Full Paper

A Disposable Biosensor for Organophosphorus Nerve Agents Based on Carbon Nanotubes Modified Thick Film Strip Electrode

Kanchan A. Joshi,[a] *Jason Tang*,[a] *Robert Haddon*,[a,b] *Joseph Wang*,[c] *Wilfred Chen*,[a] *Ashok Mulchandani*[a]*

[a] Department of Chemical and Environmental Engineering, University of California, Riverside, CA 92521, USA
[b] Department of Chemistry, University of California, Riverside, CA 92521, USA
*e-mail: adani@engr.ucr.edu
[c] Department of Chemistry and Biochemistry, New Mexico State University, Las Cruces, NM 88003, USA

Received: May 4. 2004
Final version: June 23, 2004

Abstract
A disposable biosensor based on acetylcholinesterase-functionalized acid purified multi-wall carbon nanotubes (CNTs) modified thick film strip electrode for organophosphorus (OP) insecticides was developed. The degree of inhibition of the enzyme acteylcholinesterase (AChE) by OP compounds was determined by measuring the electrooxidation current of the thiocholine generated by the AChE catalyzed hydrolysis of acteylthiocholine (ATCh). The large surface area and electro-catalytic activity of carbon nanotubes lowered the overpotential for thiocholine oxidation to 200 mV (vs. Ag/AgCl) without the use of mediating redox species and enzyme immobilization by physical adsorption. The biosensor detected as low as 0.5 nM (0.145 ppb) of the model organophosphate nerve agent paraoxon with good precision, electrode to electrode reproducibility and stability. Analysis of real water sample using the sensor demonstrated the feasibility of the application of the sensor for on site monitoring of OP compounds.

Keywords: Carbon nanotubes, Thick film strip electrodes, Biosensor, Organophosphorus insecticides, Acetylcholinesterase, Paraoxons

1. Introduction

Organophosphorus compounds (OPs) are widely used as pesticides, insecticides and chemical warfare agents [1–3]. These compounds affect the nervous system by inhibiting acetylcholinesterase (AChE) function of regulating the neurotransmitter acetylcholine. As a result of acute toxicity of these compounds, it is necessary to monitor them in food and water.

Biosensor technology is well suited for field monitoring OP compounds. Biosensors based on modulation of the AChE activity have been reported [4, 5]. Because of the high sensitivity, amperometric transducers have been the transduction principle of choice in many of these biosensors [6]. These biosensors have used either AChE alone or combined with choline oxidase. The AChE inhibition in the single and bienzyme system are monitored through the electrochemical oxidation of thiocholine or *p*-aminophenol, and hydrogen peroxide, respectively. The approach based on the electrochemical oxidation of thiocholine produced due to the acetylcholinesterase (AChE) catalyzed hydrolysis of thiocholine ester, acetylthiocholine, is largely preferred due to the inherent simplicity and robustness of a single enzyme system [7].

Because the inhibition of AChE by OP compounds is irreversible, a cheap disposable biosensor which will eliminate the enzyme reactivation maneuver is highly desirable [6]. Single-use biosensors, based on advanced microfabrication technology, are particularly attractive for field deployment due to their extremely low cost and compatibility with hand-held analyzers. Screen-printed electrodes (SPEs), in particular, can combine ease of use and portability with simple, inexpensive fabrication techniques. The thick-film, screen-printing, technology is capable of mass producing extremely inexpensive, and yet highly reproducible biosensor strips.

Carbon nanotubes are an important class of material due to their unique electronic, metallic and structural characteristics [8]. The possibility of the promotion of electron transfer reactions at a lower overpotential due to their structure dependent metallic character and their high surface area provide ground for unique biochemical sensing systems [9]. The electrochemistry of CNT modified electrodes has been studied and it has been shown that they have stable electrochemical behavior and catalyze the electrochemical reaction of dopamine, epinephrine, ascorbic acid [10], NADH [11], cysteine, glutathione [12], and hydrogen peroxide [13].

The high surface area and the acidic sites created due to purification of CNTs with oxidizing acids have been exploited for the adsorption and entrapment of biological molecules [14–16] for applications as biosensors [17–19]. The fabrication and evaluation of carbon-nanotube (CNT)-derived screen-printed disposable electrochemical sensors based on a CNT ink has been reported [20]. The cost of CNT might be an important factor in such an application. A disposable OP biosensor based on AChE and CHO enzymes covalently attached to multiwalled CNT-modified

 © 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim DOI: 10.1002/elan.200403118

screen-printed electrode was recently reported [17]. While elegant, the use of bienzyme and the tedious and time consuming covalent immobilization of the biological sensing elements are the limitations of the biosensor reported.

