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Enzyme-based Electrochemical Biosensors

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1. Introduction

A biosensor can be defined as a device incorporating a biological sensing element connected to a transducer to convert an observed response into a measurable signal, whose magnitude is proportional to the concentration of a specific chemical or set of chemicals (Eggins 1996). According to the receptor type, biosensors can be classified as enzymatic biosensors, genosensors, immunosensors, etc. Biosensors can be also divided into several categories based on the transduction process, such as electrochemical, optical, piezoelectric, and thermal/calorimetric biosensors. Among these various kinds of biosensors, electrochemical biosensors are a class of the most widespread, numerous and successfully commercialized devices of biomolecular electronics (Dzyadevych et al., 2008). In this chapter, we will focus on the enzyme-based electrochemical biosensors since enzyme electrodes have attracted ever-increasing attentions due to the potential applications in many areas.

Enzyme-based electrochemical biosensors have been used widely in our life, such as health care, food safety and environmental monitoring. Health care is the main area in the biosensor applications, such as monitoring blood glucose levels and diabetics by glucose biosensors. Besides, the reliable detection of urea has potential applications for patients with renal disease either at home or in the hospital. Industrial applications for biosensors include monitoring fermentation broths or food processing procedures through detecting concentrations of glucose and other fermentative end products. The sensitive detection of phenolic compound is an important topic for environmental research because phenolic compounds often exist in the wastewaters of many industries, giving rise to problems for our living environment as many of them are very toxic.

This chapter is on the enzyme-based electrochemical biosensors, which will begin with a section for enzyme immobilization methods due to their important roles in biosensors. The next section will focus on the recent advances in enzyme-based electrochemical biosensors. Nanomaterials play an important role in recent development of enzyme-based biosensors, thus some popular fabrication methods of nanomaterials will be briefly described towards their applications in nanomaterials synthesis. The emphasis of this chapter is on the recent advances particularly nanomaterials-based biosensors. Some important and intelligent nanomaterials including gold, ZnO, carbon nanotube and polypyrrole will be presented in a way to the current achievements in enzyme-based electrochemical biosensors. The last section of this chapter will discuss challenges currently faced to practical applications.

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2. Enzyme immobilization methods

In order to make a viable biosensor, the biological component has to be properly attached to the transducer with maintained enzyme activity. This process is known as enzyme immobilization. Biosensors are usually designed with high enzyme loading to insure sufficient biocatalyst activities, and the enzymes are provided with an appropriate environment to sustain their activities. The local chemical and thermal environment can have profound effects on the enzyme stability. The choice of immobilization method depends on many factors, such as the nature of the biological element, the type of transducer used, the physicochemical properties of the analyte and the operating conditions in which the biosensor is to function, and overriding all these considerations is necessary for the biological element to exhibit maximum activity in its immobilized microenvironment (Singh et al., 2008). A detailed information on advantages and drawbacks of different methods for enzyme immobilization could be found in the literature (Buerk 1993; Eggins 1996; Nunes & Marty, 2006). Generally, there are 4 regular methods for enzyme immobilization and they are briefly described as shown below:

1. **Adsorption:** It is the simplest and fastest way to prepare immobilized enzymes. Adsorption can roughly be divided into two classes: physical adsorption and chemical adsorption. Physical adsorption is weak and occurs mainly via Van der Waals. Chemical adsorption is stronger and involves the formation of covalent bonds. Many substances adsorb enzymes on their surfaces, eg. alumina, charcoal, clay, cellulose, kaolin, silica gel, glass and collagen. For this method, there are good examples in the section of 3.2.1 of this chapter, in which physical adsorption is mostly used for enzyme immobilization in ZnO-based glucose biosensors.
2. **Entrapment:** It refers to mixture of the biomaterial with monomer solution and then polymerised to a gel, trapping the biomaterial. However, this method can give rise to barriers to the diffusion of substrate, leading to the reaction delay. Besides, loss of bioactivity may occur through pores in the gel. The gels commonly used include polyacrylamide, starch gels, nylon, silastic gels, conducting polymers, etc.
3. **Covalent bonding:** In this method, the bond occurs between a functional group in the biomaterial to the support matrix. Some functional groups which are not essential for the catalytic activity of an enzyme can be covalently bonded to the support matrix. It requires mild conditions under which reactions are performed, such as low temperature, low ionic strength and pH in the physiological range.
4. **Cross-linking:** For this method, usually, biomaterial is chemically bonded to solid supports or to another supporting material such as cross-linking agent to significantly increase the attachment. It is a useful method to stabilize adsorbed biomaterials. Glutaraldehyde is the mostly used bifunctional agent. The agents can also interfere with the enzyme activity, especially at higher concentrations.

3. Enzyme-based electrochemical biosensors

3.1 Fabrication techniques for nanomaterials

Recent years witness the vigorous applications of various nanomaterials in the development of biosensors. Nanomaterials are generally referred to the materials with dimensions ranging from 1 to 100 nm, which have some special physicochemical characteristics resulting from their "small" size structures. Nanomaterials make contribution to the

improvement of the performance and stability of enzyme electrodes in the electrochemical biosensors, which can be fabricated by many various techniques. The generally used techniques for nanomaterials in biosensor applications are described briefly as follows.

Wet chemical route, also called chemical solution deposition, is one of the most widely used to fabricate nanomaterials, especially nanoparticles. For wet chemical route, solution of chemical species will be involved during the process, which thus differs from dry chemical route. Briefly, it uses a liquid precursor, usually a solution of organometallic powders, dissolved in an organic solvent. Chemical reactions then occur in order to get purposeful product(s). It is a quite common method to be used for nanomaterials fabrication, especially in the application of electrochemical biosensors.

The vapor-liquid-solid method is based on a mechanism for the growth of nanostructural materials with one-dimension from chemical vapor deposition, such as nanowires. It is generally very slow for a crystal to grow through direct adsorption of a gas phase onto a solid surface. During vapor-liquid-solid process, this problem is overcome by inducing catalytic liquid alloy phase to rapidly adsorb a vapor to supersaturation levels, and thus crystal growth can subsequently occur from nucleated seeds at the liquid-solid interface. The physical characteristics of nanowires grown in this manner is closely associated with the size and physical properties of the liquid alloy.

Hydrothermal synthesis is a method to synthesize crystalline materials from high-temperature aqueous solutions at high vapor pressures. The chemical reaction occurs in a vessel, which is separately from ambient environment. Hydrothermal synthesis will drive those hardly-dissolved compounds under normal conditions to dissolve in the solution under special conditions followed by recrystallization. The method can be used for the large crystal growth with high quality, where good control over composition is required. This method has been used for the fabrication of nanomaterials with low-dimensions.

The sol-gel process, strictly, belongs to a wet-chemical technique (chemical solution deposition) for material fabrication. This process uses a chemical solution as the precursor for an integrated network (or gel) of either discrete particles or network polymers. The sol evolves towards the formation of a gel-like system with two phases (a liquid phase and a solid phase), whose morphologies range from discrete particles to continuous polymer networks. A drying process is generally required to remove the remaining liquid phase, during which a significant amount of shrinkage and densification occur. The precursor sol can be either deposited on a substrate to form a film or used to synthesize powders. The sol-gel approach is a cheap and low-temperature technique that allows for the fine control of the product's chemical composition.

