國　立　交　通　大　學

生 物 科 技 學 院

生 化 工 程 研 究 所

博士論文

以矯正模組與奈米金於電流式生物感測器之研究

Researches of Amperometric Biosensors Using Correction Module and Gold Nanoparticles

研 究 生： 林 岳 暉

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中 華 民 國 九 十 八 年 七 月

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國立交通大學 生化工程研究所

五鼎生物技術股份有限公司 研發部

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以矯正模組與奈米金於電流式生物感測器之研究

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摘要

以網印碳電極(screen-printed carbon electrode；SPCE)為架構的電流式生物感測器，是相當適合於以化學、生物或物理的工程技術，將酵素、氧化還原媒介物、抗體和奈米材料等，予以修飾其上，以強化其在作為醫療診斷、食品安全確保與環境監控時所需要的靈敏性。在本研究中，分別研究開發可運用在 SPCE 的一矯正模組與一奈米金(Au nanoparticles；AuNPs)修飾技術。

分別以有無葡萄糖氧化酶(glucose oxidase；GOD)之網印碳電極(GOD(+)-SPCE 與 GOD(-)-SPCE)作為複合電極，再以外加尿酸與調整血球容積比(hematocrit ratio；Hct%)方式進行葡萄糖測試溶液的電流法測定，進行矯正模組研究。在以 YSI 2300 STAT Plus 作為參考法比較之下，全血中葡萄糖濃度受到尿酸干擾的關係性，以及 Hct%，可藉本矯正模組使其更為準確。利用 GOD(+)-SPCE 與 GOD(-)-SPCE 之間的電流差值，ΔCurrent 的計量，可降低大部分尿酸/生化物質的干擾。而所建立內插法方式可用於矯正 Hct%在血糖檢測時的干擾；這內插法之 Hct%矯正，在葡萄糖濃度高於 110mg dL^{-1} 且同時 Hct%低於 35%時更顯重要。這樣的實用性可用於增進為糖尿病患族群服務時，全血量測的準確性。這方法也可提供給其他全血樣品，於進行電流式量測時降低干擾之用。

另一方面，該 AuNPs 修飾之 SPCE，係以 13-nm AuNPs 搭配 1,1-二茂鐵甲酸(ferrocenedicarboxylic acid；FeDC)以增強電流訊號；其可用於大腸桿菌 O157：H7 (*Escherichia coli* O157:H7)電流式免疫電極，與偵測加保扶(carbofuran)/穀胱甘肽(glutathione)之酪胺酸酶(tyrosinase；Tyr)電極。在 SPEC 的工作極上，AuNPs、FeDC 與抗體(antibody；Ab)的共修飾(SPCE/AuNPs/FeDC/Ab)，確實提昇了電流訊號；如此，可以偵測到在 10^2~10^7 菌落形成單位(colony-forming units；CFU) mL^{-1} 範圍的 *E. coli* O157:H7 濃度。在磷酸緩衝液與牛奶樣品測試中的，可推算出每片 AuNPs/FeDC-SPCE 免疫電極的偵測極限，分別為 6 CFU 與 50

CFU。此電流式免疫感測器的重點優勢為可於 1 小時完成牛奶樣品的檢測。相似地，AuNPs 與 FeDC 在 SPCE 與 Tyr 的共修飾 (SPCE/AuNPs/FeDC/Tyr)，可以分別偵測到 4.52-45.2 nM 的加保扶與 12.5~100 μM 的穀胱甘肽。該 SPCE/AuNPs/FeDC/Tyr 試片的應用性與光譜法相較，毫不遜色。本研究所使用的酪胺酸酶為每試片 2 活性單位(unit；U)，相較於先前的電流式酪氨酸酶感測器研究，用量僅約 1/30。矯正模組的基本架構與金奈米粒搭配二茂鐵的修飾，在網印碳電極之應用相當具潛力，將可整合成為一系統以應臨床檢測與環安監測的需求。

關鍵字：電流式生物感測器、網印碳電極、血糖、奈米金、大腸桿菌 O157：:H7、酪胺酸酶

Researches of Amperometric Biosensors Using Correction Module and Gold Nanoparticles

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Abstract

Amperometric biosensors constructed from screen-printed carbon electrode (SPCE) are amenable to chemical, biological or physiological engineering with modifying agents such as enzyme, redox mediators, antibodies and nano-material to impart the requisite sensitivity for medical diagnostics, food quality assurance and environmental monitoring in complex matrices of the biosensors. In this study, we developed a correction module and a modification technique with gold nanoparticles (AuNPs) for SPCE.

The SPCE test strips with and without glucose oxidase (i.e., GOD(+)-SPCEs and GOD(-)-SPCEs) were used for multiple test strips and the amperometric currents of glucose-testing solutions with various spiked uric acid concentrations and hematocrit ratios were measured for the study of correction module. By establishing the interference relationships between glucose concentrations and uric acid concentrations as well as Hct% values and with appropriate correction module, the whole blood glucose determinations could be made to be more accurate and comparable to those determined by the reference YSI method. Specifically, the use of ΔCurrent value, the current difference between GOD(+)-SPCE and GOD(-)-SPCE measurements, would reduce most of the uric acid/biochemical interferences. An interpolation method was also established to correct for the blood glucose determinations with Hct% interferences. The Hct% corrections using the interpolation method are especially important and necessary for those blood samples with glucose concentrations higher than 110 mg dL^{-1} and Hct% values lower than 35%. A practical solution can be developed to improve the accuracy of determinations for whole blood glucose and to serve the diabetic community better. This simplified approach also will be applied to reduce interferences for amperometric determinations of other analytes in the whole blood sample.

On the other hand, 13-nm diameter AuNPs coupled with ferrocenedicarboxylic acid (FeDC) are modified onto the SPCE for the enhancements of amperometric current for the *Escherichia coli* O157:H7 (*E. coli* O157:H7) immunosensors and the tyrosinase (Tyr)-based carbofuran/glutathione (GSH) biosensor. The modification of AuNPs, FeDC and antibodies (Ab) on the working electrode of SPCE (SPCE/AuNPs/FeDC/Ab) certainly enhances the mediated-amperometric signals. The concentrations of *E. coli* O157:H7 from 10^2 to 10^7 CFU mL^{-1} can be detected. The detection limit is 6 CFU per strip in phosphate buffer and 50 CFU per strip in milk, respectively. One of important advantages of our SPCE/AuNPs/FeDC/Abs immunosensing strips can be utilized for the determination of *E. coli* O157:H7 during 1 h. Similarly, the modification of AuNPs, FeDC and Tyr on the working electrode of SPCE (SPCE/AuNPs/FeDC/Tyr), can be enhance the efficiency for the determination of carbofuran and GSH; the linear concentration range of carbofuran and GSH is 4.52 to 45.2 nM and 12.5 to 100 μM, respectively. The applications of SPCE/AuNPs/FeDC/Tyr strips were quite as good as the spectrophotometric methods of carbofuran and GSH. The using tyrosinase was only 2 Unit per strip that was 1/30 compared with the past studies. The basis of correction module and the modifications of AuNPs/FeDC provides a potential for further applications of amperometric SPCE, which will be incorporated into an integrated system for the requirements of diagnosis testing and environmental/safety monitoring.

Keyword: amperometric biosensors, screen-printed carbon electrode, blood glucose, gold nanoparticles, *Escherichia coli* O157:H7, tyrosinase

Content

Overview

Biosensors are well suited to delivering on-site detection, identification and monitoring in a variety of security and defense situations. These are applied mainly in medical diagnostics, environmental diagnostics and the food industry (Kauffmann, 2003; Song et al., 2006). Biosensors comprise a biological or biologically derived sensing element intimately associated with or integrated within a physicochemical transducer (Ziegler and Göpel, 1998; O'Connell and Guilbault, 2001; Malandain et al., 2005). Sensing elements are analytical devices that determine biochemical and physiological change based on the biologically active compounds (e.g., enzyme) modified on the surface of sensing elements. Transducers, required pieces of analytical devices that convert the biological and chemical changes into the practical electronic data, make use of these sensing elements to provide different types of transducers.

The reaction between the bioactive substance and the species produce a product in the form of a biological or chemical substance, heat, light or sound; then a transducer such as a thermistor, photocounter, semiconductor, sound detector or electrode changes the products of the reaction into usable data (Muhammad-Tahir and Alocilja, 2003; Mehrvar and Abdi, 2004). Alternatively, based upon the type of transducers, there are electronic biosensors (electrical or electrochemical), optical biosensors (fluorescent, surface plasmon resonance, or Raman), and piezoelectric biosensors (quartz crystal microbalance). Electrochemical biosensors are more amenable to miniaturization, have compatible instrumental sensitivity and can even operate in turbid solution and emerge as the most commonly used biosensor in diagnosis test and environmental/safety monitoring(Lei et al., 2006; Song et al., 2006).

Depending on the electrochemical property measured by a detector system, the electrochemical biosensors can be divided into conductometric, potentiometric and amperometric biosensor (O'Connell and Guilbault, 2001). Early techniques of electrochemical biosensors in the analysis of species involved reactions that took place in a bulk solution with catalysis and samples. In recent years, however, the biosensor techniques have provided alternative systems that allowed the reactions without adding reagents to take place at a surface of an electrode. Because the reagents have been already coated, immobilized or packaged in the sensing systems, the biological and chemical sensor with minimum human intervention. The recent improvement of electrochemical biosensor techniques has continued to advance the performance of the conventional analytical system, eliminate slow preparation and the use of expensive reagents. The modern concept of amperometric biosensors represents a rapidly expanding field of instruments to determine the concentration of substrates and other parameters of biological interest since the

invention of a precise, convenient and low-cost biosensor for blood glucose (Clark and Lyons, 1962). In fact, amperometric systems possess linear concentration dependence and measure changes in the current on the working electrode due to the direct oxidation or reduction of the products of a biochemical reaction in direct or indirect system (Mehrvar and Abdi, 2004). Therefore, amperometric biosensors also have the advantages of being more speedy response, greater simplicity, low-cost and disposable in comparison to optical, calorimetric and piezoelectric biosensor, even the conductometric and potentiometric biosensors (Prodromidis and Karayannis, 2002; Lei et al., 2005; Belluzo et al., 2008).

Situations of amperometric biosensors

The development of amperometric biosensors was based on the change of oxygen or hydrogen peroxide. The direct electron transfer between enzyme and a common electrode was observed to be too slow science the redox center of enzyme was usual buried in a thick protein shell that slowed the electron transfer (Renedo et al., 2007). To establish an electron transfer, researchers used the electro-active compound, mediators, to access the redox center of enzyme, to act as the charge carriers, and then to reduce or oxide at inert material (e.g., aurum, platinum, palladium, carbon electrodes) working electrodes of amperometric biosensors. Finally, the amperometric meter are used to detection them. For ensuring high electron transfer efficiency between the mediators and the electrode, a redox mediator is advantageously comprised in the paste. These mediators were chosen as mediators to improve the selectivity, linear range and response time of biosensors (Mehrvar and Abdi, 2004).

In the early eighties, considerable attention has been focused on the production of carbon (graphite)-based amperometric biosensors, as carbon is a versatile and inexpensive electrode material (Gilmartin and Hart, 1995). Such devices were amenable to chemical and biological engineering with modifying agents such as polymeric membranes, enzymes and redox mediators, to impart the requisite selectivity for analyses in complex matrices (Wedge et al., 1999; Yang et al., 2004; Hsu et al., 2006). Screen-printing is a thick-film process usually referred to as an ink, which has been used for the production of miniature, robust and cheap electronic circuits. It is easy to produce relatively inexpensive devices using polymeric materials are cheaper and easier to process. The process was adapted for the production of amperometric biosensors and has had a huge impact on their commercialization. Multiple repeat patterns are usually designed onto a single screen, further enhancing production speed. Today, inks based on carbon and many metals are widely available for low temperature applications. The majority of the most successful electrochemically-

based devices to date have used the technique (Renedo et al., 2007).

The attractive concept is that the enzyme can be readily immobilized in the electrode matrix by simple mixing with the electrode components: graphite and binding agent (e.g., paraffin, Teflon, epoxy etc.). The resulting biosensor may be shaped as a cartridge for multiple-use or as a single use strip by screen printing the "bio-paste" (Renedo et al., 2007). Most of amperometric biosensors employed though are the screen printed carbon electrodes (SPCE), based also on carbon/graphite electrodes dispersed in a suitable polymer matrix and the enzyme/additional reagent/membrane micro-dispensed onto the electrodes, where the miniaturized sensing part allows micro-volumes of samples to be analyzed and the sensing element is disposable (Wedge et al., 1999; Renedo et al., 2007).

Limitations of amperometric biosensors

A well-known example is the glucose meters used by diabetics to monitor their blood glucose levels (Turner et al., 1999). Nowadays, a vast majority of the whole glucose meters are based on amperometric SPCE biosensor technology (Newman and Turner, 2005; Heller and Feldman, 2008). The inherent small size and simple construction of the electrochemical transducer and instrument are ideally suited for point-of-care biosensing (Lee, 2008). An international standard ISO 15197 have been suggested by national standards organizations for the company that manufacturers self-testing glucose meters. But most of developed cost-effective glucose monitoring system is still not to meet target accuracy of ±5%, the goals of American Diabetes Association science 1994 (American Diabetes Association, 1994.).

Besides glucose, a wide variety of amperometric biosensors have been developed for the measurements of some other key metabolites, proteins, nucleic acids and microbe. They have been used in a wide range of applications from medical diagnostics, food quality assurance, environmental monitoring and industrial process control to biological warfare agent detection (Dennison and Turner, 1995; Prodromidis and Karayannis, 2002; Kauffmann, 2003; Malandain, et al., 2005). Not surprisingly, great efforts have been devoted to their commercialization. Nevertheless, unlike the glucose meters, limited success has been achieved for the requirements of the molecular diagnostics and pathogen detection. This might a major barricade in the application of biosensors. Most biosensors reported in the literature work very well in laboratories, however may meet series problems in test real samples. As a result, it is essential to develop novel surface modification approaches in order to avoid non-specific adsorption at surfaces (Lin et al., 2004; Belluzo et al., 2008).

The usefulness of amperometric biosensors should be popularly. But the amperometric techniques still have had some limitations: affect selectivity, sensitivity, limited dynamic range, electrochemically active interferences in the sample and weak long-term stability when the techniques face the diversity of determination (Mehrvar and Abdi, 2004; Renedo et al., 2007; Belluzo et al., 2008). Therefore, the researches and applications of novel materials or methods for amperometric biosensor become to the important strategies of researchers in order to break through these limitations.

Researches for the improvements of SPCE biosensors

Currently, considerable attention has been focused on the production of SPCE-biosensors, as carbon is a versatile and inexpensive electrode material. Such SPCE-biosensors are amenable to chemical, biological or physiological 'engineering' with modifying agents such as redox mediators, antibodies and nano-material to impart the requisite sensitivity for medical diagnostics, food quality assurance, environmental monitoring in complex matrices of the biosensors (Mehrvar and Abdi, 2004; Renedo et al., 2007; Belluzo et al., 2008). In view of the manifolds of amperometric SPCE-biosensors, the following reviews of improvement would focus on the glucose biosensors, immunobiosensors and pollutant biosensors. There will be more opportunities that the results can apply to mass production and combine the advantages of SPCE for the requirements of diagnosis testing and environmental/safety monitoring.

●Glucose biosensor

Glucose is oxidized by the enzyme and the electrons involved in the redox reaction relayed to the electrode through a mediator, resulting in electric currents that are proportional to the level of glucose in sample solutions. A great number of the disposable biosensors used in the analysis of glucose are based on electrochemical determination of enzymatic-generated hydrogen peroxide (H_2O_2). Oxidation or reduction of H_2O_2 generally requires high potentials at bare electrodes, which implies a very poor sensitivity (Mehrvar and Abdi, 2004; Newman and Turner, 2005; Heller and Feldman, 2008). For this reason, most of the glucose sensors use mediators that enable the reduction of H_2O_2 at low potentials, thereby avoiding any kind of electrochemical interferents. A method for glucose determination at low potentials using glucose oxidase (GOD)-SPCE employ copper and CuO to modify the electrode which leads to a significant decrease in oxidation and reduction over-potentials (Luque et al., 2005). Osmium complex mediators and Cobalt phthalocyanine mediators have also been used in the development of GOD-SPCE for the oxidation

for the determination of glucose in serum (Zhang et al., 2001; Crouch et al., 2005). Furthermore, GOD is an oxygen-dependent enzyme which complicates the analysis of blood samples from capillary or vein, for this reason other enzymes, glucose dehydrogenase (GDH) have also been used in the construction of disposable glucose sensors (Razumienė et al., 2003). Ferrocene derivatives are widely used as mediators with GDH glucose biosensors.

Commercial glucose electrodes are required to operate in whole blood. As such, these electrodes can suffer from imprecision and inaccuracy due to the effect of erythrocytes red blood cells and other interfering species (Chance et al.,1999; Tang et al., 2000; Magner, 2001). It can be understood that, in the absence of any compensation for hematocrit (Hct), the amperometric electrodes will exhibit negative biases as Hct is increased. A number of strategies have been employed in reducing the Hct effect of glucose electrodes. The electrode is overlaid with a porous membrane having a pore size of less than the diameter of an erythrocytes red blood cell (typically 7 μm) (McAleer et al., 2001). In theory, erythrocytes are then retained on the membrane surface and only plasma reaches the electrode. However, later workers recognized that the use of such membranes was not conducive to economic large-scale manufacture of electrodes and incorporated the sensor active components (enzyme or mediator) together with the membrane-forming materials in a single screen-printable formulation. A nitrocellulose-based SPCE using two separate reagent zones and hexamineruthenium (III) chloride ($[Ru(NH_3)_6]^{3+}$) as a mediator effectively eliminates interference from both easily oxidable species and hematocrit in the glucose determination of glucose in blood (Cui et al., 2000). A porous conducting carbon layer, which is impregnated with a mixture including glucose oxidase and a ferrocene redox mediator, excludes erythrocytes and is consequently capable of operating acceptably in venous, capillary, arterial and neonatal blood over a wide hematocrit range of 20-70% (Forrow and Bayliff, 2005).

In order to improve the properties of SPCE glucose biosensor, new materials and construction techniques have been developed over the last few years (Luo et al., 2006; Guo and Wang, 2007; Shen et al., 2007; Wang and Lin, 2008). A sensor based on multi-wall carbon nanotubes (MWCNTs) immobilized with glucose oxidase and upon SPEC was reported (Guan et al., 2005). The MWCNT modified electrode system acted as an efficient biosensor, since it had higher sensitivity and wider linear response range than the typical glucose electrochemical biosensor. A quantitative analysis of benchmark amperometric system using SPCE/copper nanoparticles plated SPCE and hexacyanoferrate redox couple [$Fe(CN)_6^{4-/3-}$] was demonstrated with the determination of glucose in plasma (Hsu et al., 2006). The modifications of Iron nanoparticles coupled with [$Fe(CN)_6^{4-/3-}$] were significant enhancement of electron transfer on SPCE glucose biosensor (Lee et al., 2005). A study was introduced an approach for fabrication of glucose biosensor based on

drop-coating GOD on SPCE modified with ferricyanide-Fe_3O_4 magnetic nanoparticles mixture and exhibited a fast response (<15 s) with high sensitivity and good repetition (Lu and Chen, 2006). A mixture of ferricyanide and chitosan oligomers, acting as an electron mediator, was drop-coated onto the surface of SPCE, and glucose oxidase was then layered on, forming a disposable GOD/ferri-COs glucose biosensor (Lee et al., 2006). The biosensor was found to exhibit a rapid amperometric response (response time was less than 20 s), low detection limit (ca. 1.38 mM), good reproducibility and high sensitivity.

●Immunobiosensors

Immunoassays (IAs), which use specific antigen antibody complex, are very important in many fields, for examples, biological and medical research, diagnostic medicine, genetics, forensics, drug and pesticide testing (Ziegle and Göpel, 1998; Prodromidis and Karayannis, 2002; Kauffmann, 2003; Song et al., 2006; Lei et al., 2006; Belluzo et al., 2008). IAs with amperometric detection can offer enhanced sensitivities and reduced instrumentation costs compared to their optical counterparts, and an increasing effort was made during the last decade lo link the specificity of bioaffinity assays with the sensitivity and low detection limits afforded by modern electrochemical techniques. SPEC can exactly contribute to develop miniaturized, easy to handle, reliable and inexpensive IAs devices, which produce results within a few minutes (Mehrvar and Abdi, 2004; Renedo et al., 2007.).

An amperometric immunosensors based on SPCE for the determination of 17-estradiol was constructed from anti anti-estradiol antibodies labelling with alkaline-phosphatase (ALP) and using *p*-aminophenyl phosphate as substrate (Pembertona et al., 2005). The theophylline immunosensor based on the principles of hexacyanoferrate (II)-loaded liposome for amperometric signal amplification is composed of SPCE and immunochromatographic nitrocellulose membrane immobilized anti-theophylline antibody for the analysis of theophylline used to prevent and treat lung diseases (Lee et al., 1999). A direct sandwich immunosensor based on amperometry was employed to detect V*ibrio cholerae* O1 (Rao et al., 2006). A rapid method can be applied to measure α-1-fetoprotein (AFP) in human serum by use of horse-radish peroxidase-labeled AFP antibody in chitosan membrane and one-step sandwich ELISA-based immunobiosensor with disposable SPCE technique (Yu et al., 2004).

●Pollutant biosensor

Enzymatic sensors constructed with SPCE present a great number of applications in the

analysis of pollutants such as pesticides (Dennison and Turner, 1995; Marty et al., 1998; Tothill, 2001; Mulchandani et al., 2001). The determination of organophosphates and carbamates with these kinds of pesticide sensors is often based on enzymatic inhibition processes. Among the most commonly used enzymatic disposable biosensors in pesticide determination are those based on enzymatic inhibition of acetylcholinesterase (AChE) (Marty et al., 1998; Renedo et al., 2007.). A method of immobilization employed to deter mine aldicarb and paraoxon using Prussian Blue as the mediator of AChE-SPCE amperometric biosensor (Suprun et al., 2005). Sol-gel immobilization of bienzymatic sensor, AChE and cytochrome P450 MB-3 fixed on a SPCE, was elaborate for sensitive determination of parathion and paraoxon (Waibel et al., 2006).

The determinations of certain phenolic compounds widely used enzymatic biosensors in the amperometric analysis based on the immobilization of tryrosinase (Tyr) (Sapelnikovaet al., 2003; Tanimoto de Albuquerque and Ferreira, 2007). In one case, Tyr was immobilized on the surface of two types of Au-screen printed four-channel electrode arrays (graphite-coated-Au- and Carbopack C-coated-Au) by entrapment in a redox hydrogel composition (Sapelnikova et al., 2003)

For those reasons, we attempt to construct some approaches to decrease interferences and promote the selectivity, sensitivity and limited dynamic range for amperometric SPCE biosensor. In this study, we utilized novel calibration module and biochemical/physical modification methods on SPCE-biosensors in order to break through these limitations of diagnosis test and environmental/safety monitoring.

Aims of the study

1. Decrease interferences and Hct effect of glucose SPCE-Biosensor.

Diabetes is a serious world problem. It is currently estimated that there are over 180 million diabetics worldwide (WHO, 2004). There are three types of diabetes:

Type 1 diabetes usually affects the young and occurs when the pancreas no longer produces any (or very little) insulin (approximately 10%).

Type 2 diabetes commonly affects people from middle agers to elders, and occurs when the pancreas doesn't produce enough insulin or when the ability of using insulin efficiently by the body is low (approximately 90%).

Gestational diabetes is a temporary condition that occurs during pregnancy.

Diabetes is one of the dominant causes of death led to by disease. The high level of blood glucose associated with diabetes slowly damages blood vessels, resulting in a variety of complications, including incidence of heart disease, kidney disease, non-traumatic limb

amputation, adult blindness and erectile dysfunction etc. These complications can be put off and even avoided (Food and Drug Administration, 2008). Constant innovations in glucose monitoring technologies are being driven by the need for more frequent testing in order to better understand each patient's glucose level trends and to enhance personal treatment protocols. These demonstrated that patients could reliably measure their blood glucose levels at home and improve control of their glucose levels. The increasing awareness of the burden of diabetes on the whole-world healthcare system is expected to spark further development of better glucose monitoring solutions. (Newman and Turner, 2005)

Most of published blood glucose monitoring systems is also based on amperometric method of detection for the past few years. An international standard ISO 15197 have been suggested by national standards organizations for the company that manufacturers self-testing glucose meters that recommends accuracy of ±20 mg dL^{-1} for glucose values under 100 mg dL^{-1} and ±20% for higher glucose values. Even new glucose monitoring technologies are working toward creating a system that is more accurate, easy to use, portable, stylish, low-cost and painless. But the developed cost-effective glucose monitoring system is still not to meet target accuracy of ±5%, the goals of American Diabetes Association (ADA) science 1994 (American Diabetes Association, 1994).

- To figure out the limitations and basic performances of the selected two-electrode SPCE.
- To analyze the effects of biochemical and Hct% interferences on GOD(+)-SPCE and GOD(-)-SPCE.
- To simplify the complexity of biochemical and Hct% interferences in the samples and apply the samples to the feasibility study of model for interference decrease.
- To analyze and collect the parameters for the compensation requirements of Hct% effect.
- To use the model of interference decrease for the glucose measurements of whole blood samples and verify the practicality of model.

