

# 國立交通大學

生物科技系所

碩士論文

利用酪氨酸酶電化學感測器檢測環境荷爾蒙

**Detection of Endocrine Disrupting compounds  
by a Tyrosinase Biosensor**

研究生：趙偉志

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中華民國九十六年七月

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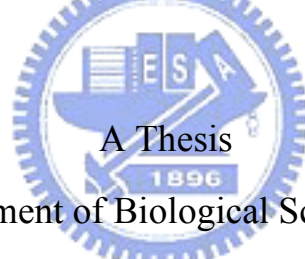
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國立交通大學

生物科技系所

碩士論文



Submitted to Department of Biological Science and Technology

National Chiao Tung University

in partial Fulfillment of the Requirements

for the Degree of

Master

in

Biological Science and Technology

July 2007

Hsinchu, Taiwan, Republic of China

中華民國九十六年七月

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### 中文摘要

內分泌干擾物，或者稱之為環境荷爾蒙，這一類的化學物質存在於自然環境中或者是由工廠中所排放出，這一類的物質藉由模擬人體內正常的荷爾蒙，去與體內的荷爾蒙接受器作用，進而造成我們人體的內分泌不正常，他會直接刺激或抑制我們體內內分泌的機制，造成我們人體的一些病變，他最主要會造成的疾病有：乳癌，睪丸癌，男性精子數目下降，早熟，生殖和發育的不正常等等....。因此發展出一種快速而且簡單的檢測方式，便使用了酪氨酸酶(tyrosinase)，藉由包埋的方式固定在碳膠電極(SPCE)的表面上形成一個電流式的生物感測器，藉由電化學的方法來做為我們的檢測方式。Bisphenol A, diethylstilbestrol, paraben 和 3-aminophenol 在文獻中說明了它們具有環境荷爾蒙的性質，因此便拿來做為檢測的物質。在電化學實驗中所施加的工作電位為-0.2V，結果所得到的檢量線範圍分別為 0.1 ~ 20  $\mu\text{M}$ ，10 ~ 100  $\mu\text{M}$ ，1 ~ 50  $\mu\text{M}$  和 20  $\mu\text{M}$  而偵測極限為 20 nM，0.4  $\mu\text{M}$ ，20 nM and 5  $\mu\text{M}$  分別對 Bisphenol A, diethylstilbestrol, paraben 和 3-aminophenol 我們所用來做檢

測的感測器它具有良好的連續性測試再現性(R. S. D = 5.8%)。



# Detection of Endocrine Disrupting compounds by a Tyrosinase Biosensor

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## Abstract

Endocrine disrupt compounds (EDCs) or environmental hormones are steroid-like compounds that exist in nature or are produced during industrial process. EDCs, once ingested, may interfere with the functions of normal endocrine system in both wildlife and humans. Hence, the development of a quick and easy operating biosensing device is necessary. In this study, an amperometric biosensor for the detection of endocrine disrupt compounds based on tyrosinase entrapped within gelatin film is designed and developed. The suspected endocrine disrupt compounds, including bisphenol A, diethylstilbestrol (DES), paraben and 3-aminophenol, were tested on the developed biosensor. Amperometric response of biosensor to EDCs in a phosphate buffer at pH 6 was measured as a function of concentrations at fixed bias voltage of  $-0.2$  V versus Ag/AgCl. The electrode gives linear response ranges of  $0.1 \sim 20$   $\mu\text{M}$ ,  $10 \sim 100$   $\mu\text{M}$ ,  $1 \sim 50$   $\mu\text{M}$  and  $20 \sim 100$   $\mu\text{M}$  for bisphenol A, diethylstilbestrol (DES), 3-aminophenol and paraben , respectively. The detection limit for bisphenol A, diethylstilbestrol (DES), 3-aminophenol and paraben are 20 nM, 0.4  $\mu\text{M}$ , 20 nM and 5  $\mu\text{M}$ , respectively. Moreover, the developed biosensor exhibits a good reproducibility with a relative standard deviation (R.S.D) of 5.8 %.

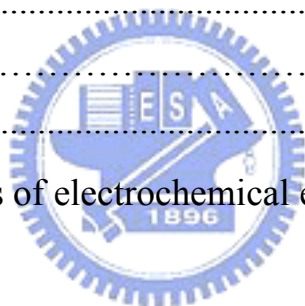
# Acknowledgment

首先感謝 袁俊傑老師在我碩士班的這兩年期間給予我的指導及幫助,讓我能在碩士班的這兩年中獲得不少的收穫,以及實驗室學長姐們對於我們學弟妹的照顧,使我的研究生這兩年的生活過的更加的充實豐富,以及感謝口試委員 楊裕雄老師和 吳東昆老師給予我寶貴的意見,最後感謝我的家人及女友對於我的支持及鼓勵。



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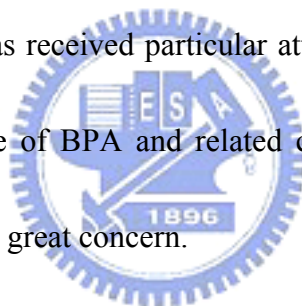
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## Introduction

Endocrine disrupting compounds (1-5), either generated naturally or synthetically, were shown to disrupt the endocrine systems of human and animals. EDCs affect the endocrine system of hosts by mimicking or antagonizing the effects of hormones, altering the pattern of synthesis and the metabolism of hormones or by changing the level of hormone receptors. There is growing concern about synthetic EDCs in the household products, because they may interrupt the development of offspring, alter the metabolic system, and cause cancer. In the cosmetics, there are more than 3000 chemicals were used. Among them, more than 100 chemicals are known causing allergic responses in human. There is a big difference in regulating the usage of these chemicals in different countries. Some countries require the cosmetics and skin care products be labeled completely; while most of countries just ignore the potential threat of these chemicals. However, many chemicals used in household cosmetics influence human health even in a small amount. Thus, it is extremely important to pay attention to the usage of EDCs in the cosmetics, and household products

For example, bisphenol A (6-8), diethylstilbestrol (9-12), paraben (13-15) and 3-aminophenol (16, 17), are widely found in baby bottles, pharmacological products, cosmetics and hair dye, respectively. BPA is a solid chemical compound with a low

volatility and a solubility of 120-300 mg/l in water (18), whereas it can be greatly solubilized in a basic solution. Accordingly, about 50% of BPA in the environment associate with sediments or soils. BPA has been found to mimic the female hormone, estrogen, disrupting the chemical messenger system in the body even in a low level. BPA also exhibited estrogenic effect to the human breast cancer cell line MCF-7. It may exhibit its hormonal effects in as low as 2-5 ppb or (2-5  $\mu\text{g/l}$ ) (5). In addition, BPA is broadly used in industries as a precursor to manufacture polycarbonate and numerous plastic wares. The release of BPA from the plastic wares and materials used to line food and drink cans has received particular attention because of its biological disrupting effect. The leakage of BPA and related compounds from bottles, dental fillings and sealants is also the great concern.



Diethylstilbestrol (9), a synthetic estrogen, was widely used by pregnant women in the 1950s and 1960s. Millions of pregnant women were given DES hope to prevent miscarriages and other problems. However, increasing reports indicated that women who took the drug exhibited an increasing risk of breast cancer (19-21). Girls whose mothers were given DES during pregnancy, termed DES daughters, were discovered to be at increased risk in malformations of the reproductive organs, infertility and miscarriages. DES daughters were also found to have increasing rate of cervical and vaginal cancers. DES sons, on the other hand, are predisposed to testicular

abnormalities, such as abnormally small testes and failure of the testes to descend into the scrotum, which increases the risk of testicular cancer. Apparently, women and the children of women who took DES are at risk for certain health problems. As a result, in 1971 the Federal Drug Administration (FDA) issued a warning that DES should not be given to pregnant women.

Parabens, such as propyl-paraben, ethyl-paraben and butyl-paraben, are commonly used in cosmetics and personal care products, including shampoos, skin creams and deodorants, as preservatives to suppress the growth of bacteria and fungi. However, they have been found to mimic estrogen in vitro and might function as endocrine disruptors. Parabens in Leave-on products, such as facial makeup and skin lotions, are of greatest concern because of the long exposure time and opportunity for ingestion via skin or mouth into the bloodstream. The estrogenic effects of parabens induces the risk of human for breast cancer (22). The role of parabens in the progression of breast cancer, however, is unknown. Currently, there is a trend to reduce the usage of paraben in the cosmetics, because of its potential in endocrine disrupting.

3-aminophenol and related compounds are aromatic amines that are widely used in hair dye and pesticide (17). Some of these aromatic amines have been found to associate with formation of bladder cancer in workers of the dye factories (23). Hair

dyes containing aromatic amines has been investigated by scientists for their potential carcinogenic effect. Aminophenol was reported to increase incidence of producing micronuclei in the bone marrow and splenocytes of mice (24, 25). Application of aminophenols in hair dyes results in human systemic exposure and may pose a potential genotoxic hazard to human health.

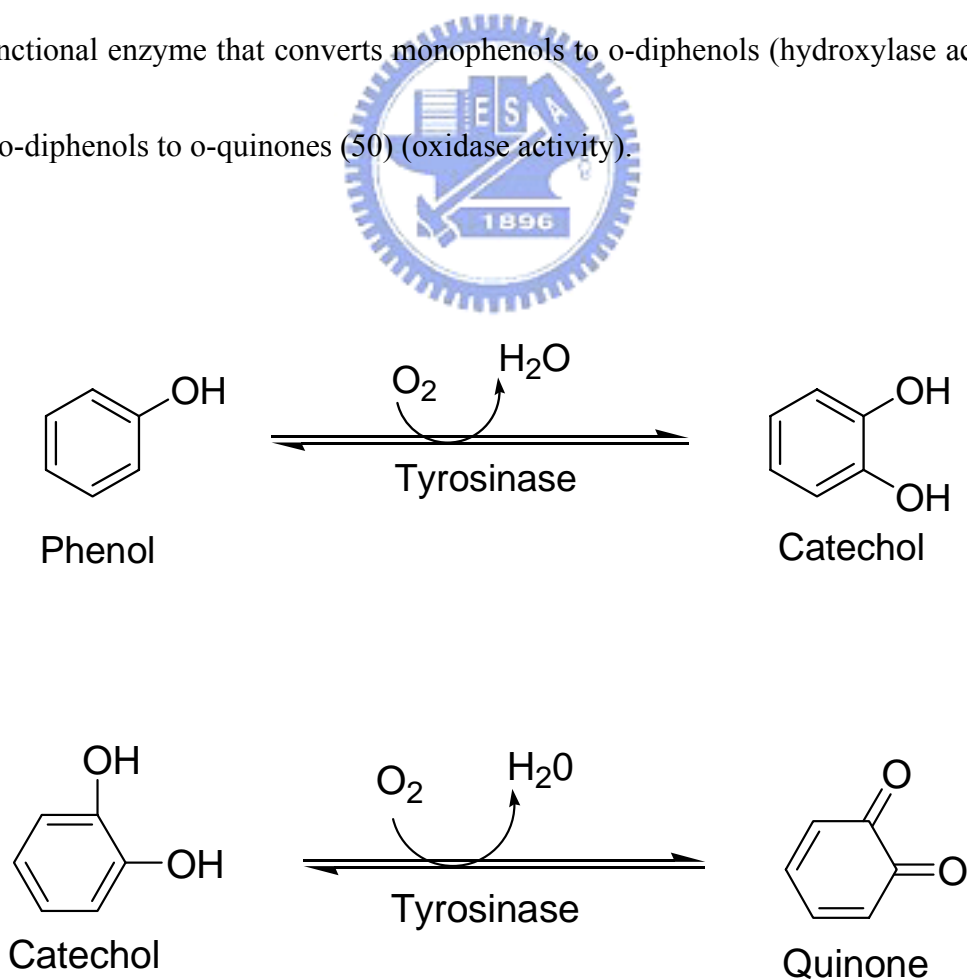
Conventionally, gas chromatography–mass spectrometry (GC–MS) (26, 27) and capillary electrophoresis (CE) (28-30) are two main analytical tools for the separation and measurement of EDCs. However, these methods are time-consuming and expensive. Thus, a quick and easy method for the detection of EDCs seems to be necessary.



Biosensor is an analytical device by integrating the immobilized biologically active material, such as protein, enzyme, antibody, cells or tissues, with a transducer. The main function of a transducer is to convert the physical or chemical change produced by biologically active material into an electric signal. Based on the type of transducer used, biosensors have been divided into optical (31, 32), calorimetric, piezoelectrical (33, 34) and electrochemical biosensors (35, 36). Electrochemical biosensors are based on the detection of electrochemically active species consumed and/or generated in the biological or chemical reactions. There are three types of electrochemical biosensors, such as, conductometric (37-39), potentiometric (40, 41),

and amperometric biosensors (42-46), are commonly developed. Screen-printing carbon paste electrode (SPCE) is very suitable for the fabrication of these types of biosensor due to its simplicity, low-cost, high reproducibility, and efficiency in mass production (47, 48). SPCE also exhibits a wide window potential, low background current, and suitability for different types of analysis (49), exhibiting a great potential in the construction of disposable sensing system.

In this thesis, we propose to fabricate a tyrosinase-based EDC biosensor for the detection of EDCs. Tyrosinase, also called polyphenol oxidase (EC 1.14.18.1), is a bifunctional enzyme that converts monophenols to o-diphenols (hydroxylase activity) and o-diphenols to o-quinones (50) (oxidase activity).



The o-quinones can be electrochemically reduced to o-diphenols under a low over-potential (9, 51) without any electron transfer mediator. Many EDCs, such as bisphenol A, Diethylstilbestrol, paraben and aminophenol, contain a phenolic functional group, by which a simple amperometric biosensor can be used for the measurement of phenolic EDCs.

Immobilization of biomolecules on a transducer is the key to the development of biosensors (52, 53). Although, the activity of immobilized enzyme may not be as high as it is in the solution, it can be stabilized to avoid thermal inactivation on often denaturing conditions (54). Enzyme can be adsorbed on electrode surface, by electrostatic interaction, Van der Waal's force, hydrogen bonding. Physical adsorption (55, 56) is easy to prepare. The enzyme may retain its active conformation through physical adsorption. However, since the interaction is weak, it may not be stable under different temperatures, pH values and ionic strengths. Even worse, enzymes may leak out from the electrode. Adsorption can be used as the first step for downstream treatment, such as covalent modification and cross linking.

Affinity interactions between the enzyme and its substrate, a receptor protein and its ligand, or antigen-antibody pairs are often characterized by high association constants of the resulting complexes, which enabled the use of specific recognition interactions to construct protein layers on solid supports. In order to increase lifetime



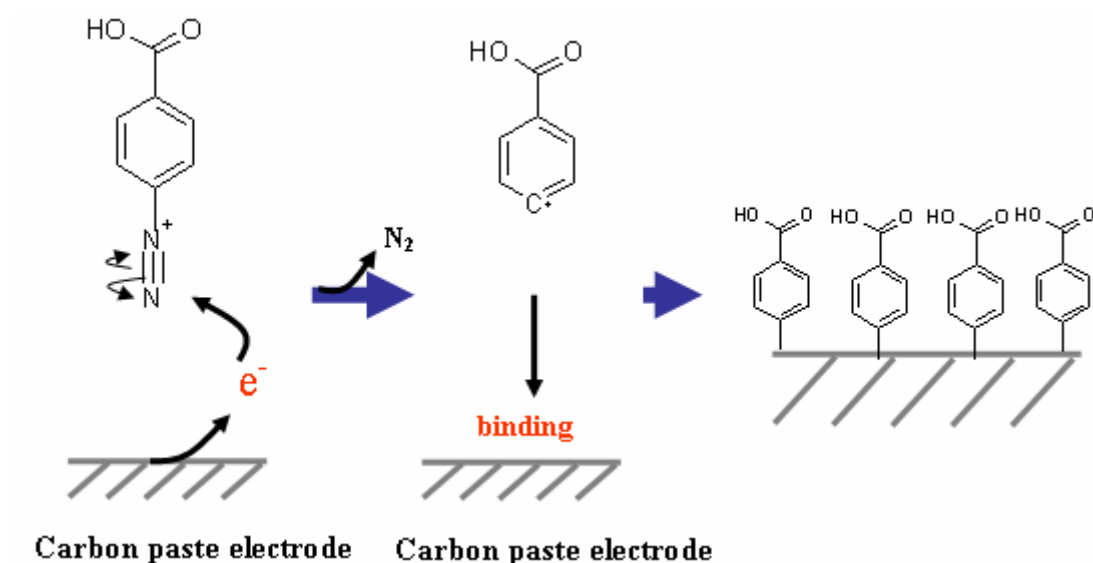
of enzyme electrodes, a covalent linkage between enzyme and transducer is necessary (57). The hydroxyl groups of Ser, Thr and Tyr residues, the sulfhydryl group of cysteine and amino group of Lys and Arg are potential groups for cross-linking (58). The advantages of this type of immobilization are low diffusion rate and good stability under adverse conditions.

Enzymes can be immobilized by enzyme using intra- or intermolecular cross-linking of enzyme molecules (59, 60). This method is based on the formation of three-dimensional links between the biological material and bi- or multi- functional reagents. The resulting modified biological material is completely insoluble in water and can be adsorbed onto a solid surface. Glutaraldehyde (61) is one of the mostly used cross-linking reagents among cross-linking reagents for protein covalent bounding. Configuration based on multiple-layer deposition using glutaraldehyde as a cross-linking agent for enzyme immobilization is one of the most reported methods for the development of planar biosensing devices (59, 60). The resulting 3D network may affect the kinetics of entrapped enzymes and diffusion rate of substrates and products. An optimization between immobilization, preserving activity and maintaining the appropriate diffusion characteristics, should be performed.

Enzymes can also be trapped in to matrixes, such as gelatin (62-65), PVA-SbQ (66), silica sol-gel (67, 68), polyurethanes (69, 70), polyvinyl alcohol (71-73) and

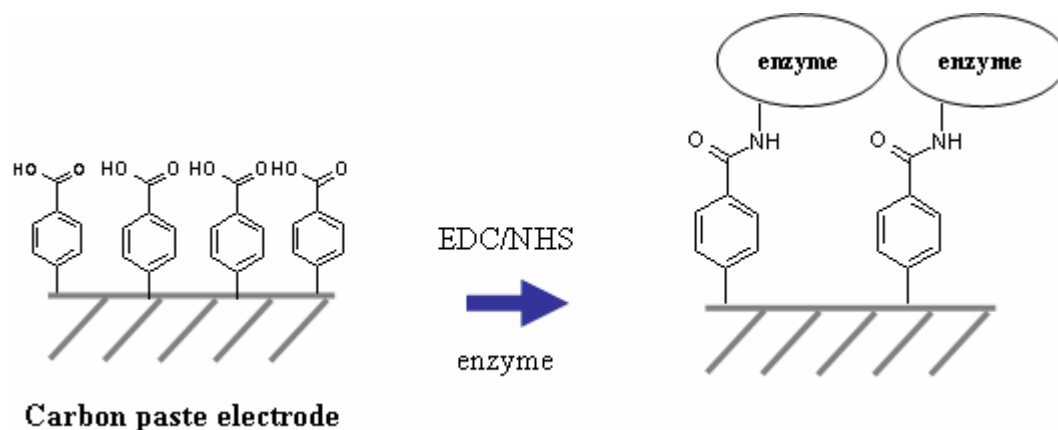
carbon paste (74, 75), in which they can be well protected. One step preparation and good reproducibility are also advantages of this technique. However, enzyme entrapment may induce the probability of substrate access to the active site of enzymes. Therefore, the apparent activity of enzymes may decrease especially when the substrate is large. The leakage of entrapped enzymes may also pose a severe problem of this technique.

The electrochemical reduced diazonium cation has been reported to used as a bringing compound in the covalent bridging of enzymes on the surface of electrode (76-78). The electrochemically activated diazonium cation can covalently bind to surface of the graphitic carbon electrodes (Scheme1). Electrodeposition of diazonium cation can be demonstrated by direct and immobilization of proteins on the surface of electrodes.



(Scheme1)

This reaction (EDC/NHS) was triggered directly onto diazonium-modified proteins.



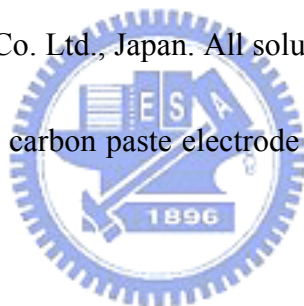
(Scheme2)

Screen-printed carbon paste electrode is commonly used in the fabrication of commercial biosensors. In this thesis, the development of a tyrosinase-based EDC biosensor is proposed for the measurement of endocrine disrupting compounds. An optimal condition for the construction of the tyrosinase/gelatin screen printed carbon paste electrode. An electrochemically reduced diazonium cation was also used to covalently immobilize tyrosinase on the surface of SPCE.