Thin films are thin material layers ranging from fractions of a nanometre to several micrometres in thickness. There are many popular deposition techniques for thin film deposition, such as evaporation, sputtering, chemical vapor depositions, etc. For example, evaporation in vacuum involves two basic processes: evaporation of a hot source material and then condensation of the material vapor on the cold substrate surface in the form of thin film. The average energy of vapor atoms reaching the substrate surface is generally low (i.e. tenths of eV) and thus normally results in a porous and little adherent material. Sputtering entails the bombardment of a target with energetic particles (usually positive gas ions), which causes some surface atoms to be ejected from the target. These ejected atoms deposit onto the substrates in the vicinity of the target. The target can be kept at a relatively low temperature, and sputtering is especially useful for compounds or mixtures. Chemical

vapor deposition is done through exposure of the substrate to one of several vaporized compounds or reactive gases. A chemical reaction occurs initially near the substrate surface, producing desired material as it condenses on the substrate forming a layer of thin film. Commercial techniques often use very low pressures of precursor gas.

3.2 Typical nanomaterials used in biosensors

3.2.1 ZnO

Among nanomaterials, ZnO has attracted much attention due to wide range of applications. ZnO as a wide band gap (3.37 eV) semiconductor plays an important role in optics, optoelectronics, sensors, and actuators due to its semiconducting, piezoelectric, and pyroelectric properties. Nanostructured ZnO not only possesses high surface area, nontoxicity, good biocompatibility and chemical stability, but also shows biomimetic and high electron communication features, making it great potential applications in biosensors. More importantly, as a biocompatible material, it has a high isoelectric point (IEP) of about 9.5. This makes it suitable for adsorption of proteins with low IEPs, as the protein immobilization is primarily driven by electrostatic interaction. ZnO with various nanostructures by same or different fabrication techniques has been widely used for enzyme immobilization in recent years. Fig. 1 gives some examples to show various ZnO nanostructures in different shapes by several various synthesis techniques.

Wet chemical route is quite a popular method to fabricate various ZnO nanostructures, such as nanoparticles, nanorods and nanosheets. It had been proposed to use these ZnO nanostructures as platform for cholesterol oxidase (ChOx) immobilization via physical adsorption. For example, using ZnO nanoparticles for enzyme immobilization, the prepared biosensor had a high and reproducible sensitivity of 23.7 $\mu\text{A}/\text{cm}^2\cdot\text{mM}$, detection limit of 0.37 nA and linear dynamic range from 1 to 500 nA (Umar et al., 2009). Recently, an ultra-sensitive cholesterol biosensor was developed using flowerlike ZnO nanostructure, in which ChOx was immobilized to the surface of modified electrode via physical adsorption followed by the covering of Nafion solution. Such biosensor exhibited a very high and reproducible sensitivity of 61.7 $\mu\text{A}/\text{cm}^2\cdot\text{mM}$ with a Michaelis-Menten constant (K_M) of 2.57 mM and fast response time of 5 s (Umar et al., 2009). A H_2O_2 biosensor was prepared using waxberry-like ZnO microstructures consisting of nanorods (8-10 nm) by wet chemical method (Cao et al., 2008). Such kind of ZnO microstructures with high surface area could provide the platform for the reduction of H_2O_2 by contributing excess electroactive sites and enhanced electrocatalytic activity. The transport characteristics of the electrode were controlled by diffusion process, and the prepared biosensor had a much wider linear range from 0.15 to 15 mM.

Glucose biosensors were also reported using ZnO nanocombs as platform by vapor-phase transport (Wang et al., 2006). For enzyme immobilization, glucose oxidase (GOD) were physically adsorbed to the nanocomb modified Au electrode, followed by Nafion solution covered on the surface of the modified electrode. The prepared biosensor had a diffusion-controlled electrochemical behavior. The covered linear range was from 0.02 to 4.5 mM and the reported sensitivity was 15.33 $\mu\text{A}/\text{cm}^2\cdot\text{mM}$. The value of K_M was as low as 2.19 mM. Using a similar technique, Weber et al. obtained ZnO nanowires with a typical length of 0.5-2 μm and a diameter of 40-120 nm, which were grown on the substrate with an array of ZnO nanowires (Weber et al., 2008). Physical adsorption was also adopted to immobilize GOD onto the electrode. This kind of biosensor had a linear trend (0.1-10 mM). A reagentless

