



A novel organophosphorus hydrolase-based biosensor using mesoporous carbons and carbon black for the detection of organophosphate nerve agents

Joon Hwan Lee^a, Jae Yeon Park^b, Kyoungseon Min^b, Hyung Joon Cha^c, Suk Soon Choi^d, Young Je Yoo^{a,b,*}

^a Graduate Program in Bioengineering, Seoul National University, Seoul 151-744, Republic of Korea

^b School of Chemical and Biological Engineering, Seoul National University, Seoul 151-744, Republic of Korea

^c National Research Laboratory of Molecular Biotechnology, Department of Chemical Engineering, Pohang University of Science and Technology, Pohang 790-784, Republic of Korea

^d Department of Biological and Environmental Engineering, Semyung University 579 Sinwoul-dong, Jecheon-City, Chungbuk 390-711, Republic of Korea

ARTICLE INFO

Article history:

Received 23 September 2009

Accepted 9 October 2009

Available online 14 November 2009

Keywords:

Organophosphorus hydrolase

Organophosphate pesticides

Carbon nanotube

Biosensor

Mesoporous carbon

ABSTRACT

To detect organophosphate chemicals, which are used both as pesticides and as nerve agents, a novel biosensor based on organophosphorus hydrolase was developed. By using mesoporous carbon (MC) and carbon black (CB) as an anodic layer, the sensitivity of the sensor to p-nitrophenol (PNP), which is the product of the organophosphorus hydrolase reaction, was greatly improved. The MC/CB/glass carbon (GC) layer exhibited an enhanced amperometric response relative to a carbon nanotube (CNT)-modified electrode because it promoted electron transfer of enzymatically generated phenolic compounds (p-nitrophenol). The well-ordered nanopores, many edge-plane-like defective sites (EDSSs), and high surface area of the MC resulted in increased sensitivity, and allowed for nanomolar-range detection of the analyte paraoxon. Thus, MCs are suitable for use in real-time biosensors. Under the optimized experimental conditions, the biosensor had a detection limit of 0.12 μM (36 ppb) and a sensitivity of 198 nA/ μM for paraoxon.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Organophosphorus (OP) compounds are neurotoxic substances that are used as pesticides, insecticides and chemical warfare agents. In view of the major concerns regarding the toxicity of these compounds, early and easy detection of OP compounds is important for protecting water resources and food supplies, for defense against terrorist activity, and for monitoring detoxification processes (Mulchandani et al., 1999a,b). Currently, there are many laboratory-based techniques for detecting OP compounds. These include gas chromatography, high performance liquid chromatography, and immunoassays. However, these techniques are time-consuming and expensive. Moreover, they require a qualified and experienced staff, and cannot be used for continuous monitoring (Jaffrezic-Renault, 2001).

As alternatives to these methods, enzyme-based biosensors using cholinesterase and organophosphorus hydrolase (OPH) have been widely studied. Biosensors based on the inhibitory effects of OP compounds on cholinesterase activity have greater sensitivity and a lower detection limit than OPH biosensors, but the indirect sensing mechanism they use has drawbacks that include poor selectivity, an irreversible response, and a multi-step proto-

col (involving substrate addition and incubation) (Mulchandani et al., 2001a,b). It has become clear that OPH-based amperometric biosensors are well-suited to the demands of on-site environmental monitoring and rapid detection of chemical warfare agents (Mulchandani et al., 2001a,b).

Recently, amperometric biosensors based on the immobilization of OPH onto screen-printed carbon electrodes and/or glassy carbon (GC) electrodes have gained much attention. They are more direct, faster, simpler, and more selective than indirect acetylcholinesterase biosensors and other OPH-utilizing sensors that detect OP compounds by detecting protons or chromophoric products (Mulchandani et al., 1999a,b). Although OPH-based approaches have advantages such as selectivity for target compounds and fast response times, their sensitivity is lower and their detection limits are much higher than those of inhibition methods (Jaffrezic-Renault, 2001; Simon et al., 2006).

