Development of reduced graphene oxide based nanocomposites for electrochemical biosensing applications

Xiaoyun Bai

Hong Kong Baptist University

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Development of Reduced Graphene Oxide Based Nanocomposites
for Electrochemical Biosensing Applications

BAI Xiaoyun

A thesis submitted in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

Principal Supervisor: Dr. SHIU Kwok Keung
Hong Kong Baptist University
November 2014
DECLARATION

I hereby declare that this thesis represents my own work which has been done after registration for the degree of PhD at the Hong Kong Baptist University, and has not been previously included in a thesis or dissertation submitted to this or any other institution for a degree, diploma or other qualifications.

Signature: __________________

Date: _________________
Abstract

The modification of electrodes is always an important task in electrochemical detection of electroactive and biological molecules. Chemically modified electrodes can offer improved selectivity and sensitivity for the target analyte, which greatly enhance the electrode performance. Various materials such as conducting polymers, metal nanoparticles and carbon nanomaterials have been exploited and widely used for the modification of electrodes. Electrochemical or spontaneous deposition, electrostatic adsorption, layer-by-layer self assembly and covalent binding have also been developed for electrode modification and offer improved performance.

Both Prussian blue (PB) and toluidine blue O (TBO) are excellent redox mediators and very popular in electrode modification. PB has shown strong catalytic property for the reduction of hydrogen peroxide, but the application in biosensor fabrication is limited for its instability at neutral pH. Graphene, as a single-atom-thick carbon material, is considered an ideal platform for designing composite nanomaterials for high-performance electrochemical or electrocatalytic devices. The combination of PB with reduced graphene oxide (RGO) and poly(toluidine blue O) (PTBO) will greatly improve the stability of PB. An amperometric biosensor based on glassy carbon (GC) electrode modified with reduced graphene oxide, PB and poly(toluidine blue O) was developed. Experimental results showed that the GC/RGO/PB/PTBO modified electrode offered an excellent electrocatalytic activity toward the reduction of hydrogen peroxide due to the possible synergistic effects of the PB-PTBO composite material. After codeposition of glucose oxidase (GOD) and chitosan (CHIT)
coating, the resulting GC/RGO/PB/PTBO/CHIT-GOD electrode exhibited excellent response to glucose with a sensitivity of 59 mA M$^{-1}$ cm$^{-2}$, a low detection limit of 8.4 μM and a linear range from 0.02 to 1.09 mM at a detection potential of +0.2 V vs. Ag|AgCl reference.

Reduced graphene oxide – gold nanoparticles composites were prepared by depositing gold nanoparticles (AuNPs) on the surface of reduced graphene oxide with different RGO-to-AuNPs weight ratios. The resulting composite materials were characterized morphologically and optically by scanning electron microscopy and UV-Visible absorption spectroscopy. Electrocatalytic effect of different composites toward the reduction of hydrogen peroxide has been investigated. The results demonstrated that RGO-AuNPs composites displayed high stability and catalytic effect for the analysis of hydrogen peroxide compared with that of RGO and AuNPs alone, demonstrating the possible synergistic effects of the RGO-AuNPs composite materials. Additionally, direct electron transfer of glucose oxidase was achieved after codeposition of GOD and chitosan. The glassy carbon electrode modified with RGO-AuNPs/CHIT-GOD material exhibited an excellent catalytic effect for glucose detection with a sensitivity of 34 mA M$^{-1}$ cm$^{-2}$ at a detection potential of −0.3 V vs. Ag|AgCl.

A reduced graphene oxide - gold nanoparticles - Prussian blue (RGO-AuNPs-PB) composite was prepared by spontaneous deposition of PB on RGO-AuNPs surface. The GC/RGO-AuNPs-PB modified electrode offered good electrocatalytic activity toward the reduction of hydrogen peroxide, indicating the possible synergistic effects of the RGO-AuNPs-PB composite material. Direct electron transfer of glucose oxidase can be realized after codeposition of GOD and chitosan coating on GC/RGO-AuNPs-PB electrode. The resulting
GC/RGO-AuNPs-PB/GOD-CHIT biosensor exhibited an excellent amperometric response to glucose with a sensitivity of 84 mA M$^{-1}$ cm$^{-2}$, and a linear range from 13 μM to 2.0 mM at +0.1 V vs. Ag/AgCl.
Acknowledgment

First and foremost, I would like to show my deepest gratitude to my supervisor Dr. Shiu Kwok-Keung for his support and encouragement during my Ph.D. study at HKBU. Dr. Shiu has given me valuable advice and patient guidance throughout my study and research.

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<tr>
<td>AA</td>
<td>Ascorbic acid</td>
</tr>
<tr>
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<td>TBO</td>
<td>Toluidine blue O</td>
</tr>
<tr>
<td>TTF-TCNQ</td>
<td>Tetrathiafulvalene-tetracyanoquinodimethane</td>
</tr>
<tr>
<td>UA</td>
<td>Uric acid</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
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<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>Area of the electrode</td>
</tr>
<tr>
<td>c</td>
<td>Concentration</td>
</tr>
<tr>
<td>D</td>
<td>Diffusion coefficient</td>
</tr>
<tr>
<td>ΔE_p</td>
<td>Peak separation</td>
</tr>
<tr>
<td>I_p</td>
<td>Peak current of the redox couple</td>
</tr>
<tr>
<td>n</td>
<td>Number of electrons</td>
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<tr>
<td>Γ</td>
<td>Surface coverage</td>
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<td>v</td>
<td>Scan rate</td>
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Chapter 1 Introduction

1.1 Electrochemical biosensors

A biosensor is an analytical device for converting biological response into a measurable signal [1,2]. The biological response can be converted into different types of signal including optical [3,4], mass spectrometric [5], magnetic [6], radioactive [7] and electrochemical [1]. Different types of biosensors based on these signal measurements have been developed.

In recent decades, the development of biosensors has become more and more significant, because of the wide application of biosensors in diagnosis and monitoring of drugs, diseases, environment, foods, cell cultures and proteomics, etc. [8]. The design of biosensors requires highly selective interactions based on the specific binding affinity between compounds immobilized on the sensors with the target molecules [9]. Additionally, the cost and the possibility of miniaturization are also important and challenging for the design of biosensors in order to satisfy the high demand of biosensors in point-of-care testing. Electrochemical biosensor is a very suitable candidate for the design of biosensors and plays an important role in various fields including biological analysis, health-care and environmental monitoring due to the low-cost, rapid response, ease for miniaturization and modification [10]. Besides, electrochemical sensor was the first type of successfully commercialized biosensors [11]. Electrochemical techniques will be utilized in electrochemical biosensors for quantitative detection of biological analytes according to the electrical signal produced which is related to the concentration of the biological analyte [10].
1.1.1 Definition and principles of electrochemical biosensors

Electrochemical biosensor is a biosensor which converts the biological or chemical response between biomolecules into a measurable electronic signal. On the whole, the setup for electrochemical biosensors consists of bioreceptors, interface architecture, transducer element and computer software, as displayed in Fig. 1.1 [2]. Firstly, the bioreceptor (a) binds to the analytes from cells, blood, food or other samples via specific binding or chemical reaction between biomolecules and bioacceptors. Specific biological reaction takes place at the interface architecture (b) and sends the corresponding signal to the transducer element (c). Then the signal picked up by the transducer is converted to an electronic signal and amplified. Lastly, the amplified signal is processed through the computer software (d) with the information of physical parameters and presented in the display (e) [2].

For the construction of a successful electrochemical biosensor, the processes for (a) and (b) are especially important. It is because the sample matrix is very complex, including all kinds of biomolecules which may introduce high influence on the selectivity and stability of the bio-reception interaction. All of these require that the catalyst must be highly efficient, stable and specific for the target analyte present in the sample solution under normal storage conditions. Variations in the operating parameters such as temperature, oxygen content, pH and stirring will also affect the biosensor response. Thus, operating parameters should be controlled independently to ensure minimal pre-treatment for the sample analysis. The biological sample is susceptible to foreign matters, and the detection is usually operated under clinical situations. The probe should be of good biocompatibility, non-toxicity and small size. In addition, the electrochemical
biosensor should be sensitive, inexpensive, and portable for practical applications [1,2].

Fig. 1.1 Typical components of biosensors [2].

1.1.2 Types of electrochemical biosensors

Electrochemical biosensors offer several unique advantages in the interface design, biological recognition and signal processing, including low volume, small size, low cost and applicable for point-of-care testing. The advantages greatly facilitate the wide applications of electrochemical biosensors in biomedical, food and environmental fields. According to the nature of the bio-recognition elements immobilized on the electrode surface, the electrochemical biosensor can be
classified into three types, namely electrochemical enzyme-, deoxyribonucleic acid- (DNA-) and immuno-based sensors [1].

1.1.2.1. Enzyme-based electrochemical biosensors

Enzyme-based electrochemical biosensors have received considerable attention due to the potential applications in environmental, foods, manufacturing and clinical monitoring [12-14]. These biosensors can be obtained through the immobilization of enzymes onto an electrode surface, which will quantitatively detect the analyte. Because of the specificity of enzymes for the recognition of target substrates, the selectivity of electrochemical biosensors based on enzymes has been greatly improved after incorporating enzymes with the substrates [15]. In addition, it is usually difficult for the direct electron transfer (DET) of enzymes on the surface of electrodes. The applications of electroactive mediators (such as ferrocene and its derivatives [16], poly(toluidine blue o) [17] ) or small molecules (including oxygen [18,19]) in order to shuttle electron transfer efficiently between enzymes and electrode surface have been realized [15]. Recently, research effort focuses on the immobilization and stability of the enzyme for the development of enzyme-based electrochemical biosensors [10]. Generally, the procedures for the immobilization of enzyme include entrapment of enzymes with a permeable membrane [20] or with a polymeric matrix [21,22] or within self assembled monolayers (SAMs) [23,24], covalent binding [25,26] and bulk modification of entire electrode material [27,28]. The enzyme immobilization procedure plays an important role in enzyme recovery, enzyme stability and selectivity as well as reduction of inhibition [25].
1.1.2.2. DNA electrochemical biosensors

Since electrochemical DNA biosensor was firstly proposed by Millan and Mikkelsen, it has attracted intense attention for the application in the recognition and monitoring of analytes in food, plant, soil and water samples such as mutagenic pollutants, carcinogens and drugs [29,30]. DNA electrochemical biosensors can be easily designed through the immobilization of a single-stranded DNA (ssDNA) on the electrode surface to recognize the target DNA sequence via hybridization [29] and offer an inexpensive, accurate and simple platform for patient diagnosis, environmental analysis and food monitoring [31]. The detection of DNA usually employs labelled or label-free methods. Labelled methods are more popular due to the improved performance with high sensitivity and selectivity [32]. Usually, redox active molecules are used as the labels and provide electrochemical signal for detection. Organic dyes, enzymes, anticancer agents, metal complexes and nanoparticles have been employed for electrochemical detection [33]. Label-free electrochemical DNA biosensors usually detect the changes in the biorecognition interface from ssDNA to dsDNA [34,35] or the electrochemical oxidation of guanine bases [36,37].

1.1.2.3. Electrochemical immunosensors

Electrochemical immunosensor is based on the change of electrochemical signal with the antibody-antigen (Ab-Ag) recognition reaction [38], which is vital for the determination of biochemical targets related to health concerns spanning from bacterial species in food to cancer antigens in patient serum [10]. Electrochemical techniques are very popular in the application of biochemical analysis due to high sensitivity, compatibility with miniaturization, and cost-effectiveness [38]. Most antigens and antibodies are intrinsically inert
Some labels are needed to promote an electrochemical reaction through the formation of immunocomplex with specific components [38]. The label electrochemical immunosensors offer improved performances such as high sensitivity and selectivity when compared with label-free immunosensors [39]. The combination of the sensitive electrochemical method with high enzyme sensitivity and activity greatly improves the performance of electrochemical immunosensors and provides a route for the development of immunosensors for practical applications [39].

1.1.3 Chemically modified electrodes

Chemically modified electrodes (CME) offer important applications in electrochemical analysis and detection. Different from ordinary electrodes (such as bare gold, platinum and glassy carbon (GC) electrodes), CME is an electrode bound with specific reagent or coated with a thin film. The thin-film chemical modifiers ranging from a molecular monolayer to a few micrometers-thick multilayer provide the electrode with the desirable electrical, optical, transport, chemical and electrochemical properties [40]. These properties greatly improve the selectivity and sensitivity of the electrode employed for analytical purposes.

1.1.3.1. Methods of modification

Different methods can be used for electrode modification, including chemisorption, covalent attachment and polymer film coating. On the other hand, chemical and electrochemical treatments are also recognized as effective means for electrode modification. Electrodes after chemical or electrochemical treatment will contain various functional groups (such as hydroxyl and carboxyl groups) or oxide film. For example, when carbon electrode is oxidized at highly positive potentials, oxygenated groups will be introduced on the electrode surface as
shown in Fig. 1.2 [41]. The functional groups can effectively improve the sensitivity and selectivity of the electrodes, and also provide reactive sites for further modification of the electrode.

![Chemisorption](image)

**Fig. 1.2 Oxygenated groups of carbon electrode after electrochemical treatment [41].**

Chemisorption refers to the spontaneous attachment of specific species on the electrode surface when the electrode is immersed in a bulk solution. Fabrication of modified electrodes can be realized through the adsorption of target species such as organic species [42] and nucleotides [43]. The species will adsorb strongly and irreversibly onto the electrode surface and form a monolayer [44]. For example, the interaction between pyrolytic graphite (PG) or GC electrode and organic molecules such as 9,10-phenanthraquinone, PQ with \( \pi-\pi \) stacking make the organic molecules irreversibly adsorb onto the carbon electrode, resulting in the formation of PQ monolayer [45].
Covalent attachment is possibly the earliest method employed for electrode modification. Examples include pretreatment and covalent synthesis. Desirable functional groups (such as hydroxyl or amino groups) are introduced through the oxidation of electrode material, and then the modifier molecules will bond to the electrode surface through interactions with the linking agents. Cyanuric chloride and organosilanes are the most commonly used linking agents [40].

Polymer films are three-dimensional (3-D) structures and offer good chemical stability and strong mechanical strength. Physical and chemical methods are usually employed for polymer film coating. Examples of physical coating methods include dip-coating [46], droplet evaporation [47] and spin coating methods [48]. Polymer films can be obtained by evaporation of the solvent of a polymer solution. Polymer coating is easy but the surfaces of the polymer films prepared are usually rough and uncontrollable. Electroactive polymers can be prepared by electrodeposition and electropolymerization. Polymers films such as polyvinylferrocene (PVF) can be deposited onto the electrode surface when they are electrochemically reduced or oxidized to give the corresponding insoluble forms [49]. On the other hand, heterocyclic compounds containing amino or hydroxyl groups such as poly(toluidine blue O) [50] can form polymer films from monomers through electrochemical reactions. Many polymer films are conductive and electroactive, and have good adhesion to the electrodes.

1.1.3.2. Materials for modification

In order to improve the electrochemical properties of the modified electrodes, many materials have been utilized. Nanomaterials including carbon nanomaterials, metals nanoparticles, nanometer oxides, nanocomposites and organic electroconductive polymers are suitable for electrode modification because of the
unique optical, mechanical, catalytical and magnetical properties. The composite materials usually retain the original chemical and electrochemical properties, and may show distinct physical, mechanical and chemical properties different from the original component [51]. Organic electroconductive polymers can be used to immobilize biological elements at the electrode surface [52], and also provide a 3-D matrix for the efficient electrical communication between redox centers of biological elements and the electrode. Metals (such as gold, platinum, silver, etc.) and carbon materials (including carbon fibers, graphite, graphene and carbon nanotubes (CNTs)) serve as electrode material and supporting substrates owing to the excellent mechanical and electrical properties [53]. Carbon materials show excellent chemical inertness, low electrical resistivity and pure crystal structure, offering wide potential window and high signal-to-noise ratio [54].

1.2 Electrochemical sensors for the detection of hydrogen peroxide and glucose

1.2.1 Hydrogen peroxide electrochemical sensors

Hydrogen peroxide (H$_2$O$_2$) is of great importance and plays wide and universal applications in our daily life. Firstly, as a side product of many enzymatic reaction involving oxidase enzymes and the substrate of the horse radish peroxidase (HRP) reaction, H$_2$O$_2$ participates in several biological events as well as intracellular pathways [55]. Secondly, it is a simple molecule but with a great significance in the food, environmental, textile, pharmaceutical and clinical fields [56-59]. Besides, as a waste product of industry and power stations, H$_2$O$_2$ can exist in rain and ground waters and pose a risk to the environment and health [60]. Therefore, the development of a sensitive, reliable, rapid, accurate and
cost-effective method for the determination of $\text{H}_2\text{O}_2$ is of practical importance for both industrial and academic purposes. Electrochemical biosensor is considered to be a suitable candidate for this purpose due to the unique properties of high sensitivity and selectivity, simplicity, fast response and convenient operation [61].

Generally, the electrochemical determination of $\text{H}_2\text{O}_2$ utilizes enzyme-based and non-enzymatic biosensors. Due to the catalysis effect and specificity between enzyme and the substrate, enzyme-based electrochemical biosensors have received considerable attention due to the high sensitivity and selectivity [62]. Heme proteins including HRP, myoglobin (Mb) and hemoglobin (Hb) are very popular in the fabrication of enzyme-based $\text{H}_2\text{O}_2$ biosensor [55]. Usually, the redox activity of heme proteins at different potentials is controlled by the iron center of the porphyrin and prosthetic groups. Basically, the most efficient fabrication of $\text{H}_2\text{O}_2$ biosensors based on the heme protein is to establish direct electron transfer (DET) between the protein and electrode, which can offer better selectivity over other methods. However, the redox center of the heme protein is usually shielded by the polypeptides, which increases the electron transfer distance between the heme protein and the electrode and greatly limits the fabrication of $\text{H}_2\text{O}_2$ biosensors utilizing the DET of heme proteins [55]. In order to facilitate the DET of heme proteins, many methods including conducting polymer [63], self-assembly monolayer [64], silica sol-gel [65] and various materials (such as metal nanomaterials [66], CNT [67] and graphene [68]) have been developed and used for the fabrication of enzyme-based biosensors. The application of these methods and materials greatly enhances the DET between the enzyme and the electrode as well as the immobilization amount of enzyme, which improves the performance of enzyme-based electrochemical $\text{H}_2\text{O}_2$ sensors [61].
addition, modifying electrodes with redox mediators is another popular method to realize the electron transfer between heme proteins and the electrode. Usually, ferrocene, metal hexacyanoferrates, methylene blue, hydroquinone and toluidine blue O (TBO) have been introduced as electron-shuttling mediators between heme proteins and the electrode. Though the mediator is favorable for the improvement of electron-shuttling between the enzyme and the electrode, some drawbacks such as easy loss from the electrode through diffusion and contaminations form sample matrices greatly affect the performance and life time of the biosensor [61]. Besides, the immobilization of enzyme is also a challenge for the fabrication and development of enzyme-based H$_2$O$_2$ biosensors.

Recently, the development of nonenzymatic H$_2$O$_2$ biosensor has received considerable attention. Various materials, especial nanomaterials such as metal nanoparticles, CNTs, graphene and various metal hexacyanoferrates have been employed to fabricate nonenzymatic H$_2$O$_2$ sensors [60,69-71]. Nonenzymatic H$_2$O$_2$ biosensors based on nanomaterials can improve the electrochemical performance of the electrode and exhibit good catalytic reduction or oxidation toward H$_2$O$_2$, which should result from the unique properties of nanomaterials including large surface area, good conductivity and chemical stability, and excellent catalytic activities [69,72,73].

1.2.2 Glucose electrochemical sensors

Enzyme biosensors are the most common and well recognized biosensors, and have attracted a lot of attention. Many kinds of enzymes have been used for biosensor research, such as GOD, HRP, alcohol dehydrogenase (ADH) and uricase [74]. Among them, biosensors based on GOD have been used for commercial products and play an important role in clinical testing.
As we all know, diabetes is one of the important causes leading to death in the world. The normal concentration of human blood glucose is between 4.4 and 6.6 mM [75]. Blood glucose concentration at lower or higher level will lead to diabetes. Therefore, a device which monitors the concentration of blood glucose is important to diagnose and manage the diabetes [76].