## Material and Methodology

### *Reagents*

Tyrosinase (EC 1.14.18.1) was purchased from Sigma. Bisphenol A (BPA), diethylstilbestrol (9), paraben, aminophenol were from Fluka. Sodium dihydrogenphosphate, disodium hydrogenphosphate and potassium dihydrogenphosphate were from Showa. Sodium chloride was from Amresco. Potassium chloride was from ICN. Potassium phosphate, dibasic was from Riedel-de Haën. Poly vinyl alcohol functionalized with pyridinium methyl sulfate (PVA-SbQ) was from Toyo Gosei Kogyo Co. Ltd., Japan. All solution was prepared by de-ionized distilled water. Screen-printed carbon paste electrode (SPCE) was from ApexBichem (Hsinchu, Taiwan).



All electrochemical experiments were performed on an electrochemical analyzer CHI 440 instrument (CH Instruments) connected to a personal computer. Electrochemical measurement was performed using SPCE as working electrode, Au as counter electrode and Ag/AgCl as reference electrode in a beaker containing 10 ml buffer solution (Appendix). The solution in the beaker was stirred at a fixed rate.

### *Preparation of enzyme electrode*

Gelatin (also called gelatine) was purchased from Yukuri pure chemicals.

Gelatin is a linear polypeptide that consists of different amounts of 18 amino acids and offers a unique set of characteristics in terms of gel-forming properties and hydrophilicity. Tyrosinase was immobilized in a gelatin matrix crosslinked with formaldehyde. Screen printed carbon paste electrodes (SPCE) were pretreated by cyclic voltammetry (CV) with a potential range from -1.0 V to 1.0 V and scanning rate of 200 (mV/s) for 5 cycles.

The 1%, 3%, 5%, 7%, 9% gelatin stock solutions were prepared by dissolving gelatin powder in warm dd H<sub>2</sub>O. To prepare tyrosinase electrodes (79), 10  $\mu$ l formaldehyde solution was first added to 1 ml gelatin solution to generate a gelatin pre-mixture. Next, mix tyrosinase stock solution (20 U/ $\mu$ l) with above gelatin pre-mixture in a ratio of 1:2. Finally, 6  $\mu$ l of the above mixture was dropped on the surface of the SPCE and allowed to dry at 4 °C for 24 h.

An amperometric biosensor consists of three electrodes in a system. The first electrode is the working electrode, by which the electrochemical responses are measured. A number of noble metals are currently available for making working electrodes, such as platinum, gold, silver, palladium, rhodium and iridium. The second electrode is the reference electrode that is taken as the reference standard against which the potential of the other electrodes present in the electrochemical cell can be measured. The third electrode is the counter or auxiliary electrode, which serves as a

source for electrons so that current can be passed from the external circuit through the electrochemical cell. Amperometric measurement is performed by applying a potential between a reference and working electrode and result in a cathodic or anodic current, which is co-related to the concentrations of electroactive analytes in the solution.

### ***Enzyme immobilization by diazonium cation***

A diazonium cation was first obtained by mixing 8 ml CMA (30 mM) 1 ml HCl (2 M) 1 ml  $\text{NaNO}_2$  (2 M) in a 10 ml electrochemical cell. The aniline derivative of CMA was diazotated in an aqueous solution for 10 min under stirring in ice-cold water. The diazonium solution prepared was then immediately used to perform electro-addressing on the surface of SPCE. Electro-addressing was achieved by directly depositing on the surface of carbon paste electrode. Ten cyclic voltammograms were carried out within a potential range of -0.7 V to 0.8 V at a scan rate 200 mV/s. After ten cyclic voltammogram, the electrode was then immersed in 0.1 M MES (2-morpholinoethanesulfonic acid) buffer, pH 6.0, containing 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) (0.4 mg/ml) and N-hydroxysulfosuccinimide (NHS) (0.6 mg/ml) with stirring for 1 h. After reaction, the electrode was rinsed quickly with rinsed dd  $\text{H}_2\text{O}$  and incubated overnight

in the phosphate buffer (pH 6.0) containing tyrosinase (1 mg/ml) at 4 °C for 24 h to allow enzymes to cross-link to the carboxylic group of CMA.

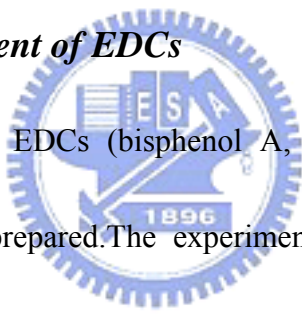
### ***Cyclic Voltammetry (CV)***

The linear sweep voltammetry (80, 81) (LSV) is a simple technique for electrochemical analysis. The potential range is scanned in one direction, starting at the initial potential and finishing at the final potential. A more commonly used variation of this technique is cyclic voltammetry (CV) (82, 83), which is usually used to study the electrochemical properties of enzyme electrode. In CV, the direction of the potential is reversed at the end of the first scan. Thus, the waveform is usually of the form of an isosceles triangle (Fig. 1a). The scan begins from the left hand side of the current/voltage plot where no current flows. As the voltage is swept further to the right (to more reductive values) a current begins to flow and eventually reaches a peak before dropping (Fig. 1b).

The characteristic peaks in the cyclic voltammetry are closely related to properties of the diffusion layer near the electrode surface. These parameters, including concentration gradients for the reactant and product at different times corresponding to the potential value (during the forward and reversed scans, respectively), and the achievement of a zero reactant surface concentration, affect the

characteristics of cyclic voltammograms. Note that the continuous change in the surface concentration is coupled with an expansion of the diffusion layer thickness ( $\delta$ ). The resulting current peaks thus reflect the continuous change of the concentration gradient with the time. Hence, the increase of the peak current corresponds to the achievement of diffusion control; while the current drop (beyond the peak) exhibits a  $t^{-1/2}$  dependence (independent of the applied potential). For the above reasons, the reversal current has the same shape as the forward one.

### ***Amperometric measurement of EDCs***



Stock solution of 1 M EDCs (bisphenol A, diethylstilbestrol, paraben and 3-aminophenol) were fresh prepared. The experimental set up for electrochemical reaction is conventional three-electrode system with working electrode, counter electrode, reference electrode of tyrosinase electrode, gold and Ag/AgCl, respectively. The working potential was set at -0.2 V versus Ag/AgCl. After reaching a steady state background current, a given concentration of the EDC sample solution was injected into a reaction chamber containing 10 ml PBS solution using microsyringe. The measurement was stop when the reductive current reached a steady state. The response time was determined by at the time when analyte was added from the time when 95% steady state current was reached (84) (Fig. 3).



## Result and Discussion

### *Generation and characterization of enzyme electrode*

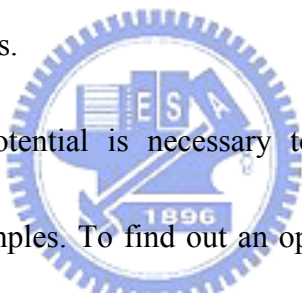
Before the construction of tyrosinase-based enzyme electrode, SPCE was pretreated with cyclic voltammetry within a potential range from -1.0 V to 1.0 V in PBS buffer for 5 cycles. The effect of different percentages of gelatin in the encapsulation of tyrosinase was studied by repetitively determine the electrochemical response of enzyme electrode to 10  $\mu\text{M}$  bisphenol A. Therefore, the reproducibility of the enzyme electrode generated by using different percentages of gelatin (0.67%, 2%, 3.33%, 4.67% and 6%) to 10  $\mu\text{M}$  bisphenol A was determined. The relative standard derivation (R.S.D) for EDC biosensors by entrapping tyrosinase in 0.67%, 2%, 3.33%, 4.67% and 6% gelatin were 35.52%, 28.06%, 13.66%, 2.86% and 3.15%, respectively (Fig. 4). This result suggests that the EDC biosensor fabricated by a Tyrosinase/4.67% gelatin matrix exhibited a good reproducibility for 10  $\mu\text{M}$  bisphenol A. Thus, enzyme electrode fabricated by entrapping tyrosinase in 4.67% gelatin matrix on SPCE was used in subsequent experiments.

The electrochemical response of enzyme electrode was studied by repetitively determine its response to 1  $\mu\text{M}$  bisphenol A 50 times. The overall average response current of the fabricated enzyme electrode was  $113 \pm 6.5$  nA with a relative standard derivation of 5.8% (Fig. 5). The result showed that the average response current of the

first 10 reactions is 114 nA. The response reduced slightly after 50 measurements.

The average response current of the last 10 reactions was 109 nA. The signal lost from the first 10 reactions to last 10 reactions is about 4.4%.

The optimal pH range for the electrochemical response of developed enzyme electrode was also studied in the pH range of 3 ~ 8 by using 10  $\mu$ M bisphenol A as substrate (Fig. 6). The result showed that the response current of enzyme electrode increased with the increase of pH and reached maximum at pH 6. Therefore, pH 6 was chosen in the following experiments to determine the enzyme biosensor to various EDCs and phenolic compounds.



An optimal working potential is necessary to avoid noise generated from electroactive impurities in samples. To find out an optimal working potential for the determination of EDCs the electrochemical response of bisphenol A were performed under different working potentials (0, -0.1, -0.2, -0.3, -0.4 and -0.5 V). Bisphenol A (1 or 10  $\mu$ M) was added into a 10 ml reaction cell, followed by an amperometric measurement. As shown in Fig. 7 the maximum response to 10  $\mu$ M BPA occurred at -0.2 V and -0.3 V. The maxima currents at working potentials at -0.2 V and -0.3 V were  $1615 \pm 25$  nA and  $1593 \pm 72$  nA, respectively. In contrast, in the buffer, the electrochemical responses of enzyme biosensor were  $38.9 \pm 5.8$  nA and  $118 \pm 12.1$  nA at working potentials at -0.2 V and -0.3 V, respectively. These results indicate that

working potential at 0.2 V is optimal for detection of EDCs.

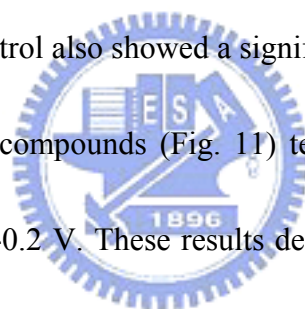
### ***Cyclic voltammograms of enzyme electrode***

The CV of enzyme electrode and bare SPCE in a pH 6.0 PBS buffer was performed within the potential range of -1.0 V ~ 1.5 V with a scanning rate of 100 mV/s (Fig. 8). The result showed that the enzyme electrode and SPCE exhibits similar cyclic voltammograms without substance, suggesting that the immobilization of tyrosinase did not affect the basal electrochemical response of SPCE. The cyclic voltammograms of bisphenol A on the enzyme electrode was carried out in 1X PBS buffer, pH 6.0. BPA stock solution (1 M) was freshly prepared by dissolving in methanol. The CV was performed within a potential range of -0.7 V ~ 0.8 V with a scan rate of 200 mV/s. Ten cycles of cyclic voltammetry in the presence of 1 mM bisphenol A were performed and illustrated in Fig. 9. The result showed that the enzyme electrode exhibited a significant increase in the reductive current at -0.2 V, which was consist with the precious observation.

Tyrosine, catechol, tyramine, neurotransmitters (dopamine, norepinephrine) and endocrine disrupting compounds (bisphenol A, diethylstilbestrol, paraben, 3-aminophenol) (Fig. 10), are consisting of phenolic group, that may be recognized by tyrosinase and exhibit electrochemical response. Stock solutions of above phenolic

compounds (100 mM) were freshly prepared in each experiment. The cyclic voltammogram of 1 mM each of dopamine, norepinephrine, catechol, tyrosine and EDCs was performed in 1X PBS buffer, pH 6.0 in a 10 ml reaction chamber. The CV was carried out between potential range -1.0 V ~ 1.0 V for tyramine (10 mM). The potential range of -1.0 V ~ 1.0 V was set for dopamine, norepinephrine, catechol and tyrosine. The scan rate of cyclic voltammograms was set at 200 mV/s.

The cyclic voltammograms of bisphenol A, 3-aminophenol, paraben and diethylstilbestrol exhibited that an oxidative peak at 0.61 V, 0.80 V, 1.02 V and 0.55 V, respectively. Diethylstilbestrol also showed a significant reductive peak at 0.012 V. All the endocrine disrupting compounds (Fig. 11) tested in this study exhibited no significant reductive peak at -0.2 V. These results demonstrated that these chemicals can be easily determined at a working potential of -0.2 V without any interference.

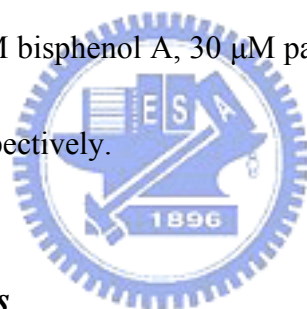


### ***Amperometric measurement of endocrine-disrupting compounds***

The experimental set up for electrochemical reaction is a three-electrode system with the SPCE, gold electrode and Ag/AgCl as the working, counter and reference electrodes, respectively. The working potential was set at -0.2 V vs. Ag/AgCl. Once the background current reached a steady state, the electrochemical reaction was started by adding 10  $\mu$ l stock EDCs solution into the electrochemical reaction cell containing 10 ml 1X PBS buffer, pH 6.0 with a constant stirring. The result

demonstrated that the EDCs were electrochemical inactive at the working potential of -0.2 V (Fig. 12).

The tyrosinase-based biosensor was fabricated by entrapping 40 U tyrosinase on the surface of SPCE by 4.67% gelatin crosslinked with formaldehyde. The step responses of fabricated enzyme electrode to repetitively added 1  $\mu\text{M}$  bisphenol A (Fig. 13a), 30  $\mu\text{M}$  paraben (Fig. 13b) or 5  $\mu\text{M}$  3-aminophenol (Fig. 13c) was determined. The results showed that the average response currents to 1  $\mu\text{M}$  bisphenol A, 30  $\mu\text{M}$  paraben and 5  $\mu\text{M}$  3-aminophenol were 116 nA, 46 nA and 443 nA, respectively. The average response time to 1  $\mu\text{M}$  bisphenol A, 30  $\mu\text{M}$  paraben and 5  $\mu\text{M}$  3-aminophenol were 73 s, 193 s and 52 s, respectively.



### ***Calibration plots of EDCs***

By using fabricated EDC biosensor the linear dynamic range of various EDCs was also determined. As shown in Fig. 14, shows the steady state current-dependent calibration curves for bisphenol A (Fig. 14a), 3-aminophenol (Fig. 14b), paraben (Fig. 14c) and diethylstilbestrol (Fig. 14d) were determined. The responses of enzyme electrode to bisphenol A, 3-aminophenol, paraben and diethylstilbestrol were found to be linear in the range of 1  $\mu\text{M}$  ~ 20  $\mu\text{M}$  ( $r^2 = 0.999$ ), 10  $\mu\text{M}$  ~ 100  $\mu\text{M}$  ( $r^2 = 0.947$ ), 20  $\mu\text{M}$  ~ 100  $\mu\text{M}$  ( $r^2 = 0.994$ ) and 1  $\mu\text{M}$  ~ 50  $\mu\text{M}$  ( $r^2 = 0.993$ ), respectively. The developed tyrosinase-based amperometric biosensor exhibited a wider dynamic range

for the detection of phenolic compounds than that of biosensors developed in recently reports (1, 85). The lowest detection limit ( $S/N \geq 3$ ) of the developed biosensor for bisphenol A, diethylstilbestrol, paraben and 3-aminophenol were 20 nM, 0.4  $\mu\text{M}$ , 5  $\mu\text{M}$  and 20 nM, respectively (Fig. 15).

Based on the regulation of EC SCF, the maximum amount of bisphenol A is permitted to transfer from the plastic into 1 kg of food is 3 mg or 3 ppm. No more than 0.8% of parabens are allowed to be added in cosmetic product as preservatives; whereas the U.S. FDA suggests that cosmetic should not contain more than 1% parabens. 3-Aminophenol, commonly used in the hair dye and pesticides, is limited to be used in a concentration more than 20 ppm. Apparently, the developed tyrosinase-based biosensor in this study is suited to detect the EDCs in household products.

### ***Application of tyrosinase-based biosensor for the detection of other phenolic compounds***

Dopamine (DA) and norepinephrine play an important role in regulating functions of central nervous, cardiovascular, renal and hormonal systems (86, 87). Dopamine and norepinephrine also function in drug addiction and Parkinson disease. The analytical determination of dopamine (DA) in biological fluids has gotten considerable attention. However, in the electroanalysis of dopamine in sera, ascorbic

acid and uric acid are major problems, because they exhibit similar oxidative potential to that of dopamine on SPCE. Thus determination of DA in sera by direct electrochemical oxidation of DA is difficult.

The use of enzyme electrodes is a highly selective sensing device for the detection of biological analytes. Therefore, the development of tyrosinase-based biosensor may reduce the interference exhibited by ascorbic acid and uric acid in sera. The cyclic voltammograms of 1 mM dopamine and 1 mM norepinephrine were performed within potential range of  $-1.0\text{ V} \sim 1.0\text{ V}$  (Fig. 16). Dopamine and norepinephrine exhibit an anodic peak current of 0.5 V and 0.5 V, respectively; whereas, their cathodic peak current of 0.1 V and 0.1 V, respectively. The enzyme electrode exhibited a linear dynamic range of  $1\text{ }\mu\text{M} \sim 40\text{ }\mu\text{M}$  ( $r^2 = 0.998$ ) (Fig 17) for dopamine. The detection limit for dopamine was 100 nM ( $S/N \geq 3$ ) (Fig. 18). The linear dynamic range for norepinephrine was between 1 and 100  $\mu\text{M}$  (Fig 19); while the detection limit of norepinephrine was 200 nM (Fig 20)

Phenolic compounds are widely used and usually release into environment. Catechol is widely used as a photographic developer, as an intermediate in rubber and lubricating oils and as a precursor or intermediate in pharmaceuticals. Catechol is also an important probe in chemical manufacturing process. A reputable international agency has classified catechol as a carcinogen that may possibly cause cancer to

human.

By using developed tyrosinase-based biosensor, catechol could be determined at -0.2 V versus Ag/AgCl by direct reduction of quinone. The cyclic voltammograms of 1 mM catechol was also performed within potential range of -1.0 V ~ 1.0 V (Fig. 21), exhibiting the oxidative and reductive peak currents of 0.4 V and 0.05 V, respectively. The amperometric I-t curve of 1  $\mu$ M catechol was also performed (Fig 22). The average response current for 1  $\mu$ M was 748 nA; while average response time was 50 s. The developed biosensor exhibited a linear dynamic range of 0.1  $\mu$ M to 10  $\mu$ M (Fig. 23). The detection limit was 20 nM ( $S/N \geq 3$ ) (Fig. 24).

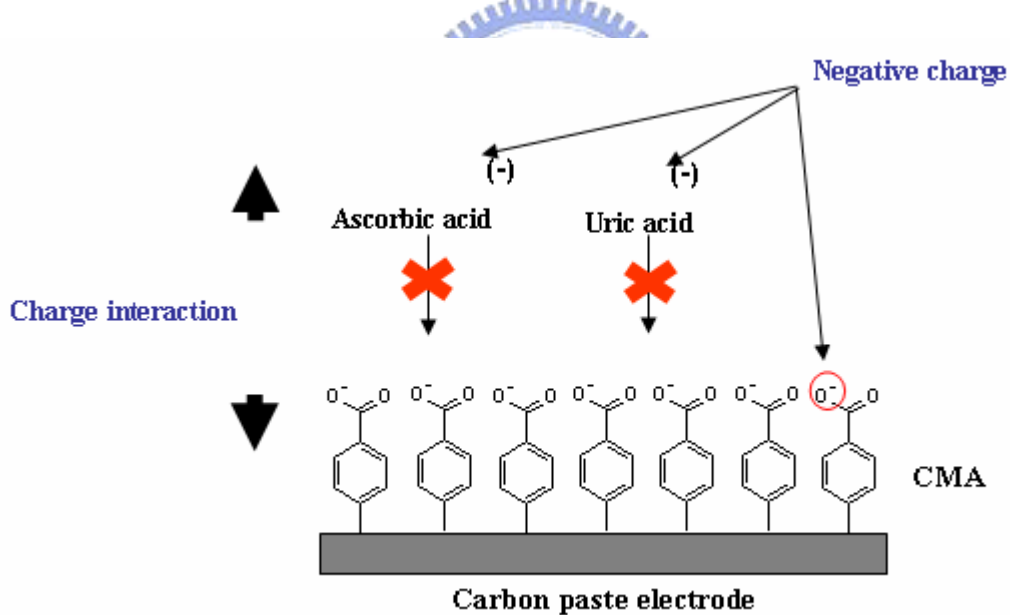
### ***Stability of tyrosinase biosensor after long-term storage***

The long term stability after storage was performed on three discrete tyrosinase-based biosensors. The electrochemical responses of these biosensors to 10  $\mu$ M bisphenol A after storage for 1, 5, 7, 10, 12, 19, 21 and 31 days were determined in 1X PBS, pH 6.0 (Fig. 25). The responses of the developed tyrosinase-based biosensor remained at around 1400 ~ nA/10  $\mu$ M bisphenol A within first week after 10 days storage, however, the activity of developed biosensor decreased about 2%. Even often 31 days storage, about 85% activity of developed biosensor remained (Fig 25).