To overcome these problems, CNT has been utilized as an electrode material in amperometric biosensors. CNT has superior properties that include a larger surface area and more electrocatalytic activity (Wang, 2005). Deo et al. (2005) significantly enhanced the amperometric anodic signal from OP compounds using a CNT-modified electrode. It was also demonstrated that pyrolytic graphite electrodes with edge-plane-like defective sites (EDSSs) were able to replace CNT-modified glassy carbon in biosensing applications, yielding advantages in reactivity, cost and simplicity of preparation (Banks and Compton, 2005).

* Corresponding author. Tel.: +82 2 880 7411; fax: +82 2 887 1659.

E-mail address: yjyoo@snu.ac.kr (Y.J. Yoo).

As an alternative to the carbon materials mentioned above, Zhou et al. (2008a) substituted highly ordered mesoporous carbon for CNTs as an electrode material for the electrochemical sensing of NADH, and reported that the mesoporous carbon-modified electrode showed improved sensitivity and stability in the oxidative measurement of electroactive compounds. Highly ordered MC has received much attention owing to its extremely well-ordered pore structure, its high specific pore volume and its high specific surface area, all of which make it suitable for use in catalysis (Joo et al., 2001), energy storage (Lee et al., 2006), and sensing (Zhou et al., 2007), among other uses. Hence, MC has potential advantages for a variety of advanced applications. Despite this potential, there have been relatively few studies on the use of MC in enzyme-based biosensor applications (Zhou et al., 2008b).

To our knowledge, this is the first application of an MC-modified electrode for the detection of organophosphorus compounds. We present an advanced electrochemical biosensing platform for the detection of organophosphate nerve agents based on MC, and this platform can also be used in other electro-oxidation-based sensors and biosensors.

2. Experimental

2.1. Materials

Single-wall carbon nanotubes (swCNTs, purity >90%, 1–10 nm diameter, arc-discharge process) were purchased from Il-jin-nanotech. Co. Ltd. (Korea). Carbon black (Ketjenblack, Carbon® ECP-600JD, surface area; 1270 m²/g, average particle size; 34 nm) was purchased from AkzoNobel corporation. Stock solutions of paraoxon (10 mM, Sulpelco) were prepared in deionized water and diluted as required in potassium phosphate buffer (0.05 M; pH 7.4). Potassium phosphate buffer was used as the cell electrolyte. Nafion® (5 wt.% in lower aliphatic alcohols) was purchased from Aldrich. All other chemicals were of analytical reagent grade. MC was kindly supplied by Professor Jongheop Lee at Seoul National University.

2.2. Purification of organophosphorus hydrolase (OPH)

Recombinant *Escherichia coli* expressing OPH with a 6×-his tag was supplied by Professor Hyung Joon Cha of Pohang University of Science and Technology. Organophosphorus hydrolase was obtained according to the method described by Kang et al. (2006) and purified with Ni-NTA resin (Qiagen). The volumetric activity of the purified enzyme was 86 unit/mL. Enzyme activity was determined by measuring the absorbance due to p-nitrophenol (PNP) released from paraoxon at 400 nm in a UV/vis spectrophotometer (UVIKON 930, KONTRON Instrument).

2.3. Electro-analytical measurements

All electroanalysis was performed with a three-electrode system (working electrode, Ag/AgCl reference electrode, Pt counter electrode). All electroanalytical measurements were performed using a potentiostat/galvanostat (Autolab PGSTAT 302N, Eco Chemie), which was controlled by GPES software (Eco Chemie). The cyclic voltammetry and chronoamperometry experiments were carried out in potassium phosphate buffer of 5 mL working volume in a 20 mL cell at room temperature. In the cyclic voltammetry experiments, the scan rate was 50 mV/sec and the scan range was –0.2 V to +1.2 V. The concentrations of paraoxon and PNP (the enzymatic product of paraoxon), were determined by chronoamperometry. The applied potential was set at +0.9 V based on the cyclovoltammetric analysis. After the background signal reached a steady state, aliquots of samples and standards were added to

the cell as it was being stirred at 300 rpm, and the current was measured.