2. Enhance the sensitivity and limited dynamic range by the nano-materials modification on the SPCE-biosensors.

Nanotechnology has recently become one of the most exciting forefront fields in analytical chemistry. A wide variety of nanomaterials, especially nanoparticles with different properties have found broad application in many kinds of analytical methods (Wang, 2005; Wang and Lin, 2008). Owing to their tiny size (normally in the range of 1-100 nm), nanoparticles exhibit unique

chemical, physical and electronic properties that are different from those of bulk materials, and can be used to construct novel and improved sensing devices; especially, electrochemical sensors. The important functions provided by nanoparticles include the characteristics of excellent conducting capability and high surface-to-volume ratio, the immobilization of biomolecules, the catalysis of electrochemical reactions, the enhancement of electron transfer between electrode surfaces and proteins, labeling of biomolecules and even acting as reactant (Luo et al., 2006; Guo and Wang, 2007).

Amperometric biosensors are attractive tools and have received considerable attention because they would be easy and economical to mass production, they are robust, and they achieve excellent detection limits with small analyte volumes. Furthermore, the availability of a variety of new materials with unique properties at nanoscale dimension, such as AuNPs, has attracted widespread attention in their utilization for the bioassay (Yáñez-Sedeño and Pingarrón, 2005; Guo and Wang, 2007). The large surface-to-volume ratios and active sites of AuNPs constitute part of the driving force in developing nano-sized electrocatalysts. Thus, AuNPs modified electrochemical interface behaving as nanoelectrode ensembles have been widely used as enhancing catalytic interface for the development of amperometric immunosensors. In principle, the electroanalytical detection limit at a nanoelectrode ensemble can be much lower than that at an analogous macrosized electrode because the ratio between the faradaic and capacitive currents is higher (Penner and Martin, 1987).

An extremely important challenge is that the protein of most enzymes of amperometric biosensors cannot be oxidized or reduced at an electrode at any potential, science the redox center is electrically insulated by a protein shell. In order to achieve this task, artificial mediators have been utilized. More recently, the modification of electrode surfaces with the AuNPs will provide a microenvironment similar to that of the redox-proteins in native systems and gives the protein molecules more freedom in orientation, thereby reducing the insulating effect of the protein shell for the direct electron transfer through the conducting tunnels of AuNPs (Guo and Wang, 2007).

- To figure out the feasibility of the disposable selected-SPCE for the detection of *Escherichia coli* O157:H7 (*E. coli* O157:H7) using immunobiosensors and the determinations of carbofuran and glutathione using tyrosinase.
- To analyze the effects of AuNPs and ferrocenedicarboxylic acid (FeDC) modifications on these two SPCE-biosensing systems.
- To analyze and collect the parameters of two modified biosensing systems for the amperometric process.

- To verify the enhancement of sensitivity and limited dynamic range of two modified biosensing systems.
- To use the two modified biosensing systems for the measurements of real samples.

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Reduction of the interferences of biochemicals and hematocrit ratio on the determination of whole blood glucose using multiple SPCE test strips

Abstract

A practical approach to reduce the interferences of biochemicals and hematocrit ratio (Hct%) on the determination of whole blood glucose using multiple screen-printed carbon electrode (SPCE) test strips is described. SPCE test strips with and without glucose oxidase (i.e., GOD(+)-SPCE strips and GOD(-)-SPCE strips) were used and the chronoamperometric currents of test glucose solutions with various spiked uric acid concentrations and hematocrit ratios were measured. By establishing the interference relationships between glucose concentrations and uric acid concentrations as well as Hct% values and with appropriate corrections, the whole blood glucose determinations could be made to be more accurate and comparable to those determined by the reference YSI method. Specifically, the use of ΔCurrent value, i.e., the current difference between GOD(+)-SPCE and GOD(-)-SPCE measurements, would reduce most of the uric acid/ biochemical interferences. An interpolation method was also established to correct for the glucose determinations with Hct% interferences. The Hct% corrections using the interpolation method are especially important and necessary for those blood samples with glucose concentrations higher than 110 mg dL^{-1} and Hct% values lower than 35%. This approach should also be applicable to other biochemical determinations using similar electrochemical techniques.

Keywords: Screen-printed carbon electrode (SPCE), electrochemical biosensor, glucose, interference, hematocrit ratio, uric acid,

1. Introduction

1.1 Chronoamperometric biosensors

Chronoamperometric biosensors have high sensitivities and allow the determination of electroactive substances at concentrations as low as 10^{-9} M with a dynamic range of 3–4 orders of magnitude. The concept of an amperometric sensor for glucose was introduced by Clark and Lyons (Clark, et al., 1962). In this case, the electrode response of the GOD catalyzed glucose oxidation reaction is dependent on the oxygen concentration in the reaction medium and the product H_2O_2 is detected typically at 500-750 mV vs. the Ag/AgCl reference electrode. However, this method suffers at least two types of interferences for whole blood and serum samples. One is from physiological biochemicals such as uric acid, ascorbic acid and acetaminophen because these interferents are oxidized at the same potential range as H_2O_2 (Chen, et al., 2002; Brahim et al., 2002). The other is due to differences of Hct%, i.e., red blood cells ratio (Fogh-Andersen and D'Orazio, 1998). This is because the red blood cell fraction in the sample may affect the diffusion pathways and rates of substrates to the enzymes to produce products and electrochemical signals.

One useful solution to partially reduce the biochemical interferences is to introduce a low-oxidation potential mediator that acts as the electron acceptor of the enzymatic catalyzed oxidation (Cass et al., 1984; Quan and Wightman, 1997; Turner et al., 1999). In this approach, a mediator such as ferrocene or a quinine-type compound is added in the reaction vessel to shuttle electrons from the reduced enzyme to the working electrode. Two major advantages for using the mediated sensors are that (1) the electrochemical signals are largely independent of oxygen concentration in the sample and (2) lower oxidation potentials can be used instead of that necessary for H_2O_2. Currently, glucose electrochemical biosensors with screen-printed carbon electrodes and a mediator are commercially mass-produced for convenient and low-cost homecare applications. Electrochemical biosensors using similar design principles for hemoglobin (Green and Lias, 1989), Hct% (Kuhn et al., 1995) and uric acid (Kuo et al.,2002) are also available. Several other techniques have also been reported to eliminate the unwanted biochemical interferences. These include the preconsumption of the interfering substances by preoxidation employing horseradish peroxidase (Shin et al., 1996) or ascorbate oxidase (Maidan and Heller, 1991), and the effective separation of the electrode from the interfering substances using a highly hydrophilic polymeric analyte-regulating membrane (Xie et al., 2005) or a polycarbonate membrane coated with a negatively charged hydrogel layer (Vaidya and Wilkins, 1994). Methods of mediator-free and membrane-free biosensors were also described which provided means to measure the cathodic (i.e., reduction) current of the enzymatically liberated H_2O_2 by

metal-dispersed carbon paste electrodes (Wang et al., 1994; Wang et al., 1995). More recently, a biosensor employing carbon nanotube nanoelectrode ensembles was developed to determine the substrate concentration selectively and avoided the generation of overlapping signals from interferents (Lin et al., 2004). A continuously used amperometric biosensor for whole blood sample analysis calibrated with acetaminophen was claimed to achieve good correction of interferences by acetaminophen-specific electrodes (Schaffar et al., 1999). However, the large-scale production of these devices is expected to be more complicated and expensive than that of the SPCE strips and may not be readily suitable for homecare applications. On the other hand, it was usually found that artificially higher responses were obtained for samples with lower Hct% values, and lower responses were obtained for those with higher Hct% values. In the preferred embodiments of a US patent (Carter et al., 1997), the interferences of Hct% of an electrochemical glucose biosensor were reduced by roughly 50% by the introduction of a dummy electrode without enzyme.

1.2 Blood glucose monitoring

Diabetes is one of the leading causes of death and disability for human beings in the world. About 7% of the world's population suffers from this disease, and the healthcare costs are increasing every year. It is a chronic disease resulting from insulin deficiency and hyperglycemia with a high risk of complicated side effects development for the eyes, kidneys, peripheral nerves, heart, and blood vessels. The effective control of this disease requires quick diagnosis and early prevention. Besides a number of therapeutic drugs developed for the cure of diabetes, enormous amount of research efforts have been devoted for convenient, accurate and inexpensive determination of blood glucose concentration levels including the use of the chronoamperometric biosensors.

Anton Hubert Clemens received the first patent for a blood glucose meter called the Ames Reflectance Meter on September 14, 1971. Richard K. Bernstein, an insulin dependent physician with diabetes, was one of the first patients to monitor his blood glucose at home using a glucose meter. The first articles in the medical literature on the home blood glucose monitoring were published in 1978 (Food and Drug Administration, 2008). These demonstrated that patients could reliably measure their blood glucose levels at home and improve control of their glucose levels. In November 1986, the American Diabetes Association (ADA), the Centers for Disease Control and Prevention, the Food and Drug Administration (FDA), and the National Institutes of Health convened a Consensus Conference on Self-Monitoring of Blood Glucose. The results of

conference were that self-monitoring of blood glucose was an exciting and important tool for effective management of patients with diabetes (FDA, 2008). It was recommended that SMBG be used by patients to accomplish the following goals: (a) Keep track of their glucose levels over time, (b) Help make day-to-day decisions for managing glucose, (c) Recognize emergency situations, and (d) Educate themselves on how to manage their blood glucose levels.

Because it is not practical at the point of care testing (POCT) setting to perform sample pretreatment to reduce interferences such as in a wet biochemical process, the accuracy and precision of glucose determinations using the dry biochemical techniques are particularly important. Thus, government regulatory agencies have established the 510K and ISO15197: 2003(E) guidelines as performance criteria. In 1987, the ADA recommended that POCT electrochemical glucose determinations should fall within ±15% of those determined by regular laboratory techniques and the future goal was to reduce this variability to within ±10% for glucose concentrations between 30 and 400 mg dL^{-1}. This goal was later modified by the ADA in 1996 to be within ±5% of those determined by regular laboratory techniques (ADA, 1987 and 1996). Although the criteria in the government regulatory guidelines have not been changed so far, it is inevitable that the glucose determination specifications are expected to be more rigorous. However, to the best of our knowledge, no report has been published to date concerning attempts to reduce the biochemical and hematocrit interferences and improve the precision and accuracy of the electrochemical glucose determination (Chance and Li, 1999; Yuoh et al., 2001).

In this paper we report the results of our approach to reduce the biochemical and Hct% interferences on the chronoamperometric determination of whole blood glucose using SPCE strips. A practical solution could then be developed to improve the accuracy and precision of whole blood glucose determinations to serve the diabetic community better. This simplified approach could also be applied to reduce interferences for chronoamperometric determinations of other analytes in the whole blood sample.

2. Materials and methods

2.1 Enzyme and Chemicals

GOD (EC 1.1.3.4, Aspergillus niger, Biozyme Laboratory, code GO3A), and reagent grade L-ascorbic acid, uric acid, bilirubin, carboxymethyl cellulose sodium salt, bovine serum albumin (BSA), lyophilized hemoglobin, deoxycholic acid, acetaminophen and water-soluble cholesterol (Sigma, St. Louis, MO, USA), D-(+)-glucose, 3-acetamidophenol, Triton X-100, potassium hexacyanoferrate(III), $K_2HPO_4.3H_2O$ and KH_2PO_4 (Merck), glucose/HK and UA plus kits (Roche Diagnostics, Mannheim, Germany), uric acid standard (Randox Laboratories, Antrim, UK) and 0.9% saline (Taiwan Biotech, Taoyuan, Taiwan) were used as received.

2.2 SPCE strips and equipments

SPCE strips and venous and fresh capillary whole blood samples drawn from normal people and diabetic patients by a registered nurse were obtained from APEX Biotechnology Corporation. A CV50W voltammetric analyzer (Bioanalytical Systems, West Lafayette, IN, USA) was used for all electrochemical measurements. For comparison purposes, a YSI 2300 STAT Plus glucose and lactate analyzer (YSI Life Sciences, Yellow Springs, OH, USA), a HemoCue B-Hemoglobin analyzer (HemoCue AB, Ängelholm Sverige, Swedish), an SP870 spectrophotometer (Metertech, Taipei, Taiwan) and a KUBOTA 3110 micro hematocrit centrifuge (KUBOTA, Bunkyo-ku, Tokyo, Japan) were used for glucose, hemoglobin, uric acid and Hct% determinations, respectively.

2.3 Construction of the SPCE test strips with and without GOD

The SPCE test strips were constructed according to a described protocol (Huang and Shen, 2005). Conducting films of silver and carbon ink were screen-printed consecutively on a flat surface of a PVC board to form a two-electrode system and the board was dried by hot air. An about 0.27-mm-thick electrically insulating layer was then applied to the flat board surface to cover most of the electrode area and allowed only the connecting and electrochemical reaction portions to be partially exposed. For SPCE test strips containing GOD, a solution with the following composition was added on the surface of the reaction area (% w/w): bovine serum albumin (0.5%), potassium ferricyanide (6.0%), carboxymethyl cellulose (0.5%), Triton X-100 (0.07%), phosphate buffer (pH 5.0, 92.30%) and GOD (0.63%). The test strips were dried at 50 °C for 15 min and cut from the PVC board after a reticular covering mesh had been applied on the reaction layer. For SPCE test strips without GOD, a solution with a similar formulation to that of GOD(+)-SPCE strips was added to the test strips except that BSA was used instead of GOD. The

amount of the formulation solution added onto the 12.6-mm2 reaction area was approximately 5 μL for both GOD(+)-SPCE strips and GOD(-)-SPCE strips. All GOD(+)-SPCE strips and GOD(-)-SPCE strips were freshly prepared for single use.

2.4 Initial study of the effects of biochemical and Hct% interferences

The GOD(+)-SPCE strips test strips were used to measure the oxidative cyclic voltamograms for the endogenous and exogenous biochemicals in the potential range from -400 to 400 mV at a scan rate of 100 mV s^{-1} using the CV50W voltammetric analyzer after a 20-s sample incubation period. For a healthy human being, the ADA recommends that the blood sugar level is best kept at 80–120 mg dL^{-1} before meals and at 100–140 mg dL^{-1} at bedtime when using a blood sample drawn from a vein (i.e., a whole blood sample). The numbers increase to 90–130 mg dL^{-1} before meals and 110–150 mg dL^{-1} at bedtime when using a blood sample drawn from a fingertip (ADA, 2006). Thus, the our test glucose saline solutions were prepared at concentrations of 71, 145 and 290 mg dL^{-1} to mimic the low-end and high-end glucose levels of a healthy human being and that of a diabetic. The concentrations of the biochemicals (i.e., ascorbic acid, uric acid, bilirubin, acetaminophen, 3-acetamidophenol and hemoglobin) for the interference studies were prepared according to the guidelines set by the National Committee for Clinical Laboratory Standards document EP7-A: interference testing in clinical chemistry; approved guideline (National Committee for Clinical Laboratory Standards, 2002).

2.5 Quantitation of biochemical and Hct% interferences

Sample solutions containing the biochemical interferents were prepared by spiking glucose and interfering biochemicals into reconstructed red blood cell saline (RRBC-saline) as well as oxygenated whole blood solutions. RRBC-saline solutions with designated Hct% were prepared by using a fresh venous blood sample, washing four times with three portions of saline solutions and centrifugation to remove unwanted biochemicals, and diluting with appropriate amounts of saline solutions (vide infra). For whole blood oxygenation, 2 mL whole blood in a 7-mL capped container was allowed to equilibrate with the atmosphere on a horizontal rotator for 15 min. The cap was then opened for 30 s, and the container was recapped and the contents were allowed to equilibrate for another 15 min. The sample solutions were added onto the GOD(+)-SPCE strips and GOD(-)-SPCE strips, and the SPCE strips were connected to the CV50W at 25°C. A constant potential of 300 mV was applied to the working electrode of the SPCE test strips and the oxidation currents vs. time data were recorded. Freshly prepared SPCE test strips were used for each

measurement. The accuracies for the glucose, Hct%, and uric acid determinations were checked by comparing them with those obtained with the YSI 2300 STAT Plus, the KUBOTA 3110 centrifuge, and the Roche UA plus kit/Meterteck SP870 spectrophotometer, respectively.

3. Results and discussion

3.1 Initial study of the effects of biochemical and Hct% interferences

A total of eight endogenous and exogenous biochemicals were chosen for this study: glucose, ascorbic acid, uric acid, hemoglobin, bilirubin, cholesterol, acetaminophen and acetamidophenol. Fig.1 shows selected cyclic voltamograms of some of these biochemicals at various concentrations using the GOD(+)-SPCE strips in the potential range from –400 to 400 mV after 20-s sample incubation. The current readings of the oxidative cyclic voltamograms at various concentrations of these biochemicals at 100, 200, 300 and 400 mV are listed in Table 1. It is noted that the endogenous biochemical concentrations in a normal, healthy person are generally as follows: glucose, below 150 mg dL^{-1}; ascorbic acid, below 5 mg dL^{-1}; uric acid, below 10 mg dL^{-1}; bilirubin, below 5 mg dL^{-1}; cholesterol, below 200 mg dL^{-1}; hemoglobin, below 10 g L^{-1} (Burtis and Ashwood, 1999). Thus, excluding glucose, the higher (than normal) working concentrations chosen for fresh hemoglobin (20 g L^{-1}) and the rest of the biochemicals (300 and 20 mg dL^{-1}, respectively) for this initial study represent quite stressed physiological conditions. From the current readings, it is observed that at 300 mV, most of the potential interfering biochemicals show relatively the greatest interfering signals. It was found that the degree of biochemical interferences for glucose determination is roughly in the order uric acid ~ ascorbic acid > hemoglobin > bilirubin > cholesterol. For the exogenous biochemicals, acetaminophen has a higher degree of interference. However, the effect would be greatly reduced after this material has been excreted from the body.

For subsequent chronoamperometric measurements of various concentrations of glucose and uric acid in saline solutions, a potential of 300 mV was used and the tenth second current readings after 20-s sample incubation were collected, as shown in Fig. 2. Under these conditions, the current signals for glucose measurements in the 50–550 mg dL^{-1} concentration range in phosphate buffer (pH 5.0) and whole blood (Hct% of 40%) were linear with good correlation coefficients, as shown in Fig. 3.

One common complication for diabetic patients is anemia due to hemodialysis (Ma et al., 1999; Anatole et al., 1998). In this case, abnormally low Hct% values were frequently observed for whole blood samples. Thus, it is important to evaluate the determination of blood glucose at

low Hct% using SPCE strips. The Hct% interference effects on the current measurements using GOD(+)-SPCE strips at several glucose concentrations are shown in Fig 4. As expected, higher current readings were observed for glucose concentrations with lower Hct% values. An exponential decay relationship, i.e., $I=ke^{-b[Hct\%]}$, could be established between the current readings and the Hct% values with correlation coefficients $R^2 \geq 0.9088$. For a given glucose concentration, the current responses at various Hct% values and two uric acid concentrations are shown in Fig 5 with the use of GOD(-)-SPCE strips. A similar result to that of the above-discussed GOD(+)-SPCE strips was observed and corroborated well with those reported earlier (Carter et al., 1997). The normal adult hematocrit ranges are 39–51% for men and 36–44% for women (Minetti, 1997). Outside this range, the glucose determinations using this technique would give larger errors, particularly for those with lower Hct%.

3.2 Elimination of interferences of uric acid/biochemicals and Hct%

Three kinds of current signals are defined in this study: (1) $I_{(s+i)}$ is the current reading obtained with the GOD(+)-SPCE strips and includes that of glucose with biochemical and hematocrit interferences, (2) Ii is obtained with GOD(-)-SPCE strips and is due to biochemicals with minor hematocrit interferences and (3) the calculated corrected current, $\Delta I=I_{(s+i)}-I_i$. Note that the hematocrit interference might not be completely eliminated by just calculating ΔI because the degree of hematocrit interferences in glucose determination is usually greater than that of other biochemicals (vide infra). To minimize the interferences of Hct% completely, additional interpolation was exercised (vide infra).

The tenth second $I_{(s+i)}$ and I_i values were obtained after an initial 20-s sample incubation period. Fig 6 shows the current (i.e., $I_{(s+i)}$ and I_i) plots against spiked uric acid concentrations in two sets of RRBC-saline glucose solutions, i.e., 93 mg dL^{-1} (RBC/solution volume ratio: 46%) and 316 mg dL^{-1} (RBC/solution volume ratio: 42%). The solutions were spiked with various amounts of uric acid. It is observed that the slopes of both $I_{(s+i)}$ and I_i plots against uric acid concentrations are similar and the slopes of both ΔI plots against uric acid concentrations are close to zero. This implies that after uric acid concentration corrections, the glucose oxidation currents are independent of uric acid concentration, if Hct% values are not very different.

The ΔI values are also plotted in Fig 7. In an attempt to eliminate Hct% interferences, a series of glucose solutions were prepared with various concentrations and Hct% values. The $I_{(s+i)}$ and I_i readings were recorded in the same way as those of Figure 6, and corresponding ΔI values were calculated. Fig 7 shows plots of ΔI values against Hct% values at four blood glucose

concentrations. From these plots, it is observed that for a glucose concentration of 75.7 mg dL^{-1}, the current readings are similar in the Hct% range tested. However, for glucose concentrations of 170.4, 318.0 and 462.0 mg dL^{-1}, the current readings are increasingly higher at lower Hct% values, as expected (vide supra). Additional efforts to eliminate Hct% interferences are described later.

3.3 Practical applications

Previously a correlation for the determination of whole blood glucose between the more convenient YSI method and that of the more accurate photometric hexokinase method (Schmidt, 1961) was established in our laboratory using 96 plasma samples. The correlation equation was $[glucose]_{YSI} = 1.064[glucose]_{hexokinase}-12.63$, with $R^2 = 0.9781$. For convenience, the YSI method was used for the practical applications discussed in this section.

The above-discussed methods to eliminate biochemical and Hct% interferences were applied to 37 preclinical human samples (16 from men and 21 from women). The experimentally obtained $I_{(s+i)}$ and I_i values as well as calculated ΔI values are listed in Table 2. The data in column 2 (i.e., $[glucose]_{YSI}$), column 3 (i.e., $[UA]_{optic}$) and column 4 (i.e., $[Hct]_{KUBOTA}$) of Table 2 are the glucose and uric acid concentrations and the Hct% values determined directly using the methods of the YSI 2300 STAT Plus, the Roche UA plus kit/Meterteck SP870 spectrophotometer and the KUBOTA 3110 centrifuge, respectively. The uncorrected glucose concentrations in column 6 (i.e., $[glucose]_{uncorr}$) were obtained using the $I_{(s+i)}$ values (column 5) and a calibration curve was established between the data in columns 2 and 5 (Collison, et al., 1999; Tieszen and New, 2003; Kovatchev, et al., 2005), i.e., $I_{(s+i)} = 0.0613[glucose]_{uncorr} + 0.0168$. For each sample, a percentage bias value is calculated using the corresponding values in columns 2 and 6 and the sum of the absolute percentage bias values for all samples is 204.2. Similarly, the uric acid corrected glucose concentrations in column 9 (i.e., $[glucose]_{uacorr}$) were obtained using the ΔI values (column 8) and a calibration curve was established between the data in columns 2 and 8, i.e., $\Delta I = 0.0601[glucose]_{uacorr} - 0.4421$. The sum of the absolute percentage bias values for $[glucose]_{uacorr}$ is reduced to 181.7, indicating some improvement in accuracy. If the 37 samples are divided into a high-glucose group (the first 14 samples; more than 120 mg dL^{-1} glucose) and a normal-glucose group (the last 23 samples; less than 120 mg dL^{-1} glucose), it is found that the percentage bias is largely contributed by the samples from the high-glucose group, i.e., samples from the higher-risk patients.

Compared with Hct% interferences, uric acid/biochemical interferences and others such as sample per strip background interferences are relatively lower and the use of ΔI values would

eliminate most of these minor interferences. However, glucose measurements using SPCE test strips are also interfered with by Hct%, particularly for samples with high glucose concentration and low Hct% (Fig 4 and 7). For example, although both samples 27 and 36 have abnormally low uric acid concentrations (i.e., outside the 3.5– 8.5 mg dL^{-1} range) and Hct% values (i.e., outside the 36–51% range), the percentage bias values are relatively low because they were in the low glucose concentration group. On the other hand, samples 6 (Hct% 33%) and 11 (Hct% 31%) with normal uric acid concentrations but abnormally low Hct% values tend to have much greater percentage bias values.

To further improve the accuracy of the glucose measurements, we propose the following method to reduce the Hct% interferences, using sample 11 as one example. The initially determined glucose concentration of sample 11 (i.e., 244.8 mg dL^{-1}) fell between 170.4 and 318.0 mg dL^{-1}; therefore, the exponential decay equations represented by curves b and c in Fig. 4 are applicable to calculate the two current values, i.e., 11.5 μA (using the equation represented by curve b) and 22.6 μA (using equation represented by c) at Hct% = 31%. Using the determined $I_{(s+i)}$ value of 15.0 μA in Table 2 (column 5, sample 11) and by interpolation, we calculated $[glucose]_{Hct\text{-}corr}$ of sample 11 to be 217.71 mg dL^{-1} for the $I_{(s+i)}$ system. The percentage bias is reduced to 8.8 and is lower than the corresponding value, 22.4 (Table 2, column 7, sample 11), without any corrections. If the ΔI value of 13.7 μA for sample 11 and the equations represented by curves b and c in Fig 7 are used, the value of $[glucose]_{Hct\text{-}corr}$ in the ΔI system is 217.69 mg dL^{-1}. The percentage bias is also reduced to 8.8 and is again lower than the corresponding value, 17.9 (Table 2, column 10, sample 11), with only uric acid/biochemical correction, as Table 3 for sample calculations. Similarly, the respective percentage bias values of sample 6 after Hct% corrections are 3.1 and 4.9, and are lower than the respective values, 13.4 (Table 2, column 7, sample 6) and 12.5 (Table 2, column 10, sample 6). The differences of the percentage bias values of Hct% corrected values and that of Hct% uncorrected values for other samples with normal Hct% are not as large as for samples 6 and 11. Thus, hematocrit corrections are especially important for samples with abnormal hematocrit values.