The present work demonstrates the use of carbon nanotubes (CNTs) for the development of a low cost disposable biosensor for the sensitive detection of OP pesticides. The dual role of CNT, electrocatalytic activity toward thiocholine and immobilization matrix for the enzyme, is demonstrated. This led to the development of a mediator free, simple and robust single enzyme biosensor for the sensitive detection of OP compounds operating at a low overpotential.

2. Experimental

2.1. Materials

Acteylcholinesterase (Type III from Electric Eel, 1070 units/mg protein), acetylthiocholine iodide, diethyl *p*-nitro phenyl phosphate (approximately 90%, paraoxon) were from Sigma (St. Louis, MO). *N*, *N* dimethylformamide (DMF) was from Acros Organics, (NJ). The thiocholine (TCh) solution was prepared by the enzymatic reaction of AChE and acetylthiocholine iodide solution for 30 min in phosphate buffer (pH 7.4). All other chemicals and reagents were of analytical grade and were prepared with double distilled, deionized water.

Acid purified multiwall CNTs (MWCNTs) and SPEs (2 mm × 4 mm working area) were prepared according to the methods described elsewhere [21, 22, 23].

2.2. Apparatus and Procedure

Amperometric measurements were performed using a voltammetric analyzer (Bioanalytical Systems, Model LC-4C) coupled to a chart recorder (Model BD 112, Kipp and Zonen, Holland). All experiments were conducted in a three electrode electrochemical cell with a working volume of 2 mL (50 mM phosphate buffer, pH 7.4 containing 0.1 M KCl) with the SPE modified with CNTs as the working electrode, Ag/AgCl reference electrode (BAS, MF 2063) and platinum wire auxiliary electrode. The working electrode was operated at desired potential and the transient currents were allowed to decay to a steady-state value. A magnetic stirrer and a stirring bar provided the convective transport.

2.3. Modification of the Working Electrodes with CNTs

Two mg acid purified MWCNTs were suspended in 1 mL *N*,*N* dimethylformamide (DMF) with the aid of ultrasonic agitation to obtain a black suspension. A film of MWCNT was cast on the surface of the screen-printed electrode by dropping 15 μL of this solution on the electrode surface. The electrode was then kept in an oven at 80 °C for 30 minutes under vacuum to evaporate the solvent.

2.4. Immobilization of Acteylcholinesterase

Ten μL AChE solution (0.132 U) was dropped on the MWCNT modified electrode surface and allowed to dry at room temperature under a current of air. The electrode was then rinsed twice with phosphate buffer (pH 7.4) to remove the loosely adsorbed enzyme molecules and bonded MWCNTs. The electrodes were stored at 4 °C when not in use.

2.5. Determination of Relative Inhibition

To determine relative inhibition, the AChE-functionalized MWCNT-SPE electrode was immersed in a cell containing 2 mL of pH 7.4, 50 mM phosphate buffer with 0.1 M KCl under constant stirring. The potential was poised at 200 mV (vs. Ag/AgCl). After current stabilization, acetylthiocholine iodide substrate was added to a final concentration of 1.75 mM. This value of current corresponded to I_0, the current before inhibition. A known concentration of paraoxon was then dropped on to the electrode and incubated for 30 minutes. After incubation, the electrode was washed with the buffer and the response was measured again as described above, this second value corresponded to I_i, the current after inhibition. The relative inhibition (*RI*, %) was determined according to the following formula: $RI\,(\%) = [(I_0 - I_i)/I_0]\,100$ and then related to the inhibitor concentration. The I_0 and I_i values were an average of three readings.

3. Results and Discussion

OP compounds are known to inhibit the activity of AChE (Eq. 1). In the present study, the iodide salt of acetylthiocholine (ATCh) was used as the substrate to determine the modulation of AChE activity by OP nerve agents (eq. 1) by measuring the oxidation current (Eq. 3) before and after incubating with OP.

$$\mathrm{AChE} \xrightarrow{\text{Inhibitor}} \mathrm{AChE_{(inactive)}} \quad (1)$$

$$\mathrm{ATChI^-;} \xrightarrow{\text{AChE}} \mathrm{TCh + HAC + I^-} \quad (2)$$

$$\mathrm{2TCh \rightarrow TCh_{(ox)} + 2\ e^- + 2\ H^+} \quad (3)$$

3.1. Electrocatalytic Oxidation of Thiocholine at the MWCNT Modified SPE

A single use biosensor has multiple advantages because it prevents contamination across samples, prevents the denaturation of enzyme with use, produces devices with a constant sensitivity and a high reproducibility. Additionally, in the present case it eliminates the need for the enzyme reactivation due to irreversible inhibition by OPs.