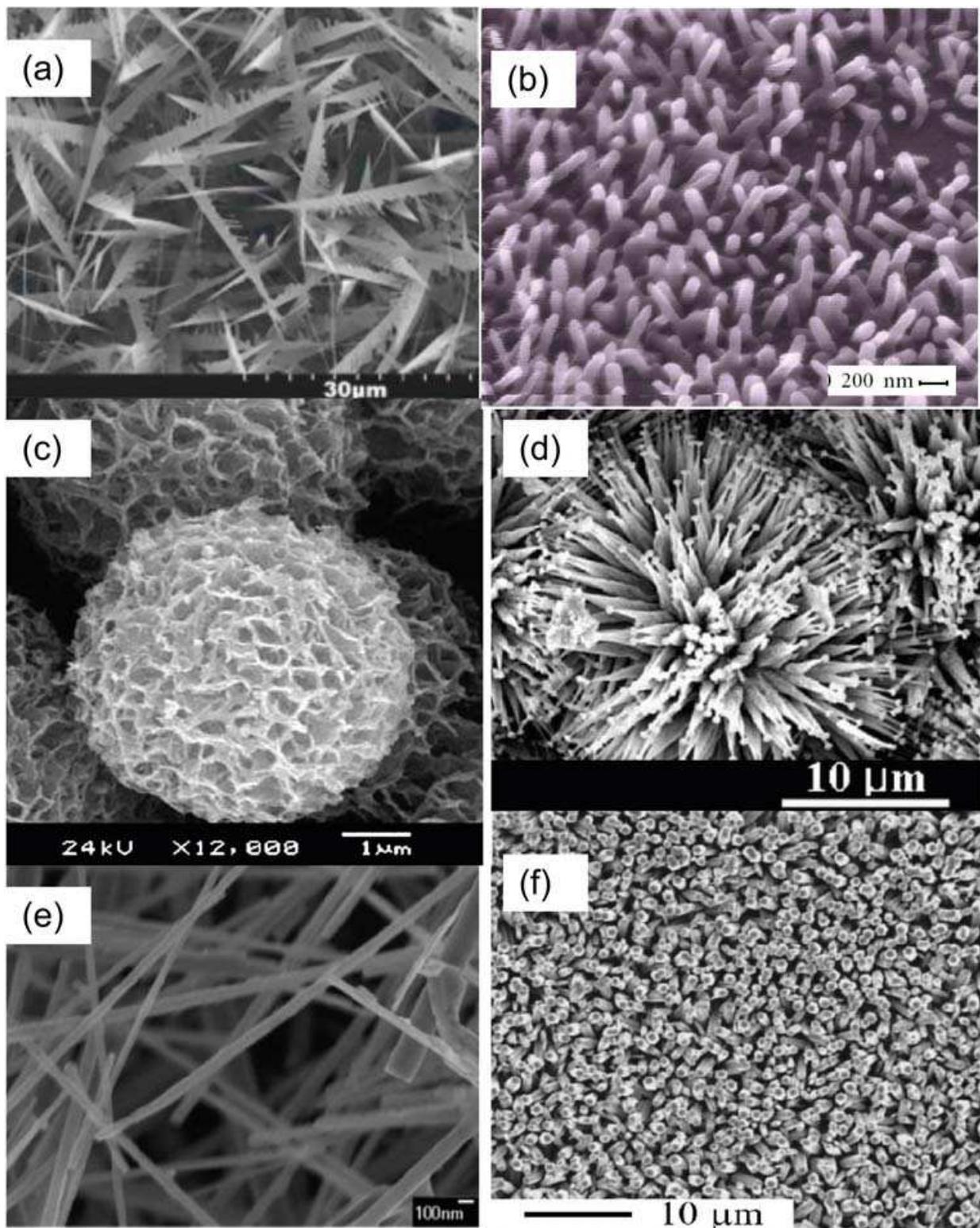


Fig. 1. ZnO nanostructure materials with various shapes. (a) nanocombs by vapor-phase-transport (Wang et al., 2006); (b) nanowires by vapor-liquid-solid (Weber et al., 2008); (c) microspheres consisting of nanosheets by wet chemical route (Lu et al., 2008); (d) nanonails by thermal evaporation (Umar et al., 2008); (e) nanowires by thermal evaporation (Zang et al., 2007); (f) nanorods by hydrothermal decomposition (Wei et al., 2006).

phenol biosensor was prepared by immobilizing tyrosinase on ZnO nanorods through electrostatic attraction and then covered by Nafion, in which ZnO nanorods were also fabricated by vapor-phase transport technique (Chen et al., 2008). Tyrosinase was adsorbed on the ZnO nanorods and its bioactivity can be well remained. Such prepared biosensor had a fast response within 5 s. The linear range of concentration spanned from 0.02 to 0.18 mM, and K_M was calculated to be as low as 0.24 mM, reflecting a high affinity of tyrosinase to phenol on ZnO nanorods and a good bioactivity (Chen et al., 2008).

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ZnO nanowires can also be obtained using thermal evaporation, in which ZnS powders were thermal evaporated under controlled conditions with Au thin film as a catalyst layer (Zang et al., 2007). GOD was immobilized onto ZnO nanowires by physical adsorption. K_M and sensitivity could be modulated in a wide range by the variation of the loading amount of ZnO/GOD onto the electrode. Umar et al. also using thermal evaporation to synthesize ZnO nanonails (Umar et al., 2008), where Zn powder was used as reaction source of Zn, and oxygen was introduced into the system. The constructed biosensor exhibited a diffusion-controlled electrochemical behavior with a linear calibration range from 0.1 to 7.1 mM. It showed a high sensitivity of 24.6 $\mu\text{A}/\text{cm}^2\cdot\text{mM}$, while K_M was relatively higher around 15 mM. Uric acid biosensor was prepared based on ZnO nanorods also by thermal evaporation (Zhang et al., 2004). Uricase with a low IEP of 4.3, was immobilized on ZnO nanorods by electrostatic attraction. The prepared biosensor had a linear range from 5 μM to 1 mM and detection limit of 2 μM . Besides, it had a lower K_M of 0.24 mM and a good thermal stability (10 - 85°C).

Among the various strategies followed, a useful and simple way for ZnO is to grown directly on electrode. This was realized in the work of Wei et al. (Wei et al., 2006), where ZnO nanorods with a hexagonal cross section were grown directly on the standard Au electrode by hydrothermal decomposition. Enzyme immobilization was done via the cover of GOD solution on the surface of the electrode. The prepared biosensor presented a quite fast response within 5 s and a high sensitivity of 23 $\mu\text{A}/\text{cm}^2\cdot\text{mM}$. It also had a low K_M value of 2.9 mM and a low detection limit of 10 μM .

ZnO matrix by sol-gel procedure was developed for tyrosinase immobilization (Liu et al., 2005). The porous and positively charged ZnO sol-gel matrix provided a moderate

microenvironment for the tyrosinase to remain its bioactivity. The so prepared biosensor had a sensitivity of 168 $\mu\text{A}/\text{mM}$, and the linear range covered from 0.15 to 40 μM (Liu et al., 2005). Another kind of matrix of ZnO/chitosan was developed for tyrosinase immobilization by dispersion of ZnO nanoparticles into the chitosan solution (Li et al., 2006). The matrix could provide a favorable microenvironment in terms of its isoelectric point for tyrosinase loading, and the immobilized tyrosinase could retain its bioactivity to a large extent. The biosensor using ZnO/chitosan matrix had a better performance than that using ZnO sol-gel matrix. K_M was calculated to be 23 μM and the detection limit was lower to be 0.05 μM (Li et al., 2006).

Different from above mentioned ZnO nanostructures, a new kind of nanostructure, nanoclusters, was proposed for a novel biosensor construction (Zhao et al., 2007). These ZnO nanoclusters doped by Co (2%) were obtained by nanocluster-beam deposition (Zhao et al., 2005; Zhao et al., 2007). Home-made electrode based on PET plate was used for enzyme immobilization instead of traditional standard electrode. Briefly, Ti ions from the plasma were implanted into PET plate, followed by a thin Au layer deposited on Ti-implanted PET substrate by magnetron sputtering. After that ZnO-based nanoclusters were directly grown on the modified PET plate. Cross-linking was used via glutaraldehyde for enzyme immobilization. The prepared biosensor had a response time within 10 s and the sensitivity was over 13 $\mu\text{A}/\text{cm}^2.\text{mM}$. ZnO porous thin films by RF magnetron sputtering was also proposed for ChOx immobilization by physical adsorption. The film was grown under high pressure (50 mTorr) so as to create native defects and therefore porous film formed. The prepared biosensor had a K_M of 2.1 mM. The wide linear range spanned from 0.65 to 10.34 mM.

In recent years, nanostructured inorganic-organic hybrid materials have emerged to fabricate biosensors by entrapping enzymes, which combine the physicochemical attributes of components to improve their features. Organic components (e.g. Nafion, chitosan) benefit the formation of defect-free inorganic membranes and make these membranes less brittle, and organic membranes can have their chemical and thermal stability improved by an inorganic phase. A H_2O_2 biosensor with good stability was developed with horseradish peroxidase (HRP) entrapped in the nanoporous ZnO/chitosan composite (Yang et al., 2005). The sensor exhibited a sensitivity of 43.8 $\mu\text{A}/\text{cm}^2.\text{mM}$, and it retained 80% of its initial current response after 40 days. It is expected that the numerous nanoscaled cavities on the surface of the microspheres are highly advantageous for the entrapment of enzymes by sequestering in the cavities or binding on the surface of the microspheres. Using this approach, Lu et al. synthesized the porous ZnO microspheres consisting of nanosheets using wet chemical route (Lu et al., 2008). Hemoglobin (Hb) was entrapped in the composite film of Hb, ZnO and Nafion. Besides the good reproducibility and long-term stability, the prepared biosensor had a sensitivity of 137 $\mu\text{A}/\text{cm}^2.\text{mM}$ and a low K_M of 0.143 mM. Other nanocomposite consisting of ZnO nanoparticles and chitosan was also reported to immobilize ChOx by physical adsorption (Khan et al., 2008).

