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A label-free electrochemical immunosensor for direct, signal-on and sensitive pesticide detection

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ABSTRACT

A new electropolymerizable monomer, [N-(6-(4-hydroxy-6-isopropylamino-1,3,5-triazin-2-ylamino)hexyl) 5-hydroxy-1,4-naphthoquinone-3-propionamide], has been designed for use in a label-free electrochemical immunosensor when polymerized on an electrode and coupled with a monoclonal anti-atrazine antibody. This monomer contains three functional groups: hydroxyl group for electropolymerization, quinone group for its transduction capability, and hydroxyatrazine as bioreceptor element. Square wave voltammetry shows that the polymer film, poly[N-(6-(4-hydroxy-6-isopropylamino-1,3,5-triazin-2-ylamino)hexyl) 5-hydroxy-1,4-naphthoquinone-3-propionamide], presents negative current change following anti-atrazine antibody complexation and positive current change after atrazine addition in solution. This constitutes a direct, label-free and signal-on electrochemical immunosensor, with a very low detection limit of *ca.* 1 pM, i.e. 0.2 ng L^{-1}, one of the lowest reported for such immunosensors. This is far lower than the detection limit required by the European Union directives for drinkable water and food samples (0.1 μg L^{-1}). The strategy described has great promise for the development of simple, cost-effective and reagentless on-site environmental monitors.

1. Introduction

Due to the increasing use of pesticides worldwide, there is a perceived threat of environmental damage as well as health issues. Atrazine (6-chloro-N-ethyl-N-[1-methylethyl]-1,3,5-triazine-2,4-diamine; ATZ) is a widely used pesticide that constitutes an excellent model for the study of small organic pollutants. It has been found to be a persistent environmental contaminant, occurring at trace levels in ground waters, and has been recognized as mutagenic, teratogenic besides having effects on the reproductive system.Many methods have been developed for atrazine analysis. The most conventional ones use GC–MS or HPLC (Dean et al., 1996; Ma et al., 2003). These techniques are very accurate but require sample pretreatment, expensive equipment and high-purity chemicals for the mobile phases. Moreover, chromatographic methods cannot be used for continuous, on site analysis (Cai et al., 2004). In the past twenty-five years, the application of immunoanalytical techniques (immunoassays and immunosensors) has increased significantly (Marinella et al., 2007). The binding properties of an antibody to an antigen have been used for the development of a broad variety of analytical techniques for high-speed environmental monitoring (Jiang et al., 2008). Enzyme-linked immunosorbent assays (ELISA) (Morozova and Levashova, 2005) are traditional solid-state methods (Lee and Kennedy, 2007). Immunosensors (Luppa et al., 2001) must be distinguished from immunoassays, where the transducer is not an integral part of the analytical system. They combine the sensitivity of the antibody–antigen interaction with fast, often direct, data acquisition possible with biosensor processes (Murphy, 2006). Immunosensors are potentially useful for the rapid detection of pesticides and now investigated with a view to environmental monitoring (Marty et al., 1995; Dennison and Turner, 1995). Depending on the transduction mechanism, they are generally classified as optical (fluorescence, chemiluminescence) (Rodriguez-Mozaz et al., 2004; Jain et al., 2004) or electrochemical (amperometric, potentiometric, conductimetric). (Yulaev et al., 2001; Grennan et al., 2003; Valera et al., 2010). These systems are sensitive, with very low detection limits (Zacco et al., 2007) (e.g. 30 pM), but need in general a label to detect the immune-reaction. However label-free transduction systems have obvious advantages. The most popular ones are optical systems based on Reflectometric Interference Spectroscopy (RIFS) (Brecht et al., 1995; Mouvet et al., 1996) and Surface Plasmon Resonance (SPR) (Bier and Schmid, 1994; Chegel et al., 1998) with two leading systems on the market:

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BIAcore (from BIAcore, Uppsala, Sweden) and IAsyse (from Fisons Applied Sensor Technology, Cambridge, UK). Nevertheless, electrochemical immunosensors (Murphy, 2006) could be competitive and revolutionize analysis, because of their simplicity and cheap technology. For pesticide detection, most of them use impedance (Hleli et al., 2006; Valera et al., 2007) or amperometry but mostly in label format (Besombes et al., 1995; Cosnier and Popescu, 1996). The use of electrogenerated polymers could avoid this drawback (Cosnier, 1999; Gerard et al., 2002). The introduction of appropriate functionalities through chemical modification of the monomer can provide polymer films with specific characteristics (Cosnier, 2003) without the help of a label.