Since the first-generation glucose biosensor was introduced by Clark and Lyons in 1962 [77], the devices for diabetes detection have been developed in the operation of glucose enzyme electrodes. The second- and third-generation glucose biosensors have been developed for glucose detection. The operation of these three types of glucose biosensors is shown in Fig. 1.3. On the whole, the measurements are usually based on the amount of reactant (O₂) consumed or product (H₂O₂) formed from the specific reaction between GOD and glucose (Equation 1.1) [78].

\[
glucose + O₂ \xrightarrow{\text{GOD}} \text{gluconic acid} + H₂O₂
\] (1.1)

In order to detect glucose, three different approaches are generally used. Natural O₂ co-substrate is utilized for the generation and detection of H₂O₂ in the first-generation glucose biosensors. Artificial mediators are used to shuttle electrons for the second-generation glucose biosensors and DET between redox centre of enzymes and electrical interface is realized for the third-generation glucose biosensors [76].

For the first-generation glucose biosensors, the redox centre (flavin adenine dinucleotide, FAD) of GOD is reduced to FADH₂ via the reaction shown in Equation (1.2) in the presence of glucose substrate. Correspondingly, glucose is converted to gluconolactone.

\[
\text{GOD (FAD)} + \text{glucose} \xrightarrow{} \text{GOD (FADH}_2\text{)} + \text{gluconolactone}
\] (1.2)
The oxidation of FADH₂ by O₂ occurs to regenerate FAD and give the product H₂O₂ (Equation 1.3).

\[
\text{GOD (FADH}_2\text{)} + \text{O}_2 \longrightarrow \text{GOD (FAD) + H}_2\text{O}_2
\]  

(1.3)

Fig. 1.3 Schematics of the first-generation (A), second-generation (B) and third-generation (D) glucose biosensors [76].

The production of H₂O₂ can be employed as signal detection means to detect the amount of analyte present. The measurement of H₂O₂ is usually simpler. The YSI probe shown in Fig. 1.4 is a typical glucose detection device. GOD is entrapped between an outer diffusion-limited / biocompatible polycarbonate membrane and an inner anti-interference cellulose acetate membrane [76,78].
However, the determination of $\text{H}_2\text{O}_2$ at ordinary electrode will require a relatively large potential which will subject to many interferences from endogenous reducing species including uric acid (UA), ascorbic acid (AA) and dopamine (DA) [79,80]. As the first reaction relies on $\text{O}_2$ as the electron acceptor, the fluctuation in $\text{O}_2$ tension and stoichiometric limitation of $\text{O}_2$ will lead to the variation in sensor response and a reduced upper limit of linearity [76]. Therefore, many researchers work on the reduction of overpotential and interference. A permselective coating is one useful strategy to reduce the interference by minimizing access of constituents to the transducer surface. By controlling the polarity, size and charge, permselective coatings offering different transport properties can be used for the discrimination of coexisting endogenous reducing species [81,82]. On the other hand, one can control the $\text{O}_2$ flux and glucose supply
using special films such as polycarbonate and polyurethane, in order to limit the mass-transport and increase the permeability ratio of O$_2$ to glucose [83,84].

The electron transfer between GOD and ordinary electrode surface is usually restricted by the thick protein layer surrounding the redox centers of GOD as an intrinsic barrier which limits the smooth direct transfer of electrons. Second-generation biosensors have been developed to improve the electrical contacts between the redox centers of GOD and ordinary electrode surface. Artificial mediators replace O$_2$ as the electron acceptor for shuttling electrons between the redox centers of the enzyme (FAD) to the electrode surface (Equation 1.4). As a result, the determination of glucose in second-generation glucose biosensors can be effectively operated with low overpotential and little interfering reactions. Furthermore, glucose detection is independent of the O$_2$ partial pressure for second-generation biosensors.

\[
glucose + \text{GOD}_{\text{ox}} \rightarrow \text{gluconic acid} + \text{GOD}_{\text{red}}
\]
\[
\text{GOD}_{\text{red}} + 2\text{M}_{\text{ox}} \rightarrow \text{GOD}_{\text{ox}} + 2\text{M}_{\text{red}} + 2\text{H}^+
\]
\[
2\text{M}_{\text{red}} \rightarrow 2\text{M}_{\text{ox}} + 2\text{e}^-
\]

Common artificial mediators such as ferricyanide, phenothiazine, ferrocene derivatives and conducting organic salts have been used for the electric connection of GOD [85,86]. For practical applications, the artificial mediators should be of low solubility in aqueous medium, offering non-toxic effects to the enzymes (GOD), with good chemical stability in acidic and alkaline conditions, and of good electrochemical properties for the electronic transfer. Even though artificial mediators are used to replace O$_2$ for electron transfer, the reaction between O$_2$ and GOD still exists, which will affect the accuracy of determination at low glucose levels. Additionally, the interfering reactions (especially ascorbate)
are not completely eliminated. Ferricyanide and ferrocene have been widely used in commercial blood glucose meters due to the effective electron transfer [78].

Different from the glucose biosensors described above, the third-generation glucose biosensor is a type of non-mediator and reagentless glucose biosensors. In this system, DET between enzymes and electrodes is achieved via the active sites of the enzyme at a low redox potential. The selectivity has been greatly improved. Similar to the first-generation glucose biosensors, the biggest challenge is to achieve effective DET at common electrodes. One route to resolve this problem is to make sure that the distance of electron transfer between the enzyme and the electrode surface is as short as possible. Through successful efforts, some new electrode materials are exploited and used for the fabrication of modified electrodes in order to shorten the distance of electron transfer. For example, tetrathiafulvalene-tetracyanoquinodimethane (TTF-TCNQ) has been used as the charge-transfer complex to accelerate the DET between the immobilized protein and the electrode surface [15,87]. The mechanism of electron transfer remains controversial and needs to be further confirmed.

1.3 Nanomaterials in electrochemical biosensors

Nanomaterials represent the materials with at least one dimension sized between 1 ~ 100 nm [88]. Since the successful detection of the reduced form of nicotinamide-adenine dinucleotide (NADH) on carbon nanotubes at low potentials, the application of nanomaterials in electrochemical biosensors has been greatly exploited [89]. Recently, more and more researches focus on the development and improvement of various materials, techniques and applications of electrochemical biosensors [10]. Nanomaterials bring new possibilities and potentials for
biosensor construction and have a big impact on the development of electrochemical biosensors [90]. The applications of nanomaterials such as graphene and CNT, in the fabrication of enzyme-based biosensors, the direct electrochemistry of redox enzymes, and the electrocatalytic detection of small molecules have been reported [91,92].

1.3.1 Graphene

1.3.1.1 Properties of graphene

Nanometer-sized materials of different structures, shapes and materials with nanosized dimensions have been synthesized. Carbon nanomaterials have been utilized widely for their unique properties such as high surface area, high-speed electron mobility, excellent heat conductivity and stability [93]. Carbon can form many allotropes including fullerenes (0-D), CNTs (1-D), graphene (2-D) and graphite (3-D) [94]. Among them, graphene is considered to be the basic building unit of other carbon materials. Graphene can form 0-D fullerence via wrapping, 1-D nanotubes via rolling and 3-D graphite via stacking [95].

Fig. 1.5 shows the spatial structures of four allotropes of carbon materials. Graphene is the thinnest known material [96], and can produce many organic compounds and new materials via covalent or non-covalent binding [94]. As a rising star in material science, graphene have received considerable attention in the study of its properties and the exploration of its application in electrochemical sensors.

Graphene is a 2-D structural crystal obtained through close packing of monolayer carbon atoms. Its thickness is only 0.35 nm and has been recognized as the thinnest material in our universe [97,98]. Additionally, the sp² hybridized carbon atoms in graphene and the diminutive bond-length (of only 1.42 Å) result
in excellent electron transport ability and mechanical strength. Wide applications in super-capacitors [99,100], fuel cells [101,102], composites [103,104], electronic equipments [105], biological monitors [106,107] and electrochemical sensors [69,108] have been developed.

1.3.1.2 Electrochemical applications of graphene

Because of the outstanding properties of graphene such as low cost, wide potential windows, large surface area, chemical stability and electron transfer ability, it plays an important role in the development of electrochemical biosensors and the detection of biomolecules. It has been reported to offer excellent catalysis towards biomolecules including $\text{H}_2\text{O}_2$, NADH, and dopamine [69]. Zhou et al. [69] reported that the electrochemical potential window of graphene ($\sim 2.5 \text{ V}$) was comparable to that of graphite and glassy carbon, and the electrochemical activity of $\text{H}_2\text{O}_2$ and NADH were greatly enhanced at graphene modified electrode. Graphene has been employed in the fabrication of enzyme-based biosensors for ethanol and glucose detection [91]. The redox centre
of the enzyme is considered to be located deeply in a hydrophobic cavity of the molecule [109]. Graphene with high specific surface area and outstanding electron mobility can shorten the distance of electron transfer between the enzyme and the electrode. The direct electrochemistry of GOD at electrode modified with functionalized graphene has been reported [109,110]. Graphene can also be used for the determination of DNA sequence, electron transfer of proteins and metal ions [91,111]. Zhou and coworkers [69] have applied graphene in the label-free electrochemical detection of DNA hybridization or damage.

1.3.2 Prussian blue (PB)

Since the mixed valence compounds were originally extolled by Robin and Day [112], the applications in electrochemistry and biosensors have been explored and developed. Transition metal hexacyanometallates is an important class of mixed valence compounds, and PB has been used as a dye for colouration [113]. Because transition metal hexacyanometallates can be electrochemically oxidized or reduced, PB and its analogues have been employed for the modification of electrodes [113]. Since Neff [114] reported that PB could serve as an electrochemical active layer through deposition on a platinum foil, the investigations and applications of PB have increased greatly [60]. Other metal hexacyanoferrates were also deposited on different electrode surface, such as ruthenium and osmium [60]. Especially, chromium hexacyanoferrate exhibited cyclic voltammetric response similar to that of PB [60].

1.3.2.1 Structure and properties of PB

The preparation of PB is very simple. Upon mixing $\text{Fe}^{2+/3+}$ with $\text{Fe(CN)}_6^{3−/4−}$ in acidic solutions (usually at pH 1.0) [60], PB will form. PB was usually deposited onto electrodes or other substrates in a layer form through
electrochemical or spontaneous deposition. The crystal structure of PB has been investigated through X-ray crystallography, neutron and electron diffraction technologies [115]. The unit cell shows alternations of Fe$^{2+}$ and Fe$^{3+}$ ions in the face centered cubic lattice [116], as shown in Fig. 1.6.

![Fig. 1.6 The unit cell of PB: (●) Fe$^{3+}$, (○) Fe$^{2+}$. [60]](image)

Electrodes modified with PB show cyclic voltammograms of the reversible redox switching of PB (Fig. 1.7) [60]. PB would be converted to the oxidized form of Berlin Green (BG) and reduced to give Prussian white (PW). PB, BG and PW can reduce O$_2$ and H$_2$O$_2$ [117,118]. The cubic structure of PB allows the diffusion of small molecules such as H$_2$O$_2$ and O$_2$ through its lattice. However, the stability of PB layer is a key factor for its application. The PB layers would be disrupted or hydrolyzed when cycling in neutral or alkaline pH, due to the strong interaction of ferric ions with hydroxyl ions at pH 6.4 or higher [116]. Many methods have been reported for improving the stability of the PB layer. For example, chemical deposition method was used for the preparation of PB and an
improvement in stability was observed at alkaline pH [119]. On the other hand, conducting or non-conducting polymers were utilized to protect and improve the stability of PB [116]. The polymers were employed for enzyme and mediator entrapment, and protein protection [120,121]. Ionic conductors (such as Nafion) were also used to enhance the response activity of PB-based biosensors through improving the transfer of electroneutral ions [122,123].

Fig. 1.7 Typical cyclic voltammogram of PB modified electrode in 0.1 M KCl [60].

1.3.2.2 Applications of PB in electrochemical biosensors

In the fabrication of electrochemical biosensors, PB is a suitable and popular material for its easy preparation, low cost and excellent catalytic effects.
Particularly, the excellent catalytic effects towards $O_2$ and $H_2O_2$ were beneficial for the development of enzyme-based biosensors. $O_2$ and $H_2O_2$ are important participants and products of enzymatic reactions. However, the detection of $H_2O_2$ requires a low overpotential in order to eliminate the interference of coexisting substances. PB can meet all these requirements and is usually referred to as an “artificial peroxidase” due to the high catalytic activity and selectivity for $H_2O_2$ determination [124]. The corresponding application of PB in biosensor fabrication was reported by Karyakin and coworkers [125]. Different enzyme biosensor systems including lactate oxidase and ethanol oxidase have been coupled with PB to offer low detection limit and interference level [122,126,127].

1.3.3 Gold nanoparticles (AuNPs)

1.3.3.1 Properties and preparation of AuNPs

When compared with the bulk materials, materials of nano-sized dimensions will display drastically different properties. Many applications in drug delivery, catalysis, chemical and biological monitoring have been exploited [128]. Additionally, the optical properties of AuNPs are closely related to the nanoparticle shape, size, local environment and aggregation state [129]. This phenomenon is shown in Fig. 1.8. In recent years, the research on AuNPs has been greatly increased. AuNPs offered high surface area ratio, excellent catalytic effects and special optical properties [130].

AuNPs were usually prepared by citrate reduction [131], Brust-Schiffrin method [132], seed growth [133], template method [134] and electrochemical methods [135]. The size, shape, colour and optical properties of AuNPs produced are closely related to the synthetic methods employed [136]. AuNPs with sizes from 16 to 147 nm in diameter can be obtained by citrate reduction through
controlling the ratio of HAuCl₄ and citric acid [137]. Brust-Schiffrin method can be used to prepare relatively stable AuNPs with sizes from 1 to 4 nm [138]. Through template method, gold materials with different shape such as nanowires [139], nanorods [140] and nanotubes [141] can be obtained.

Fig. 1.8 TEM and UV-Visible spectroscopy of different size, shape and aggregation state of gold nanorods with various aspect ratios. (Seed sample): aspect ratio 1; (a): aspect ratio: 1.35 ± 0.32; (b): aspect ratio: 1.95 ± 0.34; (c): aspect ratio: 3.06 ± 0.28; (d): aspect ratio: 3.50 ± 0.29; (e): aspect ratio: 1.35 ± 0.32; (a): aspect ratio: 4.42 ± 0.23 [129].

1.3.3.2 Applications of AuNPs in biosensors

In the construction of biosensors, AuNPs can form a useful interface for the detection of electroactive molecules such as NADH and H₂O₂ [130]. For the enzyme-based biosensor fabrication, AuNPs would retain the biomolecular activity when employed for the immobilization of biomolecules. Besides, AuNPs
would decrease the distance between redox centers of the enzymes and the surface of electrode, which would improve the DET between the enzymes and the electrode [142]. Electrochemical deposition of AuNPs on the electrode surface is an effective and simple way for the preparation of AuNPs modified electrode. For example, a gold nanoparticle modified electrode with immobilized tyrosinase enzyme was fabricated and was used for the detection of phenolic compounds [143]. On the other hand, AuNPs modified electrodes have been employed for the deposition of GOD and xanthine oxidase [144,145]. Self-assembly monolayers technology was also used for the modification of AuNPs with different enzymes [146]. Gold nanoparticles were also used for the fabrication of immunosensors [147,148] and DNA biosensor [149,150].

1.3.4 Conducting Polymers

Generally, organic compounds with effective ability of charge transfer can be divided into three groups including organic conjugated polymer/conducting polymers, organometallic species and charge transfer complexes [121,151]. Polymers were originally used as electrical insulators in many fields. With the exploitation of conductive capability, conducting polymers have attracted considerable attention in biosensor platforms and biomolecule immobilization strategies [152]. Conducting polymer contains an electron conducting backbone, resulting from the excellent electronic properties including electrical conductivity, high electron affinity, low ionization potential and low energy optical transitions [121]. These properties offer the wide applications in electrocatalysis and biosensing devices through the modification of electrode and immobilization of biomolecules.
1.3.4.1. Preparation and properties of conducting polymers

Well-ordered thin films of conducting polymers have been prepared using various methods including Langmuir-Blodgett technique and stretch-drawing [153]. These methods can provide polymers with preferentially oriented backbones along the substrate surface [153]. In addition, electrochemical method is favorable and effective to prepare conducting polymers for its high-speed, one-step construction, high reproducibility, controllable thickness and addressable deposition [154]. The thickness of the polymer film can be controlled readily through the change of the potential or current with time [121]. Besides free standing and homogeneous polymer films, copolymers and graft copolymers can also been synthesized using electrochemical methods [155]. Especially in the development of electrochemical biosensors, conducting polymers play an important role for rapid electron transfer and biomolecule immobilization.

1.3.4.2. Applications in electrochemical sensors and biomolecule immobilization

Possible entrapment of enzymes at conducting polymers can be realized during electrochemical polymerization, which makes them favorable for the fabrication of electrochemical biosensors and the immobilization of biomolecules [151]. The film thickness, spatial distribution of the biomolecules and modulation of biomolecular activity can be conveniently controlled [151]. Conducting polymers can be covalently or non-covalently modified with nanomaterials and display outstanding catalytic or affinity properties, which will exhibit great advantageous in the design of biosensors [156].

Water-soluble dyes can also form conducting polymer. Because of the good catalytic effect toward biomolecules, water-soluble dyes including methylene blue, phenazine methosulfate and toluidine blue O (TBO) are popular in the fabrication
of biosensors and the immobilization of biomolecules. Especially, TBO (Fig. 1.9) has been extensively used in the fabrication of biosensors [157-159]. TBO as a phenothiazine dye has been extensively utilized for the electrocatalysis of some biological active compounds, such as NADH [160]. TBO can form a monomeric mediator with an extended aromatic system [161,162], which is beneficial to increase the thermodynamic driving force of TBO. On the other hand, TBO can be incorporated into PTBO structures through the amine groups in position 3 [163]. PTBO can offer effective electron transfer because of its 3-D structure [164]. Furthermore, TBO can be used for the immobilization of enzymes and cofactors [165]. Yao and Shiu [17] have reported the immobilization of GOD by PTBO through electrochemical deposition. PTBO not only provides the polymer matrices to maintain the biosensor activity, but also serves as an effective redox mediator to improve electron transfer between enzymes and substrate electrode.

![Chemical structures of toluidine blue O and poly(toluidine blue O)](image)

Chitosan is the N-deacetylated derivative of chitin, and possesses distinct chemical and biological properties. The reactive hydroxyl and amino groups make it amenable to chemical modifications [166]. Fig. 1.10 shows the structure of chitosan. Chitosan is a natural cationic polyelectrolyte (pKₐ ≈ 6.5) and soluble in acidic aqueous media with pH < 6.5, displaying a positively charged surface [166].
As a natural polymer, chitosan is recognized as a suitable functional material because of the excellent properties such as biodegradability, biocompatibility, adsorption and non-toxicity [167]. Chitosan has attracted considerable attention for electrode modification, especially for enzyme immobilization [168]. Chitosan dissolves in acidic media and adheres to negatively charged surfaces or materials [169]. Thus, GOD can be immobilized on the electrode surface with chitosan. Kang et al. [170] have constructed a glucose biosensor through immobilizing GOD on the graphene surface using chitosan. Shan et al. [171] also immobilized GOD on graphene-AuNPs composite film through chitosan.

![Structure of chitosan](image)

Fig. 1.10 Structure of chitosan

### 1.4 Nanocomposites based on graphene

In recent years, graphene has attracted considerable research attention due to its unique properties including large surface area, excellent thermal conductivity, and high carrier mobility [172]. Many synthetic routes and methods such as oxidation, sonication and intercalation have been used to produce graphene and exploit the properties of graphene [172]. Generally, chemically exfoliated graphene oxide (GO) and reduced graphene oxide (RGO) possess many reactive oxygen-containing groups, which provide a wide range of possibility for the preparation of graphene-based composites or functional materials [173,174]. To date, graphene-based composites with inorganic metal or metal oxides, polymers,
organic dyes and metal hexacyanometallates have been successfully synthesized [171,175-177]. The applications of these graphene-based composites have been explored in many fields such as supercapacitors, photocatalysis, batteries and sensing platforms [172].