More complex inorganic-organic composites are also commonly prepared in biosensor development by introducing other inorganic materials (e.g. gold and multi-walled carbon nanotubes (MWCNTs)). It's well known that gold and MWCNTs have been already used for enzyme immobilization to realize direct electron transfer between active sites and electrode. Besides, the presence of biocompatible Nafion in the biocomposite film not only makes the film uniform, but also could lead to the increased activity of enzyme. Recently, a biosensor

under these approaches was prepared using the platform consisting of ZnO, MWCNTs and Nafion, which showed a very high sensitivity of $1310 \mu\text{A}/\text{cm}^2\cdot\text{mM}$ and a very low of K_M of $82.8 \mu\text{M}$ (Ma et al., 2009). The composites consisting of ZnO, Nafion and gold nanoparticles were also developed to entrap HRP for H_2O_2 biosensors (Xiang et al., 2009). The biosensor had a K_M of 1.76 mM and a low detection limit of $9 \mu\text{M}$. It showed reproducibility and good stability after one month. Other composites are also proposed consisting of ZnO crystals, gold nanoparticles and chitosan (Zhang et al., 2009). The principle of enzyme immobilization differed from the methods mentioned above. It is known that ZnO crystals with high IEP are suitable for the electrostatic adsorption of proteins with lower IEP. The positively-charged ZnO crystals and amine-derivatized chitosan could facilitate higher capability of assembling negatively charged nanogold through strong electrostatic adsorption and the covalent bonds between amine groups and gold (Zhang et al., 2009). Biocompatible nanogold could further allow HRP to be immobilized with well-remained bioactivity in addition to increased loading amount. The prepared biosensor can achieve sensitive electrochemical response to H_2O_2 at a potential of -0.2 V . Similar composites for enzyme immobilization was reported by Duan et al. (Duan et al., 2008), but the composites were mixed by the solutions of ZnO/chitosan, Hb and gold. The as-prepared biosensor has a fast response to H_2O_2 within 4 s and a detection limit of $0.097 \mu\text{M}$.

Recent advances in phenol biosensors witness the use of modern process in semiconductor industry, such as photolithograph for designed patterns. A new tyrosinase biosensor was constructed based on the covalent immobilization of tyrosinase by glutaraldehyde on the biofunctional ZnO nanorod microarrays via photolithograph (Zhao et al., 2009). The as-prepared biosensor had a ultrahigh sensitivity of $287 \mu\text{A}/\text{cm}^2\cdot\text{mM}$ and a detection limit of $0.25 \mu\text{M}$. The linearity covered a wide range from $1\text{-}150 \mu\text{M}$.

In the development of uric acid biosensor, multilayer structure was introduced toward a highly sensitive and stable uric acid biosensor. Using ZnO nanoparticles and MWCNTs, multilayer structure was realized firstly by negatively charged MWNTs cast on pyrolytic wafers, followed by decoration of ZnO nanoparticles (Wang et al., 2009). Uricase was immobilized onto ZnO nanoparticles also by electrostatic attraction, and finally PDDA layer was coated on the surface of uricase. The as-prepared biosensor had a wide linear response range of 1mM to 5 M , a high sensitivity of $393 \mu\text{A}/\text{cm}^2\cdot\text{mM}$. It also exhibited a long-term stability after 160 days.

3.2.2 Gold

Gold nanoparticles could provide a stable immobilization for biomolecules retaining their bioactivity. Moreover, electron transfer between redox proteins and electron surfaces is facilitated, which is induced by many factors, such as the high surface-to-volume ratio, high surface energy, decreased proteins-metal particles distance and the functioning as electron-conducting pathways between prosthetic groups and the electrode surface from the gold nanoparticles. Pingarron et al. recently reported a review on gold nanoparticle-based electrochemical biosensors, in which gold-based enzyme biosensor are summarized (Pingarron et al., 2008). Gold nanoparticles are normally synthesized by chemical route and electrodeposition.

The electrodes are usually modified by gold in different ways to improve the performance of the biosensor. The electrode surface could be roughened by gold nanoparticles to enhance the interaction of enzyme with the electrode. An example is the construction of

acetylcholinesterase biosensor in which electrode was modified by electrodeposited gold nanoparticles at the electrode surface after hydrolysis of acetylthiocholine by the immobilization enzyme (Shulga & Kirchoff, 2007). This method is valuable for the development of new devices for the sensitive detection of potentially dangerous and deadly neurotoxins. Carbon paste electrode could be modified by the colloidal gold consisting of pretreated graphite powder with colloid gold solution and paraffin oil (Liu & Ju, 2003). GOD was immobilized onto the modified electrode via physical adsorption. Such kind of GOD biosensor can efficiently exclude the interference of commonly coexisted uric and ascorbic acid (Liu & Ju, 2003). The similar methodology is also favored for other substrate detection, such as phenol and hydrogen peroxide (Liu & Ju, 2002; Liu et al., 2003). Gold electrode can be modified by attachment of gold nanoparticles via covalent bond. These gold nanoparticles by chemical route were self-assembled on gold electrode by dithiol via Au-S bond, where dithiol was physically absorbed on the electrode surface by putting gold electrode immersed into a dithiol ethanol solution (Zhang et al., 2005). A cystamine monolayer was then chemisorbed onto those gold nanoparticles and exposed to an array of amino groups, after that GOD was immobilized by covalently attached to the cystamine modified electrode (Zhang et al., 2005). The scheme diagram in Fig. 2 shows the steps for above procedure. The so prepared biosensor provided a linear response to glucose from 20 μM - 5.7 mM with a sensitivity of 88 $\mu\text{A}/\text{cm}^2\cdot\text{mM}$. The sensor had a good reproducibility and remained stable over 30 days.

A wide variety of matrices, including inorganic materials, organic polymers, and other commercially available solid supports, have been used for enzyme immobilization. Chitosan, as mentioned in previous part, is one of the most promising immobilization matrices due to its excellent properties. Colloidal gold nanoparticles have been also used as the matrix for the enzyme immobilization to retain the macromolecules' bioactivity. The adsorption of colloidal gold nanoparticles on the chitosan membrane could provide an assembly of gold nanoparticle multilayers and a suitable microenvironment similar to the native environment of biomolecules. Based on this approach, a disposable biosensor was fabricated for the rapid detection of H_2O_2 by entrapping HRP in colloidal gold nanoparticle-modified chitosan membrane (Liu & Ju, 2003). The biosensor was characterized with good detection precision and storage stability. Based on a similar methodology, glucose (Luo et al., 2004) and HRP (Luo et al., 2005) biosensors were prepared by self-assembling gold nanoparticles on chitosan hydrogel modified Au electrodes.

Nanocomposites by combination of gold nanoparticles with inorganic or organic nanomaterials have shown to possess interesting properties, which can be profited for the development of electrochemical biosensors. An example of such nanocomposites is a colloidal gold-CNT composite electrode using Teflon as the non-conducting binding material (Manso et al., 2007). The constructed biosensor showed significantly improved responses to H_2O_2 , and the incorporation of GOD into the new composite matrix allowed the preparation of a mediatorless glucose biosensor with a remarkably higher sensitivity than that from other GOD-CNT bioelectrodes (Manso et al., 2007). Hybrid nanocomposites of gold nanoparticles and organic materials are proposed, in which gold and PPy are fabricated by wet chemical route using HAuCl_4 and pyrrole as the reaction reagents (Njagi & Andreescu, 2007). The reaction occurs in mild aqueous conditions and doesn't involve application of an electrical potential, surfactants or solvents that could affect the biological activity. A stable nanocomposite strongly adhered to the surface of GCE electrode and

enzyme was entrapped into the matrix. The fabricated biosensor showed high sensitivity for phenol detection, fast response time, good operational stability and reproducibility (Njagi & Andreescu, 2007).

