Nanomaterial-facilitated protein direct electron transfers and their enhanced electrochemical biosensors

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Their Enhanced Biosensors

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

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Transfers and Their Enhanced Electrochemical
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SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

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School of Chemical and Biomedical Engineering

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Abstract

Nanomaterial-facilitated protein direct electron transfer (DET) has been extensively studied and widely applied in the development of electrochemical biosensors. However, the further enhancement of DET capacity necessary to develop high-performance biosensors for practical application remains a great challenge. To address this challenge, this PhD research project designs and fabricates novel nanocomposites with desirable properties to enhance protein DET capacity for the development of high-performance biosensors while exploring the fundamental insights in the effects of nanomaterial properties on protein DET behaviors and the mechanism behind enzymatic biosensing to gain fundamental knowledge.

A nanocomposite of carbonized TiO₂ nanotubes prepared by carbonizing TiO₂ nanotubes to obtain a large reaction surface area and a high level of conductivity has been used to immobilize hemoglobin and explore its DET behaviors. Compared with TiO₂ nanotubes, the nanocomposite is found to greatly enhance hemoglobin DET capacity, with the resulting biosensor exhibiting excellent bioelectrocatalytic activity toward H₂O₂ characterized by a rapid response time, a long linear detection range and a low detection limit. This study thus demonstrates that the conductivity of nanomaterials plays an important role in enhancing protein DET capacity and improving biosensing performance.

A functional nanocomposite composed of mesoporous carbon with high conductivity and a large specific surface area, glucose oxidase (GOD) and an ionic liquid with good ionic conductivity that has been filled into a microcavity to fabricate a paste microelectrode exhibits enhanced GOD DET capacity and improved glucose biosensing performance. This work provides an efficient and accurate approach to the study protein

direct electrochemistry for the further development of miniaturized biosensors.

A prepared nanocomposite of hollow sphere-structured conductive polymer/metal oxide possessing high conductivity and desirable surface charge properties is found to result in the strong electrostatic adsorption of GOD while retaining its native structure and significantly enhancing its DET capacity. More importantly, by using the nanocomposite and specially designing experiments, it reveals that the enzymatic glucose biosensing mechanism is a GOD DET process with competitive glucose oxidation and oxygen reduction to detect glucose.

A unique nanocomposite biofilm of horseradish peroxidase-Au nanoparticle-polyaniline nanowires has been fabricated by using negatively charged Au nanoparticle coated polyaniline nanowires as a supporting matrix, of which the large contact surface area, desirable surface charge properties and high conductivity greatly promote horseradish peroxidase immobilization and enhance DET capacity. Furthermore, the biofilm has been successfully employed for ischemia diagnosis, finding that the number of H_2O_2 molecule released per ischemic cell is 2.7-times of that of a normal cell.

In a brief, this research project demonstrates that the high conductivity, desirable surface charge properties and porous structure of nanomaterials play important roles in enhancing protein DET capacity and improving biosensing performance. In addition, it clearly reveals that the mechanism behind enzymatic glucose biosensing is a GOD DET process with competitive glucose oxidation and oxygen reduction to detect glucose.

Chapter 1 Introduction

1.1 Direct Electron Transfer of Proteins

Electron transfer plays an important role in biological and chemical systems. For example, in the biological system of photosynthesis, flavoproteins act as carriers of electrons to conduct the electron transfer necessary to convert light energy into chemical energy¹⁻². In chemical systems, electron transfer involves reactions via bond formation and fracture. Protein electron transfer, which is of great importance in both the basic understanding of the mechanism behind biological redox reactions and the development of novel bioelectronic devices³⁻⁶, has become one of the most active research areas.

Generally, there are two ways of achieving protein electron transfer, namely mediated electron transfer and direct electron transfer (DET)⁷. Mediated electron transfer is the process of electron transfer via the diffusion of low-molecular weight redox-reversible mediators between the active centers of proteins and an electrode surface⁸, whereas, DET is the direct transfer of electrons, as is reflected in its name, between the active centers of proteins and a supporting substrate. Unlike mediated electron transfer, DET offers the unique advantages of simplicity, the potential for miniaturization and the redox potential close to those of the redox-active centers of proteins for advanced bioelectronic devices⁹⁻¹⁰. However, it is difficult to achieve protein DET with bare electrode supports because of the relatively long distance between the active sites of proteins and an electrode surface, as is explained by Marcus theory, which is described below¹¹.

$k_{ET} = 10^{13} \exp[-\beta(r - r_0)] \exp[-\frac{-(\Delta G^0 + \lambda)^2}{4\lambda RT}]$, where k_{ET} is the DET rate; r_0 and r are the minimal and actual electron donor-acceptor distance, respectively; λ is reorganization free energy; β is a decay parameter that depends on the intervening atomic and molecular structure; and ΔG^0 is the reaction free energy. According to Marcus theory, the electron transfer rate between the active centers of proteins and the electrode surface strongly depends on the distance between them. Having the active sites deeply buried within electrically well-insulated prosthetic shells, protein DET is very unlikely to occur, or does so only at a prohibitively slow rate due to the exponential decrease in tunneling probability resulting from the relatively long distance between the active sites and the electrode surface.

Recent advances in nanoscience and nanotechnology have enabled the design and fabrication of materials on the nanometer scale with well-tailored properties for promoting applications¹²⁻¹³. The remarkable characteristics of these materials including a size comparable to that of proteins, a high surface-to-volume ratio and quantum-confined electronic properties¹⁴⁻¹⁷, have enabled their use in efficient conductive molecular wires to promote protein DET. Various nanostructured materials, such as nanoparticles¹⁸⁻²⁰, nanowires²¹⁻²³, nanotubes²⁴⁻²⁹ and porous materials³⁰⁻³⁵, prepared from carbon, metal, metal oxide, polymer or ionic liquid have been utilized to enhance protein DET capacity and enable the development of advanced applications.

1.2 Motivations

Although nanomaterial-facilitated protein DET has been extensively studied and

widely applied in the development of mediator-free biosensors, enhancing it to a level necessary for the development of high-performance biosensors remains a great challenge. In addition, the impact of the properties of nanomaterials on protein DET behaviors, as well as the mechanism behind enzymatic glucose biosensing remain unclear and therefore require greater investigation. The main motivation behind this PhD project is responding to this research need by designing and fabricating novel nanocomposites with desirable structural, electrical and chemical properties to greatly enhance protein DET capacity and improve biosensing performance while contributing to the fundamental understanding of the impact of nanomaterial properties on protein DET behaviors and the mechanism behind enzymatic biosensing.

1.3 Objectives

The objectives of this research are to design and fabricate novel nanocomposites for enhanced protein DET capacity and improved biosensing performance while identifying and describing the relationship between nanomaterial properties and protein DET behaviors and contributing to the understanding of enzymatic biosensing.

To Design and Fabricate Novel Nanocomposites with Desirable Structural and Surface Properties

To fabricate novel nanocomposites with desired structural and surface properties, various nanomaterials of different structures, such as nanoparticles, nanotubes, nanowires and hollow spheres, as well as mesoporous substrates composed of carbon, metal oxide, metal, conducting polymer and/or ionic liquid are designed and explored. The

nanostructures and their surface properties are tailored by adjusting the reaction conditions and/or applying post-treatments before being systematically investigated using various characterization techniques.

To Explore the Impact of Nanomaterial Properties on Protein DET Behaviors in the Development of High-Performance Biosensors

To explore the effects of nanomaterial properties on protein DET behaviors, various nanocomposites with desirable properties are prepared and explored as supporting matrixes to investigate protein DET behaviors and analyze the relationship between protein DET behaviors and the properties of nanocomposites. Nanocomposites that are found to enhance protein DET capacity are further utilized to construct high-performance electrochemical enzymatic biosensors.

To Reveal and Understand the Mechanism behind Enzymatic Biosensing by Specially Designed Experiments

To resolve the long-standing puzzle regarding whether enzymatic glucose biosensing involves an enzyme DET process, a unique nanocomposite with high conductivity and desirable surface charge properties is fabricated to achieve the enhanced DET capacity of GOD. Based on the resulting GOD DET behaviors, experiments are specially designed and conducted to identify the mechanism behind enzymatic glucose biosensing.

1.4 Organization

This dissertation explores the development and utilization of novel nanocomposites

for the enhancement of protein DET capacity necessary to improve biosensing performance, identifies the relationship between nanomaterial properties and protein DET behaviors as well as the mechanism behind enzymatic biosensing, and expands understanding of the nanomaterial-facilitated DET of proteins for *in-situ* selective and quantitative molecular detection in cell-based assays. Chapter 1 introduces the areas of investigation and provides an overview of the motivation and objective of this PhD research. Chapter 2 reviews the related research, including that pertaining to the types of nanomaterials and protein immobilization methods used to achieve protein DET and the applications of protein DET. Chapter 3 describes the experimental approaches, materials and instruments used in this study in detail. Chapter 4 describes nanocomposite of carbonized TiO₂ nanotubes used for enhancing the DET capacity of hemoglobin (Hb) and improving hydrogen peroxide biosensing. Chapter 5 describes a unique nanocomposite of an ionic liquid/ordered mesoporous carbon/glucose oxidase microelectrode and its use in enhancing DET capacity and high-performance glucose biosensing. Chapter 6 discusses a hollow-structured nanocomposite of conducting polymer/metal oxide-promoted DET of glucose oxidase and the mechanism behind enzymatic glucose biosensing. Chapter 7 describes the *in-situ* molecular detection of ischemic cells enabled by the enhanced DET capacity provided by a unique nanocomposite biofilm of horseradish peroxidase-Au nanoparticle-polyaniline nanowires. Chapter 8 provides a general conclusion and proposes several recommendations for future research.

Chapter 2 Literature Review

Due to their comparable size with biological molecules such as proteins and nucleic acids^{14, 17, 36}, high surface-to-volume ratio, tunable physical and chemical properties, and quantum-confinement effects³⁷⁻³⁸, nanomaterials have been widely used to facilitate protein DET for its application to advanced bioelectronic and biosensing devices³⁹⁻⁴⁰. This section first reviews the types of nanomaterials commonly used in protein DET before summarizing the methods of immobilizing protein on nanomaterials and discussing protein DET applications.

2.1 Nanomaterials for Protein DET

2.1.1 Carbon Nanomaterials

Carbon nanomaterials in their various forms ranging from zero-dimensional bulkeyballs to two-dimensional graphene (Figure 2-1) have recently attracted great interest⁴¹. Among various carbon nanomaterials, carbon nanotubes (CNTs), fascinating one-dimensional materials, might be the most widely studied because of their unique structural, mechanical and electronic properties including their high levels of electrocatalytic activity, chemical and thermal stability and tensile strength^{27, 42-43}. In particular, their conductivity and extremely small size have enabled their use as a key nanomaterial in conductive molecular wires to facilitate protein DET and as active components in advanced bioelectronic devices⁴⁴⁻⁴⁸.

Figure 2-1 Various carbon nanomaterials: zero-dimensional buckyballs, one-dimensional carbon nanotubes and two-dimensional graphene⁴⁹. Copyright (2008), with permission from Nature Publishing Group.

Shortened single-walled carbon nanotubes (SWNTs) immobilized vertically on an electrode can act as molecular wires to enable the protein (Microperoxidase MP-11) DET by reducing the electron tunneling distance between the active sites of MP-11 and the underlying electrode (Figure 2-2)⁵⁰. Although the rate constant of the heterogeneous electron transfer of MP-11 on the nanotube-modified electrodes is similar to that of MP-11 attached directly to the same platform without SWNT modification, the active density of MP-11 on the SWNT-modified electrode is much higher than that on the electrode without SWNT. This result demonstrates that the use of SWNT can efficiently facilitate the DET of MP-11 by acting as conductive molecular wires. Such an advantage is not limited to SWNTs, as multi-walled carbon nanotubes (MWNTs) have also demonstrated

utility in enhanced protein DET capacity by acting as molecular wires or nanoconnectors that electrically connect the redox centers of immobilized proteins^{24, 51-52}.

Figure 2-2 (A) Scheme showing the fabrication of aligned SWNTs on Au electrodes and the immobilization of protein microperoxidase MP-11. (B) CVs of Au/cysteamine/MP-11 (a) and Au/cysteamine/SWNTs/MP-11 (b) in PBS solution (pH 7.0) under argon gas protection at a scan rate of 100 mV s^{-1} versus Ag/AgCl⁵⁰. Copyright (2003), with permission from American Chemical Society.

Very recently, graphene composed of a single layer of carbon atoms in a two-dimensional lattice has attracted extensive attention in both theoretical research and practical application due to its high conductivity, large special surface area and tailored surface chemistry⁵³. Graphene's unique structure and properties make it a promising material for the facilitation of protein DET and high-performance electrochemical biosensors. For example, graphene functionalized by ionic liquid (a polyethylenimine-functionalized ionic liquid, PFIL), which has good dispersion in aqueous solution, high ionic conductivity in film form and a positive charge (Figure 2-3a and b) has been used as an electrode material in a study of protein glucose oxidase (GOD) DET and biosensing⁵⁴. GOD immobilized on graphene-PFIL clearly shows a pair of well-defined redox peaks, with a formal potential of ~ -0.43 V (vs Ag/AgCl in saturated KCl), while no obvious redox peaks can be found for GOD on graphite-PFIL (Figure 2-3c), suggesting that graphene plays the most critical role in achieving DET.

Regarding the mechanism behind the graphene-promoted DET of GOD, it is hypothesized that in addition to its extraordinary electron transport property, graphene offers a large number of edge planes that act as conductive molecular wires in the efficient reduction of the tunneling distance between the active redox center FAD/FADH₂ of GOD and the electrode surface. The graphene-PFIL-GOD electrode has also demonstrated high performance in glucose biosensing, with a high level of reproducibility and a linear response ranging from 2 to 14 mM (Figure 2-3d), which is exactly appropriate for determining blood sugar concentrations of 4 to 6 mM.

Figure 2-3 (A) Structure of PFIL. (B) TEM image of graphene. (C) CVs of graphene-PFIL (dashed), graphite-PFIL-GOD (dotted) and graphene-PFIL-GOD (solid) electrodes in N_2 -protected PBS solution. (D) CVs at the graphene-PFIL-GOD electrode in PBS solution with different glucose concentrations, and the calibration curve from amperometric responses at a potential of -0.49 V. Scan rate: 0.05 V s^{-1} ⁵⁴. Copyright (2003), with permission from American Chemical Society.

In addition to CNTs and graphene, other carbon nanomaterials including fullerenes⁵⁵, mesoporous carbon⁵⁶⁻⁵⁸, carbon black⁵⁹⁻⁶² and carbon fiber⁶³⁻⁶⁴, have also demonstrated a high level of capacity in protein DET and biosensings. Nevertheless, there is still a big room for carbon nanomaterials to be used in protein DET and biosensing. Although carbon nanomaterials have yet to be fully used in protein DET and biosensing, the further development of carbon nanocomposites with controllable and tailored properties will likely lead to the further enhancement of protein DET and biosensing capacity.

2.1.2 Metal Nanomaterials

The availability of different nanostructures with unique photophysical, optical and catalytic properties at nanoscale dimensions make metal nanomaterials ideal functional electrode materials in protein DET and high-performance biosensors⁶⁵⁻⁶⁸.

Among all metal materials, gold (Au) nanomaterials have been the most widely researched in protein DET studies because of their high level of biocompatibility, as well as the availability of various Au nanostructures⁶⁹. Au-nanoparticles (Au NPs) offering the benefits of easy preparation and a delicately tailored size ranging from ~1 nm to ~100 nm have effectively served as tiny conduction centers in facilitating protein DET. Functionalized Au NPs at sizes of ~1.4 nm have been used to facilitate the DET of bioengineered GOD⁷⁰. Two approaches have been used to construct Au NP-GOD hybrid materials: (1) to immobilize Au NP-GOD on electrode; (2) to immobilize Au NP-FAD on electrode, followed by the addition of Apo-GOD shell (Figure 2-4A and B). Electrochemical characterizations have shown that the glucose electro-oxidation current increases as glucose concentration increases up to 120 mM (Figure 2-4B and C). The electron transfer rate constant for the Au NP-GOD hybrid molecules is 5000 s^{-1} , seven times higher than that of native GOD with O_2 as the electron acceptor (700 s^{-1}), demonstrating the rapid rate of the DET of GOD. This much higher DET rate and higher level of electrocatalytic activity can be attributed to the small size and excellent electron transfer capability of Au NPs characteristics that allow them to act as efficient electrical relaying elements. As the use of dithiolate molecules as spacers in bridging Au NPs and bulk electrodes causes the significant overpotential (+0.40 V vs. -0.45 V vs SCE) of glucose electro-oxidation due to the introduction of an electron tunneling barrier, further

research should be conducted into the means of eliminating the overpotential by using highly conductive spacers.