Thiol containing compounds are known to undergo electrochemical oxidation processes at solid electrodes,

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

but the oxidations occur at relatively high potentials. To overcome the problem of thiocholine oxidation at a higher potential on conventional electrodes [24–26], mediators such as cobalt (II) phthalocyanine (CoPC) [27–29], Prussian blue [30] and tetracyanoquinodimethane [31, 32], substrate oxidation, pulsed electrochemical detection, use of mercury electrodes, derivatization and others have been used [33].

To determine the optimum potential for the sensor operation, hydrodynamic voltammetric studies were carried out with 2 mM thiocholine at an unmodified SPE and MWCNT-SPE. The working electrode was operated at a desired potential and the transient currents were allowed to decay to a steady-state value. The response for the buffer solution not containing thiocholine (blank) was subtracted from the response for thiocholine. As shown in Figure 1, a significant response was obtained starting from 0.1 V, the response increased substantially after 0.4 V and leveled off around 0.6 V at the MWCNT-SPE. In comparison, the response was significantly lower for the entire range studied at the unmodified electrode. This large increase in current at the MWCNT-SPE can be attributed to the catalytic behavior of CNT toward thiocholine. The electrocatalytic oxidation of thiocholine, which also contains a thiol moiety, by the use of a MWCNT modified electrode, is in agreement with recently reported use of carbon nanotubes for the detection of thiols such as cysteine and glutathione [12]. Since the operation of a sensor at a low potential is analytically more desirable to reduce the electrochemical interferences for high selectivity, a low operating potential of 200 mV was chosen to demonstrate the applicability of this sensor for the sensitive detection of thiocholine.

3.2. Response of the Screen-Printed Electrode Modified with MWCNT to the Substrate

Figure 2 shows the calibration plot for the sensor obtained by successive additions of the substrate. The response of the sensor was linear from 5 μM – 430 μM ($r^2 = 0.999$) with a sensitivity of 6.018 mA/M. In comparison, the response of AChE immobilized directly on the screen-printed electrode was only 5% of that for the MWCNT-SPE demonstrating the contribution of CNTs in improving the sensitivity.

The apparent Michaelis-Menten constant, K_m^{app} for ATCh can be determined form the electrochemical Eadie-Hofstee form of the Michaelis-Menten equation [34]

$$i = i_{max} - K_m^{app}\ (i/C\ )$$

where i is the steady-state current, i_{max} is the maximum current measured under conditions of enzyme saturation and C is the ATCh concentration. From the slope of the plot of current vs. (current / concentration of ATCh), the K_m^{app} for AChE was determined to be 0.66 mM (Figure 2, inset). The concentration of ATCh equal to 1.75 mM ($> 2\,K_m^{app}$) was used for the determination of the maximum activity of AChE before and after inhibition by the model OP, paraoxon.

3.3. Analytical Characteristics of the Biosensor

The biosensor showed good precision and operational stability for the measurement of ATCh. A low relative standard deviation (RSD) of 2.9% was observed for six repeated measurements of 1.75 mM ATCh with the same electrode. The electrode was washed thoroughly between measurements. No difference in background current was observed for repeated measurements. Similarly, a low RSD of 6% for eight different electrodes for the measurement of