In this work, we describe an original electrogenerated polyquinone film functionalized by a hydroxyatrazine moiety for label-free electrochemical detection of atrazine. First, the synthesis and characterization of a new multifunctional monomer is described. [N-(6-(4-hydroxy-6-isopropylamino-1,3,5-triazin-2-ylamino)hexyl)-5-hydroxy-1,4-naphthoquinone-3-propionamide] (JUG-HATZ) contains three functional groups: the hydroxyl group for electropolymerization, the quinone group to be used as transducer, and hydroxyatrazine as bioreceptor element. Electropolymerization of JUG-HATZ leads to poly[N-(6-(4-hydroxy-6-isopropylamino-1,3,5-triazin-2-ylamino)hexyl)5-hydroxy-1,4-naphthoquinone-3-propionamide], poly(JUG-HATZ). By this method, the quinone and the hydroxyatrazine functions are preserved.

The quinone function is well known to be particularly sensitive to its chemical environment, in terms of pH or ionic strength (Rubin et al., 2010) Therefore, the quinone group of poly(JUG-HATZ) can be used as a redox sensor for chemical or electrochemical modifications of its vicinity. These can be generated by heavy molecules, such as antibodies, or charged molecules immobilized at the vicinity of the film surface, influencing the diffusion layer by steric hindrance or electrostatic effects (Piro et al., 2007; Reisberg et al., 2008). The working principle of this sensor is illustrated in Fig. 1 and the polymer structure, poly(JUG-HATZ), is presented in Fig. 2. As shown, poly(JUG-HATZ) is able to bind to α-ATZ, i.e. the antibody directed towards unmodified atrazine, due to the cross-reactivity of α-ATZ for HATZ. After complex formation (HATZ/α-ATZ), the faradic current of the quinone group should decrease (Fig. 1, step 2). The electrode modified by poly(JUG-HATZ) where HATZ is complexed by α-ATZ, poly(JUG-HATZ/α-ATZ), is then utilized to detect ATZ in solution. Indeed, α-ATZ preferentially binds to ATZ, so a displacement equilibrium should occur (Fig. 1, step 3) between ATZ in solution and HATZ incorporated in the polymer. Addition of free atrazine removes the complexed antibodies from the electrode surface leading to an increase in the current. This *signal-on* system allows detection of one of the lowest atrazine concentrations (1 pM) recorded in the literature for this kind of electrochemical immunosensor.

2. Experimental

2.1. Chemicals and biological

5-Hydroxy-1,4-naphthoquinone (juglone), 1-naphtol (1-NAP) and lithium perchlorate were purchased from Aldrich; silver nitrate ($AgNO_3$) and succinic acid ($(CH_2–COOH)_2$) from Fluka; cyanuric chloride ($ClCN)_3$), isopropylamine $(CH_3)_2CHNH_2$) and N-Boc-1,6-diaminohexane ($C_{11}H_{24}N_2O_2$) from Alfa Aesar; N,N′-dicyclohexylcarbodiimide (DCC) from Acros and 4-(dimethylamino)pyridine (DMAP) from Aldrich. All other reagents used (NaOH, HCl) and solvents, acetonitrile (ACN), methanol (MeOH), dichloromethane (DCM), ethyl acetateand toluene, were PA (practical grade). Phosphate buffer saline (PBS, 0.137 M NaCl; 0.0027 M KCl; 0.0081 M Na_2HPO_4; 0.00147 M KH_2PO_4, pH 7.4) was provided by Sigma. Aqueous solutions were made with bi-distilled or ultrapure (MilliQ) water. Glassy carbon (GC) working electrodes (area, 0.07 cm^2) were from BASInc, Tokyo, Japan; (α-ATZ), monoclonal anti-atrazine antibody (M_w = 150 kDa) from Thermo Scientific, USA. Immune sera against OVA and α-NEF were obtained by immunizing mice of C57BL/6 strain (H-2b haplotype) with relevant protein antigens. 2 i.p. injections (a priming followed by a boost) were made with 300 μg of the antigen in 0.2 mL of PBS per mouse.

