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CARBON NANOTUBES (CNTs) FOR THE DEVELOPMENT OF ELECTROCHEMICAL BIOSENSORS

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1. ABSTRACT

Carbon nanotube (CNT) is a very attractive material for the development of biosensors because of its capability to provide strong electrocatalytic activity and minimize surface fouling of the sensors. This article reviews our recent developments of oxidase- and dehydrogenase-amperometric biosensors based on the immobilization of CNTs, the co-immobilization of enzymes on the CNTs/Nafion or the CNT/Teflon composite materials, or the attachment of enzymes on the controlled-density aligned CNT-nanoelectrode arrays. The excellent electrocatalytic activities of the CNTs on the redox reactions of hydrogen peroxide, nicotinamide adenine dinucleotide (NADH), and homocysteine have been demonstrated. Successful applications of the CNT-based biosensors reviewed herein include the low-potential detections of glucose, organophosphorus compounds, and alcohol.

2. INTRODUCTION

Carbon nanotubes (CNTs) have been exploited for the development electrochemical and biological sensors (1-52) since they were first introduced in 1991 (53) because of their capability to provide strong electrocatalytic activity and minimize surface fouling of the sensors. Carbon nanotubes have been known to promote electron-transfer reactions of cytochrome c (2), NADH (1, 6), norepinephrine (3, 28), ascorbic acid (4), carbohydrates (22), xanthine oxidase (30), catalase (31), tryptophan (32), dopamine (4, 33), and thyroxine (34). The CNT-based electrode has also been demonstrated to provide direct electron transfer of hemoglobin immobilized on the electrode and maintain the bioactivity of the hemoglobin for the reduction of H_2O_2 (23). This is attributed to their electronic structure, high electrical conductivity, and redox active sites. Carbon is also a versatile electrode material that can undergo various chemical and electrochemical modifications to produce suitable surfaces for high electrode responses. Carbon electrodes have a wide useful potential range, especially in the positive direction, due to the slow kinetics of carbon oxidation.

The measurement principle of oxidase- and dehydrogenase-based amperometric biosensors often relies upon the immobilization of the oxidase or dehydrogenase enzymes on the surface of various electrodes and the detection of the current associated with the redox products in the biological reactions. Having recognized the superior catalytic activities of CNTs, many have focused on the development of electrode materials and methods for constructing CNT-based biosensors. Three major approaches for developing CNT-based biosensors are 1) casting of CNT thin films, from the suspensions of CNTs in solvents, on electrode surfaces (1-5, 23-43), 2) using CNTs as paste electrodes or electrode composites (6, 11, 44-47), and 3) using aligned CNT as substrates for immobilization of enzymes (7, 9-10, 48-52). We have been at the forefront in investigating all the three techniques for developing biosensors based on CNTs. This article reviews our recent successful development of CNT-based amperometric biosensors either by the immobilization of CNTs (1, 5, 11), co-immobilization of CNTs and enzymes (6, 8), or attachment of enzymes on controlled-density aligned CNT-nanoelectrode arrays (7, 9, 10). We have demonstrated the capabilities of CNTs to promote the oxidation/reduction (redox) of hydrogen peroxide and nicotinamide adenine dinucleotide (NADH), which are involved in a wide range of amperometric biosensors associated with oxidase and

dehydrogenase enzymes, respectively. The applications of CNT-based biosensors examined in our laboratories include the low-potential detections of glucoses (5, 6, 10), organophosphorous compounds (8), and alcohols (6). In addition, we have explored the electocatalytic properties of CNTs and their applications for the detection of thiol-containing homocrystiene (11), a major biomarker for a wide range of diseases (55).

Significant research and development efforts have been devoted to produce reliable glucose sensors for *in vitro* or *in vivo* applications because of the high demand for blood glucose monitoring. To increase the selectivity and sensitivity of amperometric biosensors, artificial mediators and permselective coatings are often used in biosensor fabrications. Artificial mediators are used to shuttle electrons between the enzyme and the electrode to allow operation at low potentials, at which interference during the detections of the target species are minimized (56). However, the stability and toxicity of some mediators limit their *in vivo* applications. Permselective membranes can also be used to eliminate but not completely reject the interferences (57). The ability of CNTs to reduce the overvoltage for the redox reactions of hydrogen peroxide and NADH, allowing low-potential amperometric detections of respective glucoses and alcohols with no significant interferences, has therefore been our focused for the development of mediator-free and membrane-free biosensors (58).

Organophosphorus (OP) insecticides are highly toxic because of their capacity to inhibit acetylcholinesterase (AChE) enzyme activity within nerve tissues. The OP compounds have also been used as extremely potent neurotoxic chemical-warfare agents (CWAs). The upsurge in terrorist activities generates tremendous demands for innovative sensors capable of detecting OP compounds in major CWAs. The new sensors must be field-deployable and real-time as opposed to the gas, liquid, and thin-layer chromatography coupled with different types of detectors and spectroscopy that are commonly used for OP detections. The significant catalytic effect of CNTs on the redox reactions of hydrogen peroxide has also been utilized in our lab to develop a new disposable CNT-modified screen printed biosensor for rapid assay of OP compounds with enhanced sensitivity (8).

Because of the extremely usefulness of CNT in imparting higher sensitivity and stability to electrochemical measurements of many important analytes associated with amperometric biosensors, we have intensively focused on developing amperometric biosensors based on CNTs. Our work, reviewed herein, includes investigating of the electrolytic activities of CNTs to various bio-reactions, as well as the fabrications and the applications of the CNT-based biosensors.

3. FABRICATIONS OF CNTs BASED BIOSENSORS

3.1. Biosensors based on immobilization of CNTs

CNTs have shown potential for applications in chemical/biological sensors and nanoscale electronic devices. A major barrier for developing such CNT-based devices is the insolubility of CNTs in most solvents. For example, less than 0.1 mg of MWCNTs can be dissolved in 1 mL of N,N-dimethylformamide (DMF) (37). This challenge has been addressed through covalent modification (59, 60) or noncovalent functionalization (61, 62) of the CNTs. A "wrapping" of CNT in polymeric chains has improved the solubility of CNTs without impairing their physical properties (63). Similar to other polymers used to wrap and solubilize CNTs, Nafion, a well-known perfluorosulfonated polymer, bears a polar side chain. Nafion also has unique ion-exchange, discriminative, and biocompatibility properties, thus it has been used to modify electrode surfaces and to construct amperometric biosensors (64, 65). In our work (5), Nafion in phosphate buffer or alcohol has been used to solubilize CNTs. Increasing the Nafion content from 0.1 to 5 weight percent (wt. %) results in dramatic enhancement of the solubility of both single-wall and multiwall CNTs. The CNT/Nafion association does not impair the electrocatalytic properties of CNTs with respect to the redox reaction of hydrogen peroxide. The Nafion-induced solubilization of CNT thus permits a variety of applications, including the modification of electrode surfaces for preparing amperometric biosensors (5, 23, 33).