1.4.1 Graphene synthesis and functionalization

Graphene can be synthesized by mechanical exfoliation [178], chemical vapour deposition (CVD, thermal CVD [179,180] and plasma enhanced CVD [181]). On the other hand, RGO can be prepared by thermal [182] and chemical reduction [173,183]. Novoselov and coworkers [184] have achieved the isolation of single layer graphene through mechanical exfoliation, producing graphene samples with highest quality, purity and electronic quality, but it is not suitable for mass throughput and is time-consuming [96]. Mild exfoliation of graphite can produce defect-free/defect-less graphene, but it is also of low-yield [185,186]. CVD is a potential mass-throughput method for graphene preparation in electronic applications [187]. The size of graphene produced with CVD can be controlled when graphene grows on the surface of substrates such as Cu and Ni [188,189]. However, graphene prepared by CVD is not suitable for research due to the low purity. By contrast, reduced graphene oxide (RGO) is very popular for graphene synthesis, because it offers mass production and is simple and economical. Graphene oxide can be obtained through extensive chemical attack of graphite crystals. GO sheets are composed of graphene-like aromatic domains and decorated by many oxygen-containing functional groups such as ether, diol, hydroxyl and epoxy groups [190,191]. Examples of oxygen-containing functionalities of GO is shown in Fig. 1.11. These functional groups play important roles for further functionalization of graphene. Reduction of GO by
chemical reductants or electrochemical methods will result in electrically conductive materials known as RGO [190,192]. Graphene reduced from GO contains abundant structural defects and functional groups which would benefit the applications of graphene in electrochemistry [91].

![Oxygen-containing functional groups of graphene oxide](image)

**Fig. 1.11 Oxygen-containing functional groups of graphene oxide [193].**

On the other hand, agglomerate phenomenon is very commonly observed during the synthesis of nanomaterials such as graphene. It is possible due to graphene agglomeration and reformation of graphite through the $\pi-\pi$ stacking interactions and weak van der Waals forces. Functionalization of graphene is an effective method to improve the dispersion of graphene. GO contains many oxygen-containing functional groups such as hydroxyl, carboxyl and epoxy groups [194]. Fig. 1.12 shows the preparation of isocyanate modified graphene through the interaction between oxygen-containing functional groups and isocyanate dispersed in aprotic solvents [195].
Fig. 1.12 Interactions between isocyanates and GO through oxygen-containing functional groups of GO [195].

1.4.2 Composites based on graphene and their preparation

According to the materials and properties, there are three different kinds of graphene-based composites, namely graphene-polymer composite, graphene-metal/metal oxide composites and graphene-transition metal cyanides composites [172]. Graphene-polymer composites can form layered graphene-polymer films [172], graphene-filled polymer composites and polymer-functionalized graphene nanosheets [172]. Graphene is considered to be a potential filler of polymer matrices, which can enhance the thermal, mechanical and electronic properties of polymers. Layered graphene-polymers display layered structures, which offer specific applications of graphene-polymer composites in photovoltaic and directional load-bearing membranes [172]. In contrast to the layered graphene-polymers films, the distribution of graphene in graphene-filled
polymer is random, and the dispersity and bonding model with polymers are the key factors to influence the composite properties [172]. Different methods including in situ polymerization, melt blending and solution mixing have been applied to prepare graphene-polymer composites in order to achieve optimal properties of composites [196-198]. Besides, graphene can also be used as templates to prepare graphene-polymer composites via covalent or non-covalent functionalization. Covalent functionalization is mainly realized through the interaction between oxygen-containing groups of graphene and the functional groups of the polymers such as –NH₂ and –OH. For example, the preparation of GO-polyvinyl alcohol composite can be achieved via esterification between the –COOH groups of GO and the –OH groups in polyvinyl alcohol [199]. \( \pi-\pi \) stacking or electrostatic interaction play important roles for the non-covalent functionalization of graphene-polymers [200], such as the preparation of graphene-TBO composite [176].

On the other hand, metal nanoparticles-graphene composites are also considered to be a potential candidate to develop magnetic, optoelectronic and catalytic materials [201]. For the synthesis of metal nanoparticles, chemical reduction is the most popular method employed and has been widely used for the preparation of nanoparticles of gold, silver and platinum employing reduction agents such as sodium borohydride and sodium citrate [172]. In addition, electrochemical deposition is an attractive method for the preparation of graphene-metal composite films (such as graphene-AuNPs) on some substrates. Ordered metal nanoparticles patterns can also be obtained using porous templates. The thickness of the composite film and the size of metal nanoparticles can be controlled [172].
Besides, graphene-transition metal cyanides such as graphene-Prussian blue composite can also be prepared through self-assembly or electrochemical deposition methods to achieve good catalysis, excellent chemical stability and large surface area [176].

1.4.3 Properties and applications of graphene-based composites

Nanocomposites have attracted considerable interest that range from energy storage to biosensing due to the combination of unique properties of individual nanomaterials and possibly synergistic effects [202]. The composites of graphene with other materials also offer increased surface area, enhanced capability and improved stability, which are recognized as synergistic effects. Integration of graphene with some other materials including metal nanoparticles, redox mediators and conducting polymers can be employed for the preparation of chemical sensors. Guo et al. [203] pointed out that nanocomposites could offer large electrochemically active surface area, which was favorable for the adsorption of biomolecules and was effective to accelerate the electron transfer between electrode and analyte. Xue et al. [204] synthesized an advanced hybrid electrode material poly(diallyldimethylammonium chloride) with AuNPs (PDDA-AuNPs) offering better performance for the oxidation of uric acid. Additionally, PDDA functionalized graphene nanosheets displayed an enhanced capability for enzyme immobilization and a strong electrocatalytic activity for the reduction of nitric oxide after combining with room temperature ionic liquids (RTIL) [205].
1.5 Research objectives

As a novel carbon material, graphene has attracted considerable research interest. Due to its unique properties including large surface area, excellent mechanical and conductive properties and good thermal stability, graphene has been widely used for the developments of solar cells, super-capacitors, liquid crystals and biosensors. In order to obtained better performance and wider applications, graphene-based composites such as graphene-polymer and graphene-metal nanoparticles composites have been explored. This thesis work mainly focuses on the synthesis, preparation and applications of graphene-based composites. Different properties of graphene-based composites including optical, structural and electrochemical properties have been characterized. Electrochemical biosensors based on these graphene-based composites have been fabricated and the electrocatalytic effects toward $\text{H}_2\text{O}_2$ have been examined. Lastly, glucose biosensors have been developed based on the graphene-based composites after immobilization of GOD and the electroanalytical behaviors of the resulting biosensors employed for glucose detection have been investigated. It is assumed that the electrochemical properties and enzymatic activities of glucose oxidase remain unchanged and are not affected by the electrode modification. The electroanalytical performance of the resulting glucose biosensors would reflect the combined effects of the materials employed for the electrode modification.

In Chapter 2, a reduced graphene oxide/Prussian blue/poly(toluidine blue O) composite was prepared through electrochemical deposition of Prussian blue (PB) and poly(toluidine blue O) (PTBO) on the surface of reduced graphene oxide (RGO). The structural, optical and electrochemical properties of the resulting composite were examined. The electrochemical catalytic effects towards $\text{H}_2\text{O}_2$ at
different modified electrodes were investigated. Experimental results showed that
the GC/RGO/PB/PTBO modified electrode offered the best electrocatalytic
activity toward the reduction of hydrogen peroxide when compared with
electrodes modified with RGO-PB, RGO/PTBO and RGO materials, indicating
the possible synergistic effects of the PB-PTBO composite material. The electrode
showed high sensitivity and good stability for the analysis of hydrogen peroxide
in phosphate buffer solution (pH 7.4). After codeposition of glucose oxidase
(GOD) and chitosan (CHIT) coating, the resulting GC/RGO/PB/PTBO/
CHIT-GOD electrode exhibited excellent response to glucose with a sensitivity of
59 mA M⁻¹ cm⁻², a low detection limit of 8.4 µM and a linear range from 20 µM
to 1.09 mM at a detection potential of +0.2 V vs. Ag/AgCl reference.

On the other hand, Chapter 3 reports the investigation of reduced graphene
oxide-gold nanoparticles (RGO-AuNPs) composites with different weight ratios.
The resulting composite materials were characterized morphologically and
optically by scanning electron microscopy (SEM) and UV-Visible absorption
spectroscopy, which indicated that of RGO-AuNPs composite with different weight
ratio showed different properties including size, shape and aggregation state and
optical property. Cyclic voltammetry and amperometric measurements were
employed to investigate the electrocatalytic effect of different composites toward
the reduction of hydrogen peroxide. Experimental results demonstrated that
RGO-AuNPs composites displayed good sensitivity, high stability and catalytic
effect for the analysis of hydrogen peroxide, demonstrating the possible synergistic
effects of the RGO-AuNPs composite materials. Additionally, direct electron
transfer of GOD was achieved after codeposition of GOD and CHIT. The glassy
carbon electrode modified with RGO-AuNPs/CHIT-GOD material exhibited an
excellent catalytic effect for glucose detection with a sensitivity of 34 mA M\(^{-1}\) cm\(^{-2}\) at a detection potential of \(-0.3\) V vs. Ag/AgCl reference.

In Chapter 4, we describes a simple procedure to prepare a novel amperometric biosensor based on reduced graphene oxide - gold nanoparticles - Prussian blue (RGO-AuNPs-PB) composites through spontaneous deposition of PB on RGO-AuNPs surface. The RGO-AuNPs-PB composite was characterized by SEM, X-ray photoelectron spectroscopy and voltammetric techniques. Experimental results showed that the GC/RGO-AuNPs-PB modified electrode offered good electrocatalytic activity toward the reduction of hydrogen peroxide, indicating the possible synergistic effects of the RGO-AuNPs-PB composite material. The electrode showed high sensitivity and good stability for the analysis of hydrogen peroxide in phosphate buffer solution (pH 7.4). In addition, direct electron transfer of GOD can be realized after codeposition of GOD and CHIT coating on GC/RGO-AuNPs-PB electrode. The resulting GC/RGO-AuNPs-PB/GOD-CHIT electrode exhibited an excellent amperometric response to glucose with a sensitivity of 84 mA M\(^{-1}\) cm\(^{-2}\), a low detection limit of 13.0 \(\mu\)M and a linear range from 13 \(\mu\)M to 2.0 mM at a detection potential of +0.1 V vs. Ag/AgCl reference.

Lastly, Chapter 5 provides a comparison among different attempts for the fabrication of electrochemical biosensors based on different RGO-based composites. Possible future work in the development of RGO-based composites has been proposed.
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Chapter 2 Electrochemical biosensor based on reduced graphene oxide modified electrode with Prussian blue and poly(toluidine blue O) coating

2.1 Introduction

Prussian blue (PB) has shown excellent catalytic effect towards the oxidation-reduction of some low molecular-weight molecules such as oxygen and hydrogen peroxide due to its special three-dimensional network structure which allows the diffusion of the small molecules [1,2]. PB has been recognized as an artificial peroxidase [3-7] and has been utilized as modifying materials in the construction of biosensors for the detection of H$_2$O$_2$ [8-10]. However, the application of PB in biosensor fabrication was limited for its high solubility at neutral and basic solutions [9]. It has been reported that the conducting polymer films offered long-term operational stability, and the three-dimensional structure of polymer film prepared by electropolymerization would benefit the catalytic activity and sensitivity of the biosensor [11]. Therefore, polymer films such as poly(3,4-ethylenedioxythiophene) and polyaniline were used as coatings to enhance the catalytic capability of PB [2,12].

Graphene has emerged as a single-atom-thick and two-dimensional carbon material and has attracted considerable attention for its unique property such as high surface area, superior electric conductivity and mechanical strength [13,14]. These properties are favorable for the applications of graphene in energy-storage materials [15,16], liquid crystal devices [17], polymer composites [18] and electrochemical applications [19]. Moreover, graphene produced by the reduction
of graphene oxide possessed some characteristic functional groups such as hydroxyl (-OH) and carboxyl (-COOH) groups, which would benefit the preparation of composites. Many reports described that nano-composites offered superior properties in optical, magnetic, electrical and chemical characteristics. Graphene-based composites have been applied in many fields including optical devices, catalyst development and sensors fabrication [20,21].

In the present work, a biosensor based on RGO-PB composite was fabricated. Poly(toluidine blue O) (PTBO) was electrodeposited onto the surface of the RGO-PB composite material. The resulting sensor displayed excellent catalytic activity toward the reduction of \( \text{H}_2\text{O}_2 \), attributing to the synergistic effects between PB and PTBO at RGO modified electrodes. The sensor also possessed good performance in stability and selectivity. After codeposition of glucose oxidase (GOD) and chitosan (CHIT) coating, the sensor can also be applied as an amperometric glucose biosensor showing great sensitivity.

2.2 Experimental

2.2.1 Reagents and apparatus

Reduced graphene oxide (RGO) was purchased from XF Nano Material Co. Ltd. (Nanjing, China). Glucose oxidase (GOD) (Type X-S, from Aspergillus niger), \( \beta \)-D(\(+\))-glucose, \( \beta \)-Nicotinamide adenine dinucleotide reduced diopotassium salt (NADH), chitosan (CHIT), dopamine hydrochloride (DA), uric acid (UA) and toluidine blue O (3-amino-7-dimethylamino-2-methyl-thiazinium chloride, TBO) were obtained from Sigma. Ascorbic acid (AA) and acetaminophen (AP) was obtained from Aldrich. Hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) and potassium hexacyanoferrate(III) (\( \text{K}_3\text{Fe(CN)}_6 \)) was purchased from International
Laboratory. All other reagents were of reagent grade and were used without further purification. Phosphate buffer solution (pH 7.4) consisting of 0.02 M NaH$_2$PO$_4$ and 0.02 M Na$_2$HPO$_4$ was used as the supporting electrolyte. All solutions were prepared with deionized water.

All electrochemical experiments were carried out by a CHI6012B electrochemical Analyzer (CH instruments, Inc., USA). A traditional three-electrode system employing bare or modified glassy carbon working electrode, a platinum wire counter electrode and Ag|AgCl reference electrode was used. All potentials were quoted versus the Ag|AgCl reference electrode. Surface characterization of the modified electrodes including RGO, RGO/PB, RGO/PB/PTBO and PB/PTBO were performed with a scanning electron microscope (SEM) (LEO, Electron Microscopy Inc., Cambridge, UK). Ultraviolet-visible absorption spectra were recorded using a Cary 100-Scan UV-Visible spectrophotometer (Varian Inc., USA).

2.2.2 Preparation of modified electrodes

Glassy carbon electrodes (GC, Bioanalytical Systems, Inc., 3 mm in diameter) were carefully polished with 0.3 and 0.05 μm alumina slurry on a microcloth (Buehler, USA), followed by ultrasonication in ethanol and deionized water for 2 min. 2.0 mg RGO was dispersed in 1 mL deionized water by ultrasonication for 1 h to form a homogenous suspension. The electrodes were allowed to dry under a nitrogen stream and used for modification immediately. 5 μL of 2.0 mg/mL RGO suspension was dropped onto the surface of glassy carbon electrode and was allowed to dry in air.

RGO/PB film modified electrodes were prepared in solution containing 2.5 mM K$_3$Fe(CN)$_6$ + 2.5 mM Fe(NO$_3$)$_3$ + 0.1 M KCl by cyclic voltammetry between
zero and +0.4 V for 30 cycles with a scan rate of 30 mV/s. Electropolymerization of TBO on the RGO/PB modified electrode was carried out by cyclic voltammetry in 0.5 mM TBO solution containing 0.1 M KNO₃ between −0.4 and +1.2 V for 20 cycles at 50 mV/s. After electrodeposition, the electrodes were thoroughly washed by doubly distilled water and were allowed to dry at 50 °C for 1 h.

2.2.3 Scanning electron microscopic (SEM) and spectroscopic characterization

Glassy carbon disks of 3 mm in diameter were obtained by wire-cutting a glassy carbon rod (Atomergic Chemetals Corp.) into pieces of 2 mm thick, and then mounted onto a Teflon sheath. The disk electrode was polished as usual and modified with different materials (RGO, PB, and PTBO) as described above. The modified electrode obtained was rinsed with pure methanol and then deionized water. The glassy carbon disks modified with the desired materials were detached from the Teflon sheath and subjected to scanning electron microscopic studies.

Indium tin oxide (ITO) glass slides were cleaned in ultrasonic bath with ethanol and deionized water for 10 min before use. ITO surface deposited with different materials (RGO, PB, and PTBO) was thoroughly rinsed with deionized water before subjected for SEM and spectroscopic measurements.

2.2.4 Fabrication of glucose biosensor and amperometric measurements

RGO/PB/PTBO based glucose biosensors were prepared by immobilization of glucose oxidase on GC/RGO/PB/PTBO modified electrodes through electropolymerization of TBO in the presence of GOD or by evaporating an aqueous solution of GOD on GC/RGO/PB/PTBO electrode. On the other hand, GC/RGO/PB/PTBO/CHIT-GOD biosensor was prepared by casting 5 μL of 10 mg/mL glucose oxidase dissolved in 3% chitosan on the surface of the GC/RGO/PB/PTBO electrode. The resulting biosensors were allowed to dry at
room temperature. The biosensors obtained were stored in phosphate buffer solution (pH 7.4) at 4 °C before use. The electrode showed good stability for glucose analysis within one week.

The steady state amperometric response to hydrogen peroxide and glucose was measured by voltammetric technique in a 0.02 M phosphate buffer solution (pH 7.4) under gentle stirring of around 100 rpm at the desired potentials. The current response was recorded on successive addition of the substrates.

2.3 Results and discussion

2.3.1 Characterization of different modified electrodes

Scanning electron microscopy (SEM) was employed to characterize the morphology of electrodes modified with different materials (RGO, RGO/PB, RGO/PB/PTBO and PB/PTBO), and the SEM images of modified glassy carbon electrodes are displayed in Fig. 2.1. Fig. 2.1A shows the external modality of RGO. The layer-by-layer structure of RGO was observed, showing the stacks of wrinkled multilayer graphene [11,14,15]. On the other hand, the surface of RGO became rough and loose after the deposition of PB, as shown in Fig. 2.1B. Occasionally, some PB clusters were observed. The presence of oxygen-containing functional groups at RGO might favor the nucleation of PB [22-24], and the interaction between them improved the stability of the PB film to some extent [25]. Fig. 2.1C shows the SEM image of the RGO/PB/PTBO modified surface. A uniform PTBO film covered completely the RGO/PB surface. On the contrary, the PB/PTBO composite film revealed completely different structures on bare glassy carbon surface, as shown in Fig. 2.1D. A flat and compact film was observed. The results indicated that the layer-by-layer structure
of RGO can be used as a template for the fabrication of three-dimensional structures. Glassy carbon and ITO slides deposited with the same materials (RGO, PB, and PTBO) showed very similar SEM characteristics.

Fig. 2.1 SEM images of glassy carbon discs deposited with different materials. (A) RGO; (B) RGO/PB; (C) RGO/PB/PTBO; (D) PB/PTBO.

2.3.2 UV-Visible spectroscopic characterization

Fig. 2.2 displays the UV-Visible absorption spectra of RGO, RGO/PB and RGO/PB/PTBO materials deposited on ITO surface. No absorption peak was observed for the RGO modified surface between 450 and 800 nm, as shown in Fig.
2.2(a). The RGO-PB material was characterized by a broad absorption peak at around 730 nm (Fig. 2.2(b)), corresponding to the mixed-valence charge-transfer absorption of PB [20,26]. On the other hand, the characteristic absorption of PB at 730 nm was not apparent when PTBO was deposited on the RGO/PB surface. Interestingly, two new absorption peaks were observed at 605 and 650 nm, as shown in Fig. 2.2(c). These two absorption peaks should correspond to the absorption of TBO moieties. The results suggested that the RGO-PB modified surface was completely covered by PTBO film, consistent with the SEM results shown in Fig. 2.1.