Figure 2-4 (A) Assembly of Au-NP-GOD hybrid molecules by two approaches. (B) TEM image of a hybrid molecule. (C) CVs corresponding to the electro-oxidation of glucose by the hybrid molecules with different glucose concentrations: (a) 0, (b) 1, (c) 10, (d) 20 and (e) 50 mM. (D) Laviron's plots corresponding to the peak-to-peak separation between the reduction and oxidation waves of the CVs of the hybrid molecules as a function of scan rate (logarithmic) with the different dithiol spacers as bridges (a) spacer 1, (b) spacer 2 and (c) spacer 3⁷⁰. Copyright (2003), with permission from The American Association for the Advancement of Science.

Compared with zero-dimensional Au NPs, three-dimensional porous Au nanofilm, with its interconnected metallic network, large active surface area and high level of porosity, may be more effective in protein immobilization and protein DET facilitation due to its greater capacity for protein adsorption, ability to penetrate to the active center of proteins for enhanced DET capacity, and provision of a suitable microenvironment for retaining protein biological activity. An electrochemical study of a three-dimensional macroporous Au film for Hb DET research has shown that the Hb adsorbed on the film can undergo rapid DET, with the oxidation and reduction peak potentials located at 0.27 V and 0.09 V (vs SCE), respectively (Figure 2-5)⁷¹. In addition, the amount of Hb adsorbed on the porous film is found to be almost ten times larger than that on a flat Au surface. The finding that the DET rate constant of Hb on the porous Au film is 0.95 s^{-1} , much higher than that of 0.49 s^{-1} on Au NPs⁷², suggests that three-dimensional porous Au nanomaterial is a superior candidate in the enhancement of protein DET capacity.

Figure 2-5 (A) Fabrication procedure for the three-dimensional porous Au nanostructure and the process of the DET between Hb and the Au film. (B) SEM image of the three-dimensional Au film. (C) CVs of the three-dimensional Au film in PBS in a) without and b,c) with 0.2 mg mL⁻¹ and 0.6 mg mL⁻¹ Hb, respectively; scan rate of 100 mV s⁻¹. Copyright (2005), with permission from Wiley - VCH Verlag GmbH & Co. KGaA.

In addition to Au nanomaterials, many other metal nanomaterials including silver, platinum and palladium nanomaterials have also demonstrated enhanced protein DET capacity⁷³. For example, silver nanoparticles have been found to enhance the DET capacity of myoglobin (Mb) by inducing a change in the heme-group to bring the active sites close to the electrode surface, thus greatly increasing the Mb DET rate and improving the bioelectrocatalytic activity toward hydrogen peroxide.

2.1.3 Semiconductor Nanomaterials

Figure 2-6 (A) TEM image of titanate nanotubes (TNT). SEM images of TNT (B) and titanate nanoparticles (TNP) (C). CVs of TNT and Mb-TNT (D) and TNP and Mb-TNP electrode (E) measured in 0.1 M buffer solution (pH 5.5) with scan rate of 0.1 V s^{-1} . (F) Model for Mb accommodation (above) and possible mechanism for TNT-enhanced DET of Mb (bottom)²⁸. Copyright (2005), with permission from American Chemical Society.

Semiconductor materials such as metal oxide have unique properties, including a high level of chemical and physical stability, low cost and ease of fabrication that make them appropriate for use in a wide variety of electronic devices⁷⁴⁻⁸⁰. When semiconductors are at the nanodimension scale, they constantly exhibit remarkable electronic, optical and chemical properties, which may allow them to serve as functional electrode materials in enhancing protein DET capacity by efficiently shortening the electron tunneling distance between the active sites of the protein and the electrode surface⁸¹⁻⁸³.

Having a high level of biocompatibility and a hydrophilic nature, metal oxides may be able to provide a friendly microenvironment that allows proteins to retain their native structure while facilitating DET⁸⁴⁻⁸⁵. Titanium dioxide (TiO_2), one of the most commonly used oxide materials has been demonstrated to facilitate rapid electron transfer and to increase the catalytic activity to a level necessary for high-performance biosensors. Titanate ($\text{H}_2\text{Ti}_3\text{O}_7$) nanotubes (TNT), which have a typical cylindrical morphology with the barlike nanotubes (Figure 2-6A), have been used as an efficient host matrix to immobilize and study Mb DET behavior by using TiO_2 nanoparticles (TNP) as a control condition²⁸. Whereas TNT film consists of aggregated nanotubes forming a porous layer electrode surface (Figure 2-6B), TNP film contains randomly accumulated particles forming ill-ordered porous film and some nanoparticles aggregating into larger particles (Figure 2-6C). TNT can retain the native structure of Mb and promote fast Mb DET rate, as reflected in the well-defined, nearly reversible redox couple. In contrast, TNP causes Mb to lose its native conformation to a great extent and fails to facilitate fast Mb DET, as reflected in the quasireversible redox peaks and diffusion-controlled behavior when the

scan rate is above 1 V s^{-1} (Figure 2-6D and E). In addition, the average surface concentration of electroactive Mb in TNT film is around 2.0 nmol cm^{-2} , much higher than that in TNP film (1.3 nmol cm^{-2}). The enhanced DET capacity of Mb on TNT has been attributed to the aqueous-like microenvironment provided by the much more hydrophilic nature of TNT, the high affinity between negatively charged TNT and positively charged Mb, and the greater number of encapsulated Mb molecules from the two-end opened nanochannels of TNT (Figure 2-6F).

Figure 2-7 SEM (A) and TEM (B and C) images the mesoporous TiO_2 . (D) CVs of different electrodes in N_2 -protected PBS solutions at 50 mV s^{-1} : a) GOD, b) GOD/ TiO_2 nanoparticle, c) mesoporous TiO_2 and d) GOD/mesoporous TiO_2 . (E) Steady state response of the GOD/mesoporous TiO_2 biosensor on successive injection of 0.15 mmol glucose at -0.45 V and the inset is the corresponding calibration curve³³. Copyright (2008), with permission from Wiley - VCH Verlag GmbH & Co. KGaA.

Compared with nanotubes and nanoparticles, mesoporous structures can provide a large specific surface area and a high level of porosity, both of which help reduce the diffusion distance that the substrate must travel to access the electrode, as well as the distance between the active sites of the immobilized proteins and the electrode for enhanced DET capacity. A novel mesoporous TiO₂ structure prepared using the carbon nanotube as the hard template has been used to achieve GOD DET and glucose biosensing³³. The mesoporous TiO₂, which has a specific surface area of 343 m² g⁻¹ and a pore centered at 7.8 nm, composed of slack ultra-thin single crystals of anatase TiO₂ (Figure 2-7A-C). GOD adsorbed on the mesoporous TiO₂ exhibits a pair of well-defined redox peaks of values consistent with reported values for the active center FAD/FADH₂ of GOD, while GOD itself and GOD adsorbed on TiO₂ nanoparticles do not show any obvious redox peak (Figure 2-7C). The electron transfer rate constant for GOD on the TiO₂ material is 3.96 s⁻¹, which is much higher than that for GOD on MWNTs (1.53 s⁻¹) and SWNTs (0.3 s⁻¹). Moreover, the surface coverage of GOD on the mesoporous TiO₂ (2.57×10⁻¹⁰ mol cm⁻²) is much higher than that on Au NPs (0.098×10⁻¹⁰ mol cm⁻²) and CdS quantum dots (QDs, 0.154×10⁻¹⁰ mol cm⁻²) (33). The enhanced GOD DET capacity on mesoporous TiO₂ can be attributed to the mesoporous structure having a pore size appropriate for reducing the distance between the active sites of the hosted GOD molecules and the electrode. When used in glucose biosensing, the GOD/mesoporous TiO₂ has a response time of less than 10 s and a linear response range up to 1.2 Mm (Figure 2-7E).

Many other metal oxide nanomaterials with good catalytic activity and stability can be used to achieve protein DET for biosensing applications. For example, using ZnO

nanodisks for superoxide dismutase⁸³, MnO₂ nanoparticles for GOD⁸⁶, MnO₂ nanosheets for myoglobin⁸⁷, ZrO₂ nanoparticles for Hb⁸⁸ and GOD⁸⁹, Co₃O₄ nanoflakes for Hb⁹⁰, NiO nanoparticles for GOD⁹¹ and Fe₃O₄ nanoparticles for Hb⁹² have been shown to enhance the DET capacity of the immobilized proteins for the development of high-performance biosensors. Other semiconductor nanomaterials that have special electronic properties associated with their tailored size and display quantum- confinement effects, such as QDs⁹³⁻⁹⁴ can also act as conductive electrical relaying elements in facilitating protein DET for high-performance biosensors⁹⁵⁻⁹⁷. For example, CdS QDs used as a supporting matrix to immobilize Hb have demonstrated a rapid rate of DET and a high level of electrocatalytic activity toward H₂O₂ with a low apparent Michaelis-Menten constant of 112 μ M⁹⁸.

2.1.4 Conducting Polymers

Conducting polymers, a type of organic polymers possessing a π -conjugated system, have attracted great attention over the past few decades due to their intrinsically conductive nature and their possible substitutability for traditional metallic and semiconducting materials⁹⁹⁻¹⁰⁰. Because conducting polymers also exhibit a wide range of novel electrochemical properties, they have been widely used as electrode materials in protein DET based biosensors.

Among all the conducting polymers, polyaniline (PANi) has been one of the most studied because of its interesting electrochemical and electrical properties¹⁰¹. Used in biosensing applications, polyaniline has the advantages of allowing for impressive signal amplification and the elimination of electrode fouling. As it also provides a suitable

environment for protein immobilization and accommodation, it is a promising material for protein DET and biosensing. A PANi nanotube array fabricated by employing anodic aluminum oxide as a template has been utilized as the supporting matrix for the DET of GOD and glucose biosensing (Figure 2-8A)¹⁰². The highly ordered PANi nanotube array has an outer diameter of 250-300 nm (Figure 2-8B). GOD molecules immobilized on a PANi nanotube array are uniformly distributed and their resulting composite retains the nanotube structure (Figure 2-8C), which can reduce the electron tunneling distance between the active center of GOD and the electrode while enabling the free and rapid diffusion of substrates and products inside the nanotubes. GOD on the PANi shows a pair of well-defined and symmetrical redox peaks (Figure 2-8D), consistent with the redox characteristic of FAD/FADH₂ of GOD. The apparent electron-transfer rate constant is estimated to be $\sim 5.8 \text{ s}^{-1}$, which is much higher than that of GOD on CNTs ($\sim 1.5 \text{ s}^{-1}$) and boron-doped CNTs ($\sim 1.56 \text{ s}^{-1}$)¹⁰², indicating that the PANi can facilitate the DET of GOD more efficiently than other substrates. The GOD/PANi structure also exhibits good electrocatalytic activity toward glucose, as reflected by its anodic peak at $\sim 340 \text{ mV}$ and the complete disappearance of its cathodic peak (Figure 2-8D), a rapid response of $\sim 3 \text{ s}$, a low detection limit $\sim 0.3 \text{ }\mu\text{M}$ and a useful linear range of 0.01 to 5.5 mM (Figure 2-8E).

Figure 2-8 (A) Scheme showing the processes for fabrication of ordered PANi nanotube array and electrochemically entrapping GOD. SEM images of ordered polyaniline nanotube array (B) and GOD/nanoPANi (C). (D) CVs of the PANi (a) and GOD/PANi in PBS (pH 5.5) in the absence of (b) and presence of (c) 5 mM glucose. Scan rate of 10 mV s^{-1} . (E) Response of the GOD/PANi to glucose at -0.3 V and the corresponding calibration curve¹⁰². Copyright (2009), with permission from American Chemical Society.

Polypyrrole (Ppy), another conducting polymer widely used in bioelectronic devices

has the characteristics of ease of preparation, a high level of chemical stability and a high level of biocompatibility¹⁰³⁻¹⁰⁴, making it an effective matrix for protein immobilization and DET enhancement¹⁰⁵⁻¹⁰⁹. Electropolymerized Ppy film has been shown to enhance the DET capacity of quinohemoprotein alcohol dehydrogenase by acting as a conductive molecular wire tightly inserted into the structure of the protein, thereby promoting efficient DET between the active sites of the protein and electrode¹¹⁰. Very recently, a derivative of Ppy called poly-(pyrrole propylic acid) (Ppa) with abundant carboxyl groups has also been successfully utilized to achieve proteins DET and construct sensitive biosensors¹¹¹⁻¹¹³. Ppa has been electropolymerized to form a film that entraps Hb by using Hb as the dopant and by the process of co-electropolymerization¹¹⁴. The resulting Ppa film retains the native structure of Hb and promotes the DET of Hb by shortening the electron tunneling distance, while the Ppa/Hb film demonstrates high performance in H₂O₂ biosensing.

Many other conducting polymers, such as polythiophene and its derivatives, have also been shown to be effective host matrices in facilitating the protein DET¹¹⁵. The advancement of nanotechnology and material science will lead to the discovery of even more novel conducting polymers with various nanostructures that are of high utility in protein DET based bioelectronic devices and applications.

2.1.5 Biomolecules

Advances in modern material chemistry have allowed for the development, design, synthesis and manipulation of many natural nanomaterials beyond those described above, such as DNA molecules¹¹⁶⁻¹¹⁷ and lipids¹¹⁸⁻¹²⁰. The well-defined structures and properties

of diverse natural nanomaterials have enabled their use in advanced applications in various bioelectronic devices. DNA is a natural nanoscale material that contains genetic instructions and information essential to all known organisms^{116, 121}. Recently, DNA has shown to promote the DET of protein Endo III, a DNA glycosylase that repairs damaged DNA chains¹²² as well as the DET of photolyase, a flavoprotein containing two noncovalently bound chromophores that can make use of light energy to split mutations in damaged DNA¹²³⁻¹²⁴. Lipid membranes in living organisms, which consist of approximately 50% proteins and 50% phospholipids, always provide the binding sites for proteins necessary to enable their normal functioning. These lipid membranes have been utilized to promote the DET of proteins, including the DET of *M. tuberculosis* catalase-peroxidase, peroxidases and Mb¹²⁵.

2.1.6 Nanocomposites

Nanocomposites composed of different kinds of nanomaterials have been attracting increasing attention due to their high level of performance in various applications, especially in electrochemical biosensors^{39, 111}. Due to the synergistic nature of their individual components, nanocomposites can sometimes provide a better platform for enhancing protein DET capacity and constructing high-performance biosensors compared to other materials.

A nanocomposite composed of carbon-coated ZnO nanowire array (C-ZnO) offering the advantages of the chemical stability and a high level of conductivity of carbon and the large specific surface area and good electron transportation capacity of the one-dimensional ZnO nanowire array has been designed and fabricated to facilitate the DET

of GOD and construct sensitive glucose biosensors¹²⁶ (Figure 2-9A and B). The C-ZnO clearly promotes the GOD DET, as reflected from the pair of well-defined redox peaks of the CV curve (Figure 2-9C), compared to the weak peak of GOD on plain ZnO. The direct electron transfer rate constant of GOD on C-ZnO is $\sim 4.7 \text{ s}^{-1}$, a rate much larger than that of GOD immobilized on SWNTs, MWNTs or TiO_2 . It appears that the nanocomposite combines the advantages of a large specific surface area from ZnO nanowire array and the good conductivity from the carbon coating in the efficient facilitation of the DET of GOD. When used in glucose biosensing, the GOD/C-ZnO substrate demonstrates a response time of 5 s, sensitivity as high as $35.3 \mu\text{A mM}^{-1} \text{ cm}^{-2}$ and a linear response range from 0.01 to 1.6 mM.

Figure 2-9 SEM (A) and TEM (B) of C-ZnO. (C) CVs of different electrodes in N₂-protected buffer solutions at a scan rate of 0.5 V s⁻¹¹²⁶. (D) A CdTe-CaCO₃/PEs sphere and the inset is a confocal image. (E) Schematic illustration of the DET mechanism. (F) CVs of Hb-CdTe-CaCO₃/PEs (a), HB-CaCO₃/PEs (b) and CdTe-CaCO₃/PEs (c) and HB-CdTe-MPS/PAH (d) in 0.1 M pH 7.0 PBS at a scan rate of 0.2 V s⁻¹¹³⁰. Copyright (2009), with permission from Elsevier and copyright (2008), with permission from Wiley - VCH Verlag GmbH & Co. KGaA.

A nanocomposite of Hb-CdTe-CaCO₃/polyelectrolyte has been synthesized by the stepwise layer-by-layer method and used to study the DET behaviors of Hb and applied to H₂O₂ biosensing³⁰. The CaCO₃ microspheres have been shown to act as a matrix for both the QDs and the Hb molecules, and to provide an optimized microenvironment and orientation for the immobilized Hb, resulting in positive synergistic effects within the nanocomposite. Moreover, the polyelectrolytes within the nanocomposite work as linkers to help form the QD shell, while the QD shell functionalizes as a conductive layer tethering into the redox center of Hb and facilitating the electron-transfer process (Figure 2-9D and E). The high rate of Hb DET on the composite is reflected in the much higher electron-transfer rate constant (10.36 s⁻¹) compared to that of Hb on other substrates (Figure 2-9F). This nanocomposite with immobilized Hb also promotes high-performance H₂O₂ biosensing, providing a detection limit as low as 2.5 μM and a linear range from 5 to 45 μM.