Because CNTs are insoluble in most solvents, previously reported CNT-modified electrodes have relied on casting a CNT/acid solution onto a surface of electrodes (1, 66), a procedure that is not compatible with the immobilization of biocomponents. Therefore, we have developed a new and simple method for preparing effective CNT-based biosensors from CNT/Teflon composite material (6) and carbon nanotube paste (CNTP) (11). Both carbon nanotube composites offer convenient bulk modification for the preparation of reagentless and renewable biosensors.

Teflon has been used as a binder for graphite particles for various electrochemical-sensing applications (62, 63). Our approach relies on CNTs as the sole conductive component rather than as the modifier cast on other electrode surfaces. The bulk of CNT/Teflon composites hence serve as a reservoir for the enzymes, in the same manner as their graphite-based counterparts. The preparation is very simple: a certain amount of CNTs is hand-mixed in the dry-state with granular Teflon to obtain a desired composition of CNT/Teflon. The CNT content of the new composites has a large effect upon their electrochemical behavior. Too high Teflon (<30 wt% CNTs) leads to high resistance to a nearly insulating matrix and low sensitivity, while too high CNT (>75 wt% CNT) leads to an operation beyond the mass-limiting plateau associated with the shift of the voltammetric signal. Therefore, the CNT content of 40 to 60 wt% is suggested.

For another preparation of reagentless and renewable CNT based biosensors, multi-wall carbon nanotubes (MWCNTs) were used in place of carbon powder and mixed with mineral oil to make carbon nanotube paste (CNTP) electrodes (11). Specifically, the MWCNTs were first stirred in 2 M nitric acid for 20 hours

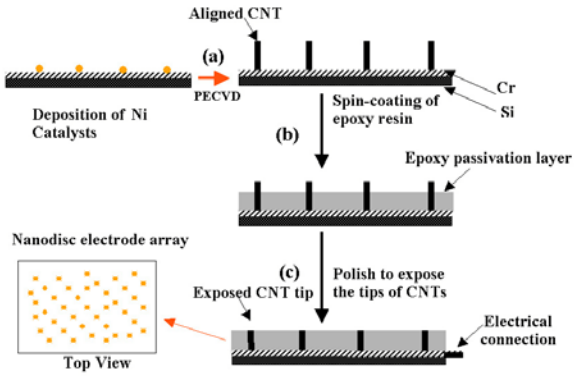


Figure 1. Fabrication scheme of a low-site-density aligned CNT nanoelectrode array.

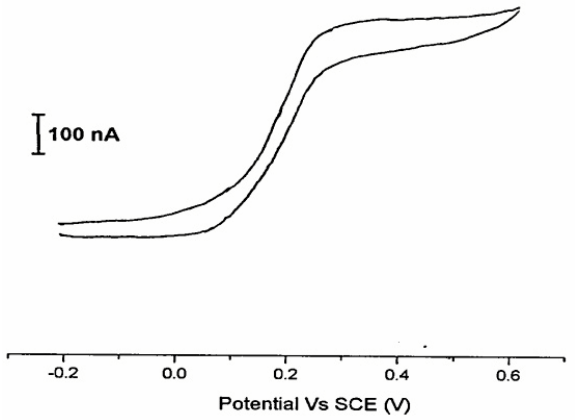


Figure 2. Cyclic voltammograms of the carbon nanotubes nanoelectrode arrays in 4 mM $K_3Fe(CN)_6$/0.5 M KNO_3 solution at the scan rate of 20 mV/s (from Ref. 10).

to remove residual metal ions. The most effective paste composes of 60 wt. % of CNTs and 40 wt. % mineral oil. The CNTP electrode has been found to be more favorable in enhancing the voltammetric sensing of homocysteine than a traditional carbon paste electrode. Besides homocysteine detections, the carbon nanotube paste electrodes have also been used for other biosensing applications (44-46).

3.2. Controlled-density aligned CNTs-nanoelectrode arrays

Although vertically aligned CNTs have good material properties (79) (e.g., good electrical conductivity, ability to promote electron transfer reactions) and are of the right size (20 to 200 nm) for nanoelectrode arrays (NEAs), they lack the right spacing. To make each nanotube work as an individual nanoelectrode, the spacing needs to be sufficiently larger than the diameter of the nanotubes to prevent the diffusion layer overlap from the neighboring electrodes (80).

Recently, a nonlithography method that allows the fabrication of low site density aligned CNT arrays with an interspacing of more than several micrometers has been developed (81). From these low-site-density CNTs, the NEAs consisting of millions of nanoelectrodes with each electrode being less than 100 nm in diameter were successfully fabricated (7, 9, 10). Since the total current of the loosely packed electrode arrays is proportional to the total number of individual electrodes, having the number of the electrodes up to millions is highly desirable. The size reduction of each individual electrode and the increased total number of the electrodes result in improved signal-to-noise ratio (S/N) and detection limits (82, 83).

In growing the low-site density aligned carbon nanotube nanoelectrode arrays (CNT-NEAs), Ni nanoparticles were randomly deposited on a 1-cm^2 Cr-coated silicon substrate by applying a pulse current to the substrate in $NiSO_4$ electrolyte solution. The size and the site density of the Ni nanoparticles were controlled by the amplitude and the duration of the pulse current. On these Ni particles, the CNTs were grown (Figure 1(a)) in the plasma-enhanced chemical vapor deposition (PECVD) system. The aligned CNT arrays had a site density of 1 x 10^6-3 x $10^6/cm^2$, a length of 10 to 12 μm, and a diameter of 50 to 80 nm. Epon epoxy resin 828 was used as the passivation layer and m-phenylenediamine (MPDA) as a hardener (Figure 1(b)). The protruding part of the CNTs beyond the polymer resin was mechanically removed by polishing with a lens. Then the electrical connection was made on the CNT-Si substrate to make the CNT nanoelectrode arrays (Figure 1(c)). The excellent stability of the epoxy layer permits a long life time (more than several weeks) of the CNT-NEAs. The CNT-NEAs fabricated based on this design effectively use the open ends of CNTs for electrochemical sensing. These open ends of the CNTs have fast electron transfer rates similar to a graphite edge-plane electrode, while the side-walls have very slow electron transfer rates similar to the graphitic basal plane (84, 85). Enzymes can be attached on the tips of these CNTs to fabricate biosensors based on specific needs.

Cyclic voltammetry was used to study the electrochemical behavior of the CNT-NEAs. Figure 2 shows the cyclic voltammogram of the CNT-NEAs in 4 mM $K_3Fe(CN)_6$/0.5 M KNO_3 solution at a scan rate of 20 mV/s. The sigmoidal-shape of the voltammogram is a sign of nanoelectrode behavior (radial diffusion). The steady-state current arises because the electrolysis rate is approximately equal to the rate of diffusion of analyte to the electrode surface (86). The scan-rate-independent limiting current behavior was observed up to 500 mV/s, indicating that there is no diffusion layer overlapping between the electrodes because most of the CNTs are separated from their nearest neighbors for at least 5 μm, much larger than the diameter of each nanotube (50 to 80 nm).

