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Research Article

Bienzymatic *Acetylcholinesterase* and *Choline Oxidase* Immobilized Biosensor Based on a Phenyl Carboxylic Acid-Grafted Multiwalled Carbon Nanotube

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Bienzymatic *acetylcholinesterase* (AChE) and *choline oxidase* (ChOx) immobilized biosensor based on a phenyl carboxylic acid-grafted multiwalled carbon nanotube (MWNT) modified glass carbon electrode (GCE) and carbon-screen printed electrode (SPE) was fabricated for acetylcholine detection in human blood samples. Phenyl carboxylic acid-modified MWNT supports were prepared by electrochemical polymerization of 4-carboxyphenyl diazonium salts, which were synthesized by an amine group and sodium nitrite, on the surface of the MWNT-modified GCE and SPE in 0.1 M PBS. The successful fabrication of the AChE-ChOx-immobilized biosensor was confirmed via scanning electron microscopy (SEM), X-ray photoelectron spectroscopy (XPS), electrochemical impedance spectroscopy (EIS), and cyclic voltammetry (CV). The sensing range of the biosensor based on a GCE and SPE was 1.0~10 μM and 10~100 μM, respectively. The interfering effect of 0.1 M L-ascorbic acid, 0.1 M L-cysteine, and 0.1 M uric acid to 0.1 M acetylcholine was 3.00%, 9.00%, and 3.00%, respectively. Acetylcholine in a human blood sample was detected by the AChE-ChOx-immobilized biosensor.

1. Introduction

Acetylcholine (ACh) is one of many neurotransmitters in the autonomic nervous system [1]. It acts on both the peripheral nervous system and central nervous system and is the only neurotransmitter used in the motor division of the somatic nervous system. ACh is also the principal neurotransmitter in all autonomic ganglia. ACh functions as a neuromodulator in both the peripheral nervous system and the central nervous system. In the central nervous system, ACh has a variety of effects as a neuromodulator upon plasticity, arousal, and reward. ACh has an important role in the enhancement of sensory perceptions on waking [2] and in sustaining attention [3]. Damage to the cholinergic (acetylcholine-producing) system in the brain has been shown to have a plausible association with the memory deficits associated with Alzheimer's disease [4]. ACh has also been shown to promote REM sleep [5]. Based on the above, the determination of ACh content in human blood samples is of great importance for the prediction of nervous diseases.

Hou et al. [6] reported that an AChE/ChOx bienzyme amperometric acetylcholine biosensor based on gold nanoparticles (AuNPs) and multiwalled carbon nanotubes (MWCNTs) has been developed by a self-assembly process in combination with a sol-gel technique, and it exhibited a wide linear range, high sensitivity, and fast amperometric response of 0.005–0.4 mM, 3.395 μA/mM and within 15 s, respectively. Deng et al. [7] also reported about the amplified electrochemiluminescence of quantum dots by electrochemically reduced graphene oxide for nanobiosensing of acetylcholine. This biosensor showed linear response ranges and detection limits of 10–210 μM and 8.8 μM for choline and 10–250 μM and 4.7 μM for acetylcholine, respectively. Upadhyay group [8] also reported about an AChE-ChOx-immobilized biosensor based on a gold-platinum bimetallic nanoparticles modified GCE for detection of organophosphate pesticides,

carbamates, and nerve agents. Paraoxon ethyl, sarin, and aldicarb could be detected up to 150–200 nM, 40–50 nM, and 40–60 nM, respectively, at the 30–40% inhibition level of AChE enzyme and followed linearity over a wide range of concentrations. In addition, Pundir et al. [9] reported that an AChE-ChOx biosensor was fabricated by coimmobilizing AChE/ChOx onto a nanocomposite of carboxylated multiwalled carbon nanotubes (c-MWCNTs) and zirconium oxide nanoparticles (ZrO_2NPs) electrodeposited on the surface of a GCE (AChE-ChOx/c-MWCNT/ZrO_2NPs/GCE) for detection of choline. The serum choline level, as measured by the biosensor, was 9.0 to 12.8 μmol/L (with a mean of 10.81 μmol/L) in apparently healthy persons and 5.0 to 8.4 μmol/L (with a mean of 6.53 μmol/L) in persons suffering from Alzheimer's disease. As shown by the above reported paper, for increasing sensitivity to the AChE-ChOx-immobilized biosensor, MWNT supports with metallic nanoparticles (NPs) were used as an electron transfer mediator; however, the metallic NPs-modified MWNT supports have a disadvantage for immobilization of biomolecules because they lack a functional group.

In previous papers [10–15], vinyl monomers with various functional groups were grafted by radiation-induced graft polymerization onto the surface of MWNTs in order to apply biosensor supports. These functionalized vinyl polymer-grafted MWNT supports exhibited good sensitivity and good stability for biomolecules; however, an aromatic polymer with bifunctional properties, such as an immobilization site with biomolecules, and electron transfer mediator properties based on a conjugated bond could not be introduced onto the surface of the MWNTs by radiation-induced graft polymerization.

Recently, we grafted phenyl carboxylic acid onto different electrodes, such as indium tin oxide (ITO), gold (Au), and glassy carbon electrode (GCE). Subsequently, an electrochemical DNA sensor (E-DNA sensor) based on the phenyl carboxylic acid-modified GCE was fabricated by the immobilization of probe DNA. The fabricated E-DNA sensor can detect the influenza virus (type A). The current density of the E-DNA sensor was also evaluated by cyclic voltammetry when the probe DNA and target DNA were hybridized [16]. It was found that 4-carboxyphenyl diazonium salts could be polymerized onto surface of the GCE as a phenyl carboxylic acid back bone. The phenyl carboxylic acid polymer is one of the categories of conjugate polymer, and this polymer can be used as an electron transfer mediator on the electrode in order to increase electron transfer, while the carboxylic acid group of this polymer can be used to immobilize biomolecules, such as enzymes.

In this study, we fabricated bienzymatic immobilized biosensors based on a phenyl carboxylic acid-grafted multiwalled carbon nanotube (MWNT) modified glass carbon electrode (GCE) and carbon-screen-printed electrode (SPE) for acetylcholine detection in human blood samples. We used *acetylcholinesterase* (AChE) and *choline oxidase* (ChOx) for acetylcholine detection in human blood. The successful fabrication of the AChE-ChOx-immobilized biosensor was confirmed by scanning electron microscopy (SEM), X-ray photoelectron spectroscopy (XPS), electrochemical impedance spectroscopy (EIS), and cyclic voltammetry (CV). The sensing range and the interfering effects of L-ascorbic acid, L-cysteine, and uric acid were evaluated as well as the actual acetylcholine content of human blood samples.

2. Experimental

2.1. Reagents. *Acetylcholinesterase* (AChE, type VI-S, lyophilized powder, 200–1,000 units/mg protein), *choline oxidase* (ChOx, lyophilized powder, ≥10 units/mg solid), 4-aminobenzoic acid, sodium nitrite ($NaNO_2$), acetylcholine chloride, L-ascorbic acid, L-cysteine, uric acid, and potassium hexacyanoferrate(II) trihydrate were from Sigma-Aldrich (Korea). MWNTs (95%; length, 10 μm) were from Hanwha Nanotech Co., Ltd. (Korea). Hydrogen chloride (HCl) was from Samchun Pure Chemical Co., Ltd. (Korea). Potassium ferricyanide ($K_3Fe(CN)_6$) and potassium chloride (KCl) were from Duksan Pharmaceutical Co., Ltd. (Korea). Phosphate buffer solution (PBS, pH = 7.0) was prepared by mixing NaH_2PO_4 and Na_2HPO_4. Glassy carbon electrode (MF-2012) was from Bioanalytical Systems, Inc. (USA). Silver paste (ELECTRODAG 479SS) of conductive ink used for primary wire, a carbon paste (ELECTRODAG PF-407C) for printing the counter electrode and the working electrode, and nonconductive ink (ELECTRODAG ML25265) for coating unnecessary portions of the electrode reaction were used for fabrication of the screen-printed electrode and from Acheson Colloids Co. (USA). Ag/AgCl ink (011464 Ag/AgCl ink for reference electrode) was from Bioanalytical Systems, Inc. (USA). Screen printer equipment (HC-SM1-3020) and silk print edition (Mesh = 350) were from Hyochang Machinery Co., Ltd. (Korea). Substrate for printing was polyvinyl chloride (PVC) film or polyethylene terephthalate (PET) film. Drawer UV light firearms were from Forcelamp Co. Ltd. (Korea). All other chemicals were of analytical grade. Water was purified using a Millipore purification system (Millipore, MA, USA).