![Fig. 2.2 UV-Visible absorption spectra of ITO glass modified with different materials. (a) RGO; (b) RGO/PB; (c) RGO/PB/PTBO](image-url)
2.3.3 Electrochemical behaviors of the modified electrodes

The electrochemical properties of different modified electrodes were examined by cyclic voltammetry in degassed 0.02 M phosphate buffer solution (pH 7.4), and the results are shown in Fig. 2.3. No characteristic redox peaks were observed for both the bare glassy carbon (Fig. 2.3(a)) and GC/RGO modified electrode (Fig. 2.3(b)). The GC/RGO electrode showed a larger current response when compared with the bare GC electrode. This was probably due to the larger surface area and changes in double layer capacitance and consequent increase in capacitance current in the presence of RGO deposits [27]. After electropolymerization of PTBO, the GCE/RGO/PTBO electrode showed two separate quasi-reversible waves at about $-0.22$ and $+0.02$ V (Fig. 2.3(c)), corresponding to the redox reactions of the heterocyclic nitrogen atom of the TBO monomer and those of the nitrogen bridges connecting TBO monomers, respectively [11,28]. On the other hand, Fig. 2.3(d) shows the voltammetric response of the GC/RGO/PB modified electrode. A pair of well-defined redox peaks was observed, corresponding to the PB materials deposited on the GC/RGO surface [4]. The formal potential ($E^\circ$), taken as the average value of the cathodic and anodic peak potentials [13], was of about $+0.14$ V (vs. Ag|AgCl reference). The ratio of current intensity for the cathodic and anodic processes was close to 1, indicating a quasi-reversible process. After PTBO was deposited on the GC/RGO/PB surface (Fig. 2.3(e)), two additional reduction waves were observed at around $-0.3$ and $-0.05$ V, corresponding to the redox reactions involving TBO moieties [11,28]. The cathodic peak at around $-0.05$ V overlapped with the reduction of PB deposits. On the other hand, the anodic peak located at around $+0.1$ V should be resulted from both the PTBO polymer and the PB deposits.
great enhancement in the current response might be due to the synergistic effect between PB and PTBO. Similar observations of synergistic effects have been described previously by Zhang and Gorshi [29,30]. These authors proposed that the combination of carbon nanotubes (CNT) and redox mediator TBO offered improved electronic and ionic transport capacity and electron self-exchange in the polymer film.

Fig. 2.3 Cyclic voltammograms of different modified electrodes in degassed phosphate buffer solution (pH 7.4) at a scan rate of 50 mV/s vs. Ag|AgCl.

Additionally, the voltammetric response of the GC/RGO/PB/PTBO electrode was examined as a function of scan rate in 0.02 M degassed phosphate buffer solution (pH 7.4). As shown in Fig. 2.4, the experimental results showed that the peak separation increased gradually with increasing scan rate while the peak
currents were linearly proportional to the square root of scan rate for both the cathode and anode processes, indicating a diffusion-confined process on the electrode [31].

![Cyclic voltammograms of GC/RGO/PB/PTBO at the scan rates of 20, 50, 70, 100, 120, 150, 170 to 200 mV/s. Inset: plot of peak current ($i_p$) vs. square root of scan rate. Supporting electrolyte: degassed phosphate buffer solution (pH 7.4).]

The surface coverages of PB and TBO moieties on the modified electrodes were estimated coulometrically and the results are listed in Table 2.1. The surface coverage of PB moieties deposited on bare glassy carbon and GC/RGO electrodes was about $2.9 \times 10^{-7}$ and $5.0 \times 10^{-7}$ mol/cm$^2$, respectively. The presence of RGO materials offered a large increase in the PB coverage of PB. This might be due to the higher surface area and the improved electrical conductivity of the RGO.
surface. Similarly, an increased TBO coverage was also obtained at the GC/RGO electrode, as compared to bare glassy carbon. However, there was no significant difference in the TBO coverage after deposition of PB on the GC/RGO surface.

Table 2.1 Surface coverage of PB and TBO moieties at the modified electrodes

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Γ (PB) nmol cm⁻²</th>
<th>Γ (TBO) nmol cm⁻²</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC</td>
<td>288</td>
<td>140</td>
</tr>
<tr>
<td>GC/RGO</td>
<td>503</td>
<td>315</td>
</tr>
<tr>
<td>GC/RGO/PB</td>
<td>/</td>
<td>320</td>
</tr>
</tbody>
</table>

2.3.4 Electrocatalytic reduction of H₂O₂ at the GC/RGO/PB/PTBO electrode

Hydrogen peroxide plays an important role in food industry, pharmaceutical and clinical. It is one of the side products of biochemical reaction involving different oxidase enzymes [32], and can be either oxidized or reduced directly at ordinary electrodes at high overpotential. The GC/RGO/PB/PTBO modified electrode was applied for electrocatalytic reduction of H₂O₂. Fig. 2.5(a) shows the cyclic voltammogram of the GC/RGO/PB/PTBO electrode in pure supporting electrolyte. Two cathodic peaks were observed at around −0.24 and −0.07 V. Upon addition of 4 mM H₂O₂, a significant increase in the cathodic current was observed, as shown in Fig. 2.5(b). This indicated a strong electrocatalytic effect toward H₂O₂ reduction at the GC/RGO/PB/PTBO modified electrode.
Fig. 2.5 Cyclic voltammograms of GC/RGO/PB/PTBO electrode in degassed phosphate buffer solution (pH 7.4) in the absence (a) and presence (b) of 4 mM H$_2$O$_2$ at 50 mV/s.

Fig. 2.6 displays the steady-state amperometric responses of different modified electrodes upon successive additions of H$_2$O$_2$ to degassed phosphate buffer solution (pH 7.4) at zero applied potential. The current response for 10 μM H$_2$O$_2$ was almost undetectable at bare glassy carbon (curve a), while small current responses of 0.013, 0.014 and 0.028 μA were recorded at the GC/RGO (curve b), GC/RGO/PTBO (curve c) and GC/RGO/PB (curve d) electrodes, respectively. On the other hand, the GC/PB/PTBO electrode gave a much higher increase in the plateau current of 0.22 μA when 10 μM H$_2$O$_2$ was added, as shown in Fig. 2.6(e), possibly due to the combined synergistic effects of mediated redox processes of PB with PTBO film. The GC/PB/PTBO electrode showed much enhanced
response as compared to electrodes with either PB or PTBO alone. The response sensitivity of different modified electrodes employed for hydrogen peroxide detection is displayed in Table 2.2. The GC/RGO/PB/PTBO electrode offered a sensitivity of 420 mA M$^{-1}$ cm$^{-2}$ as compared to the sensitivity of 20 and 40 mA M$^{-1}$ cm$^{-2}$ for the GC/RGO/PTBO and GC/RGO/PB electrodes, respectively. Observations of similar synergistic effects have been reported for CNT-PB [26] and CNT-PTBO [28,33,34] composite materials employed for the detection of hydrogen peroxide [26], glucose [28] and NADH [33,34].

![Figure 2.6](image_url)

**Fig. 2.6** Amperometric response recorded at different electrodes with successive addition of 10 μM H$_2$O$_2$ in 0.02 M phosphate buffer solution (pH 7.4) under stirring at zero applied potential vs. Ag|AgCl. (a) Bare glassy carbon; (b) GC/RGO; (c) GC/RGO/PTBO; (d) GC/RGO/PB; (e) GC/PB/PTBO; (f) GC/RGO/PB/PTBO.
Furthermore, the GC/RGO/PB/PTBO electrode offered the highest current response of 0.33 μA for the same H₂O₂ concentration of 10 μM (curve f). The GC/RGO/PB/PTBO electrode showed some improvement in the current response as compared to the GC/PB/PTBO electrode (Fig. 2.6(e)). The GC/RGO/PB/PTBO electrode offered a sensitivity of 420 mA M⁻¹ cm⁻² as compared to the sensitivity of 310 mA M⁻¹ cm⁻² for the GC/PB/PTBO electrodes. Further increase in current response (Fig. 2.6(e)) was probably due to the increased surface area of the RGO materials. The results indicated that there were interactions between RGO and the PB-PTBO composite film, which greatly enhanced the amount of PB and TBO assembled on electrode and provided a large catalytic surface area [33,34]. In addition, the PTBO film also served as a mediator expressing strong catalysis toward the reduction of H₂O₂. Significant improvement in the electrocatalytic activities toward H₂O₂ reduction was observed for the GC/RGO/PB/PTBO electrode. Synergistic effects observed for the PB-PTBO composite material were possibly resulted from π-π stacking interaction coupled with ionic interactions [34,35], and improved electronic and ionic transport capacity and electron self-exchange in the polymer film [29,30,35].

Table 2.2  Sensitivity of electrode systems employed for H₂O₂ detection

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Sensitivity (mA M⁻¹ cm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC</td>
<td>0.07</td>
</tr>
<tr>
<td>GC/RGO</td>
<td>18</td>
</tr>
<tr>
<td>GC/RGO/PTBO</td>
<td>20</td>
</tr>
<tr>
<td>GC/RGO/PB</td>
<td>40</td>
</tr>
<tr>
<td>GC/PB/PTBO</td>
<td>310</td>
</tr>
<tr>
<td>GC/RGO/PB/PTBO</td>
<td>420</td>
</tr>
</tbody>
</table>
Fig. 2.7 shows the corresponding calibration curve for H$_2$O$_2$ detection at the GC/RGO/PB/PTBO electrode. With the addition of H$_2$O$_2$, the current response reached stable current within 5 s. The current response exhibited a good linear correlation over the concentration range from 5 to 600 μM (R = 0.996) with the sensitivity of 420 mA M$^{-1}$ cm$^{-2}$ and a detection limit of 1.5 μM for a signal-to-noise ratio of 3. The relative standard deviation (RSD) for the amperometric detection of 10 μM H$_2$O$_2$ at the GC/RGO/PB/PTBO electrode was determined to be of 5.6 % for successive measurements at six different electrodes prepared identically.

Fig. 2.7 Calibration curve for H$_2$O$_2$ detection at the GC/RGO/PB/PTBO electrode in 0.02 M phosphate buffer solution (pH 7.4) under stirring at zero applied potential vs. Ag/AgCl.
Additionally, the anti-interference ability of the GC/RGO/PB/PTBO electrode was investigated and the results are shown in Fig. 2.8. The amperometric responses resulted from the addition of 10 μM of UA, NADH, DA and AA at the GC/RGO/PB/PTBO electrode was negligible when compared with that observed for 10 μM H₂O₂, indicating a high selectivity and anti-interference property of the modified electrode. The response characteristics of the GC/RGO/PB/PTBO electrode employed for hydrogen peroxide detection is comparable to other electrode systems employed for H₂O₂ detection reported in the literature [4,25,36,37]. Experimental results indicated that the GC/RGO/PB/PTBO electrode offered high sensitivity and good stability for the detection of H₂O₂ at zero potential in phosphate buffer solution.

Fig. 2.8 Anti-interference property of the GC/RGO/PB/PTBO electrode with addition of 10 μM UA, 10 μM NADH, 10 μM DA, 10 μM AA, and 10 μM H₂O₂ in 0.02 M phosphate buffer solution (pH 7.4) under stirring at zero applied potential vs. Ag|AgCl.
2.3.5 Amperometric detection of glucose at GC/RGO/PB/PTBO/GOD electrode

The GC/RGO/PB/PTBO electrode offered excellent performance for the reduction of H$_2$O$_2$. The electrode was also employed for the fabrication of glucose biosensor. Biopolymer chitin and its derivative chitosan (CHIT, poly(D-glucosamine)) have wide applications in medical and food industry for their high biocompatibility, high mechanical strength, easy manipulation, low cost, excellent film-forming ability and good solubility in acidic medium [38]. The construction of biosensors with chitosan coatings has been reported in the literature [39,40,41,42,43]. For our work, GOD as a representative of model enzyme was immobilized onto the GC/RGO/PB/PTBO electrode with chitosan for fabricating the respective glucose biosensor.

The electrocatalytic behaviors of the RGO/PB/PTBO based glucose biosensors were examined. The biosensor obtained by entrapment of glucose oxidase during electropolymerization of TBO was designated as GC/RGO/PB/PTBO-GOD in the work, while GC/RGO/PB/PTBO/GOD corresponded to the biosensor obtained by evaporation/deposition of GOD on GC/RGO/PB/PTBO electrode. Fig. 2.9 shows the amperometric response of the modified electrodes on successive addition of glucose in stirred air-saturated phosphate buffer solution (pH 7.4) at an applied potential of +0.2 V. The chitosan coated biosensor (GC/RGO/PB/PTBO/CHIT-GOD) displayed good catalytic activity upon successive addition of 20 μM glucose (curve a). On the other hand, the biosensor obtained by codeposition of PTBO and GOD (GC/RGO/PB/PTBO-GOD) showed almost no signal upon successive addition of 20 μM glucose. Reasonable catalytic response was only observed until when 500 μM glucose was added to the electrolyte, as shown in curve b of Fig. 2.9. This
indicated that the chitosan coating offered significant improvement in the electrocatalytic response of the glucose biosensor. Chitosan might serve as protective coating to improve the stability and catalytic activity of the PB and TBO moieties.

Fig. 2.9 Amperometric response for glucose at different modified electrodes in stirred air-saturated phosphate buffer solution (pH 7.4) at an applied potential of +0.2 V. (a) GC/RGO/PB/PTBO/CHIT-GOD for successive addition of 20 μM glucose; (b) GC/RGO/PB/PTBO-GOD for successive addition of 500 μM glucose; (c) GC/RGO/PB/PTBO/GOD for successive addition of 20 μM glucose.

However, the biosensor obtained by evaporation/deposition of GOD on GC/RGO/PB/PTBO electrode (GC/RGO/PB/PTBO/GOD) only displayed a sloping trace of amperometric response upon addition of glucose (curve c of Fig. 2.9).
2.9). The response showed good discrimination between signal and noise of the measurements. Immobilization of GOD on the modified electrode through direct deposition/evaporation did not seem to be an effective means for enzyme immobilization.

Fig. 2.10 shows the amperometric response of the GC/RGO/PB/PTBO/CHIT-GOD electrode upon successive addition of glucose in stirred air-saturated phosphate buffer solution (pH 7.4) at an applied potential of +0.2 V. The current response reached steady-state within 10 s, and the current increased with increasing glucose concentration. The calibration curve for glucose detection at the GC/RGO/PB/PTBO/CHIT-GOD electrode shown in the inset figure indicated a linear range from 20 to 1090 μM (R = 0.9995, n = 34) with a detection limit of 8.4 μM for a signal-to-noise ratio of 3. The sensitivity was calculated to be 59 mA M⁻¹ cm⁻². The relative standard deviation (RSD) of the current response for 20 μM glucose at the GC/RGO/PB/PTBO/CHIT-GOD electrode was 3.5 % for 16 successive measurements, indicating good reproducibility and high enzymatic activity of the biosensor. A comparison in the electrode response between the present (GC/RGO/PB/PTBO/CHIT-GOD) biosensor with other glucose biosensors reported in the literature [7,4,44,45] showed that the present biosensor offered reasonable detection of glucose as shown in Table 2.3.
## Table 2.3 Comparison of biosensor systems employed for glucose detection

<table>
<thead>
<tr>
<th>electrode</th>
<th>pH</th>
<th>potential (V)</th>
<th>Line range (mM)</th>
<th>Detection limit (μM)</th>
<th>Sensitivity (mA M⁻¹ cm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCE/MWNT/HRP+GOx/PTBO [7]</td>
<td>pH 7.4 PBS</td>
<td>-0.1</td>
<td>0.1-1.2</td>
<td>30</td>
<td>113</td>
</tr>
<tr>
<td>GCE/PB/GO [4]</td>
<td>pH 6.0 PBS</td>
<td>+0.1</td>
<td>0.1-13.5</td>
<td>0.343</td>
<td>15.28</td>
</tr>
<tr>
<td>GCE/(P-PB/GOx)_3 [44]</td>
<td>pH 7.4 PBS</td>
<td>-0.1</td>
<td>0.1-11</td>
<td>10</td>
<td>3.7</td>
</tr>
<tr>
<td>Au/(PB-GOD-PTBO)_n [45]</td>
<td>pH 7.4 PBS</td>
<td>-0.1</td>
<td>0.1-10</td>
<td>10</td>
<td>/</td>
</tr>
<tr>
<td>GCE/RGO-PB-PTBO/GOD [this work]</td>
<td>pH 7.4 PBS</td>
<td>+0.2</td>
<td>0.02 -1.09</td>
<td>8.4</td>
<td>59</td>
</tr>
</tbody>
</table>

MWNT: multi wall carbon nanotube; HRP: horseradish peroxidase; GOD or GOx: glucose oxidase; PTBO: poly(toluidine blue O); PB: Prussian blue; GO: graphene oxide; P-PB: Prussian blue nanoparticles protected by poly (diallydimethyl-ammonium chloride); RGO: reduced graphene oxide.

![Amperometric response of GC/RGO/PB/PTBO/CHIT-GOD electrode for successive addition of glucose in air-saturated phosphate buffer solution (pH 7.4) at +0.2 V. Inset: calibration curve for glucose detection.](image)

Fig. 2.10 Amperometric response of GC/RGO/PB/PTBO/CHIT-GOD electrode for successive addition of glucose in air-saturated phosphate buffer solution (pH 7.4) at +0.2 V. Inset: calibration curve for glucose detection.
The practical application of glucose biosensors will be affected by interfering species such as ascorbic acid (AA), dopamine (DA), uric acid (UA) and nicotinamide-adenine dinucleotide (NADH) [46]. Therefore, the selectivity and anti-interference ability of the biosensor at +0.2 V was also investigated for 0.1 mM glucose and the results are shown in Fig. 2.11.

![Graph showing anti-interference property of the GC/RGO/PB/PTBO/CHIT-GOD biosensor with addition of 0.1 mM UA, 0.1 mM NADH, 0.1 mM DA, 0.1 mM AA, and 0.1 mM glucose in stirred air-saturated phosphate buffer solution (pH 7.4) at an applied potential of +0.2 V.](image)

As shown in Fig. 2.11, the current responses of 0.1 mM UA and 0.1 mM NADH were negligible. After addition of 0.1 mM DA and AA, the current responses showed significant increase in the negative direction for the oxidation
of DA and AA at +0.2 V. However, addition of glucose of the same concentration caused greater change in current response in the positive direction resulted from the reduction of H₂O₂ generated from the enzymatic reaction. It was evident that the influences of the interference substances were negligible, indicating a high selectivity and good anti-interference ability of the biosensor. The stability of the glucose biosensor was also investigated. The response current for oxidation of 0.1 mM glucose still retained about 90% of the initial value of current response after one week. The biosensor showed very good stability.

2.4. Conclusions

Through successful deposition of PB and PTBO onto the RGO modified glassy carbon electrodes, a sensor for the amperometric detection of hydrogen peroxide has been developed. The RGO/PB/PTBO composite electrode exhibited excellent electrocatalytic characteristics towards the reduction of H₂O₂ and great anti-interference ability at zero applied potential. The enhanced electrochemical performance of the GC/RGO/PB/PTBO electrode could be due to the special structure, high surface area and good conductivity of RGO. The PTBO film served both as a protecting film and an electron mediator, and the modified electrode showed high stability at phosphate buffer solution (pH 7.4). The presence of both the PB and PTBO moieties offered combined synergistic effects for the electrocatalytic reduction of H₂O₂. Furthermore, a glucose biosensor was constructed after the immobilization of GOD. The results showed that the biosensor based on the GC/RGO/PB/PTBO/CHIT-GOD system displayed high sensitivity and good selectivity. The present approach provides a novel way to maintain the electrocatalytic activity of PB film in neutral and alkaline solution,
and offers a new approach for the fabrication of biosensors with the hybrid polymer film.
References


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8853-8857.


**2001**, *73*, 915-920.


Chapter 3 Investigation of the optimal weight contents of reduced graphene oxide-gold nanoparticles composites and theirs application in electrochemical biosensors

3.1 Introduction

Graphene has attracted enormous interest because of the excellent properties, such as high surface area, superior electric conductivity and good mechanical strength [1,2]. The special two-dimensional (2-D) single-atom-thick structure of graphene also plays an important role in the fabrication of materials with different spatial structures. Graphene is also recognized as the basic building block of carbon materials [3,4] and has been utilized in the development of energy-storage materials [5,6], liquid crystal devices [7], polymer composites [8] and electrochemical applications [9,10]. However, the irreversible agglomerates of graphene are readily formed due to the van der Waals interactions [11]. In order to reduce the aggregation, some molecules and polymers such as polyvinylpyrrolidone [12], octadecylamine [13] have been used to modify and protect graphene. The hybridization of graphene with some inorganic particles has also been recognized as an ideal method for reducing the aggregation of graphene, because of the formation of some new graphene-based nanocomposites [11]. Some researches demonstrated that graphene favoured the dispersion and stabilization of metal nanoparticles, such as Pt, Au and Pd [11,14].

On the other hand, gold nanoparticles (AuNPs) offered many applications in biosensor fabrication and enhanced analytical performance because they provided a suitable microenvironment for retaining the biomolecular activity and allowed
direct electron transfer between redox proteins and electrode surface without redox mediators [15]. The high surface area and good electron conductivity of AuNPs facilitated electron transfer and served as a suitable candidate for biosensor fabrication [16].