4. ELECTROCATALYTIC ACTIVITIES OF CNTs

4.1. CNTs in promoting redox reaction of hydrogen peroxide

Hydrogen peroxide is involved in a wide range of biosensing applications associated with oxidase enzymes including those for detecting glucose (5, 6, 10) and organophosphorous compounds (8). We have exploited the capability of CNTs to promote redox activity of hydrogen

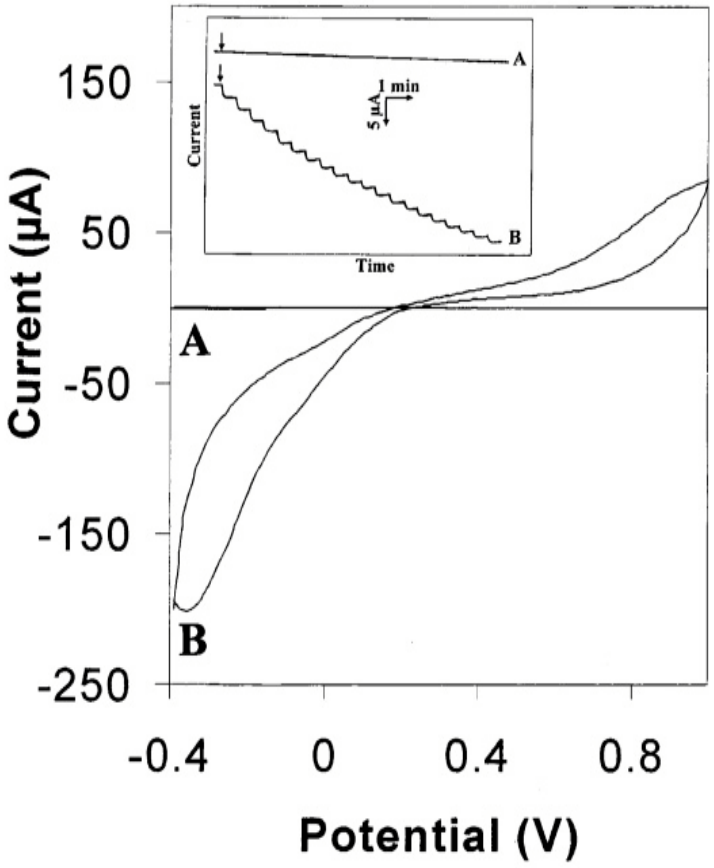


Figure 3. Cyclic voltammograms of $5x10^{-3}$ M hydrogen peroxide at unmodified (A), and MWCNT/Nafion-modified (B) GC electrodes. Operating conditions: scan rate, 50 mV/s; electrolyte, phosphate buffer (0.05 M, pH 7.4). Upper inset shows the amperometric response after increasing hydrogen peroxide concentration for 1x10-3 M incrementally while potential was held at 0.0 V (from Ref. 5).

peroxide. Specifically, the electrocatalytic activity of CNTs for hydrogen peroxide oxidation/reduction was studied using CNT/Nafion (from a 0.5 wt. % Nafion solution containing 2 mg/mL of CNTs) coated on a glassy carbon (GC) electrode surface. Figure 3 displays cyclic voltammograms for 5 x 10^{-3} M hydrogen peroxide recorded at a bare GC electrode (A) and the CNT/Nafion-modified GC electrode (B). Significant oxidation and reduction currents starting around +0.20 V are observed on the CNT/Nafion-coated electrode, while none are observed at the bare GC electrode. The CNT/Nafion-coated electrode offers a marked decrease in the overvoltage for hydrogen peroxide reaction and hence allows low-potential amperometric detection. The inset of Figure 3 shows the amperometric response at 0.0 V to successive additions of hydrogen peroxide. While the modified electrode (B) responds very rapidly and favorably to the changes in hydrogen peroxide concentration, no response is observed at the bare GC electrode (A). Results also indicate that Nafion does not impair the electrocatalytic properties of CNT. Also, the sensings at the CNT/Nafion-coated electrode were reproducible and not affected by regenerating the electrode surface.

Similar substantial lowering of the overpotential and significantly improved current signals for hydrogen peroxide by CNTs have also been found at CNT/Teflon electrodes, obtained by packing 60/40 wt.% of CNT/Teflon into the electrode cavity of a glass sleeve with a copper wire as the electrical contact. Figure 4 compares hydrodynamic voltammograms (HDV) for 1 mM hydrogen peroxide at the graphite/Teflon (a) and CNT/Teflon (b) electrodes. Compared to the graphite/Teflon electrode, the CNT/Teflon electrode responds more favorably to hydrogen peroxide over the entire potential range (0.0 to 1.0 V) with significant response starting at +0.20 V. The Teflon binder is proven to not impair the electrocatalytic properties of CNTs.

4.2. CNTs in promoting redox reaction of NADH

β-Nicotinamide adenine dinucleotide (NADH) is a cofactor in several hundred enzymatic reactions of NAD^+/NADH-dependent dehydrogenases. The electrochemical oxidation of NADH has thus been the subject of numerous studies related to the development of dehydrogenase-based amperometric biosensors (69). Problems inherent to such anodic detection are the large overvoltage encountered for NADH oxidation at ordinary electrodes (70) and surface fouling associated with the accumulation of reaction products (71). CNTs have thus

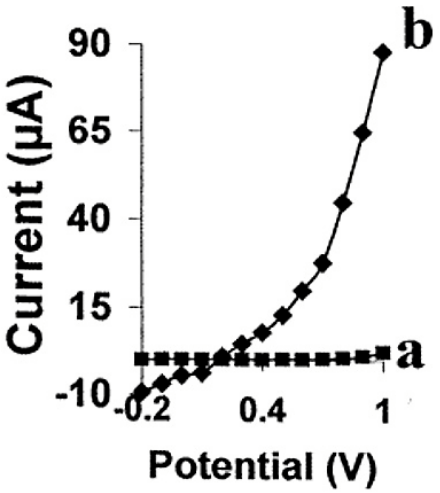


Figure 4. Hydrodynamic voltammograms for 1 mM hydrogen peroxide at the 60:40 wt% graphite/Teflon electrode (a) and the 60:40 wt% MWCNT/Teflon electrode (b). Operating conditions: potential, +0.1 V; supporting electrolyte, phosphate buffer (0.05 M, pH 7.4); stirring rate, 400 rpm (from Ref. 6).