4. Conclusions

In “Elimination of interferences of uric acid/biochemicals and Hct%”, only glucose solutions with spiked uric acid and adjusted Hct% were examined to illustrate the interference effects, i.e., the sample background complexity of blood glucose determination was somewhat simplified. However, the results of the simplified exponential decay models were still quite useful in practical

applications, because by only determining the uric acid concentrations and Hct% values for the samples in Table 2, we could make significant correction improvements for the glucose determinations. From the results discussed in this section, Hct% corrections have been found to be more important for low Hct% samples. Corrections with uric acid/ biochemicals interferences, although evident, may not be as drastic as those with Hct% corrections. The present approach should also be applicable to other analytes that can be determined by SPCE electrochemical techniques.

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Figures

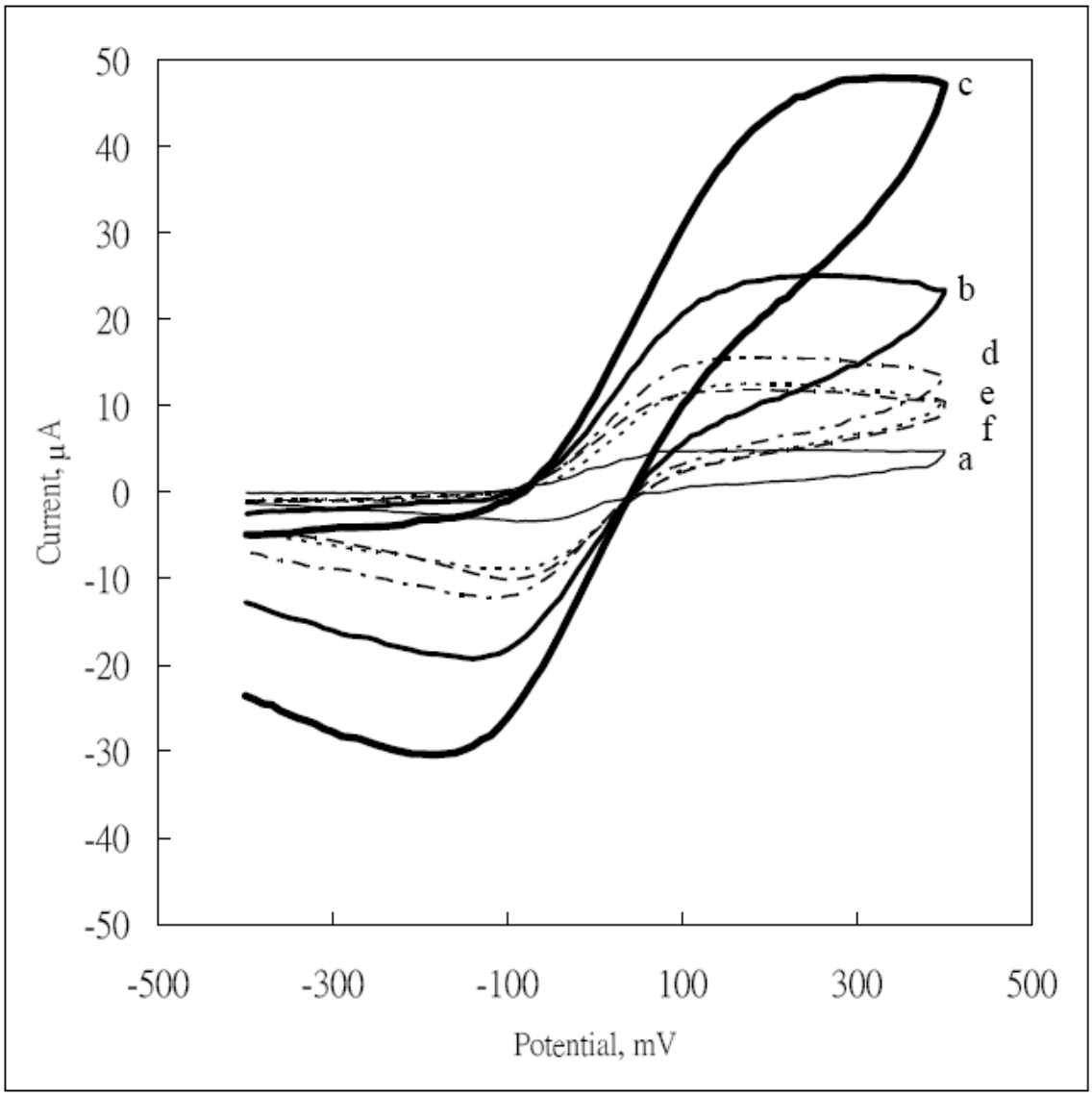

Fig. 1 The voltamograms of glucose and biochemicals in saline solutions on the GOD(+)-SPCE strips after 20 seconds sample incubation (scanning rate, 100mV s^{-1}). (a) control, glucose: 0 mg dL^{-1}, (b) glucose: 100 mg dL^{-1}, (c) glucose: 200 mg dL^{-1}, (d) ascorbic acid: 20 mg dL^{-1}, (e) uric acid: 20 mg dL^{-1}, (f) hemoglobin: 20 g L^{-1}.

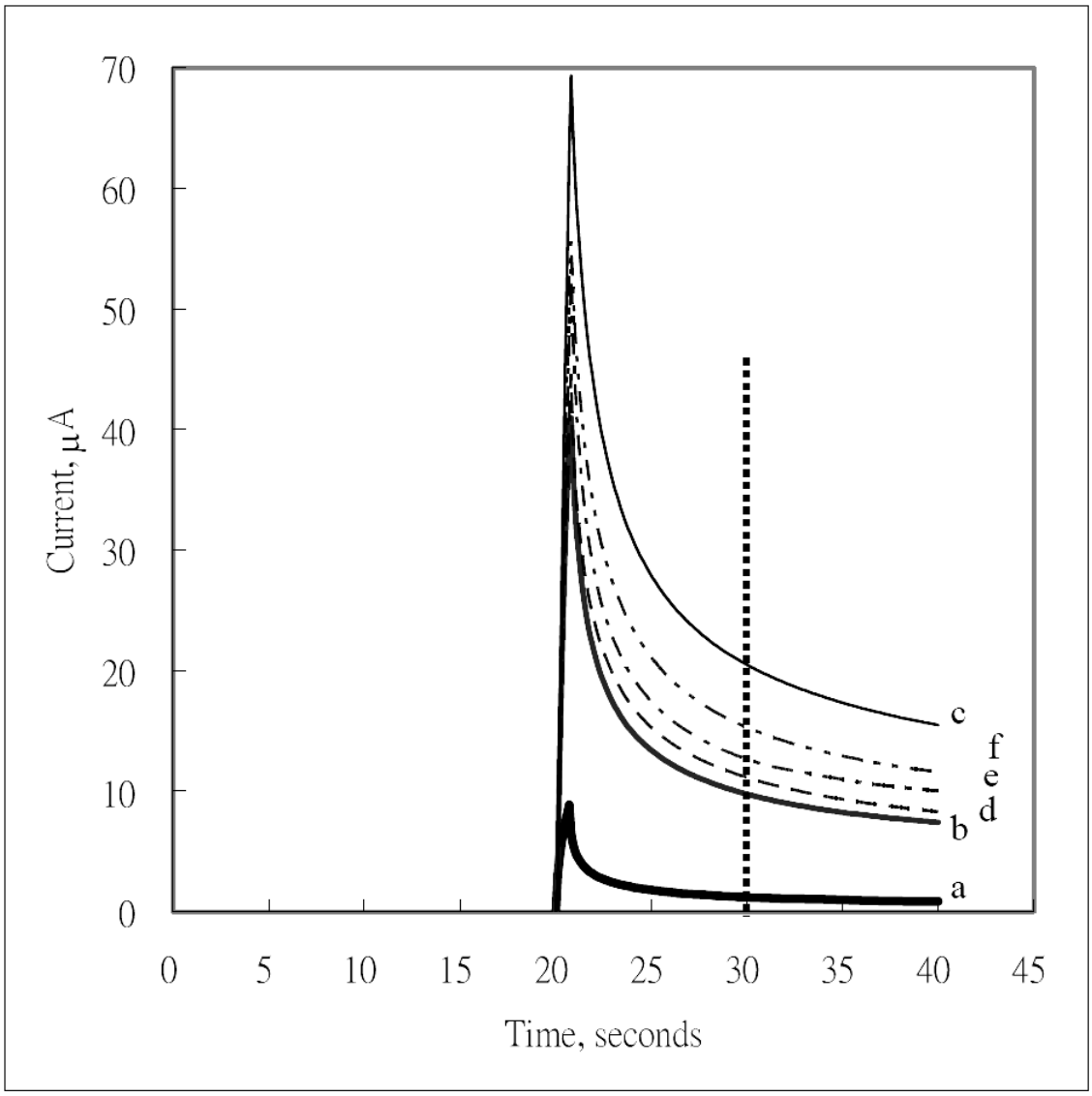

Fig. 2 Plots of the chronoamperometric currents (μA) of saline solutions containing different glucose and uric acid concentrations vs. time at 300 mV on the GOD(+)-SPCE strips after 20 s incubation. Glucose: (a) 0, (b) 71 and (c) 145 mg dL^{-1}; Uric acid: (d) 5, (e) 10 and (f) 20 mg dL^{-1}.

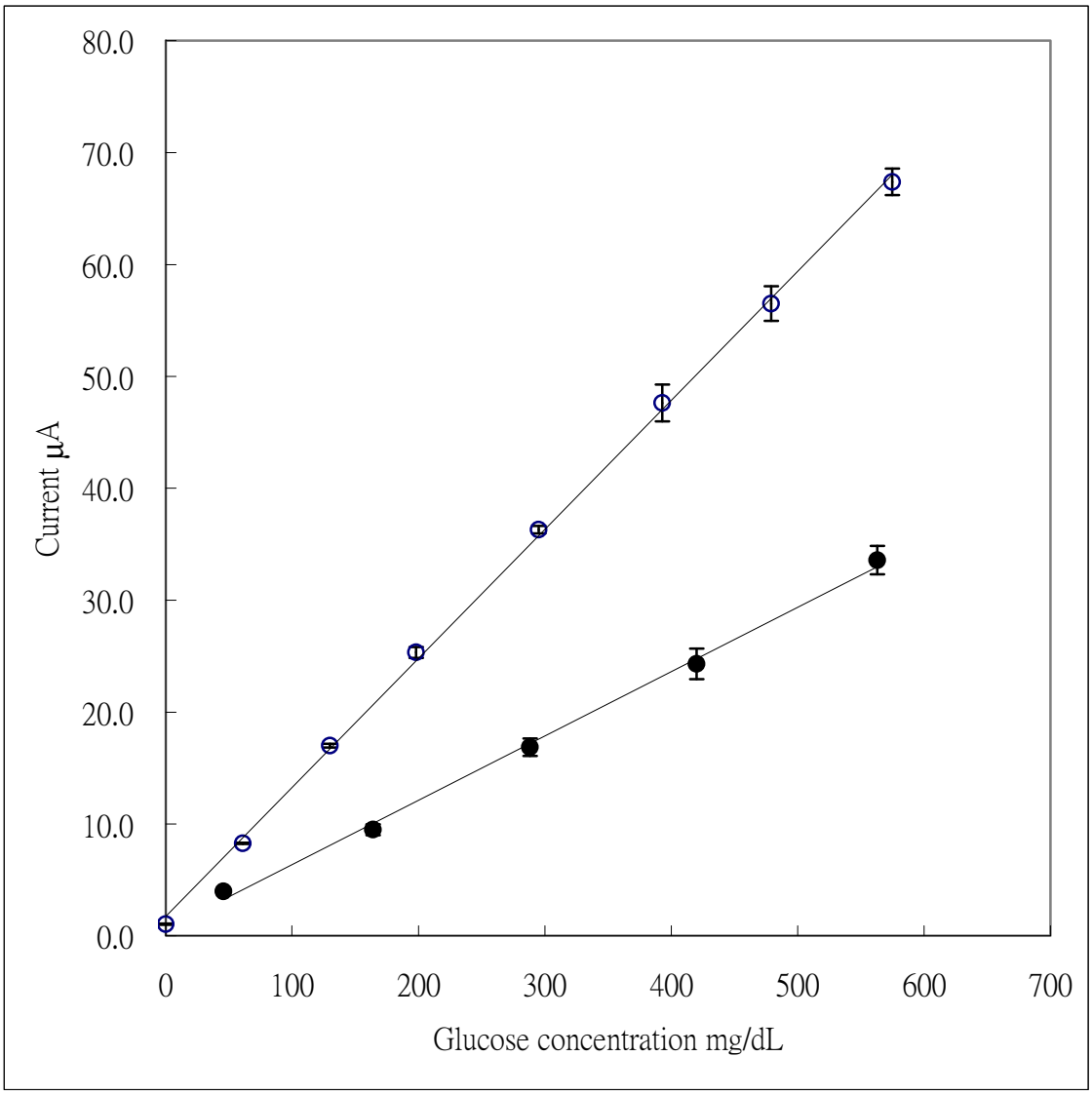

Fig. 3 Linear correlations of currents and glucose concentrations (50-550 mg dL^{-1}) in saline and whole blood (Hct 40%) samples measured with GOD(+)-SPCE strips. (○) : saline samples; (●) : whole blood samples. Error bars were standard deviation values (n = 5).

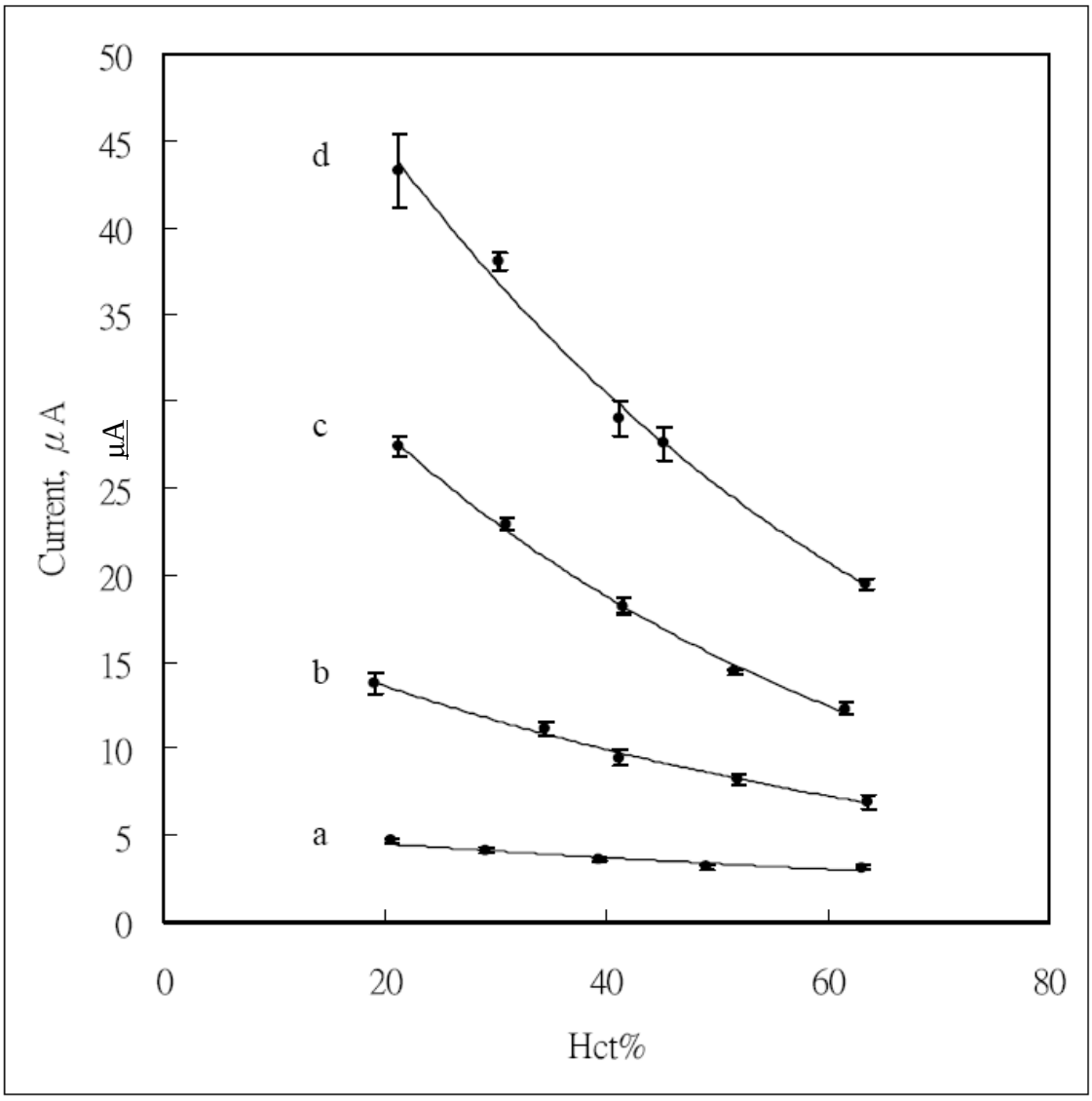

Fig. 4 Plots of whole blood chronoamperometric current readings (μA) vs. Hct% at various glucose concentrations using GOD(+)-SPCE strips. Glucose concentrations: (a) 75.7, (b) 170.4, (c) 318.0, (d) 462.0. Uric acid concentration was 6.5 mg dL^{-1}. The best fitted equations were: (a) $Y = 5.491e^{-0.0097X}$ ($R^2 = 0.909$), (b) $Y = 18.599e^{-0.0156X}$ ($R^2 = 0.994$), (c) $Y = 42.482e^{-0.0204X}$ ($R^2 = 0.997$) and (d) $Y = 64.524e^{-0.0186X}$ ($R^2 = 0.993$). Error bars were standard deviation values (n = 5).

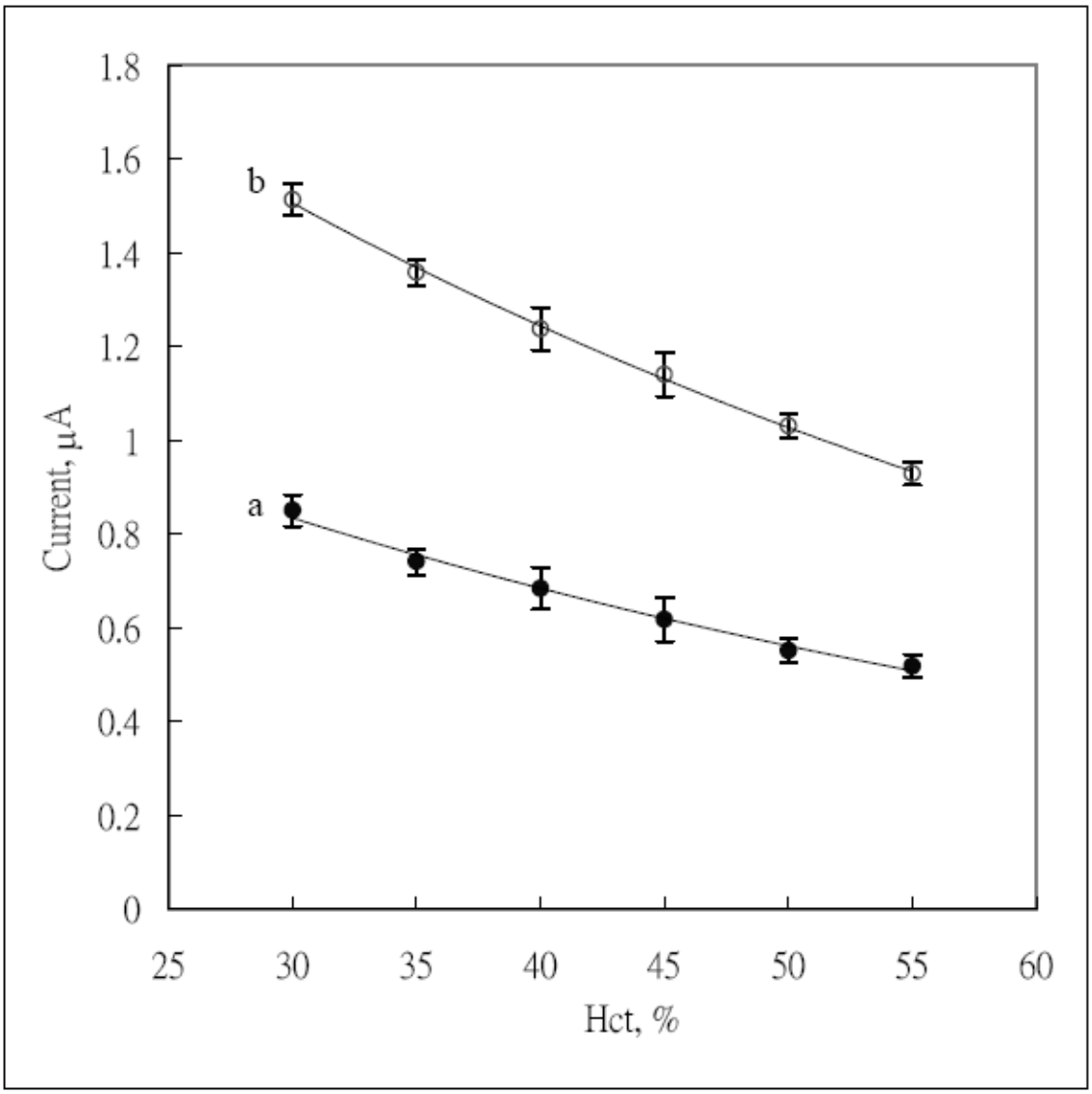

Fig. 5 Plots of whole blood chronoamperometric current readings (μA) vs. Hct% at a glucose concentration of 85 mg dL^{-1} using GOD(-)-SPCE strips. Uric acid concentrations in the whole blood were (a) 4.6 and (b) 16.3 mg dL^{-1}. Best fitted equations were: (a) $Y = 1.512\ e^{-0.0198X}$ ($R^2 = 0.992$) and (b) $Y = 2.674\ e^{-0.0191X}$ ($R^2 = 0.992$). Error bars were standard deviation values (n = 5).

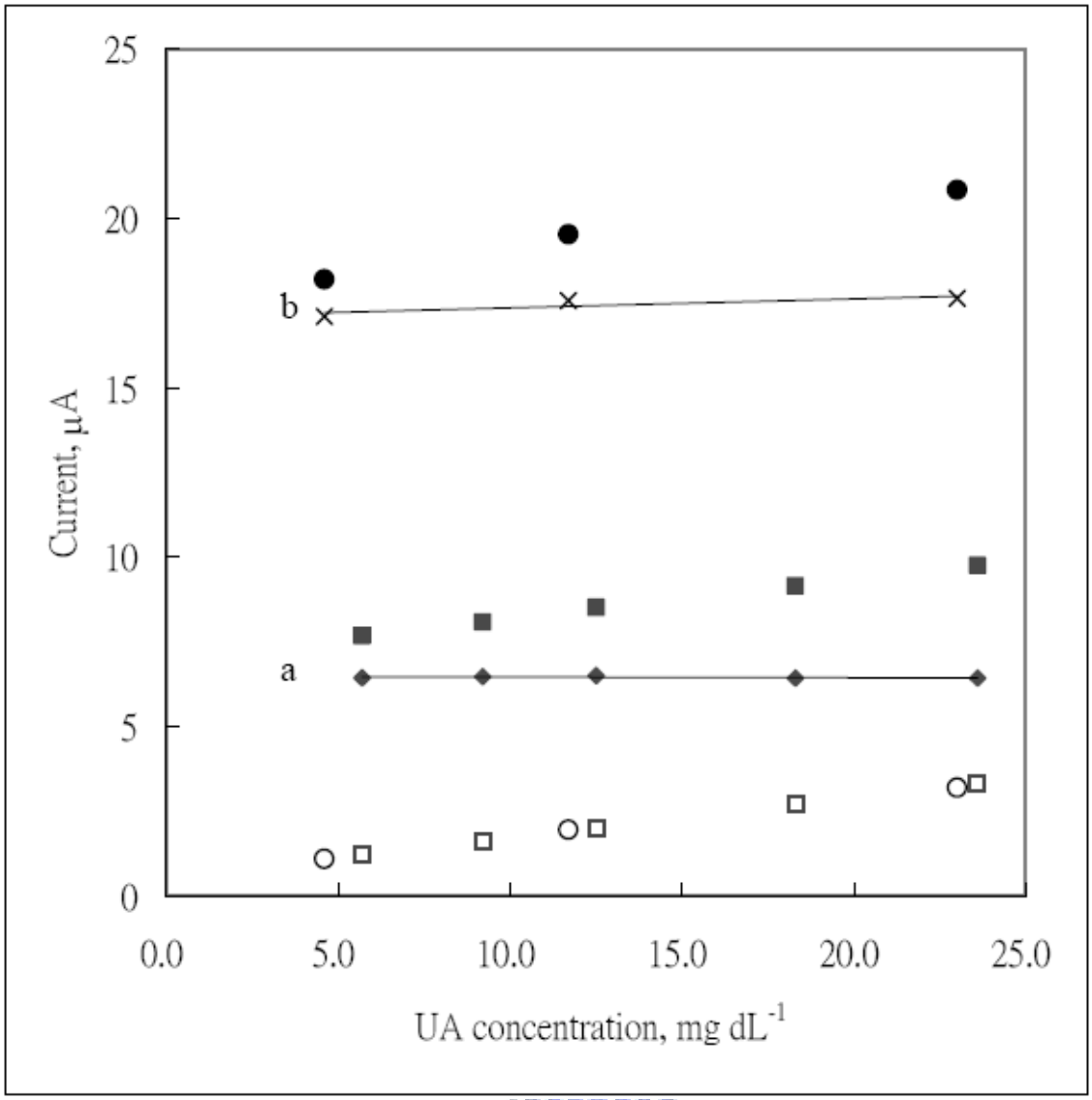

Fig. 6 Effects of spiked uric acid concentrations on the RRBC-saline chronoamperometric current readings. The 10th second $I_{(s+i)}$ and $I_{(i)}$ readings were recorded after 20 seconds sample incubation and ΔI calculated. (a) glucose 93 mg dL^{-1}: $I_{(s+i)}$ (■), $Current_{(i)}$ (□), ΔI (◆); best fitted equation for ΔI: Y = -0.002X + 6.481. (b) glucose 316 mg dL^{-1}: $I_{(s+i)}$ (●), $I_{(i)}$ (○), ΔI(×); best fitted equation for ΔI: Y = 0.027X + 17.089. The range of all standard deviations is 0.053~0.192 (n = 5).