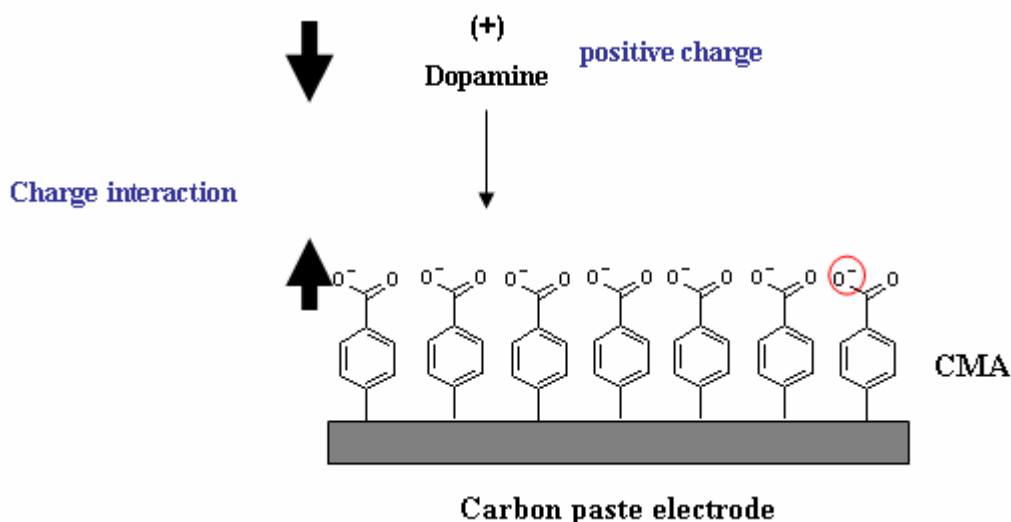


## *Development of tyrosinase based biosensor by Diazonium cation immobilization*

Electrochemical deposition of diazonium cation was performed by cyclic voltammetry. The cyclic voltammogram was performed by immersing SPCE in diazonium cation solution (24 mM). The potential range was set between -0.7 V and 0.8 V for ten cycles. As shown in Fig 26, the reduction currents were gradually decreased during ten scan cycles. To demonstrated diazonium cation was deposited on the electrode surface, we determine whether modified SPCE can catalyze redox reaction of ferricyanide differentially under different pH value (Diagrams 1 + 2)



(Diagram 1)



**(Diagram 2)**

The modified electrode contains carboxylic groups, which have a pKa of 4.2. The surface of the electrode have negative charged at pH 7.0. Therefore, negative charged substances, such as ascorbic acid, uric acid and ferricyanide, may be repelled from the surface of electrode, reducing the electron transfer rate. In contrast, dopamine, a positively charged compound at pH 7.0. Interestingly, the cyclic voltammograms ferricyanide, ascorbic acid and uric acid exhibited a high oxidative peak current and broaden oxidative peak on the modified electrode than that on the bare electrode (Fig. 27). This result suggests that the SPCE was really modified by diazonium cation.

In pH 7.0, the 4-carboxyphenol groups were deprotonated and presented negative charge. However, at pH 2.0, the carboxylic group of diazonium cations remained protonated, reducing the repelling force to the ferricyanide. Therefore, the reductive and oxidative peak currents re-appeared in the cyclic voltammogram (Fig.

28). In comparison, bare SPCE exhibited similar cyclic voltammograms of ferricyanide at both pH 2.0 and 7.0 (Fig. 28).

Subsequently, tyrosinase can be immobilized on the surface of modified electrode by EDC/NHS (76, 77). The generated tyrosinase/CMA/SPCE was then tested for its electrochemical reactivity to 10  $\mu$ M bisphenol A (Fig. 29). The average response current of tyrosinase/CMA/SPCE to 10  $\mu$ M bisphenol A is  $1907.5 \pm 52.3$  nA with a relative standard derivation of 2.74 % (Fig. 29). Compare with gelatin entrapment, tyrosinase immobilized on the diazonium matrix exhibited a much faster response time and higher electrochemical response (Table. 1).

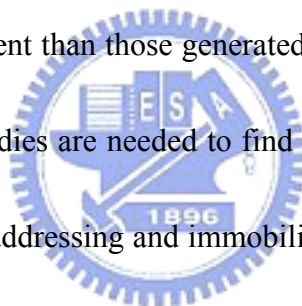


## Conclusions

We have developed a tyrosinase-based biosensor by immobilizing tyrosinase on the surface of SPCE in a 4.67% gelatin matrix. The reproducibility and pH profile of developed tyrosinase-based biosensor was then characterized. The developed biosensor exhibited a maximum electrochemical response to bisphenol A at around pH 6.0. The detection of EDCs and other phenolic compounds by the tyrosinase-based biosensor can be optimally determined at -0.2 V without much interference from the analytes. The developed biosensor exhibits electrochemical responses of 116 nA, 46 nA and 443 nA to 1  $\mu\text{M}$  bisphenol A, 30  $\mu\text{M}$  paraben and 5  $\mu\text{M}$  3-aminophenol, respectively. The average response time to 1  $\mu\text{M}$  bisphenol A, 30  $\mu\text{M}$  paraben and 5  $\mu\text{M}$  3-aminophenol were 73 s, 193 s and 52 s, respectively. The bisphenol A, 3-aminophenol, paraben and diethylstilbestrol could be detected on the developed tyrosinase electrode linearly in the range of 1  $\mu\text{M}$  ~ 20  $\mu\text{M}$  ( $r^2 = 0.999$ ), 10  $\mu\text{M}$  ~ 100  $\mu\text{M}$  ( $r^2 = 0.947$ ), 20  $\mu\text{M}$  ~ 100  $\mu\text{M}$  ( $r^2 = 0.994$ ) and 1  $\mu\text{M}$  ~ 50  $\mu\text{M}$  ( $r^2 = 0.993$ ), respectively. The lowest detection limit ( $S/N \geq 3$ ) for bisphenol A, diethylstilbestrol, paraben and 3-aminophenol were 20 nM, 0.4  $\mu\text{M}$ , 5  $\mu\text{M}$  and 20 nM, respectively. These results demonstrated that the developed tyrosinase-based biosensor exhibited a better detection limit, sensitivity and long-term storage stability than those of other reported tyrosinase-based biosensors (Table. 2, Table. 3 and Table. 4) (1, 79, 85).

However, other phenolic compounds, such as catechol and phenol, in environment may cause interference to the developed tyrosinase-based biosensor during the electrochemical reactions. In the future, an integration between the developed biosensor and a capillary electrophoresis method, which is to separate EDCs from mixtures in advance of the detection, may largely improve the detection of EDCs in the environment.

The feasibility of immobilization of tyrosinase on the surface of SPCE by diazonium cation has been demonstrated in this study. We find that a faster response time and higher response current than those generated by the tyrosinase/gelatin-SPCE biosensor. However, more studies are needed to find out an optimal condition for the diazonium salt-based electro-addressing and immobilization of enzyme on the surface of SPCE. This technique exhibits a great potential for the future fabrication of micro- or even nano-biosensors.



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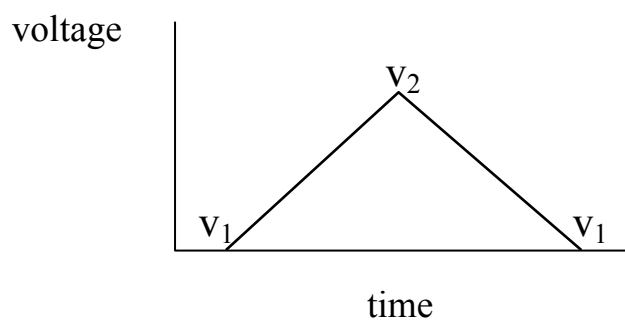
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(a)



(b)

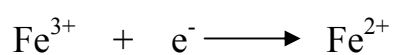
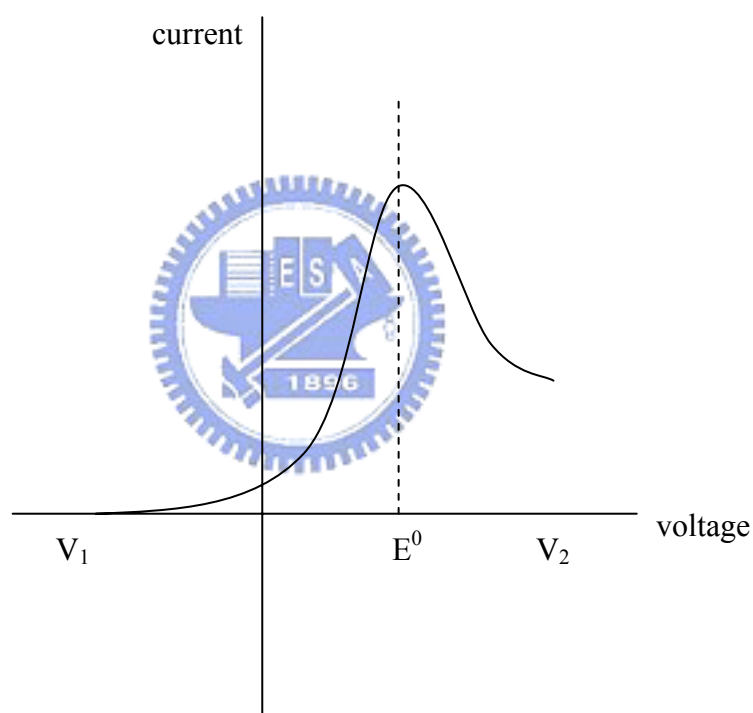


Figure 1. Response of a redox couple during a potential cycle

(a) Cyclic voltammetry consists of scanning linearly the potential of a stationary working electrode. Depending on the information sought, single or multiple cycles can be used. (b) A redox potential scan is chosen for first half cycle. As the applied reductive potential reached  $E^0$  for the redox reaction, the current was increased, until the reductive peak was reached.

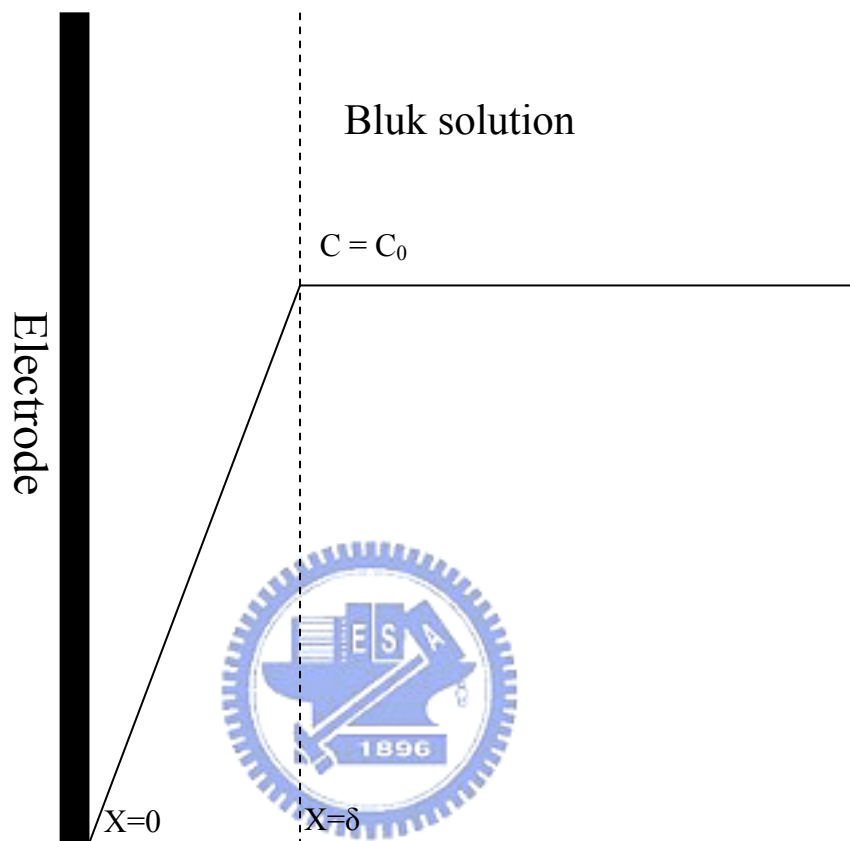
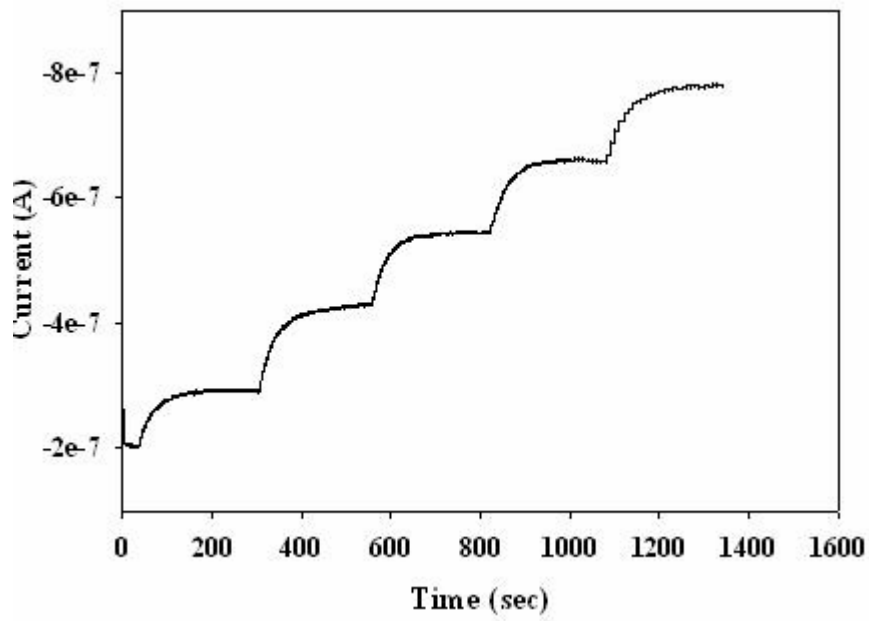


Figure 2. The Nernst diffusion layer

The electrode is presented by the blue bar on the left side of the picture. The x-axis is the distance from the electrode. The  $x = 0$  is the electrode surface. The y-axis is the concentration of substrate in the bulk solution. The diffusion layer thickness  $\delta$ , in normal stirred solution the thickness of diffusion layer was between 0.01 mm and 0.001 mm.

(a)



(b)

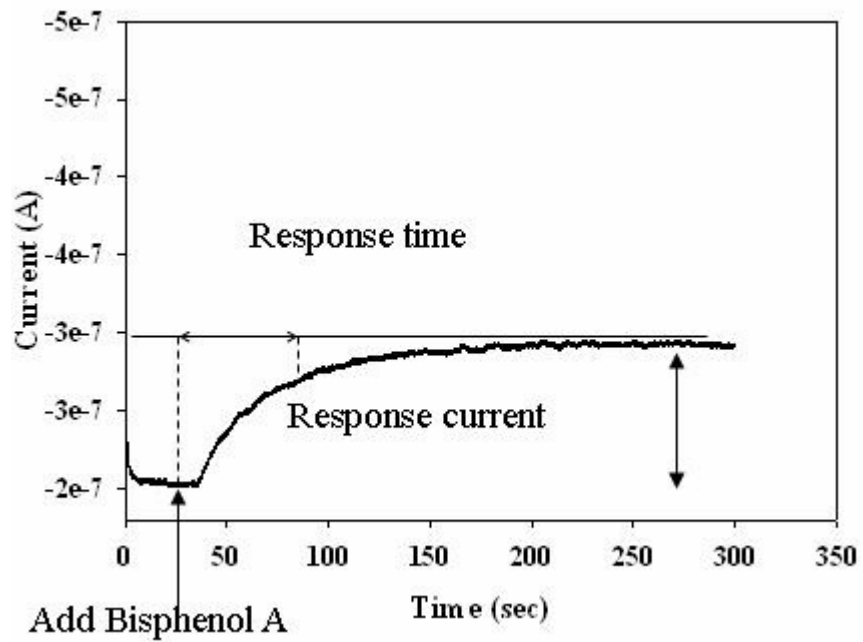




Figure 3. Step responses of tyrosinase biosensor to sequentially added 1  $\mu\text{M}$  bisphenol A

Endocrine disrupting chemicals sensing strip was fabricated by entrapping 40 U tyrosinase by gelatin. (a) The step responses to 1  $\mu\text{M}$  bisphenol A was performed in PBS buffer, pH 6.0 by repetitively adding 1  $\mu\text{M}$  bisphenol A. Working potential is -0.2 V vs. Ag/AgCl. (b) The response time was determined after responses current reaching a steady state. The response time is determined by the interval between the time of adding bisphenol A and the time reaching 95% steady state current.



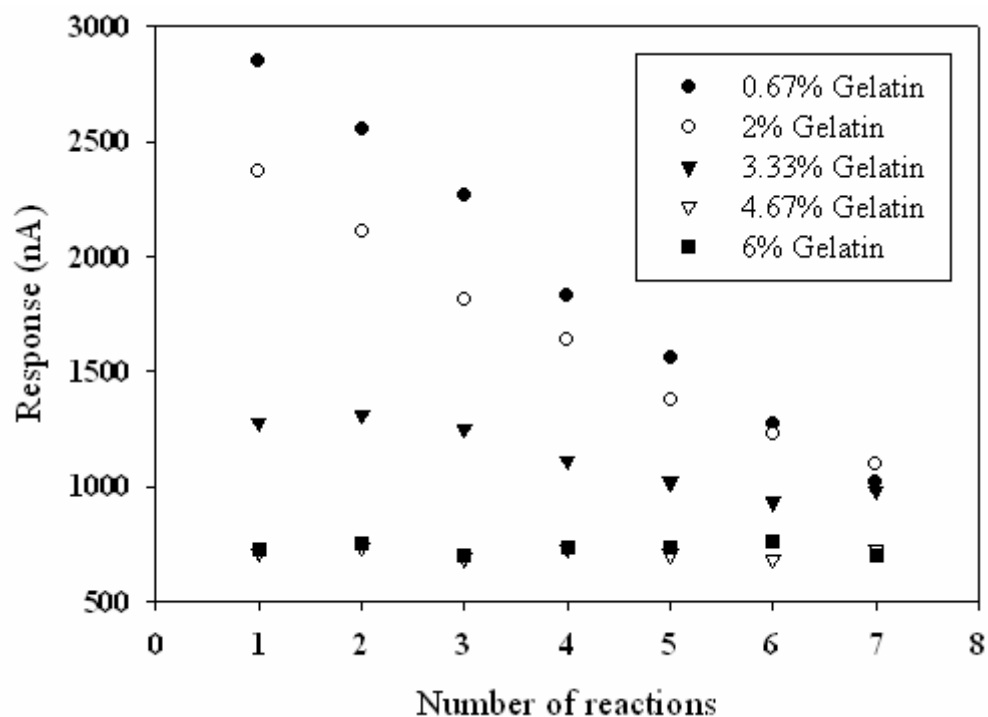


Figure 4. Stability of tyrosinase biosensor entrapped by different percentage gelatin

Tyrosinase biosensor were fabricated by entrapping tyrosinase in (-●-) 0.67% gelatin (-○-) 2% gelatin (-▼-) 3.33% gelatin (- ▽-) 4.67% gelatin (-■-) 6% gelatin on the surface of SPCE. The electrochemical response of tyrosinase biosensor to 10  $\mu$ M bisphenol A was determined in PBS, pH 6 with stirring. The working potential was -0.2 V versus Ag/AgCl. Seven reactions were recorded.