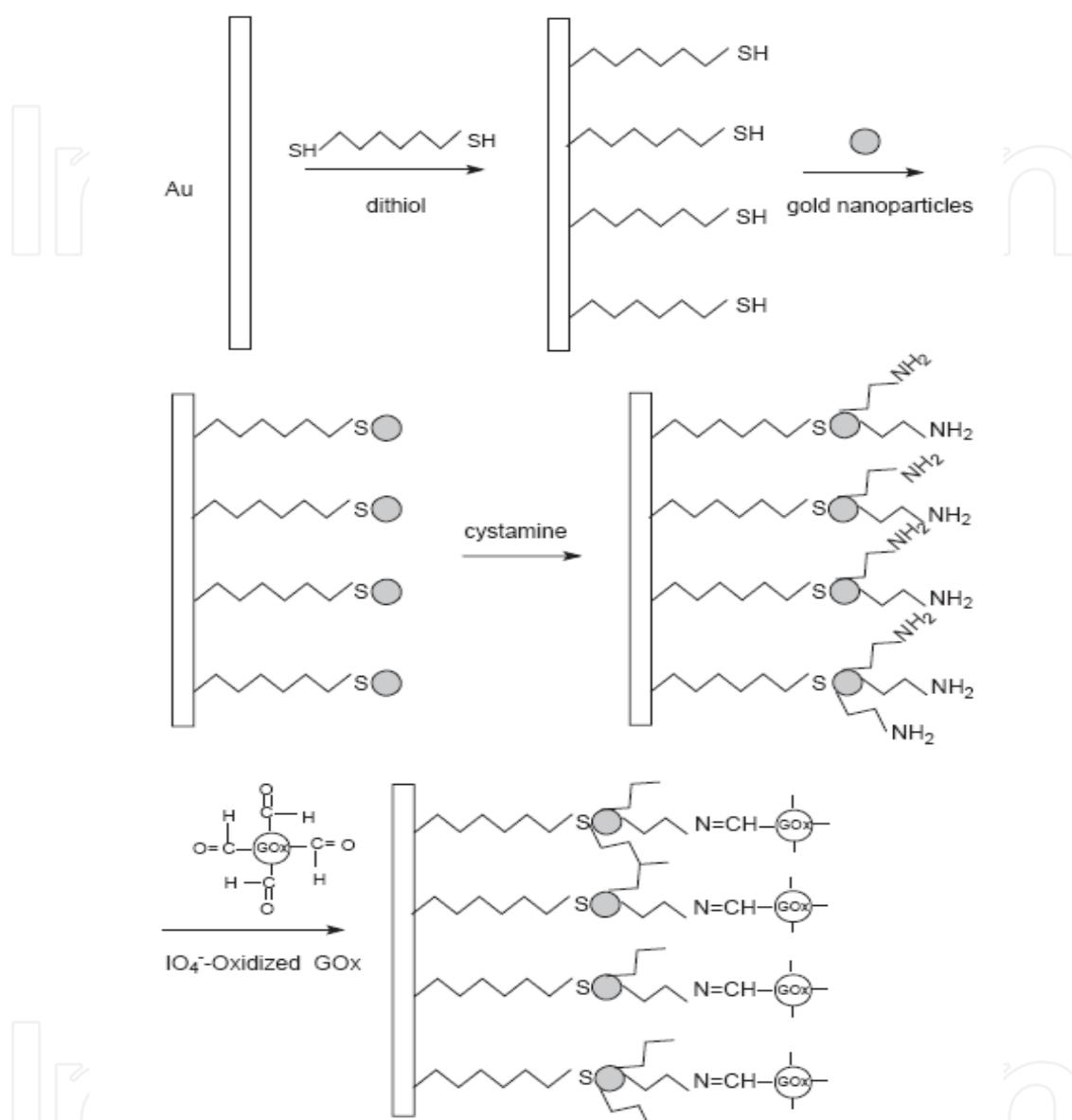


Fig. 2. Stepwise assembly of dithiol, gold, cystamine, IO₄⁻ oxidized GOD on a gold electrode, from paper (Zhang et al., 2005)

Enzymes deposited in ordered monolayer or multilayer systems have an important significance for fabrication of biosensors and bioelectronic devices. Layer-by-layer self-assembly technique based on electrostatic interaction attracts extensive interest due to its simplicity of the procedure, wide choice of the composition and thickness of the layer on the molecular level (Yang et al., 2006). This technique was originally developed by Decher and coworkers (Decher et al., 1992; Lvov et al., 1993) for linear polyelectrolytes and later extended to proteins, enzymes, nanoparticles, and so on (Feldheim et al., 1996; Caruso et al., 1997; He et al., 1998). Using this technique, a glucose biosensor was constructed, in which PMMA dendrimers with modified gold nanoparticles were alternated with

poly(vinylsulfonic acid) layers on ITO electrodes (Crespilho et al., 2006). The method of cross-linking was chosen for enzyme immobilization (Crespilho et al., 2006). Other glucose biosensor by layer-by-layer self-assembled technique could also be realized consisting of different multilayer films with chitosan, gold nanoparticles and GOD (Wu et al., 2007). A method of layer-by-layer covalent attachment of enzyme molecules was proposed to overcome the unstability occurring in the layer-by-layer self-assembly technique caused by the driving force of electrostatic interaction. Such kind of biosensor was prepared by construction of multilayer films consisting of glucose oxidase and gold nanoparticles using cysteamine as a cross-linker based on two covalent reactions: Schiff bases reaction between aldehyde-group of IO_4^- -oxidized GOD and amino-group of cysteamine, and covalent bond between gold nanoparticles (GNPs) and sulphhydryl of cysteamine (Yang et al., 2006). Layer-by-layer construction of GOD/GNPs multilayer film on an Au electrode were shown in Fig. 3. The constructed biosensor exhibited a good stability and long lifetime up to 4 weeks.