2.4. Electrode preparation and modification

A glass carbon electrode (GCE, 3 mm in diameter with a 9-mm surface diameter) was polished with 0.05 μm alumina powder and rinsed thoroughly with acetone (50%), then washed successively with double-distilled water in an ultrasonic bath. The total time of sonication did not exceed 2 min during any of the washing steps to protect the electrode surface. A low-contour porcelain mortar and pestle (PM-9, Vision Lab.) was used to turn the MC substances into a uniform fine powder, and the powder that passed through the testing sieve (aperture; 45/325 μm) was gathered. The MC and/or CNT layer on the GCE was prepared by first sonicating the carbon solution (1 mL buffer and 10 mg MC and/or CNT; or 1 mL buffer and 5 mg CNT) for 30 min, and then casting 10 μL of it onto the GCE surface (Fig. S1 in Supplementary data). The amount of CNT was determined as an optimum by an independent experiment (data not shown). CB was added to the solution in the amounts indicated in the text. For biosensing experiments, the enzyme was immobilized by casting a 10 μL solution of OPH (86 IU/mL) in Nafion® (0.5% in ethanol) onto the CNT or MC-modified GC electrode (Fig. S1 in Supplementary data). The enzyme-modified electrode was dried at room temperature and was kept in a refrigerator at 4 °C before it was used for further study.

3. Results and discussion

3.1. Determination of working potential of the MCs-modified electrode for PNP detection

Hydrolysis of certain OP nerve agents (such as paraoxon and parathion) at the OPH layer (Fig. S1 layer A in Supplementary data) generates p-nitrophenol, which is electroactive. The OPH-based amperometric biosensing of OP nerve agents relies on the anodic detection of this enzymatically liberated PNP by the electrode (Mulchandani et al., 1999a,b). The oxidation current (which is the sensor signal) measured at a fixed-potential anode should be directly proportional to the concentration of PNP formed. It is important to find the proper working potential to obtain the strongest signal and to minimize interference from direct oxidation of the substrate. Fig. S2 (Supplementary data) depicts hydrodynamic voltammograms for 10 μM PNP and paraoxon at the GC electrode modified with MC. In both cases, the responses start above +0.8 V. The MC-coated electrode exposed to PNP displays a steeper increase in the current up to +0.9 V, with a slower increase thereafter. On the other hand, a substantially weaker response (18 nA for 10 μM paraoxon and 125 nA for 10 μM PNP) is observed in the presence of paraoxon, demonstrating good selectivity for PNP. Thus the MC-based amperometric sensor responds specifically to the enzymatic product. All of the subsequent amperometric detections were carried out at a potential of +0.9 V, which offered the highest signal-to-noise ratio and the maximum signal. This working potential is similar to those reported in previous studies (Chough et al., 2002; Deo et al., 2005; Mulchandani et al., 1999a,b, 2001a,b).

3.2. Optimization of MC-modified electrode for anodic detection of PNP

Since the electrochemical reactivity of the modified electrode is strongly dependent upon the levels of surface modifiers such as MC, CNT, and Nafion®, and enzyme (OPH) (Deo et al., 2005), we first examined anodic detection of PNP with different levels of MC loaded onto the GCE.