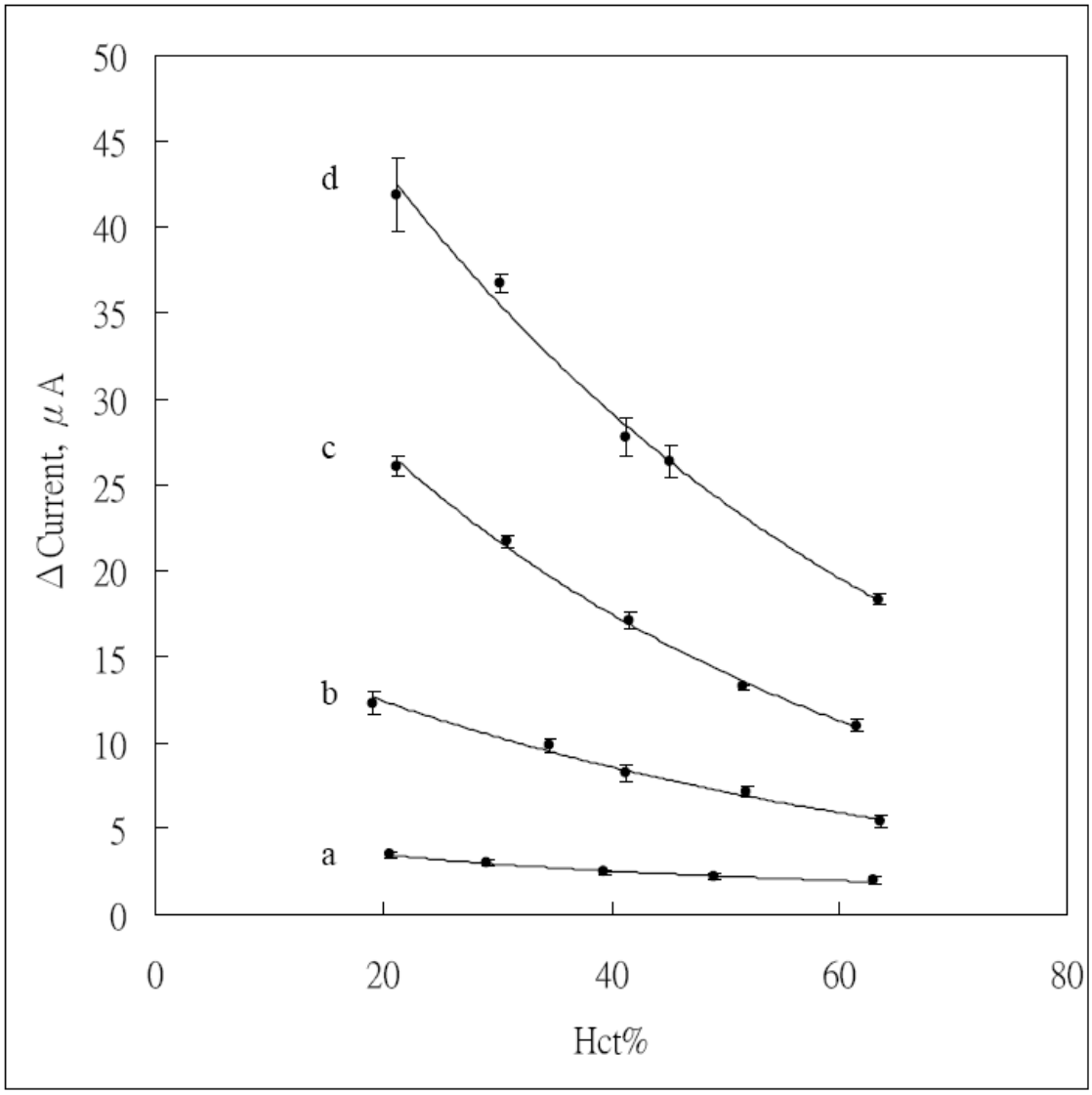

Fig. 7 Plots of whole blood chronoamperometric ΔCurrent (ΔI values, μA) vs. Hct% at various glucose concentrations. The 10th second $I_{(s + i)}$ and $I_{(i)}$ readings were recorded after 20 seconds sample incubation and ΔI calculated. Glucose concentrations: (a) 75.7, (b) 170.4, (c) 318.0, (d) 462.0. Best fitted equations: (a) $Y = 4.419e^{-0.0133X}$ ($R^2 = 0.909$), (b) $Y = 17.958e^{-0.0184X}$ ($R^2 = 0.990$), (c) $Y = 42.023e^{-0.0219X}$ ($R^2 = 0.998$) and (d) $Y = 64.696e^{-0.0199X}$ ($R^2 = 0.995$). Error bars were standard deviation values (n = 5).

Tables

Table1. The current readings (μA) at various concentrations of selected biochemicals using the GOD(+)-SPCE strips at scanning potentials of 100, 200, 300, and 400 mV; * indicates biochemical interferences.

Biochemicals	Concentrations		**Scanning voltage** (mV) and current (μA)			
			100	**200**	**300**	**400**
Saline			4.7	4.8	4.8	4.8
Glucose	71	mg dL^{-1}	20.6	24.7	24.9	23.4
	145		30.6	43.5	47.8	47.2
	290		46.2	67.9	81.1	88.2
Ascorbic acid*	10	mg dL^{-1}	8.8	8.9	8.7	7.7
	20		14.6	15.6	15.1	13.5
Uric acid*	10	mg dL^{-1}	7.7	8.1	7.9	7.1
	20		11.3	11.8	11.4	10.6
Bilirubin*	10	mg dL^{-1}	6.7	6.5	6	5.5
	20		7.9	7.2	6.7	6.2
Cholesterol	150	mg dL^{-1}	5.1	4.9	4.8	4.7
	300		4.2	4.2	4	3.8
Fresh Hemoglobin*	10	g L^{-1}	7.7	7.8	7.5	6.9
	20		11.5	12.5	11.7	10.7
Lyophilized Hemoglobin	10	g L^{-1}	4.9	4.8	4.8	4.8
	20		5.1	5	4.9	4.8
Acetaminophen*	10	mg dL^{-1}	5.5	7.7	12.6	12.4
	20		5.3	9.7	18.4	20.9
3-Acetamidophenol	10	mg dL^{-1}	4.9	5	4.8	5
	20		4.7	4.8	4.8	4.8

Table 2. Comparisons of the $I_{(s+i)}$ and ΔI readings as well as uncorrected and corrected glucose concentrations for 37 pre-clinical human samples.

Sample No.	Reference methods			GOD(+)-SPCE only			GOD(+)- and GOD(-)- SPCE		
	$[Glucose]_{YSI}$	$[UA]_{optic}$	$[Hct]_{KUBOTA}$	$I_{(s+i)} = 0.0613x[Glucose]_{uncorr} + 0.0168$			$\Delta I = 0.0601x[Glucose]_{uacorr} - 0.4421$		
	mg dL^{-1}	mg dL^{-1}	%	$I_{(s+i)}$	$[Glucose]_{un}$	|BIAS%|	△I	$[Glucose]_{ua}$	|BIAS%|
1	352.0	7.1	39.0	21.2	345.4	1.9	20.5	348.7	0.9
2	330.0	9.1	44.0	21.3	347.5	5.3	20.1	341.1	3.4
3	329.0	2.5	43.0	20.5	333.9	1.5	20.0	340.0	3.4
4	298.0	4.0	43.0	18.0	292.6	1.8	17.0	290.1	2.6
5	270.0	2.4	45.0	14.1	230.1	14.8	13.7	234.7	13.1
6	258.0	4.4	33.0	18.0	292.6	13.4	17.0	290.1	12.5
7	257.0	6.7	44.0	15.0	243.7	5.2	14.3	244.7	4.8
8	248.0	5.4	38.0	15.4	250.2	0.9	14.7	252.7	1.9
9	248.0	8.4	43.0	15.5	251.9	1.6	14.7	251.3	1.3
10	232.0	5.0	43.5	13.1	213.3	8.1	12.5	214.7	7.5
11	200.0	4.0	31.0	15.0	244.8	22.4	13.7	235.8	17.9
12	175.0	3.6	45.5	9.5	154.0	12.0	8.8	154.0	12.0
13	175.0	4.6	37.0	11.6	188.8	7.9	10.7	185.9	6.2
14	134.0	4.0	39.0	6.5	106.2	20.8	6.0	106.6	20.4
sub-sum						117.4			)7.9
15	112.0	4.8	43.0	6.5	106.5	5.0	6.2	109.8	2.0
17	102.0	4.4	45.0	6.4	104.4	2.3	5.8	103.6	1.6
18	101.9	3.8	42.0	6.2	101.2	0.7	5.7	102.4	0.5
19	98.7	5.3	41.0	6.5	105.6	7.0	6.0	106.4	7.8
20	98.5	3.7	38.0	6.4	104.2	5.8	5.5	99.5	1.0
21	95.6	4.3	38.0	6.2	101.4	6.1	5.7	102.3	7.0
22	92.5	5.6	44.0	6.0	98.0	6.0	5.5	98.5	6.4
23	89.9	5.2	40.0	5.4	87.6	2.5	5.0	90.5	0.7
24	89.6	7.1	44.0	5.7	92.3	3.0	5.1	91.9	2.6
25	89.4	6.4	51.0	5.0	81.8	8.5	4.8	87.0	2.6
26	87.0	3.9	41.0	5.4	88.1	1.3	5.0	90.2	3.7
27	86.5	3.4	36.0	5.4	87.7	1.4	4.9	89.6	3.6
28	85.0	4.1	41.0	5.1	82.8	2.6	4.5	81.7	3.9
29	84.8	5.4	45.0	5.3	86.9	2.5	4.7	85.4	0.7
30	82.2	9.0	47.0	5.3	86.2	4.9	4.6	83.8	2.0
31	81.0	6.6	41.0	5.1	83.3	2.9	4.6	83.9	3.5
32	80.2	4.7	42.0	4.7	76.4	4.7	4.1	75.8	5.4
33	78.0	3.7	42.0	4.5	73.0	6.4	4.0	74.4	4.6
34	72.3	3.2	42.0	4.5	73.8	2.0	4.0	74.0	2.4
35	70.5	6.2	51.0	4.5	72.4	2.6	3.9	72.0	2.1
36	69.0	2.7	33.0	4.4	71.4	3.5	3.8	70.6	2.3
37	67.0	4.0	39.0	4.1	66.5	0.8	3.5	65.0	3.0
sub-sum						86.8			73.8
sum						204.2			31.7

Table 3. The calculation of $[Glucose]_{Hct\text{-}corr.}$ for samples No. 11 & 6

No	System	$[Glucose]_{Hct\text{-}corr.}$;(mg dL^{-1})	BIAS %
11	$I_{(s+i)}$	$= \{ \frac{[15.0 - (18.599\, e^{-0.0156*31})]}{[(42.482\, e^{-0.0204*31}) - (18.599\, e^{-0.0156*31})]} * (318.0-170.4)\} + 170.4$ $\fallingdotseq$ 217.7	8.8
	ΔI	$= \{ \frac{[13.7 - (17.958\, e^{-0.0184*31})]}{[(42.023\, e^{-0.0219*31}) - (17.958\, e^{-0.0184*31})]} * (318.0-170.4)\} + 170.4$ $\fallingdotseq$ 217.7	8.8
	$[Glucose]_{YSI}$	200	
6	$I_{(s+i)}$	$= \{ \frac{[18.0 - (18.599\, e^{-0.0156*33})]}{[(42.482\, e^{-0.0204*33}) - (18.599\, e^{-0.0156*33})]} * (318.0-170.4)\} + 170.4$ $\fallingdotseq$ 266.7	3.1
	ΔI	$= \{ \frac{[17.0 - (17.958\, e^{-0.0184*33})]}{[(42.023\, e^{-0.0219*33}) - (17.958\, e^{-0.0184*33})]} * (318.0-170.4)\} + 170.4$ $\fallingdotseq$ 270.7	4.9
	$[Glucose]_{YSI}$	258	

Disposable amperometric immunosensing strips fabricated by Au nanoparticles-modified screen-printed carbon electrodes for the detection of foodborne pathogen *Escherichia coli* O157:H7

Abstract

A disposable amperometric immunosensing strip was fabricated for rapid detection of *Escherichia coli* O157:H7. The method uses an indirect sandwich enzyme-linked immunoassay with double antibodies. Screen-printed carbon electrodes (SPCE) were framed by commercial silver and carbon inks. For electrochemical characterization the carbon electrodes were coupled with the first *E. coli* O157:H7-specific antibody, *E. coli* O157:H7 intact cells and the second *E. coli* O157:H7-specific antibody-conjugated with horseradish peroxidase (HRP). Hydrogen peroxide and ferrocenedicarboxylic acid (FeDC) were used as the substrate for HRP and mediator, respectively, at a potential +300 mV *vs.* counter/reference electrode. The response current (RC) of the immunosensing strips could be amplified significantly by 13-nm diameter Au nanoparticles (AuNPs) attached to the working electrode. The results show that the combined effects of AuNPs and FeDC enhanced RC by 13.1-fold. The SPCE immunosensing strips were used to detect *E. coli* O157:H7 specifically. Concentrations of *E. coli* O157:H7 from 10^2 to 10^7 colony-forming units (CFU) mL^{-1} could be detected. The detection limit was approximately 6 CFU per strip in PBS buffer and 50 CFU per strip in milk. The SPCE modified with AuNPs and FeDC has the potential for further applications and provides the basis for incorporating the method into an integrated system for rapid pathogen detection.

Keywords: *Escherichia coli* O157:H7, screen-printed carbon electrode, immunosensor, electrochemistry, nanoparticles

1. Introduction

In the last 20 years, *Escherichia coli* O157:H7 has been an important foodborne pathogen in a variety of foods worldwide. This strain of *E. coli* is classified as an enterohemorrhagic bacterium with the ability to cause hemorrhagic colitis with symptoms such as bloody diarrhoea, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpurea (Ho et al., 2004). The monitoring of *E. coli* O157:H7 with conventional procedure (Johnson et al., 1995; Meng et al., 2001; Ho et al., 2004) could take 2-3 days. These methods are time-consuming, which delays the introduction of remedial measures. Therefore, a method for rapid detection of this pathogenic microorganism in food and water would aid the prevention of infection, illness, and economic loss.

The field of electrochemical biosensing has grown rapidly, and biosensors for detecting antigens and nucleic acids have been reported (Bakker, 2004; Mehrvar and Abdi, 2004). Several electrochemical methods for the detection of bacterial contamination are known; for example, the amount of cells electrostatically attaching on carbon electrodes (Morisaki et al., 2000), the detection of glucose catabolism (Palenzuela et al., 2004), or the endogenous enzymatic activity of bacteria (Yemini et al., 2007). Additionally, a broad class of immunosensing techniques with improved specificity also was reported (Patel, 2002; Rasooly and Herold, 2006). Pathogens have been detected using amperometric immunoassay procedures in which the immunoreagents are immobilized on the transducer surface (Zhou et al., 2002; Chemburu et al., 2005; Rao et al., 2006). Reproducible methods of quantifying *E. coli* O157:H7 were recently studied based on an immunoassay combined with a liposome-base fluorescent-labeling method (Ho et al., 2004), a quartz crystal Au piezoelectric electrode (Su and Li, 2004), a self-assembled monolayer-based surface plasmon resonance sensor (Subramanian et al., 2006), and a fluorescent tracer-digital camera image transport system (Ligler et al., 2007). With these methods, *E. coli* O157:H7 cells could be detected at concentrations between 10^2 and 10^8 colony-forming units (CFU) mL^{-1}. However, the expense of the sensor materials or infrequent measuring instruments would limit out-of-laboratory applications for economic and fast screening.

Due to recent developments in biosensor technology, horseradish peroxidase (HRP) are often conjugated with antigen-antibody complex (Zhou et al., 2002; Chemburu et al., 2005; Castañeda et al., 2007) on the electrodes of immunosensor as reporters which react with substrate to generate electrons to be detected. And the AuNPs modified electrodes also can be used to amplify the detection signal, improve the electron transducer and reduce the limitation

on detection in electrochemical biosensors (Daniel and Astruc, 2004; Willner et al., 2007). Here, we present an approach for the quantification of *E. coli* O157:H7 that combines amperometric detection with a disposable immunosensing strip. The system was designed for the rapid, sensitive detection of bacterial cells, such as *E. coli* O157:H7, utilizing AuNPs and ferrocenedicarboxylic acid (FeDC)-modified, disposable screen-printed carbon electrode (SPCE).

2. Materials and methods

2.1 Apparatus

Cyclic voltammetric and amperometric determinations were performed with a CV50W voltammetric analyzer (Bioanalytical Systems, West Lafayette, IN, USA) connected to a personal computer and a PalmSens/Personal Digital Assistant, hand-held, battery-powered instrument (Palm Instruments, BZ Houten, Netherlands) for data collection and calculation.

2.2 Reagents and solutions

G-451 carbon/graphite ink and silver-resin inks were purchased from ERCON (Wareham, MA, USA). Sodium citrate, ethanol, and H_2O_2 were obtained from Merck (Darmstadt, Germany). FeDC, chlorauric acid ($HAuCl_4$), glutaraldehyde, glycerol, glycine, sodium chloride (NaCl), sodium carbonate, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), bovine serum albumin (BSA), Triton X-100, phosphate-buffered saline (PBS) and were obtained from Sigma-Aldrich (St. Louis, MO, USA). Monoclonal anti-*E. coli* O157:H7 antibody (ab20976) and horseradish peroxidase-conjugated polyclonal anti-*E. coli* O157:H7 antibody (ab20425) were purchased from Abcam (Cambridge, UK). The buffer or solutions used in this study were prepared as follows: PBS buffer (0.1 M PBS, pH 7.2), blocking buffer (67 μM glycine in 0.1 M PBS buffer, pH 7.2), substrate solution (88 μM H_2O_2 in 0.1 M PBS buffer, pH 7.2) and FeDC solution (5 mM FeDC in 95% ethanol).

2.3 Preparation of AuNPs

The AuNPs were prepared as described by Lei et al. (2004), but with minor modifications. A volume of 2.5 mL of 38.8 mM sodium citrate was quickly added to 25 mL of boiling 1 mM $HAuCl_4$ with vigorous stirring for 10 min, giving a color change from blue to red-violet. The mixture was boiled continuously and stirred for another 10 min and then removed from the heater. Colloidal Au particles with an average diameter of 13-nm were produced. The prepared

AuNPs were stored at 4ºC.

2.4 Fabrication of AuNPs-modified SPCE (SPCE/AuNPs)

An electrochemical immunosensing strip based on SPCE was made (Fig. 1, lower half) by printing the SPCE strips sequentially with silver-resin inks and carbon/graphite inks, using a system consisted of automated screen-printer and oven (BUILT-IN Corp, Taichung, Taiwan) to print electrodes and dry organic solvent in inks. The SPCE consisted of a 9.8 mm^2 carbon surface working electrode and a 9.8 mm^2 carbon surface counter/reference electrode. The distance is 0.5 mm between the two electrodes. A volume of 10 μL of 2.5 mM glutaraldehyde (Hart et al., 2005), was dropped onto the working electrode area, incubated for 1 h at 4°C and washed twice with PBS buffer. A volume of 10 μL volume of AuNPs solution was dropped onto the glutaraldehyde-treated working electrode, incubated for 1 h at 4°C, and then washed twice with PBS buffer.

2.5 Preparation of microbial sample

The bacterial cultures, including *E. coli* O157:H7 (ATCC 43894), *E coli.* K12 (ATCC 15153), *Listeria monocytogens* (ATCC 19114), *Salmonella choleraesuis* (ATCC 13311) and *Vibrio parahaemolyticus* (ATCC 27519), used in this study were obtained from the Bioresources Collection and Research Center (Food Industry Research and Development Institute, Hsinchu, Taiwan). The pure cultures were grown in brain/heart infusion broth (Difco Laboratories, Detroit, MI, USA) at 37°C for 24 h before use. A conventional spread-plating method was used for bacterial counts.

For the detection using real samples, the cultured *E. coli* O157:H7 cells were inoculated into sterilized milk and subsequently treated by the method (Yamaguchi et al., 2003) for eliminating lipids and proteins. Briefly, proteinase K (0.25 mg; Promega, Madison, WI, USA) and 50 μL of 0.1% Triton X-100 were added to 100 μL samples of milk, and then incubated at 37°C for 10 min. After incubation, 900 μL of 150 mM $NaCl_{(aq)}$ was added to the samples and the mixture was centrifuged at 12,000 × *g* for 10 min. The bacteria-containing pellets were collected and re-suspended in 100 μL of 150 mM $NaCl_{(aq)}$ and used for detection of bacteria.

2.6 Immunosensor fabrication, recognition and sensing procedures

Each working electrode of the SPCE/ AuNPs was incubated with 10 μL of the monoclonal

anti-*E. coli* O157:H7 antibodies (100 ng mL^{-1}) for 15 min at 37°C, coated with 10 μL of FeDC solution for 10 min at 42°C, and blocked with 10 μL of blocking buffer for 60 min at 4°C (Fig. 1, upper half). Between each step, each electrode was washed twice with PBS buffer. A volume of 10 μL sample was applied to the immunosensing strip on the working electrode and incubated for 15 min at 37°C. A volume of 10 μL of HRP-conjugated polyclonal anti-*E. coli* O157:H7 antibody (100 ng mL^{-1}) was applied to the immunosensing strip and incubated for 15 min at 37°C. The electrodes then were washed twice with PBS buffer to remove non-specific binding between antigens and antibodies. All amperometric experiments were performed in 10 μL of substrate solution applied to the electrochemical reaction area of the AuNPs/FeDC-SPCE immunosensing strip and incubated for 40 s at 25°C. A fixed potential of +300 mV *vs.* counter/reference electrode was applied after the incubation period. Two kinds of current signals were measured in this study: (i) the response current (RC) (sum of current signals collected per 0.1 s during the following 50 s); and (ii) ΔCurrent (difference in the average RC when bacteria were present and the average RC when bacteria were absent).

There were five group, groups a to f, to investigate the effects of different modification for immunosensing detection of SPCE strips. Groups a to f, were with the first monoclonal anti-*E. coli* O157:H7 antibody attached. Groups a and b were fabricated without AuNPs and FeDC, i.e., [AuNPs(–)/FeDC(–)]. Group c was fabricated with FeDC but without AuNPs [AuNPs(–)/ FeDC(+)] and groups d and e were fabricated with AuNPs but without FeDC [AuNPs(+)/ FeDC(–)]. Group f was fabricated with both AuNPs and FeDC [AuNPs(+)/FeDC(+)]. A volume of 10 μL sample containing the intact cells of *E. coli* O157:H7 (6.36×10^7 CFU mL^{-1}) were incubated in the immune-recognizant processes for groups b, c, e and f [*E. coli* O157:H7(+)] and the sample that contained no bacteria was used for groups a and d [*E. coli* O157:H7(–)]. Later, all groups were treated with the HRP-conjugated polyclonal anti-*E. coli* O157:H7 antibody.

2.7 Measurement procedure for plate ELISA

The plate ELISA was performed according to the indirect sandwich method (Kim et al., 2005) with slight modifications. Each well of a Maxisorb plate (Nunc, Naperville, IL, USA) was coated with a volume of 100 μL of monoclonal anti-*E. coli* O157:H7 antibody (1 μg mL^{-1}) prepared in carbonate buffer (50mM, pH 9.6) and incubated overnight at 4°C. After washing with PBS buffer, a volume of 200 μL of 3% BSA was added to block for 30 min at 25°C. After washing, a volume of 100 μL of PBS buffer containing bacteria and 3% BSA was added and

incubated for 30 min at 25°C. After washing, a volume of 100 μL of HRP-conjugated polyclonal anti-*E. coli* O157:H7 antibody (200 ng mL^{-1}) was added and incubated for 30 min at 25°C. After washing again, the enzymatic reaction was developed using ABTS as the substrate for 30 min at 25°C. The absorbance at 450 nm was measured with an ELISA plate reader (Molecular Devices Corp., Sunnyvale, CA, USA).

2.8 Safety considerations

All bacteria were handled according to the rules appropriate for biosafety level 2 microorganisms (no special hazard) as defined by the U.S. Centers for Disease Control and Prevention.