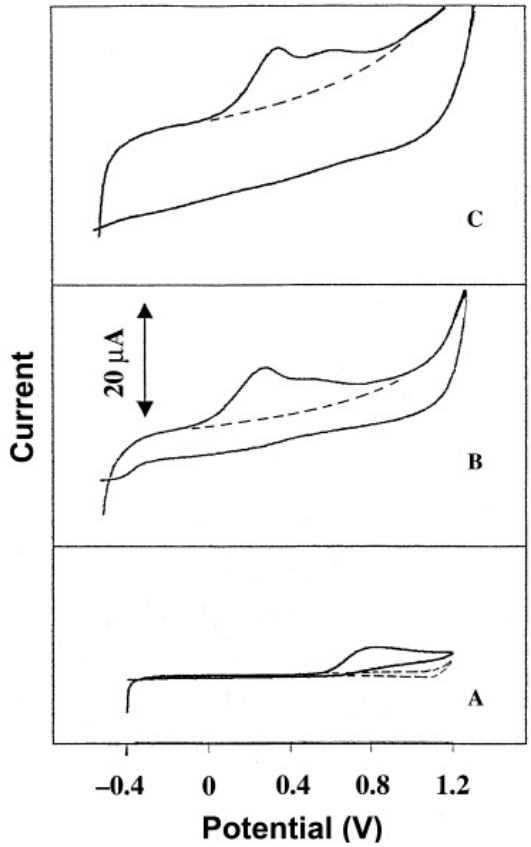


Figure 5. Cyclic voltammograms for 5x10-3 M NADH at unmodified (A), MWCNT-modified (B), and SWCNT-modified (C) GC electrodes. Operating conditions: scan rate, 50 mV/s; electrolyte, phosphate buffer (0.05 M, pH 7.4). Dotted lines represent the background response (from Ref. 1).

been investigated in our recent work (1, 6) to reduce the overpotential for NADH oxidation and to alleviate surface fouling issues.

In our previous study (1), single-wall and multi-wall CNTs were dispersed in concentrated sulfuric acid, and each was subsequently cast on a glassy carbon electrode. Figure 5 shows the cyclic voltammograms of NADH measured at unmodified (A), MWCNT-modified (B), and SWCNT-modified (C) glassy carbon electrodes. Both modified electrodes yielded an approximately 2-fold larger NADH peak than did the unmodified electrode. Such electrocatalytic behavior is perhaps due to the oxygen-rich groups on the CNT surface, introduced during the acid dispersion. Figure 6 shows that successive additions of 1 x 10^{-4} M NADH result in increasing response detected at the CNT-modified electrode (B) but no response at the unmodified electrode (A) when the detection potential was kept low (i.e., 0.3 V). This fast response at the CNT-modified electrodes occurred within 10s. The amperometric responses of $5x10^{-3}$ M NADH at the CNT-modified electrodes were also stable; the decay of the signal was less than 10% and 25% after a 60-min period at the MWCNT-modified and SWCNT-modified electrodes, compared with 75% and 53% at the graphite-coated and acid-treated electrodes, respectively. This shows the capability of CNTs in resistance to the fouling effects and preventing the diminishing of signals in successive cyclic voltammetric detections.

The capabilities of CNT to remarkably decrease overvoltage for the NADH oxidation and reduce the surface fouling effects suggest the great promise of CNTs for developing highly sensitive, low-potential, and stable amperometric biosensors based on dehydrogenase enzymes.

4.3. CNTs in promoting oxidation of thiol-containing homocysteine

Homocysteine is a major biomarker for a wide range of diseases (55), thus its detection has been the subject of great interests in biomedical filed. We have successfully used a CNT paste electrode, prepared as in section 2.1, for the voltammetric sensing of homocysteine. Figure 7 compares the cyclic voltammograms for the responses of (a) blank solution (0.05 M phosphate buffer, pH 7.4) and (b) 160 μM of homocysteine at (A) the CNTP and (B) the carbon paste electrodes at the scan rate of 100 mV/s. The inset of Figure 7 shows that there was a linear response (current at +0.64 V) to the addition of homocysteine on the CNTP electrode, while at the traditional carbon paste electrode the response was negligible. Many interesting aspects were drawn from the cyclic voltammograms. First, at the CNTP electrode a well-defined signal emerged at +0.28V and reached a plateau at +0.64V, while at the carbon paste electrode only a slight increase in the oxidation current was found at +0.4V. Second, the oxidized species of homocysteine underwent chemically irreversible reactions because no reduction peak was found at reversed potential scanning. Hence, the overall oxidative reaction process can be expressed as

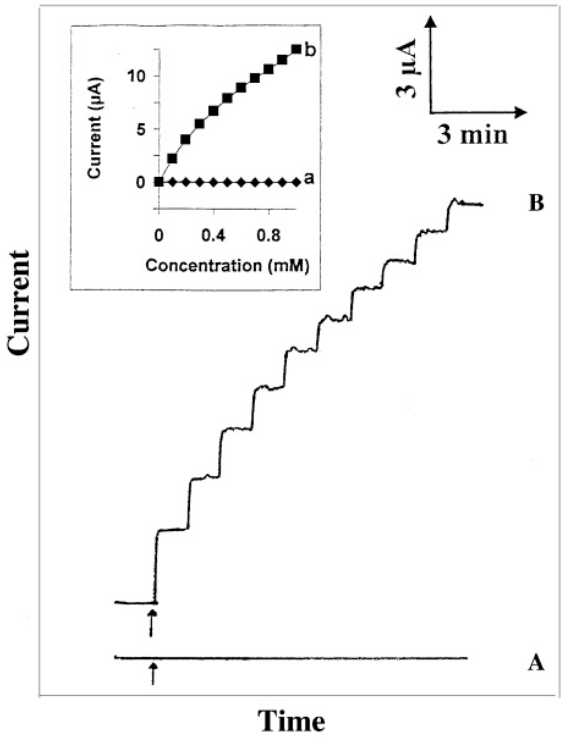


Figure 6. Current-time recordings obtained after increasing the NADH concentration of 1 x 10^{-4} M (each step) at unmodified (A) and MWCNT-modified (B) GC electrodes. Inset shows the corresponding calibration curve. Operating conditions: potential, +0.3 V; stirring rate, 500 rpm; electrolyte, phosphate buffer (0.05 M, pH 7.4) (from Ref. 1).