2.2. Preparation of AChE-ChOx Biosensor Based on GCE and SPE. The AChE-ChOx biosensor based on a GCE and SPE was fabricated as follows (Figure 1). In order to introduce the conjugated aromatic polymer and carboxylic acid group onto the surface of MWNTs, 4-carboxyphenyl diazonium salts were first synthesized by the diazotization reaction of 4-aminobenzoic acid (5.0 mmol) and sodium nitrite ($NaNO_2$, 5.0 mmol) in 0.5 mol HCl solution (10 mL).

Prior to modification, the bare GCE was polished with α-Al_2O_3 powder and then rinsed with double distilled water and cleaned ultrasonically in ethanol and water for 3 min. MWNTs were ultrasonicated at room temperature in a 3 : 1 mixture of sulfuric acid and nitric acid. The obtained MWNTs (10 mg) were dispersed in 5 mL of a 1 : 1 mixture of distilled water and ethanol with the aid of ultrasonic agitation to give a black suspension. The cleaned GCE was dipped into the obtained black suspension. MWNTs were deposited electrochemically on the surface of the polished GCE at a constant potential of −0.57 V for 5 min and dried naturally at room temperature to form MWNT film. 10 μL of the

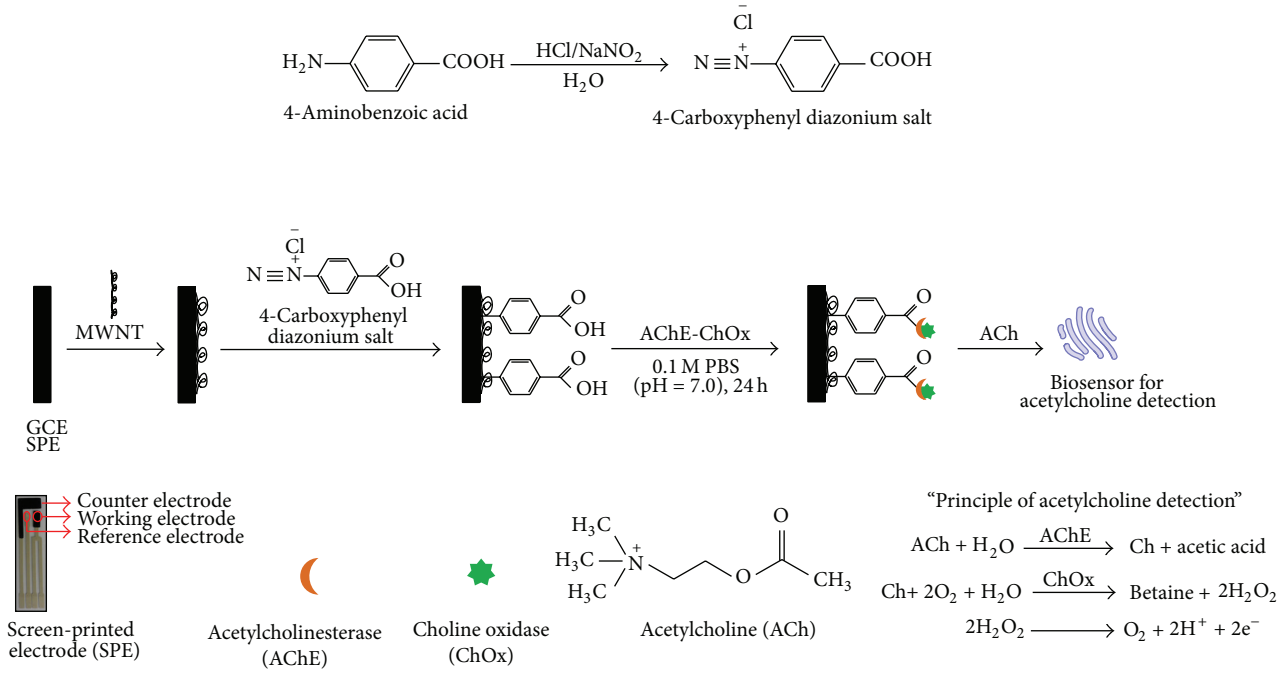


Figure 1: Schematic fabrication of the AChE-ChOx-modified biosensor based on a GCE and SPE.

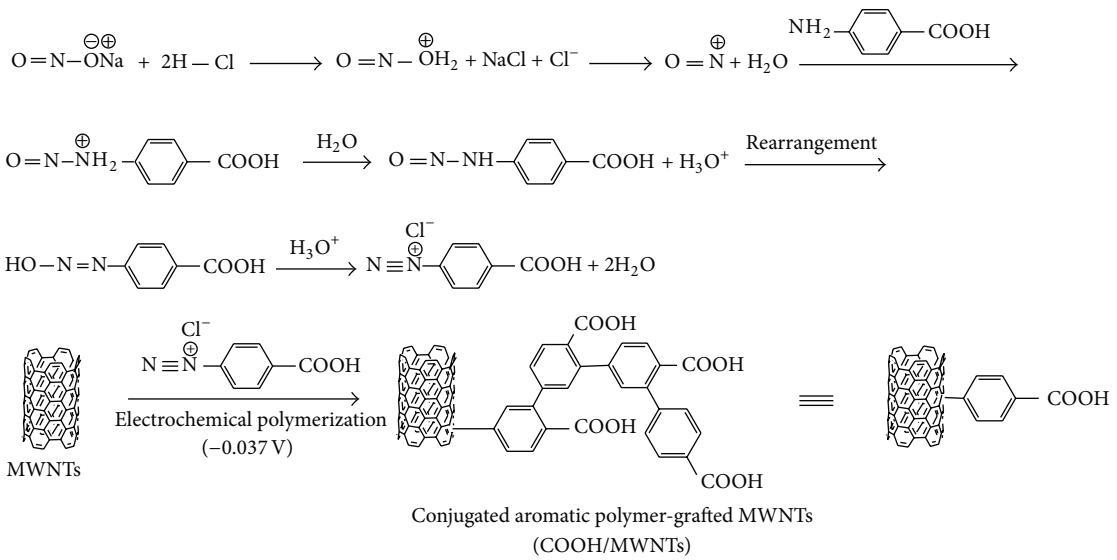


Figure 2: Possible synthesis mechanism of the conjugated aromatic polymer-grafted MWNTs using 4-carboxyphenyl diazonium salts.

black suspension was dropped onto the working electrode of the SPE. Synthesized 4-carboxyphenyl diazonium salts were deposited electrochemically on the surface of the MWNT modified GCE and SPE at a constant potential of −0.037 V for 5 min and dried naturally at room temperature.