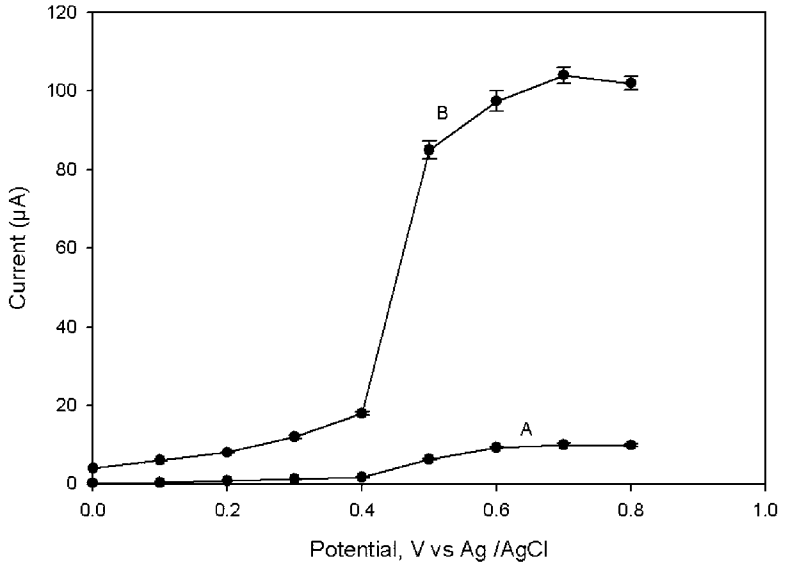

Fig. 1. Hydrodynamic voltammogram for 2 mM thiocholine at A) Unmodified SPE and B) MWCNT modified SPE. Measurement conditions: 50mM phosphate buffer containing 0.1 M KCl, pH 7.4

 © 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

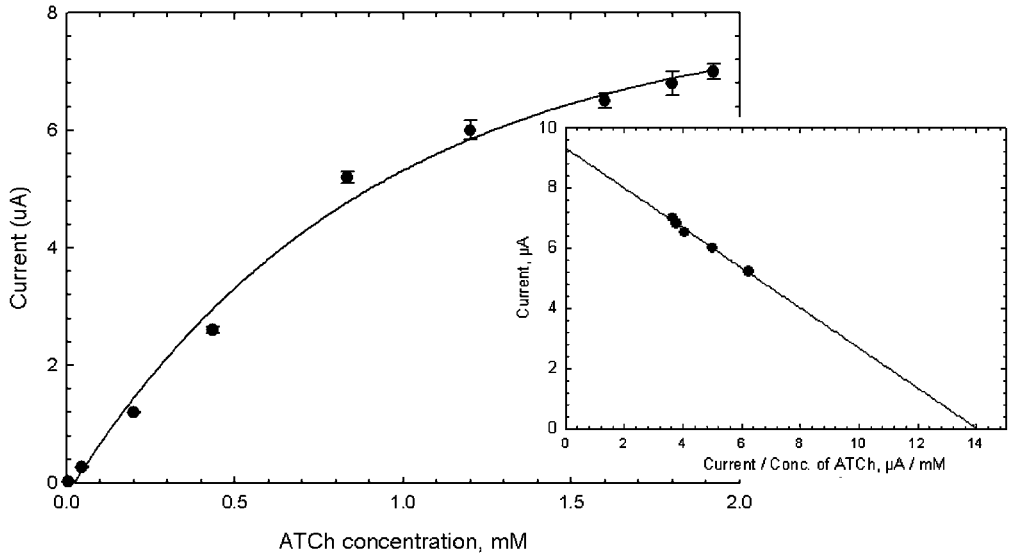

Fig. 2. Calibration plot of ATCh substrate for AChE immobilized on screen-printed electrode modified with CNT. Measurement conditions: 50 mM phosphate buffer containing 0.1 M KCl, pH 7.4; applied potential 200 mV (vs. Ag/AgCl). Inset: electrochemical Eadie-Hofstee plot.