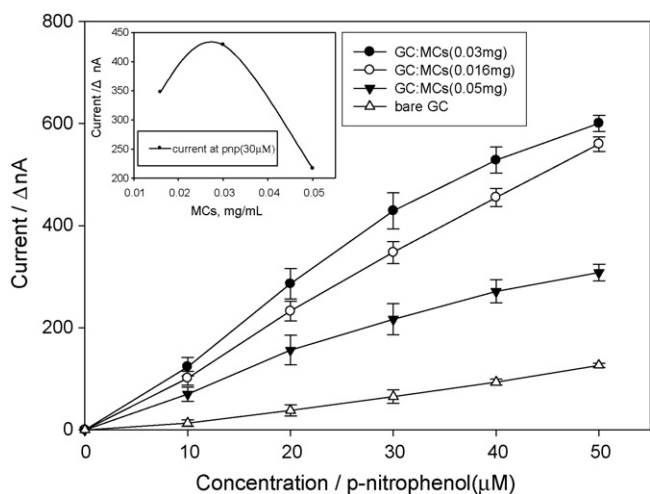


Fig. 1. Calibration plots resulting from amperometric measurement of p-nitrophenol, with levels increasing in 10- μ M increments. The left inset shows the effect of surface loading of CMK on the response to 30 μ M p-nitrophenol. Operating potential: +0.9 V (vs. Ag/AgCl); electrolyte: phosphate buffer (0.05 M, pH 7.4); stirring rate: 300 rpm.

Fig. 1 shows that there was an optimum level of MC within the limited working surface of the GCE. The response to PNP increases slowly with increased MC surface loading up to 0.016 mg, then starts to decrease above 0.03 mg. Since the glass electrode has an exposed conducting area only 3 mm in diameter, the casting of the conducting material onto the GCE could enlarge this reactive surface beyond the limit required to maintain low resistance. Other loading values were determined to be 0.5% Nafion®, 0.286 U OPH and 0.05 mg CNT.

By incorporating CB into the electrode, the sensitivity of the MC-modified electrode was heightened. Fig. 2 compares the p-nitrophenol calibration plots (over the 10–70 μ M range) of different electrodes. When using larger catalysts (such as metal oxides and rod-type mesoporous carbons) within the limited working surface of the electrode, carbon additives can be used as electron-carriers to enhance conductivity (Jang et al., 2003). Although the CB-enhanced surface gave elevated anode signals due to the large active surface area of CB (Fujiwara et al., 2006), there was also an optimum value of CB-loading within the limited working surface of the GCE. The response to p-nitrophenol was maximal when the

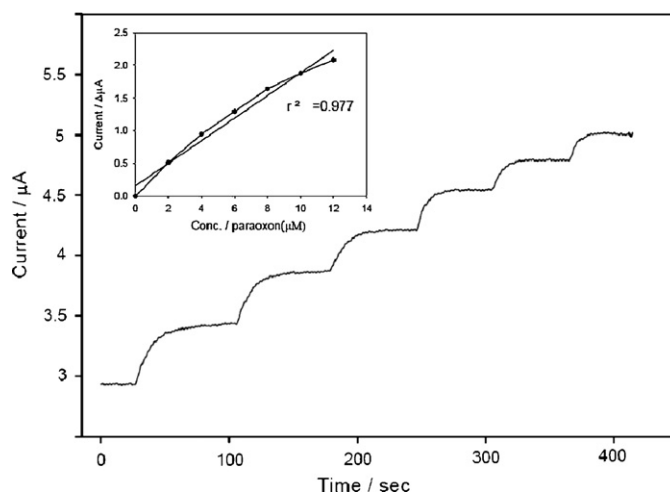


Fig. 3. Current–time curves for the OPH/MC (0.03 mg)/CB (0.013 mg)-modified GC electrode exposed to paraoxon, with levels increasing in 2- μ M increments. Also shown (left inset): the corresponding calibration plot. Other conditions are the same as in Fig. 1.

CB surface loading was 0.013 mg, and started to decrease above 0.026 mg. However, CB alone did not yield a stronger signal relative to the electrode with the mixture of MC and CB. We assumed that the small diameter of the CB particles (34 nm) allowed them to fit into spaces in the MC and thereby decrease the internal resistance and increase the conductivity of the electrode surface.