In order to immobilize AChE and ChOx, a COOH/MWNT modified GCE was immersed in a mixture of AChE solution (1 mg/mL) and ChOx solution (5 mg/mL) at 4°C for 24 h. On the other hand, mixture of 10 μL of AChE solution (1 mg/mL) and 10 μL of ChOx solution (5 mg/mL) was dropped on the COOH/MWNT modified SPE. The prepared enzyme electrode was rinsed clean with distilled water, dried, and stored at 4°C until use.

2.3. Fabrication of Screen-Printed Electrode (SPE). The screen-printed electrode was fabricated as follows (Figure 1). Silver paste of conductive ink was printed for primary wire on polyvinyl chloride (PVC) film or polyethylene terephthalate (PET) film using a patterned silk-screen and then dried for 15 min at 93°C using a dry oven. The counter electrode was

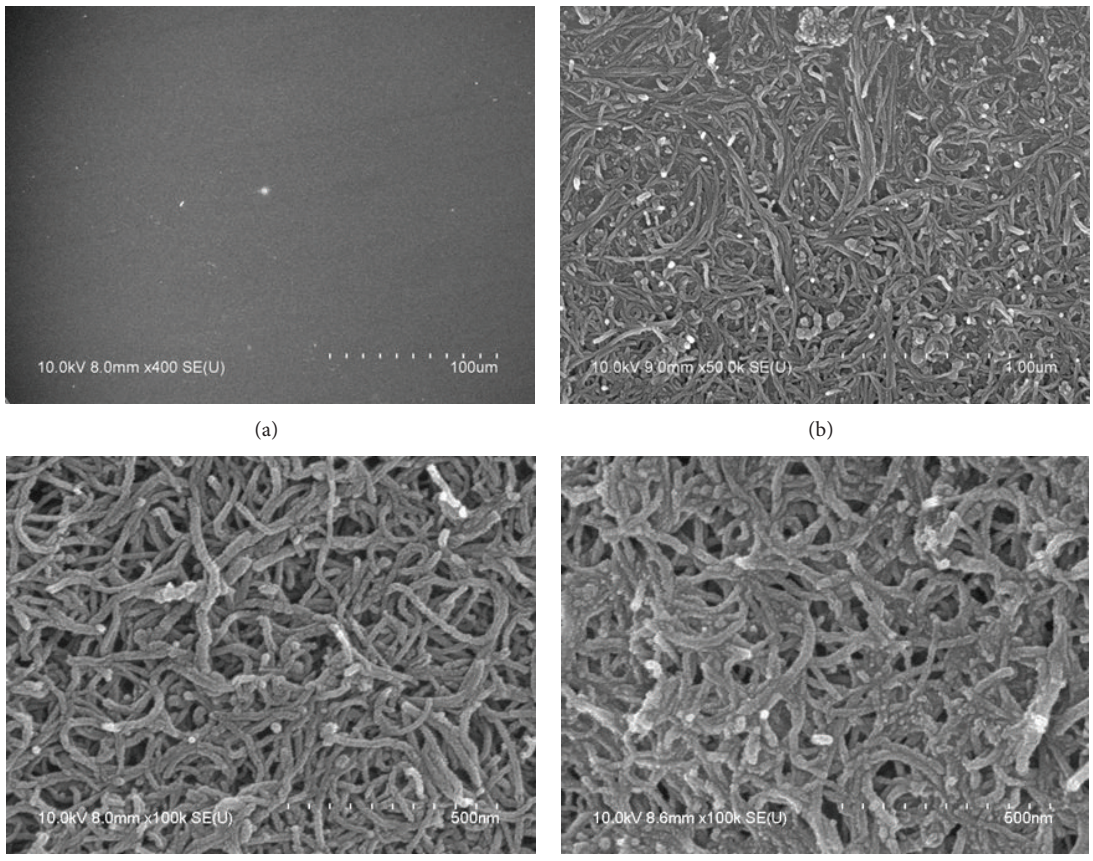


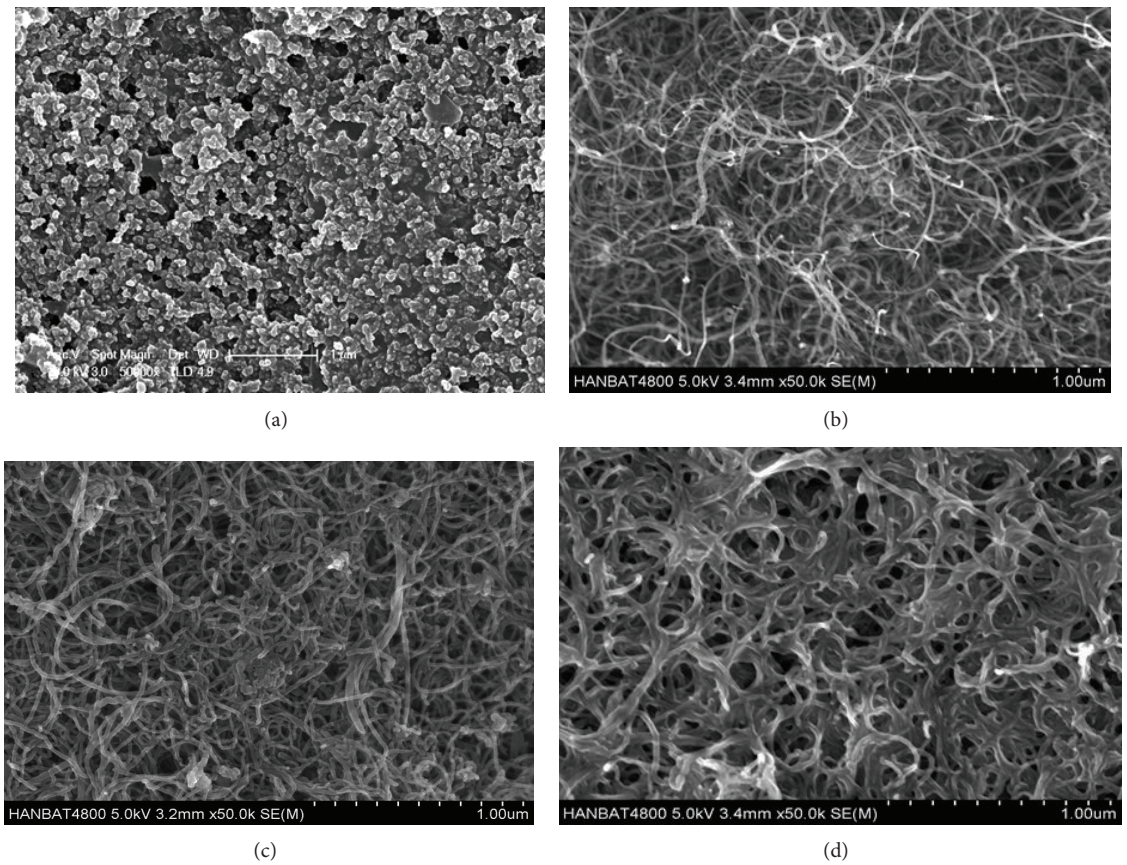
Figure 3: SEM images of the carbon plate as model electrode (a), MWNT-modified electrode (b), COOH/MWNT-modified electrode (c), and AChE-ChOx/COOH/MWNT-modified electrode (d).

printed with conductive carbon paste and then dried for 30 min at 90°C in a dry oven. The reference electrode was printed with Ag/AgCl reference electrode ink and dried for 10 min at 80°C using a dry oven. As with the counter electrode, the working electrode was printed with carbon paste, and then it was dried for 15 min at 93°C. For the final step to produce the screen-printed electrode, the electrode was printed with nonconductive ink and cured for 3 min in drawer UV light firearms to coat any unnecessary part in the electrode reaction so that it would avoid contact with the electrolyte measurement solvent. The diameter of the exposed working electrode, reference electrode, and counter electrode area in the prepared screen-printed electrode was 0.2 cm, 0.1 cm, and 0.48 cm^2, respectively.

2.4. Instrumentation. Cyclic voltammetry (CV) was performed using a VersaSTAT 3 potentiostat/galvanostat (AMETEK PAR, USA) and a conventional three-electrode system comprising a composite-coated glassy carbon (diameter, 3.0 mm) working electrode, a platinum wire counter electrode, and an Ag/AgCl (saturated KCl) reference electrode. Screen-printed electrode with a 2.0 mm (diameter) working electrode, Ag/AgCl ink reference electrode, and carbon paste counter electrode was fabricated with a screen-printer produced by Hyochang Machinery Co., Ltd. (Korea). Electrochemical impedance spectroscopy (EIS) was performed using a PP240 and IM6ex (ZAHNER-Elektrik GmbH & Co. KG, Germany). EIS measurements were performed in the presence of PBS (pH = 7.0) containing 1.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1 : 1) mixture as a redox probe in the frequency range between 50 mHz and 100 kHz at the formal potential of +20 mV. Surface properties were characterized by atomic force microscopy (NP-AFM, PROBES Co., Ltd., Korea), scanning electron microscopy (FE-SEM (S-4800), Hitachi Co., Ltd., Japan), contact angle (PHOENIX-300, Surface Electro Optics Co., Ltd., Korea), and X-ray photoelectron spectroscopy (MultiLab. ESCA 2000, Thermo Fisher Scientific Inc., USA).

3. Results and Discussion

3.1. Fabrication of the AChE-ChOx-Immobilized Biosensor by Electrochemical Polymerization of 4-Carboxyphenyl Diazonium Salts. 4-Aminobenzoic acid can be easily converted to 4-carboxyphenyl diazonium salts by the diazotization reaction in acid aqueous solution at 0°C (Figure 2). The conjugated phenyl polymer with carboxylic acid was grafted



(a) (b) (c) (d)

Figure 4: SEM images of the bare SPE (a), MWNT-modified SPE (b), COOH-modified SPE (c), and AChE-ChOx-modified SPE (d).

onto the MWNT surface of the electrode by electrochemical polymerization as shown in the following reaction:

$$\text{HOOC-C}_6\text{H}_4\text{N}_2^+ + \text{e}^- \longrightarrow \text{N}_2 + \text{HOOC-C}_6\text{H}_4^\bullet \quad (1)$$

$$\text{Electrode} + \text{HOOC-C}_6\text{H}_4^\bullet \longrightarrow \text{Electrode-C}_6\text{H}_4\text{COOH} \quad (2)$$

$$\text{Electrode-C}_6\text{H}_4\text{COOH} + n\ \text{HOOC-C}_6\text{H}_4^\bullet \longrightarrow \text{Electrode-grafted polymer} \quad (3)$$

The conjugated phenyl polymer-grafted MWNTs can be used as electron transfer supports on electrodes. Furthermore, carboxylic acid placed onto the supports on the electrode can interact easily with biomolecules via hydrogen bonding interaction.

SEM images of the carbon plate as model electrode (Figure 3(a)), MWNT-modified electrode (Figure 3(b)), COOH/MWNT-modified electrode (Figure 3(c)), and AChE-ChOx/COOH/MWNT-modified electrode (Figure 3(d)) were prepared. In the MWNT-modified electrode (Figure 3(b)), the surface morphology of the MWNTs appears as crystal-like form, while the surface of the COOH/MWNTs, which means the conjugated phenyl polymer-grafted MWNTs, exhibits a smooth-like form because of the grafted polymers (Figure 3(c)). The surface morphology of the AChE-ChOx/COOH/MWNT-modified biosensor demonstrates a polymer-like connection structure partially among the MWNTs because of the loading of bienzymes. Based on these results, we concluded that the AChE-ChOx/COOH/MWNT-modified biosensor based on a glass carbon electrode was fabricated successfully for detection of acetylcholine.

Screen-printed electrodes have inherent advantages such as miniaturization, versatility, low cost, and the possibility of mass production as biosensors. Consequently, we fabricated the SPE as described in Section 2 and applied an AChE-ChOx-modified biosensor as a base electrode. From the SEM images of the bare SPE (Figure 4(a)), MWNT-modified SPE (Figure 4(b)), COOH-modified SPE (Figure 4(c)), and AChE-ChOx-modified SPE (Figure 4(d)), it can be seen that the surface morphology of the AChE-ChOx-modified SPE demonstrates a polymer-like connection structure partially among the MWNTs because of the loading of bienzymes. As a result, we concluded that the AChE-ChOx-modified biosensor based on the SPE was fabricated successfully for detection of acetylcholine.

AFM-3D images of the carbon plate as model electrode (Figure 5(a)), MWNT-modified electrode (Figure 5(b)), COOH/MWNT-modified electrode (Figure 5(c)), and AChE-ChOx/COOH/MWNT-modified electrode (Figure 5(d)) show that surface roughness increased after modification,

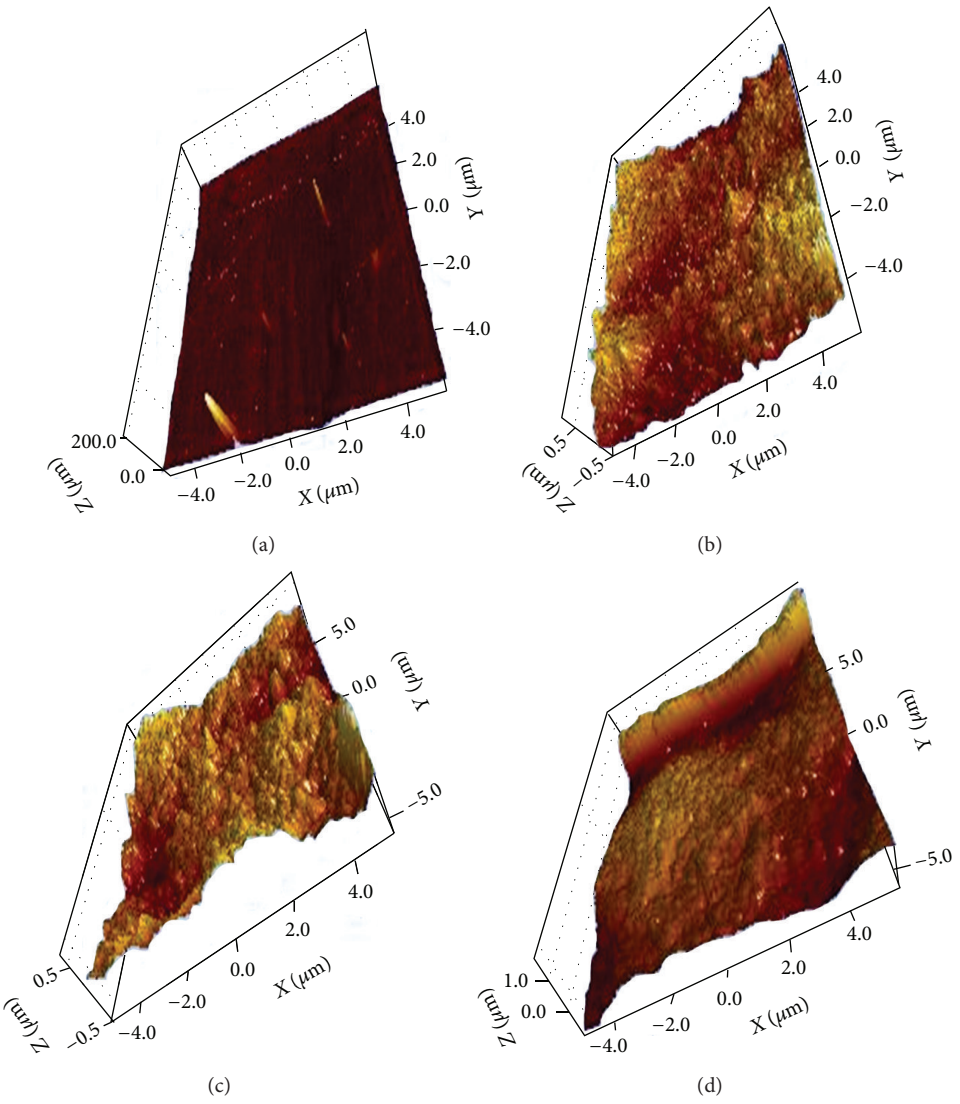


Figure 5: AFM-3D images of the carbon plate as model electrode (a), MWNT-modified electrode (b), COOH/MWNT-modified electrode (c), and AChE-ChOx/COOH/MWNT-modified electrode (d).