These examples clearly demonstrate that nanocomposites are superior substrates in the enhancement of the DET capacity of proteins. As such, nanocomposites will continue to attract increasing attention in research into protein DET and high-performance biosensing.

2.2. Methods of Protein Immobilization

The ability to design and prepare nanomaterials with desirable chemical and physical properties is very important in achieving the DET of proteins with electrodes, as the way in which proteins are immobilized onto nanomaterials determines the DET capacity of proteins as well as the performance of bioelectronic devices. The improper

immobilization of proteins can lead to the failure to achieve DET and/or the partial or complete loss of biocatalytic activity¹²⁷⁻¹²⁸. Despite the development of many effective protein immobilization methods¹²⁹⁻¹³⁴, no one universal method is suitable for the immobilization of all types of proteins. The choice of an optimal immobilization approach should be determined by consideration of whether the physiological, chemical and structural properties of the protein and nanomaterials will allow them to be a good match. This section summarizes the five most widely used methods of protein immobilization: physical adsorption, covalent binding, membrane embedment, *in-situ* polymerization and layer-by-layer assembly.

2.2.1. Physical Adsorption

Proteins can physically adsorb onto nanomaterials via physical trapping, intermolecular forces, the polar effect and ionic binding. The major advantage of protein immobilization by physical adsorption is its simplicity, as the method generally involves two steps: contact, in which proteins in buffer solution adsorb onto nanomaterials via intermolecular forces, and washing, in which loosely binded protein molecules are removed¹³⁵.

Using this approach, many types of proteins have been successfully immobilized on various nanomaterials, such as CNTs, Au nanoparticles, mesoporous TiO₂ and macroporous Au film^{33, 136-140}. For example, mesoporous TiO₂ with a specific surface area of 343 m² g⁻¹ and a uniform porous distribution of 7.8 nm matches well with the dimensions of the GOD molecule (7.0 nm × 5.5 nm × 8.0 nm)³³. As expected, the amount of GOD loading on the mesoporous TiO₂ material can reach up to 39.3 wt% (GOD

concentration of 10 mg ml⁻¹ in buffer solution). In addition, the immobilized GOD molecules exhibit rapid DET rate, while the GOD/mesoporous TiO₂ substrate has great utility in the high-performance biosensing of glucose.

However, the physical adsorption method has several drawbacks, two of which are the susceptibility of the adsorption process to changes in pH, ionic strength and temperature and the susceptibility of the adsorbed proteins to being removed from the nanomaterials during the measurement process¹³⁵. Despite these drawbacks, the physical adsorption approach has been demonstrated to have wide applications, and shows promise in future applications.

2.2.2. Covalent Binding

With their assembled functional units of amino acids, proteins can be covalently bound to the surface of nanomaterials through accessible functional groups. The major advantage of covalent binding compared with other protein immobilization methods is its stability. The three basic steps in this method are (1) the activation of the surface of nanomaterials via functionalization, (2) the coupling of the proteins, and (3) the removal of loosely bound proteins¹⁴¹⁻¹⁴². The functional groups available in proteins for covalent binding and the functionalities of the required functional groups of nanomaterials are summarized in Table 2.2.1.

The amine and thiol groups are the two major chemistries used in covalent bonding approaches. Lysine residues of the amine group are the most commonly used amino acids in protein due to their omnipresence on the exterior of the protein surface, allowing for the facile activation of reactions¹⁴³⁻¹⁴⁵. Aldehyde and N-Hydroxysuccinimide (NHS) are

the corresponding two agents for coupling with amine groups, forming stable labile Schiff's bases and amide bonds, respectively. Cysteine is an amino acid that contains a unique functional group of thiol¹⁴⁶⁻¹⁴⁷, with which maleimide reacts rapidly to form stable thioether bonds within a specific pH range (usually 6.5-7.5). Protein HRP has been successfully immobilized on conducting polymer 5,2:5,2-terthiophene-3-carboxylic acid by using Ethyl-3-(3-dimethyl-amino-propyl)-carbodiimide as an activating agent to form peptide bonds between the amine groups of the HRP (lysine residues in the peptide chain) and the carboxylic acid groups of the conducting polymer¹⁴⁸. HRP covalently bonded on the conducting polymer has exhibited a high rate of DET and a high level of electrocatalytic response toward hydrogen peroxide.

Table 2.2.1. Functional groups in proteins and the required functionalities on nanomaterial surface.

Amino acids	Functional side group	Required surface
Aspartic acid	-COOH	Amine
Glutamic acid		
Serine	-OH	Epoxy
Threonine		
Lysine	-NH ₂	Carboxylic acid, Active ester, Epoxy, Aldehyde
Hydroxyl-Lysine		
Cysteine	-SH	Maleimide, Pyridyl disulfide, Vinyl sulfone

Although covalent binding can offer much stability in protein immobilization, it might cause a decrease in or even total loss of the bioactivity of the immobilized protein, leading to its limited application in protein DET research and bioelectronic devices.

2.2.3. Membrane-Like Embedment

The membrane-like embedment method is an efficient yet simple approach for protein immobilization that offers the advantages of being easy to prepare, requiring only a small amount of enzyme, and being able to preserve the integrity of the enzyme while promoting substrate diffusion through a membrane-like structure¹⁴⁹.

The use of a sol-gel based membrane-like structure in protein immobilization has recently drawn great interest due to its easy preparation, chemical inertness, tunable porosity, low-temperature encapsulation and swelling behavior¹⁵⁰⁻¹⁵³. Among various sol-gel based membrane-like structures, a silicate sol-gel matrix might be the most famous promising due to its ability to maintain a large content of water in the membrane and to be performed at room temperature, which is necessary for retaining the native structure of proteins. One type of membrane-like structure, a silica sol-gel solution prepared by hydrolyzing tetraethylorthosilicate at room temperature, has been applied to immobilize Hb on the electrode surface¹⁵⁴. After partial hydrolysis of the sol-gel solution, a porous matrix of silica is formed that promotes the diffusion of analyses from solution to the electrode surface, while the aqueous microenvironment retains the native structure of the entrapped Hb, which demonstrates a high rate of DET as reflected from the nearly reversible CV peaks corresponding to the active center $\text{Fe}^{(\text{III})}/\text{Fe}^{(\text{II})}$ redox couple of Hb.

Surfactants with a polar (or charged head group) and at least one nonpolar tail (usually a long hydrocarbon chain) can also be used to form functional and stable membrane-like structures for immobilizing protein while providing a natural-like environment^{120, 155}. A mixture of dimyristoylphosphatidylcholine vesicle and protein spinach photosystem I cast on an electrode can form a lipid bilayer membrane-like structure on an electrode¹²⁰ while retaining its native conformation and, more importantly, enabling rapid and reversible DET.

Although it offers the advantages described above, the membrane-like embedment method faces the major drawback that few nanomaterials are appropriate for membrane construction. Nevertheless, material science and nanotechnology research may soon identify new types of nanomaterials appropriate for membrane construction.

2.2.4. *In-Situ* Polymerization

Some conducting polymers can be electro-synthesized on an electrode surface in biocompatible conditions such as a neutral pH solution and room temperature¹⁵⁶⁻¹⁵⁸. More importantly, biomolecules such as proteins at a nanoscale size can be readily used as doping agents during the electropolymerization process, an approach that has the advantage of allowing simultaneous property adjustment and protein immobilization¹¹⁴.

In-situ polymerization of conducting polymer Ppy has been carried out to immobilize protein quino-hemoprotein alcohol dehydrogenase by using the protein as a doping agent¹¹⁰. Briefly, the polymerization solution is prepared by mixing the monomer pyrrole and the protein in a solution, followed by *in-situ* polymerization at a positive potential. The prepared protein/Ppy electrode can rapidly travel the DET pathway via the Ppy chain.

Nevertheless, it should be noted that *in-situ* polymerization for protein immobilization has the drawbacks of providing poor orientation of entrapped protein and reducing the conductivity of the resulting polymerized film.

2.2.5. Layer-by-Layer Assembly

Layer-by-layer (LBL) assembly, as its name indicates, is the process of the sequential adsorption of positive or negative charged species by the alternatively dipping of the substrate into the solutions, followed by the washing of the excess material after each adsorption step¹⁵⁹⁻¹⁶⁰. The advantages of LBL assembly are its simplicity, provision of the ability to precisely control thickness in nanoscale, and use in a variety of available species¹⁶¹⁻¹⁶².

Many types of proteins exhibit a polyelectrolyte nature in buffer solution and a characteristic that permits their immobilization on the electrode surface by LBL assembly. One substrate that has been constructed by LBL assembly is layered GOD/SWNT film¹⁶³. In neutral pH, both GOD (from *Aspergillus Niger* with an isoelectric point of 4.2) and acid-treated SWNTs have a negative charge, and thus repel each other. Fortunately, the process can be adjusted by changing the pH. By conducting it in a 0.1 M acetate buffer solution at pH 3.8, GOD can be successfully immobilized on the SWNT surface, with the resultant GOD in layered film demonstrating a rapid rate of DET with the electrode.

Protein molecules do not need to be used as electrolytes as polyelectrolyte LBL films can be constructed and used to immobilize proteins. Although this method is similar to that of physical adsorption, it may allow for better retention of the conformation and bioactivity of proteins. For example, Mb entrapped in LBL film constructed using

polyelectrodes and inorganic nanoparticles has shown a high level of DET capacity with a rapid electron transfer rate¹⁶⁴.

2.3 Applications of Protein DETs

The redox processes of proteins are ubiquitous in nature. Gaining understanding of the electron transfer process and exploring the intrinsic mechanisms of the electron transfer-related redox reactions of proteins allow for the deep understanding of complex biological systems necessary to utilize redox biological processes in practicable applications in bioelectronic devices^{139, 165-166}. This section summarizes the three main objectives of examining the DET of proteins, namely, gaining understanding of fundamental biological redox reactions, developing biosensors and developing biofuel cells.

2.3.1. Understanding Fundamental Biological Redox Reactions

Photosystem II (PS II) is a large transmembrane protein complex that can facilitate the conversion of photons into chemical energy in cyanobacteria, algae and higher plants by catalyzing the light-driven oxidation of water to oxygen and the reduction of plastoquinone (Figure 2-10A). Strong knowledge of the redox reaction behaviors of these protein-bound cofactors is very important in achieving efficient electron transport across biological membranes. By immobilizing the bound redox cofactors of PS II in dimyristoylphosphatidylcholine (DMPC)-based lipid films, the redox mechanisms of the bound redox cofactors of PS II can be investigated in the absence of light¹⁶⁷ (Figure 2-10B). Using manganese cluster-depleted PS II as the control condition, it has been found

that a midpoint potential of -0.29 V vs and 0.2 V vs NHE of reversible redox peaks in the CV curve at pH 7.5 can be assigned to quinone cofactors and the oxygen-evolving manganese cluster (Mn_4), respectively (Figure 2-10C and D). These findings reveal a proton-gated electron-transfer process for the PS II system. Such DET research thus provides detailed fundamental knowledge for understanding redox processes in the electron transfer pathways of light-harvesting systems.

Figure 2-10 (A) Schematic illustration of the mechanism of the DET process of PS II. (B) The setup of the PS II/lipid film electrochemical device. (C and D) Background-subtracted forward and reverse square wave voltammograms of PS II in DMPC films in pH 7.5 buffer solution for (C) a scan in the negative direction and (D) a scan in the positive direction¹⁶⁷. Copyright (2006), with permission from American Chemical Society.

To protect the integrity of the genome, which is constantly being assaulted and damaged, an impressive repair network has evolved in DNA-mediated DET of base excision repair proteins as the first step in DNA damage detection. Endo III featuring a [4Fe-4S] cluster is a DNA glycosylase that repairs damaged pyrimidines of DNA chain base pairs. The redox activity of Endo III, especially the DET between it and its bound DNA, plays an important role in the DNA repair process. To better understand this process, the DET behavior of Endo III with regard to the potential shift associated with DNA binding has been studied using highly oriented pyrolytic graphite (HOPG) electrodes with and without DNA modification (Figure 2-11A and B)¹²². On a HOPG electrode with DNA, Endo III exhibits a DET process with a quasi-reversible redox couple that has a midpoint potential of ~20 mV versus NHE, while this signal is absent for Endo III on a HOPG electrode without DNA (Figure 2-11C). In addition, a significant negative shift in potential occurs for the $3^+/2^+$ couple of Endo III [4Fe-4S] cluster on DNA binding, clearly indicating that DNA binding stabilizes the oxidized 3^+ form of the cluster, whereas without DNA its 2^+ form is more stable. As long as the DNA duplex is well stacked, a quasi-reversible redox couple with a significant negative shift in potential activates the protein toward oxidation and forces the protein from the DNA, demonstrating that the redox electrochemistry of DNA binding proteins, especially their DET capacity is of great importance in the DNA repair process.

Figure 2-11 Schematic representation of electrochemistry for Endo III on a HOPG electrode without (A) and with (B) DNA modification. (C) CVs of Endo III at a HOPG electrode with DNA modification¹²². Copyright (2006), with permission from American Chemical Society.

2.3.2. Developing Biosensors

Protein DET-based electrochemical biosensors, referred to as third-generation biosensors, possess several advantages^{83, 149, 168}. They can operate within a potential window closer to the redox potential of proteins, and are therefore less prone to interfering reactions. In addition, because they can eliminate the use of mediators in the reaction sequence, their detection process is simple yet efficient. Moreover, their

properties can be adjusted to those desired by using novel, well-tailored functional nanomaterials.

The development of an efficient way to quantify benzydamine, a nonsteroidal anti-inflammatory drug molecule widely used in human health care, is of great importance. Human flavin-containing monooxygenase 3 (hFMO3) is a microsomal phase I drug metabolizing protein that can enzymatically catalyze benzydamine. Thus, a third-generation biosensor employing hFMO3 is a very attractive prospect for benzydamine identification. hFMO3 has been covalently immobilized on a dithio-bismaleimidoethane functionalized Au electrode (Figure 2-12A and B) and on a surfactant didodecylammonium bromide functionalized glassy carbon electrode (GC/DDAB/hFMO3) for benzydamine sensing¹⁶⁹. The resulting immobilized hFMO3 on both electrodes exhibits a pair of redox peaks with a midpoint of -280 mV, demonstrating DET behavior, and hFMO3 coverage of $\sim 3.4 \times 10^{12}$ per cm^2 , exactly the size of a sub-monolayer. The biosensor shows a high level of performance in benzydamine sensing, providing a Michaelis-Menten constant of $\sim 44 \mu\text{M}$ and a linear range up to $40 \mu\text{M}$ (Figure 2-12C).

Figure 2-12 (A) Schematic representation of the immobilization of hFMO3 on a dithio-bismaleimidoethane functionalized Au electrode. (B) Homology model of hFMO3 with cysteine residues in orange and FAD cofactor in yellow (left) and Superimposed structures (right) of yeast (green, PDB:2GV8) and bacterial (cyan, PDB:2 VQ7) FMO with hFMO3 model (blue). (C) Calibration curve of the biosensor (GC/DDAB/hFMO3) towards benzydamine¹⁶⁹. Copyright (2010), with permission from American Chemical Society.

Superoxide anions (O_2^-) are a type of reactive oxygen species of a class of oxygen-containing molecules that play an important role in biological activities. Excessive generation of O_2^- can lead to apoptotic and necrotic cell death, as well as pathogenesis of a panel of clinically distinct disorders such as diabetes and cancer. Therefore, the

development of a sensitive and selective biosensor for O_2^- analysis is very important. A biosensor has been designed and fabricated by immobilizing a biomimetic superoxide dismutase, manganese(II) phosphate ($Mn_3(PO_4)_2$), on highly conductive TiO_2 nanoneedles. The detection mechanism is schematically shown in Figure 2-13 A-C¹⁷⁰. The as-built biosensor exhibits a high level of O_2^- sensing and can provide good selectivity, a rapid response of ~5 s, a long linear range of 0.5 μM to 1.5 mM, and a low detection limit of 170 nM (Figure 2-13 D). Using the biosensor, it has been found that cancer cells can generate a much higher concentration of O_2^- than that of the normal cells (Figure 2-13E), indicating that O_2^- might be a biomarker of cancer.

