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Electrochemistry Communications 7 (2005) 1371–1374
electrochemistry communications
www.elsevier.com/locate/elecom

Electrochemical and optical bioassays of nerve agents based on the organophosphorus-hydrolase mediated growth of cupric ferrocyanide nanoparticles

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Received 9 September 2005; received in revised form 23 September 2005; accepted 26 September 2005
Available online 3 November 2005

Abstract

A new biometallization route for detecting low levels of organophosphorus nerve agents based on organophosphorus-hydrolase (OPH)-stimulated formation of cupric-ferrocyanide (CuFeCN) nanoparticles is described. The growth and accumulation of these nanoparticles onto the surface of carbon paste electrodes, coupled with their favorable redox activity, allow the amplified electrochemical detection of nerve agents. The results clearly demonstrate the applicability of the biocatalytic formation and surface preconcentration of CuFeCN nanoparticles as a new and attractive bioamplification route and its potential for monitoring of a wide range of biocatalytic transformations.

Keywords: Biometallization; Nanoparticles; Nerve agents; Cupric ferrocyanide; Electrochemical properties; Organophosphorus-hydrolase; Nanomaterials

1. Introduction

The unique catalytic properties of metal nanoparticles have recently been exploited for amplified detection of biological interactions [1,2]. For example, the catalytic enlargement of gold nanoparticles has been employed for amplified optical [3], electrochemical [4], or microgravimetric [5] transduction of DNA hybridization. Recent efforts have focused on exploiting the biocatalytic growth of gold nanoparticles for sensitive monitoring of enzymatic reactions [6–9]. Such biometallization protocols rely on the reductive enlargement of gold particles by an enzymatically liberated reducing agent. Colorimetric biosensing of glucose [6], alcohol [7], or neurotransmitters [8] has thus been accomplished through the hydrogen-peroxide, NADH- and hydroquinone-induced enlargement of the gold particles, respectively. An optical test for acetylcholine-esterase (AChE) inhibitors, based on the inhibition of the AChE-stimulated growth of gold nanoparticles, has also been reported [9]. All of the above biocatalytic assays have relied on colorimetric monitoring of the growth of gold nanoparticles.

The present communication reports on the electrochemical biosensing of organophosphorus (OP) nerve agents based on the organophosphorus-hydrolase (OPH)-stimulated formation of cupric-ferrocyanide (CuFeCN) nanoparticles. Metal hexacyanoferrates have generated considerable attention owing to their attractive electrochromic, electrocatalytic, photomagnetic or ion-exchange properties [10]. For example, the low-potential electrocatalytic detection

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doi:10.1016/j.elecom.2005.09.019

of hydrogen peroxide at Prussian-Blue modified electrodes has been exploited for highly selective biosensing of glucose [11]. Yet, to our knowledge, there are no reports on the biocatalytic formation of CuFeCN nanoparticles and its utility for amplified biodetection.

OPH catalyzes the hydrolysis of a wide range of OP pesticides and chemical warfare agents [12]. Because OPH offers direct monitoring of its OP substrates, rather than indirect (inhibition-based) biodetection, OPH-based biosensors show considerable promise for a wide range of security and environmental surveillance applications [13,14]. OPH-based enzyme electrodes commonly rely on the amperometric (anodic) monitoring of *p*-nitrophenol product of nitrophenyl substituent OPs (e.g., methyl parathion, paraoxon, fenitrothion, parathion) [13]. In the following sections, we will illustrate that the nitrophenol enzymatic product of OPH accelerates the formation of CuFeCN nanoparticles and that the accumulation of these nanocrystals onto a carbon electrode transducer leads to an amplified voltammetric detection of OP nerve agents.

2. Experimental

2.1. Reagents

Paraoxon, parathion, methyl parathion (from Supelco), *p*-nitrophenol, potassium ferricyanide, cesium nitrate (Aldrich), potassium citrate, cupric sulfate (Sigma) and potassium nitrate (Fisher), were used without further purification. Organophosphorus hydrolase (OPH; specific activity 82 U mL^{-1} measured using paraoxon as substrate) was produced according to a previously described method [1]. Graphite powder (Grade 38, 1–2 μm) was purchased from Fisher Scientific (Fair Lawn, NJ) while paraffin oil was obtained from Fluka. Stock solutions of the OP pesticides (100 ppm in acetonitrile) were used for preparing the sample solutions. All solutions were prepared using ultrapure water (>18 MΩ) from a Pure Lab Ultrawater system (Elga, Chandler, AZ).