and it is known from the literature that higher values for surface roughness can be explained by the inclusion of surface modifying materials. The surface of the MWNT-modified electrode (Figure 5(b)) showed a significant increase in surface roughness compared with that of the carbon plate electrode (Figure 5(a)). For the COOH/MWNT-modified electrode (Figure 5(c)), the surface roughness changed to a smooth roughness form because of the conjugated phenyl polymer grafted onto the surface of the electrode, while the roughness height of the surface increased compared with the MWNT-modified electrode because of the grafted polymer via electrochemical polymerization. The surface roughness of the AChE-ChOx/COOH/MWNT-modified electrode (Figure 5(d)) changed dramatically to a smooth plate form because of immobilization of bienzymes on the surface of the electrode. Furthermore, the altitude of the AChE-ChOx/COOH/MWNT-modified electrode increased considerably compared with that of the COOH/MWNT-modified electrode (Figure 5(c)). This result means that the AChE-ChOx-modified biosensor based on a GCE for acetylcholine detection was fabricated successfully.

AFM-3D images of the screen-printed electrode (Figure 6(a)), MWNT-modified electrode (Figure 6(b)), COOH/MWNT-modified electrode (Figure 6(c)), and AChE-ChOx/COOH/MWNT-modified electrode (Figure 6(d)) show that the surface roughness of the modified electrode changed from rigid form to smooth form because of the conjugated phenyl polymer grafted onto the surface of the electrode. Furthermore, the altitude of the modified electrode increased in accordance with modification order in the surface of the electrode. This means that the AChE-ChOx-modified biosensor based on a SPE for acetylcholine detection was fabricated successfully.

The contact angles of the carbon plate as model electrode (Figure 7(a)), MWNT-modified electrode (Figure 7(b)), COOH/MWNT-modified electrode (Figure 7(c)), and AChE-ChOx/COOH/MWNT-modified electrode (Figure 7(d)) show that the contact angle of the carbon plate

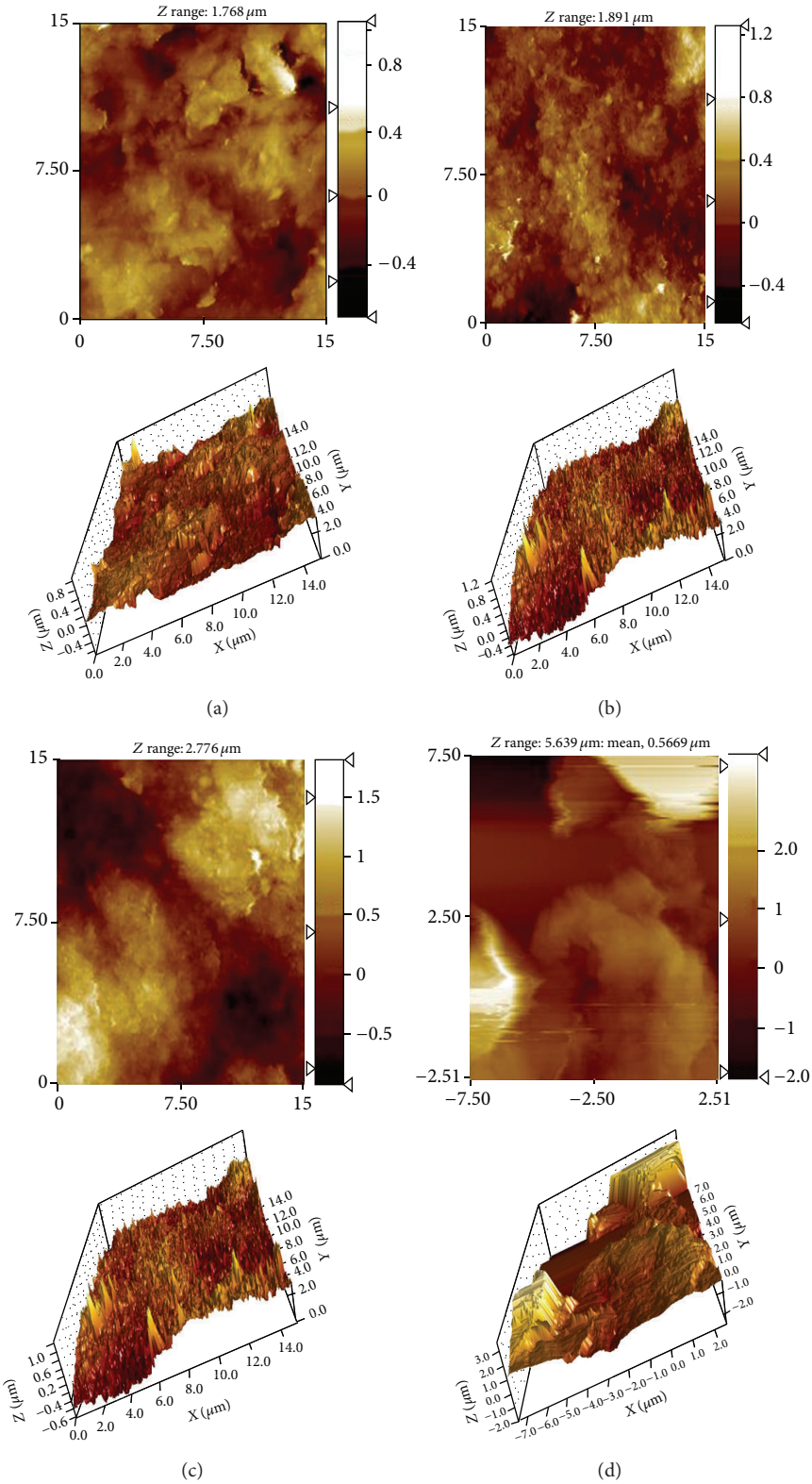


Figure 6: AFM-3D images of the screen-printed electrode (a), MWNT-modified electrode (b), COOH/MWNT-modified electrode (c), and AChE-ChOx/COOH/MWNT-modified electrode (d).