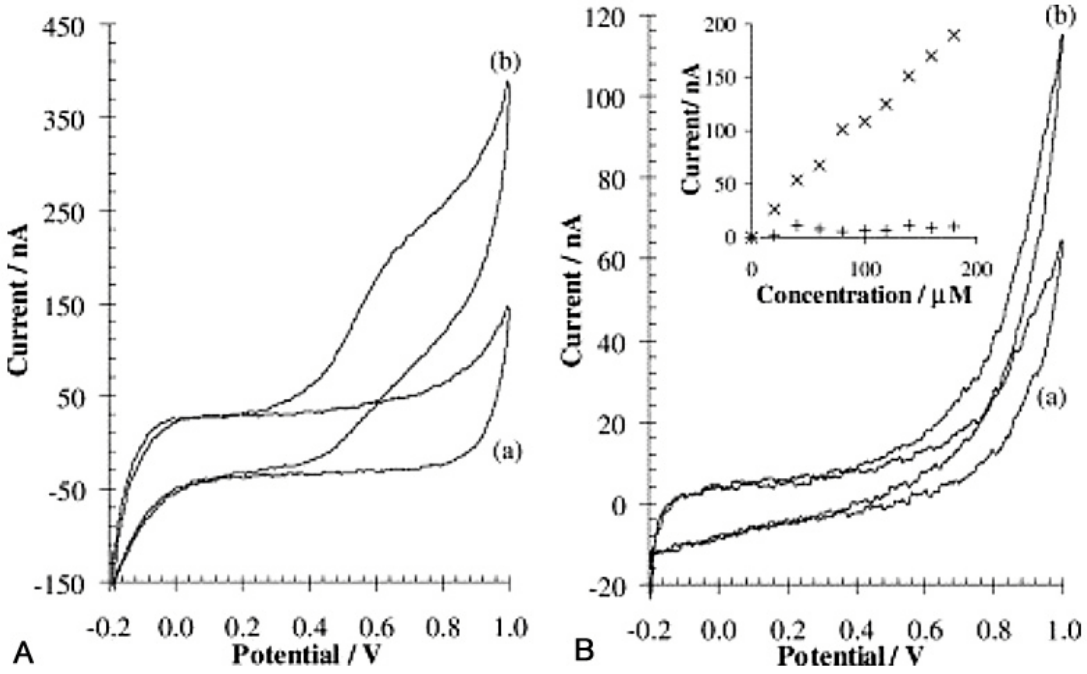


Figure 7. Cyclic voltammograms for the responses of (a) blank solution (0.05 M phosphate buffer, pH 7.4) and (b) 160 μM of homocysteine at (A) CNTP and (B) carbon paste electrodes; scan rate of 100 mV/s. Inset: the corresponding standard addition plot obtained at (+) the carbon paste and (×) the CNTP electrodes (from Ref. 11)

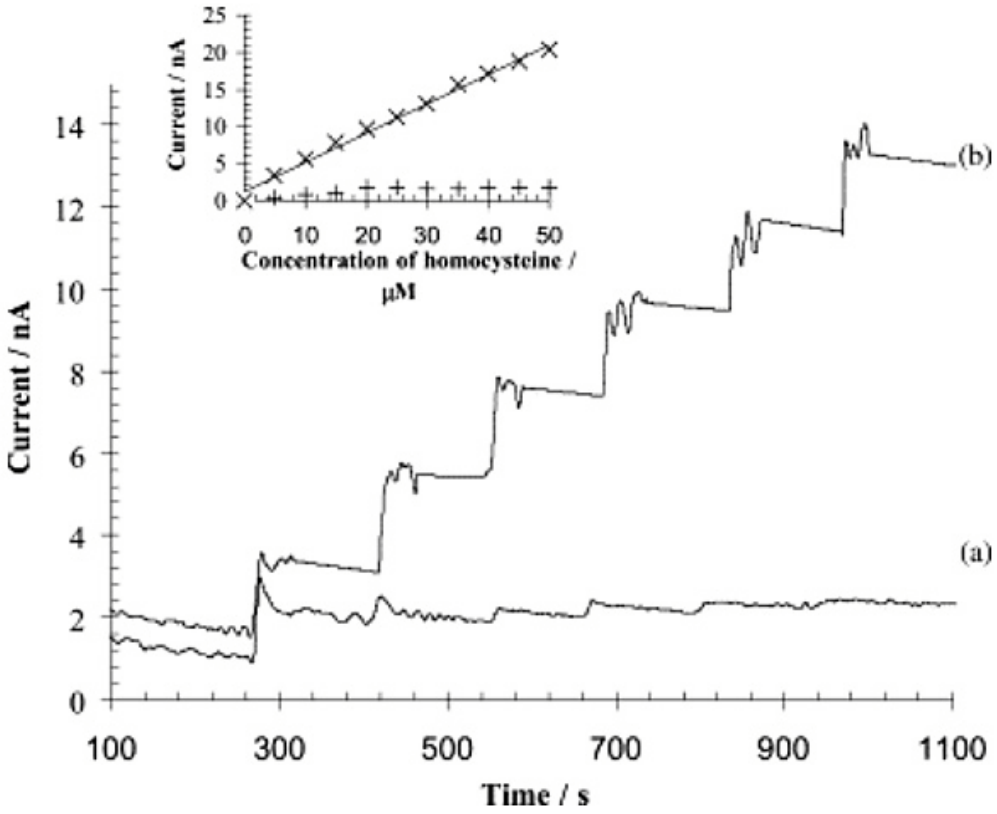


Figure 8. Fixed potential (+0.7 V) response at (a) the carbon paste and (b) CNTP electrodes. Inset is the corresponding standard addition plots at (+) the carbon paste and (×) CNTP electrodes (from Ref. 11).

$$2RSH \rightarrow 2RS\bullet + 2e^- + 2H^+ \rightarrow RSSR$$

where one proton, one electron oxidation of homocysteine first occurs to generate the radical species RS•, which subsequently undergoes rapid demerisation to form disulfide homocysteine species (55). The consumption of the homocysteine on both electrode surfaces is evidently demonstrated by the 30-35% decay of the oxidation current over the period of one hour. Yet, the oxidation of homocysteine at the CNTP electrode produced responses that were more stable over time than those at the carbon paste electrode. Third, the oxidative reaction of homocysteine is diffusion controlled. Like the traditional carbon paste electrode, the CNTP electrode can be regenerated easily by mechanically polishing the surface. Figure 8 shows the amperometric calibrations upon successive addition of 5 μM homocysteine obtained at a fixed potential +0.7 V at (a) carbon paste, and (b) CNTP electrodes. At the CNTP electrode, the detection limit of homocysteine was found to be 6.5 μM in 0.05 M phosphate buffer (pH 7.4). Marked enhancement of the signals was obtained at the CNTP electrode compared to those obtained at the carbon paste electrode, attributed by the electrocatalytic activity of CNTs toward the oxidation of thiols on homocysteine. Such electrocatalytic activity toward thiols was confirmed by performing the similar cyclic voltammetry experiment on other thiol-containing species, including cysteine, glutathione, and n-acetylcysteine. Similar to the finding using homocysteine, the oxidation of these thiol-containing species at the CNTP electrode occurred at lower overpotential and the signal-to-noise was higher than those observed at the traditional carbon paste electrode. This encourages favorable use of CNTP electrode for detecting other important thiol-containing biomolecules over common carbon paste electrodes.

5. APPLICATIONS OF CNT-BASED AMPEROMETRIC BIOSENSORS

CNTs display a marked electrocatalytic activity toward redox reactions of hydrogen peroxide, and hence are promising for the development of biosensors in connection with oxidase enzymes, including sensors for glucose and organophosphorus compounds. Likewise, the electrocatalytic properties of CNTs in reducing the overpotential for the redox reaction of NADH suggest their potential use in dehydrogenase-based amperometric biosensors such as those for alcohol detection (6).