Recently, many electrochemical biosensors based on the composites of nanomaterials have been developed, including polymer-graphene composites [17,18], redox mediators with carbon nanotubes [19,20] and metal particles-graphene composites [21,22]. Nanomaterial composites frequently offered improved electron transfer characteristics. Moreover, graphene (or reduced graphene oxide (RGO)), usually produced by the reduction of graphene oxide, possesses some characteristic functional groups such as hydroxyl (-OH) and carboxyl (-COOH) groups [23]. These functional groups were usually utilized as active sites for the nucleation and growth of metal nanoparticles in order to prepare RGO-based composites [24]. Composites of AuNPs and graphene have been synthesized by various methods such as in-situ growth [14,24] and electrochemical deposition [25], and the application of the composites in the fabrication of electrochemical sensors have been reported [26,27]. Composite nanomaterials offered improved electron transfer characteristics resulted from possible synergistic effect [19,26,28]. Many reports demonstrated that RGO-AuNPs composites with good dispersity can be obtained through controlling the loading amount of the AuNPs on the graphene surface [24,27]. However, only few researches focus on the impact of weight ratio of RGO and AuNPs on the properties of composites obtained, such as the particles size, dispersity, optical and electrochemical properties.
In this work, AuNPs were prepared through the reduction of HAuCl₄ by sodium citrate. Reduced graphene oxide-gold nanoparticles (RGO-AuNPs) composites were obtained through nucleation and growth of gold particles on the surface of reduced graphene oxide. The optimal weight contents of RGO and AuNPs in the formation of RGO-AuNPs composites was investigated and characterized by SEM, ultraviolet-visible spectroscopy and electrochemical techniques. The RGO-AuNPs composites displayed excellent catalytic activity toward the reduction of H₂O₂, attributing to the synergistic effects between RGO and AuNPs. Additionally, glucose oxidase (GOD) was immobilized on the GC/RGO-AuNPs surface through electrostatic interactions between chitosan coating and RGO-AuNPs materials. The resulting sensor achieved direct electron transfer of GOD and was applied for the detection of glucose. The sensitivity for glucose detection was roughly two times higher than that reported by others for similar electrode system [24].

3.2 Experimental

3.2.1 Reagents and apparatus

Reduced graphene oxide (RGO) was purchased from XF Nano Material Co. Ltd. (Nanjing, China). Glucose oxidase (GOD) (Type X-S, from Aspergillus niger), β-D-glucose, chitosan (CHIT), dopamine hydrochloride (DA), uric acid (UA), gold(III) chloride trihydrate (HAuCl₄·3H₂O) were obtained from Sigma. Citric acid trisodium salt dihydrate and ascorbic acid (AA) were obtained from Aldrich. Hydrogen peroxide (H₂O₂) and potassium hexacyanoferrate (K₃Fe(CN)₆) were purchased from International Laboratory. All other reagents were of reagent grade and were used without further purification. Phosphate buffer solution (pH
7.4) consisting of 0.02 M NaH$_2$PO$_4$ and 0.02 M Na$_2$HPO$_4$ was used as the supporting electrolyte. All solutions were prepared with deionized water.

The morphologies and surface structures were characterized by a scanning electron microscope (SEM) (LEO, Electron Microscopy Inc., Cambridge, UK) operated at 20 kV. The SEM samples were prepared by placing 5 μL sample dispersion onto a glassy carbon substrate, and allowing them to dry in air. Ultraviolet-visible absorption spectra were recorded using a Cary 100-Scan UV-Visible spectrophotometer (Varian Inc., USA). All electrochemical experiments were carried out at room temperature using a single compartment, three-electrode cell with the modified electrode as working electrode, a platinum wire and Ag|AgCl as auxiliary and reference electrodes, respectively. All potential were measured and reported versus the Ag|AgCl reference electrode (sat. KCl). Cyclic voltammetry and amperometric measurements were performed on a CHI6012B electrochemical workstation (CH instruments, Inc., USA).

3.2.2 Preparation of RGO-AuNPs composite materials

RGO-AuNPs composite solution was prepared according to Natan [29]. Typically, 0.2 mL HAuCl$_4$ (20 mg/mL) and 0.1 mL RGO (2 mg/mL) solutions were added to 20 mL of boiling deionized water with vigorous stirring. Then, 0.8 mL sodium citrate solution (10 mg/mL) was rapidly added to the RGO-HAuCl$_4$ mixture. The resulting mixture was stirred and heated for 15 min with a color change from pale yellow to pink. The resulting mixture was immediately collected by centrifugation and thoroughly washed with deionized water. The resulting product was re-dispersed in 0.2 mL deionized water under sonication to produce a colloidal suspension (designated as Composite B in this study). A series of RGO-AuNPs composites with various weight ratios of RGO and AuNPs were
prepared. The weight ratios of the composite mixtures can be easily controlled by changing the volume of precursor materials (RGO, HAuCl₄ and sodium citrate) employed for preparation. The compositions of mixtures employed for the preparation of different RGO-AuNPs composites are listed in Table 3.1 (assuming complete conversion of HAuCl₄ to AuNPs). In addition, pure AuNPs were synthesized similarly without the addition of RGO.

3.2.3 Preparation of modified electrodes

Glassy carbon electrodes (GCE, Bioanalytical Systems, Inc., 3 mm in diameter) were carefully polished with 0.3 and 0.05 μm alumina slurry on a microcloth (Buehler, USA), followed by ultrasonication in ethanol and deionized water for 2 min. Modified electrodes were prepared by adding 5 μL of the target suspension (RGO, AuNPs or RGO-AuNPs composites) on the GC electrode surface and were allowed to dry in air.

For the preparation of glucose biosensors, 5 μL 3% chitosan (CHIT) solution containing 10 mg/mL glucose oxidase (GOD) was cast onto the surface of GCE/RGO-AuNPs modified electrode and was allowed to dry at room temperature. The biosensors were stored at 4 °C before use.

The steady state amperometric response to H₂O₂ and glucose were measured by voltammetric technique in a degassed 0.02 M phosphate buffer solution (PBS, pH 7.4) under gentle stirring of around 100 rpm at the desired potentials. The current response was recorded on successive addition of the substrates.
Table 3.1 Preparation of RGO-AuNPs composites

<table>
<thead>
<tr>
<th>Samples</th>
<th>Composition of preparation mixture (mg)</th>
<th>Composition of resulting RGO-AuNPs composite (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(RGO: HAuCl₄: sodium citrate)</td>
<td>RGO</td>
</tr>
<tr>
<td>Composite A</td>
<td>0.2 : 4 : 4</td>
<td>0.2</td>
</tr>
<tr>
<td>Composite B</td>
<td>0.2 : 4 : 8</td>
<td>0.2</td>
</tr>
<tr>
<td>Composite C</td>
<td>0.2 : 4 : 16</td>
<td>0.2</td>
</tr>
<tr>
<td>Composite D</td>
<td>0.2 : 2 : 8</td>
<td>0.2</td>
</tr>
<tr>
<td>Composite E</td>
<td>0.2 : 8 : 8</td>
<td>0.2</td>
</tr>
<tr>
<td>Composite F</td>
<td>0.2 : 8 : 16</td>
<td>0.1</td>
</tr>
<tr>
<td>Composite G</td>
<td>0.2 : 2 : 4</td>
<td>0.4</td>
</tr>
<tr>
<td>AuNPs</td>
<td>0 : 4 : 8</td>
<td>0</td>
</tr>
</tbody>
</table>

3.3 Results and discussion

3.3.1 Effects of preparation composition

The deposition of the gold nanoparticles (AuNPs) on the surface of reduced graphene oxide (RGO) produced a 3-D structure, which greatly enhanced the surface area and offered good electrochemical properties. On the other hand, the size, density and morphology of the resulting reduced graphene oxide - gold nanoparticles (RGO-AuNPs) composites changed with the different weight ratio of RGO and AuNPs employed in the preparation. To explore the effects of preparation compositions on the physical and chemical properties of the RGO-AuNPs composites, different RGO-AuNPs composites were synthesized (as detailed in Table 3.1) and various techniques (SEM, UV-Visible and electrochemical techniques) were employed for characterization. Fig. 3.1 shows the SEM images of RGO-AuNPs composites prepared with different preparation compositions (RGO, HAuCl₄ and sodium citrate). The amount of sodium citrate was in excess and should be enough to convert all HAuCl₄ reagents to give
AuNPs. RGO materials gave a flake-like thin wrinkling film structure, as shown in Fig. 3.1(h), similar to those reported in the literature [12,30]. On the other hand, gold nanoparticles were well dispersed on the glassy carbon surface, as shown in Fig. 3.1(i). The average size of AuNPs was about 26 nm.

RGO-AuNPs composites prepared with different compositions displayed very different morphologies, as evidenced from the SEM images shown in Fig. 3.1(a)-(g). For all the composites prepared in this study, different degree of aggregation was observed, as compared to the well dispersed AuNPs deposited on bare glassy carbon shown in Fig. 3.1(i). Most of the AuNPs were located at the edge of RGO, and only few AuNPs distributed on the RGO surface. It might be due to the high density of edge-plane-like defective sites on RGO surface, which provided many favourable sites for AuNPs deposition [30].

It has been reported in the literature that large amount of sodium citrate would produce AuNPs with smaller particles [31]. For Composite A (Fig. 3.1(a)), the AuNPs displayed a nano-fiber structure with about 300 nm long and about 75 nm wide. Only few spherical AuNPs with average diameter of about 63 nm dispersed on the surface of RGO layers. The average size of spherical AuNPs obtained from different preparation compositions is shown in Table 3.2. On the other hand, Composite B (Fig. 3.1(b)) showed a very different structure as compared to Composite A. The appearance of the AuNPs was like rice grains with an average size of about 45 nm stacked on the RGO surface to form a relatively uniform porous network structure. In contrast, most of the AuNPs of Composite C (Fig. 3.1(c)) decreased obviously in size and the particles were spherical with an average diameter of 32 nm. However, some AuNPs were of nano-fiber shape with dimensions of 145 nm by 37 nm.
Fig. 3.1 SEM images of glassy carbon discs deposited with different RGO-AuNPs composites prepared with different weight ratio of RGO, HAuCl₄ and sodium citrate. (a) Composite A; (b) Composite B; (c) Composite C; (d) Composite D; (e) Composite E; (f) Composite F; (g) Composite G; (h) RGO; (i) AuNPs.

All AuNPs in Composite D (Fig. 3.1(d)) were spherical but with very different diameters. Some of the AuNPs with small diameter of about 37 nm were well dispersed on the surface of RGO layers. However, most of the AuNPs aggregated and formed large gold particles with a diameter of about 147 nm.
Composite E (Fig. 3.1(e)) displayed AuNPs of very different morphologies, including wafer-like shape with a diameter of about 300 nm, plate-type shape with a width of about 150 nm and the rod-like shape with a length of about 1 μm. Besides, spherical particles with the size of about 140 nm were also observed in Composite E, which was much larger than the AuNPs in Composite D and Composite B. On the other hand, all AuNPs gathered on the RGO surface in Composite F, showing the low dispersity and high intensity (Fig. 3.1(f)). No AuNPs were observed outside of the RGO layers. Besides, the AuNPs displayed spherical shape with a smaller diameter of about 35 nm. Composites G gave smaller and spherical AuNPs, which were well dispersed on the surface of RGO layers with a relatively uniform size of about 50 nm.

Table 3.2 Average size of spherical AuNPs of RGO-AuNPs composites

<table>
<thead>
<tr>
<th>Samples</th>
<th>Composition of resulting RGO-AuNPs composite (mg)</th>
<th>SEM characteristics of resulting composite</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RGO</td>
<td>AuNPs</td>
</tr>
<tr>
<td>Composite A</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>Composite B</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>Composite C</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>Composite D</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>Composite E</td>
<td>0.2</td>
<td>4</td>
</tr>
<tr>
<td>Composite F</td>
<td>0.1</td>
<td>2</td>
</tr>
<tr>
<td>Composite G</td>
<td>0.4</td>
<td>2</td>
</tr>
<tr>
<td>AuNPs</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

Interestingly, the morphologies of the resulting composites depended on the HAuCl₄-to-citrate weight ratio employed in the preparation solutions. It has been
reported that the aspect ratio (the length-to-height ratio) of the particles increased with the H\textsubscript{Au}Cl\textsubscript{4}-to-citrate weight ratio in the growth solution [32]. AuNPs in Composites A and E were prepared in solutions with a high H\textsubscript{Au}Cl\textsubscript{4}-to-sodium citrate weight ratio of 1. As shown in Fig. 3.1(a) and 1(e), most of the AuNPs particles were non-spherical and the particle size was not uniform. The particle size of Composite A was about 63 nm. Composite E gave some gold nano-rods with a length of about 1 \( \mu \)m and width of about 95 nm. The particle size of Composite E was estimated to be about 140 nm.

On the other hand, Composites B, F and G were prepared with a H\textsubscript{Au}Cl\textsubscript{4}-to-citrate weight ratio of 1:2, and gave smaller and spherical AuNPs with diameters from 35 to 50 nm. At this H\textsubscript{Au}Cl\textsubscript{4}-to-citrate weight ratio, the dispersity of AuNPs on RGO layer was greatly improved on increasing RGO content (Composite F < Composite B < Composite G), as shown in Fig. 3.1(f), 1(b) and 1(g). These results can be reasonably explained based on the considerable active sites and large surface area provided by RGO for AuNPs deposition [30]. The microstructure and dispersion of AuNPs can be controlled by varying the compositions of the preparation mixture such as RGO and H\textsubscript{Au}Cl\textsubscript{4}. By contrast, most of the AuNPs of Composites C and D were spherical in shape (Fig. 3.1(c) and 1(d)), resulted from the lower H\textsubscript{Au}Cl\textsubscript{4}-to-citrate weight ratio of 1:4. The particle size of Composite C and D was about 32 and 37 nm, respectively.

The particles size and morphology of AuNPs were influenced by the amount of H\textsubscript{Au}Cl\textsubscript{4} addition. When the amounts of RGO and sodium citrate reagents used for preparation were fixed, the density and the size of AuNPs in the composite increased with the weight of H\textsubscript{Au}Cl\textsubscript{4} reagent employed (Composite D < Composite B < Composite E). On the other hand, the amount of sodium citrate
reagent employed in the preparation also affected the particle characteristics. The length of AuNPs decreased with the HAuCl₄-to-citrate weight ratio and the particles varied from nano-rod to nano-sphere on increasing citrate concentration in the preparation solution (Composite A > Composite B > Composite C). Variation of particles from nano-rod to nano-sphere was also observed on decreasing HAuCl₄ concentration (Composite E > Composite B > Composite D).

In summary, the particles size and shape of AuNPs obtained were controlled by the HAuCl₄-to-citrate weight ratio. Either increasing the citrate amount or decreasing the HAuCl₄ amount will favour the formation of spherical particles with smaller size. On the contrary, decreasing the citrate amount or increasing the HAuCl₄ amount will produce non-spherical particles with larger size. The mechanism of AuNPs formation has been reported in the literature [33]. It has been proposed that the formation of AuNPs involved a fast nucleation process and a diffusion controlled growth [34]. The nucleation rate and the number of gold nuclei would increase with increasing citrate concentration during the original nucleation process. Then the number of gold nuclei would keep constant and small particles would be produced in the following diffusion-controlled growth process. On the other hand, the dispersity and density of AuNPs on the surface of RGO layers were determined by the amount of RGO in the preparation mixture. The oxygen functionalities of RGO were responsible for the nucleation of AuNPs located on the RGO layers [35]. Goncalves et al. [35] demonstrated that the AuNPs might have a good dispersity along the surface of RGO layers if a high density of oxygen-containing functional groups was present on RGO. Therefore, large amount of RGO would be favorable for the high dispersity of AuNPs due to
the introduction of many oxygen functional groups, which was consistent with the results shown in Fig. 3.1.

3.3.2 Spectroscopic characterization of all kinds of RGO-AuNPs composites

AuNPs are known to have strong surface plasmon resonance which will give a red solution and an absorption band at around 520 nm [36,37]. Fig. 3.2 displayed the UV-Visible spectra of different RGO-AuNPs composites obtained by varying the composition of the preparation mixture. Pure AuNPs gave an absorption maximum at 528 nm, as shown in Fig. 3.2(i). RGO offered no characteristic absorption between 350 and 700 nm [38], as shown in Fig. 3.2(h). All seven RGO-AuNPs composites prepared in this study showed strong absorption peaks from 530 to 570 nm (Fig. 3.2), corresponding to the surface plasmon resonance of gold nanoparticles. The results confirmed the successful deposition of AuNPs on the RGO surface. However, different RGO-AuNPs composites showed slight variation in the solution color and absorption maxima, as listed in Table 3.3. Composites A and E showed very flat characteristic absorption with maxima at around 565 and 547 nm (Fig. 3.2(a) and 2(e)), respectively. This indicated an apparent red-shift as compared to pure AuNPs. Besides, Composite E also had absorption at around 655 nm, corresponding to the longitudinal mode absorption of AuNPs [39]. The significant difference in the absorption maximum might result from the difference in the size and shape of the gold nanoparticles obtained.
Fig. 3.2 UV-visible absorption spectra of RGO-AuNPs suspensions prepared with different weight ratio of RGO, HAuCl₄ and sodium citrate. (a) Composite A; (b) Composite B; (c) Composite C; (d) Composite D; (e) Composite E; (f) Composite F; (g) Composite G; (h) RGO; (i) AuNPs. Inset shows the color of the composite suspensions.

Generally, the aggregation of nanoparticles will lead to the formation of larger particles, which will give a red shift in the surface plasmon resonance [40]. On the other hand, nanoparticles with a higher aspect ratio would also bring about a red-shift in the characteristic absorption [40]. Therefore, AuNPs in Composites A and E might have a higher aspect ratio and bigger size. The AuNPs in the other composites showed characteristic absorption at around 532 nm, reflecting the spherical shape and relatively smaller size, consistent with the SEM images displayed in Fig. 3.1. The peak absorbance reflected the total amounts of AuNPs.
present in the RGO-AuNPs composites. As evidenced from Fig. 3.2, Composite B offered a maximum yield of AuNPs among the RGO-AuNPs composites obtained. In the presence of excess sodium citrate, complete conversion of HAuCl$_4$ to AuNPs was assumed. Accordingly, 2 mg of AuNPs would be produced, and the coverage of AuNPs at GC electrode modified with Composite B was estimated to be 144 $\mu$mol/cm$^2$. This composite was selected for electrode modification, and was employed for H$_2$O$_2$ catalysis and glucose detection.

Table 3.3 Spectroscopic characteristics of RGO-AuNPs composites

<table>
<thead>
<tr>
<th>Samples</th>
<th>Composition of resulting RGO-AuNPs composite (mg)</th>
<th>Spectroscopic characteristics of resulting composite</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RGO</td>
<td>AuNPs</td>
</tr>
<tr>
<td>Composite A</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>Composite B</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>Composite C</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>Composite D</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>Composite E</td>
<td>0.2</td>
<td>4</td>
</tr>
<tr>
<td>Composite F</td>
<td>0.1</td>
<td>2</td>
</tr>
<tr>
<td>Composite G</td>
<td>0.4</td>
<td>2</td>
</tr>
<tr>
<td>AuNPs</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

3.3.3 Electrochemical characterization of different modified electrodes

The electrochemical properties of different RGO-AuNPs composites obtained were characterized by cyclic voltammetry in 1 mM K$_3$Fe(CN)$_6$ solution at 50 mV/s. As shown in Fig. 3.3, a pair of well-defined quasi-reversible redox peaks of ferricyanide ions was observed at bare glassy carbon electrode, giving a formal potential of +0.32 V and a peak separation ($\Delta E_p$) of 81 mV (Fig. 3.3(a)).
For electrodes modified with either RGO (Fig. 3.3(b)) or AuNPs (Fig. 3.3(c)) alone, the peak current for \( \text{K}_3\text{Fe(CN)}_6 \) exhibited a slight enhancement as compared to that for bare GCE. This might be due to the larger electroactive surface area available for electron-transfer of \( \text{Fe(CN)}_6^{3/-4} \) [41] at electrodes modified with RGO or AuNPs. However, the GCE/RGO and GCE/AuNPs electrodes displayed larger peak separation of 89 and 108 mV, respectively. Negatively charged RGO and AuNPs might offer hindrance for the diffusion of \( \text{Fe(CN)}_6^{3/-4} \) toward the electrode surface.

