Enhanced stability of enzyme organophosphate hydrolase interfaced on the carbon nanotubes

Valber A. Pedrosa⁴, Sheetal Paliwal⁴, Shankar Balasubramanian⁴, Dhruti Nepal⁵, Virginia Davis⁶, James Wild⁷, Erlan Ramanculov⁸, Aleksandr Simonian⁴,∗

Abstract

In this paper we demonstrate that SWNTs and a covalent immobilization strategy enable very sensitive sensors with excellent long term stability. Organophosphorus hydrolase (OPH) functionalized single and multi-walled carbon nanotube (CNT) conjugates were exploited for direct amperometric detection of paraoxon, a model organophosphate. The catalytic hydrolysis of paraoxon produces equimoles of p-nitrophenol; oxidation was monitored amperometrically in real time under flow-injection (FIA) mode. OPH covalently immobilized on single-walled carbon nanotubes (SWNTs) demonstrated much higher activity than OPH conjugated to multi-walled carbon nanotubes (MWNTs). The dynamic concentration range for SWNT-OPH was 0.5–8.5 μmol L⁻¹ with a detection limit of 0.01 μmol L⁻¹ (S/N = 3). In addition to this high sensitivity, the immobilized OPH retained a significant degree of enzymatic activity, and displayed remarkable stability with only 25% signal loss over 7 months. These results suggest that covalent immobilization of OPH on CNTs can be used for specific immobilization with advantages of long term stability, high sensitivity, and simplicity.

1. Introduction

The proper allocation of proteins and enzymes onto interfaces plays a key role in the development of sensitive and stable biosensors. The main challenge is to design functional biocompatible materials and interfacial structures, which meet two significantly different criteria: (i) stable attachment of enzymes while maintaining their activity and function as close as possible to their native state and (ii) excellent optical or electrical signal propagation. Carbon nanotubes meet these requirements, their structure and chemistry enable both covalent and noncovalent attachment schemes and their optical and electrical properties are unparalleled [1,2].

There has been significant interest in CNT/OPH-based biosensors in the past few years, but a clear approaches nanotube type and immobilization scheme selection have yet to emerge. Recent research achievements have demonstrated carbon nanotubes’ (CNTs) capability to not only provide an excellent scaffold for proteins but to also significantly enhance the operational characteristics of electrochemical sensors. Performance characteristics of CNT-based biosensors are affected by a combination of the nanotube chemistry, immobilization method and detection platform. Carbon nanotubes can be classified into two general categories, single-walled carbon nanotubes (SWNTs) and multi-walled carbon nanotubes (MWNTs). Within each category, the thermal, electrical and mechanical properties of individual nanotubes can vary widely. Purified single-walled carbon nanotubes (SWNTs) are attractive for their higher surface area for protein interaction and low electrical percolation thresholds. Multi-walled carbon nanotubes (MWNTs) are attractive due to their easier dispersibility and lower cost. This is considered one of the key obstacles impeding more rapid development of not only CNTs-OPH sensors, but also CNTs biosensors in general [1]. SWNT synthesis typically results in a mixture of semiconducting and metallic nanotubes with diameters ranging from 0.6 to 1.8 nm and a broad distribution of lengths. In one of the earliest investigations of CNTs-OPH sensors, Deo et al. demonstrated enhanced amperometric biosensing of organophosphates using a simple film casting procedure. First, a dispersion containing Nafion and MWNTs was cast onto a glassy carbon electrode; OPH was then immobilized on the surface by simply casting a film from OPH and Nafion onto the nanotube surface [3]. More recently, Liu et al. demonstrated real time organophosphate detection by...
using AC dielectrophoresis to connect individual and small bundles of single-walled carbon nanotubes across electrode pairs [4]. An OPH suspension was then dropped onto the aligned nanotubes and allowed to dry. This method provided two key advantages. First, the use of aligned small bundles increased the interfacial contact volume between the OPH and CNTs. Second, in a subsequent step, the metallic SWNTs were selectively burned out to increase the sensitivity of the sensor to electrostatic variations in the environment. Laotthanachareon et al. mixed oxidized MWNTs with a suspension of crude enzyme based OPH cross linked enzyme crystals (CLEC-OPH), BSA and glutaraldehyde (GA). The mixture was then deposited on a glassy carbon electrode [5]. This resulted in similar sensitivity, but higher detection limits than sensors prepared using purified OPH. In parallel, several research groups have been pursuing acetylcholinesterase (AChE) based carbon nanotube sensors with promising results [6–8]. The primary limitation to these sensor types is their lack of selectivity; they detect total AChE inhibition from numerous possible sources including pesticides, heavy metals and detergents.