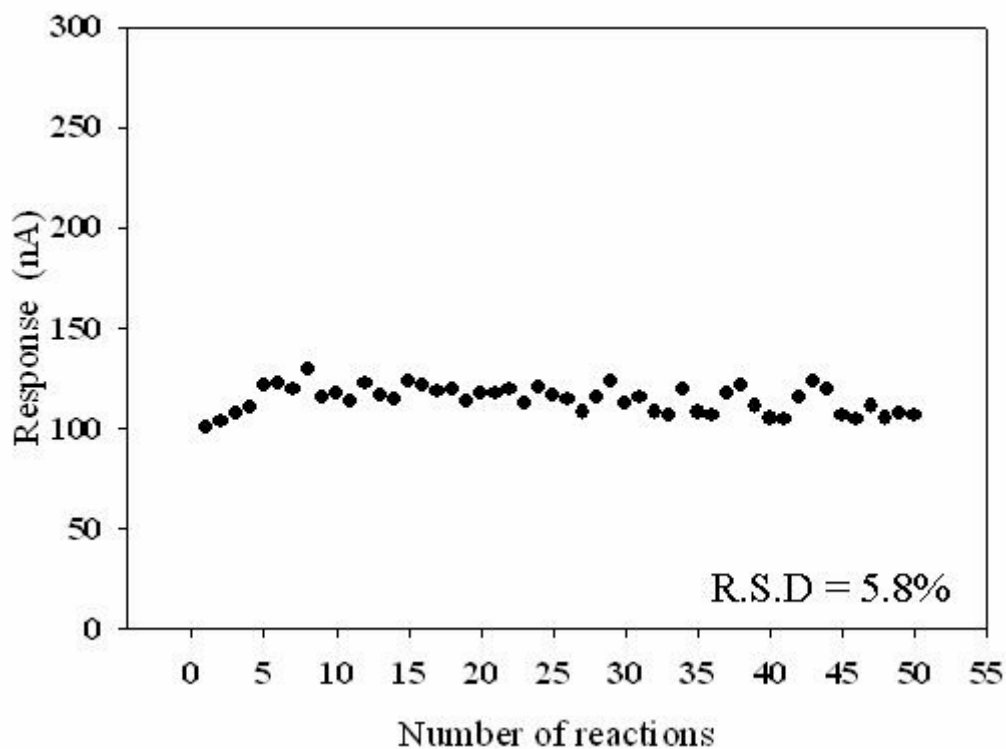


Figure 5. Reproducibility of tyrosinase biosensor with tyrosinase entrapped in 4.67% gelatin

Tyrosinase biosensor was entrapped in 4.67% gelatin and coated on a SPCE. The electrochemical response of tyrosinase biosensor to 1  $\mu$ M bisphenol A was determined in 1X PBS, pH 6.0. The working potential was -0.2 V vs. Ag/AgCl. Fifty reactions were recorded.

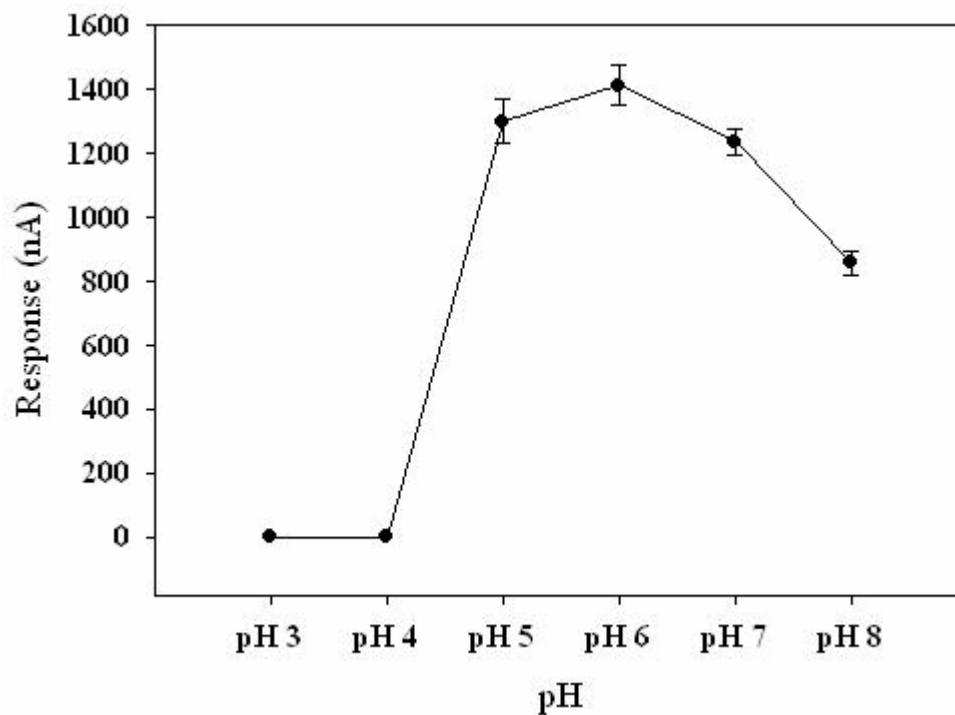


Figure 6. Effect of pH on the response of the tyrosinase biosensor

The effect of pH on the response of tyrosinase biosensor to 10  $\mu\text{M}$  bisphenol A was determined in PBS under different pH values (pH 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0). Working potential was -0.2 V vs. Ag/AgCl. Each data is presented as mean  $\pm$  S.D. and obtained from three independent.

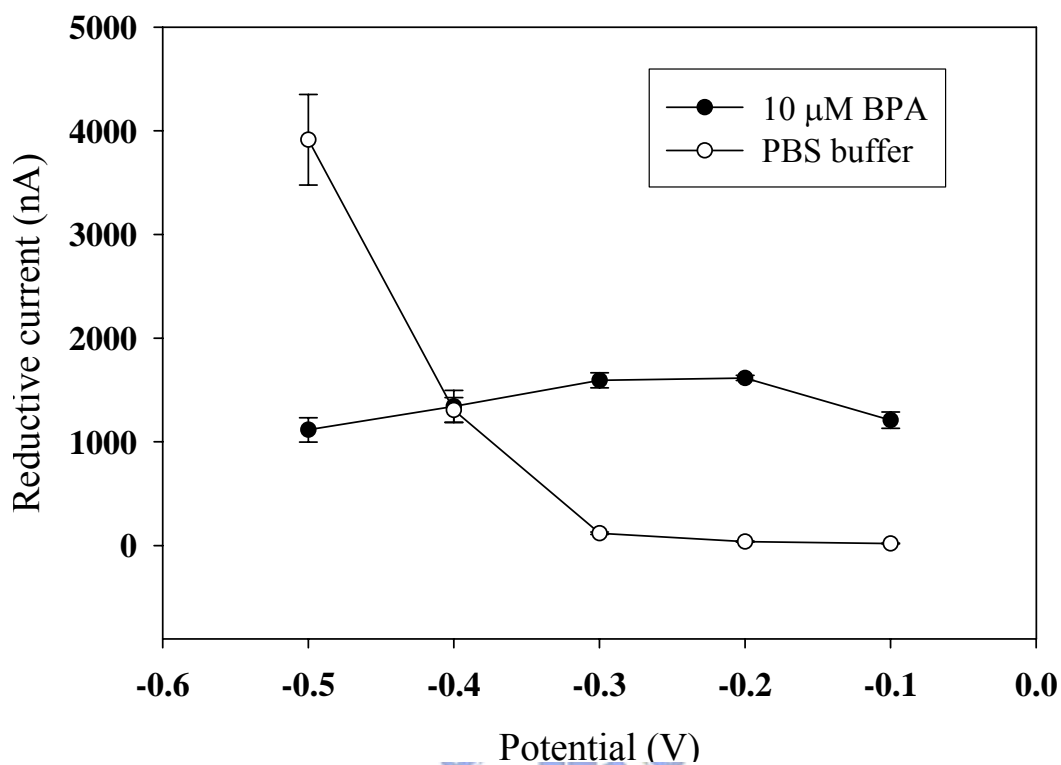


Figure 7. Amperometric response of tyrosinase biosensor to 10  $\mu$ M bisphenol A at different applied voltages

Tyrosinase biosensor was fabricated by entrapping 40 U tyrosinase via 7% gelatin. The electrochemical responses of tyrosinase biosensor to 10  $\mu$ M bisphenol A (-●-) and buffer only (-○-) at different working potential (-0.1 V, -0.2 V, -0.3 V, -0.4 V and -0.5 V) were measured. The reactions were performed in PBS, pH 6.0 at different voltages vs. Ag/AgCl. Each data obtained from three independent measurements.

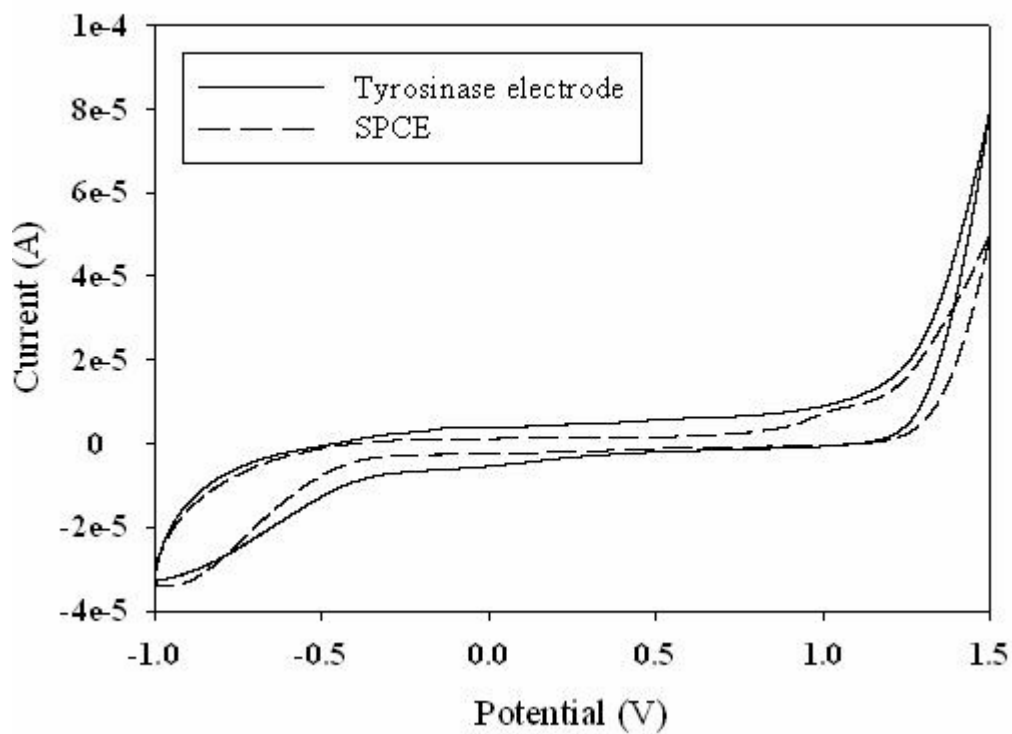


Figure 8. Cyclic voltammograms of tyrosinase biosensor and SPCE

The electrochemical response of SPCE (dot line) and tyrosinase biosensor (solid line) was performed in 1X PBS, pH 6.0. CV was set for -1.0 V ~ 1.5 V vs. Ag/AgCl with a scanning rate of 100 mV/s.

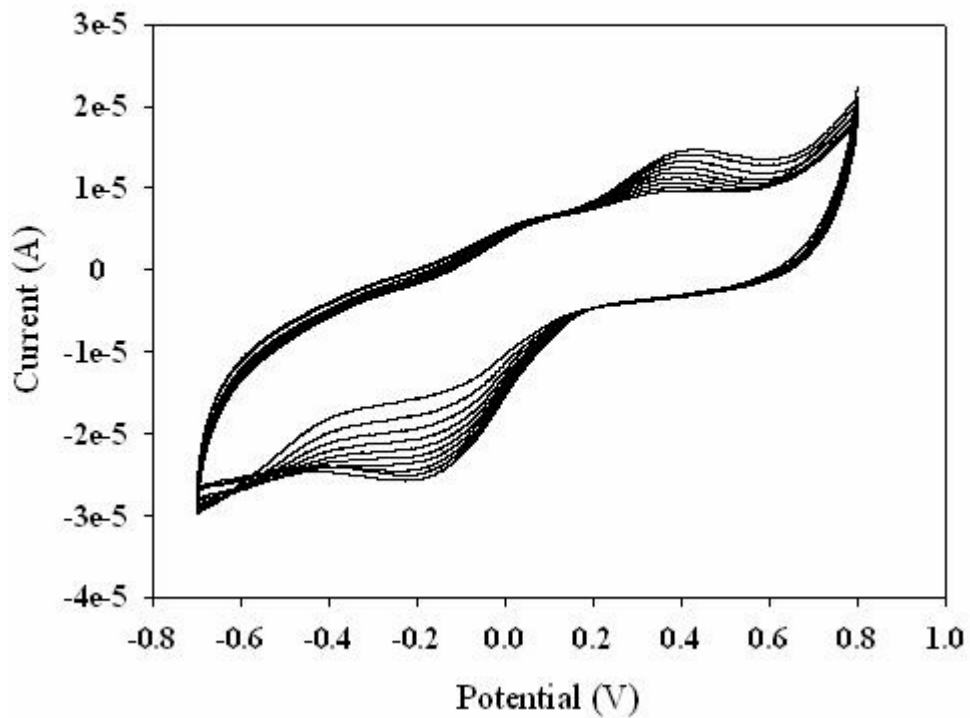
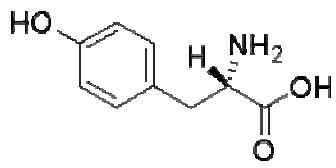


Figure 9. Cyclic voltammograms of tyrosinase biosensor in the presence of bisphenol A

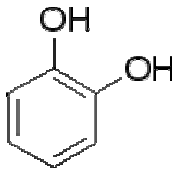
The cyclic voltammograms of tyrosinase biosensor to 1 mM bisphenol A was performed in 1X PBS, pH 6.0. Scanning range was set for -0.7 V and 0.8 V vs. Ag/AgCl with a scanning rate of 200 mV/s for 10 cycles.

(a)



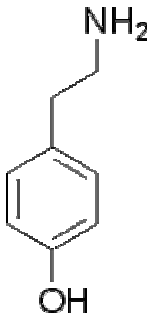
Tyrosine

(b)



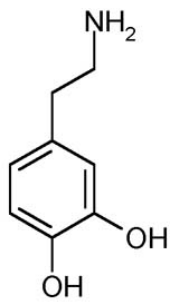
Catechol

(c)

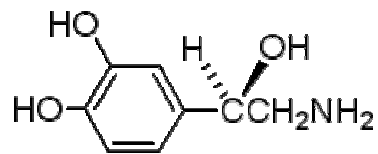


Tyramine

(d)



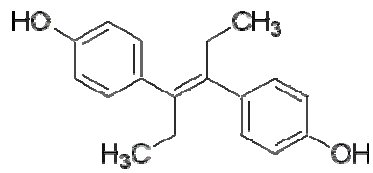
Dopamine



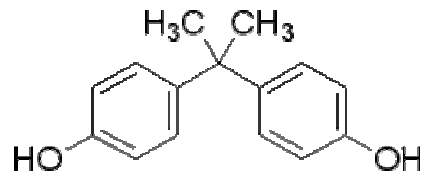
Norepinephrine



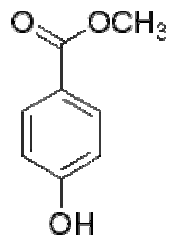
(e)



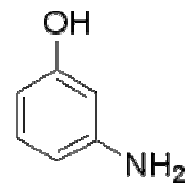
Diethylstilbestrol



Bisphenol A



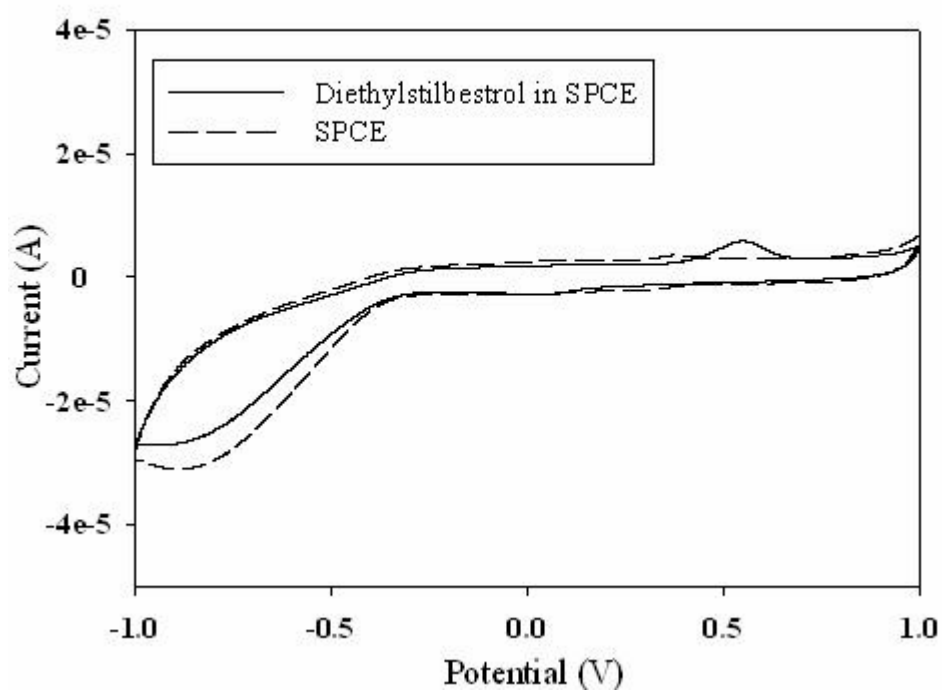
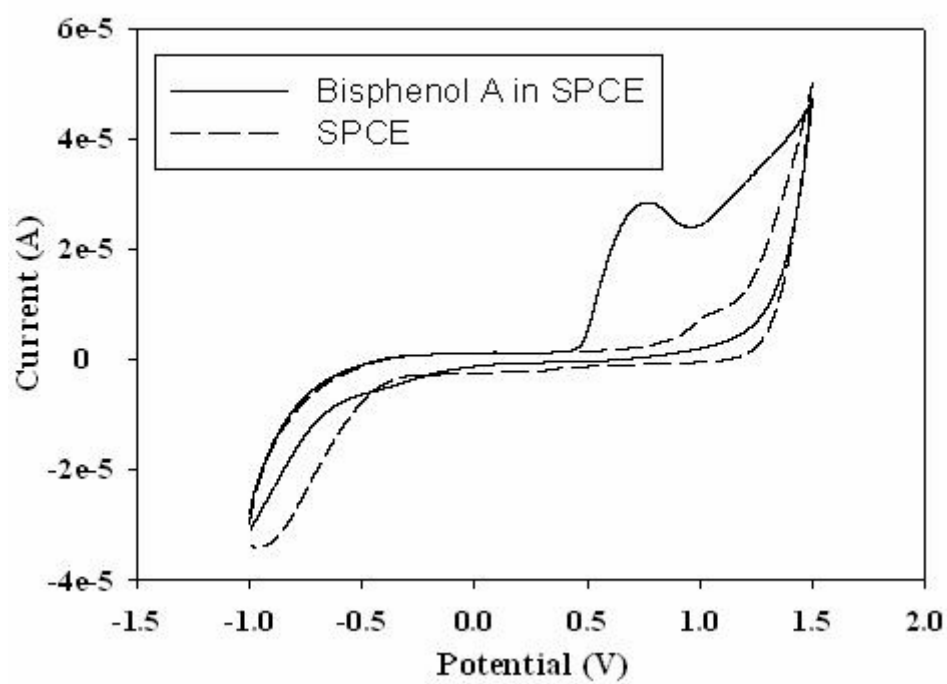
Paraben



3-Aminophenol

Figure 10. Structure of endocrine disrupting compounds and phenolic compounds

The chemical structure of (a) tyrosine (b) catechol (c) tyramine (d) dopamine and norepinephrine and (e) endocrine disrupting compounds.