5.1. Glucose biosensors

Co-immobilization of CNTs and enzymes opens the door to a wide range of biosensing applications. For example, co-immobilization of glucose oxidase (GO_x) enzyme and CNTs/Nafion composite has been investigated in our laboratory to develop a glucose biosensor. The CNT/Nafion/GO_x modified glassy carbon (GC) electrode (prepared by coating a 0.5 wt% Nafion solution containing

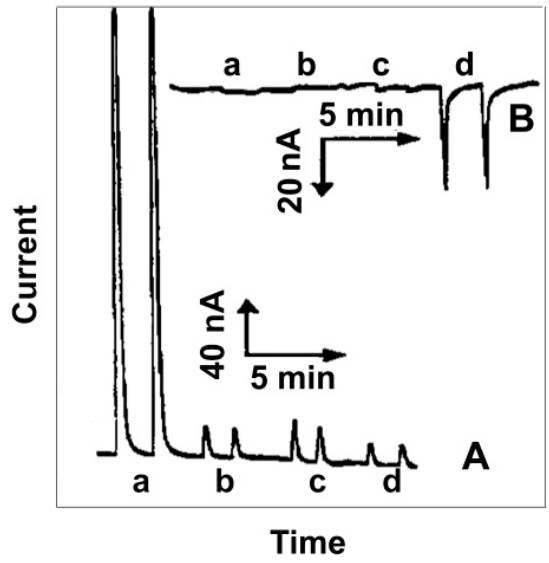


Figure 9. Flow-injection signals for 2 x 10^{-4} M acetaminophen (a), 2 x 10^{-4} M ascorbic acid (b), 2 x 10^{-4} M uric acid (c), and 1 x 10^{-2} M glucose (d), at the Nafion/GOx modified GC electrode (A) at +0.8 V, and the MWCNT/Nafion/GOx modified GC electrode (B) at -0.05 V, and flow rate of 1.25 mL/min (from Ref. 5)

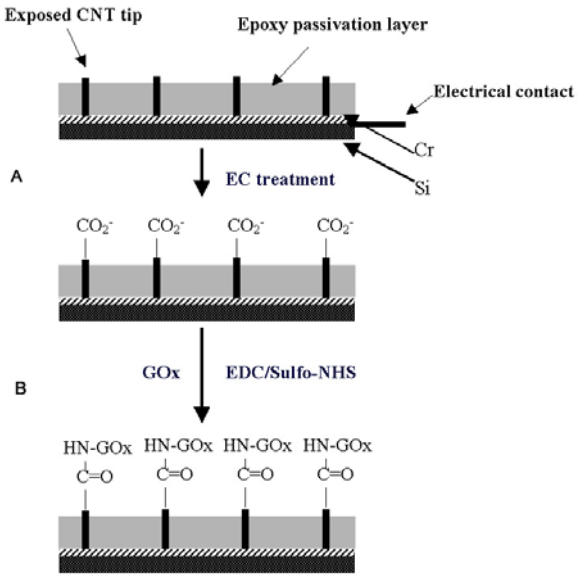


Figure 10. Fabrication of a glucose biosensor using the CNT-nanoelectrode arrays: (A) electrochemical treatment of the CNT-NEAs for functionalization (B) coupling of the enzyme (GOx) to the functionalized CNT-NEAs (from Ref. 10).

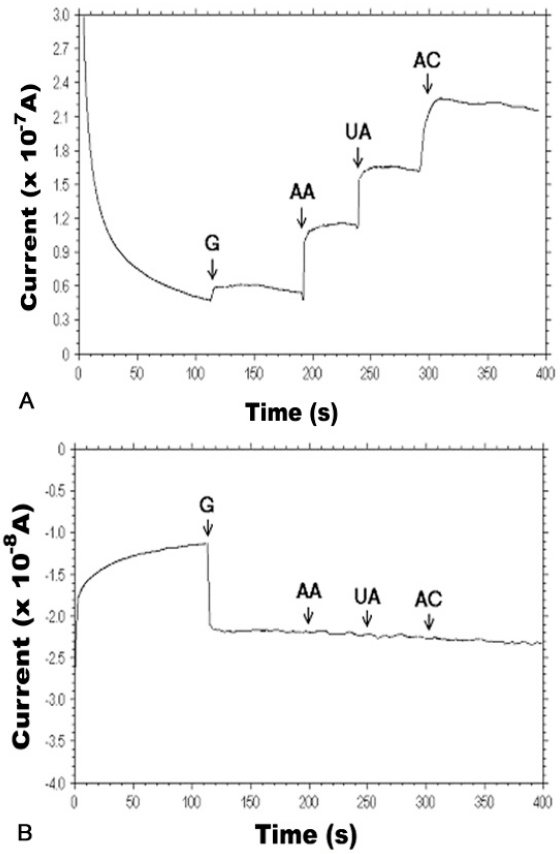
-0.2V (b). Electrolyte: 0.1 M phosphate buffer/0.1 M NaCl (pH 7.4) (from Ref. 10).

2 mg/mL of CNTs on a GC electrode) has been used in flow-injection system to measure glucoses (5). Figure 9 compares the amperometric responses for relevant physiological levels of glucose, ascorbic acid, acetaminophen, and uric acid at the CNT/Nafion/GO_x modified GC electrode (B) and Nafion/GO_x modified GC electrode (A). In Figure 9, the accelerated electron-transfer reaction of hydrogen peroxide at the CNT/Nafion/GO_x modified GC electrode allows for glucose measurements at very low potentials (i.e., -0.05 V) where interfering reactions are minimized. As a result, a well-defined glucose signal (d) is observed, while the signals of acetaminophen (a), uric acid (b), and ascorbic acid (c) are negligible. No such discrimination is obtained at the Nafion/GO_x biosensor (without the CNT) (A) held at +0.80 V, where large oxidation peaks are observed for all interferences, indicating that the permselective (charge-exclusion) properties of Nafion are not adequate to fully eliminate anionic interferences. In short, the coupling of the permselective properties of Nafion with the electrocatalytic action of CNT allows for glucose detection with effective discrimination against both neutral and anionic redox constituents. Similarly, the CNT/Nafion-coated electrodes have also been demonstrated to dramatically improve the signal of dopamine in the presence of the common ascorbic acid interference (5).

Attachment of glucose oxidase [GO_x] enzymes (10) has also been performed on carbon nanotube nanoelectrode arrays (CNT-NEAs) by an electrochemical method to oxidize CNTs to create carboxylic acid groups (17), followed by the enzyme immobilization on the open-ends of CNTs by carbodiimide chemistry using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide(EDC) as the coupling agent (78). The procedure is illustrated in Figure 10. Figure 11 compares amperometric responses for 5 mM glucose (G), 0.5 mM ascorbic acid (AA), 0.5 mM acetaminophen (AC), and 0.5 mM uric acid (UA) at the GO_x modified CNT-NEAs and the potentials of +0.4V (a) and –0.2V (b). Well-defined cathodic and anodic glucose responses were obtained at the CNT/GO_x-based biosensor at both potentials. However, the glucose detection at lower operating potential (-0.2 V) was significantly less influenced by the interferences, indicating high selectivity towards the glucose substrate. Such a highly selective response to glucose was obtained at the CNT/GO_x-based biosensor without the use of mediators and permselective membranes. The linear response to glucose at the CNT/GO_x-based biosensor was up to 30 mM, which is higher than the 15 mM required for practical use in the detection of blood glucose, and the steady state was reached within 20 to 30 seconds. The limit of detection, based on a signal-to-noise ratio of 3, was 0.08 mM. Gao and coworkers (52) have used align carbon nanotube array as a substrate for coating polypyrrole thin film, followed by electrochemically entrapping GO_X enzymes in the film.

5.2. Organophosphorus compound biosensors

Organophosphorous (OP) compounds are very toxic and are thus widely used as pesticides and chemical-warfare agents (CWAs). Recently, we have successfully used MWCNTs in developing an amperometric biosensor for OP compounds (8). Specifically, the MWCNT was used to modify screen-printed carbon electrodes, which were subsequently co-immobilized with acetylcholinesterase



Figures 11. Amperometric responses for 5 mM glucose (G), 0.5 mM ascorbic acid (AA), 0.5 mM acetaminophen (AC), and 0.5 mM uric acid (UA) at the GOx-modified, CNT-nanoelectrode array and the potentials of +0.4V (a) and -0.2V (b). Electrolyte: 0.1 M phosphate buffer/0.1 M NaCl (pH 7.4) (from Ref. 10).

(ACHE) and choline oxidase (CHO) enzymes. The MWCNT-modified electrode has demonstrated a significant catalytic effect for the redox reaction of hydrogen peroxide, leading to the development of a novel biosensor for the assay of OP compounds with enhanced sensitivity.

The ACHE enzyme is known to play an important role in cholinergic transmission as a catalyst for the rapid hydrolysis of the neurotransmitter acetylcholine to acetate and choline as follows:

$$\text{Acetylcholine} + H_2O \xrightarrow{ACHE} \text{Acetate} + \text{Choline} \qquad \text{(i)}$$

However, in the presence of OP compounds, the rate of choline production is reduced. The capability of OP compounds to inhibit ACHE activity is well known (72-76) and thus is being exploited in developing biosensors for OP compound detection. In our work, the amperometric biosensor for OP compounds is based on co-immobilization of ACHE and CHO on a printed CNT electrode. In the biosensor based on ACHE/CHO enzymes, choline produced in reaction (i) serves as a substrate for the CHO enzyme in the presence of oxygen to produce hydrogen peroxide as follows:

$$\text{Choline} + O_2 \xrightarrow{CHO} \text{Betaine Aldehyde} + H_2O_2 \qquad \text{(ii)}$$

The hydrogen peroxide produced in reaction (ii) can be detected amperometrically, and the amperometric response is negatively proportional to the amount of an OP compound that is introduced into the system.

The inhibition of catalyst ACHE by OP compounds is an irreversible process; once exposed to the OP compounds, the enzyme is inactivated, and the sensor can be reused only after an appropriate enzyme reactivation (77). Therefore, we have attempted to develop biosensors that are low-cost and disposable using screen-printed carbon electrodes (8). To make the sensor, the suspension of MWCNTs in N, N-DMF was cast on the surface of screen-printed carbon electrodes to form a thin-film of CNTs. The screen-printed carbon only serves as a conducting base for the CNT electrode. An electrochemical method was used to oxidize CNTs to create a carboxylic acid group (17). Both enzymes, ACHE and CHO, were then co-immobilized on the CNTs via carbodiimide chemistry by forming amide linkages between their amine residues and carboxylic acid groups on the CNT surface (78). The optimum biosensor was found to contain a loading of 0.8 mU ACHE and 1.5 U CHO on the electrode, with a 2 mM ACH as the substrate.

An amperometric method was employed to study the sensor response time of the ACHE inhibition after the spike of methyl parathion, as a representative OP compound. In Figure 12a, the amperometric response was rapid (i.e., within 30 seconds) after the ACH addition, reflecting the fast diffusion of enzyme substrates and the products (as the CNTs are membrane-free porous). When methyl parathion was successively added to the test area of the biosensor, the response decreased significantly and rapidly in the first 10 minutes and more slowly thereafter. The significant inhibition effect of methyl parathion to the catalytic activity of ACHE reduces the production of hydrogen peroxide, leading to low signals.

In addition to methyl parathion, the inhibition effects of the other two OP compounds were investigated using the CNT modified, ACHE/CHO immobilized electrode. Figure 12b compares the enzyme activities with a function of time after the spike of each OP compound, including (A) chlorpyrifos, (B) fenitrothion, and (C) methyl parathion.

The enzyme activity is the ratio of I_i (a steady-state current obtained in the presence of a given OP compound) to I_o (that obtained in the absence of the OP compound). After spiking with OP compounds, the enzyme activity decreases with time. The high inhibition effect of OP compounds can be correlated to low enzyme activity of ACHE. Their inhibition effects are in the following ascending order: methyl parathion > fenitrothion > chlorpyrifos.

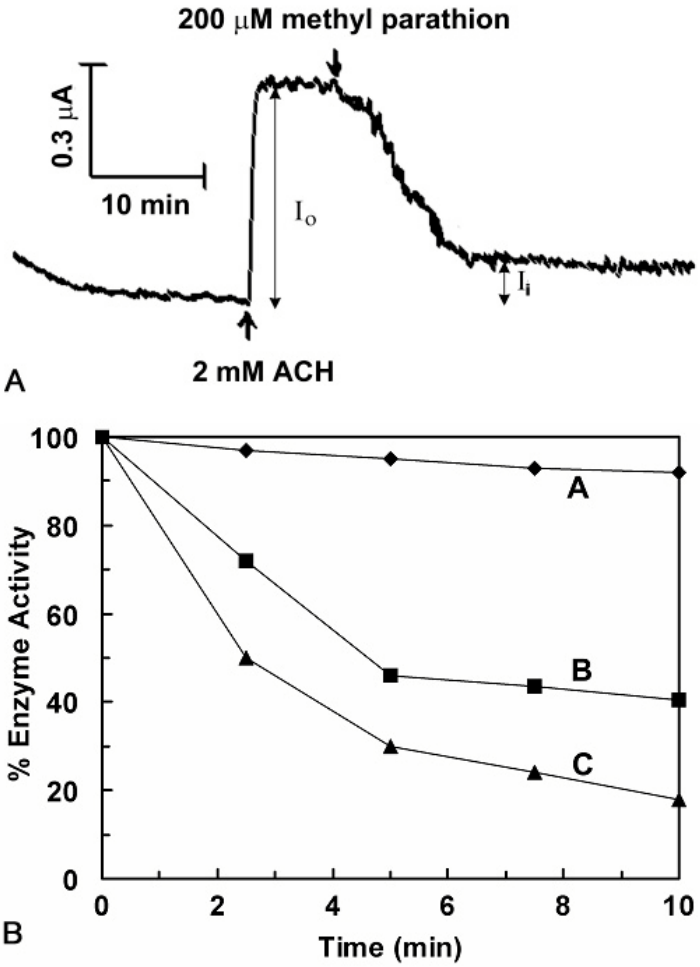


Figure 12. (a) Amperometric response of methyl parathion at the CNT/ACHE/CHO immobilized screen-printed biosensor. Operating conditions: 0.1 M phosphate buffer/0.1 M NaCl (pH 7.4), potential of +0.50 V. (b) Inhibition effects by three organophosphates, (A) chlorpyrifos, (B) fenitrothion, and (C) methyl parathion on the enzyme activity, measured with the CNT/ACHE/CHO immobilized screen-printed biosensor. Other conditions are as in (a). (from Ref. 8).