In this research, we provide key information on the effects of both nanotube type and immobilization scheme on sensor performance. We compare electrochemically based sensing of organophosphates using covalently immobilized OPH on both single-walled carbon nanotubes and multi-walled carbon nanotubes. Two functionalization approaches were used to enable covalent immobilization of OPH. In both cases, the immobilization was conducted in dispersions of oxidized nanotubes. Performing immobilization while the nanotubes are still dispersed increases the interfacial volume for OPH carbon nanotube interaction compared to immobilizing the OPH on deposited films. The first immobilization approach involved a direct coupling of ethylene-diamine with the carboxyl groups on oxidized nanotubes to introduce amino groups via amide formation (EDC-NHS). The second approach involved a direct coupling of the carboxyl group to the amine group (APTES) modified with glutaraldehyde (GA). Scanning electron microscopy (SEM), transmission electron microscopy (TEM), and Fourier transform infrared spectrometry (FT-IR) were used to characterize the CNT-OPH conjugates. Flow-injection amperometric (FIA) detection of paraaxon, a model organophosphate, was used to evaluate sensor sensitivity. The catalytic activity of the immobilized OPH on CNTs was determined spectrophotometrically by monitoring p-nitrophenol formation indicated by increased absorbance at 405 nm. The activity of OPH immobilized on SWNTs using EDC-NHS chemistry was higher than that achieved using MWNTs and with either EDC-NHS or APTES-GA. The SWNTs-OPH sensors provided not only high sensitivity but unprecedented stability with only 25% signal loss after 7 months.

2. Experimental

2.1. Material and equipment

MWNTs (purity 95%, length 1–5 μm, diameter 30 ±10 nm) prepared by a CVD process were purchased from Nanolabs. HiPCO SWNTs (Rice University) were purified by a thermal oxidation–acid extraction cycle. Thermogravimetric analysis using a TA Instruments Q500 under air showed >99.5% SWNT purity; both Transmission Electron Microscopy (Zeiss EM 10) and Raman spectroscopy (Renishaw InVia Reflex) also indicated very high purity. Organophosphorus hydrolase was purified according procedure described elsewhere [9]. Paraaxon was obtained from ChemService (West Chester, PA); N-hydroxysuccinimide (NHS) was obtained from Acros–Organics (through Fisher Scientific); 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), Nafion solution and 3-aminopropyltriethoxy silane (APTES), and analytical grade reagents and chemicals were from Sigma–Aldrich (St. Louis, MO) and used as received. Water used for preparation of aqueous dispersions was from a Millipore Direct-Q Water system (resistivity, 18 MΩ cm).

A Shimadzu (Thermo-Electron Corp., Waltham, MA) FT-IR spectrophotometer at room temperature was used to obtain spectra in the range of 500–4000 cm⁻¹ using potassium bromide (KBr) pellets. The spectrometer was equipped with a Globar source, a KBr beam splitter, and a broadband DTGS detector. Each spectra was acquired over 100 scans and the background was corrected using KBr pellets. Raman scattering studies were conducted on a Renishaw InVia Reflex Raman Microscope (Hoffman Estates, IL) equipped with a 50× objective, and excitation wavelengths of 514 nm (Arion laser) and 785 nm (Diode laser). Raman samples were prepared by spreading freeze dried powders on double-sided tape which was mounted on a glass slide. In order to firmly attach the samples in flat layer, a clean glass cover slip was pressed over it for 30 s.