Fig. 2 summarizes the amperometric measurement of p-nitrophenol from all of the modified electrodes. The slope of the current in Fig. 2 indicates the sensitivity (nA/ μ M) of the electrode. Under optimum conditions, the MC/CB-coated surface offers dramatically enhanced sensitivity over the others. This is consistent with the idea that the higher level of EDSs in the MC are primarily responsible for the enhanced catalytic activity of the MC/GE versus the CNT/GE (Banks and Compton, 2005; Banks et al., 2004, 2005; Yu et al., 2009). The surface composed of both MC and CB offered a further dramatic enhancement of sensitivity toward p-nitrophenol because MC not only has more surface area while its conductivity was enhanced by CB, it also contains more EDSs than do CNTs. MC has a higher BET surface area (1205 m²/g) and a larger pore volume (1.176 cm³/g) than do CNTs (217 m²/g, 0.839 cm³/g). Furthermore, transmission electron microscopic (TEM) images of the MC showed that hexagonal arrays of carbon rods, 7 nm in diameter and 3 nm apart, possessed a delicate and ordered surface structure with a pore size of $\sim$ 4.2 nm (Joo et al., 2001). The Raman spectrum of MC indicates that without purification or end-opening (which are usually required in CNT applications), MC contains more EDSs than do CNTs (Zhou et al., 2008a; Ferrari and Robertson, 2000; Jia et al., 2007). The EDS level is the main factor affecting oxidation signal levels at the working surface of an anode electrode, and was thought to be the primary reason for the increased sensitivity of the MC/CB-coated electrode.

3.3. Characterization of the OPH/MC/CB-modified amperometric biosensor

With the goal of creating an improved biosensor, a bi-layer approach, with an OPH layer on top of the MC layer, was used. A trial in which OPH and MC were pre-mixed to form a single layer gave a much weaker signal. Fig. 3 displays the current-time amperometric signals obtained with the OPH/MC/CB-modified GC exposed to paraoxon at micro-molar concentrations. The resulting calibration plot (shown in the left inset of Fig. 3) displays high linearity for paraoxon (correlation coefficient 0.977) up to 8 μ M,

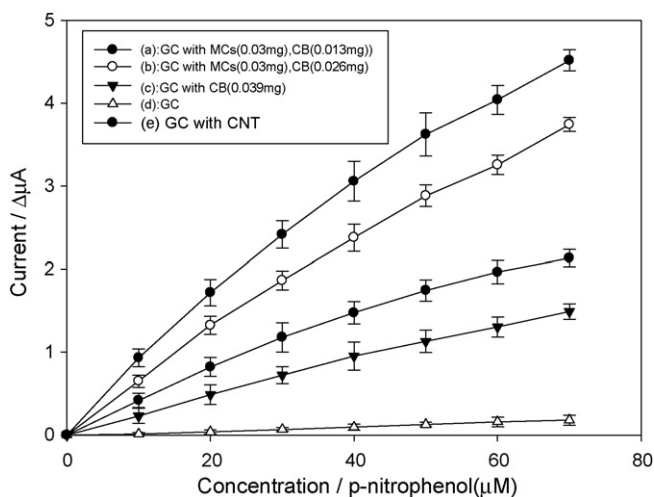


Fig. 2. Calibration plots resulting from amperometric measurement of p-nitrophenol by various MC- and CB-modified electrodes; p-nitrophenol levels increase in 10- μ M increments. The conditions are the same as in Fig. 1.

Table 1
Performance of various sensors.

Type	Substrate	Sensitivity (nA/ μ M)	Linear range (μ M)	Detection limit (μ M) (S/N = 3)	R^2	Response time	Ref.
Thick-film	Methyl parathion Paraoxon	2.83 1.67	5–40	0.07 0.09	0.99<	<10 s	Mulchandani et al., 1999a,b
Carbon paste + Nylon net	Paraoxon	12	0.02–0.18	0.02	0.99<	–	Chough et al., 2002
Amperometric flow injection	Paraoxon	2.29	1–10	0.1	0.99<	<40 s	Wang et al., 2003b
CNT-modified electrode with amperometric flow injection	Paraoxon	25	0.25–4	0.15	0.95<	<10 s	Deo et al., 2005
MC and CB modified electrode	Paraoxon	198	0.2–8	0.12	0.985<	<10 s	This work

with a slight curvature thereafter. Also, the reproducible signal curves demonstrated that the OPH/MC/CB-modified GC offered a favorable response time, with ~ 10 s required to attain steady-state currents. This response is significantly faster than that of inhibition (AChE)-based amperometric OP biosensors. (Jaffrezic-Renault, 2001; Simon et al., 2006).

