Electrochemical Sensing of H$_2$O$_2$ at Flavin Adenine Dinucleotide/Chitosan/CNT Nanocomposite Modified Electrode

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In this study, multiwalled carbon nanotubes (MWCNTs) were dispersed in chitosan (Chi) solution, and the dispersion was used to modify glassy carbon electrode (GCE) and indium tin oxide coated glass electrode. Further, MWCNTs/Chi thin-film modified electrodes were used for the immobilization of flavin adenine dinucleotide (FAD) from an aqueous solution. FAD could be immobilized very strongly onto the MWCNTs/Chi film, and the modified electrode was very stable in a wide pH range of 1–12. The FAD-attached MWCNTs/Chi modified GCE (FAD/MWCNTs/Chi/GCE) was used to test its electrochemical and electrocatalytic properties. Moreover, the FAD/MWCNTs/Chi/GCE was used for the electrocatalytic reduction of hydrogen peroxide (H$_2$O$_2$) at low potential in neutral buffer solution.

Since their discovery by Iijima,[1] carbon nanotubes (CNTs) are attracting the attention of scientists due to their outstanding properties such as high mechanical strength but ultralight weight, high surface area, and thermal and chemical stability.[2] CNTs have also recently emerged as building blocks of novel nanoscale structures and devices.[3] CNTs can be viewed as a rolled-up sheet of graphite, a sheet formed of hexagons of carbon atoms. Depending on the detailed geometry, nanotubes have a variety of electronic properties that can be exploited for a variety of applications. Further, nanotubes have been functionalized to be biocompatible and to be capable of recognizing proteins.[4-7]

Recently, CNTs have attracted much attention in electrocatalysis and biosensing applications. They are suitable for the modification of various electrodes due to their high electronic conductivity for the electron transfer reactions and better electrochemical and chemical stabilities in both aqueous and nonaqueous solutions.[8] Furthermore, construction of efficient electrochemical sensors using the CNT-modified electrodes is very promising in that they promote electron-transfer reactions in several small biologically important molecules and large biomolecules.[9,10]

Hydrogen peroxide (H$_2$O$_2$) is used as an oxidant, disinfectant, and bleaching agent in various industrial and household applications.[11] The broad occurrence and application of H$_2$O$_2$ therefore require the use of suitable analytical methods for its determination from trace concentration levels up to the levels typically found in food, pharmaceutical, clinical, industrial, and environmental samples. H$_2$O$_2$ also plays a key role as the product of the enzymatic reaction in coupled enzyme systems.[12,13] Flavin adenine dinucleotide (FAD) is a cofactor in the enzymes D-amino acid oxidase, glucose oxidase, and xanthine oxidase. The flavin-containing adenine group is capable of undergoing oxidation–reduction reactions and can accept either one electron in a two-step process or can accept two electrons at once.[14-16]

In this article, FAD has been electrochemically immobilized onto a multiwalled carbon nanotubes (MWCNTs)/chitosan (Chi) film modified electrode. FAD was irreversibly attached and not easily leached out of the modified electrode surface. Scanning electron microscopy (SEM) and atomic force microscopy (AFM) were used for the surface characterization of MWCNTs/Chi and FAD/MWCNTs/Chi film modified electrodes. The electrochemical and electrocatalytic properties of the FAD/MWCNTs/Chi/glassy carbon electrode (GCE) were investigated by cyclic voltammetry and amperometry. Finally, the FAD/MWCNTs/Chi modified electrode was used as an electrochemical sensor for the detection of H$_2$O$_2$ in neutral buffer solution.

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Experimental

Chemicals and reagents.— FAD disodium salt, MWCNTs (o.d. 10–15 nm, i.d. 2–6 nm, and length from 0.1 to 10 μm), and Chi (from crab shells) were purchased from Sigma-Aldrich (St. Louis, MO). Hydrogen peroxide (30%, w/w), potassium ferrocyanide, sulfuric acid (H$_2$SO$_4$, assay 95%), nitric acid (HNO$_3$, assay 60%), and sodium hydroxide (NaOH, purity 93%) were purchased from Wako Pure Chemicals (Osaka, Japan). Sodium acetate and sodium dihydrogen phosphate (NaH$_2$PO$_4$) were obtained from E-Merck (Darmstadt, Germany), and other chemicals were of analytical grade and used without further purification. Double distilled water was used in all experiments. Diluted H$_2$O$_2$ standard solutions were freshly prepared directly before use.

Apparatus.— Electrochemical measurements were performed with a CHI750A electrochemical work station (CH Instrument Inc.). Indium tin oxide (ITO) coated glass electrodes were purchased from Merck Display Technologies, Ltd. (Darmstadt, Germany). The ITO and GCE were used as working electrodes. ITO substrates were cleaned by using a detergent, diluted nitric acid, and then finally rinsed with distilled water. Platinum wire was used as the counter electrode. All the cell potentials were measured with respect to a Ag/AgCl [KCl (saturated)] reference electrode. Amperometry measurements of H$_2$O$_2$ were performed on a bipotentiostat model CHI750A (TX) having an analytical rotator model AFMSRK with MSRX speed control (rotatable disk electrode, PINE Instruments). A model S-3000H scanning electron microscope (Hitachi Scientific Instruments, London, U.K.) was used for surface image measurements. The AFM images were recorded with a multimode scanning probe microscope system operated in tapping mode using a Being Nano-Instruments CSPM-4000 (Ben Yuan Ltd., Beijing, China). Electrochemical impedance spectroscopy (EIS) measurements were performed using an impedance measurement unit, IM6ex ZAHNER, Messsysteme (Kroanch, Germany). All experiments were carried out at room temperature.

Electrode modification.— Chi solution (0.5%) was prepared in 5% acetic acid, and this solution was used for the preparation of MWCNT dispersion. MWCNT dispersion (2 mg/mL) was prepared and sonicated for about 10 min, and then 10 μL of dispersion was cast onto the surface of the GCE. The MWCNTs/Chi modified electrode was dried in an oven at 50°C for 30 min; this way, a MWCNTs/Chi modified electrode was obtained. Thereafter, the MWCNTs/Chi/GCE was cycled (from 0.2 to −0.4 V) in a 0.1 M H$_2$SO$_4$ solution containing 1 mM FAD for 40 cycles at a scan rate of 0.05 V/s. Then, the FAD-immobilized MWCNTs/Chi film modified electrode was thoroughly washed using distilled water and dried using nitrogen gas. For comparison studies, 10 μL of 0.5% Chi was cast on a GCE and dried in an oven.
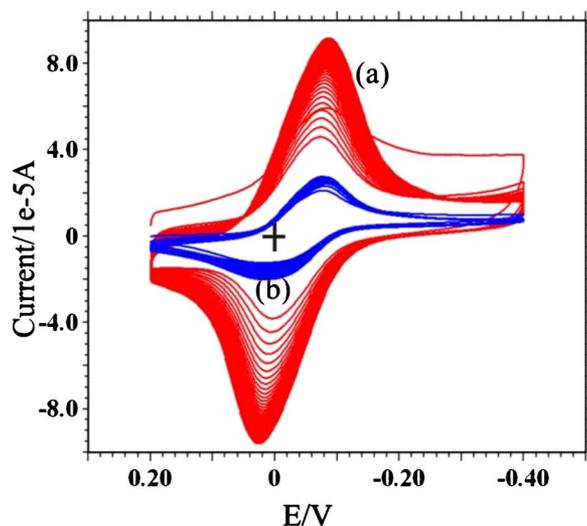
Figure 1. (Color online) (a) CVs of FAD deposition onto the MWCNTs/Chi/GCE and (b) onto the Chi/GCE from 0.1 M H_2SO_4 (pH 1.0) containing 1 mM FAD. Scan rate = 50 mV/s.

Figure 2. (Color online) CVs recorded in different pH solutions using the FAD/MWCNTs/Chi/GCE: (a) pH 1, (b) 7.0, and (c) 13.0. Scan rate = 50 mV/s.

Results and Discussion

Electrochemical deposition of FAD.— Figure 1a shows the consecutive cyclic voltammograms (CVs) of FAD adsorption onto an MWCNTs/Chi film modified electrode from pH 1.0 solution containing 1 mM FAD. The CVs of the electrochemical deposition of the FAD film onto an MWCNTs/Chi/GCE was characterized by the FAD redox couple in the scanning potential region between 0.2 and −0.4 V. The continuous increase in anodic and cathodic peak currents of the FAD redox couple indicated the surface deposition of FAD molecules (Fig. 1a). After the 40th cycle, FAD adsorption reached saturation. For comparison, in the same condition, FAD was deposited onto a Chi film modified GCE; from the CVs, it was concluded that the adsorption of FAD is very slow, and a sluggish redox behavior was observed (Fig. 1b). It was clearly confirmed that the MWCNTs/Chi nanocomposite increases the surface area of the electrode and its biocompatible nature is responsible for the higher loading of FAD molecules onto the electrode surface. After FAD adsorption, the FAD/MWCNTs/Chi modified electrode was thoroughly washed with distilled water and dried at room temperature for an hour.

Chi is a natural and biocompatible polysaccharide with glucosamine as the basic unit. The amounts of amine and hydroxyl groups in their glucosamine units are important for some bioengineering applications. Besides, using Chi as a dispersant is a requirement for environmental protection.[17,18] Earlier investigations have revealed interactions of CNTs with Chi via their π-π stacking and hydrophobic interactions.[19] In addition, electrochemical measurements also revealed that electrons can be hoppingly transported in a Chi-coated CNT electrode, indicating that CNTs coated with Chi can accept and transport electrons.[17,18] In this study, the MWCNTs/Chi nanocomposite film has shown excellent electron transport between the electrode and FAD. Biomolecules have been trapped within conducting polymers as dopant molecules when the appropriate conditions are met.[20] As shown in Fig. 1a, redox currents of FAD increase with cycle numbers, which demonstrates the adsorption of FAD molecules on the surface of the MWCNTs/Chi film modified electrode by electrostatic interactions.[15] FAD adsorption rate is higher on the MWCNTs/Chi modified electrode than on Chi/GCE (Fig. 1b) because MWCNTs have a large surface area and good electrical conductivity, while Chi provides a compatible environment to immobilized FAD.
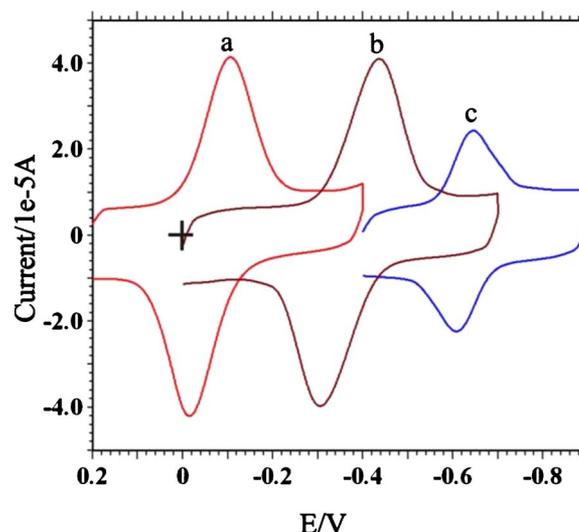
Electrochemical properties of the FAD/MWCNTs/Chi modified electrode.— A FAD/MWCNTs/Chi modified electrode was used to record CVs in different pH solutions. As shown in Fig. 2, a redox peak of FAD shows a pH-dependent response, which indicates that protons were involved in the electron-transfer reaction (Eq. 1). However, the redox peak current of FAD was decreased with increasing pH of the electrolyte, which may be due to the loss of electroactivity in the basic solution[15,16]

$$FAD + 2H^+ + 2e^- \rightleftarrows FADH_2 \qquad [1]$$

Figure 3a shows the CVs of the FAD/MWCNTs/Chi/GCE in 0.1 M phosphate buffer solution (PBS) (pH 7.0) at different scan rates. A reversible redox peak was observed and the formal potential ($E^{0'}$) was −0.36 V vs Ag/AgCl. Also, the electrochemical responses of the FAD/MWCNTs/Chi/GCE are anticipated for a diffusion-controlled redox reaction because the peak currents were directly proportional to the square root of the scan rate up to 200 mV s^{-1}
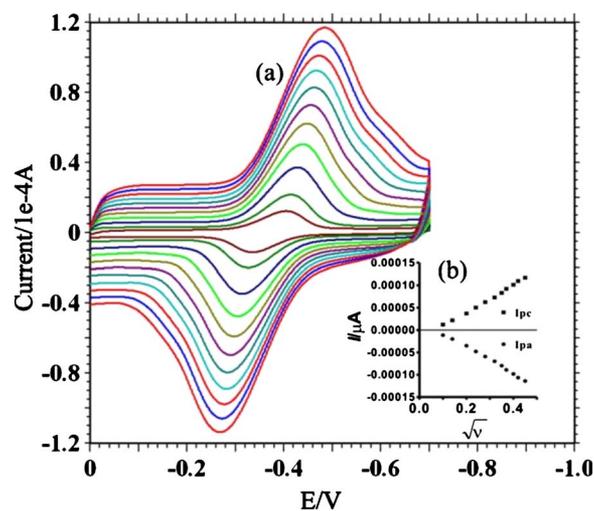

Figure 3. (Color online) (a) CVs of the FAD/MWCNTs/Chi/GCE in pH 7.0 PBS at different scan rates (the scan rates from inner to outer ranges from 10 to 200 mV s^{-1}). (b) Inset shows I_{pa} and I_{pc} vs square root of scan rate.
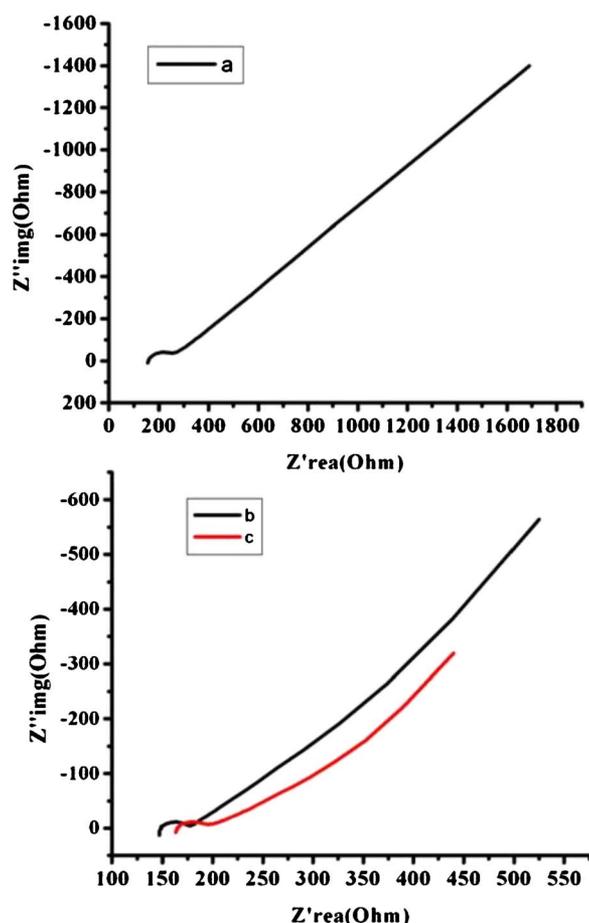
Figure 4. (Color online) Electrochemical impedance spectra of (a) the bare GCE, (b) the MWCNTs/Chi/GCE, and (c) the FAD/MWCNTs/Chi/GCE in 5 mM $[Fe(CN)_6]^{3-/4-}$ + 0.1 M PBS.

(Fig. 3b).[21] The ratio of the cathodic to anodic peak currents at various scan rates was almost constant. The surface coverage concentration (Γ) of FAD was evaluated from Eq. 2

$$\Gamma = Q/nFA \qquad [2]$$

where A (cm^2) is the area of the GCE, n (=2) is the number of electrons per reactant molecule, Q is the charge obtained by integrating the anodic peak at a low voltage scan rate (10 mV s^{-1}), and F is the Faraday constant. We assume that all of the immobilized redox centers are electroactive on the voltammetry time scale. In the present case, the calculated value of Γ_{FAD} was 1.11×10^{-4} mol/cm^2.

EIS studies.— Figure 4 shows Nyquist plots of a bare GCE, MWCNTs/Chi/GCE, and FAD/MWCNTs/Chi/GCE in the presence of 5 mM $[Fe(CN)_6]^{3-/4-}$ redox probe in pH 7.0 PBS. As shown in Fig. 4, each of the impedance spectra includes a semicircle part and a linear line part, corresponding to the electron-transfer process and the diffusion process, respectively.[22] The diameter of the semicircle represents the electron-transfer resistance (R_{et}) of the electrode surface. The Nyquist plot for a simplified Randles cell is always a semicircle. The solution resistance can be found by reading the real axis value at the high frequency intercept.[23] From the impedance spectra of the bare GCE, solution resistance was 150 Ω. As shown in Fig. 4, curve (a) is a Nyquist plot of a bare GCE in 5 mM $[Fe(CN)_6]^{3-/4-}$. A very small semicircle domain ($R_{et} = 250$ Ω) was found as the total resistance, which implied a very low electron-transfer resistance to the redox probe. The charge-transfer resistance of the bare GCE was 100 Ω. After modification with the MWCNTs/

Chi layer, the total electron-transfer resistance reaches a lower value (curve b, $R_{et} = 180$ Ω). Further, adsorption of FAD with the MWCNTs/Chi film modified electrode reached a similar value of electron-transfer resistance (curve c, $R_{et} = 200$ Ω), which indicated that FAD adsorption slightly alters the electron transfer of the electrochemical probe on the nanocomposite modified electrode. From the EIS spectra of MWCNTs/Chi/GCE and FAD/MWCNTs/Chi/GCE, charge-transfer resistances were calculated as 30 and 50 Ω, respectively. The small increase in charge-transfer resistance on the FAD/MWCNTs/Chi/GCE surface originates from the FAD monolayer. The differences in the EIS spectra of the bare GCE (Fig. 4a) and FAD/MWCNTs/Chi/GCE (Fig. 4c) indicate that the attached FAD/MWCNTs/Chi layer changes the electron-transfer resistance.

SEM and AFM studies.— Figure 5a and b shows the SEM images of Chi and FAD/MWCNTs/Chi modified electrodes, respectively. A thin film of Chi was observed on the electrode surface after the modification process (Fig. 5a). The FAD/MWCNTs/Chi film shows a highly porous structure (Fig. 5b). Figure 5 depicts AFM images of the Chi film (image a1) and FAD/MWCNTs/Chi (image b1) film modified electrodes. As we have observed in the SEM image, a highly porous thin-film-like surface was observed for the FAD/MWCNTs/Chi film modified electrode. The approximate film thicknesses were evaluated by AFM measurements as 435 and 1100 nm for Chi and FAD/MWCNTs/Chi films, respectively.

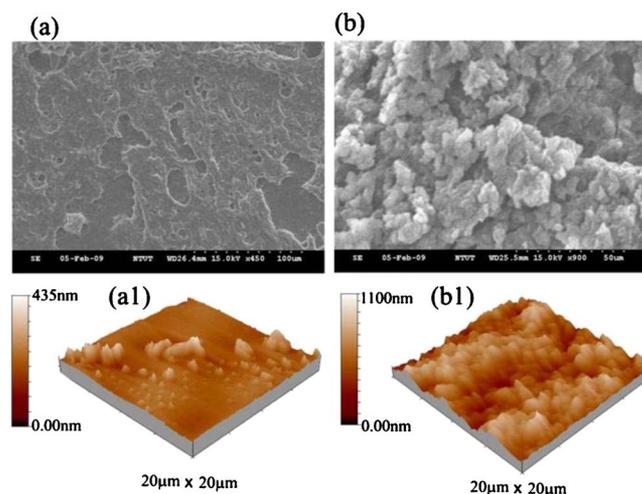**Figure 5.** (Color online) [(a) and (b)] SEM and [(a1) and (b1)] AFM images of [(a) and (a1)] Chi and [(b) and (b1)] FAD/MWCNTs/Chi films.

Electrocatalysis of H_2O_2.— To investigate the electrocatalytic activity of the FAD/MWCNTs/Chi/GCE, the electrochemical catalytic reduction of H_2O_2 was investigated by cyclic voltammetry (Fig. 6). Bare GCE could not reduce H_2O_2 at lower potential. There was no reduction peak observed at the MWCNTs/Chi/GCE in the presence of H_2O_2 in the potential range from 0.0 to -0.7 V, suggesting that MWCNTs/Chi/GCE was inactive to the direct reduction of H_2O_2 (curve g). However, at the FAD/MWCNTs/Chi/GCE, the reduction peak current at about -0.40 V was greatly enhanced in the presence of H_2O_2 corresponding to the decrease in the oxidation peak current, suggesting a typical excellent electrocatalytic reduction process of H_2O_2. The reduction peak current increased with the concentration of H_2O_2 in the solutions (curves b–f). In the absence of H_2O_2 a reversible redox peak of FAD was retained (curve a). The above results indicate that a mediated reduction of H_2O_2 takes place at the FAD/MWCNTs/Chi/GCE. According to earlier articles,[13,16] the possible electrochemical reduction mechanism of H_2O_2 at a FAD-modified electrode was presented in Eq. 3