Transmission electron microscopy (TEM) samples were prepared by placing a drop of CNTs dispersion on 200 mesh formvar coated nickel grids and air-dried overnight before imaging. Images were recorded by a transmission electron microscope operating at 60 kV (NAME LOCATION). Scanning electron microscopy (SEM) samples were prepared by dropping a small amount of CNT dispersion on a mica substrate and air-drying overnight. The samples were then coated with a thin layer of gold (~10 nm) and imaged using a JEOL (Peabody, MA) JSM 7000F field emission scanning electron microscope equipped with an energy dispersive X-ray analyzer.

Amperometric measurements were performed using a BAS CV-50W (Bioanalytical Systems, Mount Vernon, IN). All experiments were conducted in a three electrode system containing a platinum wire auxiliary electrode, a CNTs-modified GC electrode and a saturated Ag/AgCl reference electrode.

2.2. Oxidation of CNTs

A suspension of 5 mg of CNTs (MWNTs and SWNTs separately) was sonicated in a 1510 Branson Sonicator at 20 °C for 9 h in a mixture of 3:1 H2SO4 and 70% HNO3. The contents were allowed to cool and sufficient time was given to let the nanotubes sediment. The supernatant was discarded and the remaining dispersion was extensively washed with de-ionized water and centrifuged until the pH of the dispersion was near neutral. The final dispersion was then freeze dried and a dark solid product was obtained. These oxidized CNTs were used for the immobilization of OPH.

2.3. Protein immobilization on CNTs by EDC-NHS chemistry

Oxidized MWNTs or SWNTs (2 mg) were suspended in 5 mL of de-ionized water by horn sonication of the mixture for 1.5 h at 22 °C [10]. Then, 1 mL of 500 mM MES buffer solution (pH 6.1) and 2.3 mL of a 50 mg/mL NHS aqueous solution were added to each suspension. Under fast stirring, 1.2 mL of fresh EDC aqueous solution (10 mg/mL) was immediately added, and the mixture was continually stirred at room temperature for 0.5 h. The suspension was filtered through a 0.05 μm hydrophilic polycarbonate membrane and then rinsed thoroughly with the buffer solution to remove excess EDC, NHS and the by-product urea.

The activated CNTs were then re-dispersed in 9 mL of phosphate buffer solution (PBS) + 1 mL of 0.2 mg/mL OPH in 50 mM PBS solution (pH 7.4). After incubating the mixture on a platform shaker at 4 °C for 9.5 h, the nanotube suspension was centrifuged at 13,200 rpm and rinsed with buffer several times to remove any unbound protein. The enzyme/nanotube conjugate was finally suspended in 1 mL of 20 mM CHES buffer solution, pH 9.0. A part of the sample was freeze dried for characterization studies; freeze drying typically enables more ready redispersal than vacuum drying [11].
2.4. Protein immobilization on CNTs by APTES-GA chemistry

Oxidized MWNTs (2 mg) were suspended in 5 mL of 10% APTES by heating the dispersion to 60 °C for 6 h followed by stirring at room temperature for 3 h. APTES modified MWNTs were recovered by centrifugation and rinsed thoroughly with water. In the next step, 5 mL of 10% aqueous glutaraldehyde solution was added to the MWNTs-APTES suspension and the resulting dispersion was sonicated by horn for 0.5 h followed by stirring at room temperature for 2 h. The suspended nanotubes were rinsed extensively with copious amount of water until near neutral. Then, 0.2 mg/mL of OPH was added to the nanotube suspension and the mixture was gently stirred in a platform shaker overnight at 4 °C. Loosely bound OPH was removed by centrifugation followed by rinsing with 1 mL of 20 mM CHES, three times. The conjugate was finally suspended in 1 mL of CHES buffer solution (pH 7.4).