The relative inhibition of methyl parathion at the CNT modified, ACHE/CHO immobilized biosensor as a function of methyl parathion concentration was investigated using a pre-incubation method in which the biosensor was exposed to the incubation solution containing methyl parathion for 10 minutes before the change in enzyme activity was measured. Successive incubation measurements were performed with varied methyl parathion concentrations. The CNT/ACHE/CHO biosensor has good analytical characteristics for methyl parathion, including a broad dynamic linear range (up to 200 μM, $r^2 = 0.96$), high sensitivity (0.48% inhibition/μM), and low detection limit (LDL = 0.05 μM). These improved characteristics reflect the catalytic activity of CNTs that promotes the redox reaction of hydrogen peroxide produced during ACHE/CHO enzymatic reactions with their substrate, as well as the large surface area of CNT materials. The hand-held electrochemical detector (i.e., CHI1232 from CHI Instrument, Inc.) coupled with the disposable biosensor developed in this work will potentially facilitate the field screening of OP pesticides and nerve agents with fast speed, high efficiency, low cost, and small sample size needed.

5.3. Alcohol biosensors

The attractive low-potential detection of NADH, along with minimal surface fouling, makes CNTs extremely attractive for amperometric biosensors of ethanol through the incorporation of ADH/NAD^+ within the three-dimensional electrode matrix. Specifically, the reagentless biocomposite was prepared by mixing the desired amounts of the ADH enzyme and the NAD^+ cofactor with the CNT/Teflon composite to obtain the final composition of 28.5:65:1.5:5 wt% CNT/Teflon/ADH/NAD^+. The mixture was then packed firmly into the electrode cavity of a glass sleeve with a copper wire as the electrical contact. Figure 13 compares the performance at a low detection potential (i.e., +0.20 V) of the CNT/Teflon-based electrode (b) to that of graphite/Teflon-based electrode (a). Only the CNT/Teflon-based electrode (b) responds favorably to successive additions of 1 mM ethanol. The response is relatively fast (~60 s to reach steady state) and nonlinear. The greatly enhanced biosensing of ethanol at the CNT-based electrode suggests the accelerated oxidation of

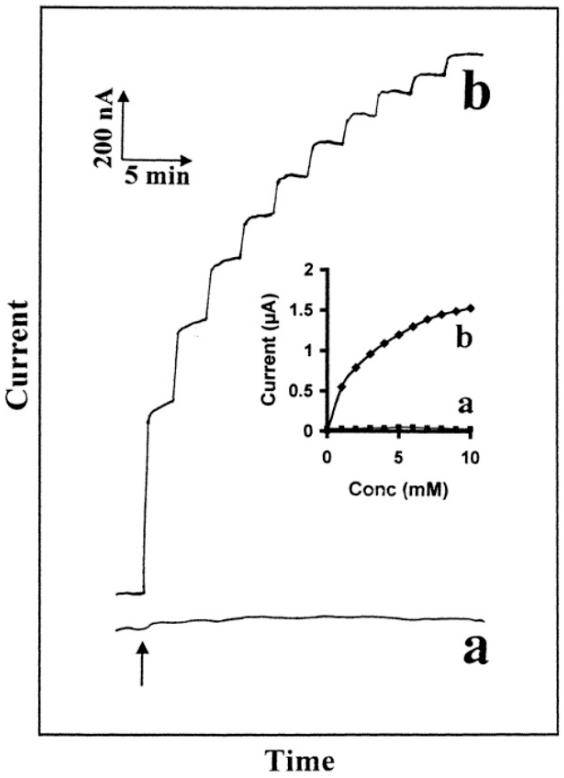


Figure 13. Current-time recordings for successive 1-mM additions of ethanol at (a) the graphite/Teflon/ADH/NAD+ and (b) the MWCNT/Teflon/ADH/NAD^+ (b). Operating potential, +0.2 V; electrode composition, 28.5:65:1.5:5 wt% carbon/Teflon/ADH/NAD^+; supporting electrolyte, phosphate buffer (0.05 M, pH 7.4); stirring rate, 400 rpm (from Ref. 6).

NADH at low-potential detection. In addition to being reagentless, the biosensor does not require a redox mediator (to shuttle the electrons from the NADH product to the surface), which is commonly used for such low-potential detection of ethanol.

6. SUMMARY AND PERSPECTIVES

The electrocatalytic properties of CNTs in promoting the redox reaction of hydrogen peroxide and NADH are being exploited in our work in developing biosensors based on oxidase and dehydrogenase enzymes, respectively. With our CNT-based biosensors, low-potential detections of alcohols, organophosphorous compounds, and glucose are made possible with many advantages over conventional devices. For many years, numerous researchers have been emphasized on the use of anti-interference layers or artificial electron mediators for improving the selectivity of amperometric biosensors. The capability of CNTs to reduce the overvoltage for the oxidation of hydrogen peroxide, NADH, and thiols allows the detection of these species at low potentials. At such low potentials, most interfering species in the test samples do not undergo oxidation, thus eliminating potential interference. Thanks to the CNTs' electrocatalytic activity, the artificial mediators to shuttle electrons between the enzymes and the electrodes are not required at the CNT-based biosensors, thereby eliminating the dependence on the mediators and enhancing the reproducibility. By eliminating interferences through the preferential detection of the target species at the CNT-based electrodes, the development of interference-free transducers will significantly simplify the design and fabrication of biosensors. CNTs also minimize the surface fouling of biosensors, thus imparting higher stability onto these devices.

While the CNT/Nafion-based biosensors use the electrocatalytic activity of CNTs and the permselectivity of Nafion to detect glucose with effective discrimination against most neutral and anionic redox constituents, the CNT/Teflon composite and CNT paste electrode allow the reagentless approach to fabricate biosensors with flexible co-immobilization of enzymes and cofactors for specific biosensing needs. Low-site-density aligned CNT nanoelectrode arrays enable the development of biosensors that consist of millions of individual electrodes, making them suitable for the highly selective detection of glucose in a variety of biological fluids (e.g., saliva, sweat, urine, and serum).

For future work, we will investigate the capability of CNTs to promote electron-transfer reactions of other biologically and environmentally important compounds. The biosensor fabrication technology demonstrated in this work holds a great future for developing routine, onsite amperometric biosensors based on both oxidase and dehydrogenase enzymes, such as those for cholesterol, alcohols, lactate, acetylcholine, choline, hypoxanthine, and xanthine. Although this article focuses on biosensors based on CNTs, other oriented conducting nanowires, e.g., oriented conducting polymer nanowires recently synthesized in our laboratory (87, 88), should also provide an alternative ideal platform for biosensing applications.

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Abbreviations: CNTs: carbon nanotubes, NEAs, nanoelectrode arrays, NADH: β-nicotinamide adenine dinucleotide, OP: organophosphorous, MWCNTs: multi-wall carbon nanotubes, ACHE; acetylcholinesterase, CHO; choline oxidase, CNTP: carbon nanotube paste, GOx: glucose oxidase.

Key Words: Carbon Nanotubes, Amperometric Biosensors, Hydrogen Peroxide, NADH, Oxidase, Dehydrogenase, Enzymes, Alcohol, Glucose, Nafion, Organophosphorous Compounds, Alcohol, Homocysteine, Thiols, Review

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