2.2. Apparatus and electrodes

All electrochemical measurements were conducted using a CHI1220 electrochemical analyzer (CH Instruments, Austin, TX), in connection to a 5 mL cell and a three-electrode configuration. A carbon paste disk served as the working electrode, with Ag/AgCl (3 M KCl, model CHI111, CH Instruments) and a platinum wire as the reference and counter electrodes, respectively. Carbon pastes were prepared by hand-mixing graphite powder and paraffin oil (8:2 ratio) for 30 min. The paste was then packed into the cavity (2 mm diameter, 2 mm deep) of a 1 mL plastic syringe holder (Becton Dickinson Co., Franklin Lakes, NJ) that served as the electrode holder; a copper wire, inserted through the opposite end, provided the electrical contact. The paste surface was smoothed on a weighing paper (VWR Scientific products, West Chester, PA) and rinsed carefully with ultrapure water prior to each experiment.

Spectrophotometric measurements were carried out using a Shimadzu UV-2501PC spectrophotometer, using quartz cuvettes with an optical path length of 1.0 cm. Prior to the spectrophotometric measurements, the nanoparticle samples were washed with water and centrifuged three times at 10,000 rpm for 3 min before being resuspended by sonication in water, to avoid the interference of other components from the reacting solution.

Scanning electron microscopy (SEM) images were obtained with a FEI XL30 EFSEM electron microscope using an accelerating voltage of 20 kV. Samples were placed in aluminum stubs and were coated with a sputtered gold layer that provided enhanced conductivity.

2.3. Procedure

Preliminary CuFeCN-nanoparticle studies were carried out using a growth solution consisting on 0.1 M cesium nitrate, 1.0 mM $CuSO_4$, 5.0 mM potassium ferricyanide and 50 μM *p*-nitrophenol in 0.1 M pH 7.3 potassium citrate buffer (adjusted using 1 M H_2SO_4). For the enzymatic reactions, initially 5.5 U L^{-1} OPH and the selected nerve agent were allowed to react for 10 min in a stirred 2 mL potassium citrate solution (0.1 M pH 7.3; adjusted using 1 M H_2SO_4). Subsequently, 0.1 M cesium nitrate, 1.0 mM $CuSO_4$ and 5.0 mM potassium ferricyanide (final concentrations) were added to this mixture to facilitate the nitrophenol-induced formation of the CuFeCN nanoparticles. The particle formation and accumulation onto the carbon-paste surface proceeded for a selected time, while stirring the solution at 100 rpm.

After the accumulation, square wave voltammetry (SWV) measurements of the particle-coated electrode were carried out in a blank (0.1 M KNO_3) supporting electrolyte medium, using a potential step of 4 mV, a frequency of 25 Hz and an amplitude of 25 mV. All measurements were performed at room temperature. Each experiment involved the use of a fresh paste electrode surface. The analytical signals used in all cases correspond to the fifth consecutive SWV scan. All data presented are the mean of four measurements.

3. Results and discussion

The new biometallization assay consists of the initial enzymatic reaction, followed by the preparation of CuFeCN nanoparticles, their growth onto the carbon-paste electrode, and transfer of the electrode (with the accumulated nanocrystals) to a blank solution for recording the voltammetric response.

The favorable electroactivity of the resulting surface-confined CuFeCN nanocrystals makes them extremely attractive for electronic transduction of biocatalytic events. Taking advantage on the catalytic effect of *p*-nitrophenol on the CuFeCN nanoparticle formation, the detection of

some OPH enzyme substrates could be implemented. For example, Fig. 1 displays square-wave CuFeCN voltammetric signals for micromolar concentrations of paraoxon (b), methyl parathion (c) and parathion (d), along with the response without the OP compound (a). The three pesticides lead to well defined voltammograms, with a single anodic peak ($E_p = +0.75$ V), corresponding to the oxidation of the surface-confined CuFeCN nanoparticles [i.e., the Cu(II)–CN–Fe(II) to Cu(II)–CN–Fe(III) electron-transfer process]. In contrast, only a negligible response is observed in the absence of these nerve agents (a). Also shown in Fig. 1 are color images of the corresponding solutions following a 30 min reaction. While a light (yellow) color is observed in the absence of the pesticides (a), the three nerve agent solutions display a darker (orange-red) color (b–d), reflecting the presence of the CuFeCN colloid. As will be illustrated below, such optical properties enable also the quantitative colorimetric analysis of the different nerve agents. Note that in contrast to biometallization assays based on the enzymatic growth of gold nanoparticles [6–9], no nanoparticle seeds are required for the biocatalytic formation of the metal hexacyanoferrate nanocrystals.