2.5. Measurement of the catalytic activity of immobilized enzyme

Catalytic activity of the immobilized OPH on CNTs was determined spectrophotometrically by monitoring p-nitrophenol formation due to enzyme hydrolysis of substrate paraoxon. The kinetic constants were determined by performing enzymatic assays with varying concentrations of substrate and constant enzyme concentrations. The kinetic assay was carried out at room temperature using 20 mM CHES (pH 9.0) buffer solution. The hydrolysis product, p-nitrophenol (ε = 17,000 M⁻¹ cm⁻¹) was monitored colorimetrically at 405 nm using Ultrospec 2100 Pro UV-Vis Spectrophotometer, Amersham Biosciences. The initial velocities were calculated and fit to the Michaelis–Menten equation (1):

\[ v_0 = \frac{V_{\text{max}} \cdot S}{K_M + (S/K_i)} \] (1)

2.6. Biosensor fabrication procedure

The glassy carbon (GC) electrode (BAS, Ø1.6 mm) was polished with 0.10 and 0.05 µm alumina slurry respectively and then ultrasonically cleaned in water for 15 min. Then 20 µL of the CNTs suspension (each batch) was cast on the cleaned electrode surface. After the solvent water was evaporated, 0.5% of Nafion solution was cast onto the modified electrode surface. The modified electrode was then dried to evaporate solvent and stored at refrigerated conditions until use. The buffer solution was 10 mM PBS (pH 7.4) which was deoxygenated with highly pure nitrogen for 5 min before any electrochemical measurements. All the electrochemical measurements were performed at room temperature.

3. Results and discussion

3.1. Characterization of protein immobilized onto CNTs

The covalent attachment of OPH on SWNTs and MWNTs was examined by FT-IR probing the vibrational changes during adsorption of proteins. Fig. 1A and B show the FT-IR spectrum of the oxidized MWNTs and SWNTs carboxylated and OPH-modified, respectively. It is well-known that oxidation of CNTs by the combination of H₂SO₄ and HNO₃ results in the formation of hydrophilic groups at defect sides and ends [12], –COOH, –C=O and –OH. All spectra for the oxidized CNTs suspension displayed a peak at 1708 cm⁻¹ corresponding to –COOH and 1631 cm⁻¹ which corresponds to –C=O. These results are consistent with previous work on CNT oxidation [13]; the exact peak positions are the result of the extent of oxidation [14]. In addition, the increased in intensity of 3434 cm⁻¹ peak clearly confirms introduction of more –OH groups after acid treatment [10]. The introduction of OPH results in the peaks at 3434 and 2857 cm⁻¹ are respectively attributed to symmetric and asymmetric –CH₂ stretching. After the covalent immobilization of OPH there are a strong band at 1210 cm⁻¹ arises which is primarily due to in-plane N–H bend of secondary amide. Fig. 1C highlights that several peaks of α-helix and amide II were observed for both SWNT-OPH and MWNT-OPH between 1600 and 1500 cm⁻¹. Similarly, peaks at 1621 and 1635 cm⁻¹ represent β-sheet conformation of the proteins while the peak at 1696 cm⁻¹ corresponds to the antiparallel β-sheet or pleated turn [10]. The bands at 1460 and 1390 cm⁻¹ are assigned to the CH₂ deformation and to vibrations of the amino acid side chains, respectively [15]. The infrared spectra are consis-
tent with the microscopy evidence of attachment of the OPH to the surface of SWNTs; since the presence of bound OPH would somewhat increase the fraction of sp\(^3\) hybridized carbons. It is also very likely that due to different active functional groups on the surface, OPH may irreversibly bind to the CNTs’ surface through cation–\(\pi\) interaction, hydrophobic interaction or \(\pi–\pi\) interactions and that this contributes to successful functionalization [16,17].