Figure 2-13 (A-C) Schematic illustration of the catalytic process of the biosensor. (D) Amperometric responses (insets) and calibration curve of the biosensor toward O_2^- at a potential of 0.0 V. (E) Electrochemical responses of the biosensor toward (a) normal cells and (b) cancer cells¹⁷⁰. Copyright (2009), with permission from The Royal Society of Chemistry.

2.3.3. Developing Biofuel Cells

Another important application of the DET of proteins is the development of enzymatic biofuel cells that utilize proteins to convert fuels such as glucose into electrical

energy¹⁷¹⁻¹⁷². Enzymatic biofuel cells represent a new kind of energy-conversion technology that can operate under moderate conditions, such as at ambient temperatures and in mild media¹⁷³⁻¹⁷⁴, and are environmentally-friendly because the biomass consumed, such as glucose and oxygen, is generally endogenous to biological systems¹⁷⁵⁻¹⁷⁶. More importantly, protein DET-based enzymatic biofuel cells offer unique advantages, including a work potential range close to the redox potential of enzymes for high power output and simplicity for miniaturization¹⁷⁷⁻¹⁷⁸.

A compartmentless glucose/O₂ biofuel cell has been constructed using SWNTs as the electrode material, protein glucose dehydrogenase (GDH) as the anode for glucose oxidation and protein laccase as the cathode for oxygen reduction (Figure 2-14A)¹⁷⁹. In the anode, methylene blue (MB) is electropolymerized on SWNTs to accelerate the turnover rate of GDH and to lower the overpotential involved in the bioelectrocatalytic oxidation of glucose. In the cathode, protein laccase undergoes DET with the assistance of SWNTs while yielding a high level of catalytic activity for the reduction of oxygen. The performance of the resulting glucose/O₂ biofuel cell is pH dependent, achieving a maximum output of power between pH 4.5 and 6. In buffer solution at pH 6.0, it can deliver an open circuit voltage as high as 0.80 V, and at 0.52 V can achieve a power density of 9.5 μWcm^{-2} (Figure 2-14B). The biofuel cell also exhibits good stability, as reflected from its retention of ~80 % of its power in the first 24 h and loss of only ~5 % per day while in continuous operation for one week (Figure 2-14C).

Figure 2-14 (A) Schematic illustration of the working principle of the compartmentless glucose/O₂ biofuel cell. (B) Polarization curve (solid dot) of the glucose/O₂ biofuel cell and the dependence of the power output (hollow triangle) on the current density. (C) Stability of the biofuel cell²⁵. Copyright (2006), with permission from Wiley - VCH Verlag GmbH & Co. KGaA.

A biofuel cell anode based on the DET of GOD on MWNTs has also been constructed by growing MWNTs on carbon papers and immobilizing GOD using poly(cation polyethylenimine) as the linker and Nafion as the entrapment and proton-conducting membrane²⁶. In buffer solution containing 20 mM glucose, the resulting anode can provide an open circuit potential of -0.4 V vs. Ag/AgCl, which is similar to the redox potential of the FAD/FADH₂ active center of GOD itself.

Chapter 3 Experimental Approaches

3.1 Materials and Equipments

3.1.1 Chemicals and Reagents

Glucose Oxidase from *Aspergillus Niger*, hemoglobin from bovine blood, and horseradish peroxidase (Type VI, essentially salt-free, lyophilized powder) were purchased from Sigma-Aldrich and used as received. When not in use, they were stored at -20 °C. Hydrogen peroxide (30% w/v), β -D(+) glucose were purchased from Sigma. Titania nanoparticles were purchased from Beijing Chemical, China. SBA-15 silica was purchased from Jilin University High-Tech. Co. Ltd., Changchun, China. 3,4-ethylenedioxythiophene (EDOT) and NiCl_2 was purchased from Aldrich. Potassium permanganate and sulfuric acid were purchase from Sigma–Aldrich. Ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate was purchase from Fluka. Nature graphite was purchased from Bay Carbon (Bay City, MI). If not specified, all other chemicals were purchased from Sigma-Aldrich and used without further purification.

All chemicals were of reagent grade and all solutions were prepared in deionized water (milliQ). Conducting polymer monomers were distilled under reduced pressure prior to use, and stored at 4°C under nitrogen when not in use. A series of phosphate buffer solutions (PBS) with different pH values were prepared by deionized water to adjust the molar ratio of $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$.

3.1.2 Equipments

Field emission scanning electron microscopy (FESEM) (JOEL JSM-6700F, Japan)

provided the morphologies and microstructure of materials.

The percentage and distribution of specific elements was detected by energy dispersive X-ray spectroscopy (JEOL EX-23000BU EDX Thermal Analyzer, Japan).

Transmission electron microscope (TEM, JEM-2100, Japan) and high-resolution transmission electron microscope (HRTEM, JEM-2100F, Japan) were used to study the crystal and porous structure of materials.

The topology and height profiles were characterized and obtained by atomic force microscopy (AFM, SPM 3100, Veeco Instruments Inc., USA).

Nitrogen adsorption/desorption experiments were carried out at 77.3 K by means of an Autosorb-1 (Quantachrome Instruments) analyzer. The surface area was calculated by using the Brunauer-Emmett-Teller (BET) equation. Pore-size distributions were calculated by the Barrett-Joyner-Halenda (BJH) method.

X-ray diffraction (XRD) (Bruker AXS, Germany) provided the XRD pattern and FTA2000 (First Ten Angstroms, Inc., USA) was used to measure the water contact angle at ambient temperature.

The thermal properties were determined by thermogravimetric analysis (TGA, Perkin Elmer Pyris Diamond thermogravimetric analyzer, USA).

UV-visible spectrophotometer (Shimadzu UV-2450, Japan) was used to measure the absorption spectra.

The fluorescence of thin film was scanned and recorded by a 2-D proteomic imaging system (ProXPRESS, Perkin Elmer, USA).

The secondary structure of protein was examined by Fourier transform infrared spectroscopy (FT-IR, Bruker EQUINOX 55 Duroscope™).

CH Instruments 760B potentiostats were used to perform most of the electrochemical experiments including electrochemical synthesis and measurement.

1260 Impedance Gain-Phase Analyzer and 1287 Electrochemical Interface system (Solartron Inc., Houston, Tex.) were used to perform impedance measurements.

3.2 Methodology

3.2.1 Electrode Preparation

Glass carbon electrodes with 3mm in diameter (GCE, CH Instruments, USA) were polished with 0.3 and 0.05 μm alumina powder sequentially, followed by thoroughly rinsing with deionized water. After sonicating in acetone and then in deionized water, the electrodes were dried at room temperature.

Protein immobilizations on nanomaterials were conducted by immersing 10 mg of these nanomaterials in a 1 mL protein solution (10mg mL^{-1} , pH 7.4, 0.01 M PBS) under ambient conditions while shaking for half an hour, followed by storage at 4 °C overnight for protein adsorption. Then, the solution became a suspension and was ready for use. For every use, the suspension was re-shaken, of which 5 μL was taken and dropped onto the surface of the pretreated GCE, followed by drying at room temperature, and then 3 μL Nafion (0.5% aqueous) solution was dropped above to form a Nafion membrane.

3.2.2 Electrochemical Techniques

Several electrochemical techniques including cyclic voltammetry, differential pulse voltammetry, amperometric i-t and AC impedance were used in this project.

In squarewave voltammetry (SWV), a squarewave is superimposed on the potential

staircase sweep and the current is measured at the end of each potential sweep such that the contribution to the current signal from the capacitive charging current can be minimized.

Cyclic Voltammetry (CV) is a type of potentiodynamic electrochemical technique, which is widely used to study the electrochemical properties of an analyte in a solution. In a CV scan, potential is ramped linearly versus time and this ramping is known as the experiments' scan rate (V s^{-1}). The potential is scanned from an initial potential toward either a high potential or a low potential. Then potential will then scan back. The current is recorded as the function of potential. The analyte studied by CV is usually redox active within the experimental potential window.

In differential pulse voltammetry (DPV), the base potential is incremented from an initial potential toward a final potential. At the same time a potential pulse is applied. The current before the potential pulse and at the end of the potential pulse are sampled. The difference of these two current samples is recorded as the function of potential. Actually, the DPV is much more sensitive to the current change near a redox potential than that of CVs.

In amperometric i-t Curve (i-t) measurement, a constant potential is applied and the current is recorded as the function of time. The applied potential is usually an oxidation overpotential or a reduction overpotential of a redox reaction. The current change related to the concentration change of the analyte can be monitored and a calibration can be generated.

In AC Impedance, the base potential is held constant at an initial potential. A sine waveform is superimposed to the base potential. The relationship between the applied

potential and the current flow is known as the impedance. The frequency is scanned from high frequency to low frequency with 12 components per decade frequency. The current and the potential are sampled and analyzed to obtain the real and imaginary impedance. Usually, the potential is always fixed at the open circuit potential of the electrochemical cell, which can be treated as a network of capacitors and resistors, which is known as an equivalent circuit. From an impedance spectrum (plotted in the real/imaginary plane), it is possible to obtain the equivalent circuit and determine the significance of the different components.

3.2.3 Electrochemical Measurements

Electrochemical measurements were carried out using CHI 760B electrochemical workstation with a three-electrode system, employing a platinum coil (wire) as the counter electrode, a silver/silver chloride (or a saturated calomel electrode (SCE)) as the reference, and the protein immobilized electrode as the working electrode. For CV testings, if not specified, all solutions were deoxygenated by bubbling highly pure nitrogen for at least 15 min and maintained under nitrogen atmosphere during the measurements.

Chapter 4 Carbonized Titanium Nanotubes Facilitated DET of Hb and Its Enhanced H₂O₂ Biosensor^a

4.1 Introduction

Nanomaterials with unique structures can facilitate protein DET due to their small size, novel physical, electronic and chemical properties¹⁸⁰. Recently, numerous nanostructured metal oxides have attracted considerable interest in the bioanalytical area because of their large specific surface area, good biocompatibility, easy of fabrication, as well as good chemical and physical stability³³. These nanomaterials, such as MnO₂ nanoparticles for GOD⁸⁶, MnO₂ nanosheet for Mb⁸⁷, ZnO nanowires for GOD¹⁸¹, ZrO₂ nanoparticles for Hb⁸⁸, Co₃O₄ nanoflakes for Hb⁹⁰, NiO nanoparticles for GOD⁹¹ and Fe₃O₄ nanoparticles for Hb⁹², have shown good catalytic activity, of which some are capable of direct electrochemistry.

TiO₂, one of the most widely used metal oxide materials in cosmetics, solar cells, batteries and paint, has already demonstrated its excellent biocatalytic behaviors¹⁸². Various TiO₂ nanostructures were used to immobilize proteins for either mechanistic study or electrochemical biosensor applications. TiO₂ anatase nanoparticles and nanotubes were used to immobilize Mb for the direct electrochemistry²⁸. Mb was also entrapped in TiO₂ nanosheet for a mediator-free H₂O₂ sensor by a DET process³⁴. High

^a Reprinted from *Biosensors & Bioelectronics*, 24 (2008), Guo, C. X., Hu, F. P., Li, C. M., Shen, P. K., Direct electrochemistry of hemoglobin on carbonized titania nanotubes and its application in a sensitive reagentless hydrogen peroxide biosensor, 819-824, Copyright (2008), with permission from Elsevier.

conductivity of a nanostructured material is essential for a fast DET process between electrode and active center of proteins¹⁸³. However, like other nanostructured metal oxide semiconductors, plain TiO₂ has poor conductivity and is not suitable for the direct electrochemistry. In our previous works, great improvement of conductivity of TiO₂ has been achieved with carbon nanotubes templated TiO₂ nanostructure³³ and polyaniline/TiO₂ nanocomposite¹⁸⁴⁻¹⁸⁵, in which TiO₂ acts as the nanostructure framework while polyaniline, the conducting polymer helps to improve the conductivity.

Here, a carbonized TiO₂ nanotube (TNT/C) with improved conductivity was synthesized by a uniform carbonization with organic polymers, and further used to study the direct electrochemistry of Hb and its applications for a mediator-free biosensor for H₂O₂.

4.2 TNT/C Preparations

TNTs were synthesized from titania nanoparticles by a mild hydrothermal method as reported¹⁸⁶. TNT/C was prepared by carbonization of TNT with the organic polymer by the following procedures¹⁸⁷. A mixture of 50.0 mg TiO₂ nanotubes and 94.7 mg poly (ethylene glycol) (MW 600) was placed in a quartz tube, through which argon gas was flowed at 500 standard cubic centimeters per minute for 60 min before heating. Then the flow rate of the argon gas was decreased to 100 standard cubic centimeters per minute and the mixture was heated for 30 min at 600 °C. The TNT/C was collected after cooling naturally to room temperature.

4.3 Morphology and Electrochemical Characterizations of TNT/C

4.3.1 Morphology Characterizations

The TNT has a highly loose bundle structure as can be seen from the FESEM image shown in Figure 4-1a. A typical TEM image of TNT is displayed in the inset of Figure 4-1a, from which the hollow nature of the nanotubes can be clearly observed. The inner diameter, outside diameter and length of the TNT ranges from 15-20 nm, 20-30 nm and 1-5 μm , respectively. The TNT/C retains the highly loose bundle structure (Figure 4-1b), but is more uniform and more porous than the TNT. The size of TNT/C bundle is around 50 nm, which is slightly larger than TNT. Clearly, the more uniform and porous structure of TNT/C should be useful in applications involved redox reactions by efficiently promoting the diffusion of analytes from electrolyte to the electrode surface.

Figure 4-1 FESEM images of TNT (a) and TNT/C (b). Inset of (a) is TEM of TNT and (b) is the high-magnification FESEM of TNT/C.

4.3.2 Electrochemical Characterizations

The electrochemical activities of TNT and TNT/C were characterized by EIS and CV in 10 mM $\text{Fe}(\text{CN})_6^{3-}$ -1.0 M KCl and the results are shown in Figure 4-2. The measured

complex impedance (Z) versus frequency, known as Nyquist Plot is shown in Figure 4-2a, in which well-defined frequency-dependent semicircle impedance curves are observed at the high frequency range followed by a straight line. The diameter of the semicircle for TNT/C is much smaller than that for TNT. Randle equivalent circuit is often used to model the complex impedance in an electrochemical cell, which is composed of the ohmic resistance of the electrolyte solution, R_s , in connection in series with parallel elements of double layer capacitance, C_{dl} , and Faraday impedance, Z_f . The parallel elements C_{dl} and Z_f versus frequencies form a semicircle over a high frequency range, and Z_f often comprises serially connected charge-transfer resistance, R_{ct} , which determines the diameter of the semicircle and Warburg impedance, Z_w from the diffusion of ions in the bulk electrolyte to the electrode surface. The values of R_{ct} obtained from Figure 4-2a are 98 and 19.6 $\Omega \text{ cm}^2$ for TNT and TNT/C electrode, respectively, indicating that TNT/C modified GCE has much faster charge transfer rate than that of TNT modified GCE.

Exchange current density (i_o) and standard rate constant (k^o) are always used to evaluate the electrocatalytic performance of an electrode material. The larger the exchange current density and standard rate constant are, the better the electrocatalysis is. i_o and k^o can be calculated from R_{ct} by using Eq. 1 and Eq. 2, derived from the Butler–Volmer relationship of current and potential¹⁸⁸.

$$i_o = RT/FR_{ct} \quad (1)$$

$$k^o = i_o/FC \quad (2)$$

where R , T , F and C are gas constant, absolute temperature, Faradic constant and concentration of the reactant, respectively. The calculated i_o s are 2.62×10^{-4} and 1.31×10^{-3} A cm^{-2} for TNT and TNT/C, respectively, while the calculated k^o s are $2.72 \times 10^{-3} \text{ s}^{-1}$ and

$1.36 \times 10^{-2} \text{ s}^{-1}$ for TNT and TNT/C, respectively, indicating much faster charge transfer rate of TNT/C than TNT.

Figure 4-2 (a) EIS and (b) CV of 10 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ in 1.0 M KCl using TNT and TNT/C modified glassy carbon electrodes (GCE).