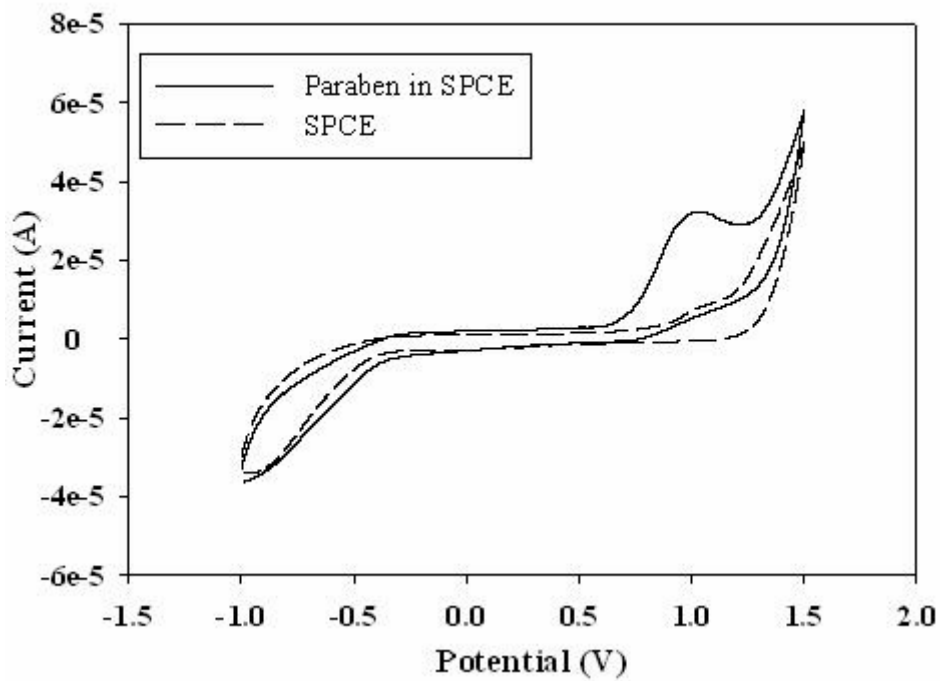
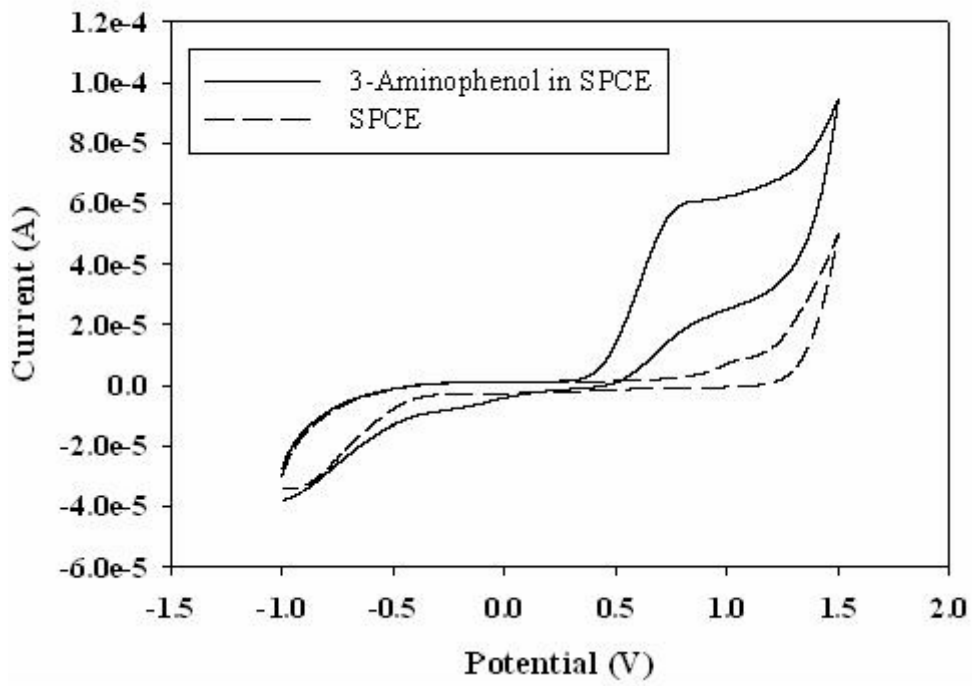
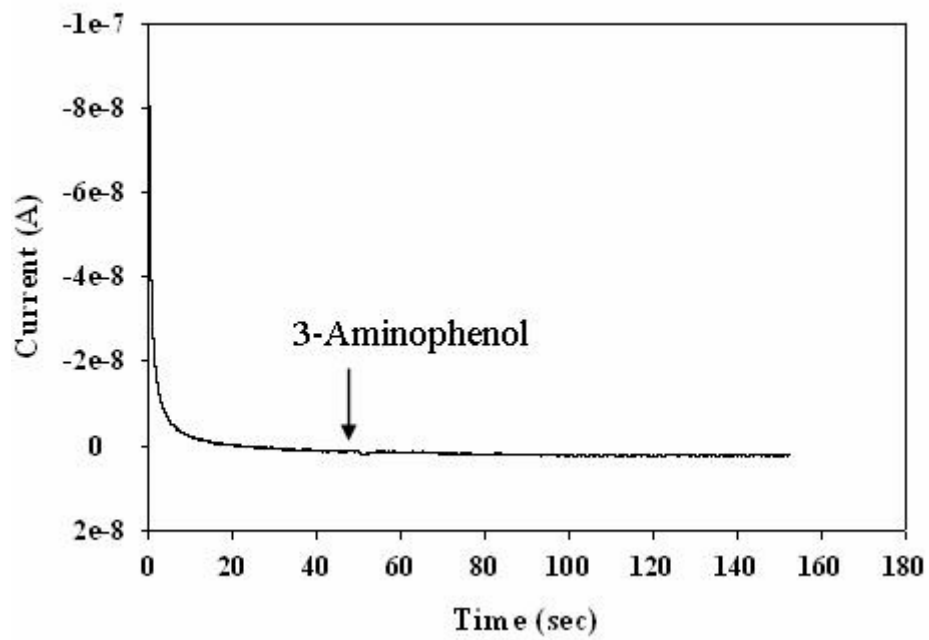
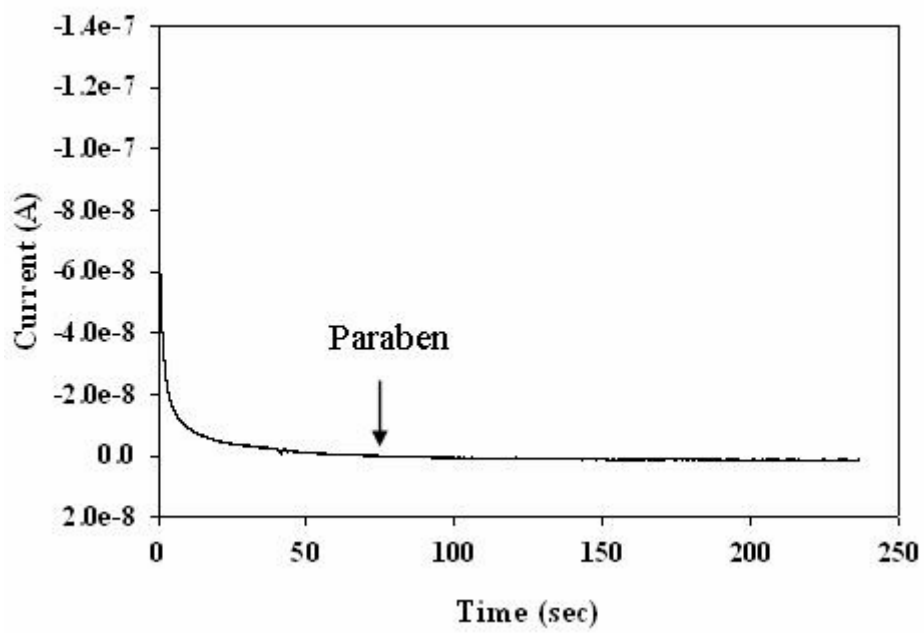


Figure 11. Cyclic voltammograms of different EDCs on the fabricated tyrosinase biosensor

The CV of SPCE (dot line) and EDCs on the SPCE (solid line) to 1 mM bisphenol A (a), diethylstilbestrol (DES) (b), paraben (c) and 3-aminophenol (d) were performed in 1X PBS, pH 6.0. Scanning range of CV was set -1.0 V ~ 1.5 V (a,c,d) and -1.0 V ~ 1.0 V (b) with a scanning rate of 200 mV/s.





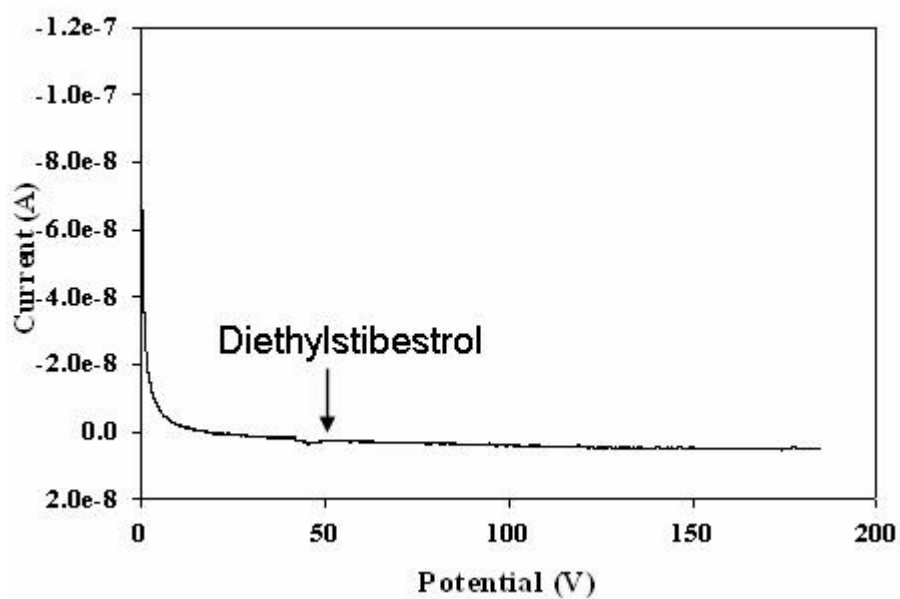
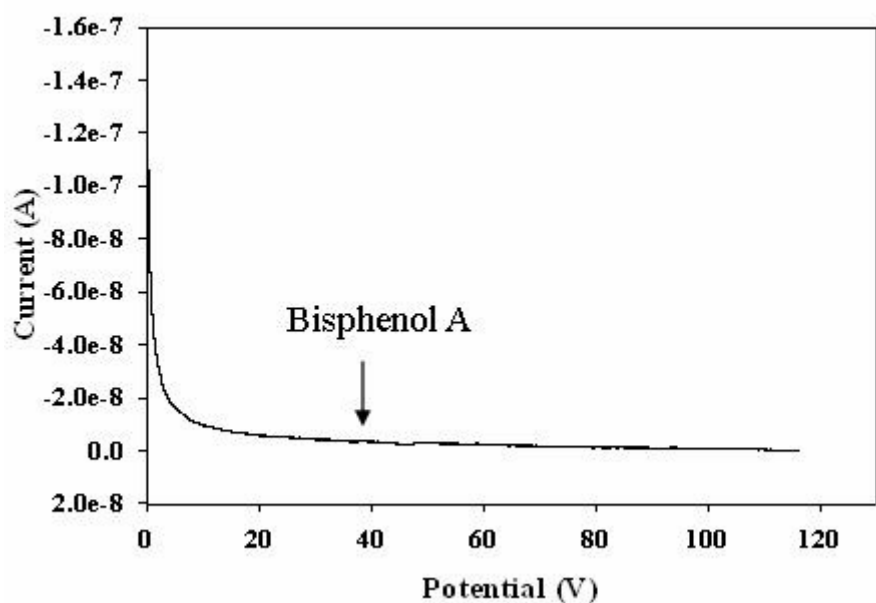
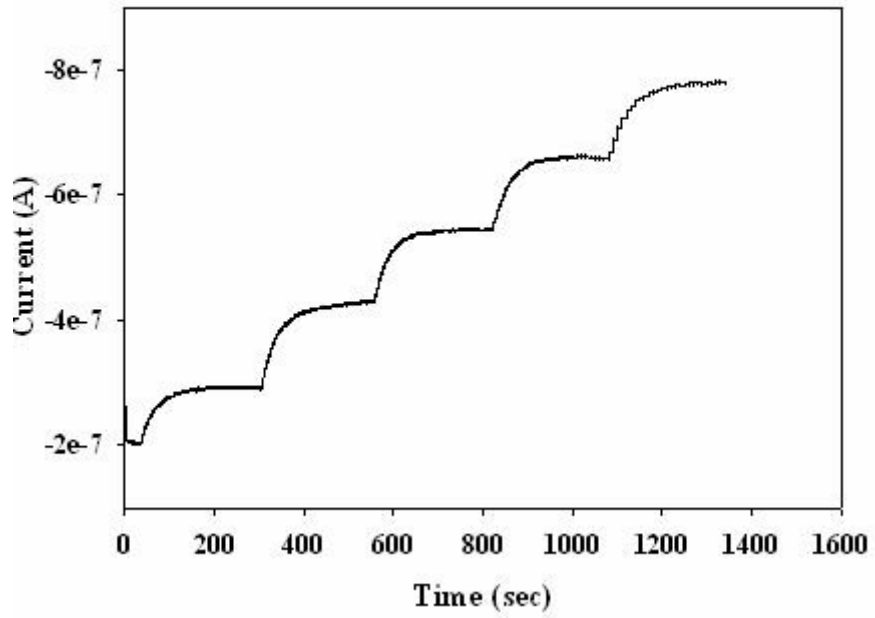


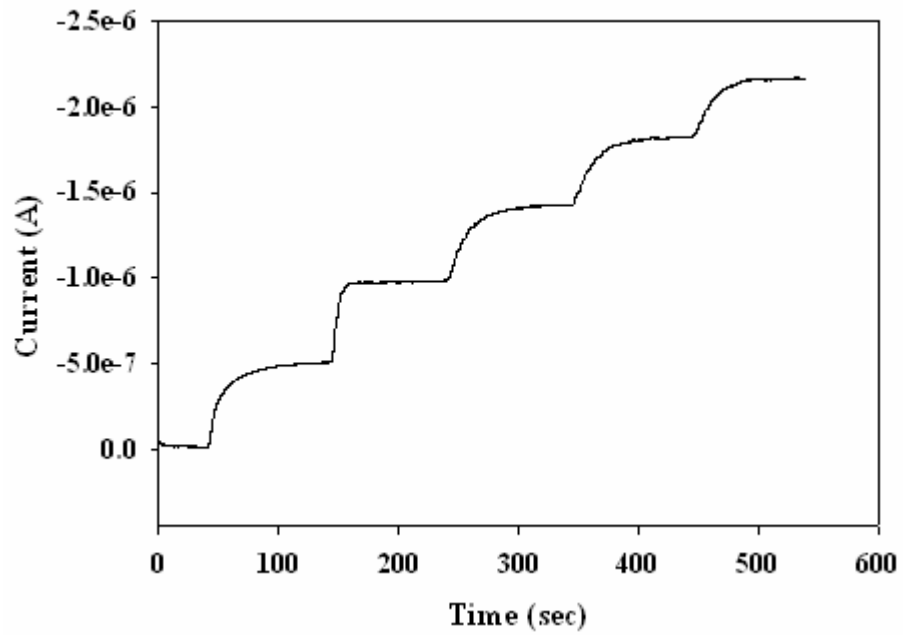
Figure 12. Response of bare SPCE to various EDCs

The *i-t* curve of SPCE to 100  $\mu\text{M}$  each of paraben (a), 3-aminophenol (b), bisphenol A (c) and Diethylstilbestrol (d) in PBS, pH 6.0. Working potential was -0.2 V vs. Ag/AgCl.

(a)



(b)



(c)

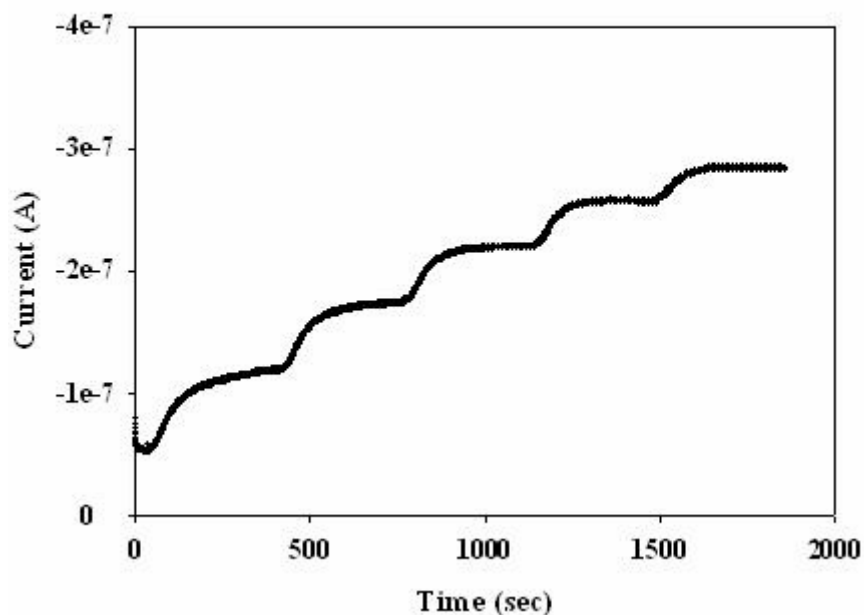
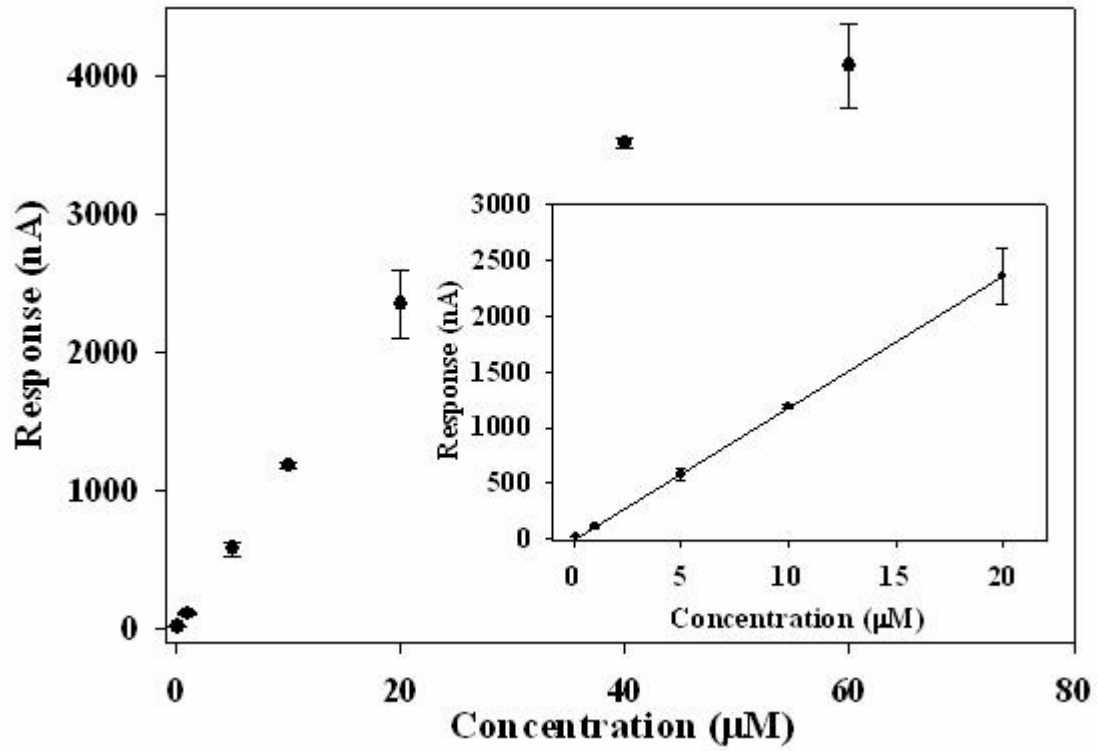


Figure 13. Step responses of tyrosinase biosensor to various EDCs

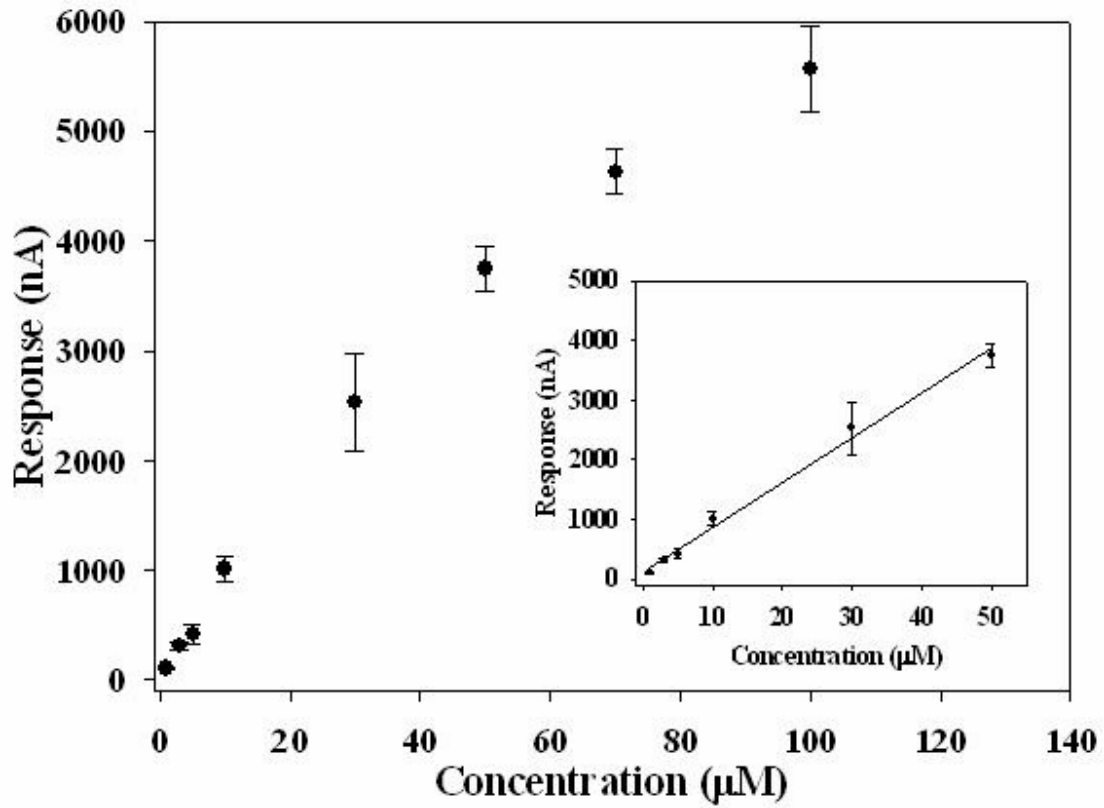
The tyrosinase biosensor was fabricated by entrapping 40 U tyrosinase in 7% gelatin on a SPCE. The step responses of tyrosinase biosensor to 1  $\mu\text{M}$  bisphenol A (a), 5  $\mu\text{M}$  3-aminophenol (b) and 30  $\mu\text{M}$  paraben (c) were performed in 1X PBS, pH 6.0. Working potential was -0.2 V vs. Ag/AgCl.



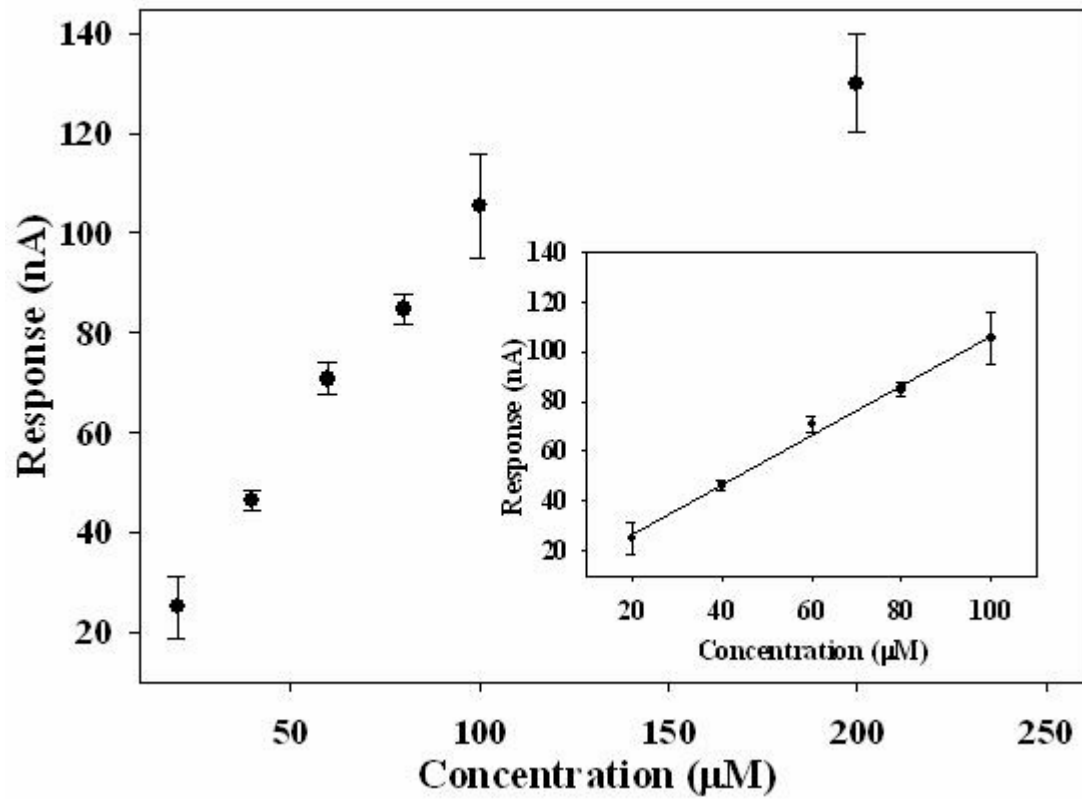
(A)



(B)



(C)



(D)

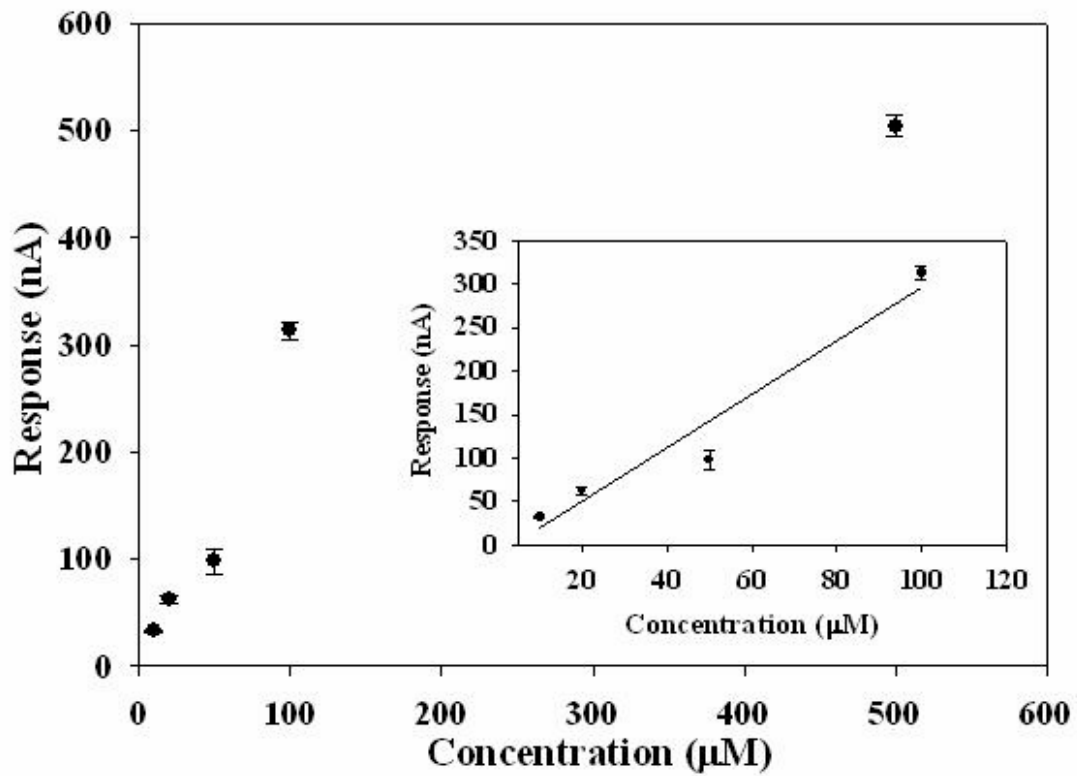
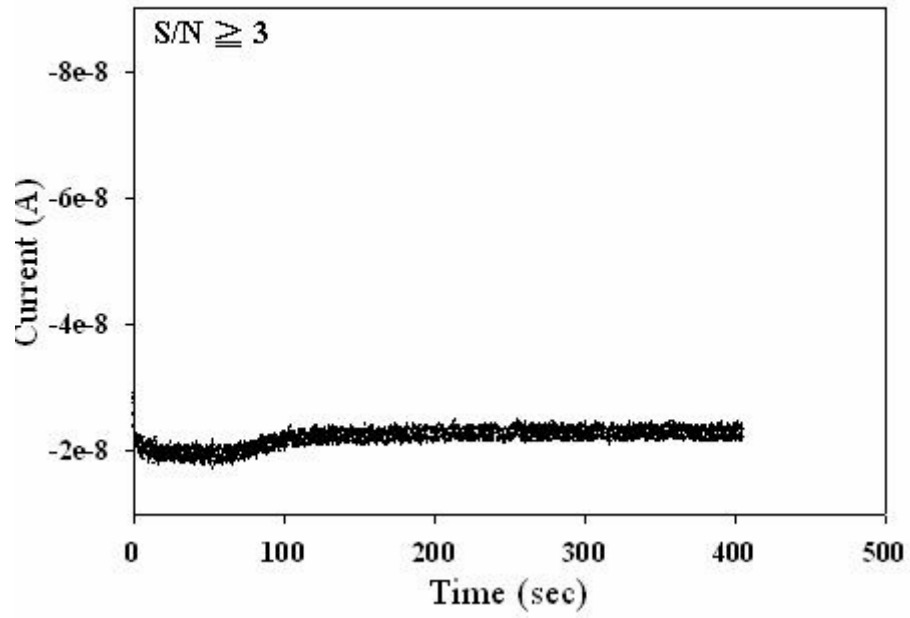


Figure 14. Linear dynamic range of various EDCs on the developed tyrosinase biosensor

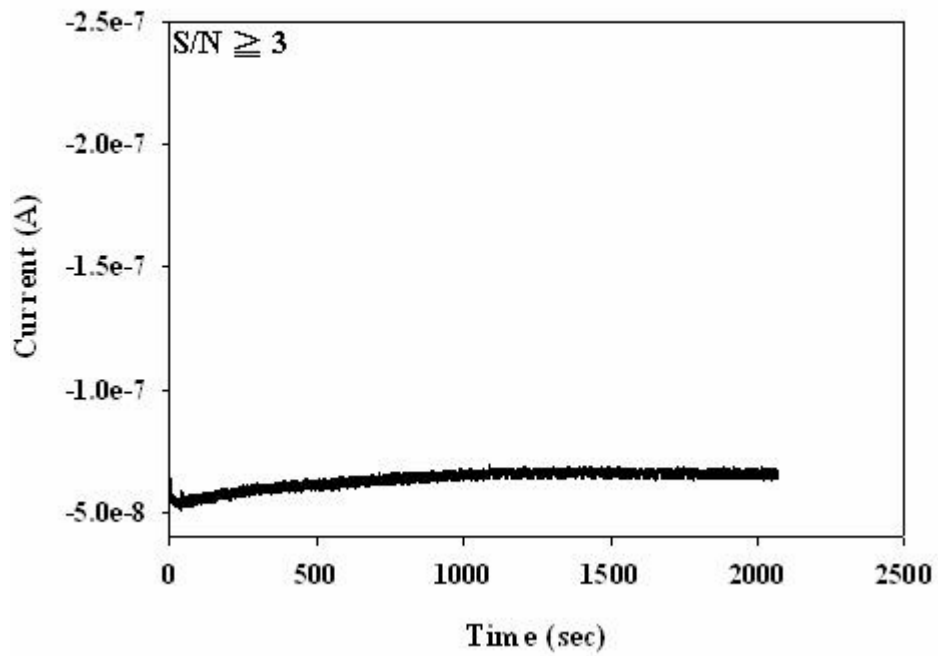
The tyrosinase biosensor was fabricated by entrapping 40 U tyrosinase in 7% gelatin on a SPCE. The reductive currents of tyrosinase biosensor to bisphenol A (A), 3-aminophenol (B), paraben (C) and diethylstilbestrol (D) were determined in PBS, pH 6.0. Working potential was -0.2 V vs. Ag/AgCl. Each data was obtained from three independent measurements.



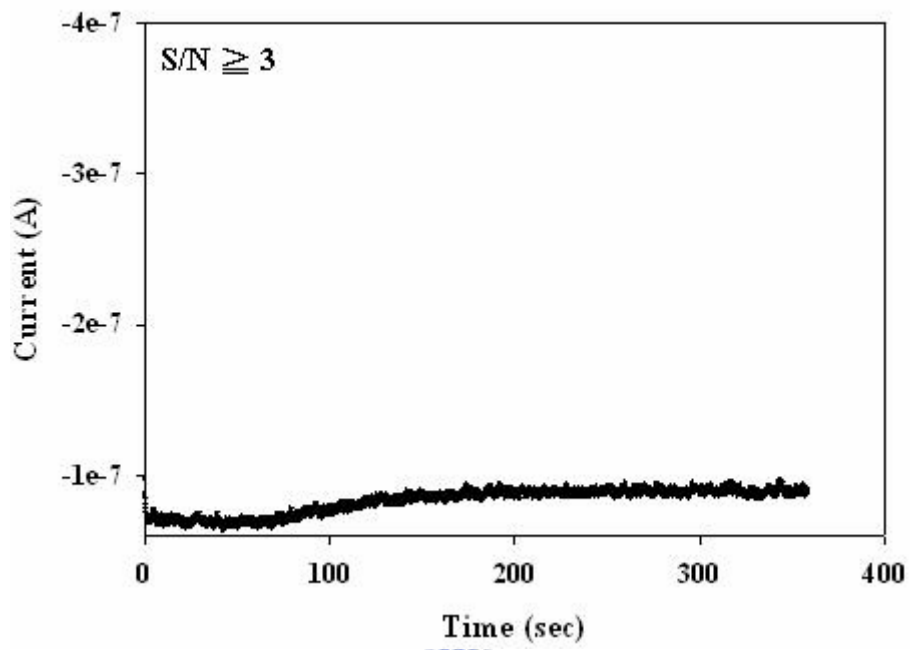
(a)



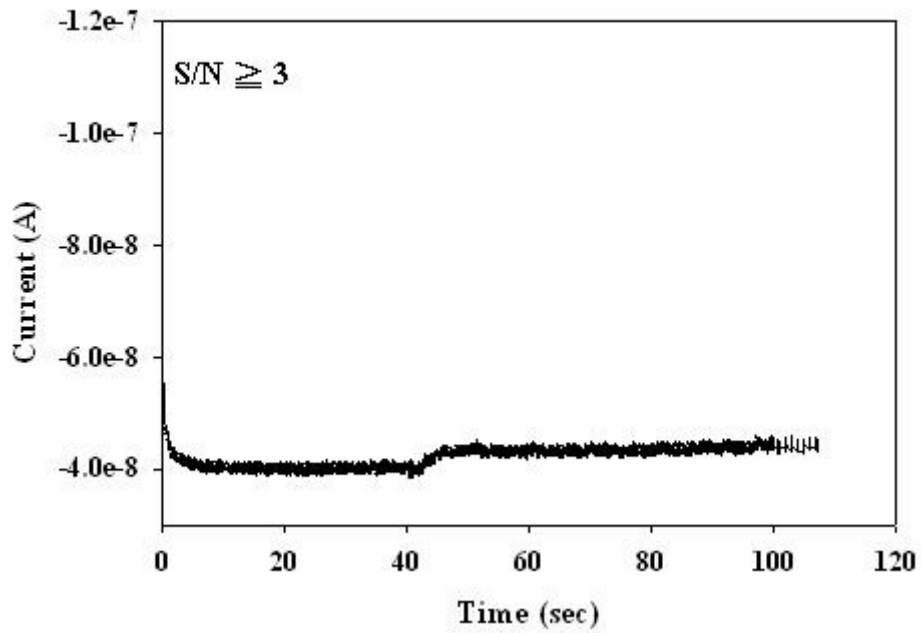
(b)



(c)



(d)

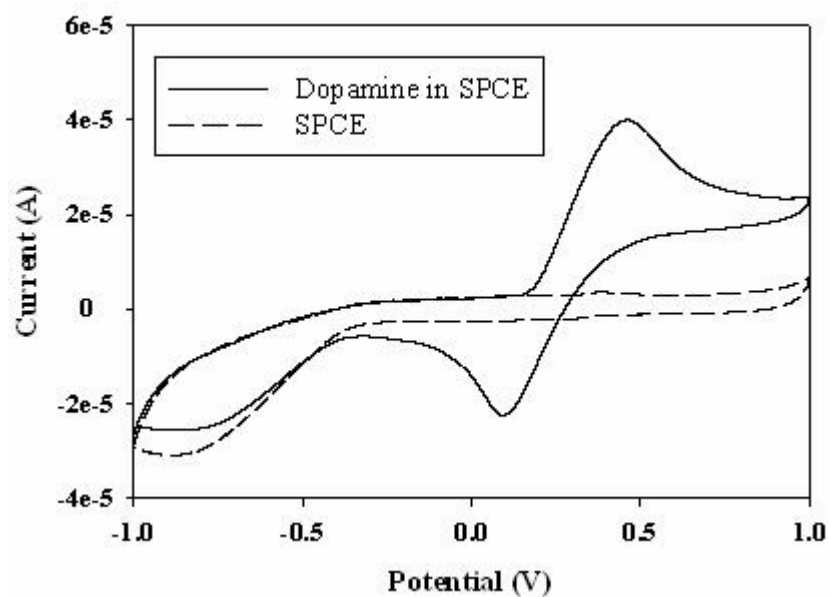


## Figure 15. Lowest detection limit of fabricated tyrosinase biosensor

The tyrosinase biosensor was fabricated by entrapping 40 U tyrosinase in 7% gelatin on a SPCE. A successful response to (a) 20 nM bisphenol A (b) 40 nM diethylstilbestrol (c) 5  $\mu$ M paraben (d) 20 nM 3-aminophenol were recorded (S/N = 3) in PBS buffer, pH 6.0. Working potential was -0.2 V vs. Ag/AgCl.



(a)



(b)

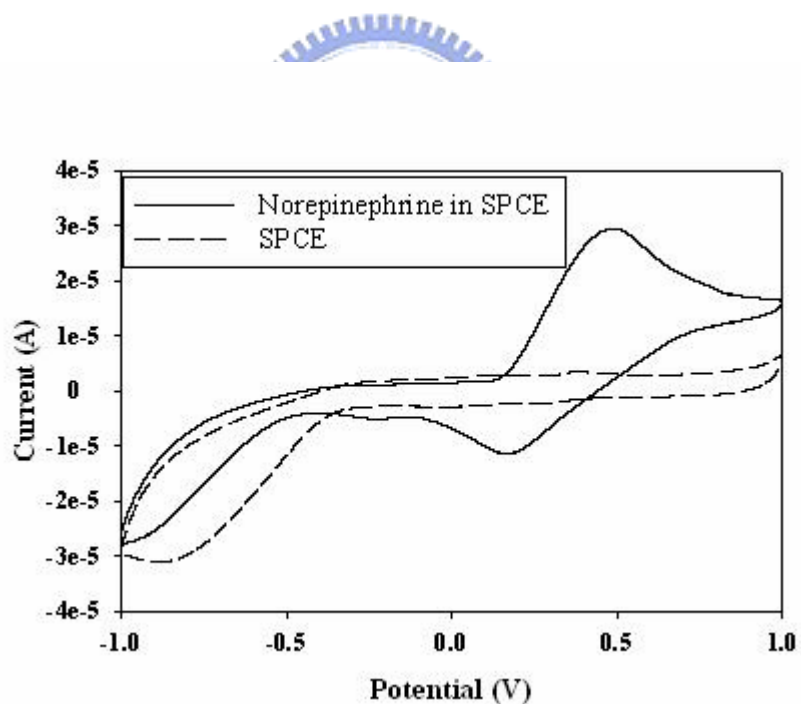


Figure 16. Cyclic voltammograms of neurotransmitter on SPCE

CV of dopamine (a) and norepinephrine (b) was performed in 1X PBS buffer, pH 6.0. CV was carried out within a potential range -1.0 V ~ 1.0 V in 1 mM dopamine and 1 mM norepinephrine. Working potential is -0.2 V vs. Ag/AgCl, and a scan rate of 100 mV/s.

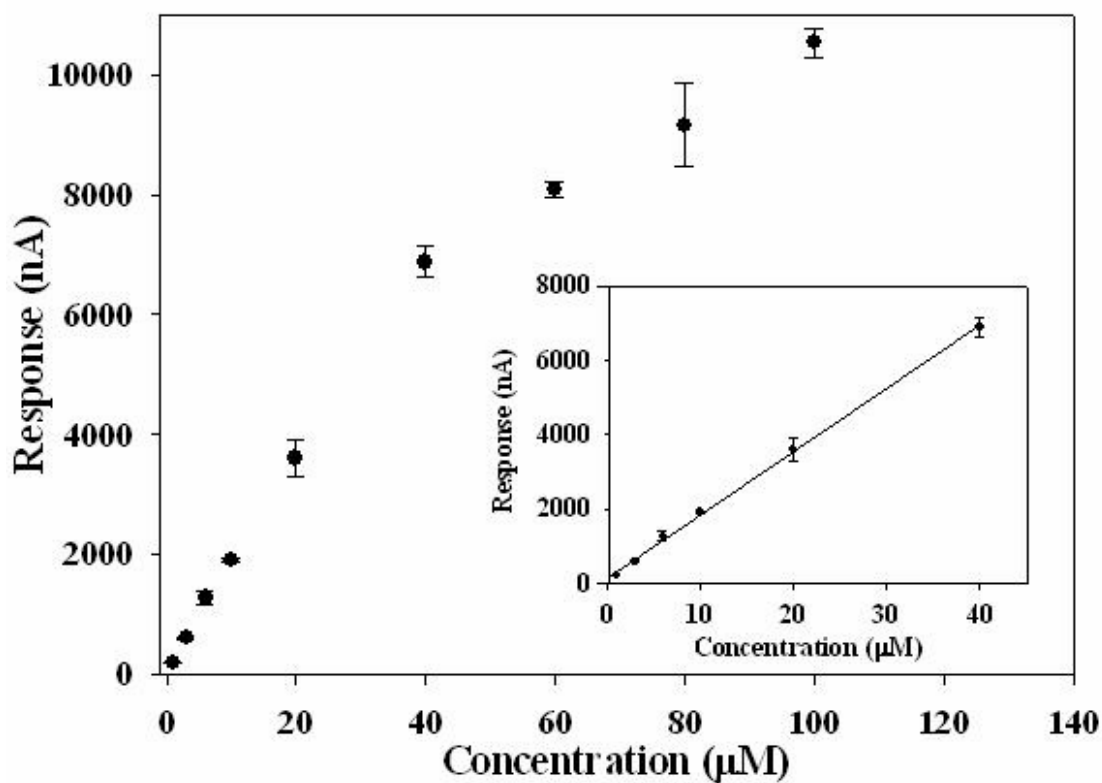


Figure 17. Linear dynamic range of dopamine of the tyrosinase biosensor

Tyrosinase biosensor was fabricated by entrapping 40 U tyrosinase in 7% gelatin on a SPCE. The reductive current of the biosensor was determined with different concentrations of dopamine (1 μM ~ 100 μM) dopamine in 1X PBS, pH 6.0. The working potential was -0.2 V vs. Ag/AgCl. Each data was obtained from three independent measurements. The linear dynamic range of dopamine was plotted as in insert panel.