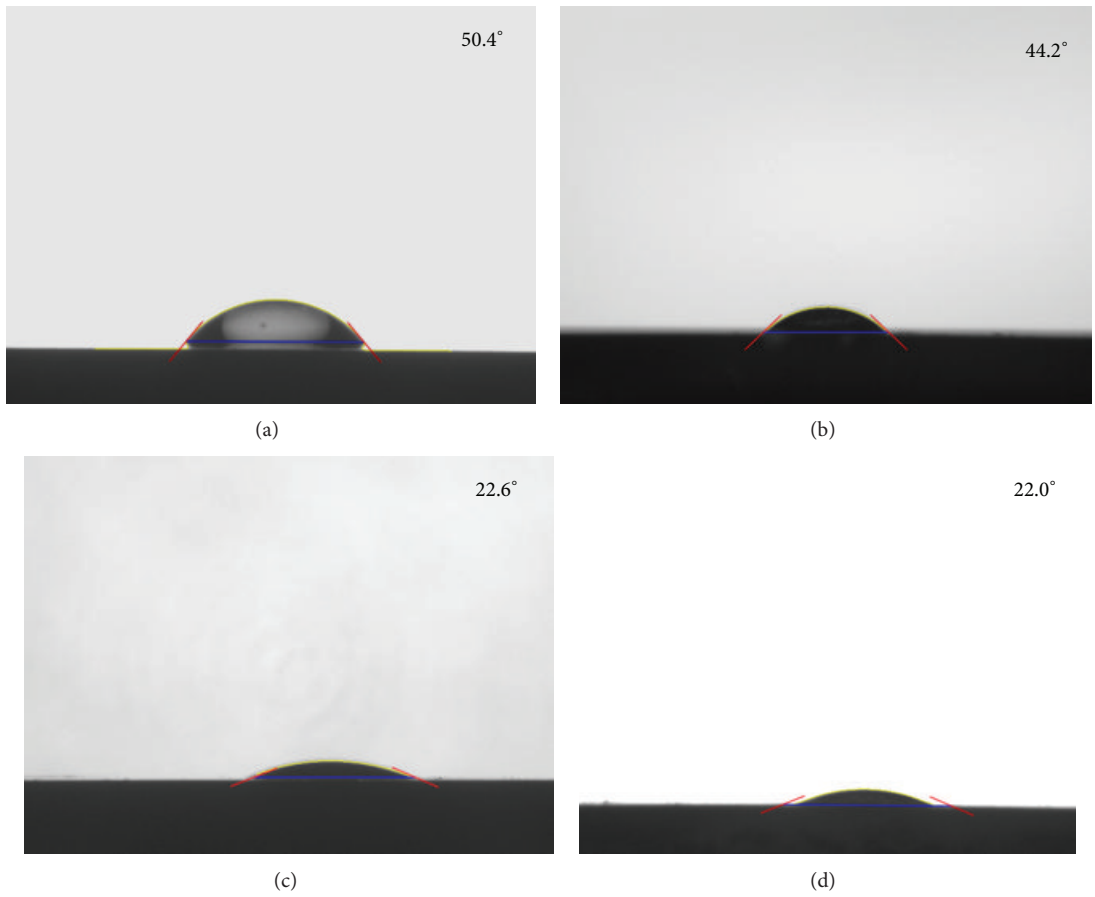


Figure 7: Contact angles of the carbon plate as model electrode (a), MWNT-modified electrode (b), COOH/MWNT-modified electrode (c), and AChE-ChOx/COOH/MWNT-modified electrode (d).

electrode surfaces was 50.4° (GCE), while the values of the contact angles for the MWNT-modified electrode, COOH/MWNT-modified electrode, and AChE-ChOx/COOH/MWNT-modified electrode were 44.2°, 22.6°, and 22.0°, respectively. The results reveal that the contact angles of the modified electrode surfaces decreased compared with that of the bare electrode, showing that the electrode surfaces were modified successfully and hydrophilicity was introduced to the surface.

XPS spectra of the C 1s of the carbon plate as model electrode (Figure 8(a)), MWNT-modified electrode (Figure 8(b)), COOH/MWNT-modified electrode (Figure 8(c)), and AChE-ChOx/COOH/MWNT-modified electrode (Figure 8(d)) were obtained. In the high-resolution XPS spectra of the carbon plate electrode (Figure 8(a)), a small amount of the >C-O group appears at 286-287 eV because of the hydroxyl group. For the MWNT-modified electrode (Figure 8(b)), a small amount of carbonyl carbon is observed because of carboxylic acid that is produced during purification of the MWNT using strong acid solution (see Section 2). After electrochemical polymerization of the 4-carboxyphenyl diazonium salts, a large amount of carbonyl peaks at 290 eV are detected because of the carboxylic acid that induces the grafted polymers. After immobilization of the AChE-ChOx, the N 1s peak at 399 eV is observed. These results show that the biosensor with AChE-ChOx based on GCE was fabricated successfully by electrochemical polymerization.

3.2. Electrochemical Properties of the Prepared Biosensor. As mentioned above, the modified MWNTs can be used as supports for biomolecule immobilization because of their preparation with physical adsorption of bienzymes (Figure 1). Change in the value of the surface electron transfer resistance (R_{et}) of the AChE-ChOx-modified biosensor based on a GCE and SPE was expected in accordance with oxidation of acetylcholine in the electrolyte (Figure 1; see the principle of acetylcholine detection). The surface electron transfer resistance (R_{et}) for the prepared AChE-ChOx-modified biosensor based on the GCE (Figure 9(a)) and SPE (Figure 9(b)) in 0.1 M PBS containing 1.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ shows that the R_{et} (Ω) for the GCE exhibited about 23 KΩ, while after modification on the GCE surface, the R_{et} (Ω) value decreased dramatically to under 10 Ω value (Figure 9(a)). This result means that the AChE-ChOx-modified biosensor based on the GCE was fabricated