Fig. 3.3 Cyclic voltammograms at different modified electrodes in 1 mM \( \text{K}_3\text{Fe(CN)}_6 \) solution at a scan rate of 50 mV/s. (a) bare glassy carbon electrode (GCE); (b) RGO modified GCE (GCE/RGO); (c) AuNPs modified GCE (GCE/AuNPs); (d) RGO-AuNPs Composite B modified GCE (GCE/RGO-AuNPs(B)).
Electrodes modified with different RGO-AuNPs composites also showed similar voltammetric behaviors in ferricyanide solutions. The peak current for the electrode modified with RGO-AuNPs Composite B (GCE/RGO-AuNPs(B)) showed a redox wave with a formal potential of +0.31 V and a peak separation of 79 mV, as shown in Fig. 3.3(d). This peak separation was almost identical to that observed at bare GCE and much smaller than those observed at GCE/RGO and GCE/AuNPs electrodes. The peak separation for the ferricyanide response observed at electrodes modified with different RGO-AuNPs composites was roughly constant (from 76 to 88 mV), as shown in Table 3.4. The corresponding cathodic peak current for Composite B was about 17 µA, larger than those observed at the GCE/RGO and GCE/AuNPs electrodes. The result can be attributed to the large surface area of RGO that increased the amount of deposition of AuNPs, which provided more electroactive sites than the electrodes modified with either RGO or AuNPs alone. Moreover, the surface roughness and porosity may contribute to the electron transfer of Fe(CN)$_{6}^{3-}/4^{-}$ species. On the other hand, the cathodic peak current of K$_3$Fe(CN)$_6$ observed for Composites of B, C and D were almost the same, which might be due to the increased surface area and surface roughness caused by RGO and AuNPs [41]. Composites E and F displayed a minimum Fe(CN)$_{6}^{3-}/4^{-}$ response among the composites examined. Aggregation of AuNPs on RGO surface and larger gold particles might result in a lower total surface area.

The electrochemically effective surface area of the modified electrode was examined by cyclic voltammetry in 1 mM K$_3$Fe(CN)$_6$ solution according to the formula given by Randles-Sevcik equation [42]:

$$I_p = (2.69 \times 10^5) n^{3/2} A D^{1/2} c v^{1/2}$$
where \( n \) is the number of electrons participating in the redox reaction; \( \nu \) is the scan rate of the potential perturbation (in V/s); \( A \) is the area of the electrode (in cm\(^2\)); \( D \) is the diffusion coefficient of the molecules in the solution (in cm\(^2\)/s); \( c \) is the concentration of the probe molecules (in mol/cm\(^3\)); and \( I_p \) is the peak current of the redox couple (in \( \mu \)A). The diffusion coefficient of \( \text{K}_3\text{Fe(CN)}_6 \) is known to be \( 7.6 \times 10^{-6} \) cm\(^2\)/s [43].

Table 3.4 Voltammetric characterization of RGO-AuNPs composites

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Peak separation (mV)</th>
<th>Electrochemical surface area (mm(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC</td>
<td>81</td>
<td>5.7</td>
</tr>
<tr>
<td>GC/RGO</td>
<td>89</td>
<td>7.1</td>
</tr>
<tr>
<td>GC/AuNPs</td>
<td>108</td>
<td>6.1</td>
</tr>
<tr>
<td>GC/RGO-AuNPs (A)</td>
<td>80</td>
<td>6.7</td>
</tr>
<tr>
<td>GC/RGO-AuNPs (B)</td>
<td>79</td>
<td>9.8</td>
</tr>
<tr>
<td>GC/RGO-AuNPs (C)</td>
<td>83</td>
<td>10.3</td>
</tr>
<tr>
<td>GC/RGO-AuNPs (D)</td>
<td>76</td>
<td>9.4</td>
</tr>
<tr>
<td>GC/RGO-AuNPs (E)</td>
<td>76</td>
<td>6.8</td>
</tr>
<tr>
<td>GC/RGO-AuNPs (F)</td>
<td>88</td>
<td>6.7</td>
</tr>
<tr>
<td>GC/RGO-AuNPs (G)</td>
<td>81</td>
<td>8.6</td>
</tr>
</tbody>
</table>

* from cyclic voltammetric measurements in 1 mM \( \text{K}_3\text{Fe(CN)}_6 \) solution with a scan rate of 50 mV/s

The effective electrochemical surface area for different RGO-AuNPs composites was determined according to Randles-Sevcik equation, and the results are displayed in Table 3.4. The effective surface area of the modified electrodes was calculated to be 5.7, 7.1, 6.1 and 9.8 mm\(^2\) for GCE, GCE/RGO, GCE/AuNPs and GCE/RGO-AuNPs(B), respectively. The results indicated that the electrode
modified with the RGO-AuNPs Composite B exhibited a large electroactive surface area, indicating more effective deposition of active materials and higher sensor sensitivity.

RGO-AuNPs Composite B was prepared from a preparation solution containing 0.2 mg RGO, 4 mg HAuCl₄ and 8 mg sodium citrate, and provided uniform sized AuNPs of 45 nm in diameter and a loose structure. The loose structure may promote the electron transfer. According to the SEM, UV-Visible and electrochemical results, Composite B offered the relatively ideal structure, optical and electrochemical properties. Therefore, Composite B was chosen as the composite material for electrode modification, and was employed for H₂O₂ catalysis and glucose detection.

### 3.3.4 Electrocatalytic behaviour of H₂O₂ at different modified electrodes

As one of side products of biochemical reaction involving different oxidase enzymes, hydrogen peroxide plays an important role in food industry, pharmaceutical preparation and clinical diagnosis. Hydrogen peroxide can be oxidized or reduced directly at ordinary electrodes. However, the redox reactions occurring at ordinary electrodes would require large overpotential which may lead to interferences [44]. Therefore, modification of electrodes is usually employed to reduce the overpotential.

Fig. 3.4 shows the cyclic voltammograms of different modified electrodes in 0.02 M phosphate buffer (pH 7.4) containing 2 mM H₂O₂. As shown in Fig. 3.4(A), a slight increase in cathodic current was observed upon addition of H₂O₂ at potentials more negative than −0.3 V, indicating the inefficient direct reduction of H₂O₂ at bare GCE. For the electrodes modified with RGO or AuNPs alone (Fig.
3.4(B) and 4(C)), reduction of H₂O₂ proceeded more effectively, as compared to bare GCE.

Fig. 3.4 Cyclic voltammograms at different modified electrodes in degassed phosphate buffer solution (pH 7.4) in the absence (a) and presence (b) of 2 mM H₂O₂ at 50 mV/s vs. Ag|AgCl. (A) bare GC electrode; (B) GCE/RGO; (C) GCE/AuNPs; (D) GCE/RGO-AuNPs.

On the other hand, reduction of H₂O₂ at the RGO-AuNPs modified electrode occurred at +0.1 V (Fig. 3.4(D)), suggesting better electrocatalytic activity toward
H₂O₂. It should be resulted from the increased active sites for electron transfer and excellent electronic conductivity of RGO and AuNPs. A pair of redox peaks was observed at around +0.51 V when AuNPs modified electrode was employed (Fig. 3.4(C) and 4(D)), indicating the presence of AuNPs. For the GCE/RGO-AuNPs (Composite B) electrode (Fig. 3.4(D)), the reduction current showed an obvious increase when compared with other modified electrodes. The great enhancement in current response and the positive shift of reduction potential might be due to the synergistic effect of RGO and AuNPs materials. The synergistic effect can be ascribed to the three-dimensional structure of RGO, which greatly facilitated the electron transfer ability [45].

In addition, the electrocatalysis of hydrogen peroxide at different modified electrodes was also examined by amperometric method. Fig. 3.5 displays the steady-state amperometric responses of modified electrodes upon successive addition of 0.1 mM H₂O₂ in degassed 0.02 M phosphate buffer solution (pH 7.4) at zero applied potential. A slight current response of about 1.6 nA was observed for 0.1 mM H₂O₂ at bare GCE (Fig. 3.5(a)). An improved current response of 15 and 19 nA were observed at the GCE/RGO (Fig. 3.5(b)) and GCE/AuNPs (Fig. 3.5(c)) electrodes, respectively. RGO and AuNPs displayed similar catalytic effects toward H₂O₂ reduction, consistent with the voltammetric responses shown in Fig. 3.4. On the other hand, the RGO-AuNPs Composite B modified electrode (GCE/RGO-AuNPs(B)) offered the highest current response of 67 nA for the same H₂O₂ concentration of 0.1 mM, as shown in Fig. 3.5(d). The results indicated a combined synergistic amplification in the presence of RGO and AuNPs. The introduction of RGO would greatly improve the effective surface area through producing a three-dimensional structure. The structure might shorten
the distance between electron transfer sites and the electrode, which would facilitate the electron transfer [19,20].

Fig. 3.5 Amperometric response recorded at different electrodes with successive addition of 0.1 mM H$_2$O$_2$ in 0.02 M degassed phosphate buffer solution (pH 7.4) under stirring at zero applied potential vs. Ag$|$AgCl. (a) Bare GCE; (b) GCE/RGO; (c) GCE/AuNPs; (d) GCE/RGO-AuNPs(B).

Additionally, the response sensitivity of different modified electrodes employed for hydrogen peroxide detection is displayed in Table 3.5. The RGO-AuNPs(B) composite electrode offered higher sensitivity of 9.5 mA M$^{-1}$ cm$^{-2}$ as compared to the sensitivity of 2.1 and 2.7 mA M$^{-1}$ cm$^{-2}$ for the GCE/RGO and GCE/AuNPs electrodes, respectively. The Composite B modified electrode offered the highest sensitivity and widest linear range as compared to
the electrodes modified with the other RGO-AuNPs composites employed in this study. It seemed that RGO-AuNPs composites with uniform, smaller and spherical AuNPs and good dispersity offered better electroanalytical behaviors. The remarkable improvement in sensitivity should attribute to the excellent electrocatalytic effect of the RGO-AuNPs composite, displaying the synergistic effects between RGO and AuNPs.

Table 3.5 Comparison of different electrode systems employed for H$_2$O$_2$ detection

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Sensitivity (mA M$^{-1}$ cm$^{-2}$)</th>
<th>Linear range (mM)</th>
<th>Limit of detection (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC</td>
<td>0.24</td>
<td>0.025 ~ 1.5</td>
<td>0.05</td>
</tr>
<tr>
<td>GC/RGO</td>
<td>2.1</td>
<td>0.025 ~ 2.0</td>
<td>0.025</td>
</tr>
<tr>
<td>GC/AuNPs</td>
<td>2.7</td>
<td>0.025 ~ 29.5</td>
<td>0.006</td>
</tr>
<tr>
<td>GC/RGO-AuNPs (A)</td>
<td>5.3</td>
<td>0.025 ~ 1.5</td>
<td>0.023</td>
</tr>
<tr>
<td>GC/RGO-AuNPs (B)</td>
<td>9.5</td>
<td>0.025 ~ 41.5</td>
<td>0.005</td>
</tr>
<tr>
<td>GC/RGO-AuNPs (C)</td>
<td>7.5</td>
<td>0.025 ~ 15.5</td>
<td>0.011</td>
</tr>
<tr>
<td>GC/RGO-AuNPs (D)</td>
<td>3.8</td>
<td>0.025 ~ 17.5</td>
<td>0.025</td>
</tr>
<tr>
<td>GC/RGO-AuNPs (E)</td>
<td>2.9</td>
<td>0.025 ~ 1.5</td>
<td>0.025</td>
</tr>
<tr>
<td>GC/RGO-AuNPs (F)</td>
<td>5.7</td>
<td>0.025 ~ 2.0</td>
<td>0.025</td>
</tr>
<tr>
<td>GC/RGO-AuNPs (G)</td>
<td>4.9</td>
<td>0.025 ~ 21.5</td>
<td>0.025</td>
</tr>
</tbody>
</table>

Based on the amperometric results, the GCE/RGO-AuNPs(B) electrode was chosen for H$_2$O$_2$ detection. The GCE/RGO-AuNPs(B) electrode offered a linear range from 0.025 to 41.5 mM for H$_2$O$_2$ detection, and a detection limit of 5 μM for a signal-to-noise ratio of 3 (Fig. 3.6). The relative standard deviation (RSD) for the amperometric detection of 0.1 mM H$_2$O$_2$ at the GCE/RGO-AuNPs(B) electrode was determined to be of 5.4% for successive measurements at four electrodes prepared identically.
Fig. 3.6 Calibration curve for H$_2$O$_2$ detection at the GC/RGO-AuNPs electrode.

The RGO-AuNPs Composite B showed the best sensitivity for the reduction of hydrogen peroxide. Firstly, the AuNPs had excellent catalyst to promote the reduction of hydrogen peroxide [46]. Secondly, the composite offered a rough surface with a higher electrochemical active area, which greatly improved the rate of catalytic reduction of hydrogen peroxide [47]. Thirdly, Composite B had a unique porous structure and large amount of uniform size AuNPs resulted from ideal composition of RGO and AuNPs materials.

### 3.3.5 Direct electrochemistry of glucose oxidase and the detection of glucose

Chitosan (CHIT) has been utilized as a biocompatible film for enzyme immobilization [48]. Chitosan can adhere to negatively charged surfaces through electrostatic interactions [48]. Glucose oxidase (GOD) was immobilized on the
surface of GCE/RGO-AuNPs composite with chitosan coating for the fabrication of GCE/RGO-AuNPs/CHIT-GOD biosensor. Fig. 3.7 shows the cyclic voltammograms of the GCE/RGO-AuNPs(B) electrode coated with chitosan. Without GOD, the electrode showed no redox peak, as shown in Fig. 3.7(a). A small reversible redox wave was observed at $-0.29 \text{ V}$ with a peak-to-peak separation of 60 mV after GOD immobilization (Fig. 3.7(b)). The redox wave should be resulted from the reversible electron transfer process of the GOD redox centre, which was deeply hidden in GOD [12]. The result demonstrated that the RGO-AuNPs Composite B provided a suitable microenvironment for enzyme loading, and the special structure facilitated the direct electron transfer of GOD.

Fig. 3.7 Cyclic voltammograms at RGO-AuNPs modified electrodes in 0.02 M degassed phosphate buffer (pH 7.4). GCE/RGO-AuNPs(B)/CHIT (a) and GCE/RGO-AuNPs(B)/CHIT-GOD (b).
Additionally, the influence of scan rate on the voltammetric response of the GCE/RGO-AuNPs(B)/CHIT-GOD electrode was also investigated. As shown in Fig. 3.8, the peak currents of GOD increased linearly with scan rate for both the cathodic and anodic processes, indicating a surface-controlled electrochemical process [49]. The GOD coverage was estimated coulometrically to be about 2.9 nmol/cm², assuming that the redox reaction involved two electrons [48]. This coverage was considerably less than that reported by others [24] for covalent binding of GOD on electrode surface.

Fig. 3.8 The influence of scan rate (of 20, 50, 70, 100, 120, 150, 170 and 200 mV/s) on the voltammetric performance of the GCE/RGO-AuNPs(B)/CHIT-GOD electrode. Inset: plot of peak current ($i_p$) vs. square root of scan rate. Supporting electrolyte: degassed phosphate buffer solution (pH 7.4).
Fig. 3.9 shows the cyclic voltammograms of the GCE/RGO-AuNPs(B)/CHIT-GOD electrode in air-saturated phosphate buffer (pH 7.4) in the absence (Fig. 3.8(a)) and presence (Fig. 3.8(b)) of 1 mM glucose. The results indicated that the baseline of the reduction decreased with the addition of glucose, resulting from the consumption of oxygen. Furthermore, the detection of glucose at the GCE/RGO-AuNPs(B)/CHIT-GOD electrode was investigated through the successive addition of 0.1 mM glucose in 0.02 M air-saturated phosphate buffer at an applied potential of $-0.3 \ V$. 

Fig. 3.9 Cyclic voltammograms at GCE/RGO-AuNPs(B)/CHIT-GOD modified electrodes in 0.02 M air-saturated buffer without glucose (a) and containing 1.0 mM glucose (b). Scan rate: 50 mV/s.
Fig. 3.10 displays the amperometric response upon successive addition of glucose at the GCE/RGO-AuNPs(B)/CHIT-GOD electrode and the resulting calibration curve of the glucose biosensor employed for glucose detection. Linear response was observed for glucose concentration from 0.1 to 1.3 mM with a detection limit of 76 μM for a signal-to-noise ratio of 3. The sensitivity was estimated to be 34 mA M$^{-1}$ cm$^{-2}$, which is roughly two times of that reported in the literature with similar electrode system [24]. The relative standard deviation (RSD) of the current response for 0.1 mM glucose at the GCE/RGO-AuNPs(B)/CHIT-GOD electrode was 6.6% for 13 successive measurements, indicating good reproducibility and high enzymatic activity of the biosensor.

![Amperometric response and calibration curve for glucose detection at GCE/RGO-AuNPs(B)/CHIT-GOD biosensor in air-saturated phosphate buffer (pH 7.4) at −0.3 V vs. Ag/AgCl.](image)

Fig. 3.10 Amperometric response and calibration curve for glucose detection at GCE/RGO-AuNPs(B)/CHIT-GOD biosensor in air-saturated phosphate buffer (pH 7.4) at −0.3 V vs. Ag/AgCl.
The selectivity and anti-interference ability of the biosensor were investigated for 0.1 mM glucose. Fig. 3.11 shows the amperometric response of the biosensor upon addition of 0.1 mM ascorbic acid (AA), dopamine (DA), uric acid (UA) and glucose. The interfering substances showed negligible response at $-0.3\ V$, indicating a high selectivity and good anti-interference ability of the GCE/RGO-AuNPs(B)/CHIT-GOD biosensor. The stability of the glucose biosensor was also investigated. The response current for 0.1 mM glucose retained about 70% of the initial value after 36 days, indicating a good stability of GCE/RGO-AuNPs(B)/CHIT-GOD biosensor.

Fig. 3.11 Anti-interference property of the GCE/RGO-AuNPs(B)/CHIT-GOD biosensor in air-saturated phosphate buffer (pH 7.4) at $-0.3\ V$ vs. Ag/AgCl.
3.4 Conclusions

RGO-AuNPs composites were prepared by reduction of gold chloride with sodium citrate in the presence of different amounts of RGO. Usually, AuNPs obtained from mixtures with a small HAuCl₄-to-citrate weight ratio will give spherical particles with smaller size. Large amount of RGO utilized in the preparation will favour the formation of AuNPs with good dispersity. The resulting composite materials were characterized by SEM, UV-Visible spectroscopy and electrochemical methods. The RGO-AuNPs composite was used for electrocatalytic reduction of hydrogen peroxide. RGO-AuNPs composites with uniform, smaller and spherical AuNPs and good dispersity usually offered better electroanalytical behaviors. A glucose biosensor was constructed after the immobilization of GOD, and direct electron transfer of GOD was observed at the composite modified electrode due to the excellent electronic conductivity and synergistic effect. The biosensor displayed a high selectivity and good stability at an applied potential of −0.3 V for the detection of glucose.
References


Chapter 4 Spontaneous deposition of Prussian blue on reduced graphene oxide - gold nanoparticles composites for the fabrication of electrochemical biosensors

4.1 Introduction

The detection of hydrogen peroxide is always attracting a lot of attention from researchers due to the importance in pharmaceutical, environmental, clinical applications and food manufacturing [1]. As a signalling molecule, hydrogen peroxide also plays an important role in regulating diverse biological process including apoptosis, vascular remodelling and immune cell activation [2-5]. Therefore, detection of hydrogen peroxide with high sensitivity, accuracy and good anti-interference properties should be well considered. Prussian blue (PB) is an ideal candidate for the detection of hydrogen peroxide due to the excellent catalysis toward the reduction of hydrogen peroxide, and therefore is referred to as an “artificial peroxidase” [6]. Furthermore, PB has also been used as an effective mediator for the preparation of composite materials and construction of electrochemical sensors due to its excellent properties including low redox potentials, enhanced electron transfer rate and good electrocatalytic properties [7-9]. However, electroanalytical performance of PB modified electrodes becomes deteriorated at neutral electrolytes due to the hydrolysis of PB, which greatly limits the application of PB for the construction of biosensors [10]. Accordingly, some measures have been taken for improving the stability of PB, such as coating the electrodes with Nafion [11], chitosan membranes [12] and conducting
polymer films [13,14]. Spontaneous deposition of Prussian blue on multi-walled carbon nanotubes has been reported [15].