2.2. Methods and apparatus

For electrochemical experiments, a conventional one-compartment, three-electrode cell was used with an Autolab PGSTAT 30. The auxiliary electrode was a platinum grid and the reference electrode a commercial saturated calomel electrode (SCE, MetrOhm). Square wave voltammetry (SWV) was used to characterize α-ATZ complexation and ATZ detection. The following parameters were used: pulse height 50 mV, pulse width 50 ms, scan increment 2 mV, frequency 12.5 Hz. The electrolytic solution was PBS, bubbled with argon for 20 min before any experiment. The SWV scans were repeated until complete stabilization of the electrochemical signal (i.e. no difference observed between two successive responses). All electrochemical experiments were conducted at room temperature.

All NMR spectra were recorded in DMSO at 298 K on a Bruker Avance III 400 MHz spectrometer. Data were processed using TOPSPIN 2.1 software (Bruker). FT-IR spectra were recorded on a NICOLET 860 Fourier transform spectrometer. Data were processed using OMNIC software (NICOLET).

2.3. Synthesis of JUG-HATZ

To a vigorously stirred suspension of 3-(5-hydroxy-1,4-dioxo-1,4-dihydronaphthalen-2(3)-yl) propanoic acid (JUG–COOH, 80 mg, 0.325 mmol) in a mixture of dry dichloromethane and dry N,N′-dimethylformamide (DCM/DMF, 5:1) were added DCC (74 mg, 0.35 mmol), 4-(6-aminohexylamino)-6-(isopropylamino)-1,3,5-triazin-2-ol hydrochloride (HATZ–NH_2·2HCl, 172.6 mg, 0.70 mmol) and a catalytic amount of DMAP (2.0 mg, 0.015 mmol). The mixture was stirred for 40 min at 65 °C after which, the white precipitate, N,N′-dicyclohexylurea, was filtered from the lemon yellow solution. DCM was then evaporated at room temperature and distilled water added to precipitate the product. The crude product, a solid brown precipitate, was further purified by column chromatography on silica gel (DCM/MeOH, 0.05:1, v/v) to afford a mixture of the positional isomers (7/3) as a yellow solid (161 mg, 65%).

FTIR/cm^{-1}: 3271 (νO–H phenol); 3114 (νN–H); 2928 (νC–C, $–CH_2–CH_2–$); 2857 (νC–H, weak, $–CH_2–$); 1727 (νC=O); 1663(νC=O, amide); 1643 (νC=O, strong, quinone); 1411 (νC–N).

^{1}H NMR (400 MHz, DMSO): δ 1.10 (6H, d, CH_3), 1.20 (4H, m, H^i, H^j), 1.30–1.40 (4H, m, H^h, H^k), 2.40 (3H, m, NH^o, H^e), 2.70 (3H, m, NH^p–H^f), 2.90 (2H, m, H^l), 3.10 (2H, m, H^g), 3.30 (1H, m, H^m), 6.80 (1H, s, H^d juglone), 7.30 (1H, d, 8.0 Hz, H^c juglone), 7.50 (1H, d, 7.5 Hz, H^a juglone), 7.70 (1H, dt, 7.5 Hz, 8.0 Hz, H^b juglone), 7.90 (1H, s, NH^q), 9.90 (1H, s, OH, atrazine), and 11.90 (1H, s, OH, naphthol). See Supplementary Information, Fig. SI.1, for letters and colors. The TOF electrospray mass spectrum (ES+) gives the expected mass: M_w calc, 496.2434 g mol^{-1}, found 496.2522 g mol^{-1}.