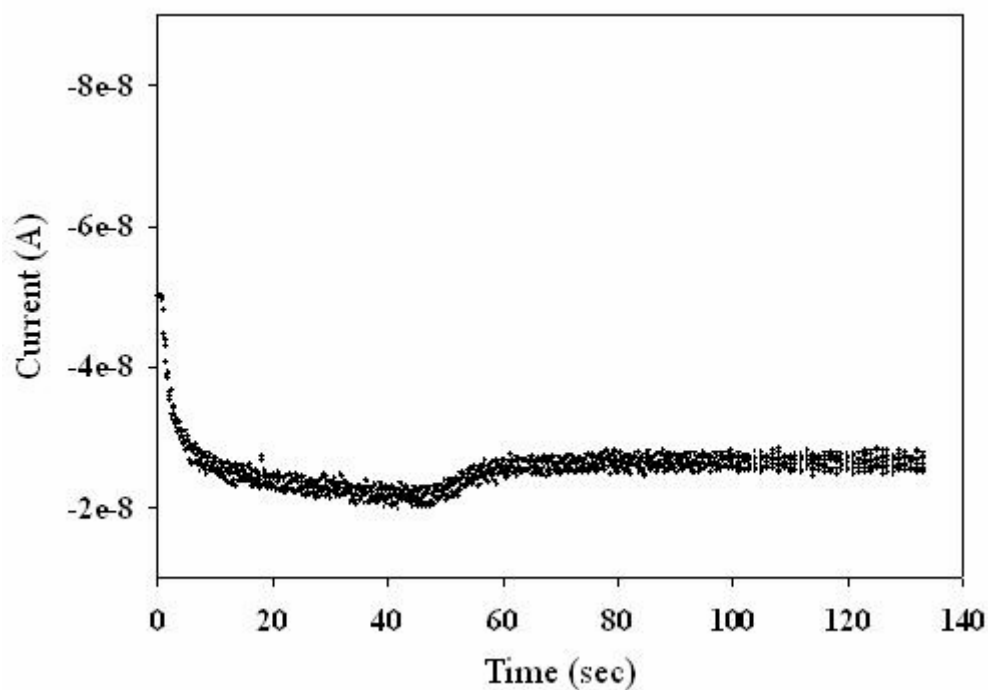


Figure 18. Lowest detection limit of fabricated tyrosinase biosensor dopamine

Tyrosinase biosensor was fabricated by entrapping 40 U tyrosinase in 7% gelatin on a SPCE. The chemical response to 100 nM dopamine in PBS buffer, pH 6.0 was recorded within 140 sec (S/N = 3). Working potential was -0.2 V vs. Ag/AgCl.

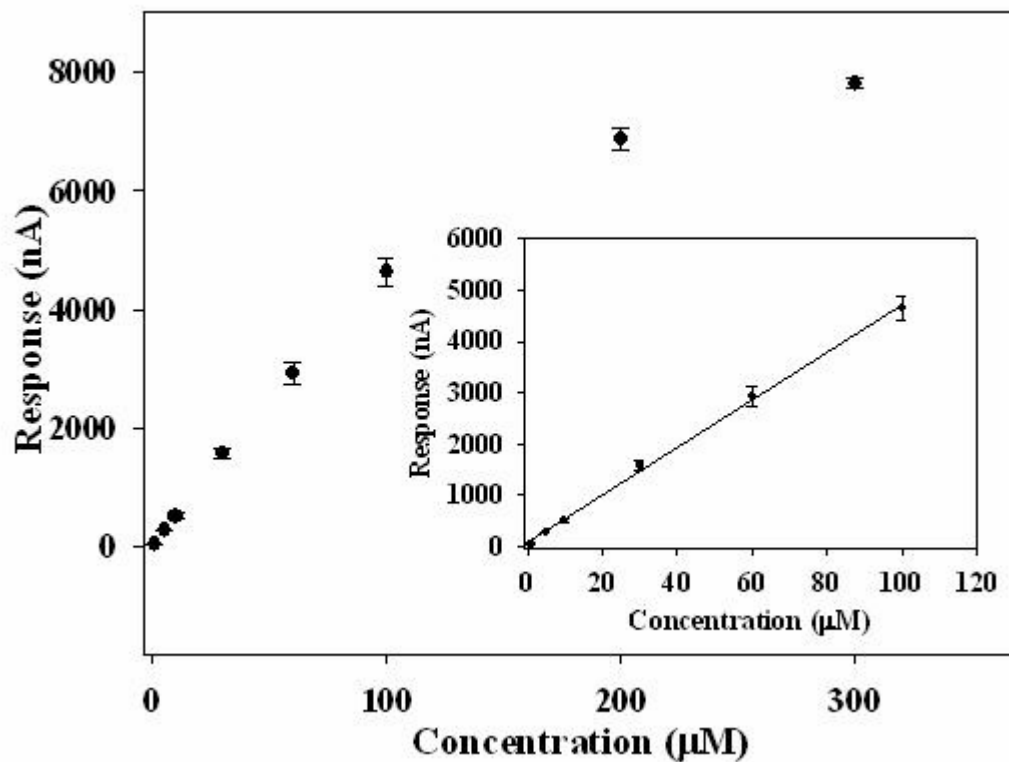


Figure 19. Linear dynamic range of norepinephrine on the tyrosinase biosensor

Tyrosinase biosensor was fabricated by entrapping 40 U tyrosinase in 7% gelatin on a SPCE. The reductive current of enzyme electrode to norepinephrine was determined with different concentration of norepinephrine (1 μM ~ 300 μM) norepinephrine in 1X PBS, pH 6.0. The working potential was -0.2 V vs. Ag/AgCl. Each data was obtained from three independent measurements.

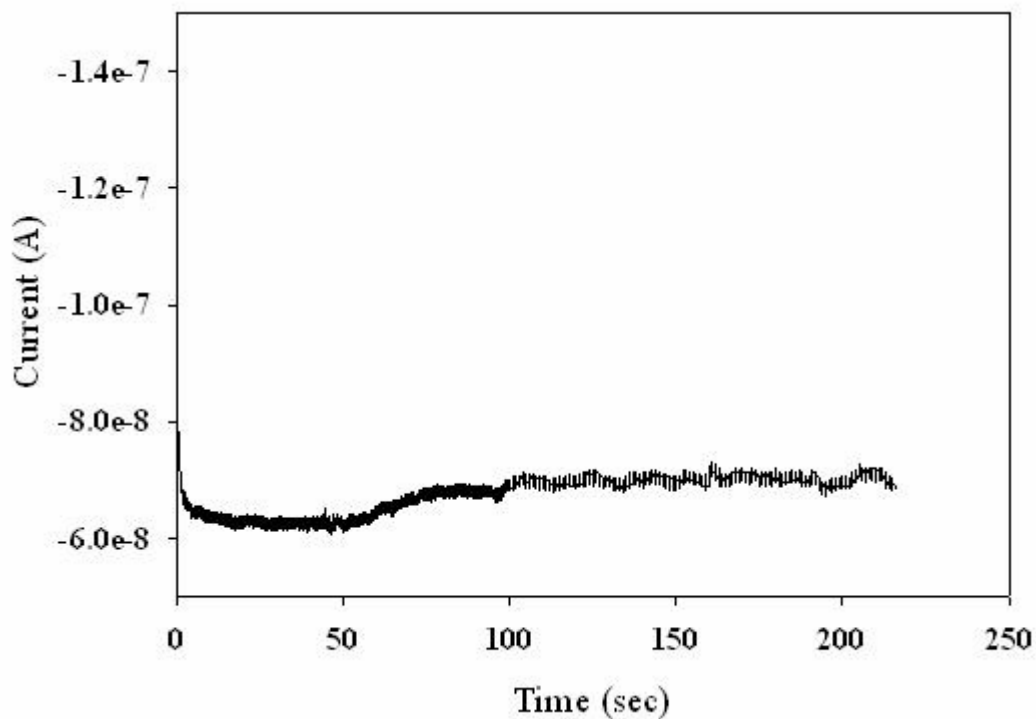


Figure 20. Lowest detection limit of fabricated tyrosinase biosensor to Norepinephrine

Tyrosinase biosensor was fabricated by entrapping 40 U tyrosinase in 7% gelatin on a SPCE. A electrochemical Response of tyrosinase biosensor to 200 nM norepinephrine in PBS buffer, pH 6.0 was recorded within 220 sec (S/N = 3). Working potential was -0.2 V vs. Ag/AgCl.

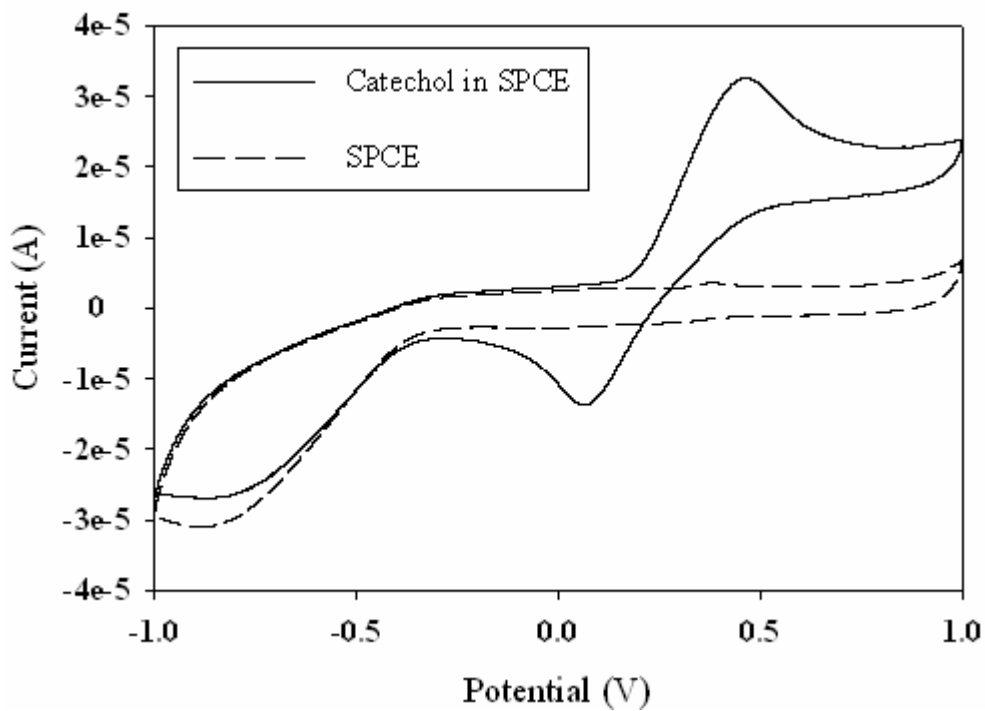


Figure 21. Cyclic voltammograms of catechol on SPCE

CV was performed in a range of -1.0 V ~ 1.0 V. The reaction was 1X PBS buffer, pH 6.0 and 1 mM catechol. The scan rate was 100 mV/s.

(dot line : SPCE, solid line : Catechol)

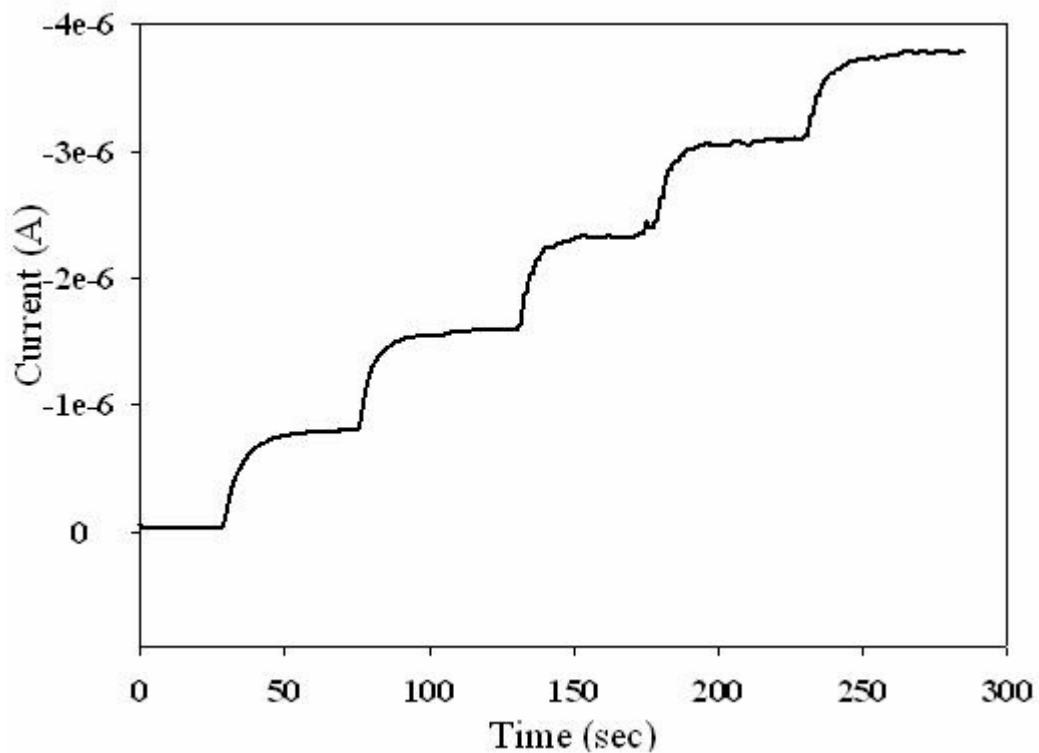


Figure 22. Step responses of tyrosinase biosensor to catechol

Tyrosinase biosensor was fabricated by entrapping 40 U tyrosinase in 7% gelatin on a SPCE. The step response of tyrosinase biosensor to 1  $\mu$ M catechol was performed in 1X PBS, pH 6.0. Working potential was -0.2 V vs. Ag/AgCl.

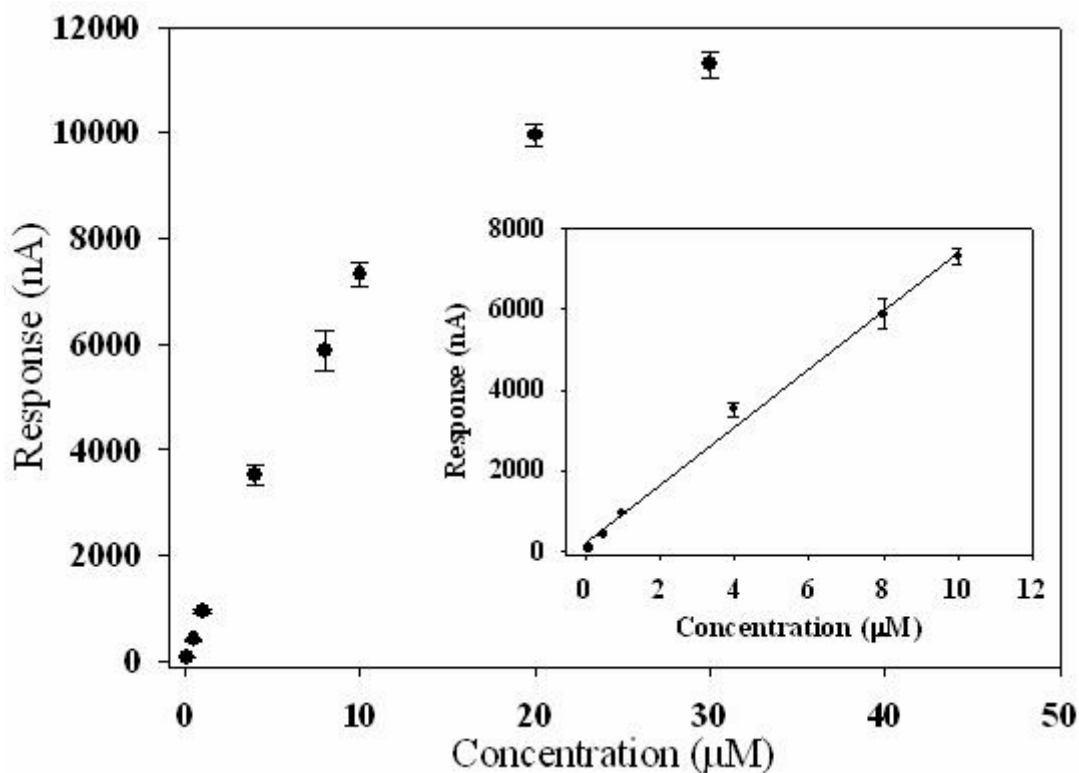


Figure 23. Linear dynamic range of catechol on the tyrosinase biosensors

Tyrosinase biosensor was fabricated by entrapping 40 U tyrosinase in 7% gelatin on a SPCE. The reductive current of enzyme electrode to catechol was determined with different concentration of catechol (0.1 µM ~ 30 µM) catechol in 1X PBS, pH 6.0. The working potential was -0.2 V vs. Ag/AgCl. Each data was obtained from three independent measurements.

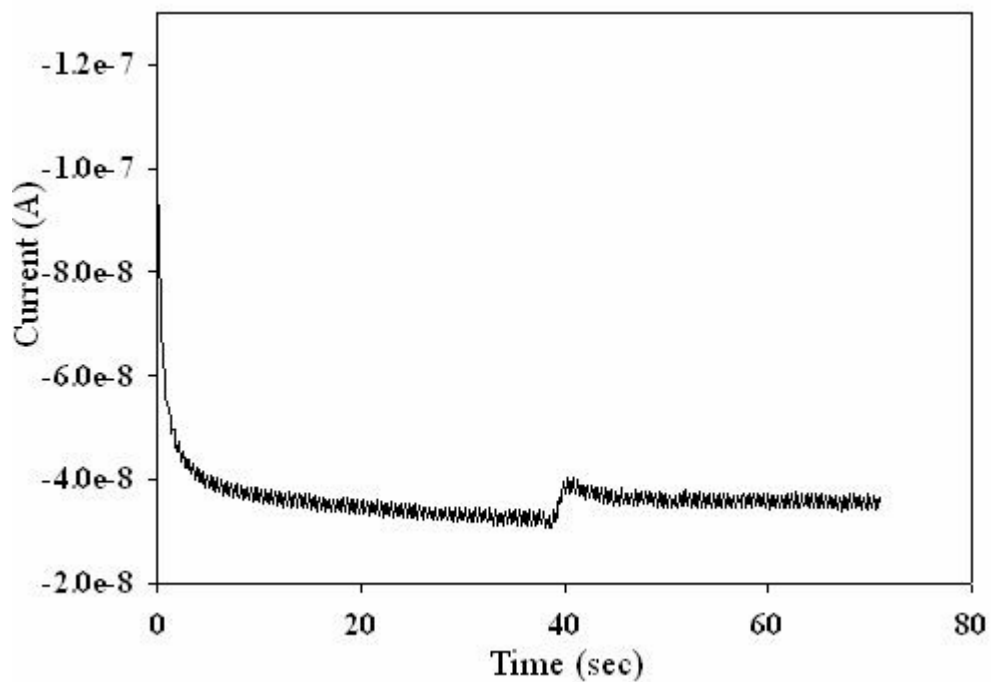


Figure 24. Lowest detection limit of fabricated tyrosinase biosensors

Tyrosinase biosensor was fabricated by entrapping 40 U tyrosinase in 7% gelatin on a SPCE. A electrochemical Response of tyrosinase biosensor to 20 nM catechol in PBS buffer, pH 6.0 was recorded within 70 sec (S/N = 3). Working potential was -0.2 V vs. Ag/AgCl.