The overall reaction for the nanoparticle generation could be described as follows:

$$2Cs^+ + Cu(Cit)^- + Fe(CN)_6^{3-} \rightarrow Cs_2Cu[Fe(CN)_6] + \text{formiate} + \text{oxalate} + CO_2 + H_2O$$

The citrate ions, initially complexed by copper, are oxidized by ferricyanide to CO_2, oxalate or formiate [15], producing the ferrocyanide ion that immediately reacts with Cu(II) in the presence of Cs(I), forming the cupric ferrocyanide nanoparticles. This reaction is relatively slow (at pH 7) but it is greatly accelerated in the presence of the enzymatically liberated *p*-nitrophenol.

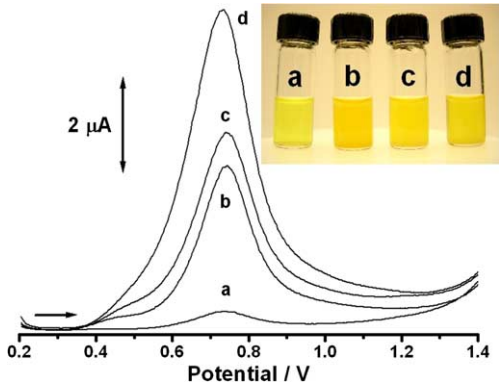

Fig. 1. Square-wave voltammograms (SWV) for enzymatically generated cupric ferrocyanide nanoparticles in the absence of the nerve agents (a) and in the presence of 25 μM paraoxon (b), methyl parathion (c) and parathion (d). Reaction medium: 1.0 mM Cu(II), 0.1 M Cs(I), 5.0 mM ferricyanide and 5.5 U L^{-1} OPH in a citrate buffer (0.1 M, pH 7.3) solution stirred at 100 rpm. Measurement solution: 0.1 M KNO_3. SWV parameters: potential step, 4 mV; amplitude, 25 mV; frequency, 25 Hz. Initial enzymatic reaction time (using OPH alone), 10 min; subsequent particle preparation and accumulation time, 15 min. Inset shows photographs of the reaction solutions in the absence of the nerve agents (a) and in the presence of 50 μM paraoxon (b), methyl parathion (c) and parathion (d) after a 30 min reaction. See Section 2 for details.

The OPH-induced accumulation of CuFeCN nanoparticles onto the electrode surface is illustrated in Fig. 2 which shows SEM images of the carbon paste electrode in the absence (A) and presence (B) of the paraoxon. Immersing the electrode in a reaction mixture not containing the nerve agent leads to few scattered and isolated nanoparticles. A dramatic increase in the coverage of the CuFeCN nanocrystals is observed in the presence of paraoxon. Such densely packed nanoparticles are of cubic shape and of an average size of 350 nm. Similar cubic-shaped nanoparticles were described earlier [16] in connection to different metal hexacyanoferrates. The surface coverage was strongly dependent upon the concentration of the pesticide and upon the reaction time (not shown). The 'background' nanocrystal coverage (without the nerve agent) increases rapidly using reaction times longer than 30 min. A 15 min reaction time was thus employed for minimizing the blank voltammetric signal in all subsequent biosensing work. The formation of the CuFeCN nanoparticles is attributed to the catalytic effect of the nitrophenol enzymatic product upon the citrate oxidation by ferricyanide in the presence of copper and cesium ions. Control experiments (not shown) indicated that all the sample components (citrate, copper, cesium and ferricyanide ions) are essential for the formation of the CuFeCN nanoparticles.