The CVs in Figure 4-2b show that the electrochemical redox reaction of $\text{Fe}(\text{CN})_6^{3-/4-}$ on the TNT/C electrode has much more reversible redox peaks and smaller peak potential separation than that of TNT electrode, indicating the former is more reversible than the latter. The results here further support that the carbon coating on TiO_2 can improve the reversibility of its catalyzed electrochemical reactions. However, although the cathodic peak current equals to its corresponding anodic one on both electrode surface, their peak

potential separations are larger than 59.5 mV. This is likely ascribed to their quasi-reversible reaction nature. The electrochemically active surface area can be estimated according to the following equation for an quasi-reversible process ¹⁸⁹,

$$i_p = (2.99 \times 10^5) n(\alpha n_a)^{1/2} A C D^{1/2} v^{1/2} \quad (3)$$

where n , A , D , v and C are the number of electrons involved in the reaction, the electrochemically active surface area, the diffusion coefficient of the reactant species in solution ($\text{cm}^2 \text{s}^{-1}$), the scan rate of the potential perturbation (V s^{-1}) and the concentration of the reactant in the bulk solution (mol cm^{-3}), respectively and α and n_a are always taken as 0.5 and 1, respectively. The redox reaction of $\text{Fe}(\text{CN})_6^{3-/4-}$ involves one-electron transfer ($n = 1$) and the diffusion coefficient (D) is $6.30 \times 10^{-6} \text{ cm}^2 \text{s}^{-1}$ ¹⁸⁹. The value of the electrochemically active surface area for TNT/C modified GCE is $7.5 \times 10^{-2} \text{ cm}^2$ in terms of the calculation from Eq. 3, which is larger than that of TNT ($3.4 \times 10^{-2} \text{ cm}^2$) by two times. The larger reaction surface area clearly comes from the more porous structure of TNT/C, which is consistent with the morphology observation in Figure 4-1. The results discussed above demonstrate that the improvement of the electrocatalysis of TNT/C is produced by both charge transfer rate and reaction surface area.

4.4 Direct Electrochemistry Behavior of TNT/C-Hb and TNT-Hb

The direct electrochemistry behavior of Hb on both TNT/C and TNT was studied. Figure 4-3 shows CVs obtained from the TNT/C, TNT/C-Hb, TNT and TNT-Hb modified GCEs in pH 5.0 acetate buffer. There is no reaction peak current observed for TNT/C or TNT modified GCEs within the potential window (curve 1 in Figure 4-3a and b). However, Quasi-reversible reaction behavior is illustrated for both TNT/C-Hb and TNT-

Hb as in curve 2 of Figure 4-3a and b, respectively, in which the TNT/C-Hb has much higher reversibility of direct electrochemistry than the TNT-Hb in terms of its much more symmetric redox wave and relative smaller peak-to-peak separation (ΔE_p) than that of TNT-Hb. The ΔE_p is 42.6 mV at scan rate 100 mV/s, which is smaller than 62.5 mV for Hb in mesoporous silica matrix¹⁹⁰ and 58.6 mV for Hb in Fe₃O₄ nanoparticles⁹² observed at the same scan rate, indicating more reversible behavior for faster electron transfer of Hb than the reported literatures can be achieved. Both cathodic and anodic peak current of CVs obtained from TNT/C-Hb is linearly proportional with scan rate from 20 mV s⁻¹ to 200 mV s⁻¹, as shown in Figure 4-3c, indicating an electrochemical redox reaction of a surface immobilized species and further proving the direct electrochemistry nature of Hb on the TNT/C surface.

The effects of ratio of Hb to TNT/C by weight ($W_{Hb}/W_{TNT/C}$) and the total amount of TNT/C-Hb on the direct electrochemistry behavior were studied. When the $W_{Hb}/W_{TNT/C}$ ratio increases, the peak current increases until the ratio is up to 1:10 (1 mg ml⁻¹:10 mg ml⁻¹), at which the current becomes almost constant (date not shown). The peak current also increases with the increase of the total amount of TNT/C-Hb increases while keeping a constant $W_{Hb}/W_{TNT/C} = 1:10$; however, no significant effect is observed after 7.08×10^{-4} g cm⁻² of TNT/C-Hb is immobilized. This possibly implies that only the Hb molecules near the TNT/C material could undergo direct electrochemistry. Therefore, 7.08×10^{-4} g cm⁻² of TNT/C-Hb with $W_{Hb}/W_{TNT/C}$ ratio of 1:10 was chosen for further studies.

Figure 4-3 CVs of (a) TNT/C (1) and TNT/C-Hb (2) modified GCEs, (b) TNT (1) and TNT-Hb (2) modified GCEs in 0.1 M pH 5.0 acetate buffer solutions at 100 mV s⁻¹, and (c) a TNT/C-Hb modified GCE in 0.1 M acetate buffer (pH 5.0) at scan rates of 20, 50, 80, 100, 150, and 200 mV s⁻¹. (d) The plot of anodic and cathodic peak currents versus the scan rate.

Square wave voltammetry (SWV), which has better signal-to-noise ratio than CVs, was often used to estimate the apparent heterogeneous electron transfer rate constant (k_{ET})¹⁹¹. The method employs nonlinear regression to analyze SWV with the single-species thin-layer SWV model¹⁹². SWVs measured with TNT/C-Hb and TNT-Hb electrodes are shown in Figure 4-4, from which the average k_{ET} values of Hb in TNT/C and TNT

obtained are 108 s^{-1} and 72 s^{-1} , respectively. The k_{ET} value (108 s^{-1}) of Hb on TNT/C is much larger than Hb on TNT and significantly greater than that on Fe_3O_4 nanoparticles (25 s^{-1}), clay film (38 s^{-1}) and on SiO_2 nanoparticle film (76 s^{-1})⁹² as well. This clearly demonstrates the excellent performance of direct electrochemistry of Hb on the TNT/C surface.

Figure 4-4 Square wave voltammograms (SWVs) of TNT/C-Hb (a) and TNT-Hb covered GCE (b) in 0.1 M pH 5.0 acetate buffer solution. SWV conditions: equilibrium time, 5 s; potential amplitude, 100 mV; step height, 4 mV; and frequency, 10 (1), 20 (2), 30 (3), 40 (4), and 50 Hz (5).

4.5 Effect of pH on the DET Behavior of Hb

The direct electrochemistry of TNT/C-Hb modified GCEs shows a strong dependence on solution pH as shown in Figure 4-5a. Voltammograms with well-defined peaks were measured in the pH range of 3.0 to 7.0. Negative shifts in both anodic and cathodic peak potentials are observed with pH increase and the changes are very reversible. The formal potential (E_p) is found to be linearly decreased with a slope of –

62.5 mV/pH when increasing pH from 3.0 to 7.0 (Inset of Figure 4-5b), which is close to the expected theoretical value of -57.6 mV/pH for a reversible proton-coupled single electron transfer process, suggesting that a single protonation accompanies electron transfer between the electrode and each of the four heme-Fe^{III}'s of Hb¹⁹³. The pH value also affects the redox peak currents of Hb (Figure 4-5b). Obviously, the maximum peak currents are observed at pH value ~5.0. This observation indicates that the assembled Hb has its highest bioactivity around the pH 5.0.

Figure 4-5 (a) CVs of TNT/C-Hb modified GCE in 0.1 M acetate buffer with pH of 3.0, 4.0, 5.0, 6.0, and 7.0 measurements at a scanning rate of 100 mV s⁻¹, and (b) plot of peak current versus pH value. Inset of b: Plot of formal potential versus the pH.

4.6 TNT-C/Hb for Sensitive H₂O₂ Detection

Hb possesses an intrinsic peroxidase activity due to its similarity with peroxidase for catalysis of reduction of H₂O₂¹⁹³. The electrocatalytic properties of the TNT/C-Hb toward H₂O₂ reduction were investigated by CV. As shown in Figure 4-6, at the TNT/C-Hb modified GCE, the reduction (cathodic) peak current at around -0.35 V increases

significantly with increase of H_2O_2 concentration (curve 3-7) accompanying the disappearance of the oxidation (anodic) peak current, demonstrating a typical electrocatalytic reduction process of H_2O_2 . However, no peak is observed at the TNT/C modified GCE in the presence of H_2O_2 , showing no reduction of H_2O_2 occurs at the TNT/C modified GCE (curve 2). Apparently, the H_2O_2 reduction is due to electrocatalysis from Hb-Fe^{II} of Hb, which can reduce H_2O_2 while being oxidized to $\text{Hb-Fe}^{\text{III}}$ and then quickly reduced back to Hb-Fe^{II} through its direct electron transfer ability with the electrode¹⁹⁴.

Figure 4-6 CVs of a TNT/C modified GCE in a 0.1 M acetate buffer solution with 1) 0 and 2) 1 mM H_2O_2 . CVs of a TNT/C-Hb modified GCE in 0.1 M acetate buffer (pH 5.0) with 3) 0, 4) 0.1 mM, 5) 0.2 mM, 6) 0.5 mM, 7) 1 mM H_2O_2 . The scan rate is 100 mV s^{-1} .

The H_2O_2 reduction on TNT/C-Hb was explored for an amperometric H_2O_2 sensor. Figure 4-7 displays the amperometric responses of different concentrations of H_2O_2 at TNT/C-Hb modified GCE with an applied potential of -0.35 V vs SCE in acetate buffer

(pH 5.0). During the measurement, the concentration change of H_2O_2 in the buffer was conducted by addition of H_2O_2 every 40 s. The injected H_2O_2 causes a stepped increase in reduction current, which is in good agreement with the CV results shown in Figure 4-6. From the steady-state measurements, the response time of less than 3 seconds is determined when the responding current reaches 95% of the steady-state current, which is much faster than Hb-based H_2O_2 sensors (> 5 s)^{21, 88}. The response linear range is up to 1.60×10^{-4} M with a detection limit of 0.92×10^{-6} M for a signal-to-noise ratio of 3. The higher sensitivity and faster catalytic response of TNT/C-Hb electrode can be attributed to the good direct electrochemistry behavior of Hb on TNT/C.

The apparent Michaelis–Menten constant (K_M), an indicator of the enzyme–substrate kinetics, is generally used to evaluate the biological activity of an immobilized enzyme¹⁹⁵. The smaller the K_M is, the higher the bioactivity will be. When the concentration of H_2O_2 is higher than 1.60×10^{-4} M, the calibration curve tends to a plateau, implying a progressive enzyme inactivation in the presence of a high concentration of H_2O_2 that coincides with Michaelis-Menten kinetics model (inset of Figure 4-7)¹⁹⁶. K_M can be obtained by analysis of the slope and the intercept of the plot of the reciprocals of the steady-state current versus H_2O_2 concentration. In this work, the apparent Michaelis–Menten constant (K_M) is found to be 87.5 μM , which is smaller than that of 112 μM for Nafion/CdS–Hb/GP electrode, 550 μM for membrane-entrapped HRP, and 230 μM for a HRP/Au colloid self-assembled monolayer electrode⁹⁸. The small apparent Michaelis–Menten constant shows a good bioactivity of TNT/C-Hb toward H_2O_2 reduction.

Figure 4-7 Current-time curve of the TNT/C-Hb modified GCE for successive additions of specific concentrations of H_2O_2 to stirred 0.1 M pH 5.0 acetate buffer at -0.35 V. Inset is the calibration curve of the prepared sensor.

As a sensor application, the stability is very critical. The stability of TNT/C-Hb was tested by continuous CV measurements for 3 hours and the amperometric response reduced less than 2.5%. The TNT/C-Hb modified GCE sensor was stored in dry condition at 4 °C. It could retain 95% of its initial amperometric response after storage for two weeks. The stability testing results demonstrate that the TNT/C-Hb based H_2O_2 biosensor has good long-term storage life time, and sustains relatively long detection time (3 hours) without significant drift, retaining the great potential application for a mediator free H_2O_2 sensor. Since Hb has the active center of heme group that can specifically catalyze H_2O_2 , the TNT/C-Hb based sensor should own high selectivity towards H_2O_2 .

4.7 Conclusion

TNT/C with highly loose and porous nanostructure was prepared by carbonization of TNT with organic polymers. A comparative study between TNT/C and TNT demonstrates that TNT/C has higher conductivity, larger electrochemical active surface area and faster direct electron transfer rate of Hb. The TNT/C-Hb modified GCE has a high electrocatalytic activity toward H_2O_2 reduction and is used for H_2O_2 sensor, which has lower detection limit ($0.92\ \mu\text{M}$) and much faster response (3 s) than reported works. The sensor also has high dynamic response range and good stability. The work reported here demonstrates that a direct electrochemistry behavior can be significantly enhanced through conductivity improvement of a nanomaterial via a simple carbon coating approach, and also renders a great potential application in mediator free H_2O_2 detection.

Chapter 5 Ionic liquid/Mesoporous Carbon Enabled DET of GOD and Its Microelectrode for Sensitive Glucose Biosensing^b

5.1 Introduction

Order mesoporous materials have aroused great interests due to their unique nanostructure with high specific surface area and pore volume, well-ordered pore structures and narrow mesopore distribution, and broad applications¹⁹⁷⁻¹⁹⁸. Among them the order mesoporous carbon (OMC) has been particularly studied due to its good electrical conductivity and unique nanostructure for electrocatalysis¹⁹⁹⁻²⁰⁰. OMC modified electrode was used to sensitively detect dopamine in the presence of ascorbic acid²⁰¹. Different electroactive biospecies on OMC modified electrodes were investigated²⁰²⁻²⁰³. Direct electrochemistry of Hb adsorbed on OMC through layer-by-layer assembly was reported⁵⁸. In addition, OMC as electrode material was used to construct high-performance electrochemical biosensors²⁰⁴. It is critical to have accurate measurements for studies of the electrocatalytic behavior of an electrode material. However, a highly porous electrode such as OMC electrode with extremely large specific surface area suffers from high interface impedance²⁰⁵. An enzymatic biosensor with immobilized non-conductive proteins makes the porous electrode more difficult in electrochemical measurements, often resulting in distorted results that have a slope as reflected from the

^b Reprinted from *Electrochemistry Communications*, 11 (2008), Sun, W., Guo, C. X., Zhu, Z. H., Li, C. M, Ionic liquid/mesoporous carbon/protein composite microelectrode and its biosensing application, 2105-2108, Copyright (2008), with permission from Elsevier. Guo, C. X. and Sun, W. contribute equally to this work.

cyclic voltammogram measurements and also deviate from the normal conditions²⁰⁶⁻²⁰⁷.

An ionic liquid (IL) is a kind of liquid electrolyte composited entirely of ions, which renders a number of prominent physicochemical properties including good ionic conductivity and wide electrochemical window for various electrochemical applications²⁰⁸. IL can also be used as an electrolyte, a modifier on the electrode surface and a binder to prepare the carbon paste electrode for broad electrochemical applications. It has been reported that the IL composite electrodes can be made from graphite²⁰⁹, MWNTs²¹⁰ and even metal oxides²⁰⁵ to fabricate sensor/biosensors²¹¹⁻²¹² or to study electrocatalytic behaviors of an electrode material²⁰⁷.

In comparison to conventional carbon paste electrodes, IL composed carbon powder microelectrode is easy to fabricate and can use less carbon material for fast and accurate electrochemical measurements²¹¹. In this work, OMC was mixed with GOD and 1-butyl-3-methylimidazolium hexafluorophosphate (BMIMPF₆), an IL to construct a paste microelectrode. The direct electrochemistry of GOD on the composite modified microelectrode was realized and its catalytic performance towards glucose was also investigated.

5.2 OMC Preparation and OMC-GOD-IL Microelectrode Fabrication

OMC was prepared by using SBA-15 silica as template and polyethylene glycol (PEG) as a carbon precursor. In more detail, 1.0 g SBA-15 silica was first dispersed in 10.0 mL PEG under ultrasonication for 1 h, followed by vacuuming at 0.1 M Pa for 2 h. The obtained mixture was then kept overnight. After removal of the redundant PEG, a viscous gel was obtained and then 1.0 mL H₂SO₄ was added in under vigorous stirring.

The obtained precursor was placed in an oven for 12 h at 150 °C, which was further pyrolyzed at 900 °C under N₂ flow for 3 h. Finally OMC was obtained by washing with 5 wt % HF to remove the silica template.

1.0 mL of 10.0 mg mL⁻¹ as-prepared OMC aqueous solution was mixed with 10.0 mg of GOD and shaken for 2 h. The mixed solution was stored at 4°C refrigerator overnight for the protein adsorption. Then the solution was dried to obtain GOD modified OMC composite (GOD-OMC). The GOD-OMC was ground with BMIMPF₆ at a ratio of 60 mg: 15 µL in an agate mortar for 15 min to produce a uniform black mixture. The microelectrode was prepared according to our previous report with a cavity diameter of 50 µm and a depth of 20 µm²¹¹. Then the GOD-OMC-IL composite was filled into the cavity by grinding the tip against small amounts of as-prepared composite on a clean glass plate.

5.3 OMC, GOD-OMC and GOD-OMC-IL Characterizations

Morphologies of the as-prepared materials are shown in Figure 5-1. OMC is discernible, flake like particles with submicron-scale particle sizes in a length of ~ 1.0 µm and channel-like pore structure (Figure 5-1A). After immobilization with GOD, OMC retains its original flake like shape, indicating the uniform GOD modification (Figure 5-1B). However, from the TEM image (inset of Figure 5-1B), the contrast of channel-like pore structure becomes weak, which should be due to the surface-adsorbed non-crystallized GOD molecules. The OMC-GOD-IL composite displays a relative uniform film with a unique structure (Figure 5-1C), in which OMC-GOD is enwrapped with BMIMPF₆ with increased diameter, illustrating that the dispersion of OMC-GOD in the

IL results in a robust uniform composite.