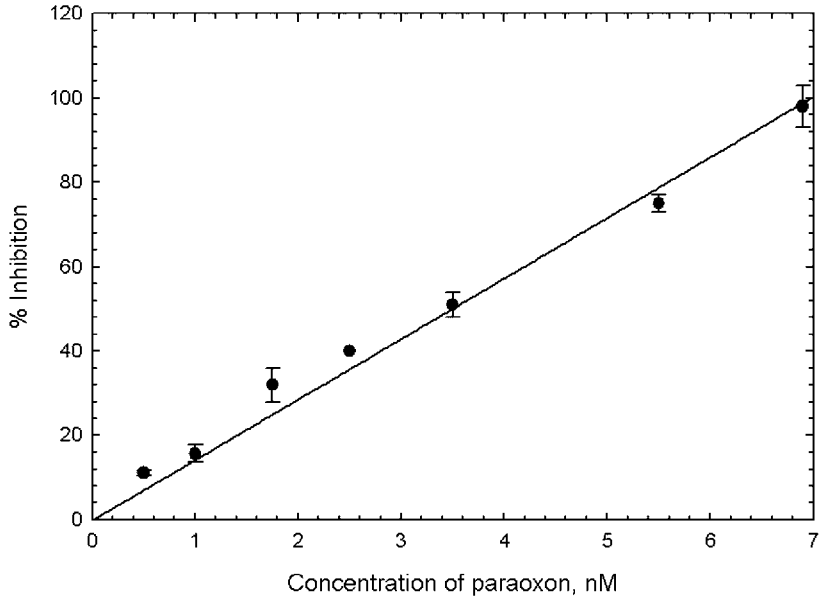

Fig. 3. Inhibition plot of immoblizied AChE by paraoxon after 30 min incubation. Measurement conditions: 50 mM phosphate buffer containing 0.1 M KCl, pH 7.4; applied potential: 200 mV (vs. Ag/AgCl); 1.75 mM acteylthiocholine substrate.

ATCh exhibited a good electrode to electrode reproducibility. When stored at 4 °C, the response was stable for seven days, showing good storage stability.

3.4. Measurement of Model OP, Paraoxon, Using the MWCNT Modified Screen-Printed Electrode

Paraoxon determination was carried out according to the three step procedure as described earlier. The response for substrate and sensitivity to inhibition by paraoxon was found to be optimal when 0.132 U of AChE was used [data not shown]. The calibration plot for the relative inhibition of AChE activity as a function of paraoxon concentration (Figure 3) displayed high linearity (slope, 14.36%/nM; correlation coefficient, 0.9859) up to 6.9 nM, and the limit of detection of 0.5 nM. This limit of detection is comparable and in some cases better than other reported values using batch procedures using similar incubation time and substrate [6]. Using the CNT-modified SPE with covalently attached AChE and CHO, Lin et al. reported a limit of detection of 50 nM for methyl parathion [17]. Based on information that paraoxon is 3.3-fold more toxic than methyl parathion (oral LD_{50} for rat) the limit of detection for methyl parathion using the present sensor could be expected to be 1.65 nM. This is thirty fold better than the

 © 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 1. Measurement of paraoxon in lake water by MWCNT-SPE.

Concentration of paraoxon spiked in lake water (ppb)	Concentration of paraoxon measured by the biosensor (ppb)
0.145	0.15 ± 0.01 ($n = 3$)
1.74	1.80 ± 0.09 ($n = 3$)

reported value. A further improvement of lower limit of detection with similar incubation time or reduction in the incubation time with similar limit of detection can be achieved by using a higher overpotential for thiocholine detection using batch protocol.

The feasibility of application of the biosensor for determination of OPs in real samples was tested. Water from Lake Elsinore, CA was filtered with a 0.22 μm filter and its pH adjusted to 7.4 from 9.2 using concentrated HCl. The water was then spiked with paraoxon and analyzed using the biosensor following the same procedure as for the measurement of the model OP, paraoxon as described previously. As shown in Table 1, the results obtained (concentrations of paraoxon in the analyzed samples were determined using the calibration plot in Fig. 3) were in good agreement (90%) with the amount spiked, demonstrating the validity of the newly developed biosensor to a practical problem.

4. Conclusions

A single enzyme biosensor which is inherently robust and simple was developed for the amperometric determination of OPs using the favorable properties of carbon nanotubes. CNTs exhibit electrocatlaytic activity toward thiocholine and posses high surface area. The use of CNT facilitated operation at a low applied potential (200 mV) without the use of a mediating redox species and immobilization of the enzyme without a membrane or any chemical treatment. The sensor showed excellent limit of detection, good precision, electrode to electrode reproducibility and stability. The feasibility of application of the sensor for the analysis of real water sample was demonstrated. This method could be extended for the detection of chemical warfare agents like VX and RVX and other OP insecticides like malathion and demeton-S. The small size, high surface area and other properties of CNT can lead to the development of novel sensors facilitating rapid, on-site monitoring of OP nerve agents with significant implications for homeland security.

5. Acknowledgements

We acknowledge the support of this work by DOD/DARPA/DMEA under grant number DMEA90-02-2-0216, MIPT2002J-A-139 from MIPT and the Office for Domestic Preparedness, U.S. Department of Homeland Security and U.S. EPA STAR Program (RD830900).