The long-term stability of the current–time curves indicated that the MC surface modifier was useful for addressing surface-fouling problems associated with the oxidation of phenolic compounds (Wang et al., 2003a). Compton et al. reported that the high density of EDSs on carbon materials may improve the resistance of carbon-based electrodes to fouling (Banks and Compton, 2005).

Fig. 4 compares all of the modified OPH-based biosensors at the nanomolar level. As shown, neither the CNT-modified GC nor the bare GC responded readily to paraoxon, unlike the OPH/MC/CB-modified GC, which showed a faster response and a linear signal. The linearity of the calibration curves of the OPH/MC/CB makes for accurate paraoxon measurements at the nanomolar level. Thus the OPH/MC/CB layer constructed in this work has properties that will allow the biosensing of OPs to become routine.

Furthermore, the detection limit of the OPH/MC/CB-modified GC for paraoxon (0.12 μ M, estimated from the signal-to-noise (S/N = 3) characteristics was extremely low (Fig. 4. inset). This indicated that the mesoporous materials are excellent adsorbents for phenolic compounds, owing to their abundant porous structure and high specific surface area (Nevskaia et al., 2004). In particular, the sensitivity difference between the MC- and CNT- modified electrodes at the lower concentrations of paraoxon makes MC/CB very attractive for the detection of the most highly toxic nerve agents. Table 1

shows this improved sensitivity along with other characteristics that were described in previous reports.

Furthermore, the detection limit of OPH-based biosensors could be further improved by genetic modification of OPH to either lower its K_M , increase its biomolecular rate constant or improve its k_{cat}/K_M for target OPs such as paraoxon (Mulchandani et al., 1999a,b).

4. Conclusion

In this work, we have demonstrated that mesoporous carbon can be combined with carbon black to create a platform for the amperometric biosensing of OP compounds that is superior to CNT-based platforms. Furthermore, we found that an MC/CB-modified electrode had greater sensitivity to phenolic compounds (p-nitrophenol) and was highly stable. The resulting OPH/MC/CB-based electrode offered sensitive and stable detection of paraoxon, and was therefore suitable for biosensing applications. This electrode exhibited the highest sensitivity to OP compounds and the similar level detection limit ever reported.

Acknowledgements

The authors would like to thank Professor Jongheop Yi at School of Chemical and Biological Engineering, Seoul National University for kindly providing the mesoporous carbon.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.10.013.

References

- Banks, C.E., Moore, R.R., Davies, T.J., Compton, R.G., 2004. Chem. Commun. (Camb.), 1804–1805.
- Banks, C.E., Compton, R.G., 2005. Analyst 130, 1232–1239.
- Banks, C.E., Davies, T.J., Wildgoose, G.G., Compton, R.G., 2005. Chem. Commun. (Camb.), 829–841.
- Chough, S.H., Mulchandani, A., Mulchandani, P., Chen, W., Wang, J., Rogers, K.R., 2002. Electroanalysis 14, 273–276.
- Deo, R.P., Wang, J., Block, I., Mulchandani, A., Joshi, K.A., Trojanowicz, M., Scholz, F., Chen, W., Lin, Y., 2005. Anal. Chim. Acta 530, 185–189.
- Ferrari, A.C., Robertson, J., 2000. Phys. Rev. B Condens. Matter Mater. Phys. 61, 14 095–14, 106.
- Fujiwara, N., Yamazaki, S., Siroma, Z., Ioroi, T., Yasuda, K., 2006. Electrochem. Commun. 8, 720–724.
- Jaffrezic-Renault, N., 2001. Sensors Basel Sensors 1, 60–74.
- Jang, J.H., Han, S., Hyeon, T., Oh, S.M., 2003. Power Sources 123, 79–85.
- Jia, N., Wang, Z., Yang, G., Shen, H., Zhu, L., 2007. Electrochem. Commun. 9, 233–238.
- Joo, S.H., Choi, S.J., Oh, I., Kwak, J., Liu, Z., Terasaki, O., Ryoo, R., 2001. Nature 412, 169–172.
- Kang, D.G., Choi, S.S., Cha, H.J., 2006. Biotechnol. Prog. 22, 406–410.
- Lee, J., Kim, J., Hyeon, T., 2006. Adv. Mater. Deerfield 18, 2073–2094.
- Mulchandani, A., Mulchandani, P., Chen, W., 1999a. Anal. Chem. 71, 2246–2249.
- Mulchandani, A., Chen, W., Mulchandani, P., Wang, J., Rogers, K.R., 2001a. Biosens. Bioelectron. 16, 225–230.
- Mulchandani, P., Mulchandani, A., Kaneva, I., Chen, W., 1999b. Biosens. Bioelectron. 14, 77–85.
- Mulchandani, P., Chen, W., Mulchandani, A., 2001b. Environ. Sci. Technol. 25, 2562–2565.