TEM further highlights the difference in attachment uniformity between SWNTs and MWNTs. While the original MWNTs sample (Fig. 2C and D) shows some dark spots from impurities,
after covalent attachment large amorphous regions are present, while other areas retain their original structure. In the case of OPH-SWNT, a network of SWNT bundles coated with an amorphous phase was observed, Fig. 2A and B. This thick protein coating hindered transmission of electrons from the SWNTs surface making it difficult to image the OPH-SWNT at the same high resolution as that achieved for OPH-MWNT. This network formation may be due to the strong interaction of OPH with the overall SWNT surface. It is important to note that solubility of OPH-SWNTs in aqueous phase was lower than for OPH-MWNT. We believe that OPH was unable to completely debundle the SWNTs, but interacted strongly with the outer surface of the SWNT bundles resulting in network formation and precipitation from the dispersion. It is also likely that network formation could have been facilitated by the SWNTs serving as a cross linker for proteins.

Imaging provides further insights into the attachment of OPH to the functionalized nanotubes. SEM images of the oxidized and OPH-functionalized SWNT and MWNT samples are shown in Fig. 3. After SWNT functionalization, the bundles show a thick coating. In the case of oxidized MWNTs (Fig. 3A and B), the structure appears to be comprised of individual MWNTs of diameter $\sim 30$ nm. This is consistent with the lower dispersibility of the oxidized SWNTs in water than the oxidized MWNTs. However, OPH deposition is less uniform than in the case of SWNTs; thick deposits of OPH are present in some locations while some of the structure appears to

---

**Table 1**

<table>
<thead>
<tr>
<th>Kinetic constants for catalytic activity of OPH.</th>
<th>SWNT (EDC-NHS)</th>
<th>MWNT (EDC-NHS)</th>
<th>MWNT (APTES-GA)</th>
<th>OPH in solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$, $\mu$mol/s</td>
<td>0.00013</td>
<td>0.00005</td>
<td>0.00003</td>
<td>0.00111</td>
</tr>
<tr>
<td>$K_M$, mM</td>
<td>0.07993</td>
<td>0.13019</td>
<td>0.10348</td>
<td>0.59960</td>
</tr>
</tbody>
</table>

---

**Fig. 4.** Activity of OPH immobilized on the CNTs.

---

**Fig. 5.** (A) Flow-injection on OPH-SWNTs electrode with sequential injection of paraoxon (A) 0.5, (B) 1.0, (C) 1.5, (D) 2.0, (E) 4.5 and (F) 8.0 $\mu$M at applied potential of 0.8 V vs Ag/AgCl and flow rate 1 mL s$^{-1}$ (inset: calibration plot from FI-analysis showing a linear response for paraoxon detection). (B) Flow-injection on OPH-MWNTs electrode with sequential injection of paraoxon in the same concentration above, (C) amperometric FIA responses using OPH-SWNT obtained for 18 injections of 4.5 $\mu$M paraoxon at applied potential of 0.8 V vs Ag/AgCl, and (D) The operational stability of OPH-SWNT (□) and OPH-MWNT (△) modified electrode at different day period.
have no coating at all. From the SEM Fig. 3C and D, it is clear that the initial oxidized dispersion consisted of small SWNTs bundles on the order of 10 nm in diameter.

3.2. Measurement of the catalytic activity of immobilized enzyme

The catalytic activities of OPH conjugated to the surface of CNTs were measured spectrometrically. Fig. 4 shows the immobilized OPH activity which is measured by monitoring the absorbance of p-nitrophenol formation at 405 nm. The data (see Table 1) is plotted in terms of initial velocity, the change in absorbance with time when the sample is exposed to the given concentration of paraaxon. Within MWNTs, OPH conjugation through EDC/NHS chemistry displayed slightly better activity compared to APTES-GA chemistry. The higher activity with EDC/NHS chemistry motivated studying the effects of this chemistry on SWNTs which are more expensive and more difficult to disperse but have better electrical properties. In particular, the promise of eventual supply of purely metallic or semiconducting SWNTs would further improve performance. The OPH conjugated to SWNTs demonstrated much higher activity compared to OPH conjugated to MWNTs. This is likely due to the more uniform deposition of OPH on the SWNTs and the formation of a SWNTs network.