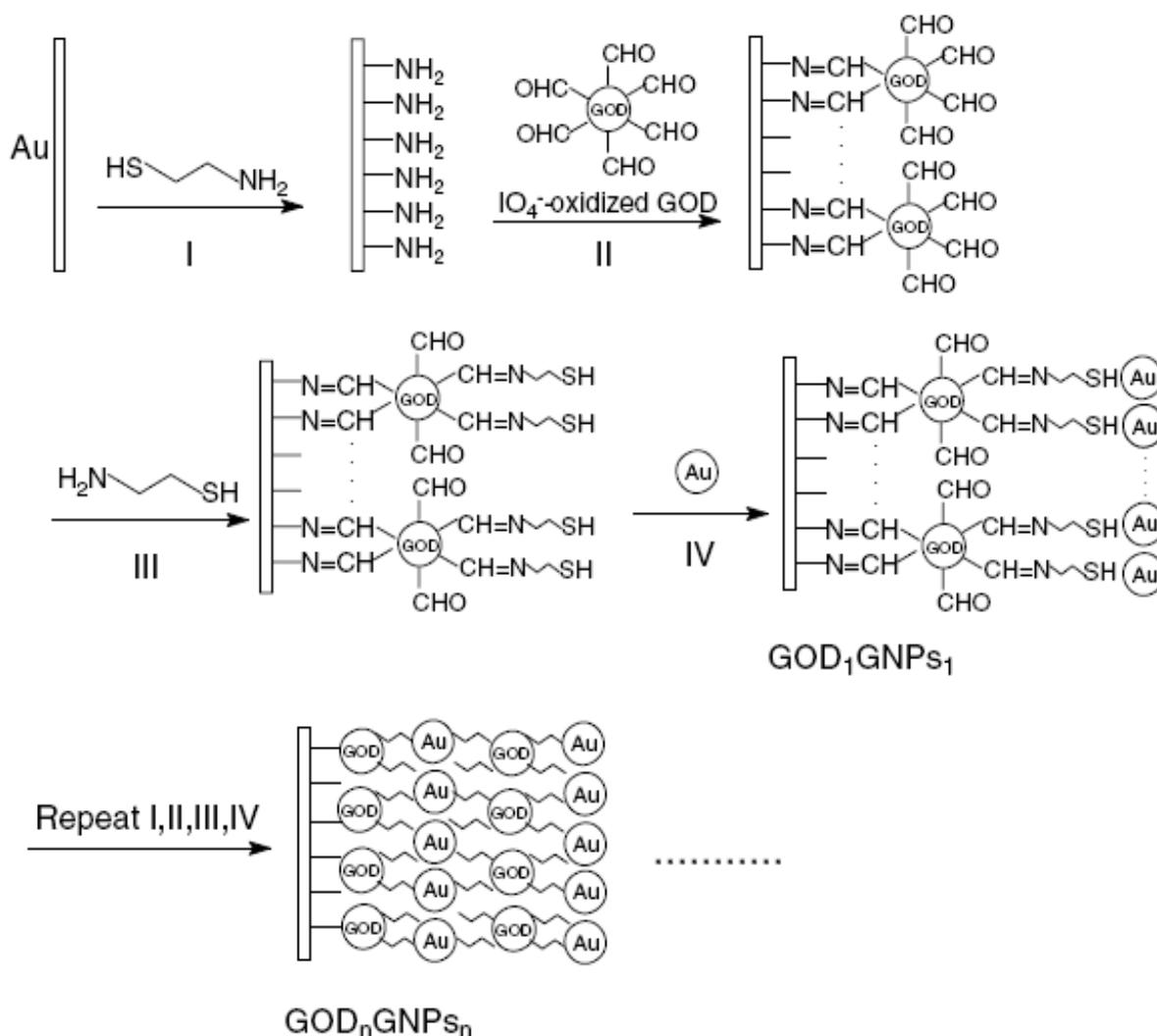


Fig. 3. Layer-by-layer construction of the GOD/gold nanoparticles multilayer films on an Au electrode (Yang et al., 2006).

Sol-gel technology provides unique means to prepare three-dimensional networks suited for the encapsulation of biomolecules. Sol-gel hybrid materials prepared by physically

encapsulating gold nanoparticles into porous sol-gel networks have been used for the fabrication of biosensors. For instance, an acetylcholinesterase biosensor was constructed, where the sol-gel derived silicate network assembling gold nanoparticles provided a biocompatible microenvironment around the enzyme molecule to stabilize its biological activity and prevent them from leaking out of the interface (Du et al., 2008).

3.2.3 CNT

CNTs are unique one-dimensional materials with unique properties such as good electrical conductivity, strong adsorptive ability and excellent bioconsistency. CNTs have led to development of many new techniques, and the applications in the biosensors have shown that CNTs have an electrocatalytic effect and fast electron-transfer rate between the electroactive species and the electrode.

A biosensor could be simply fabricated using multi-walled CNTs (MWCNTs) as immobilization platform with direct electron transfer and enhanced catalytic effect. For example, bilirubin oxidase could be immobilized directly onto MWCNTs modified glassy carbon electrodes (Weigel et al., 2007). Direct electron transfer reactions of bilirubin oxidase occur and the incorporation of MWCNTs enhances the catalytic bilirubin oxidase reaction up to a factor of 26 (Weigel et al., 2007).

An extremely robust, sensitive and selective galactose biosensor was proposed by the dispersion of single-walled CNTs (SWCNTs) into a chitosan matrix to form a stable dispersion, followed by the chemical cross-linking with glutaraldehyde and free aldehyde groups produced a substrate for covalent immobilization of galactose oxidase (Tkac et al., 2007). The detection of oxygen uptaken by galactose oxidase on chitosan/SWCNTs layer at -0.4 V was robust with a low detection limit of 25 μM .

Activating CNT surfaces is an essential prerequisite in order to effectively improve the performance of the prepared biosensors. In practical, CNT solubilization in aqueous media is essential for CNTs as supporting matrix for the immobilization of proteins. This can be achieved by the surface functionalization of CNTs with ionic or hydrophilic groups or the functionalization of CNTs with water-soluble polymers. Based on this approach, MWCNTs are modified by redox polymer, poly(vinylimidazole) complexed with $\text{Os}(4,4'\text{-dimethylbpy})_2\text{Cl}(\text{PVI-demeOs})$, resulting in the turning of MWCNT surface from hydrophobic to hydrophilic without changes of surface morphology (Cui et al., 2009). The prepared biosensor showed the enhanced sensing sensitivities induced by the redox polymer film, where the enzyme molecules was wired through the redox centers tethered on the mobile redox polymer backbones to the MWCNTs electrodes. MWCNTs could be modified by the coating of polyethylene imine (PEI) or poly(acrylic acid) (PAA) to obtain water-soluble MWCNTs (Yan et al., 2008). Recent development on the modified MWCNTs was to use O_2 plasma to treat MWCNTs, and thus oxygen contained functional groups were introduced onto their surface without influencing their bulk properties (Lee et al., 2009). Attaching metal nanoparticles to CNT and to CNT sidewalls is of interest to obtain nanotube/nanoparticle hybrid materials with useful properties. By electrostatic interaction, CNTs could be coated with gold nanoparticles and further filled with gold nanocluster after heat treatment in NH_3 (Jiang & Gao, 2003). Such heat treatment with NH_3 could make CNTs open-ended and generate functional basic groups on the inner wall of the nanotubes.

The composite of CNTs with other organic/inorganic materials has an important role in CNT-based enzyme biosensors. For instance, MWCNTs/PVP/Prussian blue (PB) composite

film were synthesized by casting films of MWCNTs wrapped with PB on Au electrodes followed by electrochemical deposition of PB on the matrix (Li et al., 2007). The modified electrode thus shows prominent electrocatalytic activity towards the reduction of hydrogen peroxidase, due to the remarkably synergistic effect of the MWCNTs and PB. Hydrogen peroxide biosensor could be also prepared by entrapping HRP in a new ormosil composite doped with ferrocene monocarboxylic acid-bovine serum albumin conjugate and MWCNTs (Tripathi et al., 2006), which exhibited a very low mass transport barrier to the substrate. Nafion and chitosan as organic materials are quite popular in the CNTs-based nanocomposites. In addition, sol-gel matrix, like titania and silica, were applied for effective enzyme immobilization (Lee et al., 2007; Tiwari & Gong, 2008). Meanwhile, metal nanoparticles of platinum were also incorporated into the composites of chitosan and MWCNTs to improve the performance of the prepared biosensor (Tsai et al., 2008).

Lactate detection is of great importance for the clinical analysis, fermentation as well as for food analysis. Enzyme-based electrochemical techniques for lactate detection is inexpensive, rapid and reliable compared to other methods, such as chromatographic and spectrometric analysis (Posner et al., 1996; Wulkan et al., 2001; Bariskaner et al., 2003; Fernandes et al., 2003). One kind of lactate biosensor was proposed by co-immobilization of lactate dehydrogenase (LDH) and Meldola's Blue on MWCNTs through cross-linking with glutaraldehyde and agglutination with mineral oil (Pereira et al., 2007). The biosensor shows a good stability after 300 times of determinations within a wide linear response range (0.1-10 mM). A MWCNT-CHIT-LDH nanobiocomposite film as a lactate biosensor was developed (Tsai et al., 2007), where MWCNT, chitosan, and LDH were mixed by a simple solvent-evaporation process. The enzyme in this kind of biosensor was entrapped in the biocomposite and the prepared biosensor showed a much fast response around 3s. In addition to MWCNT and chitosan as immobilization materials, polyvinylimidazole-Os (PVI-Os), can be also introduced into the biocomposite to form network structure (Cui et al., 2007). In the nanocomposite of chitosan/PVI-Os/MWCNT/LOD(lactate oxidase), negatively charged LOD was entrapped by a positively charged chitosan. PVI-Os was used as a leachables electron mediator due to its polymeric redox form and its positive charge could also enhance the entrapment for LOD. Negatively charged CNT was designed as a cross-linker to network chitosan and PVI-Os for the nanocomposite. The prepared biosensor showed significantly improved conductivity, stability and electroactivity for lactate detection. The sensitivity could reach $19.7 \mu\text{A}/\text{cm}^2.\text{mM}$, and the low limit of detection of $5 \mu\text{M}$. Recently, a new kind of hybrid composite for lactat biosensor was developed by introducing double-walled CNTs (DWCNTs) into alginate gel (Ma et al., 2008). DWCNTs with two concentric grapheme cylinders have attracted great interests in recent years because of their unique coaxial structure and promising mechanical, electrical, optical and thermal properties over SWCNTs and MWCNTs. LDH was prepared by pre-adsorbed on DWCNTs and then they were incorporated into alginate gel followed by Ca^+ cross-linking. The prepared lactate biosensor could greatly reduce the water loss and LDH leakage.

Recent advances in CNT-based enzyme biosensors have shown to design a biocomposite biosensor so as to detect more than one substrate. An good example was given by a bienzyme biosensor with a bienzyme-channelling configuration, where toluidine blue functionalized MWCNTs were used for enzyme immobilization (Jeykumari & Narayanan, 2009). The constructed biosensor shows a short response time ($< 2\text{s}$), good stability and anti-interferant ability. Many efforts have been made to detect the biomolecules at very low

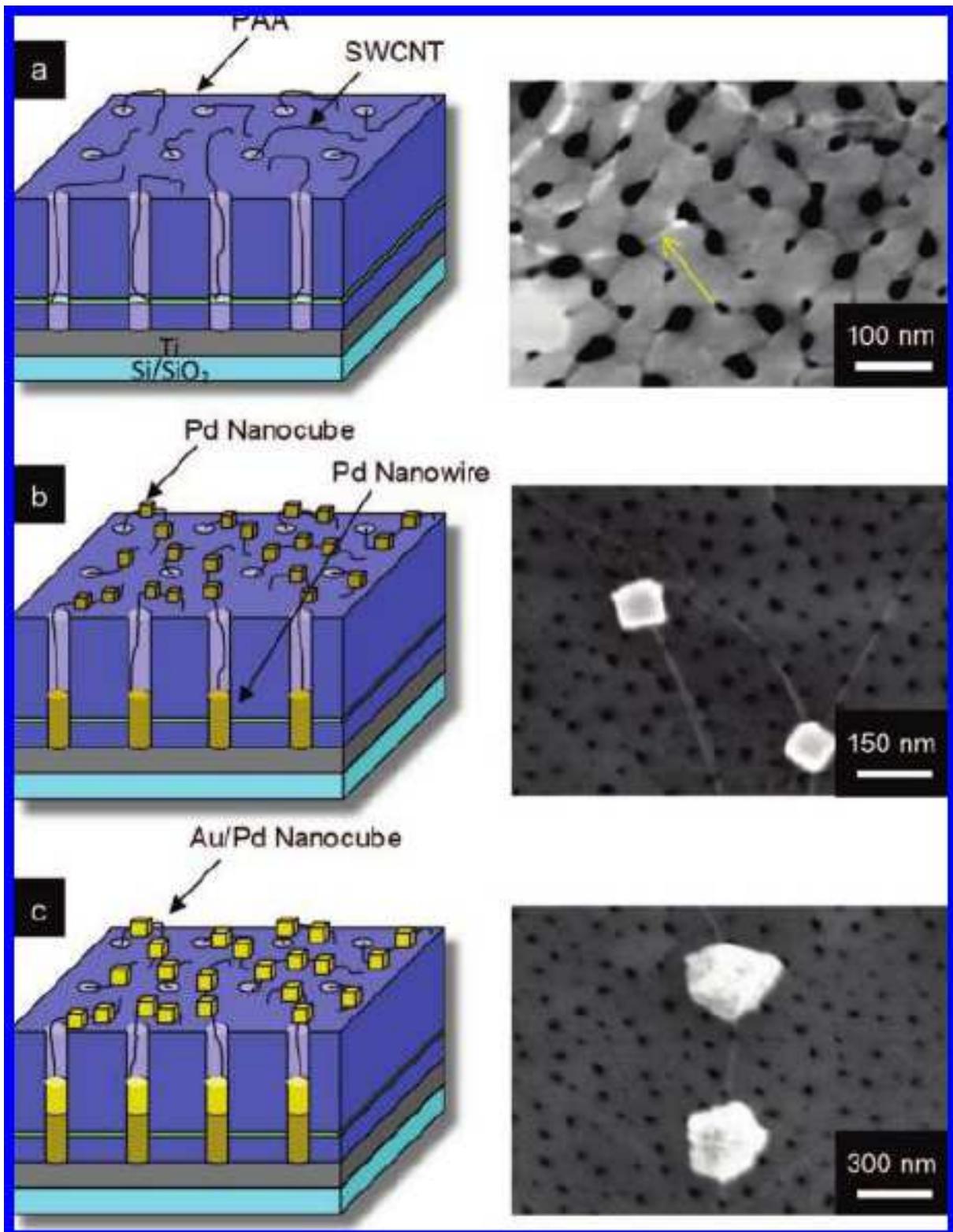


Fig. 4. Tilted cross-sectional schematics with corresponding SEM images portraying sequential fabrication process steps: (a) SWCNTs grown from the pores of the PAA via MPCVD, (b) electrodeposition of Pd to form Pd nanowires in pores and Pd nanocubes on SWCNTs and (c) electrodeposition to coat the existing Pd nanocubes with a thin layer of Au (Claussen et al., 2009).

concentrations. Networks of SWCNTs decorated with Au-coated Pd nanocubes are employed as electrochemical biosensors showing a limit of detection as low as 2.3 nM for H₂O₂, in which Au-coated Pd nanocubes were grown at the defect sites of template SWCNT networks through a simple electrodeposition process (Claussen et al., 2009). Fig. 4 shows the schematic fabrication process steps with corresponding SEM images.