Noble metal nanoparticles could satisfy the specific demands of biosensors such as disease diagnosis, cell tracking, vivo imaging and therapy monitoring, and have played an important role in the development of biosensors [16]. Gold nanoparticles (AuNPs) have attracted much attention due to the intriguing properties, including high surface-to-volume ratio, rapid electron transfer, high surface free energy and good biocompatibility [17]. These unique properties of gold nanoparticles not only provide a large loading of biomolecules, but also permit direct electron transfer between electroactive species and electrodes, and maintain good bioactivity and stability of biomolecules [18,19]. Furthermore, gold has good affinity with some functional groups such as nitrile (–CN), amino (–NH₂) and mercapto (–SH) groups [20]. The strong affinity between AuNPs and PB may improve the stability of PB in neutral conditions through the hybridization of AuNPs and PB.

Graphene is a novel material and has attracted considerable attention in electrode modification due to the excellent chemical, electronic and mechanical properties [21]. Moreover, graphene or reduced graphene oxide (RGO) produced by the reduction of graphene oxide possess some characteristic functional groups such as hydroxyl (–OH) and carboxyl (–COOH) groups, which would benefit the preparation of composite materials [22]. In the present work, RGO-AuNPs-PB composite was prepared through electrochemical deposition of AuNPs and spontaneous deposition of PB on RGO materials. PB displayed a good stability after hybridization with AuNPs due to the high affinity with AuNPs. The RGO-AuNPs-PB composite displayed an excellent catalytic activity toward the
reduction of hydrogen peroxide. Direct electron transfer of glucose oxidase (GOD) was realized after codeposition of GOD and chitosan (CHIT) coating on the RGO-AuNPs-PB composite, and the resulting modified electrode was applied as an amperometric glucose biosensor showing great sensitivity.

4.2 Experimental

4.2.1 Reagents and apparatus

Reduced graphene oxide (RGO) was purchased from XF Nano Material Co. Ltd. (Nanjing, China). Glucose oxidase (GOD) (Type X-S, from Aspergillus niger), β-D(+)-glucose, chitosan (CHIT), dopamine hydrochloride (DA), uric acid (UA) and gold(III) chloride trihydrate (HAuCl₄·3H₂O) were obtained from Sigma. Hydrogen peroxide (H₂O₂) and potassium hexacyanoferrate (III) (K₃Fe(CN)₆) were purchased from International Laboratory. All other chemicals were of reagent grade and were used without further purification. Phosphate buffer solution (pH 7.4) consisting of 0.02 M NaH₂PO₄ and 0.02 M Na₂HPO₄ was used as the supporting electrolyte. All solutions were prepared with deionized water.

All electrochemical experiments were carried out by a CHI6012B electrochemical Analyzer (CH instruments, Inc., USA). A traditional three-electrode system employing bare or modified glassy carbon working electrode, a platinum wire counter electrode and Ag|AgCl reference electrode was used. All potentials were quoted versus the Ag|AgCl reference electrode.

Surface characterization of the modified electrodes (including RGO, RGO-AuNPs, RGO-AuNPs-PB, RGO-PB, AuNPs and AuNPs-PB) were performed with a scanning electron microscope (SEM) (LEO, Electron Microscopy Inc., Cambridge, UK) at 20 kV. X-ray photoelectron spectroscopy
(XPS) was performed in an ultrahigh vacuum chamber, with a base pressure below $8 \times 10^{-7}$ Pa at room temperature. Photoemission spectra were recorded using a SKL-12 spectrometer with VG CLAM 4 multichannel hemispherical analyzer and Al/Mg excitation source. The sample for SEM and XPS analysis were prepared on glassy carbon disks of 3 mm in diameter, which were obtained by wire-cutting a glassy carbon rod (Atomergic Chemetals Corp.) into pieces of 2 mm thick, and then mounted onto a Teflon sheath. The disk electrode was polished as usual and modified with different materials (RGO, AuNPs and PB). The modified electrode obtained was rinsed with deionized water. The glassy carbon disks modified with the desired materials were detached from the Teflon sheath and subjected to scanning electron microscopic studies.

4.2.2 Preparation of modified electrodes

Glassy carbon electrodes (GC, Bioanalytical Systems, Inc., 3 mm in diameter) were carefully polished with 0.3 and 0.05 μm alumina slurry on a microcloth (Buehler, USA), followed by ultrasonication in ethanol and deionized water for 2 min. 2.0 mg RGO was dispersed in 1 mL deionized water by ultrasonication for 1 h to form a homogenous suspension. 5 μL of 2.0 mg/mL RGO suspension was dropped onto the surface of glassy carbon electrode and was allowed to dry in air.

GC/AuNPs and GC/RGO-AuNPs electrodes were prepared by immersing bare GC and GC/RGO electrodes in 2 mM HAuCl₄ solution for 200 s with a constant applied potential of $-0.2$ V. For PB spontaneous deposition, different electrodes were immersed into 0.1 M HCl + 0.1 M KCl solution (pH 1.0) containing 1 mM K₃Fe(CN)₆ + 1 mM Fe(NO₃)₃ for different period of time (from 0.5 to 3.0 h) to obtain the PB modified electrodes, similar to the procedures.
reported in the literature [15]. Fig. 4.1 displays the procedure for the preparation of GC/RGO-AuNPs composites with deposition of PB for 0.5 and 2.0 hours.

4.2.3 Fabrication of glucose biosensor and amperometric measurements

For the preparation of glucose biosensors, 7 μL 3% chitosan (CHIT) solution containing 10 mg/mL glucose oxidase (GOD) was cast onto the surface of GC/RGO-AuNPs-PB modified electrode and was allowed to dry at room temperature. The biosensors were stored at 4 °C before use.

The steady state amperometric response to hydrogen peroxide and glucose was measured by voltammetric technique in a supporting electrolyte under gentle stirring of around 100 rpm at the desired potentials. The current response was recorded on successive addition of the substrates.

Fig. 4.1 Procedure for the fabrication of GC/RGO-AuNPs composites with deposition of PB for 0.5 and 2.0 hours.
4.3 Results and discussion

4.3.1 Characterization of composite materials

The surface features of modified electrodes and their morphologies were examined by scanning electron microscopy (SEM). As shown in Fig. 4.2(a), graphene planar sheets displayed a well-defined two-dimensional structure with a thin wrinkling paper-like feature. A flat and compact AuNPs film was observed for AuNPs modified electrode, as shown in Fig. 4.2(b). Fig. 4.2(c) shows the surface structures of the RGO-AuNPs composite. Gold nanoparticles of about 50 nm in diameter were found distributed uniformly on the surface of graphene layers after deposition of AuNPs. Furthermore, some of the graphene layers stacked to form a three-dimensional structure with larger surface area. The results indicated that planar graphene sheets were well suitable as a foundation for the hybridization with other materials, and the layer-by-layer structure of RGO can be used as a template for the fabrication of three-dimensional composites. Fig. 4.2(d) shows the surface feature of the RGO-AuNPs composite after the spontaneous deposition of PB for two hours. AuNPs of 50 to 130 nm in diameter were observed and the appearance of cubic PB nanoparticles suggested the successful deposition of PB. Moreover, obvious interconnections between adjacent AuNPs and PB materials and the increase of composite particle size would greatly improve the electron conduction. PB particles were hardly seen on the RGO surface in the absence of AuNPs (Fig. 4.2(e)), while many cubic PB particles deposited on AuNPs modified glassy carbon surface (Fig. 4.2(f)). The results suggested that spontaneous deposition of PB on AuNPs were more efficient than at the RGO surface. Possibly, AuNPs served as the nucleus and as an excellent catalyst for the deposition and growth of PB [23].
In order to further confirm the chemical composition of the composites, x-ray photoelectron spectroscopy (XPS) was used to analyze the modified electrodes because elemental and chemical information of the composite can be obtained from the binding energy of a core level peak [24]. Fig. 4.3 shows the overview XPS scans of RGO and RGO-AuNPs-PB composite. Only carbon and oxygen signals were observed at RGO. On the other hand, Au, N and Fe appeared in the RGO-AuNPs-PB composite, indicating the successful deposition of AuNPs and PB on the RGO surface.
Additionally, the high-resolution XPS spectra further confirmed the detailed information of the electronic states of the elements, as shown in Fig. 4.4. The C 1s spectrum (Fig. 4.4(a)) of the RGO-AuNPs-PB composite indicated the presence of C–C (284.6 eV) and O–C=O (288.9 eV) species, which were ascribed to the aliphatic carbon [25] of RGO and confirmed the presence of oxygen-containing functional groups in RGO. These polar groups (O–C=O) might lead to the stable modified electrode by providing strong interaction with the cleaned glassy carbon electrode surface [26]. Moreover, they can also serve as the activated sites or nuclei for the deposition of AuNPs or PB. Fig. 4.4(b) shows that the Au 4f peaks appeared at binding energies of 84.3 and 84.9 eV, confirming the presence of metallic gold in the RGO-AuNPs-PB composite [27]. In addition, the high-resolution spectra for Fe 2p (Fig. 4.4(c)) show Fe peaks at 708.6 and 721.3
eV, corresponding to Fe 2p$_{3/2}$ and Fe 2p$_{1/2}$, respectively. The weak shoulder Fe 2p$_{3/2}$ peak at 710.7 eV indicated the existence of Fe$^{2+}$ ions in PB, which could offer catalytic activity for the biomolecules. On the other hand, the N 1s spectra (Fig. 4.4(d)) show peaks with binding energies of 397.8, 399.1 and 402.4 eV, possibly corresponding to the –CN groups of PB, which also confirmed the formation of PB.

Fig. 4.4 XPS spectra of RGO-AuNPs-PB. (a) C 1s spectrum; (b) Au 4f spectrum; (c) Fe 2p spectrum and (d) N 1s spectrum.
4.3.2 Electrochemical behaviors of different electrodes with PB deposits

Spontaneous deposition of Prussian blue was realized by immersing different electrodes in 0.1 M HCl + 0.1 M KCl solution (pH 1.0) containing 1 mM K$_3$Fe(CN)$_6$ + 1 mM Fe(NO$_3$)$_3$ for 2 hours. To evaluate the role of individual components for the deposition of PB, electrochemical behaviors of different electrodes (including GC/PB, GC/RGO-PB, GC/AuNPs-PB and GC/RGO-AuNPs-PB) were examined by cyclic voltammetry. A pair of well-defined redox peaks with a peak-to-peak separation of about 67 mV was observed at the GC/PB electrode (Fig. 4.5(a)), resulted from the redox processes of PB deposits. This indicated the successful deposition of PB on bare GC electrode. Moreover, the weak redox peak current of PB (~ 0.66 μA) suggested that few PB was deposited on bare GC electrode. RGO was also inefficient for the spontaneous deposition of PB, showing a pair of poorly defined and small peaks of PB of about 1.14 μA (Fig. 4.5(b)). However, the peak-to-peak separation of PB at RGO film reduced to about 60 mV, suggesting some improvement in the charge transfer rate, which might be resulted from the three-dimensional structure of RGO. AuNPs were prepared by reducing 2 mM HAuCl$_4$ solution for 200 s at a constant applied potential of –0.2 V. The coverage of AuNPs at GC electrode was estimated coulometrically to be 584 nmol/cm$^2$. Interestingly, the incorporation of AuNPs onto the GC/PB or GC/RGO-PB composites greatly enhanced the deposition of PB and influenced the electrochemical properties, indicating the presence of synergist effects between AuNPs and PB. The presence of AuNPs offered improvement in the redox behaviors of PB immobilized on AuNPs-PB (Fig. 4.5(c)) and RGO-AuNPs-PB (Fig. 4.5(d)) composites, as evidenced from the reduction of peak-to-peak separation. The peak-to-peak separation reduced from
67 mV at the GC/PB electrode to 33 mV at the GC/AuNPs-PB electrode (Fig. 4.5(c)), while the peak separation at the GC/RGO-PB electrode changed from 60 to 48 mV at the GC/RGO-AuNPs-PB electrode (Fig. 4.5(d)).

Additionally, the incorporation of AuNPs also greatly increased the deposition amount of PB on the electrodes, as evidenced by the increase in peak current of PB. The anodic peak current of PB at the GC/AuNPs-PB electrode was 16.7 μA, about 25 times of that observed at the GC/PB electrode, while the peak current of PB at the GC/RGO-AuNPs-PB electrode was 33.0 μA, about 29 times of that of that at the GC/RGO-PB electrode. The surface coverage of PB were
determined coulometrically and were found to be of 0.12, 0.68, 1.4 and 3.6 nmol cm\(^{-2}\) on bare GC, RGO, AuNPs and RGO-AuNPs modified electrodes, respectively. The electrochemical behaviors of PB deposits at different electrodes are summarized in Table 4.1.

### Table 4.1 Voltammetric characterization of different electrode systems

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Au coverage (nmole/cm(^2))</th>
<th>(E_{pa}(\text{PB})^*) (mV)</th>
<th>(I_{pa}(\text{PB})^*) ((\mu\text{A}))</th>
<th>PB peak separation* (mV)</th>
<th>PB coverage (nmole/cm(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC/AuNPs</td>
<td>584</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>GC/RGO-AuNPs</td>
<td>769</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>GC/PB</td>
<td>-----</td>
<td>297</td>
<td>0.67</td>
<td>67</td>
<td>0.12</td>
</tr>
<tr>
<td>GC/RGO-PB</td>
<td>-----</td>
<td>293</td>
<td>1.14</td>
<td>60</td>
<td>0.68</td>
</tr>
<tr>
<td>GC/AuNPs-PB</td>
<td>584</td>
<td>264</td>
<td>16.7</td>
<td>33</td>
<td>1.4</td>
</tr>
<tr>
<td>GC/RGO-AuNPs-PB</td>
<td>769</td>
<td>277</td>
<td>33.0</td>
<td>48</td>
<td>3.6</td>
</tr>
</tbody>
</table>

* cyclic voltammetric measurements in 0.02 M phosphate buffer containing 0.1 M KCl at a scan rate of 50 mV/s

Apparently, the amount of PB deposits displayed a considerable increase in the presence of AuNPs. The AuNPs contributed to the catalytic deposition of PB. The formation of PB involves two steps as shown in the following [28]:

\[
\begin{align*}
\text{Fe}^{3+} + \frac{1}{2} \text{H}_2\text{O} & \rightarrow \frac{1}{4} \text{O}_2 + \text{H}^+ + \text{Fe}^{2+} \\
\text{K}^+ + \text{Fe}^{2+} + \text{Fe(CN)}_6^{3-} & \rightarrow \text{KFe}^{III}[\text{Fe}^{II}(\text{CN})_6] 
\end{align*}
\]

(4.1) (4.2)

According to Neff [29], the AuNPs served as catalysts to promote the production of PB by reducing the standard free-energy of Reaction (4.1) [23]. On the other hand, the cyclic voltammograms of the GC/RGO-AuNPs-PB modified electrode at various scan rates were investigated. As shown in Fig. 4.6, both the redox peak currents and peak-to-peak separation increased with increasing scan rate. The
linear relationship between peak current and scan rate (from 20 to 200 mV/s) indicated a reversible and surface-confined redox process of PB in the composite film [30].

Fig. 4.6 Cyclic voltammograms of GC/RGO-AuNPs-PB electrode at various scan rates of 20, 50, 70, 100, 120, 150, 170 and 200 mV/s in degassed 0.02 M pH 7.4 PBS containing 0.1 M KCl. Inset: plot of redox peak current ($I_p$) vs. scan rate.

4.3.3 Spontaneous deposition of PB

According to the literature report [10], there was direct relationship between the activity of the PB modified electrode and the preparation conditions. GC/RGO-AuNPs electrodes with different deposition time of PB were investigated. GC/RGO-AuNPs-PB electrodes exhibited a sharp current peak (Fig. 4.5(d)), consistent with the typical structure of the inorganic polycrystal [31]. This
indicated the successful deposition of PB on the electrode. The voltammetric peak current measured at a scan rate of 50 mV/s showed a rapid linear increase with increasing deposition time, indicating the increase in PB loading, as shown in Fig. 4.7(a). The increase in peak current became slower after 2 h, which might be due to the decrease in surface area caused by the increasing particle size of the AuNPs-PB composite. In addition, the peak separation for the PB redox peak increased with the deposition time of PB. It might be caused by the increase of the thickness of PB layer, which would lead to a reduction in electron transfer rate. Furthermore, the electrocatalytic properties of GC/RGO-AuNPs electrodes with different amounts of PB deposits were also examined through amperometric response toward hydrogen peroxide at an applied potential of +0.1 V, as shown in Fig. 4.7(b). The GC/RGO-AuNPs electrode obtained by PB deposition of 2 h exhibited the strongest catalytic effect and the greatest sensitivity toward the reduction of hydrogen peroxide. Therefore, a deposition of PB for two hours was considered as the optimal time for PB deposition and was employed for the fabrication of RGO-AuNPs-PB biosensors.
Fig. 4.7 Effects of deposition time of PB on the voltammetric response of GC/RGO-AuNPs-PB electrode in the absence (a) and presence of 50 μM H₂O₂ (b) in degassed 0.02 M phosphate buffer (pH 7.4) containing 0.1 M KCl solution.
4.3.4 Electrocatalysis of $\text{H}_2\text{O}_2$ at GC/RGO-AuNPs-PB modified electrode

Hydrogen peroxide is one of the side products of many biochemical reactions. Usually, the redox processes of hydrogen peroxide at ordinary electrodes usually experience a high overpotential [22]. As a well-known “artificial peroxidase”, PB is a superior and selective electrocatalyst for the reduction of hydrogen peroxide [10]. To examine the electrocatalytic activity of the RGO-AuNPs-PB composites, electrocatalytic reduction of $\text{H}_2\text{O}_2$ at the GC/RGO-AuNPs-PB electrode was carried out, and the results are shown in Fig. 4.8.

Fig. 4.8 Cyclic voltammograms of GC/RGO-AuNPs-PB electrode in the absence (a) and presence of 0.5 mM (b); 1.0 mM (c) and 1.5 mM (d) $\text{H}_2\text{O}_2$ in degassed 0.02 M phosphate buffer (pH 7.4) containing 0.1 M KCl solution at a scan rate of 50 mV/s.
With the addition of H$_2$O$_2$, the cathodic peak current of PB increased and its anodic peak current decreased, indicating a typical electrocatalytic reduction process of H$_2$O$_2$. Moreover, the cathodic peak current of PB continuously increased with successive addition of H$_2$O$_2$. The result indicated that the RGO-AuNPs-PB composite had an efficient electrocatalytic effect toward H$_2$O$_2$ reduction.

The electrocatalytic performance of different electrodes for H$_2$O$_2$ reduction was investigated through the amperometric measurements with successive addition of H$_2$O$_2$. Fig. 4.9(a) shows that no obvious current response could be observed at the GC/PB electrode upon addition of H$_2$O$_2$, indicating a low catalytic effect toward H$_2$O$_2$ reduction at an applied potential of +0.1 V. It might be because that few PB was deposited on the bare GC electrode, which limited the electrocatalytic ability of the GC/PB electrode for H$_2$O$_2$ reduction. On the other hand, the RGO-PB modified electrode displayed an observable response to various concentrations of H$_2$O$_2$ (Fig. 4.9(b)), possibly due to the good electrocatalytic activity of RGO toward H$_2$O$_2$ [13] and the synergist effect of the RGO-PB composite. The response to the changes of H$_2$O$_2$ concentration improved rapidly at the GC/AuNPs-PB electrode (Fig. 4.9(c)) and reached highest response at the GC/RGO-AuNPs-PB electrode (Fig. 4.9(d)), indicating a strong catalytic effect of the GC/AuNPs-PB and GC/RGO-AuNPs-PB electrodes toward H$_2$O$_2$ reduction. It should be ascribed to the synergistic effect of AuNPs-PB composites and the large amount of PB deposits.
Fig. 4.9 Amperometric response recorded at different electrodes with successive addition of 50 μM H₂O₂ in degassed 0.02 M phosphate buffer (pH 7.4) containing 0.1 M KCl under stirring at an applied potential of +0.1 V vs. Ag|AgCl. (a) GC/PB; (b) GC/RGO-PB; (c) GC/AuNPs-PB and (d) GC/RGO-AuNPs-PB electrodes.