2.9 Data analysis

Each experiment was repeated five times using five different SPCE strips to test the reproducibility of the immunosensing strips. All data with mean signals and ± standard deviation (SD). Differences between groups were evaluated by the two-tailed Student's *t*-test, and $P \leq 0.05$ was considered statistically significant.

3. Results

3.1 Effect of AuNPs modification of SPCE on electrochemical characters

The results of SPCE/AuNPs and an *E. coli* O157:H7 cell bound to antibodies immobilized on the surface of a SPCE/AuNPs were confirmed by scanning electron microscope (SEM) (Fig. 2). The size of AuNPs was very uniform and approximately 13-nm in diameter on average. There were 73.6 ± 8.1 AuNPs per 0.04 μm^2 of SPCE working surface in five random samplings from the SEM images.

In other studies, FeDC was used as the mediator in the H_2O_2-peroxidase electrochemical reaction and the biosensor operated at 150-420 mV (Wring et al., 1991; Sadeghi et al., 1997). Therefore, a non-enzymatic FeDC redox system was investigated at a scan rate of 100 mV s^{-1} and a cyclic scan range of –300 mV to +300 mV to verify the effects on electrochemical characteristics of the SPCE/AuNPs. The effects of AuNPs and FeDC modification on a typical cyclic voltammogram (CV) were evaluated using 10 μL of substrate solution. On the SPCE, modification with FeDC (line c in Fig. 3), but not AuNPs (line b in Fig. 3), the CV line could be detected in the presence of H_2O_2. Moreover, the peaks of oxidation and reduction current

(i.e. CV behavior) of the SPCE treated simultaneously with FeDC and AuNPs were significantly increased (line d in Fig. 3) as using only FeDC.

3.2 Amperometric characterization of *E. coli* O157:H7 immunosensing strip

The results of RC obtained with the amperometric immunosensing processes of groups a to f were shown in Fig. 4. Values of RC for groups a, b and c were similar. In contrast, value of RC for group d was higher than those in groups a, b and c ($P < 0.01$). Values of RC were much higher for groups e and f than for group d. Additionally, the value of RC was higher for group f than for group e. Although the surface of SPCE/AuNPs could elevate the electrochemical response of the background by around 3.4-fold compared to the SPCE without AuNPs modification (group d *vs.* group a), the benefit of amplification using AuNPs modification was as much as 10.7–fold increased for *E. coli* O157:H7 (group e *vs.* group b). The amplification benefit of both AuNPs and FeDC modification (SPCE/AuNPs/FeDC) was 1.2–fold higher (group f *vs.* group e), and the benefit of FeDC modification was up to 1.1–fold higher (group c *vs.* group b). The enhancing effects of FeDC were limited in the SPCE used to detect *E. coli* O157:H7 in the immunosensing strips. Together, AuNPs and FeDC modifications could enhance the RC in the SPCE immunosensing by 13.1–fold (group f *vs.* group b) compared with unmodified electrodes.

3.3 Detection limits and specificity of *E. coli* O157:H7 immunosensing strips

Seven *E. coli* O157:H7 concentrations, from 5.75×10^1 to 5.75×10^7 CFU mL^{-1} were prepared by serial dilution in PBS buffer. A 10 μL sample of each bacterial suspension (i.e. approximately from 6×10^0 CFU to 6×10^5 CFU of *E. coli* O157:H7 cells used in each detection per strip) were taken for immunosensing to evaluate the detection range by calculation of ΔCurrent. The values of ΔCurrent of the SPCE/AuNPs/FeDC immunosensing strips that were used to detect the different concentrations of *E. coli* O157:H7 cell are shown in Fig. 5. The results show that the concentration of bacteria and ΔCurrent were highly correlated. The linear relationship was well described ($R^2 = 0.9771$) by ΔCurrent (μA) = 25.081 (log CFU mL^{-1} of *E. coli* O157:H7) – 66.171. The lower limit of detection was approximately 6×10^2 CFU mL^{-1} in PBS buffer and around 6 CFU of *E. coli* O157:H7 cells per strip.

An approximately 5×10^7 CFU mL^{-1} sample of *E. coli* O157:H7, *E. coli.* K12, *Listeria monocytogens*, *Salmonella choleraesuis* and *Vibrio parahaemolyticus* cultures was used to evaluate the specificity of the AuNPs/FeDC-SPCE immunosensing strips. In the case of *E. coli*

O157:H7, ΔCurrent reached 140.0 (± 14.7) μA shown in Fig. 6. However, the values of RC for the bacteria other than *E. coli* O157:H7 were similar to the values obtained in the absence of bacteria, indicating that there was no obvious cross-reaction of *E. coli* O157:H7-specific immunosensing strips with other bacterial species or strains.

3.4. Detection of *E. coli* O157:H7 cells in real samples

The cultured *E. coli* O157:H7 cells were inoculated into milk at concentrations of 0, 5.03×10^2, 5.03×10^3, 5.03×10^4 and 5.03×10^5 CFU mL^{-1}. These samples were used to detect *E. coli* O157:H7 by the SPCE/AuNPs/FeDC immunosensing strips and by the plate ELISA method. A volume of 10 μL sample of each concentration was evaluated. Thus, for each sample, 0, $\sim 5 \times 10^1$, $\sim 5 \times 10^2$, or $\sim 5 \times 10^3$ CFU of *E. coli* O157:H7 was applied to the immunosensing strip and the plate ELISA test. Using a concentration of *E. coli* O157:H7 with $> 5.03 \times 10^3$ CFU mL^{-1} (i.e., approximately 50 CFU per strip), the detected values of ΔCurrent were significantly higher than background (i.e., without bacteria). The values of ΔCurrent and the concentrations of *E. coli* O157:H7 from $\sim 5 \times 10^3$ to $\sim 5 \times 10^5$ CFU mL^{-1} had a positive linear correlation shown in Fig. 7. Using the plate ELISA detection the absorbance values did not vary among samples containing 0, $\sim 5 \times 10^1$, $\sim 5 \times 10^2$, $\sim 5 \times 10^3$ CFU mL^{-1} of *E. coli* O157:H7. The absorbance value differed significantly from the background only for the milk sample with a concentration of *E. coli* O157:H7 of approximately 5×10^5 CFU mL^{-1} (i.e., 5×10^3 CFU per plate well).

4. Discussion

The SPCE/AuNPs/FeDC strips were investigated in an effort to develop a rapid detection protocol for monitoring *E. coli* O157:H7 contamination in food. The recognition and sensing procedures for the *E. coli* O157:H7 immunosensing were designed without pre-incubation or pre-filtration of samples to increase or concentrate the intact cells of the pathogen. After the bacteria were recognized and bound by the first monoclonal anti-*E. coli* O157:H7 antibodies on the surface of the SPCE immunosensing strip, the HRP-conjugated polyclonal anti-*E. coli* O157:H7 antibodies were sequentially put onto the working electrode for immune recognition. Finally, HRP reacted with H_2O_2 to generate electrodes to be detected. The total analysis, including recognition, washing, and sensing processes, took ~1 h for each test. The sensitivity and specificity of the SPCE/AuNPs/FeDC immunosensing strips were examined and shown to be comparable to other conventional plate ELISA methods and biosensors.

In some biosensing methods, the major applications of Au materials, including AuNPs, have functions of conjugation (Castañeda et al., 2007), immobilization (Subramanian et al., 2006), reporters (Tang et al., 2007), or electrode/conductors (Radke and Alocilja, 2005).The electrodes modified with AuNPs were used for the detection of H_2O_2 with the peroxidase mechanism. In other studies, AuNPs with an average diameter of 20-nm (Xu et al., 2006) and 16.8-nm (Tangkuaram et al., 2007) were used to increase the sensitivity and stability of biosensors. In the present study, the results show that 13-nm diameter AuNPs on the SPCE improved electrochemical ability for oxidation/reduction and effectively shuttle electrons between the FeDC mediator and the SPCE. The AuNPs on our SPCE immunosensing strips increase the effective area of the working electrode and, as reported earlier, decrease, in part, the distances for the reductive-form mediator to diffuse to the stereo-electrode structures (Liang et al., 2005; Tang et al., 2007). In addition, a donor–acceptor pair model can explain the observed increase in the rate of electron transfer between redox enzymes and electrodes caused by AuNPs (Willner et al., 2007). Therefore, we supposed that the SPCE used in this study may have a large specific surface area and excellent conductivity due to the attached AuNPs. These attached AuNPs might overcome the long-range barriers to the formation of complexes of antibodies and bacterial cells that deliver an electron via peroxidase to the electrode.

There have been several reports of *E. coli*-specific biosensors; for example, a flow-through immunofiltration system (Abdel-Hamid et al., 1999), a PAPG/β-galactosidase method (Boyac et al., 2005), and a real-time detection of DNA sequences using a circulating-flow system with a quartz crystal microbalance (Wu et al., 2007). But complex pre-culture, cell lysing, and DNA isolation, or sensitive instruments are needed for these methods to enhance specificity or sensitivity. Recently, a few *E. coli* intact cell immunosensors have been reported with electromechanical or amperometric methodology. An impedance-dependent biosensor, which was developed with an interdigitated microelectrode array, was able to detect *E. coli* O157:H7 cells specifically at concentrations of 10^4-10^7 CFU mL^{-1} (Radke and Alocilja, 2005). A flow-through sandwich amperometric immunoassay was utilized in which intact cells were captured by antibodies immobilized on activated carbon particles, then labeled with HRP-conjugated antibodies. The quantitative working range for *E. coli* was 10-1,500 CFU mL^{-1} (Chemburu et al., 2005). In the present study, the AuNPs modification and indirect sandwich immunoassay were combined on the SPCE for immunosensing *E. coli* O157:H7 intact cells. Concentrations of *E. coli* O157:H7 in PBS buffer from 6×10^0 to 6×10^5 CFU per strip could be determined precisely using the immunosensing strips. The results indicate that

the *E. coli* O157:H7-specific immunosensors fabricated with SPCE/AuNPs/FeDC were more useful for the detection of pathogen in milk than the plate ELISA method. Moreover, the estimated detection limit of the immunosensing strips was around 50 CFU of *E. coli* O157:H7 in milk. That is, the detection limit for *E. coli* O157:H7 was about 50 CFU per strip (i.e., 5×10^3 CFU mL^{-1}) in the real sample, indicating that the detection limitation should be improved when the SPCE/AuNPs/FeDC immunosensing strips are used to detect pathogens in real samples. We propose that the protocol for bacterial cells isolated from real samples needs to be modified to increase the recovery efficiency.

The SPCE/AuNPs/FeDC immunosensing strips required a secondary labeled antibody for the amperometric detection. However, our device has the potential for commercialization because it uses a low-cost disposable electrode, it is hand-held operation, it is highly specific, and no pretreatment is needed for fast screening for *E. coli* O157:H7 contamination. The results obtained with the *E. coli* O157:H7 immunosensing strips for the detection of bacterial contamination in food are comparable to those from the plate ELISA method and other biosensing methods (Ho et al., 2004; Radke and Alocilja, 2005; Subramanian et al., 2006; Wu et al., 2007). The amperometric immunosensor for *E. coli* O157:H7 detection could be improved if the system was integrated with a lateral-flow method for rapid screening. However, the development of *E. coli* O157:H7 biosensors should continue in the pursuit of a method of detecting low levels of bacterial contamination, because a dose of *E. coli* O157:H7 as low as 10 organisms in a real sample may be harmful to humans.

5. Conclusions

Here, we describe a rapid, specific, and sensitive electrochemical technique for the detection of intact cells of *E. coli* O157:H7. The technique is based on an indirect sandwich amperometric immunoassay. The disposable SPCE/AuNPs/FeDC immunosensing strips, the key element of this detection system, were fabricated by carbon inks and modified with 13-nm diameter AuNPs and FeDC. Amperometric detection was selected because of its many benefits, including its adaptability for use in miniaturized and portable systems. The method uses a sensitive detection of HRP activity coupled with AuNPs and FeDC to amplify the amperometric effect. Interference from other bacteria was eliminated by the use of doubly specific antibodies. One of the important advantages of our SPCE/AuNPs/FeDC immunosensing strips is that approximately 50 CFU of *E. coli* O157:H7 in samples of milk can be detected in 1 h. The SPCE/AuNPs/FeDC immunosensing strip system has a potential for

further applications and provides the basis for incorporating the method into an integrated system for rapid pathogen detection.

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Figures

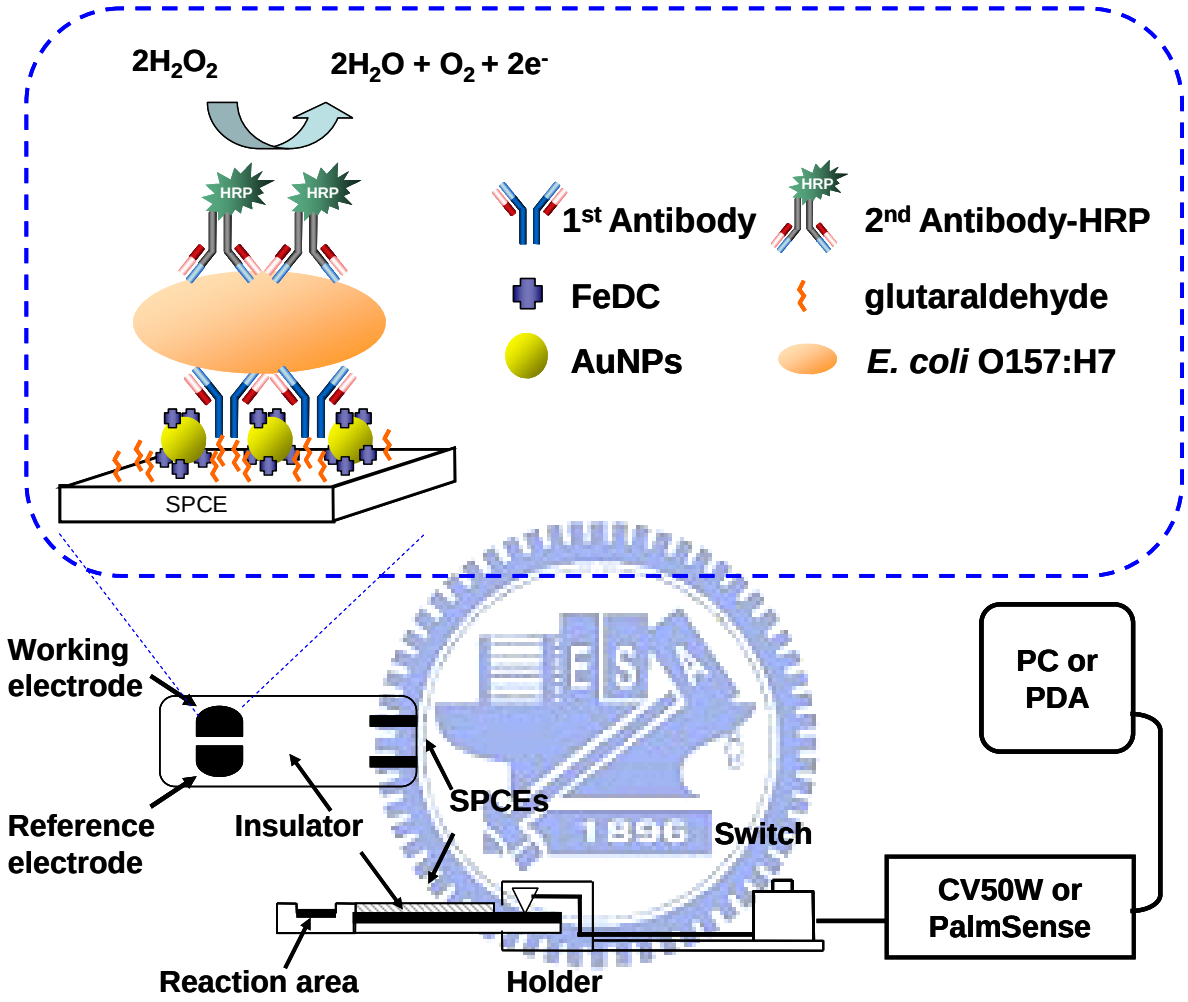

Fig. 1 A diagram of the apparatus and immunosensing processes of the AuNPs/FeDC-SPCE immunosensor system for *E. coli* O157:H7 detection. The AuNPs and FeDC were modified on the working electrode and the detection model was shown in the top panel.

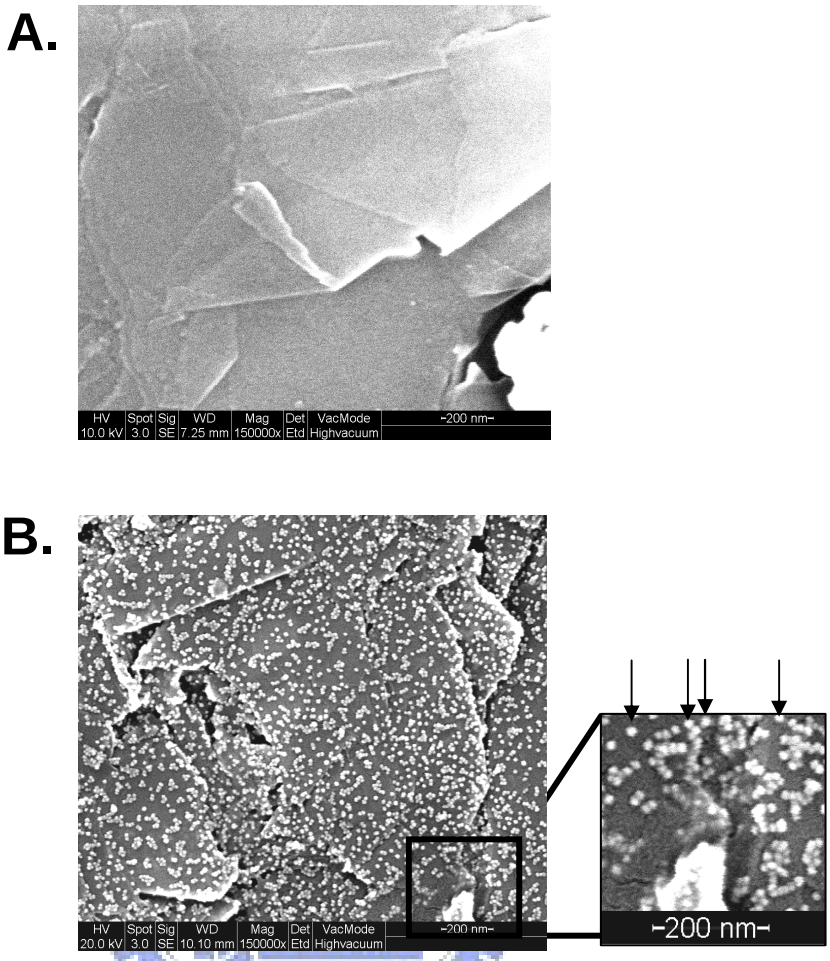

Fig. 2 Scanning electron micrographs of the SPCE. The surface of an unmodified SPCE (A), and the surface of an AuNPs-modified SPCE (B) are shown. The AuNPs attached on the surface of the SPCE are indicated by arrows. The average diameter of the AuNPs was 13-nm.

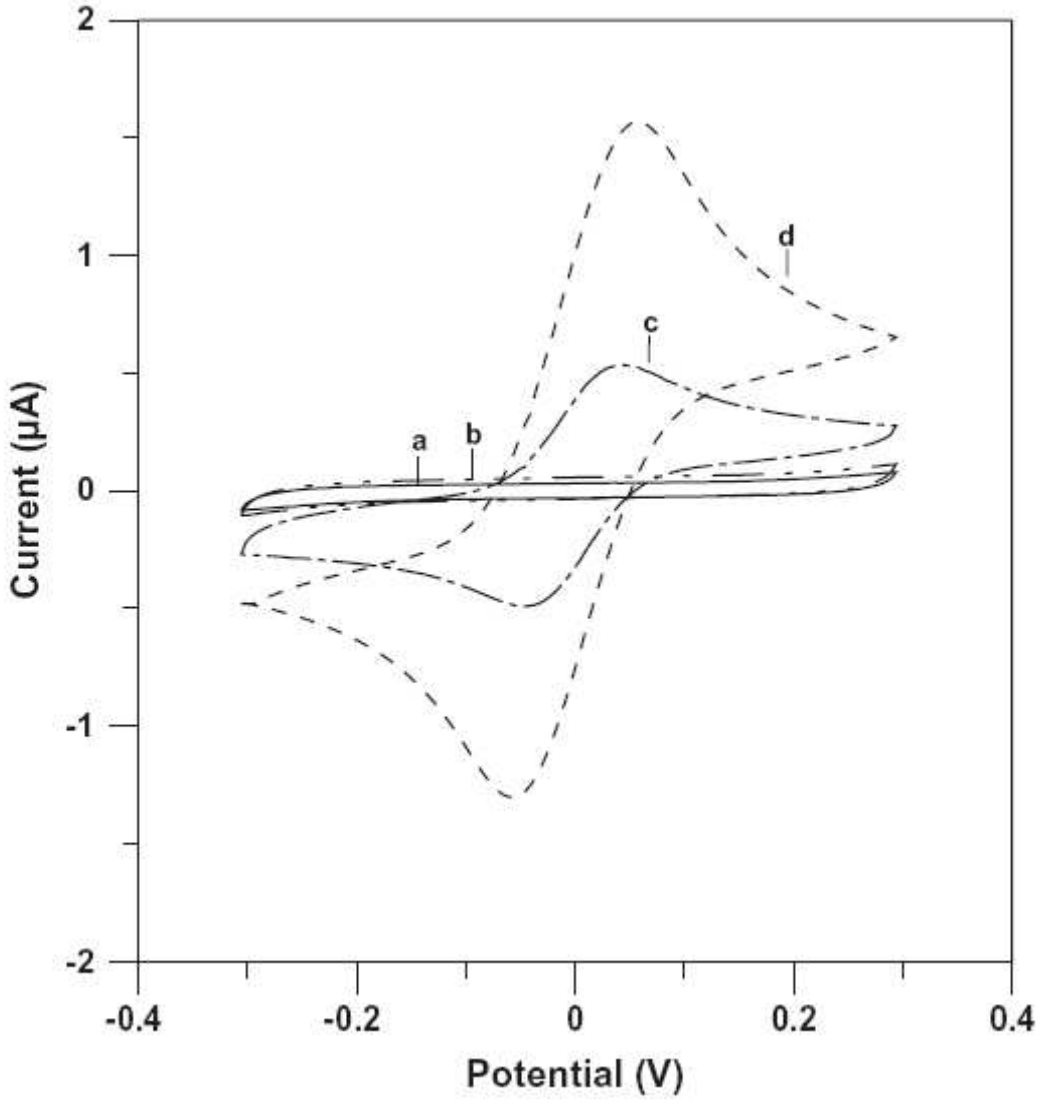

Fig. 3 Cyclic voltammograms (CV) of the SPCE strips. The typical CV was obtained in 88 μM H_2O_2 in PBS buffer, which was used as a supporting electrolyte for the SPCE formed under the following conditions: a, unmodified SPCE; b, AuNPs-modified SPCE; c, FeDC-modified SPCE; d, both FeDC- and AuNPs-modified SPCE. All of the CVs were detected under the same potential step from –300 to +300 mV *vs.* counter/reference electrode. All of scan rate was 100 mV/s.

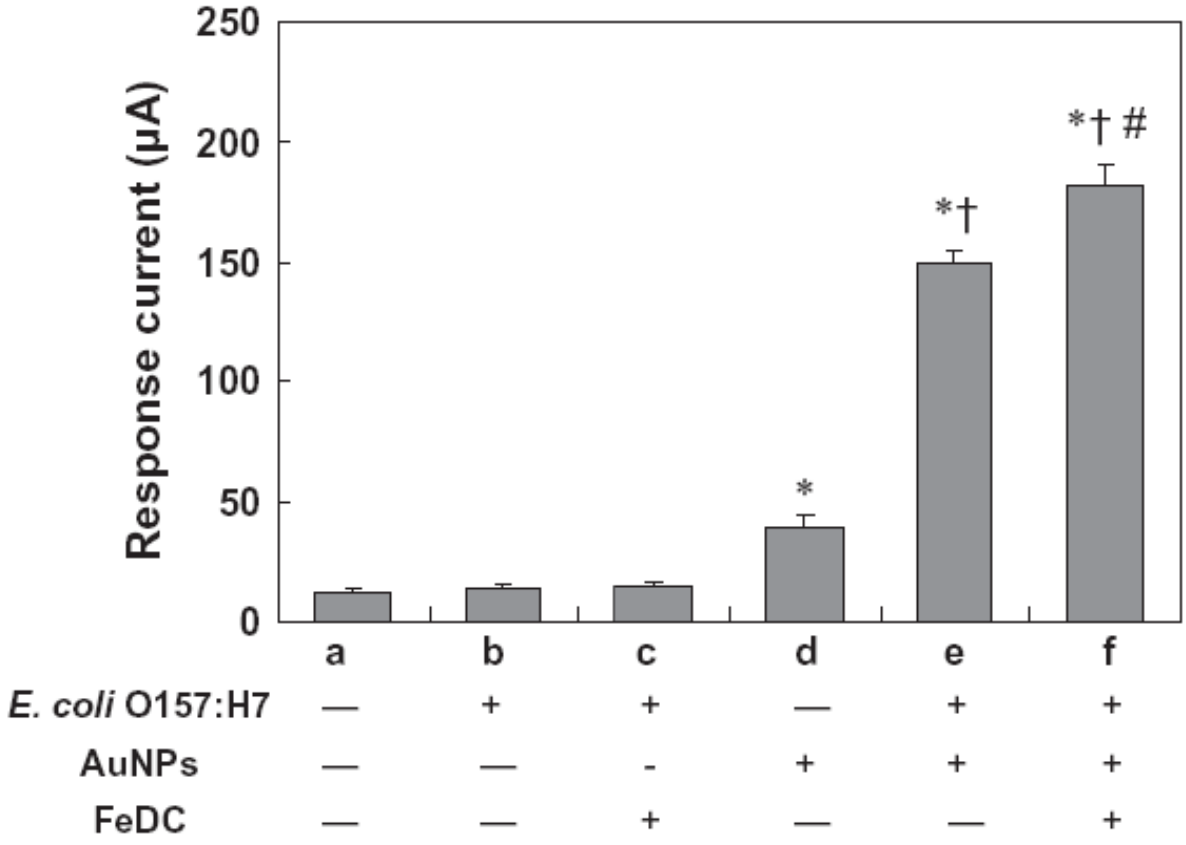

Fig. 4 Effects of AuNPs and FeDC modification on the response current in the SPCE immunosensing strips. The values of response current for the groups a to e are shown. A potential of +300 mV was applied to each working electrode (*vs*. counter/reference electrode). Each value is the mean of five independent measurements; * indicates $P < 0.01$ *vs.* groups a, b and c; † indicates $P < 0.01$ *vs.* groups a, b, c and d; # indicates $P < 0.01$ *vs.* groups a, b, c, d and e.