As the concentration of the enzymatically liberated nitrophenol is controlled by the concentration of the nerve-agent substrate, the formation of CuFeCN particles provides the basis for the quantitative electrochemical detection of low levels of the respective substrate. Fig. 3 shows square-wave voltammograms for increasing concentration of paraoxon over the 25–150 μM range (b–f), along with the corresponding blank voltammogram (a). The CuFeCN peak current increases rapidly and nearly linearly with the paraoxon concentration. A slight curvature is thus observed in the resulting calibration plot (left inset). The low detection limit of around 5 μM, estimated from the signal-to-noise characteristics of the response for the 25 μM solution (b), reflects the amplification feature of the nanoparticle. Such detection

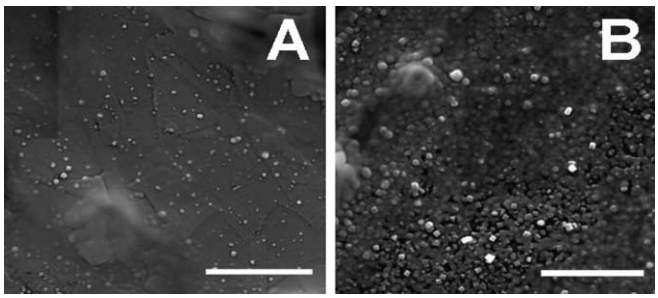

Fig. 2. SEM images of the surface of the carbon paste electrode following a 15 min immersion in the reaction mixture, in the absence (A) and presence (B) of 50 μM paraoxon. Scale, 5 μm; magnification, 3500×; acceleration voltage, 20 kV. Other conditions, as in Fig. 1.

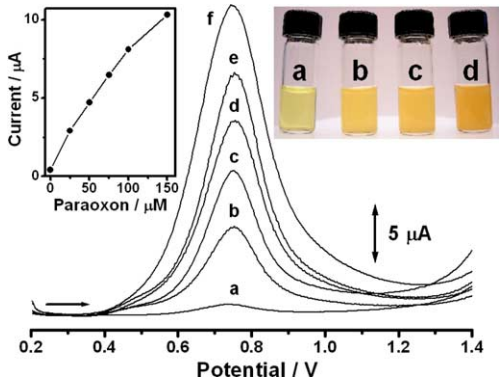

Fig. 3. Effect of the paraoxon concentration on the response of the enzymatically generated cupric ferrocyanide. Paraoxon concentration: 0 (a), 25 (b), 50 (c), 75 (d), 100 (e) and 150 (f) μM. Reaction time, 15 min. Other conditions, as in Fig. 1. Also shown (insets) are the corresponding calibration plot and photographs of reaction solutions containing different levels of paraoxon: 0 (a), 50 (b), 100 (c), and 150 (d) μM (in connection to a 45 min reaction time).

limit is similar to those reported for conventional OPH-based fiber-optic [14] or potentiometric [17] biosensors, and is slightly higher than the detection limit of amperometric OPH electrodes [13]. Note that these analytically useful signals represent the fifth scan after transferring the electrode to the blank KNO_3 solution. The CuFeCN peak increased gradually and became sharper during the first five scans after which no variations in the signal were observed (not shown). Such behavior is attributed to the cation exchange of Cs^+ with K^+, in a manner similar to that reported for Prussian Blue nanoparticle films [18]. Also shown in Fig. 3 (right inset) are color images of cuvettes containing CuFeCN particles formed in the presence of different concentrations of paraoxon. These indicate that the color intensity increases gradually upon increasing the concentration of the OP compound, and can be exploited for analogous colorimetric testing of chemical warfare agents.

4. Conclusions

In conclusion, we demonstrated a new biometallization route for detecting low levels of OP nerve agents based on the nitrophenol-mediated formation of CuFeCN nanoparticles. The process enables us to develop a novel electrochemical biosensor path for OP compounds. The results clearly demonstrate the applicability of the biocatalytic formation and accumulation of CuFeCN nanoparticles as a new and attractive bioamplification route. We are currently exploiting such enzymatically induced formation of CuFeCN nanocrystals for the sensitive monitoring of other biocatalytic transformations. Preliminary bioassays of glucose in the presence of glucose oxidase and its ferricyanide electron acceptor are very encouraging. While we are currently examining the scope and selectivity of the new bioassay, interferences from co-existing compounds that may induce the formation of CuFeCN nanoparticles could be eliminated using parallel measurements without the enzyme.

Acknowledgments

Financial support from the National Science Foundation (Grant Number CHE 0506529) and from the EPA (STAR Program) is gratefully acknowledged.

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