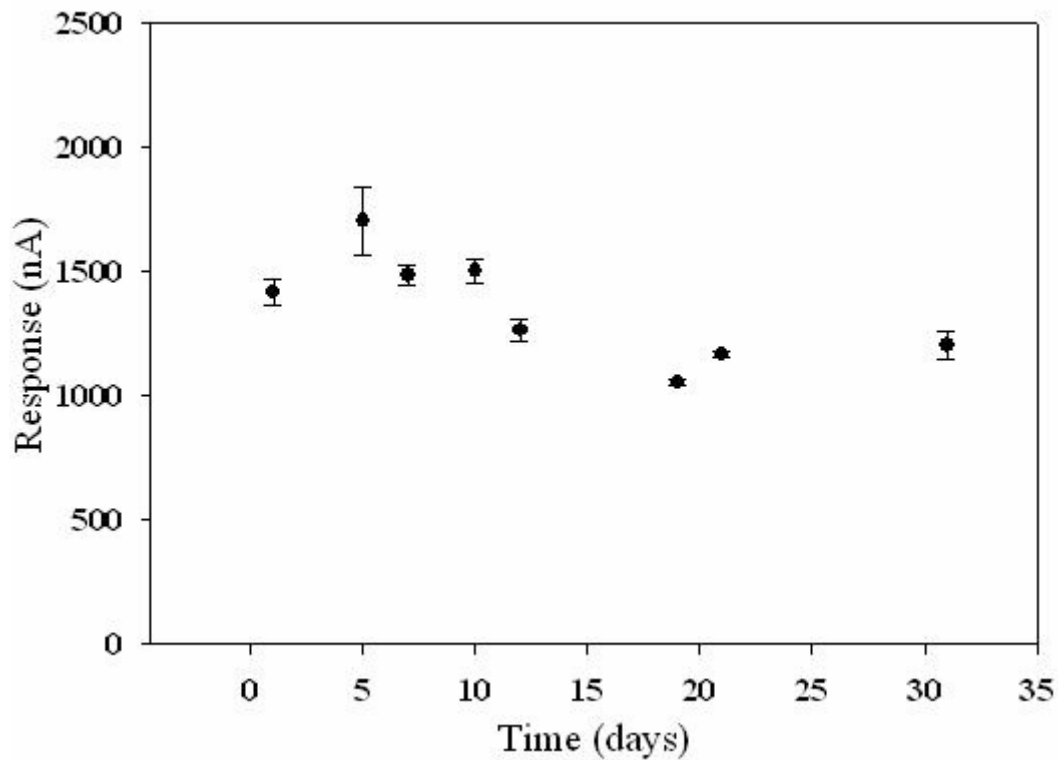


Figure 25. Long-term stability of tyrosinase biosensor after storage

Tyrosinase biosensor was stored at 4 °C. At the time indicated the tyrosinase biosensor was subjected to electrochemical reaction in the presence of 10 μM bisphenol A in PBS buffer, pH 6.0. The working potential was -0.2 V vs. Ag/AgCl. Each data was represented as mean ± S.D and obtained from three independent measurements.



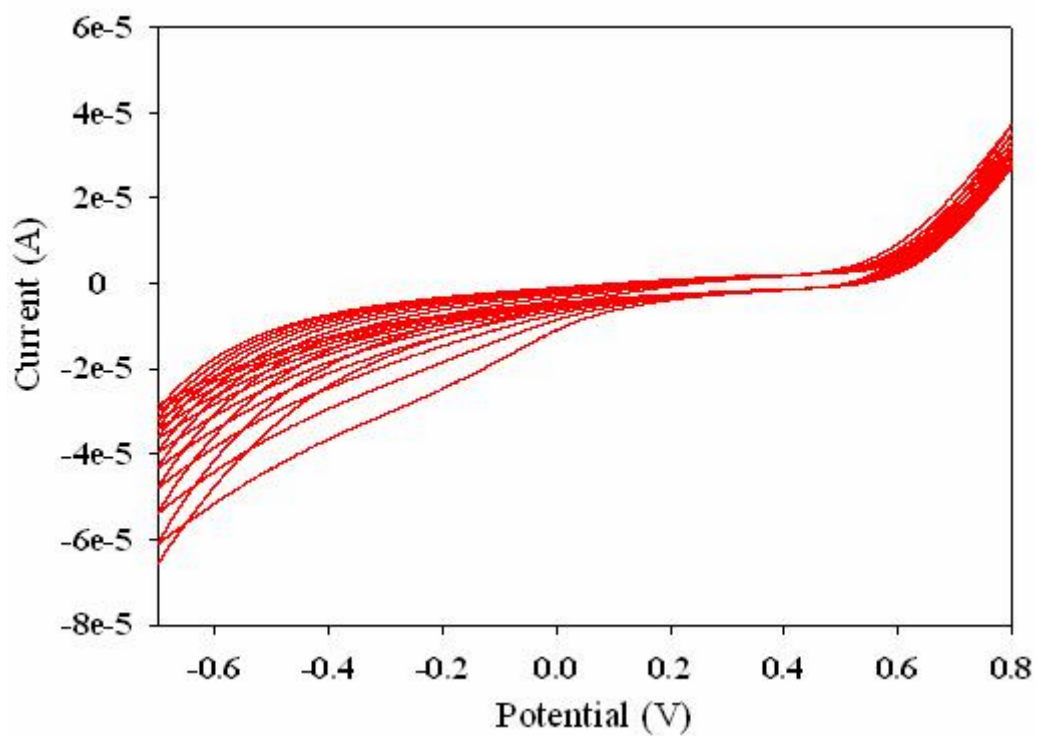


Fig 26. The cyclic voltammograms of diazonium cation

Cyclic voltammograms for the reduction of diazonium cation was carried of a scan rate of 200 mV/s for 10 scan cycles within potential of -0.7 V ~ 0.8 V.

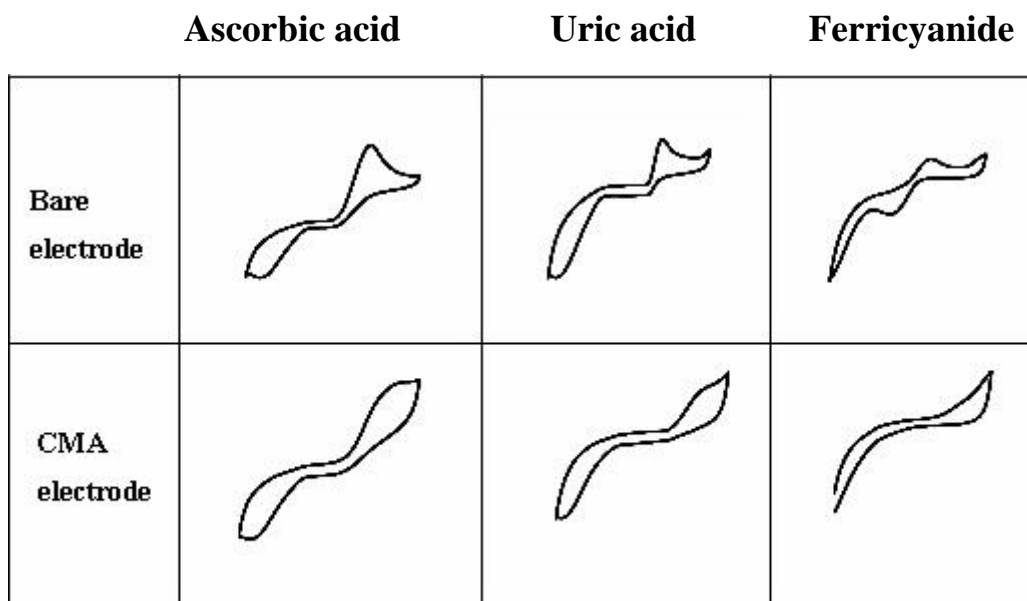
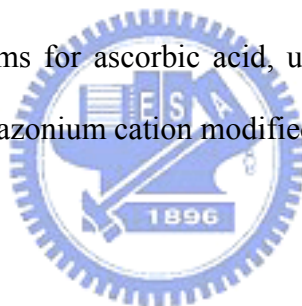
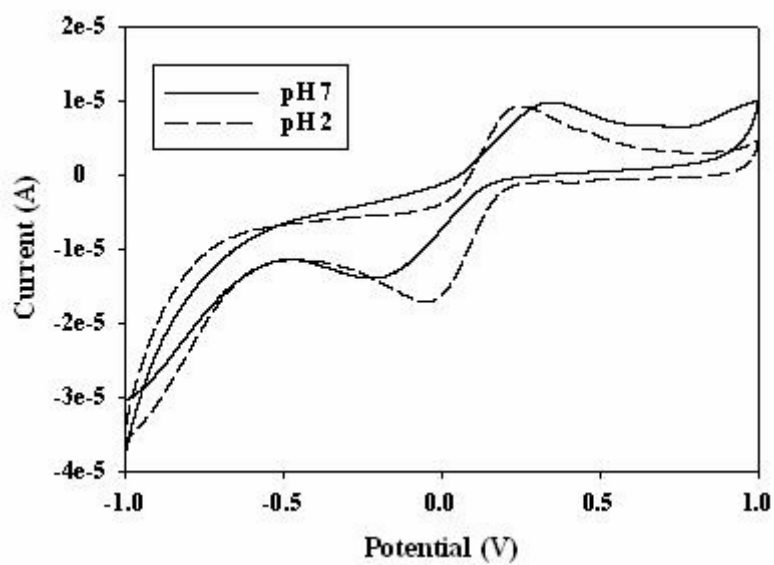


Fig 27. Cyclic voltammograms of various mediators

Cyclic voltammograms for ascorbic acid, uric acid and ferricyanide in the bare electrode and diazonium cation modified electrode.



(a)



(b)

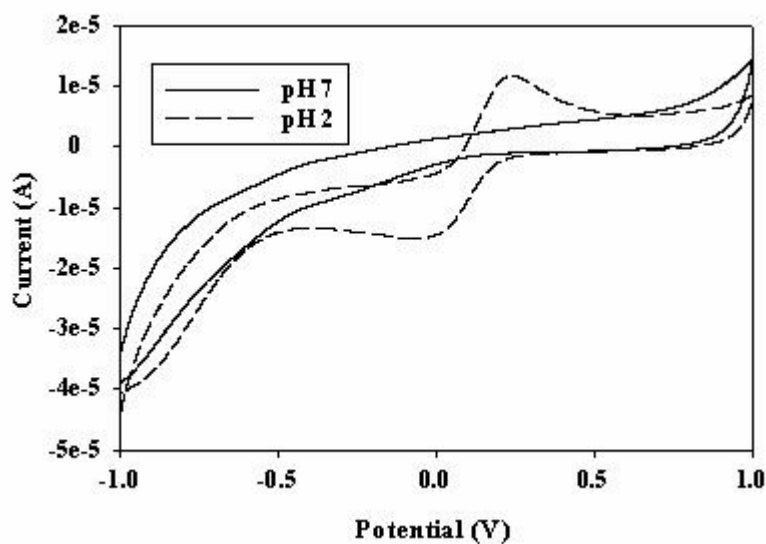


Fig 28. Cyclic voltammograms of ferricyanide on SPCE and modified electrode

CV of SPCE (a) and modified electrode (b) was performed in 1X PBS buffer, pH 6.0. CV was carried out within a potential range -1.0 V ~ 1.0 V in 1 mM ferricyanide.

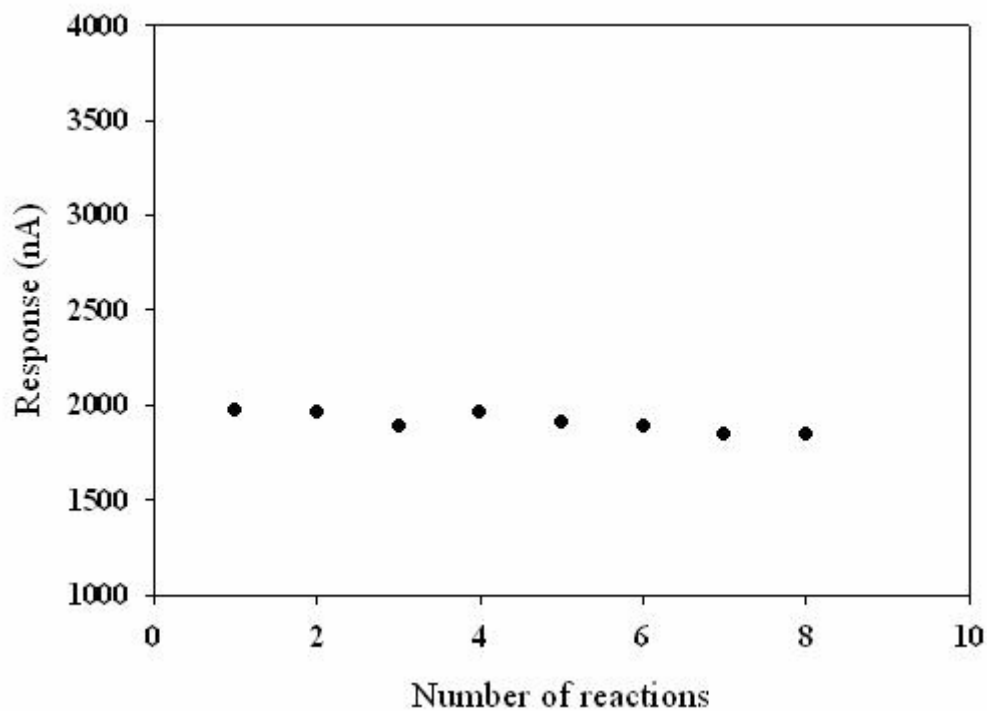
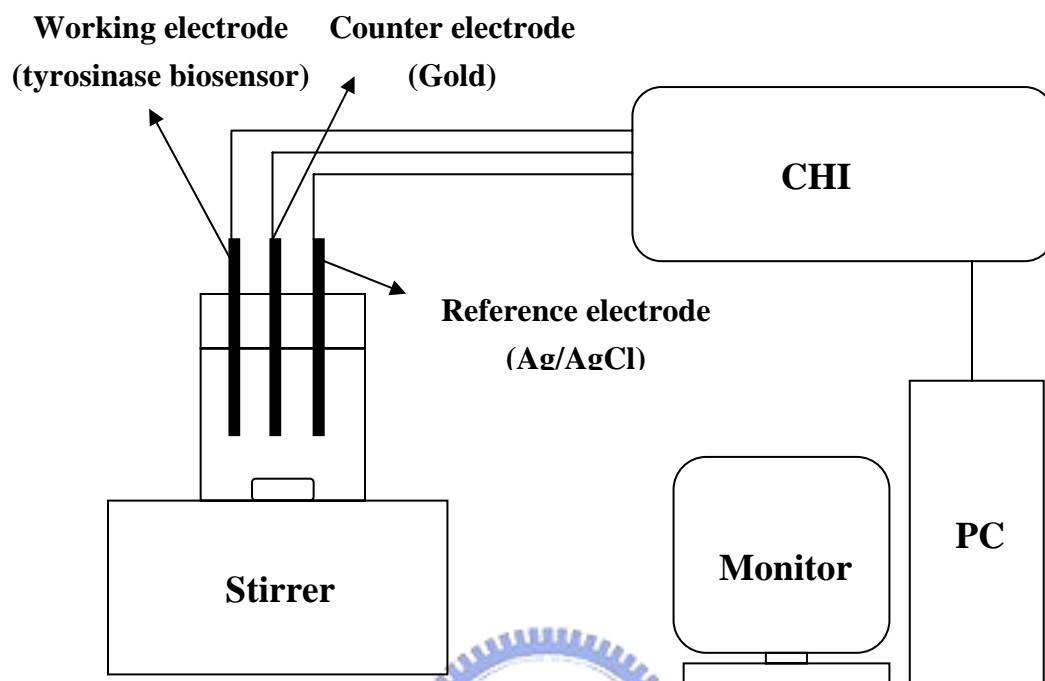


Fig 29. Successive tested on tyrosinase/CMA/SPCE electrode

Tyrosinase was immobilized by EDC/NHS. The electrochemical response of tyrosinase biosensor to 10  $\mu\text{M}$  bisphenol A was determined in PBS, pH 6.0. The working potential was -0.2 V versus Ag/AgCl. Seven reactions were recorded.

## Appendix



Appendix 1. The apparatus of electrochemical experiment

|  | <b>Response time<br/>(10 <math>\mu</math>M bisphenol A)</b> | <b>Response current<br/>(10 <math>\mu</math>M bisphenol A)</b> |
|--|---|--|
| <b>Gelatin entrapment</b>              | 161 (sec)   | 1242 (nA)  |
| <b>Diazonium cation immobilization</b> | 19 (sec)  | 1907.5 (nA)  |

Table 1. Electrochemical characters of gelatin entrapment and diazonium cation immobilization tyrosinase biosensor



| Name                  | Linear range                  | R <sup>2</sup> | Detection limit        | Sensitivity                   | Response time  |
|-----------------------|-------------------------------|----------------|------------------------|-------------------------------|----------------|
| <b>Catechol</b>       | 0.1 ~ 10<br>( $\mu\text{M}$ ) | 0.994          | 20<br>(nM)             | 748<br>(nA/ $\mu\text{M}$ )   | 50<br>(sec)    |
| <b>Bisphenol A</b>    | 1 ~ 20<br>( $\mu\text{M}$ )   | 0.999          | 20<br>(nM)             | 116<br>(nA/ $\mu\text{M}$ )   | 75<br>(sec)    |
| <b>3-Aminopheol</b>   | 1 ~ 50<br>( $\mu\text{M}$ )   | 0.993          | 20<br>(nM)             | 88.6<br>(nA/ $\mu\text{M}$ )  | 52<br>(sec)    |
| <b>Paraben</b>        | 20 ~ 100<br>( $\mu\text{M}$ ) | 0.994          | 5<br>( $\mu\text{M}$ ) | 46<br>(nA/30 $\mu\text{M}$ )  | 193<br>(sec)   |
| <b>Dopamine</b>       | 1 ~ 40<br>( $\mu\text{M}$ )   | 0.998          | 100<br>(nM)            | 203<br>(nA/ $\mu\text{M}$ )   | 5<br>(sec)     |
| <b>Norepinephrine</b> | 1 ~ 100<br>( $\mu\text{M}$ )  | 0.998          | 200<br>(nM)            | 52.2<br>(nA/ $\mu\text{M}$ )  | 91.8<br>(sec)  |
| <b>Tyramine</b>       | 1 ~ 60<br>( $\mu\text{M}$ )   | 0.989          | 50<br>(nM)             | 134.5<br>(nA/ $\mu\text{M}$ ) | 77.25<br>(sec) |

Table 2. Electrochemical characteristics of Tyrosinase/gelatin SPCE for phenolic compounds

| <b>Catechol</b>  | <b>Detection limit</b> | <b>Sensitivity</b> | <b>Response time</b> | <b>Storage stability</b>                  |
|--|------------------------|--------------------|----------------------|---|
| <b>Tyrosinase/gelatin SPCE</b>   | 20 nM                  | 748 nA/ $\mu$ M    | 50 sec               | Retain ~85%activity after 31 days storage |
| <b>Tyr-CPE</b><br>(Analytical Chemistry, Vol. 76, No. 3, February 1, 2004) | 25 nM                  | 208 nA/ $\mu$ M    | 2 min                | Retain ~57%activity after 31 days storage |
| <b>Tyrosinase/gelatin GCE</b><br>(Anal Bioanal Chem 2005 383: 1127–1132)   | 21 nM                  | 122 nA/ $\mu$ M    |                      | Retain ~77%activity after 7 days storage  |

Table 3. Electrochemical characteristics of Tyrosinase/gelatin SPCE, Tyr-CPE and Tyrosinase/gelatin GCE for catechol



| <b>Bisphenol A</b>   | <b>Detection limit</b> | <b>Sensitivity</b> | <b>Response time</b> | <b>Storage stability</b>                  |
|--|------------------------|--------------------|----------------------|---|
| <b>Tyrosinase/gelatin SPCE</b>   | 20 nM                  | 116 nA/ $\mu$ M    | 75 sec               | Retain ~85%activity after 31 days storage |
| <b>Tyr-CPE</b><br>(Analytical Chemistry, Vol. 76, No. 3, February 1, 2004)           | 150 nM                 | 21 nA/ $\mu$ M     | 3 min                | Retain ~57%activity after 31 days storage |
| <b>Poly(thionine)/gelatin GCE</b><br>(Biosensors and Bioelectronics 20 2004 367–377) | 23 $\mu$ M             | 0.4 nA/ $\mu$ M    | ~<1 min              | Retain ~56%activity after 5 days storage  |

Table 4. Electrochemical characteristics of Tyrosinase/gelatin SPCE, Tyr-CPE and Poly(thionine)/gelatin GCE for bisphenol A