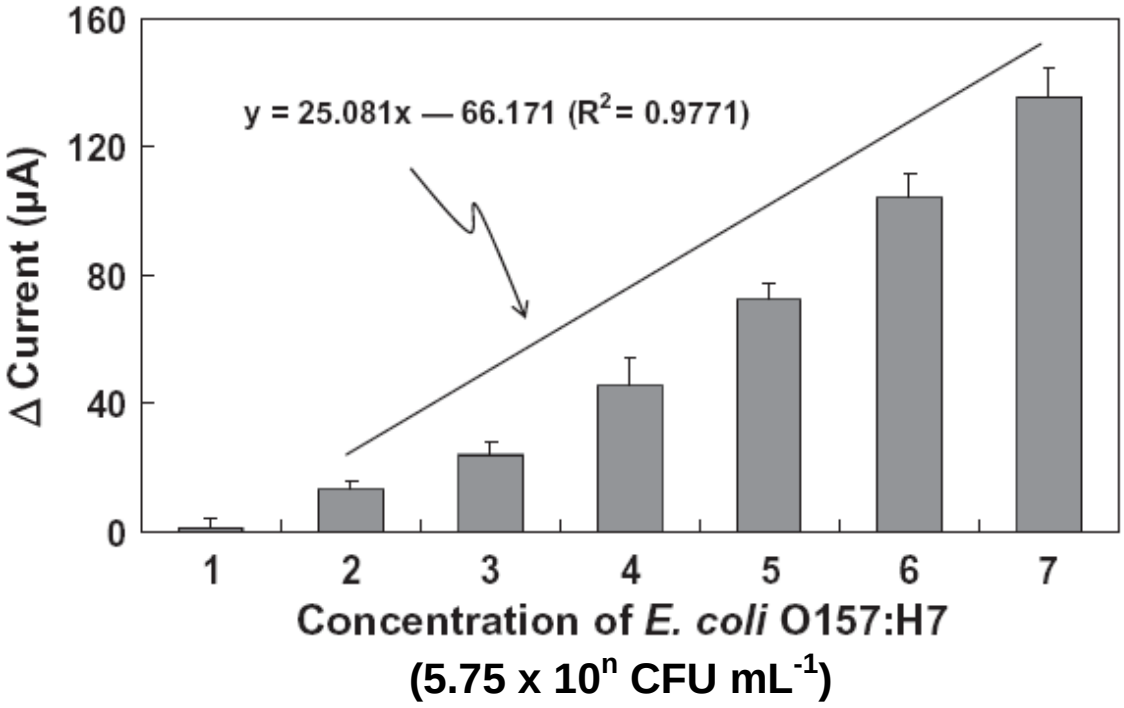

Fig. 5 The ΔCurrent due to the different concentrations of *E. coli* O157:H7 cells detected by the SPCE/AuNPs/FeDC immunosensing strips. ΔCurrent was calculated by $RC_{Bacteria(+)} - RC_{Bacteria(-)}$, where $RC_{Bacteria(+)}$ and $RC_{Bacteria(-)}$ are the mean of RC in the presence and in the absence of bacteria, respectively. Each value of RC was derived from five independent measurements. The detection limit of the immunosensing strips was approximately 6×10^2 CFU mL^{-1}; i.e., approximate 6 CFU $strip^{-1}$. A positive linear correlation was found between ΔCurrent and log *E. coli* O157:H7 cell concentrations from 5.75×10^2 to 5.75×10^7 CFU mL^{-1}.

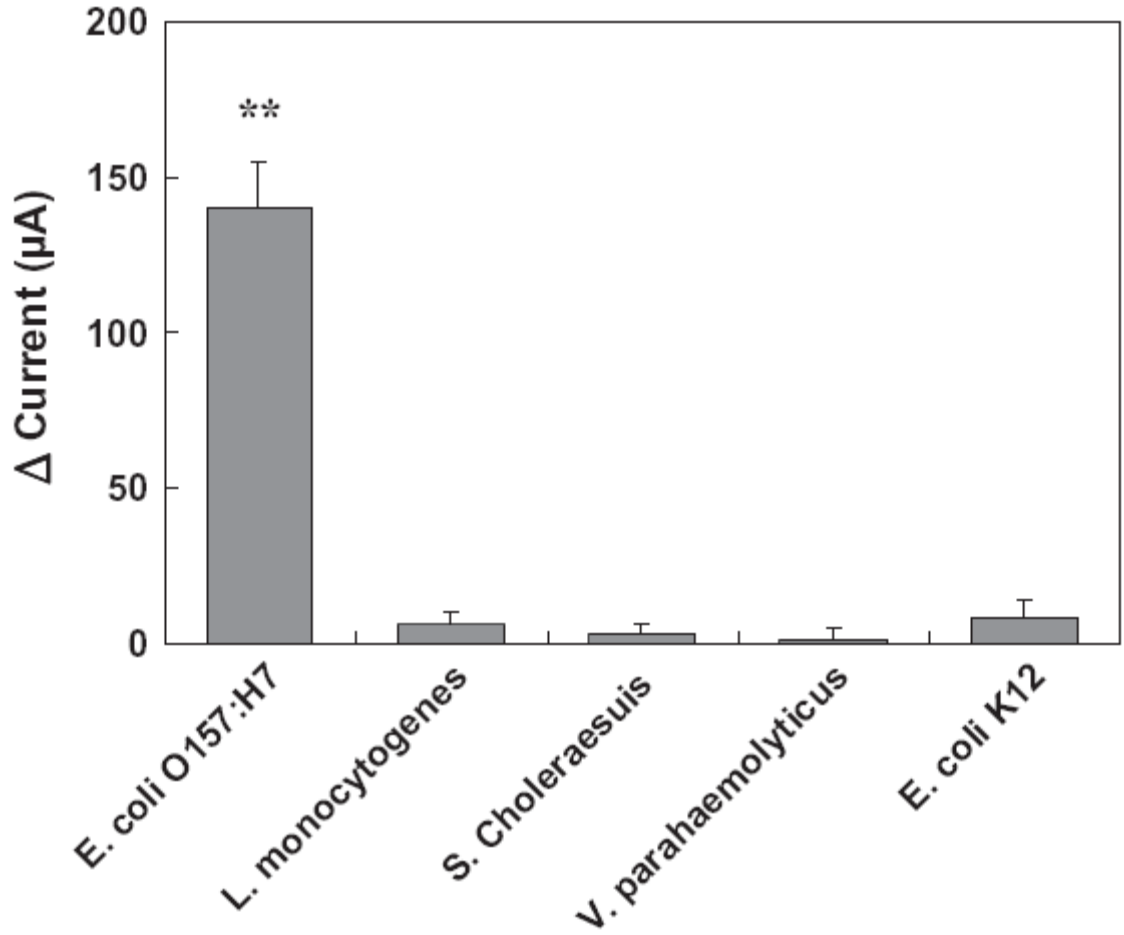

Fig. 6 Specificity of *E. coli* O157:H7 detection by the SPCE/AuNPs/FeDC immunosensing strip. The cultured bacteria, *E. coli* O157:H7, *Listeria monocytogens*, *Salmonella choleraesuis*, *Vibrio parahaemolyticus* and *E. coli.* K12 cultures at a concentration around 5×10^7 CFU mL^{-1}, were applied to the detection by the immunosensing strip. Each value was derived from five independent detections. ** indicates $P < 0.01$ *vs*. the groups of *L. monocytogens*, *S. choleraesuis*, *V. parahaemolyticus* and *E. coli.* K12.

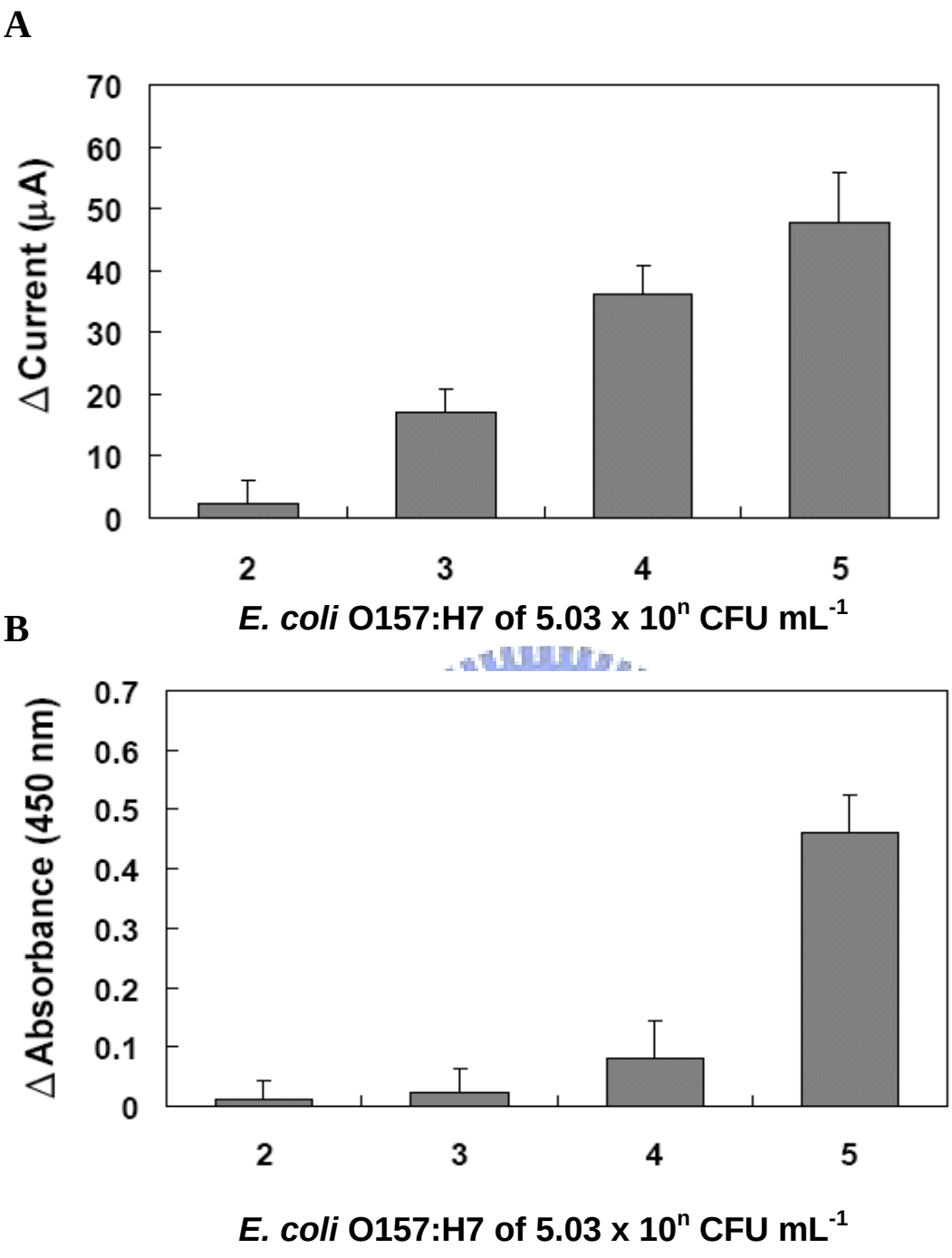

Fig. 7 Detection of *E. coli* O157:H7 in milk. Milk was inoculated with *E. coli* O157:H7 cells at concentrations of 0 (blank), 5.03×10^2 to 5.03×10^5 CFU mL^{-1}. The mean of each ΔCurrent (μA) was calculated from five independent measurements of SPCE/AuNPs/FeDC immunosensing strips. In the detection by the immunosensing strip, the values of ΔCurrent and the concentrations of *E. coli* O157:H7 from 5.03×10^3 to 5.03×10^5 CFU mL^{-1} have a positive linear correlation.

Amperometric determination of carbofuran and glutathione using screen-printed carbon electrode strips modified with gold nanoparticles, ferrocenedicarboxylic acid and tyrosinase

Abstract

Disposable amperometric screen-printed carbon electrode (SPCE) strips modified with gold nanoparticles (AuNPs, 13 nm diameter), ferrocenedicarboxylic acid (FeDC, as mediator) and tyrosinase (i.e. SPCE/AuNPs/FeDC/Tyr strips) were prepared for rapid determination of carbofuran and glutathione (GSH), at a potential -300 mV *vs.* counter/reference electrode. When catechol (400 μM) is used as the substrate for tyrosinase, the response current (RC) of amperometric measurement using SPCE/AuNPs/FeDC/Tyr strips is enhanced by 1.53 fold as compared to that without AuNPs and FeDC modifications. Carbofuran competes against catechol binding with tyrosinase and results in decreased RC which is proportional to [carbofuran], with a working linear concentration range 4.52-45.2 nM. On the other hand, GSH reacts with *o*-quinone (i.e. the oxidized product of catechol) and also results in decreased RC, with a working linear concentration range 12.5-100 μM in phosphate buffer. These methods have been successfully applied to real samples containing carbofuran and GSH in the extracts of lettuce leaves and blood samples of mice treated with N-acetylcysteine, respectively.

Keywords: screen-printed carbon electrode, tyrosinase, biosensor, electrochemistry, Au nanoparticles, ferrocenedicarboxylic acid, carbofuran, glutathione

1. Introduction

The research field of enzyme-based electrochemical biosensors development has grown rapidly with many analytical applications in clinical diagnosis, food industry, agricultural and environmental monitoring and bioassay (Cremisini et al., 1995; Newman and Turner, 2005; Mazloum-Ardakani et al., 2005; Lin et al. 2008; Zhai et al., 2009). These biosensors are promising alternatives for traditional methods by spectrophotometry and enzyme-linked immunosorbant assay (ELISA) due to their relatively low-cost, miniaturization and rapid procedures (Dennison et al., 1995).

Organophosphates and carbamates are two of cholinesterase inhibitors and represent two groups of the most commonly pesticides used because of their relatively low half-lives in the environment, although some of them exhibit high acute toxicity (Bachmann et al., 2000). Since these pesticides are toxic even at low concentrations, their on-line field determinations become especially important. Previous determinations of cholinesterase inhibitors using electrochemical biosensors were based on the inhibition of cholinesterases catalyzed thiocholine ester hydrolysis reactions (Solná et al., 2005), coupled with choline oxidation catalyzed either by choline oxidase (Cremisini et al., 1995) or by direct electrochemical means (Mulchandani et al., 2001).

Recently, the use of tyrosinase biosensors has been reported for the determination of oganophosphorus, carbamates and phenols (Sapelnikova et al., 2003; Campanella et al., 2007; Tanimoto de Albuquerque and Ferreira, 2007; Kochana et al., 2008). Tyrosinase contains an active binuclear copper center and catalyzes the oxidation of phenol to catechol and then to *o*-quinone and the reduced tyrosinase is oxidized by oxygen to its native form with the production of water (Kim and Uyama, 2005). Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) is one of the most toxic carbamate pesticides. An acetylcellulose-graphite composite tyrosinase carbon past electrode modified with cobalt(II) phthalocyanine for the determination of carbofuran was reported (Tanimoto de Albuquerque and Ferreira, 2007). However, this method required a large amount of tyrosinase (~60U) for the determination of carbofuran in a linear concentration range of 2.26-40.68 nM.

On the other hand, reduced glutathione (GSH), a tripeptide (L-*g*-glutamyl-L-cysteinyl-glycine), plays very important roles in various organisms (Haddad, 2002). GSH not only acts as a major antioxidant within the cells maintaining a tight control of various redox events, but it also acts as mediator of many physiological reactions. These include cellular signaling (e.g., in cell cycle regulation, proliferation and apoptosis) and metabolism of xenobiotics, thiol disulfide

exchange reactions, as well as being an important reservoir of cysteine (France et al., 2007). The concentration of GSH in human blood is ~1 mM (Mills and Lang, 1996). A number of analytical methods have been developed for the determination of GSH in biological samples by employing high performance liquid chromatography (Willian et al., 2001), capillary electrophoresis (Wang and Chen, 1995), gas chromatography (Takagi et al., 1996) and spectrophotometry (Shah et al., 1995). These methods offer good selectivity and detection limits. However, these analyses require expensive analytical instruments and the procedures are extensive and time-consuming. A biosensor based on the enzymatic reactions of glutathione reductase and sulfhydryl oxidase for the determination of GSH was reported with limited sensitivity in a linear [GSH] range of 0.2-10 mM (Timur et al., 2008). Another biosensor using immobilized tyrosinase carbon past electrode required a huge amount of tyrosinase (~4000 U) to determine 30 μM GSH (Huang et al., 2002).

Use of redox mediators (e.g., cobalt phthalocyanine and ferrocyanide) and gold nanoparticles (AuNPs) have been found to improve the electron transfer processes and lower the detection limits for some electrochemical biosensors (Tanimoto de Albuquerque and Ferreira, 2007; Rao et al., 2006; Lei et al., 2004). In this paper, we report our studies of using AuNPs and a mediator, ferrocenedicarboxylic acid (FeDC), modified tyrosinase screen-printed carbon electrode (SPCE) strips for the determination of carbofuran and GSH; and better sensitivities have been obtained as compared to those previously reported. A diagram of the apparatus and biosensing processes applying the redox reaction of catechol/o-quinone catalyzed by the tyrosinase-SPCE strips modified with AuNPs and FeDC is shown in Fig. 1.

2. Materials and methods

2.1 Reagents and solutions

Sodium citrate and ethanol were obtained from Merck (Darmstadt, Germany). Tyrosinase (from mushroom, EC1.14.18.1, 5370 U mg^{-1} of solid), FeDC, chlorauric acid ($HAuCl_4$), catechol, carbofuran, GSH, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), meta-phosphoric acid (MPA), N-acetylcysteine (NAC), dimethyl sulfoxide (DMSO), dibasic sodium phosphate, monobasic sodium phosphate, proline, glutaraldehyde and potassium chloride, were obtained from Sigma (St. Louis, MO, USA). All chemicals were of analytical grade and used as received. Stock solutions used in this study were prepared including 0.1 M KCl-phosphate working buffer (50 mM, pH 6.5), substrate solution of catechol (100 mM in working buffer) and FeDC solution (5 mM, in 95% ethanol). For the preparation of the biosensor strips, the following

solutions were used in working buffer: 25,000 U mL^{-1} tyrosinase, 2.5 mM glutaraldehyde solution and 1% BSA solution. All solutions were prepared with twice-distilled water obtained from a Milli-Q system (18 MΩ; Millipore, Bedford, MA, USA). E1660 silver-resin and C3451 carbon/graphite inks were purchased from ERCON (Wareham, MA, USA). ICR mice were obtained from the Laboratory Animal Center, National Taiwan University, Taiwan.

2.2 Apparatus

Cyclic voltammetric (CV) and amperometric measurements were performed with a CV50W voltammetric analyzer (Bioanalytical Systems, West Lafayette, IN, USA). The analyzer was connected to a personal computer and a battery-powered and hand-held PalmSens/Personal Digital Assistant instrument (Palm Instruments, BZ Houten, Netherlands) for data collection and calculation. The SPCE surface images were obtained with a Quanta 400 FEG scanning electron microscope (SEM) (FEI Co., Hillsboro, OR, USA) and a Cressington 108 automatic sputter coater (Cressington Scientific Instruments Ltd., England, UK). Dynamic light scatter (DLS) analyses (BI200-SM, Brookhaven Instruments Co., Holtsville, NY, USA) to determine the particle size in transparent media were performed at a scattering angle of 90°. The absorbance values of AuNPs, tyrosinase inhibition and GSH assay were measured by using the SpectraMax 190 spectrophotometer (Molecular Devices Corporation, Sunnydale, CA, USA).

2.3 Preparation and analysis of AuNPs

AuNPs were prepared according to the methods reported previously with minor modifications (Rao et al., 2006). A volume of 2.5 mL of 38.8 mM sodium citrate was quickly added to 25 mL of boiling 1 mM $HAuCl_4$ with vigorous stirring for 10 min, and a color change from blue to red-violet was observed. The mixture was heated to boil and stirred for another 10 min and then cooled at ambient temp. Colloidal Au particles were produced and stored at 4ºC in the solution. The AuNPs solution was diluted with water and analyzed at 25°C.

2.4 Fabrication of AuNPs-, FeDC- and tyrosinase-modified SPCE strips

The SPCE strips were prepared by printing on polyvinylchloride boards sequentially with silver-resin and carbon/graphite inks using an automated screen-printer system, and an oven (BUILT-IN Corp, Taichung, Taiwan) to drive off organic solvent in inks (Fig. 1). Each SPCE strip consisted of a working electrode and a counter/reference electrode, both with 9.8 mm^2

surface area. The distance between the two electrodes is 0.5 mm. A volume of 10 μL of glutaraldehyde solution used as a linker was added drop-wise onto the working electrode area, and the strip was incubated for 1 h at 4°C and washed twice with doubly distilled water. A volume of 10 μL of AuNPs solution was then added drop-wise onto the glutaraldehyde-treated working electrode, incubated for 1 h at 4°C, and followed by washing twice with water. The surface morphology of SPCE/AuNPs strip was examined by using a Cressington 108 automatic sputter coater (< 0.05 mBar, Pt coating with 20 mA, 2 min) and a Quanta 400 FEG SEM (high-vacuum: $< 4.5 \times 10^{-5}$ Pa.; voltage 20~25 kV; detectors: 50% secondary electron and 50% back-scattered electron ; resolution: 2-3 nm). For the preparation of FeDC-modified strips, to each working electrode of the SPCE strips with or without AuNPs was added 10 μL of FeDC solution, followed by incubation for 30 min at 37°C, and then washed in water and dried in air. Tyrosinase-modified SPCE strips were prepared similarly by adding the tyrosinase solutions with various activities onto the working electrodes of SPCE strips with or without AuNPs and/or FeDC modifications. All strips were stored at 4°C.

2.5 Optimization and characterization of tyrosinase-SPCE strips

The pH-dependence studies were performed in the pH range 4.5-8.0. For the studies of effects of enzyme loading amount and substrate concentration, different amounts of catechol and tyrosinase were used in the ranges 50-1,000 μM and 1-20 unit (U) per strip, respectively. In CV studies, the voltage was scanned from –600 mV to +600 mV with a scan rate 100 mV s^{-1}. Before all CV and amperometric experiments, a 50 μL catechol solution was added drop-wise onto the electrochemical reaction area of the SPCE strip and incubated for 30 s at 25°C. A fixed potential (-300 mV) *vs.* counter/reference electrode was applied for amperometric measurements after strip incubation. The sum of current signals collected every 0.1 s during the following 50 s was obtained as the response current (RC).

2.6 Determinations of carbofuran and GSH

The determination of carbofuran was performed in two steps. First, to a tyrosinase-SPCE strip was added drop-wise a volume of 5 μL of test solution and pre-incubated for 5 min. Then, a catechol solution was mixed well with the test solution and the response current (RC) was immediately collected. The determination of GSH was performed as follows: First, a volume of 12.5 μL sample was diluted in a volume of 12.5 μL of 50 mM phosphate buffer and then mixed well with a volume of 25 μL of 800 μM catechol solution. The resulting 50 μL analyte solution

was then subjected to amperometric measurement. Calibration curves were then prepared and used for quantitation.

2.7 Preparations of vegetable and blood samples

Carbofuran solutions at 0, 0.565, 1.13, 2.26 and 4.52 μM (0, 1.25, 2.5, 5.0 and 10.0 μg g^{-1}) were prepared. A volume of 1 mL of each solution was then sprayed onto the surfaces of fresh lettuce leaves (one concentration per leaf) at room temperature, and the leaves were dried in air. A piece with an area of 9 cm^2 was cut from each leaf, ground to pieces and mixed well with a volume of 1 mL 20% DMSO for 30 min. The solution was then diluted to 20 mL with phosphate buffer for amperometric and spectrophotometric measurements.

For GSH sample preparations, fifteen "imprinting control region" (ICR) mice weighing approximately 15 g were divided into 3 groups randomly. These mice were housed in a plastic suspended cage placed in a well-ventilated mice house, provided with mice pellets and water, and subjected to a natural photoperiod of 12 h light/dark cycle for 14 days. Mice in Group I served as control and were administered normal saline. Mice in Groups II and III were treated orally with 50 and 100 mg kg^{-1} body weight day^{-1} NAC in saline, respectively, to increase the GSH level (Medved et al., 2004). The GSH concentrations in whole blood of ICR mice were measured at day 0, 7 and 14 after NAC treatment. Whole blood samples were collected with heparin and then erythrocyte hemolyzed (1:1) with water. The blood samples were then mixed with 10% meta-phosphoric acid (MPA) in the ratio of 2:1 (v/v) and the mixtures were centrifuged at 13,000 rpm for 10 min at 4°C. The supernatants were collected and mixed with 50 mM phosphate buffer in the ratio of 3:17 (v/v) and then analyzed by amperometric and spectrophotometric assays.