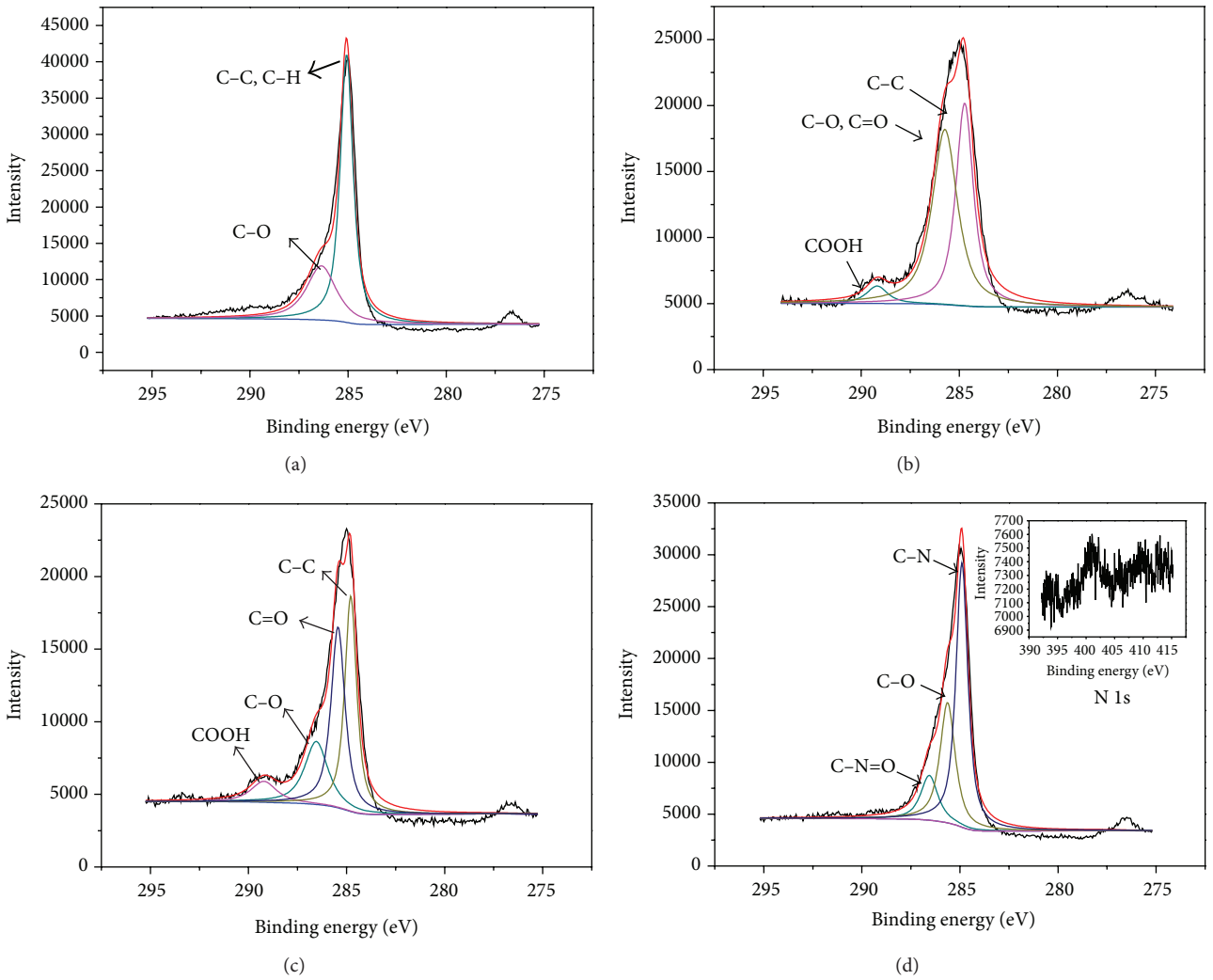


FIGURE 8: XPS spectra of the C 1s of the carbon plate as model electrode (a), MWNT-modified electrode (b), COOH/MWNT-modified electrode (c), and AChE-ChOx/COOH/MWNT-modified electrode (d).

successfully by modification and electrochemical polymerization; however, acetylcholine detection by electrochemical impedance using the prepared biosensor based on the GCE could not be performed because of the extremely large R_{et} (Ω) value. In the electrochemical impedance spectra of the prepared biosensor based on the SPE, the R_{et} (Ω) value for the fabricated SPE was about 13×10^3 KΩ because of the conductive inks with lower conductive value in 0.1 M PBS with 1.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$; however, after modification, the R_{et} (Ω) value decreased dramatically (Figure 9(b)). The R_{et} (Ω) values for the MWNT-modified electrode, COOH/MWNT-modified electrode, and AChE-ChOx/COOH/MWNT-modified electrode were determined to be 11 KΩ, 12.5 KΩ, and 12 KΩ, respectively. Actually, we expected a lower R_{et} (Ω) value for the COOH/MWNT-modified electrode because the grafted conjugated polymer could transfer electrons; however, the R_{et} (Ω) value for the MWNT-modified electrode showed a lower R_{et} (Ω) value compared with that of the COOH/MWNT-modified electrode because of side polymerization as mentioned in another paper [16].

The cyclic voltammograms of the AChE-ChOx-immobilized biosensor based on a GCE as a function of acetylcholine concentration (Figure 10) exhibit the same patterns in accordance with acetylcholine concentration, while the oxidation peaks at +0.25 V increased slightly with increasing acetylcholine concentration, suggesting that the prepared AChE-ChOx-immobilized biosensor based on the GCE could be used to detect ACh concentration through cyclic voltammetry. The linear calibration plot of current versus logarithmic acetylcholine concentration for the AChE-ChOx-immobilized biosensor under optimized experimental conditions shows that the current increased with increasing ACh concentration. The sensing range was 1.0×10^{-6}~10.0×10^{-6} M ($R^2 = 0.98$), with a detection limit of 1.0×10^{-7} M given a signal-to-noise ratio, n, of 3.

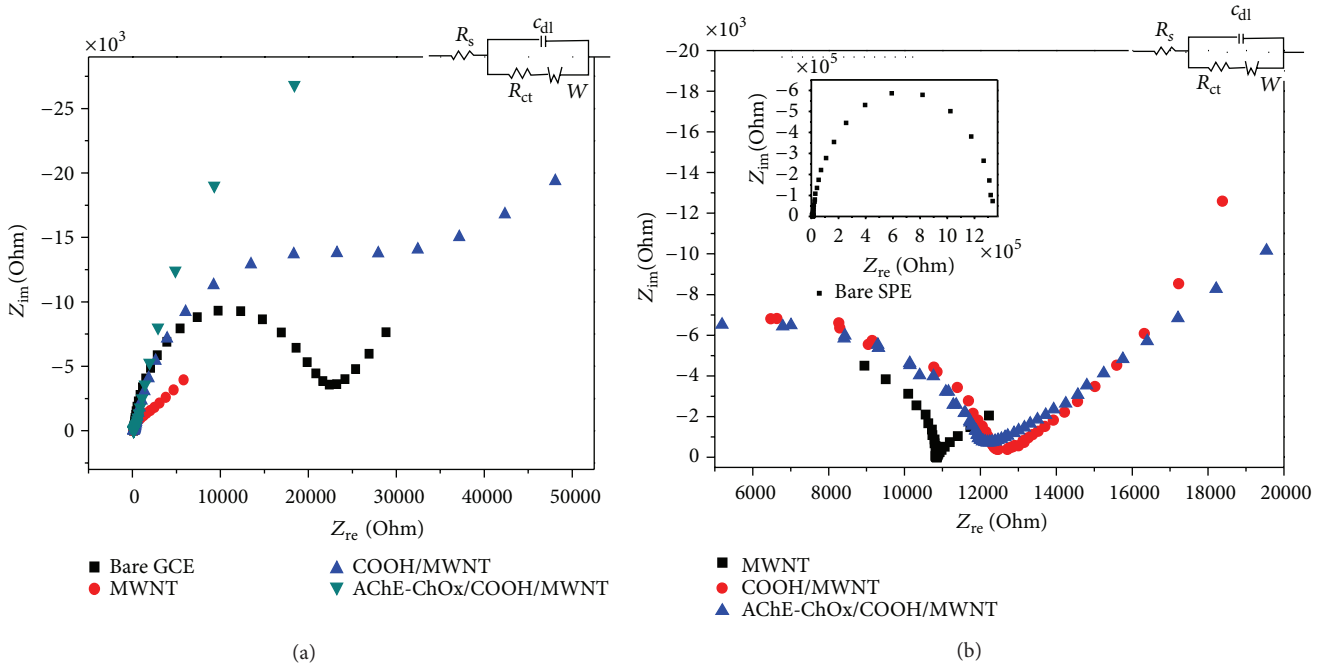


FIGURE 9: Electrochemical impedance spectra of the AChE-ChOx-modified biosensor based on a GCE (a) and SPE (b) in 0.1 M PBS buffer (pH = 7.0) containing 1.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$.

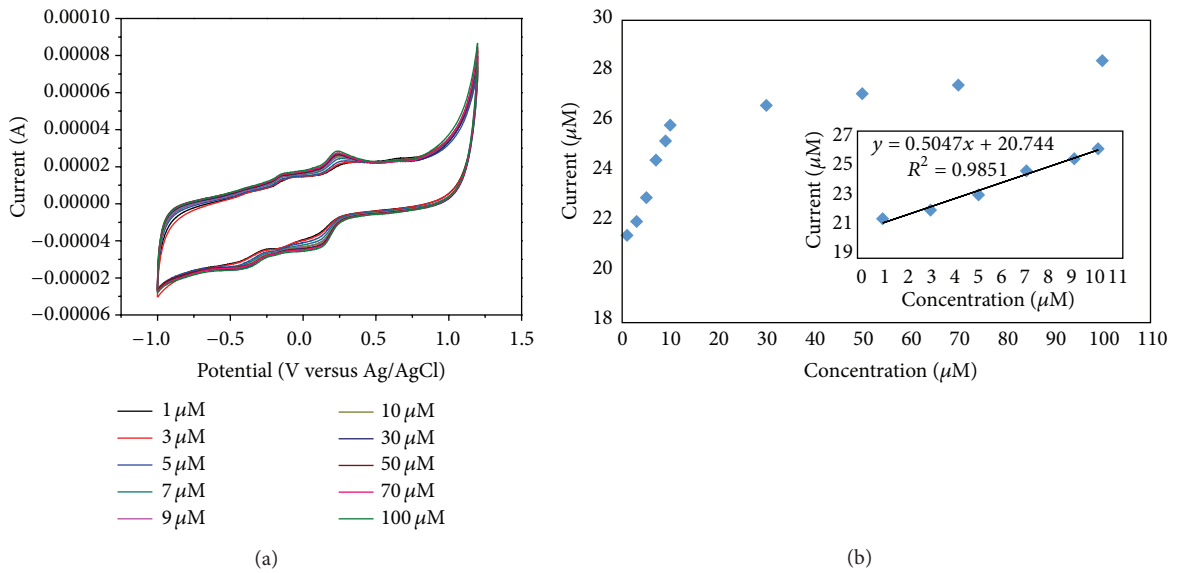


FIGURE 10: Cyclic voltammograms (CV) of the acetylcholine with the AChE-ChOx-modified biosensor based on a GCE in 0.1 M PBS (pH = 7.0) with 1 μM~100 μM acetylcholine with a scan rate 50 m/s and calibration curve.