Figure 5-1 SEM images of (A) OMC, (B) OMC-GOD and (C) OMC-GOD-IL, the insets are the high magnification SEM and TEM images.

RA-IR spectroscopy was used to verify native structure of GOD. The shape and position of the amide I ($1700\text{--}1600\text{ cm}^{-1}$) and amide II ($1620\text{--}1500\text{ cm}^{-1}$) bands of proteins could provide detailed information on the secondary structure of the polypeptide chain of proteins³³. As shown in Figure 5-2, typical amide I and amide II adsorption bands of GOD appear at 1634 and 1527 cm^{-1} (curve a of Figure 5-2) and that of GOD in GOD-OMC composite appears at 1626 and 1534 cm^{-1} (curve b of Figure 5-2) respectively, which are in a normal range of the specific group adsorption band. The RA-IR spectra change greatly after further mixed with the IL (curve c of Figure 5-2), which is due to the presence of the organic group of IL. However, the amide I and amide II bands of GOD are still observed in GOD-OMC-IL composite with similar positions, clearly indicating that GOD in the IL based composite remains its native structure.

Figure 5-2 RA-IR spectra of (a) GOD, (b) OMC-GOD and (c) OMC-GOD-IL.

5.4 Direct Electrochemistry of GOD-OMC-IL

CVs of different microelectrodes in pH 7.0 PBS solution in Figure 5-3A show that no

redox peaks appear on the control OMC-GOD-paraffin microelectrode (curve a of Figure 5-3A), indicating no direct electrochemistry occurs on the paraffin based composite. It is very possibly ascribed to the non-conductive nature of paraffin for blocking the electron transfer²¹³. The OMC-IL microelectrode does not produce, clearly due to the absence of electroactive species in the composite (curve b). The increased background current should be caused by the high capacitance current of OMC from its highly porous structure. In contrast, the CVs from the GOD-OMC-IL microelectrode show a pair of well-defined redox peaks (curve c), demonstrating direct electron transfer of GOD. IL is a binder with high ionic conductivity²¹⁴, so the DET between GOD and microelectrode was greatly improved. From the curve c the anodic (E_{pa}) and the cathodic peak potential (E_{pc}) is -0.505 and -0.415 V (vs.SCE), respectively. The peak-to-peak separation (ΔE_p) and the formal potential (E^0) were calculated to be 80 mV and -0.47 V, respectively, which were in good accordance with the typical characteristics of GOD electrochemistry [23], indicating that the successful immobilization of GOD on OMC-IL and the redox center of GOD, FAD, was reduced to $FADH_2$ and reoxidized to FAD in the redox process. Hence the combination of OMC and IL provides suitable microenvironment for GOD to achieve DET.

The influence of the scan rate on the electrochemical response of GOD-OMC-IL microelectrode was further investigated with the results shown in Figure 5-3B. With the increase of the scan rate in the range from 1.0 to 10.0 $mV s^{-1}$, the redox peak current increased simultaneously with the negative movement of reduction peak potential and the positively movement of oxidation peak potential, which was the typical quasi-reversible process.

Figure 5-3 (A) CVs of different microelectrodes in pH 7.0 0.1 M PBS with the scan rate of 8.0 mV s^{-1} . (a) GOD-OMC-paraffin microelectrode, (b) OMC-IL microelectrode and (c) GOD-OMC-IL microelectrode. (B) CVs of GOD-OMC-IL microelectrode in pH 7.0 0.1 M PBS with scan rate from inner to outer as 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 mV s^{-1} . (C) Linear relationships between the I_{pa} and I_{pc} with scan rate. (D) Plot of the steady-state current against glucose concentration.

The relationship of redox peak current with the scan rate was calibrated (Figure 5-3C) with linear regression equation as $I_{pa}(\mu\text{A}) = 0.00618 + 0.00552 \text{ } v \text{ (mV s}^{-1}\text{)}$ ($\gamma = 0.993$) and

$I_{pc}(\mu A) = -0.00727 - 0.00509v$ ($mV s^{-1}$) ($\gamma = 0.993$), indicating a surface-controlled thin-layer electrochemical behavior. The surface concentration of the electroactive proteins (Γ^*)²¹⁵ was estimated based on the formula of $Q = nF\Gamma^*$ and the average value of Γ^* was calculated as $7.05 \times 10^{-8} \text{ mol cm}^{-2}$, which was much higher than the reported value¹⁸. The relationship of redox peak potential with the scan rate was further investigated with the linear regression equations as $E_{pa}(V) = -0.473 + 0.0212 \ln v$ ($mV s^{-1}$) ($\gamma = 0.995$) and $E_{pc}(V) = -0.457 - 0.0274 \ln v$ ($mV s^{-1}$) ($\gamma = 0.992$). Based on the Laviron's equation²¹⁶, the electrochemical parameters such as the electron transfer coefficient (α) and the heterogeneous electron transfer rate constant (k_s) were calculated as 0.468 and $0.135 s^{-1}$, respectively. The buffer pH also influences the electrochemical response of the microelectrode. With the increase of pH from 5.0 to 8.0, the formal potential (E^0) shifted to the negative direction with a linear regression equation as $E^0(V) = 0.0179 - 0.0727 \text{ pH}$ ($\gamma = 0.993$). The slope value was close to the theoretical value of -0.060 V pH^{-1} , indicating the same amounts of proton and electron participated in the electrochemical process³³.

5.5 Sensitive Glucose Biosensing

The fabricated GOD-OMC-IL microelectrode also showed good electrocatalytic ability to glucose oxidation. With the addition of glucose to the air-saturated 0.1 M PBS the cathodic peak current decreased gradually (Figure 5-3D) and the linear regression equation was got as $I_p(nA) = -0.18C$ ($\mu\text{mol L}^{-1}$) + 62.50 ($n = 7$, $R = 0.991$) in the range from 10.0 to 80.0 $\mu\text{mol L}^{-1}$. The sensitivity of this sensor ($0.18 \text{ nA } \mu\text{M}^{-1}$) is higher than that of the conventional sensing scheme ($0.0016 \text{ nA } \mu\text{M}^{-1}$)²¹⁷ by two orders of magnitude. When the glucose concentration was more than 100.0 $\mu\text{mol L}^{-1}$, a plateau current appeared,

indicating a Michaelis-Menten kinetic mechanism of the enzymatic reaction. By using the electrochemical version of the Lineweaver-Burk equation²¹⁸⁻²¹⁹, the apparent Michaelis-Menten constant (K_M^{app}), which gives an indication of the enzyme-substrate kinetics, was calculated as 2.42 $\mu\text{mol L}^{-1}$, which was smaller than some reported values^{18, 220}. The smaller K_M^{app} of GOD on OMC-IL indicates that the enzyme electrode possesses higher enzymatic activity and higher affinity to glucose for higher sensitivity. However, in terms of Michaelis-Menten equation²¹¹, a lower K_M^{app} causes a narrower dynamic range. That is why the sensing dynamic range is smaller than the conventional sensor reported²¹⁷.

The stability of the microelectrode was tested by continuous CV scans in the same solution for 4 h with about 2.3% decreased response. After stored in 4°C refrigerator for three week, 93.0% of its initial response remained, indicating good stability. Because GOD is an enzyme that specifically catalyzes the oxidation of glucose, the resulting biosensor should retain the high selectivity towards glucose.

5.6 Conclusion

A unique composite paste microelectrode was fabricated by mixing GOD, OMC and BMIMPF₆ followed by inserting in a microcavity. The results show that GOD retains its native structure and the DET is realized on the composite paste microelectrode, due to the high conductivity and also specific microenvironment provided by the combination of OMC and IL. The electrochemical behaviors of GOD in the composite were investigated and the paste microelectrode showed good electrocatalytic ability to glucose oxidation, thus demonstrating good sensitivity and stability for glucose biosensing.

Chapter 6 PEDOT/NiO Hollow Sphere Promoted GOD DET for Enzymatic Glucose Biosensing Mechanism Study^c

6.1 Introduction

GOD from *Aspergillus Niger* is a typical flavin enzyme with FAD (flavin adenine dinucleotide)/FADH₂ as the redox prosthetic group (active center)²²¹⁻²²³. It is known that the GOD molecule is a structurally rigid glycoprotein with a molecular weight of 152 k-186 k Da, and consists of two identical polypeptide chains, each containing a FAD redox center²²⁴⁻²²⁶. The biological function of GOD is to catalyze glucose to form gluconolactone. When GOD catalyzes glucose oxidation, the GOD-FAD is reduced to GOD-FADH₂ that can be oxidized by the electrode back to GOD-FAD with the following reactions (Eq. 1 and 2)^{70, 126}.

However, generally it is very difficult for GOD-FADH₂ to be directly oxidized electrochemically through the electrode reaction. The major barrier is that the active center FAD/FADH₂ is located deeply with depth about 13 Å in the insulative apoenzyme, so even if GOD molecules are tightly immobilized the electrode surface, the distance between either one of its two FAD/FADH₂ centers and the electrode surface exceeds the critical

^c Reprinted from *Physical Chemistry Chemical Physics*, 12 (2010), Guo, C. X., Li, C. M., Direct electron transfer of glucose oxidase and biosensing of glucose on hollow sphere-nanostructured conducting polymer/metal oxide composite, 12153-12159, Copyright (2010), with permission from Royal Society of Chemistry.

electron tunneling distance^{26, 227}. The bioactivity of GOD is its capability to oxidize glucose through FAD/FADH₂ redox center as shown in Eq. 1. However, the insulative shell of GOD blocks the electron transfer between FAD/FADH₂ redox center and the electrode surface as shown in Eq. 2 to have poor electroactivity. To enable electron transfer between the active center of GOD and the electrode, traditionally, small diffusive electrochemical redox species, called electron mediators are added either in solution or immobilized on electrode to mediate the electron transfer, and have been extensively used to fabricate glucose biosensors^{114, 173, 228}.

With both theoretical significance and practical application with inherited simplicity, glucose biosensors based on DET between the active center of GOD and electrode have fueled up extensive research efforts^{102, 229-230}. Nanostructured materials to facilitate DET of GOD is one of the most popular approaches because they can immobilize GOD molecules to effectively shorten the electron tunneling distance by their superior quantum-confined physical, electronic and chemical properties^{163, 172, 231}. Various nanomaterials reported such as graphene⁵⁴, mesoporous TiO₂³³ and conducting polymer nanowires²³² with immobilized GOD have shown clear GOD DET and their based glucose biosensors have been constructed with good performance. However, it remains great challenges to develop new nanomaterials with even higher GOD DET capacity for not only use in sensitive enzymatic sensors, but also in biofuel cells with high energy conversion efficiency²³³⁻²³⁴. In addition, the contributions from both electrode and protein to the DET of the enzyme have not been completely understood. Particularly, it is a puzzle for a long time whether the mechanism of the glucose sensing is based on the DET of GOD. The main argument is that the direct electrochemistry based glucose biosensors relies on detection of decreased amperometric

responses of oxygen reduction rather than directly sensing glucose oxidation, which is very similar to the early Clark glucose sensor that senses the oxygen after consumption for H_2O_2 formation to detect glucose concentrations²³⁵, but it is not a direct electrochemistry based glucose biosensor. Oxygen seems not functioning as an electron transfer mediator in these glucose sensors since all electron transfer mediator-assisted enzymatic sensors directly detect the glucose oxidation with proportional responses to glucose concentrations²³⁵.

In this study, a hollow sphere-nanostructured conducting polymer (Poly(3,4-ethylenedioxythiophene), PEDOT)/metal oxide composite (PEDOT-NiO) was synthesized and characterized. The electrochemical properties, particularly the DET behavior of GOD on the material were studied. A glucose biosensor based on GOD immobilized on the material was explored. Experiments were delicately designed and conducted to study the mechanism behind the enzymatic glucose biosensing, and a possible mechanism was proposed.

6.2 PEDOT-NiO Hollow Sphere Synthesis

Carbon spheres were first synthesized according the hydrothermal method reported²³⁶. In detail, glucose was dissolved in water to form a clear solution, which was placed in a teflon-sealed autoclave and maintained at 160 °C for 12 h. The products were isolated by centrifugation, cleaned by three cycles of centrifugation/washing/redispersion in water and in alcohol, and dried at room temperature. Newly prepared carbon spheres were dispersed in water with ultrasonication to give a 0.5 wt% suspension. 20 mL of this suspension was added to 20 mL aqueous solution containing 0.01 M NiCl_2 , 0.1 M urea, and 0.05 M sodium dodecyl sulfate and further stirred at room temperature for 10 min. The mixed solution was

transferred into a 50 mL stainless steel autoclave and heated at 100 °C for 8 hours. After it was naturally cooled down, the sample was taken out and rinsed with water and dried in vacuum oven for overnight. The dried sample was calcined at 650 °C for 3 hours to get NiO hollow spheres. In the formation of conducting polymer/NiO hollow spheres, 3,4-ethylenedioxythiophene (EDOT) was chosen because its polymerized form, PEDOT has good stability and high conductivity²³⁷. To deposit, enough EDOT was added by micropipette to aqueous solution containing 0.5 wt% hollow NiO spheres, and the mixture agitated for 10 min. After centrifuge, the precipitate was transferred to a vial and dried at 50 °C for 1 h, after which a solution of ammonium persulfate (0.1 M) was added. The mixture was stirred overnight for completely chemical polymerization of EDOT to form PEDOT, isolated by centrifugation with water and ethanol washing, and dried at 50 °C under vacuum overnight.

Figure 6-1 Schematic of the preparation of PEDOT-NiO hollow sphere.

6.3 Material Characterizations

The SEM image in Figure 6-2a shows the template carbon spheres have an average diameter of 250 nm. After hydrothermal hydrolysis of Ni^{2+} salt and heat-treatment in air to

remove the carbon spheres, NiO hollow spheres (NiO HS) with an average diameter of 200 nm were obtained (Figure 6-2b). The slightly smaller diameter of NiO HS in comparison with the carbon sphere template might be due to the shrinkage during the high temperature treatment¹². From the SEM image of two occasionally broken NiO spheres (inset in Figure 6-1b), it is obvious that the NiO wall is thin in a thickness of ~15 nm. After deposition of conducting polymer PEDOT via intercalation²³⁷ on NiO HS, the material (PEDOT-NiO HS) retains the shape of sphere with an average diameter of 220 nm (Figure 6-1c), indicating the thickness of PEDOT coated on NiO layer is ~10 nm. For the composite, the PEDOT loading on NiO HS is about 28% (w/w) based on thermogravimetric analysis. To get more information about the structure of PEDOT-NiO HS, TEM was used. The strong contrast difference between the edge and the inner of these spheres (Figure 6-1d) reveals that the sample consists of hollow interior, which confirms that of PEDOT-NiO HS is successfully fabricated.

Figure 6-2 SEM images of carbon spheres (a), NiO hollow spheres (HS) (b), and PEDOT-NiO HS (c). Inset of (b) shows two occasionally broken NiO spheres. (d) TEM image of PEDOT-NiO HS.

6.4 Crystal Structure and Electrochemical Property Investigations

To reveal the crystal structure of the samples, XRD measurements were carried out. Figure 6-3a shows the XRD pattern of NiO HS. According to the Powder Diffraction File, JCPDS 14-0117, all of the diffraction peaks can be readily indexed to the standard XRD values of NiO. After PEDOT deposition, except to the peaks similar to that of NiO HS, there is a broad peak at $2\theta = 24.73^\circ$ ($d = 0.36$ nm), a typical XRD peak for PEDOT²³⁸. These results clearly tell that the hollow spheres compose of metal oxides NiO and polymer PEDOT. The nitrogen adsorption/desorption measurements display that pore sizes of

PEDOT-NiO are 5 to 20 nm with large specific surface area of $234.2 \text{ m}^2\text{g}^{-1}$, which can be readily tailored to match the dimensional size of GOD ($<8 \text{ nm}$)³³ for a large amount of GOD adsorption. The electrochemical properties were characterized by EIS. The measured complex impedance (Z) versus frequency, known as Nyquist plot is shown in Figure 6-3b, which is always modeled by using Randle equivalent circuit (inset of Figure 6-3b). One of the most important parameters in the equivalent circuit is the charge transfer resistance, R_{ct} , which determines the diameter of the semicircle. The values of R_{ct} that are calculated from the results in terms of Randle equivalent circuit by using the software package ZSimpWin are 6.5 and $1.8 \text{ } \Omega \text{ cm}^2$ (surface area of GCE, 0.071 cm^2) for NiO HS and PEDOT-NiO HS electrodes, respectively, indicating that PEDOT-NiO HS has much lower charge transfer resistance.

Figure 6-3 (a) XRD patterns of NiO HS and PEDOT-NiO HS. Electrochemical impedance spectroscopy measurements in 1.0 M KCl containing 10 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ of electrodes before (b) and after (c) GOD immobilization. Inset of (b) is the Randle equivalent circuit. (d) FTIR spectra of plain GOD (1), GOD/NiO HS (2), and GOD/PEDOT-NiO HS (3).