3.3. Flow-injection amperometric (FIA) detection of paraaxon

A problem that often limits the practical utility of the biosensors prepared by physically adsorption is a loss of sensitivity and stability under operational conditions. This is often ascribed to enzyme denaturation and, especially under flow conditions, to enzyme leaching. Fig. 5A and B shows flow-injection calibration data for paraaxon over the concentration ranges 0.5–8.5 μM. The inset illustrates the proportional increase in current to paraaxon concentration, which yields highly linear calibration plots for both SWNT-OPH and MWNT-OPH immobilized process by the EDC-NHS process. The biosensor showed excellent sensitivity, $y = 0.001 \times 2.4 \mu A/μM$ for SWNTs and $y = 0.002 \times 0.17 \mu A/μM$ for MWNTs (calculated from the slopes of the linear part of the calibration curve). As can be observed from the slopes of analytical curves, the determination of paraaxon by SWNTs has a higher sensitivity when compared to MWNTs. The calculated value of detection limits was 0.01 μmol L$^{-1}$ for SWNTs and 6.4 μmol L$^{-1}$ for MWNTs. It is clearly seen that the limit of detection of the proposed method is in the same order of sensitive found in previous paper [18]. However some advantages of using this methodology include a clearly advantage of the stability and life-time of the biosensor.

The reproducibility and repeatability of the FIA systems was evaluated by using a 4.5 μM standard solution of paraaxon. The amperometric signals obtained are shown in Fig. 5C. The repeatability is expressed as the RSD (3.2%) value of the peak heights obtained for 18 injections. Fig. 5D displays paraaxon responses obtained by injecting 4.5 μM paraaxon, at different time intervals, under similar conditions. After 7 months of use, the SWNTs-OPH response decreased slightly, however the sensor retained around 75% of the initial signal. These observations suggest that covalent binding significantly improved the enzyme stability when compared with those results obtained with MWNTs-OPH. Also, based on literature information of stability using OPH physically adsorbed on surface showed a short life (less than 2 months) [19,20]. We found that the SWNTs-OPH immobilized by EDC-NHS appears to be the most useful since it offers the highest sensitivity resulting from a high current and a high signal/noise ratio; as well the stability of the system was examined over a 7-month period (using the same surfaces, with intermittent storage at 4°C).

4. Conclusions

The characterization techniques confirmed the successful immobilization of OPH on the CNTs surface. Covalent functionalization seemed to be a better choice for enzyme immobilization on SWNTs to achieve improved sensor characteristics and with higher stability. Our studies with OPH have shown that a significant degree of enzymatic activity is retained after immobilization. Sensors employing the SWNTs with covalent anchor, exhibit significantly enhanced solution–storage and operational stability. When stored for 7 months with intermittent storage at 4°C, such sensors exhibit only a 25% loss in signaling. In contrast, sensors fabricated using physically adsorbed has shown losses of 50% after 2 months of continual use. Other authors have been reported on the use of covalent immobilization showed similar improvements in sensor stability [21].

In the above-mentioned references, as well as in our work presented within, it is presumed that the covalent anchor enhances stability via the mechanism of enhancing the affinity of the enzyme for the carbon nanotube surface. The sensor exhibited high sensitivity, good reproducibility and was able to efficiently preserve the enzyme activity. This fact is a significant advantage over the other methods and encouraging for the development of biosensors using CNTs immobilized with covalent bond.

Acknowledgements

This research was supported by NSF under the Grant CTS-0330189 and by the USDA-CSREES under grant 2006-34394-16953. We also acknowledge the Richard E. Smalley Institute for Nanoscale Science and Technology at Rice University for the SWNTs. Additionally, this material was based on work which supported ALS by the National Science Foundation, while working at the Foundation. Any opinion, finding, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

References