3.2.4 Polypyrrole

Among various conducting polymers, polypyrrole (PPy) as an intelligent material plays an important role in the electrochemical biosensors for the purpose of increased electrochemical activity and sensitivity, owing to its good biocompatibility, conductivity, stability, and efficient polymerization at neutral pH as well as easy synthesis. PPy films can be easily formed from aqueous solutions by chemical or electrochemical routes, and have a high degree of selectivity due to the inherent size-exclusion property. A recently good review on the applications of polymers in electrochemical biosensors could be found in the literature (Teles & Fonseca, 2008), in which polypyrrole was highlighted.

In biosensor construction, PPy is often used as a conducting matrix and thus other organic/inorganic materials could be introduced into the matrix to further improve the performance of the biosensor. For example, stable and homogenous hybrid films consisting of PPy and copper hexacyanoferrate by electrochemical method were synthesized, aiming to obtain an electrocatalyst for H₂O₂ reduction in the presence of either Na⁺ or K⁺ ions (Fiorito et al., 2006). The constructed biosensor shows excellent catalytic properties towards H₂O₂ detection, with a performance higher than those observed for Prussian Blue and other analogues due to the electronic conductivity of the polymeric matrix (Fiorito et al., 2006).

In practical, it is important to find ways to obtain PPy polymers with desirable properties for biosensor applications by introducing various dopants. For instance, electrical conductivity can be achieved in polymer films by doping or inserting anionic or cationic species during the process of polymerization. Besides, the incorporation of a large size dopant anion, such as polyvinyl sulphonate (PVS), *p*-toluene sulphonate (*p*TS), and dodecylbenzene sulphonate (DBS) into PPy films during electropolymerization makes PPy film more porous, which is very important for the facile immobilization of enzyme (Tsai et al., 1988). According to this strategy, by using electrochemical method, PPy-PVS(polyvinyl sulphonate) nanocomposite film could be easily fabricated onto ITO electrode, and the enzyme is immobilized by cross-linking via glutaraldehyde on the hybrid film. A good performance of the biosensor was exhibited in terms of dynamic range of detection, short response time, long lifetime and stability. PPy can also be doped with alginate. Alginate hydrogel supports are usually made by cross-linking the carboxyl group of the guluronic acid residue with a solution of cationic crosslinkers such as calcium chloroxide, barium chloride, strontium, etc., and thus enzyme could retain their activity in alginate hydrogels (Martinsen et al., 1989). By taking advantages of both of alginate and PPy, a novel composite was synthesized through providing a gel by Ca⁺ cross-linking (Ionescu et al., 2005), which exhibits a greater enzyme retention as well as increased alginate stability towards the destructive effect of phosphate anions compared to the natural alginate gel. Recently, protonated sodium alginate (pSA) was also reported to be a dopant for electrogeneration of Ppy/pSA functionalized films for GOD immobilization. This was achieved via covalent bonding of carboxyl groups of the main chain of alginate with amino groups of the enzyme (Chen et al., 2008).

Layer-by-layer assembled technology has been also used in PPy-based biosensors. An example is that layer-by-layer assembled PPy and CNTs multilayer films were fabricated on Pt coated Polyvinylidene fluoride membrane, where PPy film was prepared by electrochemical polymerization and CNTs layers were coated by a vacuum filtration technique (Shirsat et al., 2008). Such multilayer structure provided an excellent matrix for the immobilization of enzyme, which possessed the favorable features of both PPy and CNTs. Cross-linking was chosen for GOD immobilization, and such prepared biosensor showed enhanced linear range, response time and sensitivity (Gade et al., 2006).

Interestingly, soluble PPy synthesized by the incorporation of sulfonate dopant anion could be well incorporated into microscopic polyacrylamide particles for glucose biosensing by concentrated emulsion polymerization method (Retama et al., 2005). The novelty of this method over conventional emulsion polymerization lies in the large volume of the aqueous dispersed phase used. The PPy/polyacrylamide microparticles showed the semi-conductivity, and GOD was immobilized in the microparticles by incorporating the enzyme into the aqueous phase of the concentrated emulsion before starting polymerization. To construct the biosensor, the obtained microparticles layer was covered and flattened around the platinum electrode surface using a dialysis membrane (Retama et al., 2005), and it showed the great interest for the application in glucose detection.

Other types of PPy nanostructures, like PPy nanotubes have been also proposed for enhanced adsorption of glucose oxidase in glucose biosensors (Ekanayake et al., 2007), where PPy nanotube array was synthesized using a solution of pyrrole and NaPF_6 at a fixed current density for 90 s. GOD was immobilized onto the electrode through physical adsorption. With this new approach, the constructed biosensor had exhibited remarkable improvement in the sensitivity, response time and linear range values.

4. Outlook

This chapter mainly presents intelligent nanomaterials (e.g ZnO, gold, CNT and polypyrrole) for construction of enzyme-based electrochemical biosensors to show the development in this area. To construct a biosensor with promising applications, it should be carefully considered to modify electrode in an effective way. The immobilization of enzyme onto the electrodes should be considered as another key step due to the important roles of the amount and bioactivity of immobilized enzyme on the performance of biosensors.

There are many challenges currently faced towards practical applications of biosensors. For example, the construction of a biosensor with a low cost is still essential when considering the commercial devices. The major application field of biosensors is medical diagnostics with commercial devices. The biosensors in other areas, such as food industry and ecology, needed to be explored deeply for more applications. Challenges also exist to find ways to improve the performance criteria including high sensitivity, wider linear range, low limit of detection, fast response and repetitive ability. Research work now still keeps continuing to investigate more effective ways to construct enzyme-based electrochemical biosensors with more perfect performance.

In the future development of electrochemical biosensors, the demands for portable and cheap biosensors with multifunctions (e.g. to detect several target analytes) will keep increasing for practical applications. Many thanks to the emergence of nanotechnology, many researchers could incorporate this technology into the biosensor construction to obtain novel structures. Miniaturization will play an important role in the trend of biosensor

development in the future. However, it may result in low current because of the decreased amount of immobilized enzyme onto the available active area. This can be overcome by the nanostructures, which enhance the sensitivity of a biosensor by one to two orders of magnitude, due to the large surface area per unit volume ratio, which allows the immobilization of a larger amount of the enzyme. Overall, electrochemical biosensors with perfect performance towards commercial systems keep a main thrust in future research.

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A biosensor is defined as a detecting device that combines a transducer with a biologically sensitive and selective component. When a specific target molecule interacts with the biological component, a signal is produced, at transducer level, proportional to the concentration of the substance. Therefore biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. Bringing together researchers from 11 different countries, this book covers a wide range of aspects and issues related to biosensor technology, such as biosensor applications in the fields of drug discovery, diagnostics and bacteria detection, optical biosensors, biotelemetry and algorithms applied to biosensing.

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