Furthermore, the electrochemical catalytic effects toward H₂O₂ at different electrodes (including bare GC, GC/RGO, GC/AuNPs and GC/RGO-AuNPs) were also investigated, and the sensitivity for H₂O₂ detection is listed in Table 4.2. The reduction of H₂O₂ hardly occurred at bare GC electrode at an applied potential of +0.1 V, showing a low sensitivity of 0.17 mA M⁻¹ cm⁻². On the other hand, H₂O₂ reduction at the modified electrodes (including GC/RGO, GC/AuNPs and GC/PB) displayed obvious improvements, indicating good catalytic activities.
toward H₂O₂ reduction at the applied potential. However, the GC/RGO-AuNPs showed a detection sensitivity of 5.5 mA M⁻¹ cm⁻², which is smaller than that of GC/RGO. It may be caused by the decrease in the number of activated sites of RGO (including –OH, –COOH) after hybridization to AuNPs, which reduced the catalytic ability toward H₂O₂ reduction. Electrodes modified with PB deposits (including GC/PB, GC/RGO-PB, GC/AuNPs-PB and GC/RGO-AuNPs-PB) usually showed better sensitivity for H₂O₂ detection. Among them, the electrodes modified with the RGO-PB and AuNPs-PB composites showed higher sensitivity due to the synergist effect of the composites. The sensitivity for H₂O₂ detection reached the highest value (456 mA M⁻¹ cm⁻²) at the RGO-AuNPs-PB modified electrode, suggesting the outstanding electrocatalytic effect toward H₂O₂ reduction.

Table 4.2 Comparison of different electrode systems employed for H₂O₂ detection

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Au coverage (nmole/cm²)</th>
<th>PB coverage (nmole/cm²)</th>
<th>Sensitivity (mA M⁻¹ cm⁻²)</th>
<th>Linear range (mM)</th>
<th>Limit of detection (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC</td>
<td>6.7</td>
<td>0.12</td>
<td>2.7</td>
<td>0.012 ~ 0.25</td>
<td>0.012</td>
</tr>
<tr>
<td>GC/RGO</td>
<td>769</td>
<td>1.4</td>
<td>312</td>
<td>0.010 ~ 1.9</td>
<td>0.010</td>
</tr>
<tr>
<td>GC/RGO-AuNPs-PB</td>
<td>769</td>
<td>3.6</td>
<td>456</td>
<td>0.002 ~ 2.65</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Amperometric measurements in 0.02 M phosphate buffer containing 0.1 M KCl at an applied potential of +0.1 V vs. Ag|AgCl.

The RGO-AuNPs-PB modified electrode offered a good linear relationship (from 0.002 to 2.65 mM), and a good reproducibility with a relative standard
deviation (RSD) of 6.7 % for the current response of 50 μM H₂O₂ on four different electrodes. Accordingly, the synergest effects of the composite might result from the effective affinity between AuNPs and the nitrile groups (–CN) of PB and the strong adsorption of PB on the RGO-AuNPs surface through the π-π interaction between RGO and –CN groups. These contributed to the high stability of PB in near-neutral solution and a long sensor lifetime which greatly enhanced the deposition amount of PB and provided a large catalytic surface area. Additionally, the special three-dimensional structure of the RGO-AuNPs-PB composite could exhibit efficient electron transfer and ion transport ability and offered an excellent electrocatalytic performance for the reduction of H₂O₂.

4.3.5 Direct electrochemistry of glucose oxidase and glucose detection

Due to the excellent performance for the reduction of H₂O₂, the GC/RGO-AuNPs-PB electrode was employed for the fabrication of glucose biosensor. Fig. 4.10 shows the cyclic voltammograms of the GC/RGO-AuNPs-PB/GOD-CHIT electrode in air-saturated phosphate buffer solution. As shown in Fig. 4.10(a), a pair of well-defined reversible redox peaks of PB between +0.2 and +0.4 V and a peak-to-peak separation of 70 mV were observed in the absence of glucose. On the other hand, weak reduction peaks were also observed at about –0.32 V, corresponding to the reversible electron transfer of redox active center of glucose oxidase (GOD). The results suggested the successful direct electron transfer of GOD at the RGO-AuNPs-PB modified electrode [32,33]. Enlarged views of the cyclic voltammetric response shows that the cathodic current of GOD at –0.36 V decreased with increasing glucose concentration (Fig. 4.10(b) versus 9(c)), indicating the oxygen consumption [33,34]. Simultaneously, the reduction peak current of PB at +0.25 V increased and the oxidation peak current of PB at
+0.32 V decreased with the higher glucose concentration, indicating good catalytic effects of PB. The increase in reduction current should ascribe to the catalytic reduction toward H$_2$O$_2$ generated from the oxidation of GOD in the presence of glucose [10].

Fig. 4.10 Cyclic voltammograms of GC/RGO-AuNPs-PB/GOD-CHIT electrode in the absence (a) and presence of 0.04 mM (b) and 0.08 mM (c) glucose in air-saturated 0.02 M phosphate buffer (pH 7.4) containing 0.1 M KCl solution at a scan rate of 50 mV/s.
Accordingly, the electrochemical reaction occurred at the GC/RGO-AuNPs-PB/GOD-CHIT electrode in the presence of glucose can be expressed as follows [30,35]:

\[
\text{glucose} + \text{O}_2 \rightarrow \text{gluconic acid} + \text{H}_2\text{O}_2
\]  

(4.3)

\[
2\text{K}_2\text{Fe}^{II}[\text{Fe}^{II}(\text{CN})_6] + \text{H}_2\text{O}_2 + 2\text{H}^+ \rightarrow 2\text{KFe}^{III}[\text{Fe}^{II}(\text{CN})_6] + 2\text{H}_2\text{O} + 2\text{K}^+
\]  

(4.4)

\[
\text{KFe}^{III}[\text{Fe}^{II}(\text{CN})_6] + \text{K}^+ + e^- \rightarrow \text{K}_2\text{Fe}^{II}[\text{Fe}^{II}(\text{CN})_6]
\]  

(4.5)

With the addition of glucose, oxygen was consumed when glucose was enzymatically oxidized to form gluconate and \( \text{H}_2\text{O}_2 \), leading to the decrease in \( \text{O}_2 \) reduction current (observed at around \(-0.36\) V). \( \text{H}_2\text{O}_2 \) generated from the enzymatic reaction was then catalytically reduced by Prussian White (PW, the reduction state of PB), and PW was then re-oxidized to PB [10]. The results suggested that the GC/RGO-AuNPs-PB/GOD-CHIT electrode could be used as an ideal glucose sensor through the detection of either the consumption of \( \text{O}_2 \) or the generation of \( \text{H}_2\text{O}_2 \).

The amperometric response of glucose at the GC/RGO-AuNPs-PB/GOD-CHIT electrode in stirred \( \text{O}_2 \)-saturated phosphate buffer (pH 7.4) is shown in Fig. 4.11. The response current increased with increasing glucose concentration. The calibration curve (inset of Fig. 4.11) for glucose detection at the GC/RGO-AuNPs-PB/GOD-CHIT electrode showed a linear range of 0.003 \(
\sim\n\) 2.0 mM (\( \text{R} = 0.998 \)) with a sensitivity of 84 mA M\(^{-1}\) cm\(^{-2}\). The reproducibility was evaluated using ten electrodes fabricated under identical conditions and the relative standard deviation (RSD) of the current response for 50 \( \mu\text{M} \) glucose was 5.5\%, demonstrating a good reproducibility. The current response maintained 96\% of the initial value for glucose detection after 4 weeks, indicating a good stability of the glucose sensor.
Fig. 4.11 Amperometric response of GC/RGO-AuNPs-PB/GOD-CHIT electrode for successive addition of glucose in O₂-saturated 0.02 M phosphate buffer (pH 7.4) containing 0.1 M KCl solution at an applied potential of +0.1 V. Inset: calibration curve for glucose detection.

Furthermore, the selectivity and anti-interference ability of the biosensor was also investigated for 0.1 mM glucose in the coexistence of 0.1 mM UA and 0.1 mM DA at an applied potential of +0.1 V, as shown in Fig. 4.12. The results demonstrated that the interference resulted from UA and DA was negligible for the detection of glucose, indicating a high selectivity and good anti-interference ability of the GC/RGO-AuNPs-PB/GOD-CHIT electrode.
Fig. 4.12 Anti-interference property of the GC/RGO-AuNPs-PB/GOD-CHIT electrode with addition of 100 μM UA, 100 μM DA and 100 μM glucose in air-saturated 0.02 M phosphate buffer (pH 7.4) containing 0.1 M KCl solution at an applied potential of +0.1 V.

Moreover, the detection of glucose in different oxygen concentration was also investigated and the experimental results are shown in Table 4.3. The GC/RGO-AuNPs -PB/GOD-CHIT electrode displayed a relatively wider linear range and higher sensitivity of 84 mA M$^{-1}$ cm$^{-2}$ for the detection of glucose in oxygen-saturated conditions. On the other hand, a narrower linear range and slightly lower sensitivity for the detection of glucose was observed under nitrogen-saturated conditions, owing to a smaller quantity of hydrogen peroxide produced from the enzymatic reaction. It indicated that the importance of oxygen
in the enzyme reaction. However, the sensitivity was very similar under either oxygen-saturated or nitrogen-saturated conditions, suggesting the good stability of the glucose biosensor.

Table 4.3 Amperometric detection of glucose at GC/RGO-AuNPs-PB/GOD -CHIT electrode under different conditions

<table>
<thead>
<tr>
<th>Electrolyte*</th>
<th>Sensitivity (mA M$^{-1}$ cm$^{-2}$)</th>
<th>Linear range (mM)</th>
<th>Limit of detection (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O$_2$-saturated</td>
<td>84</td>
<td>0.013 – 2.0</td>
<td>0.013</td>
</tr>
<tr>
<td>air-saturated</td>
<td>78</td>
<td>0.007 – 1.2</td>
<td>0.007</td>
</tr>
<tr>
<td>N$_2$-saturated</td>
<td>65</td>
<td>0.003 – 0.85</td>
<td>0.003</td>
</tr>
</tbody>
</table>

*0.02 M phosphate buffer (pH 7.4) + 0.1 M KCl KCl at an applied potential of +0.1 V

4.4 Conclusions

A novel electrochemical sensor based on graphene – gold nanoparticles – Prussian blue composite was constructed by a simple spontaneous deposition procedure. The loading of PB can be readily controlled through controlling the deposition time. The high affinity between AuNPs and PB through –CN and Au greatly improved the stability of PB in neutral condition. The RGO-AuNPs-PB composite was characterized by SEM, XPS and electrochemical methods. The RGO-AuNPs-PB composite displayed a good electrocatalytic effect toward the reduction of hydrogen peroxide at +0.1 V due to the synergist effects of RGO, AuNPs and PB. Furthermore, a glucose biosensor was further fabricated through immobilizing GOD-CHIT on the surface of the RGO-AuNPs-PB composite.
Direct electron transfer of GOD was observed at the composite modified electrode due to the excellent electronic conductivity and synergistic effect. The biosensor displayed a high sensitivity, high selectivity and good stability at an applied potential of +0.1 V for the detection of glucose.
References


Chapter 5 Summary and future work

5.1 Summary

This thesis work mainly focuses on the preparation of nanocomposites consisting of reduced graphene oxide (RGO) and the investigation of the morphological and electrochemical properties of the resulting composite materials. Nanocomposites consisting of reduced graphene oxide (RGO), Prussian blue (PB) and gold nanoparticles (AuNPs), including RGO/PB/PTBO, RGO-AuNPs and RGO-AuNPs-PB, have been prepared by electrochemical deposition, chemical reduction and spontaneous deposition methods. The composite materials have been utilized for the detection of hydrogen peroxide and the development of amperometric glucose biosensors [1,2]. Amperometric glucose biosensors have been employed as the model system to compare the performance of the biosensors utilizing different RGO composite materials. This study provides a better understanding on the effects of different RGO composite materials related to the electrode performance. This kind of information is valuable in the design and fabrication of biosensors.

For RGO/PB/PTBO, π-π stacking interaction coupled with ionic interactions between RGO, PB and PTBO not only increased the coverage of PB and PTBO, but also enhanced the stability of PB at high pH values, which greatly improved the electroanalytical properties of the RGO/PB/PTBO composite. RGO provided a chemical platform for the deposition of PB and PTBO as well as for the fabrication of a 3-dimensional (3-D) structure. The large surface area of RGO greatly increased the deposition amount of PB and PTBO, and provided a large
catalytic surface area. The resulting RGO/PB/PTBO composite exhibited an excellent electrocatalytic effect toward H$_2$O$_2$. The RGO/PB/PTBO composite was further applied to construct glucose biosensor that exhibited an excellent performance.

On the other hand, a series of RGO-AuNPs composites with different weight ratios of RGO and AuNPs have been synthesized. Oxygen-containing functional groups of RGO served as activated sites for the nucleation and growth of gold nanoparticles. Experimental results indicated that the structure, particles size, optical and electrochemical properties of RGO-AuNPs composites were closely related to the weight ratios of RGO and AuNPs employed for preparation. The RGO-AuNPs composites with different weight ratios of RGO and AuNPs displayed different electrocatalytic effects toward H$_2$O$_2$. The RGO-AuNPs composite with optimized weight ratio has been further used to construct glucose biosensor using chitosan to immobilize GOD.

Another piece of research work reports the spontaneous deposition of PB on the surface of the RGO-AuNPs composite and the utilization of the composite material for electroanalytical applications. The strong affinity between AuNPs and −CN group of PB not only enhanced the deposition speed and amount of PB, but also improved the stability of PB. The resulting RGO-AuNPs-PB composite showed greatly improved electrochemical properties and electroanalytical behaviors. In addition, the relationship between the deposition time of PB and electrochemical properties of the composite have also been investigated. The RGO-AuNPs-PB with optimized deposition time of PB was used for the detection of H$_2$O$_2$ and the construction of glucose biosensor with the immobilization of GOD using chitosan.
For a better comparison of the biosensors fabricated in this thesis work, the performances of different RGO-based composites utilized for hydrogen peroxide detection and glucose biosensing are summarized in Table 5.1 and 5.2, respectively.

Table 5.1 Summary of electrocatalytic effect of different RGO-based composites toward H₂O₂ reported in this thesis

<table>
<thead>
<tr>
<th>Biosensors</th>
<th>Detection Potential (V)</th>
<th>Sensitivity (mA M⁻¹ cm⁻²)</th>
<th>Linear range (mM)</th>
<th>Limit Detection (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC/RGO/PB/PTBO</td>
<td>0</td>
<td>420</td>
<td>0.005-0.6</td>
<td>1.5</td>
</tr>
<tr>
<td>GC/RGO-AuNPs(B)</td>
<td>0</td>
<td>9.5</td>
<td>0.025-41.50</td>
<td>5</td>
</tr>
<tr>
<td>GC/RGO-AuNPs-PB</td>
<td>+0.1</td>
<td>456</td>
<td>0.002-2.65</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 5.2 Summary of performance characteristics of different glucose biosensor reported in this thesis

<table>
<thead>
<tr>
<th>Biosensors</th>
<th>Detection potential (V)</th>
<th>Sensitivity (mAM⁻¹ cm⁻²)</th>
<th>Linear range (mM)</th>
<th>Limit detection (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC/RGO/PB/PTBO/GOD-CHIT</td>
<td>+0.2</td>
<td>59</td>
<td>0.02-1.09</td>
<td>8.4</td>
</tr>
<tr>
<td>GC/RGO-AuNPs/GOD-CHIT</td>
<td>−0.3</td>
<td>9.4</td>
<td>0.1-1.3</td>
<td>76</td>
</tr>
<tr>
<td>GC/RGO-AuNPs-PB/GOD-CHIT</td>
<td>+0.1</td>
<td>84</td>
<td>0.013-2.0</td>
<td>13</td>
</tr>
</tbody>
</table>
5.2 Future work

5.2.1 Self-assembled AuNPs monolayer on reduced graphene oxide

This thesis work describes the utilization of reduced graphene oxide in the preparation of useful composite materials for the electrochemical detection of hydrogen peroxide and the development of amperometric glucose biosensors. In particular, the RGO-AuNPs composites offered high sensitivity and excellent performance for the detection of hydrogen peroxide and glucose. Gold nanoparticles offer excellent conductivity and good biocompatibility to facilitate direct electron transfer of enzymes [3]. The properties of AuNPs including size, shape, charge and optical properties greatly rely on the preparation methods and the reducing agents employed [4], which lead to the wide applications of AuNPs in the fabrication of biosensors. For example, AuNPs was favorable for the immobilization of enzymes through the strong affinity of the AuNPs with cysteine residues and amino groups of the enzyme molecules [5]. In addition, AuNPs could maintain the enzymatic and electrochemical activity for an extended period of time, demonstrating the good compatibility of AuNPs for enzymes [6,7]. On the other hand, large surface area of AuNPs is more effective for the immobilization of enzymes.

Three approaches are usually employed in the deposition and immobilization of AuNPs on electrode surface. These include electrochemical deposition, directly coating of colloidal AuNPs, and binding AuNPs with surface functional groups to form self-assembled monolayers on electrode surface [8]. In this work, both electrochemical deposition and direct coating of AuNPs on electrode surface have been utilized. The development of electrode systems based on the self-assembly of AuNPs via interactions with different functional groups on electrode surface
will be explored in the future. Usually, different functional groups, such as –CN, –NH₂ and –SH groups, will be generated on graphene materials during the preparation of reduced graphene oxide. Thus, self-assembly monolayer of AuNPs can be formed on RGO surface through the interaction with the surface functional groups. In particular, alkanethiols can become immobilized on RGO surface through hydrophobic interaction between alkanethiols and RGO [9]. AuNPs monolayer can then be self-assembled on the electrode surface through the interaction between AuNPs and surface –SH groups of alkanethiols when the electrode immerses in colloidal AuNPs suspensions. The morphological, spectroscopic and electrochemical properties of the resulting RGO-AuNPs composites obtained through self-assembly will be examined and the electroanalytical behaviors of the resulting composites will be investigated.

5.2.2 Investigation of synergistic effects of graphene composite materials

The RGO composites (RGO/PB/PTBO, RGO-AuNPs and RGO-AuNPs-PB) described in this thesis work offered synergistic effects in the electrochemical responses. A significant enhancement in the voltammetric and electroanalytical responses has been observed for the composite material as compared to the corresponding response of individual component of the composite. Similar observations of synergistic effects have been described previously by Zhang and Gorshi [10,11]. These authors proposed that the combination of carbon nanotubes and redox mediator TBO offered improved electronic and ionic transport capacity and electron self-exchange in the polymer film. Synergistic effects observed for the PB-PTBO composite material were possibly resulted from π-π stacking interaction coupled with ionic interactions [12,13], and improved electronic and ionic transport capacity and electron self-exchange in the polymer film [10,11,13].
Better understanding of the synergistic effects should benefit the preparation of nanocomposites to obtain high performance nanomaterials for electroanalytical and biosensing applications [14].

Graphene offers an excellent performance to promote electron-transfer reaction of proteins and enhance the electrochemical reactivity of biomolecules, which makes it attractive in biosensor fabrication and electrochemical determination [15-17]. With a high hydrophobic surface and π-conjugative structure [18], graphene will interact with some materials (usually aromatic compounds, inorganic metal nanoparticles and conducting polymers) through π-π stacking, electrostatic interaction and hydrophobic interaction [19,20]. Many creative electrochemical systems that greatly promoted electron-transfer processes demonstrating the synergistic effects of graphene with different materials have been reported [14,21,22].

Various factors can introduce synergistic effects on electrochemical responses, including large surface area, specific electrode interface, excellent selectivity and stability of electrode [10,12]. The distance of electron transfer from bulk solution to the electrode will lead to different electron transfer rate and may contribute to the synergistic effect [23]. RGO-AuNPs composite materials with different alkanethiols can be prepared through self-assembly [9]. The distance of electron transfer pathway can be controlled by the length of the carbon chain of alkanethiols which may also influence the conductivity of the electrode material. The electrochemical properties (such as the peak-to-peak separation, electron transfer rate, and electrochemical surface area, etc.) of the resulting RGO-AuNPs composite materials will be examined by electrochemical techniques. Better understanding of the synergistic effects may be obtained.
References


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- Received the degree of Bachelor of Applied Chemistry from Luoyang Normal University, July 2009.
- Received the degree of Master of Analytical Chemistry from Wuhan University, July 2011.

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