2.8 Spectrophotometric assays for carbofuran and GSH

Tyrosinase inhibition assays were performed in 96-well microplates according to the modified Rezepecki and Waite method shown below (Streffer et al., 1998):

$$\text{catechol} \xrightarrow{\text{tyrosinase}} \textit{o}\text{-quinone} \quad (1)$$

$$\textit{o}\text{-quinone} + \text{proline} \longrightarrow \text{4-N-prolyl-}\textit{o}\text{-quinone} \quad (2)$$

A volume of 5 μL of phosphate buffer (50 mM, pH 6.5) or inhibitor solution was mixed with a volume of 100 μL of tyrosinase solution (20 U mL^{-1}) and pre-incubated for 5 min. Then a

volume of 105 μL substrate solution containing 0.8 mM catechol and 0.8 mM proline was added to initiate the assay reaction and the rate of formation of prolyl-*o*-quinone was immediately monitored by measuring the change in absorbance at 530 nm within 1 min. Stock solutions were stored on ice in dark containers. The measurements were performed at room temperature.

The Ellman's method was used for the spectrophotometric determination of GSH (Calvo-Marzal et al., 2006). GSH is oxidized by DTNB to give GS-SG with the formation of yellow colored 2-nitro-5-thiobenzoic acid (TNB) in the thiolate anion form (equation 3).

$$\text{DTNB} + \text{GSH} \longrightarrow \text{2-nitro-5-thiobenzonic acid} + \text{GS-SG} \qquad (3)$$

A volume of 100 μL test sample was mixed with a volume of 50 μL DTNB stock solution (2 mM) containing 50 mM sodium acetate, a volume of 100 μL Tris solution and a volume of 750 μL water to result in a final volume of 1 mL which was then mixed well and incubated at 37^{o}C for 5 min. A volume of 100 μL of the final test mixture was used for spectrophotometric measurement at 410 nm.

2.9 Data analysis

Each experiment was repeated five times using five different SPCE strips to test the reproducibility of these enzyme-modified SPCE strips. All data were reported as mean values ± standard deviations (SD). Differences between groups were evaluated by the two-tailed Student's *t*-test, and $P \leq 0.05$ was considered statistically significant.

3. Results and discussion

3.1 Preparation of AuNPs modified SPCE strips

The red AuNPs we prepared had an absorption maximum at 520 nm as a result of their surface-plasmon oscillation. The results of DLS measurements for these colloidal AuNPs indicated a mean diameter of 13 nm. SEM measurements of the AuNPs modified SPCE strips also confirmed size uniformity and the average AuNPs diameter (Fig. 2). The distribution of AuNPs on the surface of SPCE strips were quite uniform and is 72.5 ± 8.4 per 0.04 μm^2 from five randomly selected sample areas of SEM images.

3.2 Cyclic voltammograms of tyrosinase SPCE strips modified with AuNPs and/or FeDC

Cyclic voltammograms (CVs) were obtained in the presence of 100 μM catechol in 50 mM phosphate buffer at pH 6.5 with a scan rate of 100 mV s^{-1} and a scan range of -600 mV to +600 mV to observe the electrochemical characteristics of various SPCE strips prepared. These included bare SPCE strip, SPCE/Tyr strip (SPCE strip modified with10U tyrosinase per strip), SPCE/AuNPs/Tyr strip (SPCE strip modified with AuNPs and tyrosinase), SPCE/FeDC/Tyr strip (SPCE strip modified with FeDC and tyrosinase), and SPCE/AuNPs/FeDC/Tyr strip (SPCE strip modified with AuNPs, FeDC and tyrosinase) (Fig. 3). No obvious redox-peak was observed for the bare SPCE strip (curve A, Fig. 3), indicating low background current. The cathodic peak current of the SPCE/Tyr strip was observed at a potential of -591 mV (curve B, Fig. 3). For the SPCE/AuNPs/Tyr strip, the cathodic peak current was at a potential of –312 mV and was enhanced by AuNPs (curve C, Fig. 3). When SPCE/FeDC/Tyr strip was used, the cathodic peak current was at a potential of -316 mV and was enhanced by FeDC (curve D, Fig. 3). The FeDC enhancement was greater than that of AuNPs under the experimental conditions. More interestingly, a synergistic enhancement of the cathodic peak current was observed when the SPCE/AuNPs/FeDC/Tyr strip was used at a potential of -300 mV (curve E, Fig. 3), i.e. the enhancement is greater than the sum of those of AuNPs and FeDC alone.

The enzymatic oxidized product of catechol, i.e. *o*-quinone, could be reduced reportedly at a potential of -200 mV (Kulys and Schmid, 1990). However, *o*-quinone is unstable in water and could easily undergo polymerization reactions (Horowitz et al., 1970), and the redox event of catechol/quinone is irreversible. This was the reason why there was no obvious cathodic peak current of *o*-quinone in curve B, Fig. 3 at a potential of -200 mV. For the SPCE/AuNPs/Tyr strip, the distances between the catalytic center of tyrosinase molecules and the electrode surface could be effectively shortened via AuNPs which results in faster electron transfer rates between the enzyme and electrode and therefore reduction of side reaction products (Haddad, 2002). Thus, polymerization of *o*-quinone molecules in water would be reduced and both the cathodic and anodic peak currents would be enhanced. The characteristics of FeDC on the SPCE/FeDC/Tyr strip were similar to those previously reported (Lin et al., 2008; Wring et al., 1991). As a mediator, FeDC improved the redox reaction rates of catechol/*o*-quinone catalyzed by tyrosinase.

AuNPs have been used in several biosensors to function as conjugation and/or immobilization media (Huang and Kuwana, 2002; Rao et al., 2006), reporters (Tang et al.,

2007), or electrode/conductors (Radke and Alocilja, 2005), and were found to increase sensitivity and stability (Xu et al., 2006). In the present study, in order to increase the signal to noise ratio, decrease the background current and avoid unknown interferences in biological samples, a potential of -300 mV was selected as a convenient potential for further amperometric studies.

3.3 Design, amperometric characterizations and optimizations of SPCE/AuNPs/FeDC/Tyr strips

The effects of pH (4.5-8.0), [phosphate buffer] (20-80 mM) and [catechol] (50-1,000 μM) were investigated for optimum RC measurements using SPCE/AuNPs/FeDC/Tyr strips with 10U tyrosinase per strip initially as Fig. 4 and 5. An optimum condition with pH 6.5 (similar to the optimum pH range reported for the free or immobilized tyrosinase catalyzed reactions (Shan et al., 2003)) and 50 mM phosphate buffer were then chosen for practical determinations. The RC values were linear in the [catechol] range 50-400 μM and a linear least squares regression equation of RC = 2.878[catechol] (μM) + 66.572, R^2 = 0.9905, could be obtained. Beyond 400 μM, the RC values started to deviate from linearity. A [catechol] 400 μM was used for all subsequent studies. The average response current (RC) of amperometric measurements using SPCE/AuNPs/FeDC/Tyr strips is enhanced by 1.53 fold as compared to that without AuNPs and FeDC modifications.

The determination of carbofuran using SPCE/AuNPs/FeDC/Tyr strips was based on the assumption that the catechol oxidation activity catalyzed by tyrosinase was inhibited by carbofuran which resulted in decreased RC and the decreased RC was proportional to the concentration of carbofuran. Previous studies showed that a competitive inhibition was observed when a carbamate was added to the tyrosinase solution together with catechol (Rosa et al., 1997). However, simultaneous addition of analyte (i.e., inhibitor) in the presence of high catechol concentration did not give reliable analytical results. On the other hand, pre-incubation of tyrosinase with the pesticide analyte and followed by the addition of substrate (i.e., catechol) gave reasonably good analytical results (Ivanov et al., 2000). This latter approach was taken by our present carbofuran determinations.

A convenient concentration range for the determination of remained carbofuran in crops was 0.1-10 μg g^{-1} (w/w) using the present method (Evtugyn et al., 1998). Minimum amount of tyrosinase should be used in order to achieve the lowest possible detection limit located near 0.1 μg $g^{-1.}$ To determine the optimum amount of tyrosinase to be loaded on the strip for lower

detection limit, six different amounts of tyrosinase were loaded on the strips and the RC values of 0 and 0.452 μM (~1 μg g^{-1}) carbofuran were recorded. Fig. 6 shows the plots of the difference of RC (ΔRC) values between 0 and 0.452 μM carbofuran vs. amount of tyrosinase loaded on the strip. An enzyme loading of 2 U per strip was chosen for practical applications. Under the final conditions, i.e. using the SPCE/AuNPs/FeDC/Tyr strip, 50 mM phosphate buffer and 2 U tyrosinase per strip at pH 6.5, the amperometric responses (RC values) at -300 mV were obtained for different concentrations of catechol and a linear regression equation RC = 0.712[catechol] (μM) + 46.261, R^2 = 0.9689, was established as Fig. 5.

For the determination of GSH, it was found that the reductive current of catechol was greatly diminished in the presence of GSH, because the concentration of *o*-quinone produced by the tyrosinase-catalyzed oxidation of catechol was directly related to GSH concentration. The amount of the *o*-quinone reacted with GSH relative to the added catechol becomes smaller when more catechol was added, and the amount of enzyme loaded on the sensor strip drastically influences the detection limit (United States Environmental Protection Agency, 2006). The experimental conditions for the determination of GSH in the range 12.5-100 μM were the same as those of carbofuran determinations, i.e. pH 6.5 (50 mM phosphate buffer), [catechol] 400 μM, 2 U per strip tyrosinase.

3.4 Determination of carbofuran and glutathione using the SPCE/AuNPs/FeDC/Tyr strips and practical applications

Fig. 7A shows the plot of RC values as a function of [carbofuran]. A linear least squares fitted equation was obtained for carbofuran in the concentration range 4.52-45.2 nM (~ 0.01-0.1 μg g^{-1}) to be RC = -2.2954[carbofuran] (nM) + 299.39, R^2 = 0.9383. For the determination of GSH, a linear least squares fitted equation was obtained in the concentration range 12.5-100 μM to be RC = -1.4366[GSH] (μM) + 338.24, R^2 = 0.995 as Fig. 7B.

For practical applications on the determination of carbofuran, the carbofuran samples were extracted from lettuce leaves by DMSO and diluted with working phosphate buffer (20-fold volume dilution), and amperometric and spectrophotometric analyses were performed, respectively. The results for the extracted carbofuran solutions at 0 and 4.52 μM were outside the linear ranges of the both analyses as Fig. 8 A & 8B. Within the linear calibration concentration range of amperometric determinations, i.e. 90.4-904 nM, the linear plot of the determined [carbofuran] by the two methods gave a slope value of 0.7489 (R^2 = 0.9995) as Fig. 9. Thus, after appropriate extraction and dilution, the amperometric determination of

carbofuran using the SPCE/AuNPs/FeDC/Tyr strips could give reasonably reliable results as compared with those by spectrophotometry.

In whole blood samples, most of the free sulfhydryl groups are from the reduced form of GSH, cysteine, and homocysteine (Lang et al., 2001). The concentration of GSH in blood (~1 mM) is normally 20-50 times higher than other thiols such as cysteine (Lang et al., 2000). Thus, the determination of total free sulfhydryl compounds by Ellman's method would give approximately the [GSH] in blood.

For the determination of GSH in biological samples by amperometry, mice blood samples were obtained after 0, 7 and 14-days treatments (Group I, II and III mice fed orally with 0, 50 and 100 mg kg^{-1} body weight day^{-1} NAC in saline). The GSH samples were diluted with 20-fold volume dilution, and then amperometric and Ellman's (spectrophotometric) measurements were performed, respectively. In the linear concentration range of 250-2,000 μM, plot of the determined [GSH] by the two methods gave a slope value 0.8201 as Fig. 10. It was found that the GSH level of Group III was not only significantly increased 8.3% as compared with those of Group I, but also higher than that of the Group II. These data indicated that feeding high dose NAC resulted in a significant increase of blood GSH levels which could be determined rapidly and precisely using SPCE/AuNPs/FeDC/Tyr strips, with the help of linear correlation with the spectrophotometric results of the Ellman's method or by the technique of standard addition.

4. Conclusions

The preparation and characterizations of tyrosinase-SPCE strips modified with AuNPs and FeDC are reported. AuNPs could efficiently improve the electron transfer between tyrosinase and electrode surface on the biosensor strips. The use of the artificial electron mediator, i.e., FeDC, could speed up the redox reaction rate and effectively reduce the operating potential and hence enhance sensitivity of the tyrosinase-SPCE strips. The SPCE/AuNPs/FeDC/Tyr strips were useful for the determinations of carbofuran and GSH at a potential of -300 mV in 50 mM phosphate buffer, pH 6.5. The determination of carbofuran in a linear concentration range 4.52-45.2 nM with the SPCE/AuNPs/FeDC/Tyr strips were based on the quantitative inhibitory effect on tyrosinase catalyzed catechol oxidation reaction. The determination of GSH was accomplished by quantitative reduction of the concentration of catechol oxidized product o-quinone by GSH which resulted in decreased response current. A linear GSH concentration response ranged from 12.5 to 100 μM. The loading amount of

tyrosinase on the disposable SPCE/AuNPs/FeDC/Tyr strips was only 2U per strip which was more economical than those previously reported for the determination of carbofuran and GSH. Practical applications of the SPCE/AuNPs/FeDC/Tyr strips in agriculture and biological/ clinical analyses were demonstrated.

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Figures

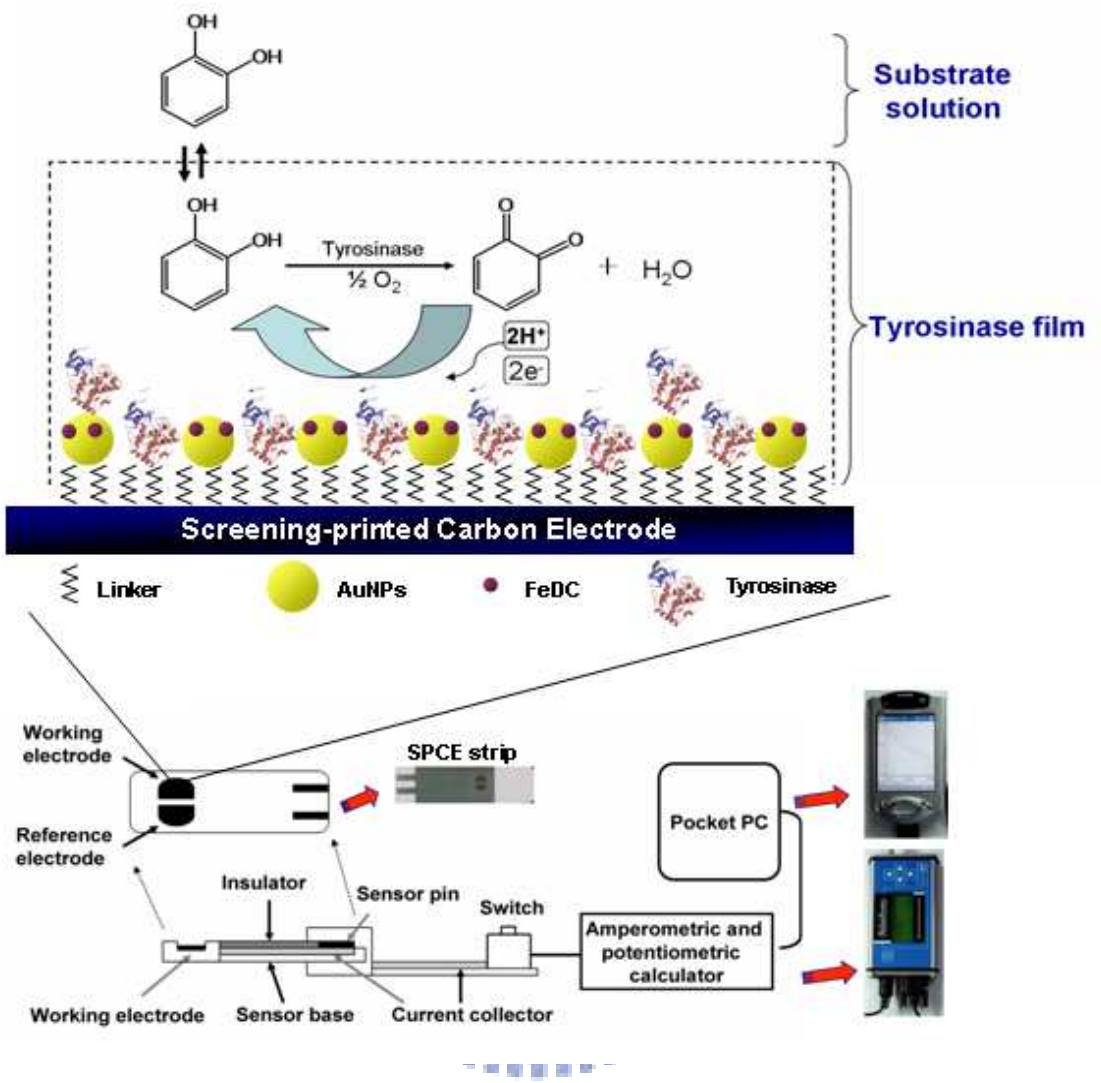


Fig. 1 A diagram of the apparatus and biosensing processes for catechol detection by the tyrosinase-SPCE system modified with AuNPs and FeDC.

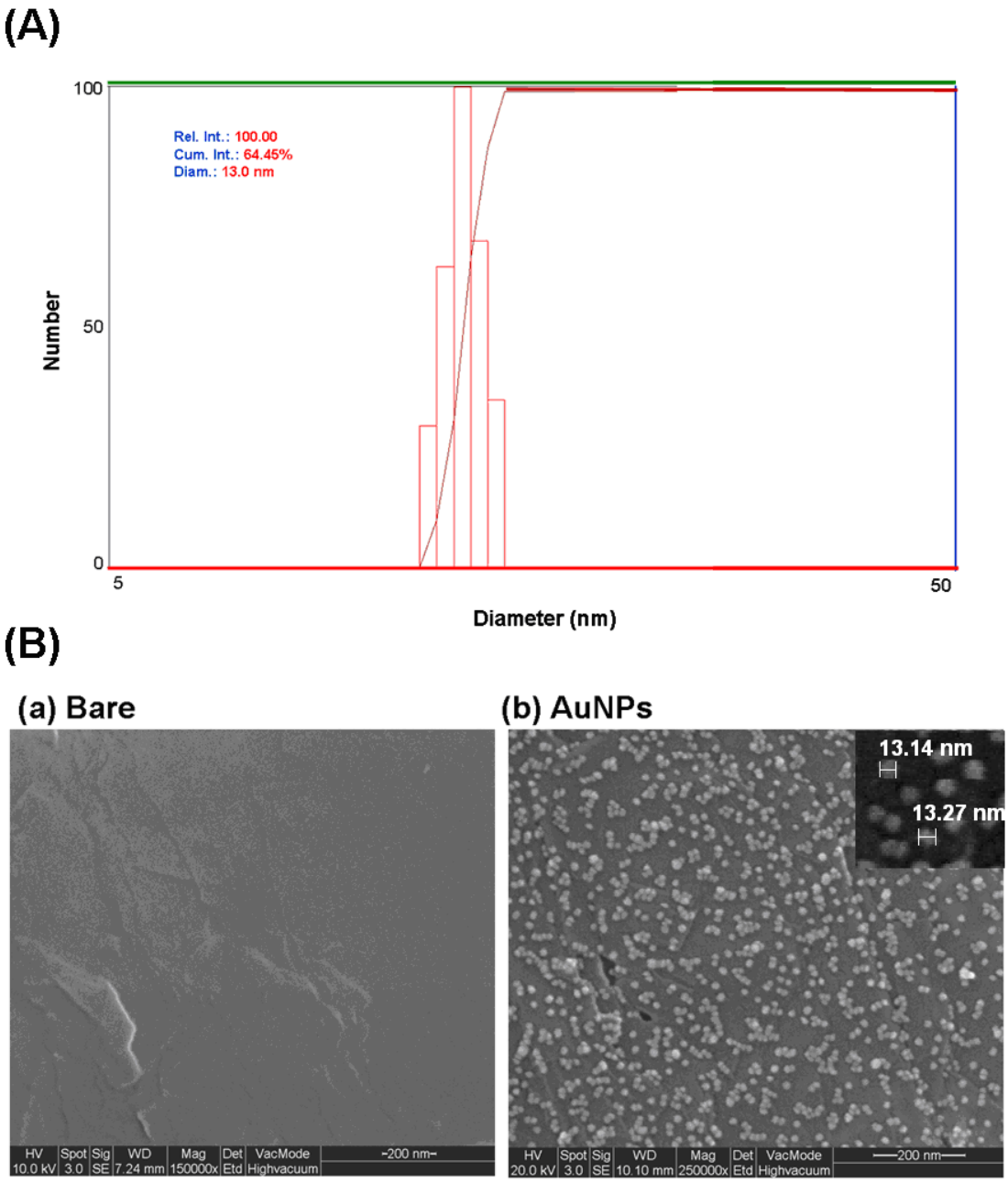


Fig. 2 Dynamic light scattering (DLS) results of colloidal AuNPs and scanning electron micrographs (SEM) of the SPCE/AuNPs. (A)The mean diameter of AuNPs was 13.0 nm measured by DLS. AuNPs solution was diluted with water and analyzed at 25^{o}C. X-axis represents the diameter of particles on a logarithm scale. Y-axis represents the relative number of collected particles. (B) A nude SPCE surface (a) and AuNPs-modified SPCE surface (b) were shown. The average diameter of AuNPs on the surface of SPCE was 13 nm.

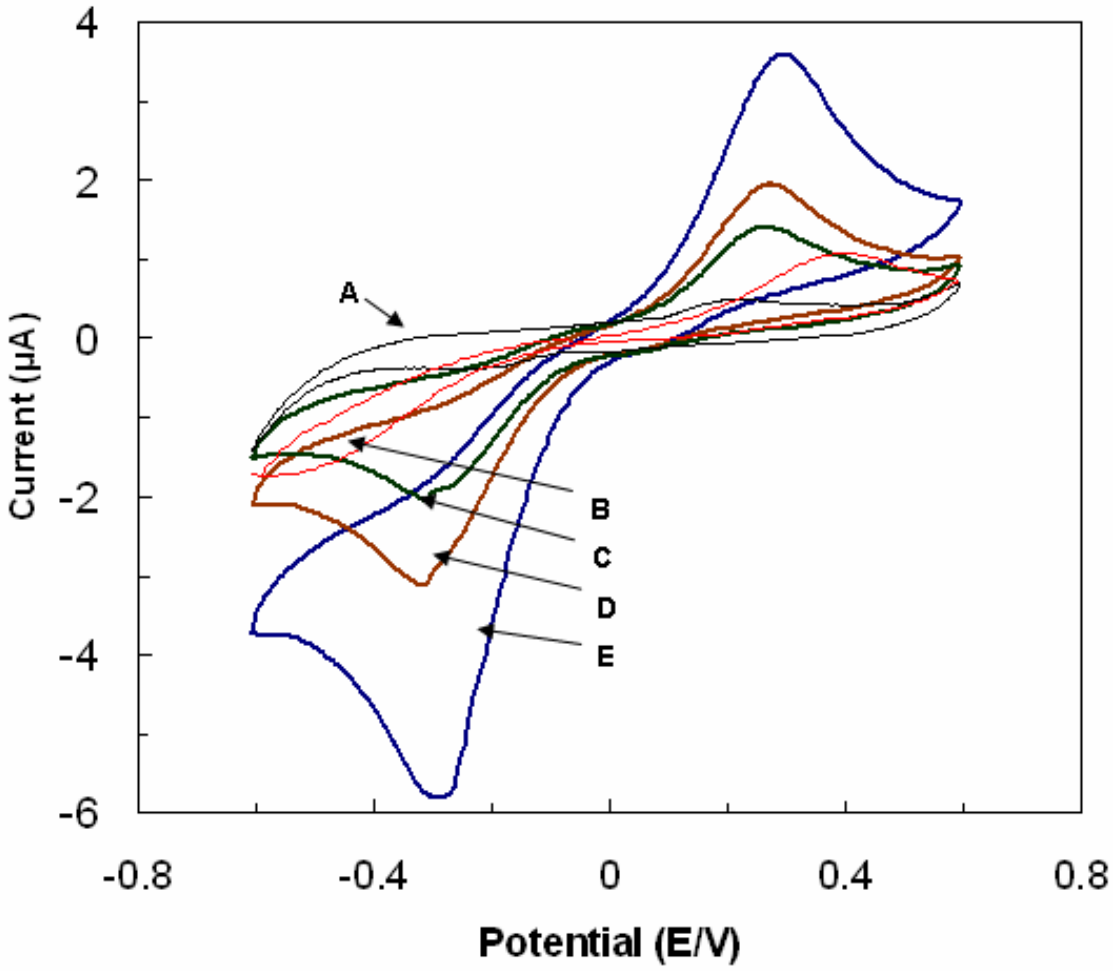
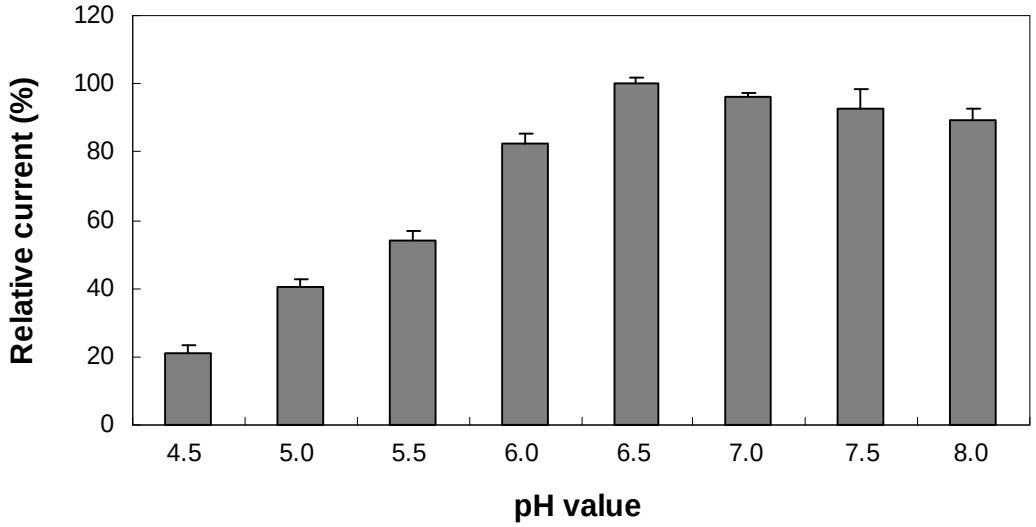


Fig. 3 Cyclic voltammograms recorded in 100 μM catechol (50 mM phosphate buffer, pH 6.5) by SPCE (A), SPCE/Tyr (B), SPCE/AuNPs/Tyr (C), SPCE/FeDC/Tyr (D) and SPCE/AuNPs/FeDC/Tyr (E) strips, respectively, at a scan rate 100 mV s^{-1}.