Electrochemical impedance spectra of the AChE-ChOx-modified biosensor based on a SPE for acetylcholine at different concentrations in 0.1 M PBS (pH = 7.0) containing 1.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ and calibration curves (Figure 11) were prepared. The calibration plot based on R_{et} (Ω) value shows that the R_{et} (Ω) value decreased with increasing acetylcholine concentration. The linear range of the R_{et} (Ω) value was 1.0×10^{-5}~10×10^{-5} M (R^2 = 0.88), with a detection limit of 5.0×10^{-6} M (n = 3).

Results were determined for acetylcholine in real human blood samples by using the AChE-ChOx-immobilized biosensor based on a GCE and SPE (Table 1). The acetylcholine level in human blood samples was detected in

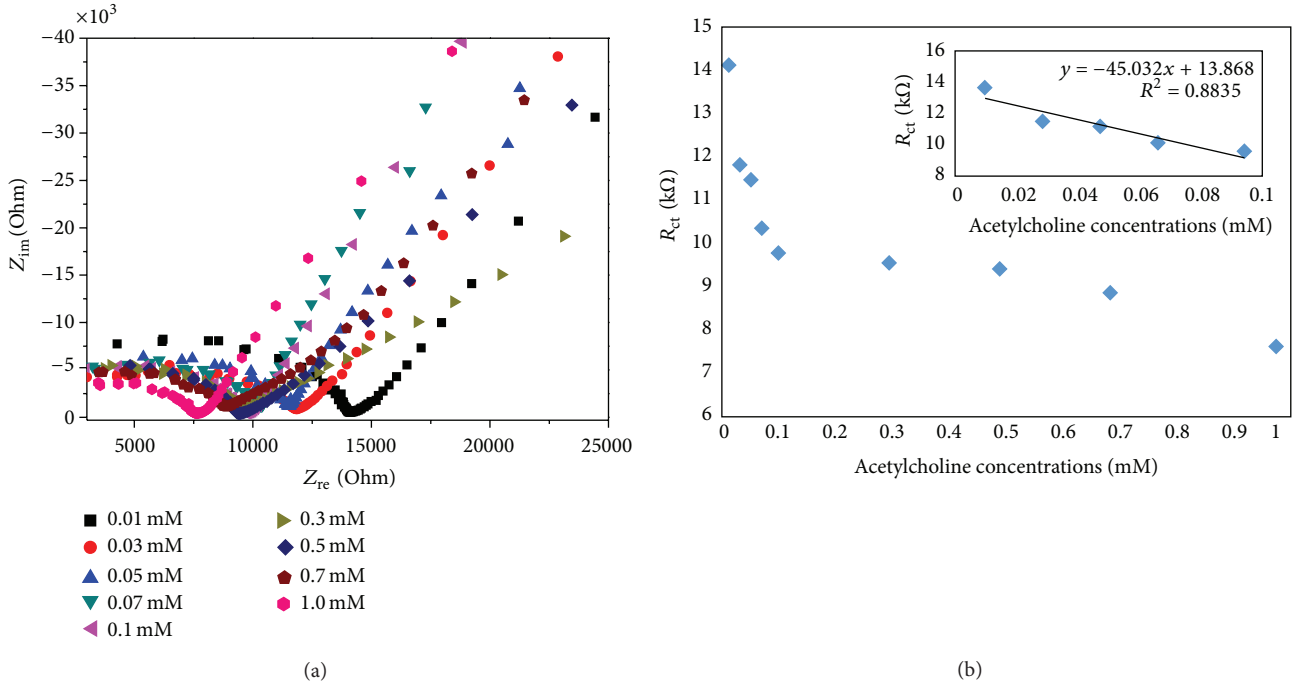


Figure 11: Electrochemical impedance spectra of the acetylcholine with the AChE-ChOx-modified biosensor based on a SPE in 0.1 M PBS (pH = 7.0) containing 1.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ and calibration curve.

the range of 40~70 μM by the prepared AChE-ChOx-immobilized biosensor based on a GCE and SPE. Meanwhile, we determined the interference effects in order to confirm the accuracy of the observed amount of acetylcholine in real human blood samples using the prepared AChE-ChOx-immobilized biosensor based on a GCE and SPE. The ratio of acetylcholine to interferents is 1 : 1. All interferents tested were present at a concentration of 1 mM. The relative responses to acetylcholine in the presence of the interferents L-ascorbic acid, L-cysteine, and uric acid were 103%, 109%, and 127%, respectively. The interference of L-ascorbic acid and L-cysteine was very small. The interference of uric acid was a little high, but there is much less uric acid in human blood samples. Based on the above results, the AChE-ChOx-immobilized biosensor based on a GCE and SPE is suitable to be applied for the detection of acetylcholine in real human blood samples.

Table 1: Results of the ACh level in human blood sample using AChE-ChOx-modified biosensor based on GCE and SPE[a].

Sex (20 years old)	ACh level (GCE) (μM)	ACh level (SPE) (μM)
Male	57.0	40.2
Female	68.5	52.1

[a]The sample is prepared by adding human blood (3.3×10^{-2} mg) in 0.1 M PBS with 1.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (10 mL).

4. Conclusions

AChE-ChOx-immobilized biosensor based on a GCE and SPE was fabricated using conjugated polymer-grafted MWNTs as follows: an AChE-ChOx-immobilized biosensor was fabricated by immobilization of bienzymatic AChE and ChOx after electrochemical polymerization of 4-carboxyphenyl diazonium salts on the surface of a MWNT-modified GCE and SPE. The sensing efficiency of the prepared biosensor for acetylcholine was investigated. From the results, the conclusions are as follows.

(1) Electrochemical polymerization of the 4-carboxyphenyl diazonium salts could introduce very simply and easily the functional group (–COOH) and conjugated aromatic backbone onto the surface of MWNTs. (2) The electrochemical polymerization of 4-carboxyphenyl diazonium salts prepared by the diazotization reaction is a very simple and easy method for preparation of good biosensor supports. (3) The sensing range of the prepared biosensor for acetylcholine is in the range of 1.0×10^{-6}~10.0×10^{-6} M ($R^2 = 0.98$) based on CV detection. (4) The sensing range of the prepared biosensor for acetylcholine is in the range of 1.0×10^{-5}~10×10^{-5} M ($R^2 = 0.88$) based on electrochemical impedance spectra. (5) Relative response of ACh to interference of L-ascorbic acid, L-cysteine, and uric acid is 103%, 109%, and 127%, respectively. (6) The prepared biosensor based on a GCE and SPE is suitable to use in measuring acetylcholine levels in real human blood samples.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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