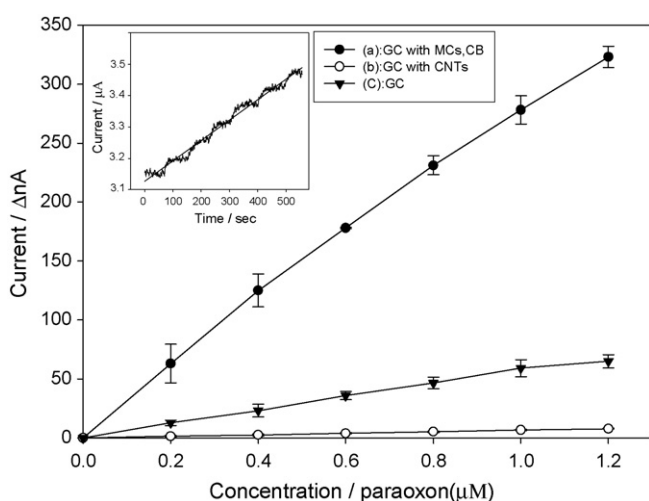


Fig. 4. Calibration curves demonstrating elevated signals from the: (a) OPH/MC (0.03 mg)/CB (0.013 mg)/GC; (b) OPH/CNTs (0.005 mg)/GC; and (c) OPH/GC with successive addition of 0.2 μ M paraoxon. Left inset: the current–time curves for the OPH/MC (0.03 mg)/CB (0.013 mg)/GC with successive addition of 0.2 μ M paraoxon.

- Nevskaia, D.M., Lopez, E.C., Munoz, V., Ruiz, A.G., 2004. *Environ. Sci. Technol.* 38, 5786–5796.
- Simon, B.P., Campas, M., Andreescu, S., Marty, J.L., 2006. *Sens. Basel Sens.* 6, 1161–1186.
- Wang, J., Deo, R.P., Musameh, M., 2003a. *Electroanalysis* 15, 1830–1834.
- Wang, J., Krause, R., Block, K., Musameh, M., Mulchandani, A., Schöning, M.J., 2003b. *Biosens. Bioelectron.* 18, 255–260.
- Wang, J., 2005. *Electroanalysis* 17, 7–14.
- Yu, J., Du, W., Zhao, F., Zeng, B., 2009. *Electrochim. Acta* 54, 984–988.
- Zhou, M., Ding, J., Guo, L.P., Shang, Q.K., 2007. *Anal. Chem.* 79, 5328–5335.
- Zhou, M., Shang, L., Li, B., Huang, L., Dong, S., 2008a. *Electrochem. Commun.* 10, 859–863.
- Zhou, M., Shang, L., Li, B., Huang, L., Dong, S., 2008b. *Biosens. Bioelectron.* 24, 442–447.