the same k_1 value at the HP/CNTs/G and HP/G electrodes, because HP molecules were just immobilized on the basal plane of the sp^2 bonded carbon atoms *via* PBASE. However, the k_1 value at the HP/CNTs/G electrode was about 3 times larger than that at the HP/G electrode. The reason for this is unclear, however, it is presumable that the chain conformation and/or network-like structure of CNTs/G at the graphene surface is involved in the acceleration of the equation 3. Compared with the two-dimensional graphene surface, CNTs modified with HP molecules might easily sway back and forth and around. In addition, π - π interaction among CNTs might stabilize HP molecules. Based on these, the k_1 value at the HP/CNTs/G electrode might therefore increase in comparison with that at the HP/G electrode. On the other hand, we also suspect the involvement of defects in CNTs for such enhancement of the k_1 value, because the D band appeared at the CNTs/G film but not graphene (Figure 3A). If the defects of CNTs are involved in the stabilization of HP, the $k_2k_3/(k_2 + k_3)$ value at the HP/CNTs/G electrode should be much larger than that at the HP/G electrode, because the defects of CNTs work as the edge plane sites. However, as mentioned above, the $k_2k_3/(k_2 + k_3)$ value at the HP/CNTs/G electrode was slightly smaller than that at the HP/G electrode. In the present work, the defects of CNTs had little effect on k_1 .

We also calculated the apparent Michaelis-Menten constant, K_M^{app} . From equation 6 and the Michaelis-Menten kinetics equation, $i = i_{23}C_S/(K_M^{\text{app}} + C_S)$, K_M^{app} is given by the following equation.

$$K_M^{\text{app}} = k_2k_3C_H/k_1(k_2 + k_3) \quad (9)$$

In consequence, the K_M^{app} values at the HP/CNTs/G and HP/G electrodes were determined to be about 1.0×10^{-4} and 3.0×10^{-4} M, respectively. These values were at least one order of magnitude lower than the that obtained at a polymerized carbon past electrode containing HP by Razumas et al. (6.4×10^{-3} M, pH 7.0).³¹ Since the K_M^{app} value at the HP/CNTs/G electrode was lower than that at the HP/G electrode, the CNTs/G film could be therefore useful as conductive nanoscaffolds for enzymes to develop highly sensitive enzyme-based electrochemical biosensors.

Effect of Microvoids in CNTs/G Hybrid Films. We further examined diffusion benefits of the microvoid within CNTs/G hybrid films (Figure 2B and C) for H_2O_2 and/or H^+ on electrochemical responses. If the CNTs/G film and graphene are excessively immobilized with the same amounts of HP molecules and diffusion of H_2O_2 and/or H^+ is not influenced by the microvoid in the CNTs/G film, the cathodic current should show roughly the same values due to the self-mediation among HP molecules.¹³ In order to demonstrate the issue, we used the HP-ML/CNTs/G and HP-ML/G electrodes, the Γ values of which were determined to be about 5.5×10^{-10} mol cm^{-2} from the integration of CV peaks (charge) at the scan rate of 10 mV s^{-1} (Supporting Information, Figure S2). As shown in Figure 4, the cathodic currents at the HP-ML/CNTs/G and HP-ML/G electrodes were larger than those

at the HP/CNTs/G and HP/G electrodes, respectively, due to the increase in the Γ value. However, the cathodic current at the HP-ML/CNTs/G electrode was 5-7 times larger than that at the HP-ML/G electrode. This difference is likely due to the contribution of microvoids in the CNTs/G film.

In the HP-ML/G electrode, the Γ value increased about 26 times compared to that at the HP/G electrode, but the cathodic current increased only about 10 times. This is because the rate-determining step for the HP-ML/G electrode is diffusion of H_2O_2 ($\leq 3.0 \times 10^{-5} \text{ M}$) or proton and/or e^- of the electron self-exchange ($\geq 3.0 \times 10^{-5} \text{ M}$), as previously reported.¹³ In fact, the catalytic current for H_2O_2 reduction at the HP-ML/G electrode significantly increased when the electrolyte solution was stirred. The HP molecules at only the interface of the HP-ML/G electrode might partially contribute to the H_2O_2 reduction because of their dense and tight immobilization on the flat surface of graphene. This means that the cathodic current doesn't increase, even if the Γ value increases. In contrast, the cathodic current at the HP-ML/CNTs/G electrode was 5-7 times larger than that at the HP/CNTs/G electrode. Also the catalytic current for the H_2O_2 reduction at the HP-ML/CNTs/G electrode slightly increased when the electrolyte solution was stirred which indicates that the rate determining step for the HP-ML/CNTs/G electrode might also be diffusion of H_2O_2 or proton and/or e^- of the electron self-exchange (Figure 5B). However, the cathodic current at the HP-ML/CNTs/G electrode was larger than that at the HP-ML/G electrode, as described above. Additionally, the cathodic current at the HP-ML/CNTs/G electrode was 15-17 times higher than that at the HP/G electrode. Thus, the microvoids in the CNTs/G film helps diffusion of H_2O_2 and/or proton to HP molecules. We can therefore conclude

that compared with the graphene electrode, the sensitivity significantly enhanced at the CNTs/G electrode due to the increase in the surface coverage of enzymes and the effect of the microvoids for diffusion of substrates. Further investigation of selectivity to analytes and interference with biological molecules will have to be assessed for the development of a practical biosensor for H₂O₂ in a human body. In addition, this work would be expected to lead to the development of not only enzyme-based highly sensitive electrochemical biosensors but also enzyme-based biofuel cells.

4. CONCLUSION

The CNTs/G film was formed by two-step CVD process. CNTs were randomly and loosely grown at the graphene monolayer, resulting in the presence of microvoids. We constructed the highly sensitive electrochemical biosensor for H₂O₂ using the CNTs/G film as the conductive nanoscaffolds for HP molecules. Compared with the HP/G electrode, the catalytic current for H₂O₂ reduction at the HP-ML/CNTs/G electrode increased about 15 times. In addition, microvoids in the CNTs/G film played a significant role in diffusion of H₂O₂ to modified HP. The CNTs/G film would be useful for not only electrochemical biosensors but also enzyme-based biofuel cells.