6.5 GOD Immobilization and Characterizations

After GOD immobilization, the surface coverage of GOD on electrodes was calculated by Nyquist plot shown in Figure 6-3c. The charge transfer resistance of hollow sphere after GOD immobilization, R_{ct}^{GOD} , is 689.5 and 299.2 $\Omega \text{ cm}^2$ for GOD/NiO HS and

GOD/PEDOT-NiO HS electrodes, respectively, which increase by a factor of ~ 100 , compared with that (R_{ct}) obtained before GOD immobilization as shown in Figure 6-3b. The surface coverage of GOD (θ) on electrode can be derived from the equation $\theta = (1 - R_{ct}/R_{ct}^{GOD}) \times 100\%$ ¹⁸³. The surface coverage of GOD confined at NiO HS and PEDOT-NiO HS electrode is 99.3% and 99.4%, respectively, indicating that the surface coverage of GOD on PEDOT-NiO HS is comparable with that on NiO HS.

To better understand the protein conformational change after immobilization, the immobilized GOD on samples was examined by the FTIR, an efficient tool to study the bioactivity related secondary structure of proteins. It is known that, the amide I and II bands among the nine characteristic vibrational bands that arise from the amide groups, together with stretching vibration of C–O band, can provide the detailed secondary structure of proteins²³⁹. Two obvious IR absorption bands centered at 1650 and 1544 cm^{-1} are observed for GOD itself (Curve 1 of Figure 6-3d), ascribing to the typical amide I and II adsorption band, respectively. An absorption band around 1100 cm^{-1} is also seen, corresponding to the stretching vibration of C–O of GOD. It is clear that amide I band has almost no change for GOD on both NiO HS (curve 2) and PEDOT-NiO HS (curve 3). On the other hand, the amide II band shows no change for GOD on PEDOT-NiO HS, whereas it has a shift of 15 cm^{-1} for GOD on NiO HS. Moreover, the stretching vibration band around 1100 cm^{-1} is still here for GOD on PEDOT-HS and it almost disappears for GOD on NiO HS. The FTIR results can provide solid evidence whether GOD is probably denatured, or partially denatured after its immobilization. Thus, the FTIR result here clearly indicates that GOD retains its native structure after its immobilization on PEDOT-NiO HS.

It is very likely that the highly biocompatible PEDOT coating can prevent GOD from immobilization-caused denature on the electrode.

6.6 Direct Electron Transfer Studies

Electrochemical behaviors of different electrodes were studied by CV. The plain GOD (curve a) and PEDOT-NiO HS (curve b) electrodes only exhibit typical capacitive CV responses caused by their double layer capacitance without any obvious redox peaks (Figure 6-4), suggesting that both the GOD and PEDOT-NiO HS are electro-inactive over this potential window. For GOD/NiO HS electrode (curve c), there is a pair of weak redox peaks with anodic (E_{pa}) and cathodic peak potential (E_{pc}) are at -0.396 and -0.488 V (vs.SCE), respectively. The peak-to-peak separation (ΔE_p) and the formal potential (E^0) are calculated as 92 mV and -0.442 V, respectively. The GOD/PEDOT-NiO HS electrode (curve d) shows a pair of well-defined redox peaks with E_{pa} and E_{pc} at -0.408 and -0.448 V (vs.SCE) respectively, corresponding to 40 mV for ΔE_p and -0.428 V for E^0 , respectively. The E^0 value of GOD on PEDOT-NiO HS is in accordance with the typical characteristics of GOD electrochemistry in neutral pH solution³³, indicating that the redox center of GOD, FAD, is reduced to FADH₂ and reversibly reoxidized to FAD for a reversible redox process as shown in Eq. 2. Based on the Laviron's equation²⁴⁰, the heterogeneous electron transfer rate constant (k_s) can be calculated when the peak-to-peak separation is less than 200 mV. From curve d of Figure 6-4, k_s of GOD on PEDOT-NiO HS is found out as 6.5 s⁻¹, much larger than that of GOD on NiO HS (3.12 s⁻¹, curve c of Figure 6-4). The electroactive protein density (Γ^* , mol cm⁻²) on the electrode surface can be estimated based on the formula of $Q=nF\Gamma^*$ ¹⁰² as 1.56×10⁻¹⁰ mol cm⁻² for GOD/PEDOT-NiO HS, which is ten-

fold higher than that of GOD/NiO HS ($0.11 \times 10^{-10} \text{ mol cm}^{-2}$). These results demonstrate the better DET capability of GOD on PEDOT-NiO HS, which is consistent with the native structure of GOD on PEDOT-NiO HS shown in Figure 6-3d.

Figure 6-4 CVs of different electrodes in 0.1 M pH 7.0 Nitrogen-saturated PBS solution at 100 mV s^{-1} . (a) plain GOD, (b) PEDOT-NiO HS, (c) GOD/NiO HS and (d) GOD/PEDOT-NiO HS.

As discussed above, the surface coverage of GOD on PEDOT-NiO HS (99.4%) is comparable with that of GOD on NiO HS (99.3%), thus the differences for k_s and Γ^* are not due to the relative amount of protein immobilized on electrodes. It is known that GOD has an isoelectric point of 4.2 to carry negative charge in a neutral solution²⁶ and PEDOT has a certain amount of positive charges²⁴¹. The electrostatic interaction between negatively charged GOD and positively charged PEDOT may push the active centers FAD/FADH₂ to access the electrode surface, thus shortening the tunneling distance for the fast direct electron transfer of the enzyme molecule. The FTIR studies show that GOD can hold its

native structure on PEDOT-NiO HS, while is probably denatured, or partially denatured on NiO HS. Moreover, the low charge transfer resistance of PEDOT-NiO HS from its low interfacial resistance (Figure 6-3b) can also enhance the electron transfer from the enzyme redox centers FAD/FADH₂ to the bulk electrode.

Figure 6-5 (a) Plots of peak current versus scan rate from CVs of GOD/PEDOT-NiO HS (from 20 to 500 mV s⁻¹). (b) Plot of peak current versus pH value of GOD/PEDOT-NiO HS at 100 mV s⁻¹. Inset of (b) is the plot of E^0 versus pH value.

The effect of scan rate on the electrochemical response of GOD on PEDOT-NiO HS in Figure 6-5a displays a linear relationship of redox peak current versus the scan rate, indicating that the GOD redox reaction is a surface-controlled electrochemical process from the FAD/FADH₂ active center of GOD on PEDOT-NiO HS³³. The electrochemical response of GOD on PEDOT-NiO HS also shows a strong dependence on solution pH (Fig. 4b), in which the formal potential (E^0) shifts negatively with increased solution pH, revealing that H⁺ involves in the electrochemical reaction of GOD. The slope of the peak current (E_p) versus pH is 60.8 mV pH⁻¹ (inset of Figure 6-5b), which can be well explained

by the redox reaction involved in the direct electrochemistry as shown in Eq. 2. In terms of Eq. 2, a theoretical value of 59.0 mV pH⁻¹ for the slope of E^0 versus pH is expected, which is very close to the measured value of 60.8 mV pH⁻¹. Hence, the DET reaction of GOD on PEDOT-NiO HS is a two-proton coupled with two-electron transfer process²³⁹. The pH value also affects the redox peak currents of GOD (Figure 6-5b), which increases as pH increases until the maximum peak current reaches around pH 7.0. With a further increase of pH, the peak current decreases, possibly indicating that the immobilized GOD has the highest bioactivity at pH 7.0.

6.7 Glucose Biosensing Mechanism Study

CV measurements with GOD/PEDOT-NiO HS electrode were conducted in air-saturated PBS containing different concentrations of glucose, showing a prominent pair of redox peaks but with much higher cathodic peak current than the anodic one. The cathodic peak current achieves the highest value in an air-saturated PBS without glucose presence (Figure 6-6a). This apparently indicates that larger cathodic current is contributed by oxygen reduction. Interestingly, no obvious cathodic current is observed on PEDOT-NiO HS electrode in the air-saturated PBS (not shown), strongly proving that GOD on PEDOT-NiO HS with DET ability can significantly catalyze the oxygen reduction. This is significantly different from the earliest Clark glucose sensors²⁴², which use a platinum electrode to measure the rate of oxygen uptake (Eq. 4) after consumption to form H₂O₂ under GOD catalysis (Eq. 3):

Apparently, the Pt electrode without GOD load in air-saturated solution without the presence of glucose can measure the largest current from oxygen reduction. Thus the oxygen reduction on the GOD/PEDOT-NiO electrode is catalyzed by GOD, very likely through the DET process of GOD as the following:

for a 4-electron transfer path of oxygen reduction²⁴², or

for a 2-electron transfer path of oxygen reduction, followed by

Followed by the redox reaction in Eq. 2, in whichever cases, the apparent net half cell reaction should be the same as Eq. 4. Figure 6-6 also presents another important fact that the cathodic peak current in the air-saturated PBS decreases with increasing the glucose concentration and can be used to detect glucose concentrations. This is very possibly ascribed to the competitive glucose oxidation also through GOD FAD/FADH₂ reaction centers by the direct electron transfer path in terms of Eq. 1 and 2.

Figure 6-6 (a) CVs of the GOD/PEDOT-NiO HS in air-saturated PBS solution with different concentrations of glucose. (b) The enlarged curves of the part shown in the rectangular of (a). Scan rate: 100 mV s^{-1} .

Figure 6-7 (a) CVs of the GOD/PEDOT-NiO HS in nitrogen-saturated PBS solution with different concentrations of glucose. (b) Curves obtained by background-subtraction from (a) to eliminate charging current effect. Inset of (b) is the calibration curve. Scan rate: 100 mV s^{-1} .

To further confirm the competitive reactions of oxygen reduction and glucose oxidation through the DET of GOD on PEDOT-NiO HS, CV measurements were conducted in nitrogen-saturated PBS containing different glucose concentrations. No anodic peak current was observed for PEDOT-NiO HS in the presence of glucose. For GOD/PEDOT-NiO HS electrode in a solution without the presence of glucose, the anodic peak current is zero; upon addition of glucose for increased concentrations, it increases accordingly (Figure 6-7a). It can be found from the calibration curve in Figure 6-7b obtained by subtracting the background that the anodic current linearly increases with the increased glucose concentration until it starts to saturate from 5 mM. This behavior is in good agreement with the enzymatic kinetic mechanism³³ and strongly supports that the glucose oxidation on the GOD/PEDOT-NiO HS electrode is based on the DET of GOD. From the background-subtraction curves (Figure 6-7b), around 0.4 μ A anodic current increase is resulted from 5 mM glucose added to nitrogen-saturated PBS, which is much lower than that of around 1.4 μ A cathodic current decrease for 5 mM glucose addition in an air-saturated PBS (Figure 6-6b). This might be caused by the competitive adsorption of a much larger glucose molecule on the GOD/PEDOT-NiO HS surface that can block much smaller oxygen molecules to be absorbed, thus resulting in larger decreased current from oxygen reduction. It should be mentioned that GOD is an enzyme that specifically catalyzes the oxidation of glucose. Therefore, biosensors utilizing GOD can retain the high selectivity towards glucose.

6.8 Glucose Biosensing Investigation

Because both in vivo and in vitro biological systems always contain oxygen, the decreased cathodic current from competitive DET reactions upon addition of glucose in air-

saturated PBS can be used to detect glucose. The response of the GOD/PEDOT-NiO HS to successive injections of a glucose solution in air-saturated PBS at -0.45 V is shown in Figure 6-8a (curve 2). Each addition of 0.18 μmol glucose results in a change in the steady state current with response time of less than 10 seconds. No obvious response is obtained at PEDOT-NiO HS electrode (curve 1), which is consisted with the above CV results.

Figure 6-8 (a) Current-time curves for successive additions of 0.18 μmol glucose to stirred 0.1 M pH 7.0 PBS at -0.45 V, (1) PEDOT-NiO HS and (2) GOD/PEDOT-NiO HS. (b) The calibration curve (current versus glucose concentration) and Lineweaver-Burk plot (current⁻¹ versus concentration⁻¹) from curve 2 of (a).

The calibration plot in Figure 6-8b obtained from curve 1 of Figure 6-8a shows a well defined, typical behavior of an enzymatic kinetic reaction with a linear regression equation as $I_p(\mu A) = 1.2 (\mu A \text{ mM}^{-1}) \times C (\text{mM})$ ($n = 5$, $R = 0.989$) in a range up to 1.5 mM, demonstrating a sensitivity of $16.9 \mu A/(\text{mM cm}^2)$, superior to that of glucose biosensors based on many other nanomaterials^{178, 227, 239}. When the glucose concentration is higher than 1.5 mM, a plateau current appears. The apparent Michaelis-Menten constant (K_M^{app}), which gives an indication of the enzyme-substrate kinetics and a smaller value means a higher enzymatic activity and higher affinity towards the substrate. By using the electrochemical version of the Lineweaver-Burk equation²⁴³, $1/I_m = 1/I_{\text{max}} + K_M^{\text{app}}/(C \times I_{\text{max}})$, where I_m is steady-state current, I_{max} is the maximum current, and C is the glucose concentration, a liner plot of $1/I_m$ vs. $1/C$ (Figure 6-8b) is obtained to calculate K_M^{app} of GOD/PEDOT-NiO HS from its intercept and slope. A value of 1.82 mM is found, which is much smaller than 8.46 mM for GOD/CNT electrode²⁴³, and 6.08 mM for a GOD/TiO₂ electrode²⁴⁴. The smaller K_M^{app} of GOD on PEDOT-NiO HS means that the enzyme electrode possesses higher enzymatic activity and higher affinity to glucose oxidation.

The stability of a biosensor is also critical. The GOD/PEDOT-NiO HS glucose sensor was tested by continuous CV measurements for 5 hours and the amperimetric response was reduced less than 2.5 %. The enzyme electrode was stored in at 4 °C when not in use. It could retain 95 % of its initial amperometric response after storage for four weeks, which demonstrates that the biosensor has good long-term life time and sustains relatively long detection time (5 hours) without significant drift.

6.9 Conclusion

A unique hollow sphere-nanostructured PEDOT/NiO composite was designed, synthesized and characterized. The electrochemical properties of the GOD immobilized on the material were investigated, demonstrating fast DET resulted from the electrostatic interaction between GOD and PEDOT and the retained native structure of GOD. Particularly, the sensing mechanism of the glucose biosensor based on the DET of GOD on the material was studied, indicating that competitive reactions through the DET path towards oxygen reduction and glucose oxidation result in significant decreased oxygen reduction current for sensitive detection of glucose. A glucose biosensor was constructed with the GOD immobilized material and exhibited high sensitivity and long term-stability. This work not only demonstrates a unique nanomaterial for enhanced direct electrochemistry of GOD and its application in high performance glucose biosensor, but also provides fundamental insights to understand the DET process, particularly the mechanism of the enzyme direct electrochemistry based glucose biosensor.

Chapter 7 Horseradish Peroxidase-Au Nanoparticle-Polyaniline Nanowire Biofilm with Enhanced Protein DET for *In-Situ* Molecular Detection of Ischemic Cells^d

7.1 Introduction

Ischemia is inadequate blood supply (circulation) to a local area due to blockage of the blood vessels and is a symptom of heart diseases, transient ischemic attacks, cerebrovascular accidents and brain tissue death²⁴⁵⁻²⁴⁶. Its important indicator is the increased H₂O₂ molecules released from ischemic tissues (cells)²⁴⁷. Currently the diagnosis of ischemic tissues (cells) mainly relies on the *ex-situ* H₂O₂ detection, which requires multiple processes including lysis of tissues (cells) to release H₂O₂, oxidation of a chemical indicator by the released H₂O₂ in the presence of fluorescence molecules followed by an absorbance measurement²⁴⁸⁻²⁴⁹. These *ex-situ* approaches have complicated procedures, use harmful chemicals such as acid, and require extended assay time. They may not able to provide results for an in time ischemia medical treatment to avoid severe damage of organs from metabolic wastes build-up²⁴⁷. Thus efficient diagnosis of ischemia by *in-situ* detection of H₂O₂ molecule is indeed highly demanded.

Selectivity and sensitivity are particularly critical for *in-situ* biosensors^{170, 250-251}. Good

^d Reprinted from *Chemical Communications*, Guo, C. X., Zheng, X. T., Ng, S. R., Lai, Y. C., Lei, Y., Li, C. M., *In-situ* molecular detection of ischemic cells by enhanced protein direct electron transfer on a unique horseradish peroxidase-Au nanoparticles-polyaniline nanowire biofilm, DOI:10.1039/C0CC04542J, Copyright (2010), with permission from Royal Society of Chemistry.

selectivity can efficiently eliminate various interferences in biological systems, while high sensitivity can detect the extremely low concentration of target molecules, which often occurs in biological systems detections²⁵². Horseradish peroxidase (HRP) has been used to provide specific detection of H_2O_2 ²⁵³, but it suffers from low sensitivity caused by its deeply embedded redox active center for difficult direct electron transfer and absorptive denaturation to result in low catalytic activity on conventional electrodes²⁵⁴⁻²⁵⁵. Therefore, construction of a superior HRP biofilm without denaturation while rendering fast direct electron transfer for high H_2O_2 electrocatalytic activity is essential to achieve a high-performance *in-situ* biosensor towards H_2O_2 .