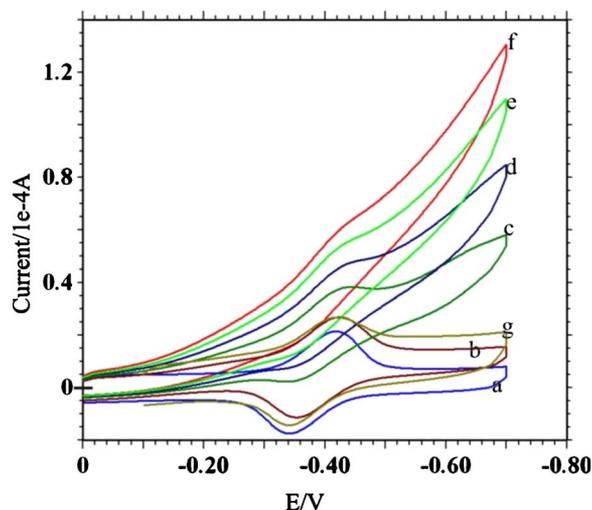$$FADH_2 + H_2O_2 \rightarrow FAD + 2H_2O \qquad [3]$$

Figure 6. (Color online) Electrocatalytic reduction of H_2O_2 in pH 7.0 PBS at the FAD/MWCNTs/Chi/GCE. (a) 0.0, (b) 50, (c) 100, (d) 150, (e) 200, and (f) 250 μM H_2O_2, and (g) MWCNTs/Chi/GCE in 250 μM H_2O_2. Scan rate = 50 mV/s.

Amperometric analysis of H_2O_2.— We attempted to study the amperometric measurement of H_2O_2 at the FAD/MWCNTs/Chi/GCE. The working potential was fixed at −0.4 V, the response current to the successive addition of different concentrations of H_2O_2 at the working electrode was measured (Fig. 7). The measured current was subtracted from the background current (8.4 μA) for the preparation of the calibration plot. In the range from 1 μM to 2.2 mM,
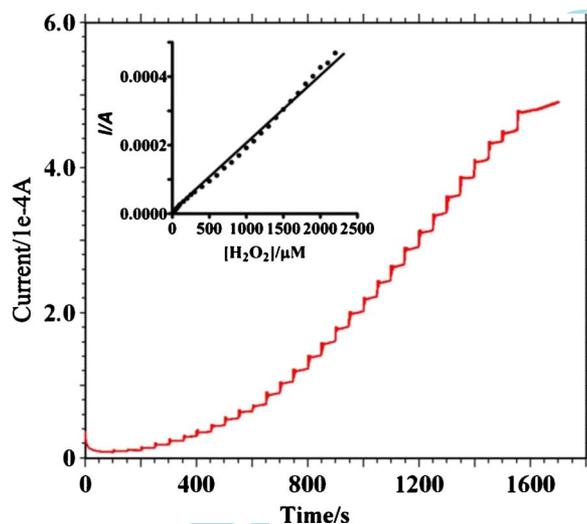

Figure 7. (Color online) Amperometric current–time curves for H_2O_2 reduction at the FAD/MWCNTs/Chi/GCE. Electrodes were held at an applied potential of −0.4 V (vs Ag/AgCl) and rotated at 1000 rpm in a 25 mL phosphate buffer solution. Concentrations were added in the range between 1 and 2200 μM H_2O_2.

the linear regression equation was $I_p(A) = 2 \times 10^{-7}x + 3 \times 10^{-6}$ ($r^2 = 0.9947$). The detection limit (S/N = 3) was 1 μM. The inset of Fig. 7 demonstrates the linear relationship of the electrocatalytic current (I_{cat}) vs H_2O_2 concentration in the wide range from 1 μM to 2.2 mM.

Stability of the biosensor.— The stability of the FAD/MWCNTs/Chi/GCE has been studied as well. Even after 100 continuous cyclic scans were carried out in the potential range from 0.0 to −0.7 V with a scan rate of 50 mV/s, no obvious change in the CVs curve could be observed. When the electrode was stored in (pH 7.0) PBS at room temperature for 4 weeks, the CV curve was still well retained, which suggested that the electrode had excellent stability.

Conclusions

A new nanocomposite film (FAD/MWCNTs/Chi) was prepared and its use in the fabrication of an amperometric biosensor for the detection of H_2O_2 was demonstrated. The FAD/MWCNTs/Chi/GCE showed excellent stability and electrocatalytic activity toward the reduction of H_2O_2 in physiological condition. The AFM, SEM, and EIS results revealed that the FAD/MWCNTs/Chi film covered the electrode surface. The electrocatalytic properties of the FAD/MWCNTs/Chi/GCE were studied by using cyclic voltammetry and amperometry. The FAD/MWCNTs/Chi/GCE has been employed as a biosensor for the determination of H_2O_2 in the range from 1 μM to 2.2 mM with a detection limit of 1 μM.

Acknowledgments

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