A



B

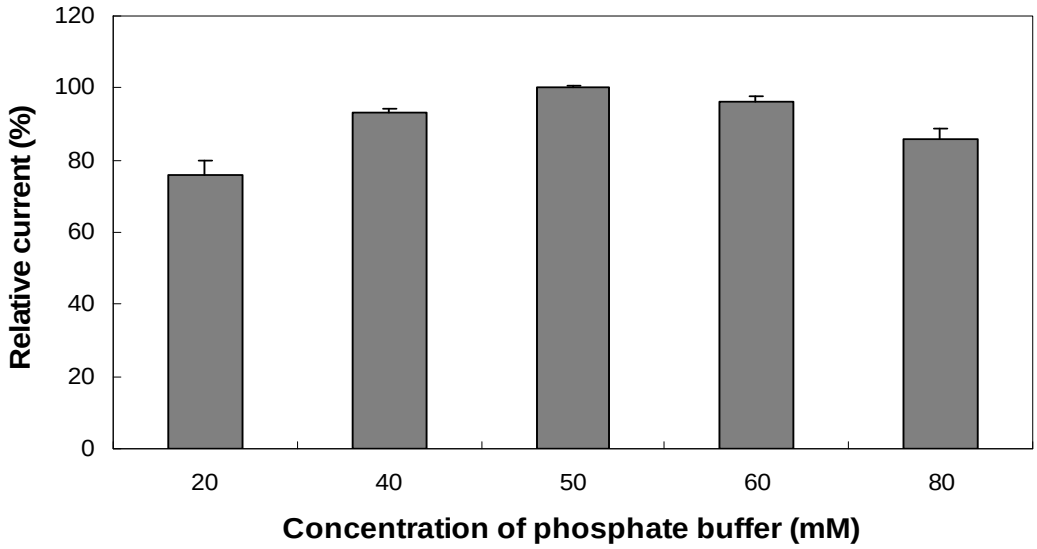


Fig. 4 Effects of reaction pH and phosphate buffer concentration for the SPCE/AuNPs/ FeDC/Tyr strips. Effects of pH and phosphate buffer concentration on the strip were tested using 100 μM catechol. The strips were modified with AuNPs, FeDC and 10 U tyrosinase per strip and the potential for the electrode was -300 mV. (A) The electrolyte was 50 mM phosphate buffer for study of pH effect. (B) The electrolyte was pH 6.5 for the study of effect of phosphate buffer concentration. The results were the average values of five independent measurements and standard deviations (SD) were shown as error bars (n = 5).

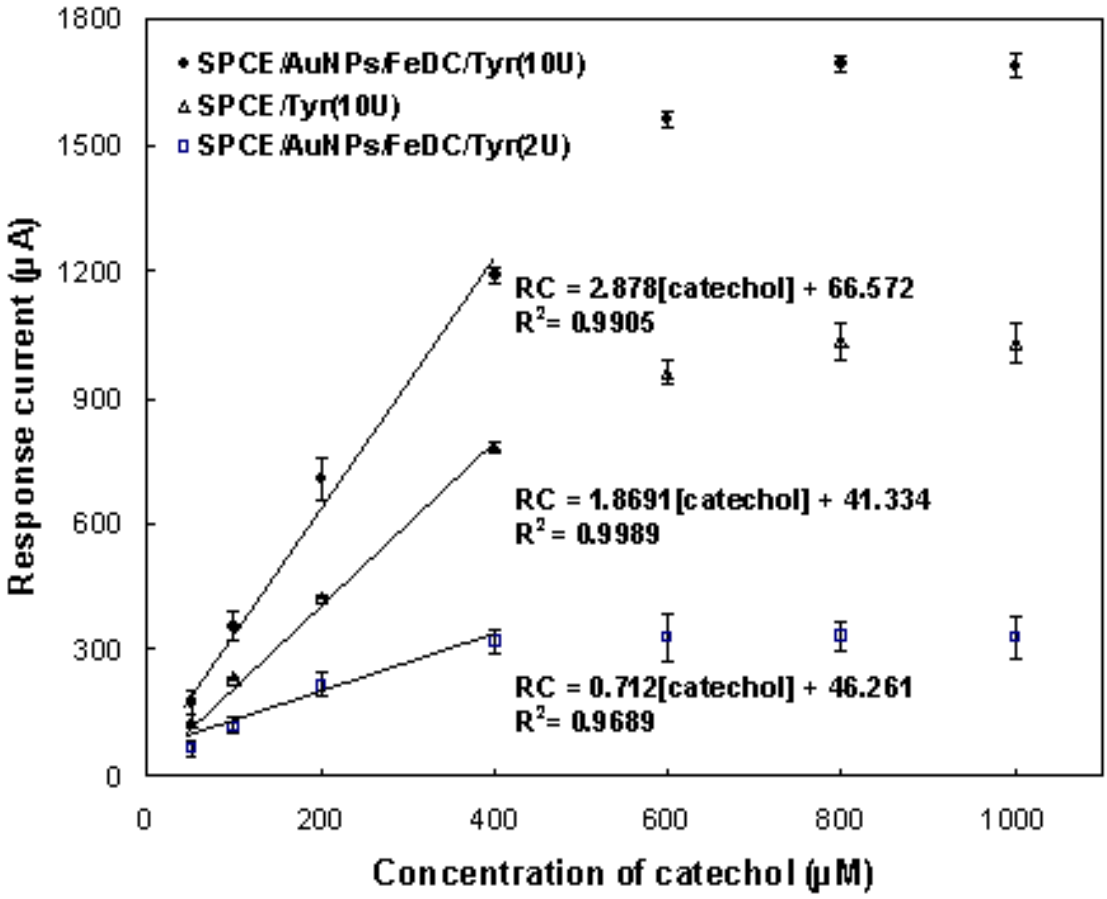


Fig. 5 The effects of substrate concentration on the RCs of SPCE/AuNPs/FeDC/Tyr strips. Amperometric measurements were recorded in 50 mM phosphate buffer (pH 6.5) containing different concentrations of catechol at an applied potential of -300 mV. The SPCE strips were modified with AuNPs, FeDC and tyrosinase (10 U (●), 2 U (□) per strip) and only with tyrosinase (10 U (Δ) per strip). Error bars were standard deviation values (n = 5).

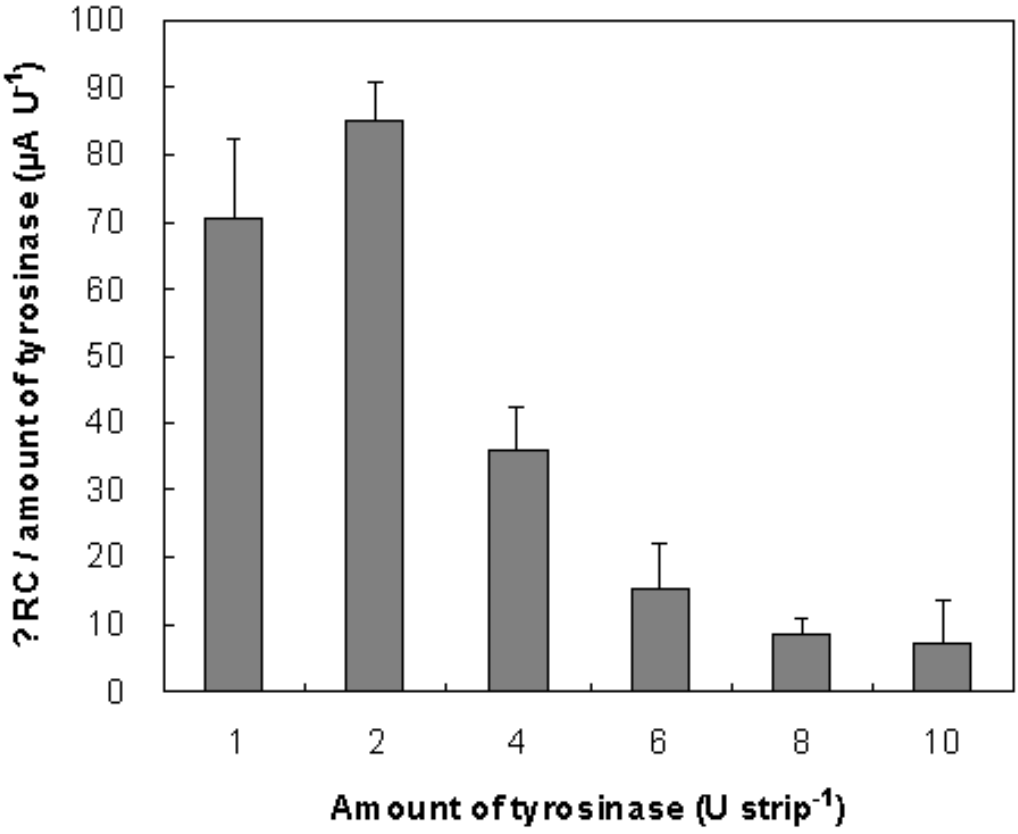
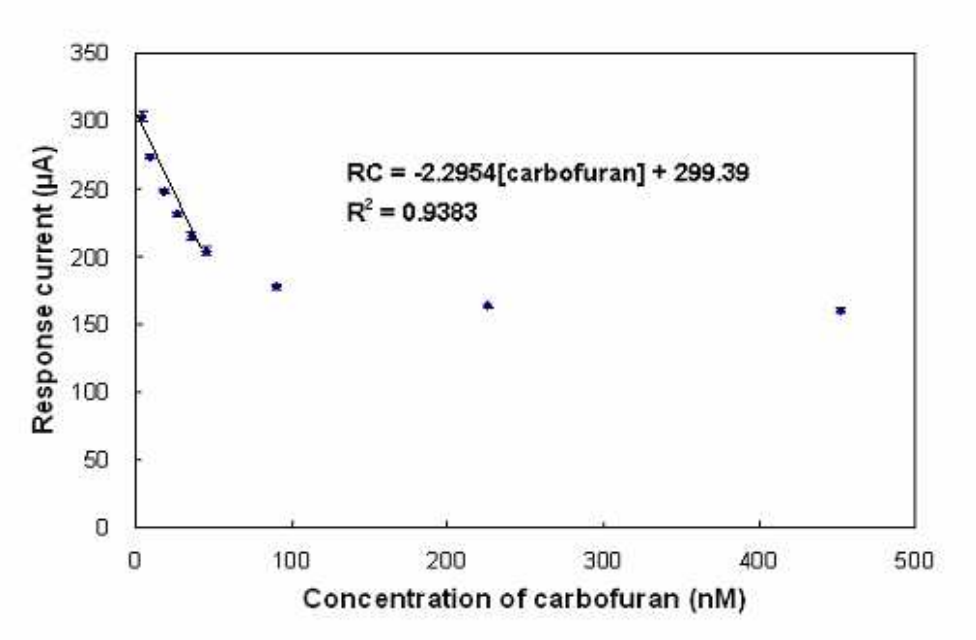


Fig. 6 Plot of ΔRC values vs. amount of tyrosinase loaded, U per strip, on the SPCE/AuNPs/FeDC/Tyr strips with 0.452 μM (=1 ppm in solution) carbofuran or without. ΔRC = the difference of RC values between 0 and 0.452 μM carbofuran. Error bars are standard deviations (n = 5).

A.



B.

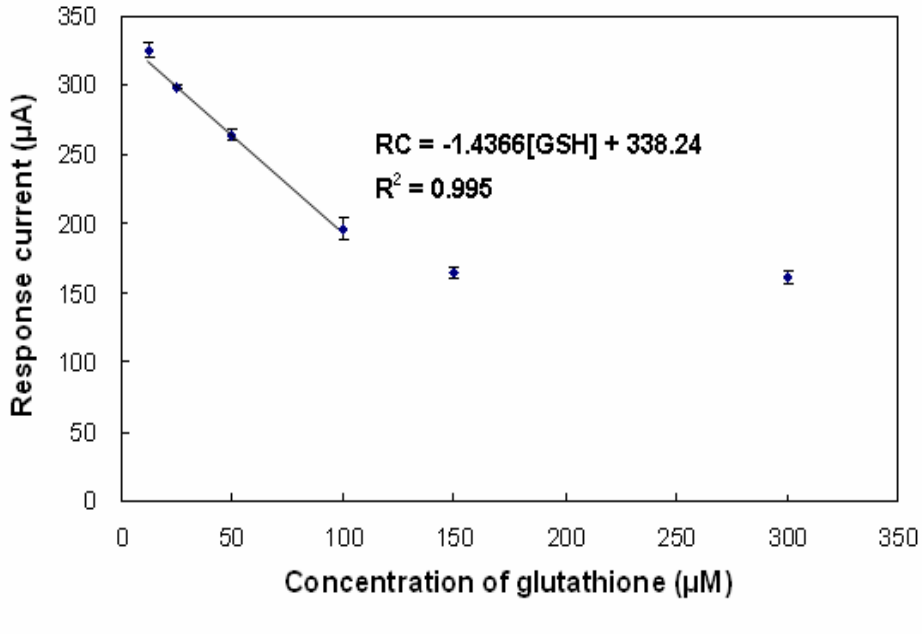
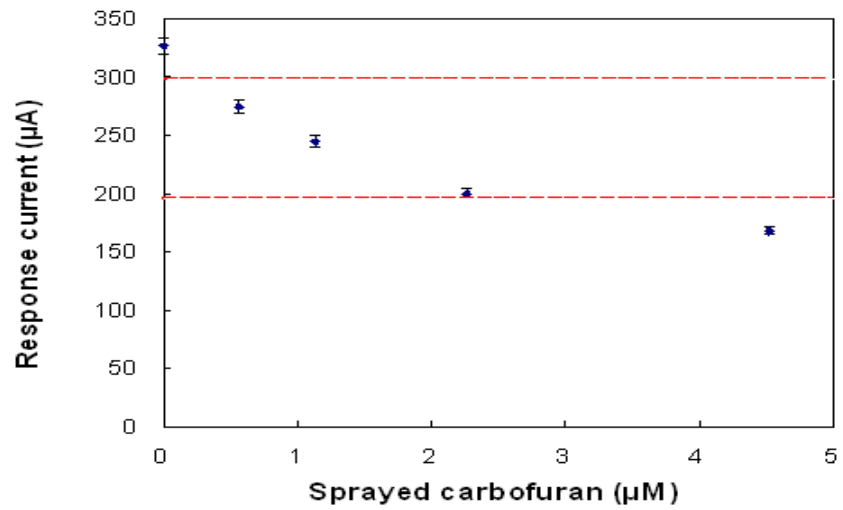


Fig. 7 Plots of RC values vs. [carbofuran] (A) and [GSH] (B) using the SPCE/AuNPs/FeDC/Tyr strips. (A) The linear least squares fitted equation for carbofuran in the concentration range 4.52-45.2 nM is: RC = -2.2954 [carbofuran] (nM) + 299.39, R^2 = 0.9383. (B) The linear least squares fitted equation for GSH in the concentration range 12.5-100 μM is: RC = -1.4366[GSH] (μM) + 338.24, R^2 = 0.9995. Error bars are standard deviations (n = 5).

A.



B.

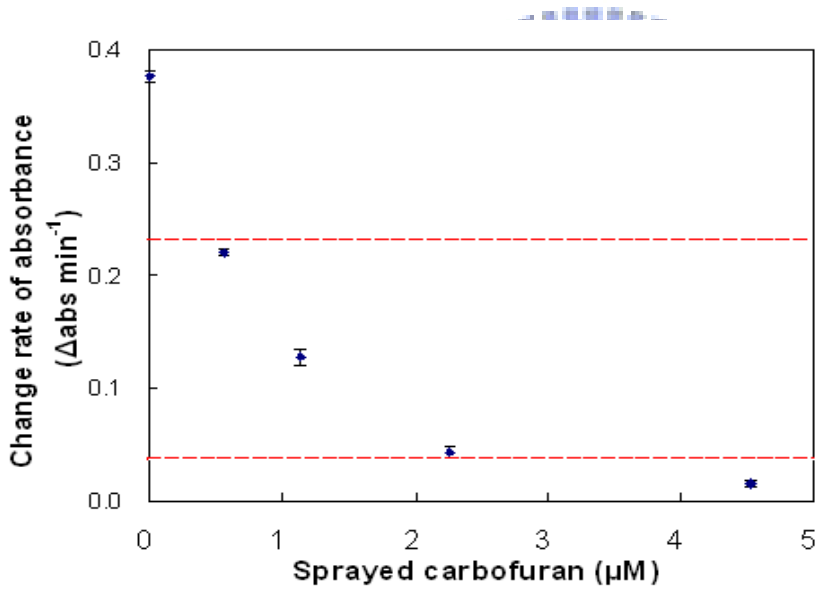


Fig. 8 The results of carbofuran determinations from the sprayed lettuce leaves by amperometric and spectrophotometric methods. The linear concentration range of amperometric determination (RC) is 196-289 μA and the linear concentration range of spetrophotometric determination (absorbance change rate) is 0.038-0.229 ΔAbs min^{-1}. Lettuce leaves were sprayed with 0, 0.565, 1.13, 2.26 and 4.52 μM carbofuran solutions, respectively. Error bars were standard deviation values (n = 5).

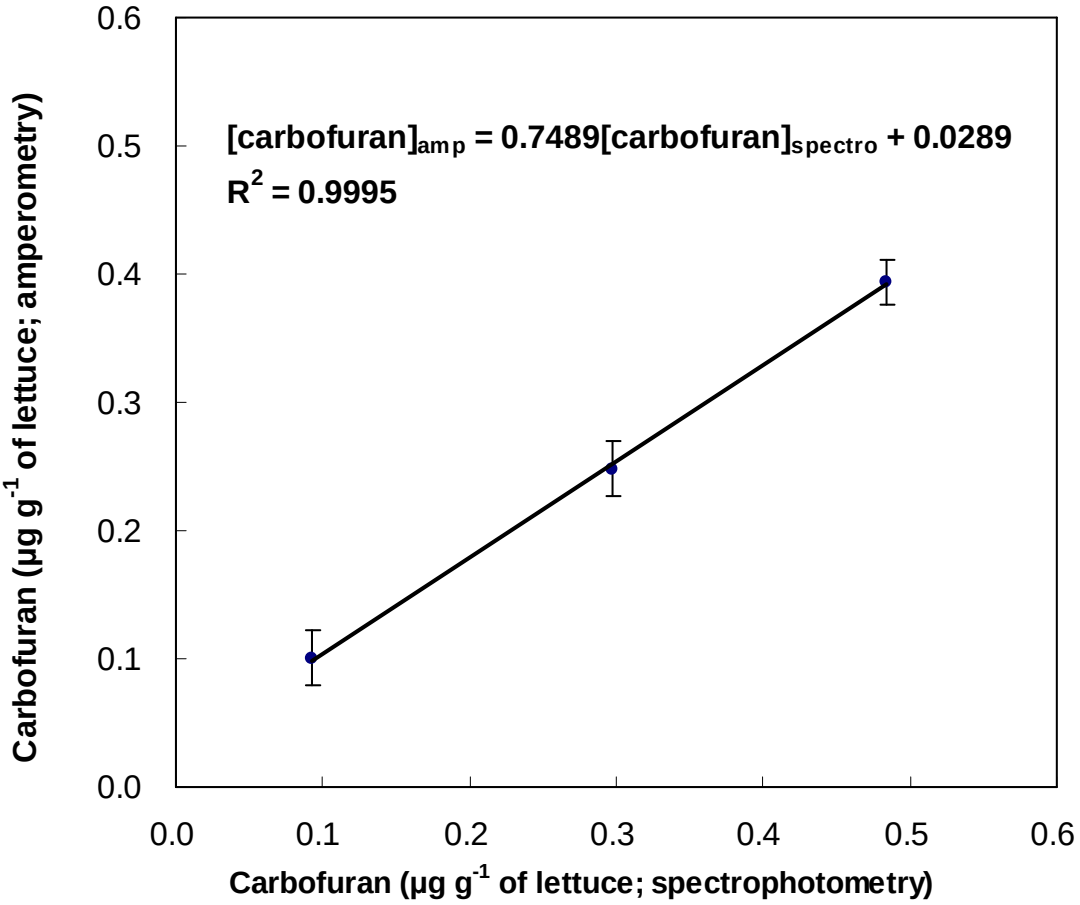


Fig. 9 Comparisons of the carbofuran concentrations on lettuces determined by the amperometric and spectrophotometric methods. The concentration values have been converted to [carbofuran](μg g^{-1}) of lettuce leaf. Error bars are standard deviations (n = 5).

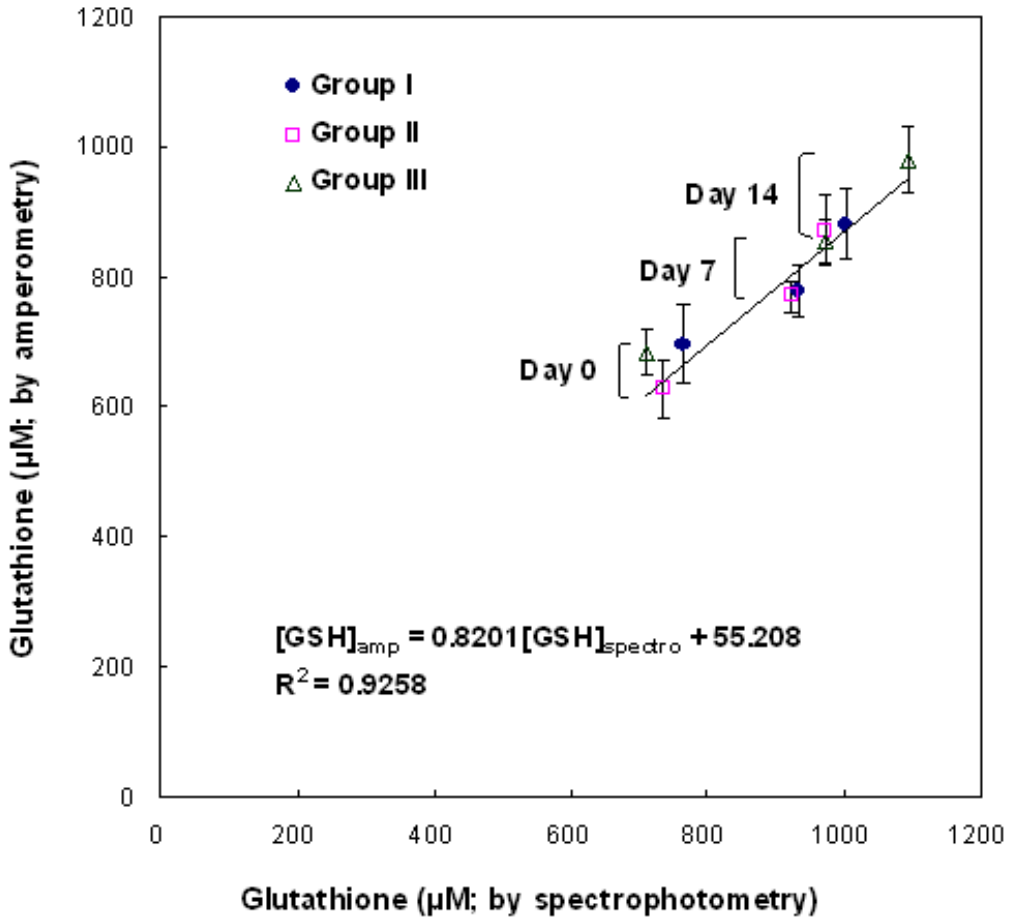


Fig. 10 Comparisons of [GSH] in whole blood samples of Group I, II and III mice determined by the amperometric and spectrophotometric methods. Mice in Group I, II and III were treated orally with NAC (0, 50 and 100 mg kg^{-1} body weight day^{-1}, respectively) in saline. The GSH in whole blood of ICR mice were measured at day 0, 7 and 14 after NAC treating. Error bars are standard deviations (n = 5). Asterisk (*) indicate $P < 0.01$.