Herein, we built a unique biofilm by immobilizing HRP (isoelectric point (pI) of 7.2) on negatively charged Au nanoparticles (Au NPs) coated polyaniline (PANi) nanowires that were directly grown on glassy carbon electrodes (HRP-Au NP-PANi), and used to quantitatively *in-situ* detect released extracellular H_2O_2 for diagnosis of ischemic cells. The directly grown PANi nanowires could provide large specific surface area while providing defect-free interfaces to eliminate high interfacial resistance. The negatively charged Au NPs (~20 nm, protected by sodium citrate) can function as efficient nanolinkers to immobilize the positive charged HRP molecules (pI of 7.2) on PANi nanowires through electrostatic adsorption in a solution with pH of 6.5 for facilitating protein direct electron transfer. The preparation and the *in-situ* detection are schematically shown in Figure 7-1.

Figure 7-1 Schematics of construction of the HRP-Au NP-PANi sensing biofilm and the *in-situ* detection.

7.2 HRP-Au NP-PANi Biofilm Preparation

PANi nanowires were grown on cleaned GCEs (surface area of 0.071 cm^2) by a two-step galvanostatic technique in 1.0 M HClO_4 containing 0.1 M aniline. It was started by applying a constant current of 0.08 mA cm^{-2} for 20 min followed by 0.02 mA cm^{-2} for 90 min . The electrodes were washed with distilled water. Au NPs coating was prepared by immersing PANi nanowires on GCEs in Au NPs solution for 24 hours at 4°C and then washed with distilled water. HRP immobilization was prepared by putting Au NP-PANi electrodes in 0.01 M phosphate buffered saline (PBS, $\text{pH } 6.5$) containing 10 mg ml^{-1}

HRP overnight at 4 °C. The electrodes were rinsed with 0.01 M PBS to remove unabsorbed HRP, and then dried with nitrogen.

7.3 Cell Culture and Measurements

Rat smooth muscle cells (SMCs) were grown in Dulbecco's Modified Eagle's Medium (DMEM) (PAA Laboratories, Pasching, Austria) supplemented with 10% heat inactivated fetal bovine serum (FBS) (PAA laboratories) and 50 U mL⁻¹ penicillin/streptomycin (Gibco). The cells were grown in a humidified incubator at 37 °C with 5.0% CO₂. Ischemia was induced as reported by clamping the gas flow cannula to cell culture wells while keeping them in nitrogen protected environment for 30 min. The SMCs were harvested by trypsinization and centrifuged to obtain a cell-packed pellet (~1 × 10⁶ cells) for the electrochemical experiments performed in 2 mL 0.01 M PBS (pH 7.0). The measurements were carried out carefully to avoid any damage to the cell pellet.

7.4 Biofilm Characterizations and Protein DET Study

The biofilm construction was investigated by SEM. Figure 7-2a shows that PANi nanowires have average diameter of 150 nm and are interconnected with each other. After the coating of Au NPs (Figure 7-2b), PANi nanowires are uniformly covered by ~20 nm-sized Au NPs, which can be attributed to the good interaction between the negatively charged Au NPs and the positively charged PANi^{232, 256}. Figure 7-2c exhibits that HRP molecules are homogenously immobilized on Au NP-PANi and the resultant composite retains the shape of nanowires. The good adsorption of HRP should come from the

electrostatic force between the negatively charged Au NPs and the positively charged HRP molecules (pI of 7.2) in solution with pH of 6.5.

Figure 7-2 SEM images of PANi nanowires (a), Au NP-PANi (b) and HRP-Au NP-PANi biofilm (c).

The possibility of HRP conformational change was examined by FTIR (Figure 7-3). Two IR absorption bands centered at 1650 and 1544 cm^{-1} are observed for plain HRP, assigned to the typical amide I and II adsorption band, respectively. An absorption band around 1082 cm^{-1} is also seen, corresponding to the stretching vibration of C–O of HRP²⁵⁷. After immobilization on Au NP-PANi, the amide I and II bands together with C–O band show no change for HRP, suggesting there is no denaturation of HRP through the electrostatic interaction.

Figure 7-3 FTIR spectra of HRP, Au NP-PANi nanowires and HRP-Au NP-PANi biofilm.

Electrochemical characterizations of the electrodes were studied by CVs. A pair of well-defined redox peaks are observed for the HRP-Au NP-PANi biofilm, while a typical capacitive CV has no obvious redox peak observed for the plain HRP and Au NP-PANi (Figure 7-4a). Obviously, the result shows that direct electron transfer process is enabled by the Au NP modified PANi. The apparent heterogeneous electron transfer rate constant is

calculated to be 3.52 s^{-1} for the HRP-Au NP-PANi biofilm, which is much larger than that of 1.85 s^{-1} and 1.52 s^{-1} for HRP on Au NPs and PANi nanowires, respectively (not shown), indicating that the enhanced direct electron transfer of HRP-Au NP-PANi biofilm. The linear relationship of redox peak currents against the scan rate in Figure 7-4b suggests that the HRP redox reaction on the biofilm is a surface-controlled electrochemical process and the redox signal really comes from the immobilized HRP²⁵⁰.

Figure 7-4 (a) CVs of different electrodes in 0.01 M N_2 -saturated PBS (pH 7.0) at 100 mV s^{-1} . (b) Plots of peak currents versus scan rate (from 5 to 250 mV s^{-1}) of the biofilm.

7.5 Sensitivity and Selectivity of the Sensing Biofilm

The electrocatalytic activity and selectivity of the biofilm toward H_2O_2 were investigated. In the presence of H_2O_2 , the onset potential starts at -0.05 V (vs. SCE) and the reduction peak current at -0.325 V increases significantly with the increase of H_2O_2 concentration (Figure 7-5a), demonstrating a typical electrocatalytic reduction process

towards H_2O_2 ²⁵⁸. The selectivity of the H_2O_2 biosensor against several kinds of potential interferences normally co-existing with H_2O_2 in biological systems was shown in Figure 7-5b, indicating that at -0.25 V the biofilm could eliminate both anodic and cathodic potential interferences, while retaining its high electrocatalytic activity towards H_2O_2 . Thus, -0.25 V was chosen in the following experiments. The amperometric responses of the biosensor towards H_2O_2 in Figure 7-5c indicate that the successive injection of H_2O_2 causes stepped increases in reduction current, and a response time of 3 s can be determined. The sensitivity of the biosensor is $117.8 \mu\text{A mM}^{-1} \text{cm}^{-2}$ (electrode surface area of 0.071 cm^{-2}) with a linear range up to 3.6×10^{-4} M and the detection limit is $0.3 \mu\text{M}$ ($\text{S/N} = 3$). The sensitivity and detection limit are much superior to these of HRP on carbon nanotubes²⁵⁹, conducting polymers¹⁴⁸, Au nanowire arrays²⁶⁰ and Au NP/graphene²⁶¹. In addition, the amperometric response of the sensor has almost the same value after 12 measurements, indicating reproducible sensitivity and signal/noise ratio. The cumulative deviation of the responses of over 8 sensors prepared with the same procedure does not exceed 6.8% (RSD). Moreover, the lifetime was tested by recording the response current for $0.1 \text{ mM H}_2\text{O}_2$ for two hours daily and it could retain 95% of its initial response for more than two weeks. These results demonstrate that the H_2O_2 biosensor can provide fast response, high sensitivity, low detection limit and good selectivity and stability, and thus could be a high-performance sensor for *in-situ* H_2O_2 detection for ischemia diagnosis.

Figure 7-5 (a) CVs of Au NP-PANi in 0.01 M PBS buffer (pH 7.0) with 1) 0 and 2) 0.5 mM H_2O_2 , and of HRP-Au NP-PANi biofilm with 3) 0, 4) 0.1 mM, 5) 0.2 mM, 6) 0.3 mM, 7) 0.5 mM H_2O_2 . (b) The selectivity profile of the biofilm. (c) Amperometric responses and the corresponding calibration curve of the biofilm for successive additions of 30 μ M H_2O_2 to 0.01 M PBS buffer (pH 7.0).

7.6 *In-Situ* Molecular Detection of Ischemic Cells

The HRP-Au NP-PANi biofilms were placed in plain 0.01 M PBS (pH 7.0), 0.01 M PBS plus normal and ischemic smooth muscle cells (SMCs), respectively to *in-situ* detect the released extracellular H_2O_2 , in which at applied -0.25 V (vs. SCE) simultaneously when injecting 0.1 mM phorbol 12-myristate-13-acetate (PMA) to induce H_2O_2 production from cells²⁶² until a plateau response achieved and then 300 U mL^{-1} catalase was injected to selectively scavenge the produced H_2O_2 ²⁵². Since the SMC used is a cell line that can keep its viability and activity in buffer solutions for several hours (250, 260), the use of PBS is good for the measurements (<30 min). However, for primary cells detection, cell culture medium is preferable as the electrolyte. The *in-situ* detections were repeated for 5 times to obtain the standard deviation and the results are shown in Figure 7-6. The increased cathodic currents are observed at the biosensors with the addition of PMA for both normal and ischemic SMCs, while a negligible response was attained at the biofilm without SMCs. In addition, after the injection of 300 U mL^{-1} catalase, the cathodic currents for both normal and ischemic SMCs decrease down to almost the background level. Therefore, it can be concluded that the increase of cathodic currents at the biosensors with both normal and ischemic SMCs should be ascribed to the reduction of extracellular H_2O_2 produced from the cells and the sensing reaction is effectively catalyzed by the biofilm. More importantly, the response to the normal and ischemic SMCs (curve a and c of Figure 7-6b) is 43 and 116 nA, respectively. With a sensitivity of $117.8\text{ }\mu\text{A mM}^{-1}\text{ cm}^{-2}$, electrode surface area of 0.071 cm^2 , cell number of $\sim 1 \times 10^6$, the volume of the electrolyte of 2 mL and the measured response current, the average number of extracellular H_2O_2 molecule released per cell can be calculated to be $\sim 1.7 \times 10^{10}$ for

ischemic SMCs, 2.7-times of that for normal SMCs ($\sim 0.62 \times 10^{10}$). In comparison to normal cells the increased H_2O_2 production from ischemic SMCs (2.7 times) detected by this *in-situ* method is consistent with that (2.5 times) obtained by using the complicated *ex-situ* approach discussed above²⁴⁹. The slightly lower H_2O_2 detected by the *ex-situ* approach is very likely to be ascribed to its extended assay time, possibly resulting in decomposition and/or loss of H_2O_2 .

Figure 7-6 (a) Amperometric responses of the biofilm in 0.01 M PBS (pH 7.0) under the injections of 0.1 mM PMA, followed by 300 U ml^{-1} catalase. (b) Plots of the current responses for normal and ischemic SMCs with addition of 0.1 mM PMA (curve a and c) followed by adding 300 U ml^{-1} catalase (curve b and d).

7.7 Conclusion

In summary, *in-situ* detecting released extracellular H_2O_2 molecules to diagnose ischemia through enhanced protein direct electron transfer is realized on a unique HRP-Au NP-PANi biofilm showing fast response (3 s), high sensitivity ($117.8 \mu\text{A mM}^{-1} \text{cm}^{-2}$), low detection limit ($0.3 \mu\text{M}$) and good selectivity and stability. This *in-situ* method can significantly reduce the assay time for in time medical treatments of ischemia. This study also demonstrates a universal approach to develop various direct electrochemistry-based protein sensors for other pathological investigations and diagnosis.

Chapter 8 General Conclusion and Further Research

8.1 General Conclusion

One of the primary motivations behind and accomplishments of this research is the development of a nanocomposite of carbonized TiO_2 nanotubes prepared by carbonizing TiO_2 nanotubes to obtain a higher reaction surface area and a better conductivity to immobilize protein Hb and explore its DET behaviors. Compared with its counterpart, plain TiO_2 nanotubes, the nanocomposite developed in this research provides superior enhancement of Hb DET capacity and exhibits excellent bioelectrocatalytic activity toward H_2O_2 when used in biosensing application, demonstrating a fast response time of 3 s, a long linear detection range of up to 100 μM , and a low detection limit of 0.92 μM . The subsequent analysis of this nanocomposite and related materials clearly demonstrate that the conductivity of nanomaterials plays an important role in enhancing protein DET capacity and improving biosensing performance.

In this study, a functional nanocomposite composed of mesoporous carbon that provides high conductivity and a large specific surface area, protein GOD, and an ionic liquid with good ionic conductivity, has been filled into a microcavity to fabricate a paste microelectrode that allows for rapid DET and high-performance glucose biosensing. The description of the development of this nanocomposite provides an efficient and accurate approach to investigating protein direct electrochemistry that has great potential in the study and the development of miniaturized electrochemical enzymatic biosensors.

A specially designed nanocomposite of hollow sphere-structured conductive polymer/metal oxide that possesses high conductivity and desirable surface charge

properties and can strongly adsorb GOD while retaining its native structure is demonstrated to have a high level of DET capacity. More importantly, delicately designed experiments reveal that the enzymatic glucose biosensing mechanism is a GOD DET process with competitive glucose oxidation and oxygen reduction to detect glucose.

A unique nanocomposite biofilm of horseradish peroxidase-Au nanoparticle-polyaniline nanowires has been fabricated by coating negatively charged Au nanoparticles on polyaniline nanowires, and then allowing for the adsorption of positively charged horseradish peroxidase. The resulting Au nanoparticle-polyaniline nanowires are found to have a large contact surface area, desirable surface charge properties and a high level of conductivity, all of which greatly promote horseradish peroxidase immobilization and DET enhancement. Furthermore, the biofilm can be successfully employed for ischemia diagnosis, finding that the number of H_2O_2 molecule released per ischemic cell is 2.7-times of that of a normal cell.

This research project not only provides the basis for the development of novel nanocomposites for enhanced protein DET capacity and high-performance biosensing in practical applications, but also expands in-depth fundamental knowledge of the effects of nanomaterial properties on protein DET behaviors, as well as of the mechanism behind enzymatic biosensing, especially enzymatic glucose biosensing.

8.2 Further Research

Although this project has fabricated several nanomaterials with unique physicochemical properties, including a high level of conductivity, desirable surface charge properties and a porous structure that enhance protein DET capacity and

biosensing performance, the full impact of nanostructure characteristics, including their size, dimensions and shapes on protein DET behaviors remains not fully understood. Systematically examining the impact of carbon nanomaterials of different dimensions, such as zero-dimensional fullerene, one-dimensional carbon nanotubes, two-dimensional graphene and three-dimensional mesoporous carbon, can lead to further understanding of protein DET behaviors.

To date, most research into protein DET has focused on the properties of nanomaterials, with few studies focusing on the means of tailoring the nano- and/or molecular structure of proteins to enhance their DET capacity. It is expected that the genetic engineering and post-modification of protein structures to promote their DET capacity with electrodes will attract more interest in research into protein direct electrochemistry and high-performance biosensors. Post-modification of protein structure means the modification of protein structure in the molecular level. Such a possible approach is to deglycosylate protein GOD shell that may reduce the tunneling distance and increase the rate of DET between the active site of GOD and electrode surface.

Nanomaterial-facilitated protein DET has been shown successful as a means of *in-situ* molecular detection in cell-based assays. Nevertheless, the capacity to detect one type of biomolecule at a time is insufficient for the development of *in-situ* biosensing applications, as several or even many biomolecules always work together in biological processes and diseases. The employment of a microarray platform by selectively functionalizing the individual electrode with the desired nanomaterial-immobilized protein would be useful for *in-situ* multiplexing detections. In addition, such a platform can also be used to monitor cell therapy effects. This approach has the great potential in

gaining fundamental understanding of physiological processes and the means of developing efficient drug-screening techniques.

Abbreviations

Full Name	Abbreviation
Direct electron transfer	DET
Glucose oxidase	GOD
Hemoglobin	Hb
Myoglobin	Mb
Horseradish peroxidase	HRP
Carbon nanotube	CNT
Nanoparticle	NP
Hollow sphere	HS
Layer-by-layer	LBL
Self-assembly monolayer	SAM
Quantum dots	QDs
Three-dimensional	3-D
Cyclic voltammogram	CV
Electrochemical impedance spectroscopy	EIS

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Patent

Three-Dimensional Cobalt-Based Porphyrin/Graphene as Efficient Electrocatalyst for Oxygen Reduction

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