6. References

[1] J. A. Compton, *Military Chemical and Biological Agents*, Telford Press, NJ **1988**, p. 135.
[2] F*ood and Agricultural Organization of the United Nations Product Yearbook* **1989**, *43*, 320.
[3] U*nited States Department of Agriculture, Agricutural Statistics*, United States Government Printing Office **1992**, p. 395.
[4] A. Mulchandani, W. Chen, P. Mulchandani, J. Wang, K. R. Rogers. *Biosens. Bioelectron.* **2001**, *16*, 225.
[5] M. Trojanowicz, *Electroanalysis* **2002**, *14*, 1311.
[6] S. Sole, A. Merkoci, S. Alegret, *Crt. Rev. Anal. Chem.* **2003**, *33*, 89.
[7] G. Turdean, C. S. Mosneag,I. C. Popescu , *ACH-Models Chem*. **2000**, *137*, 519.
[8] H. B. Ray, A. A. Zakhidov, W. A. de Heer, *Science* **2002**, *297*, 787.
[9] S. Sotiropoulou, N. A. Chaniotakis, *Anal. Bioanal. Chem.* **2003**, *375*, 103.
[10] H. Luo, Z. Shi, N. Li, Z. Gu, Q. Zhuang, *Anal. Chem*. **2001**, *73*, 915.
[11] M. Musameh, J. Wang, A. Merkoci, Y. Lin, *Electrochem. Commun*. **2002**, *4*, 743.
[12] X. N. Cao, L. Lin, Y. Z. Xian, W. Zhang, Y. F. Xie, L. T. Jin, *Electroanalysis* **2003**, *15*, 892.
[13] J. Wang, M. Musameh, Y. Lin, *J. Am. Chem. Soc.* **2003**, *125*, 2408.
[14] R. J. Chen, Y. Zhang, D. Wang, H. Dai, *J. Am. Chem. Soc.* **2001**, *123*, 3838.
[15] J. J. Davis, M. L. H. Green, H. A. O. Hill, Y. C. Leung, P. J. Sadler, J. Sloan, A. V. Xavier, S. C. Tsang, *Inorg. Chim. Acta* **1998**, *272*, 261
[16] F. Balavoine, P. Schultz, C. Richard, V. Mallouh, T. W. Ebbesen, C. Mioskowski *Angew. Chem.*, *Int. Ed.* **1999**, *38*, 1912
[17] Y. Lin, F. Lu, J. Wang, *Electroanalysis* **2004**, *16*, 145.
[18] A. Guiseppi-Elie, C. Lei, R. H. Baughman, *Nanotechnology* **2002**, *13*, 559.
[19] J. Z. Xu, J. L. Zhu, Q. Wu, Z. Hu, H. Y. Chen, *Electroanalysis* **2003**, *15*, 219.
[20] J. Wang, M. Musameh, *Analyst* **2004**, *129*, 1.
[21] C. N. R. Rao, R. Sen, B. C. Satishkumar , A. Govindaraj, *Chem. Commun.* **1998**, *15*, 1525.
[22] H. Hu, B. Zhao, M. A. Hamon, K. Kamaras, M. E. Itkis, R. C. Haddon *J. Am. Chem. Soc*. **2003**, *125*, 14893.
[23] A. Mulchandani, P. Mulchandani, W. Chen, J. Wang, L. Chen, *Anal. Chem*. **1999**, *71*, 2246.
[24] J. L. Marty, N. Mionetto, R. Rouillon, *Anal. Lett.* **1992**, *25*, 1389.
[25] J. L. Marty, N. Mionetto, T. Noguer, F. Ortega, C. Roux, *Biosens. Bioelctron.* **1993**, *8*, 73.
[26] J. L. Marty, N. Mionetto, S. Lacorte, D. Barcelo, *Anal. Chim. Acta* **1995**, *311*, 265
[27] P. Skladal, *Anal. Chim. Acta* **1991**, *252*, 11.
[28] J. P. Hart, I. C. Hartley, *Analyst* **1994**, *119*, 259.
[29] M. K. Halbert, R. P. Baldwin, *Anal. Chem.* **1985**, *57*, 591.
[30] F. Ricci, F. Arduini, A. Amine, D. Moscone, G. Palleschi, *J. Electroanal. Chem.* **2004**, *563*, 229.
[31] D. Martorell, F. Cespedes, E. M. Fabreagas, S. Alegret, *Anal. Chim. Acta* **1997**, *337*, 305.
[32] J. Kulys, E. D'costa, *Biosens. Bioelctron.* **1991**, *6*, 109.
[33] P. C. White, N. S Lawrence, J. Davis, R. G Compton, *Electroanalysis* **2002**, *14*, 89.
[34] N. F. Almeida, A. K. Mulchandani, *Anal. Chimi. Acta* **1993**, *288*, 353.

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim