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General approach for electrochemical detection of persistent pharmaceutical micropollutants: Application to acetaminophen

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ABSTRACT

We propose in this work a general and versatile methodology for electrochemical monitoring of persistent pharmaceutical micropollutants. The system presented is based on an electroactive and electropolymerized hapten (mimetic molecule of the pollutant to be detected) and a specific antibody that competitively binds either the hapten or the pollutant. The current delivered by the device depends on this competitive equilibrium and therefore on the pollutant's concentration. The determination of the pharmaceutical product operates within minutes, using square wave voltammetry without labeling or addition of a reactant in solution; the competitive hapten/antibody transduction produces a “signal-on” (a current increase). Applied to acetaminophen, this electrochemical immunosensor presents a very low detection limit of ca. 10 pM, ($S/N=3$) and a very high selectivity towards structural analogs (aspirin, BPA, and piperazine) even in a mixture.

1. Introduction

Acetaminophen (N-acetyl-p-aminophenol, APAP), an antipyretic commonly known as paracetamol, is one of the major pharmaceuticals and personal care products (PPCPs) detected in the aquatic environment. For this reason, its concentration is used as an indicator of human activity (Vystavna et al., 2013). Today, there is a need to break away from current analytical methods such as high performance liquid chromatography (Santos et al., 2013) and chromatographic techniques coupled to mass spectrometry (Buchberger, 2011; Nebot et al., 2007), unsuitable for real-time monitoring or fast response in case of suspected contamination. Due to their simplicity and cheap technology, electrochemical methods constitute an efficient alternative (Gooding, 2006). In addition to their high sensitivity, they can be easily implemented to achieve compact, portable and low consumption energy devices and satisfy many requirements for on-site wastewater monitoring. Actually, most of the electrochemical sensors developed to detect pharmaceutical products in water are based on the electro-oxidation of pollutants and require high potentials. They are easy to design but present a risk of parasitic oxidation phenomena associated with the oxidation of undesired targets with similar oxidation potential. Moreover, side-oxidation may lead to fouling of the electrodes due to the formation of dimers or other degradation products, which decreases sensitivity. Another strategy is to use systems able to provide specific and quantitative information using a specific receptor acting as a recognition element and immobilized on the electrode surface; most often, this specificity is achieved using a biomolecule (Thevenot et al., 2001). That is the case of electrochemical aptasensors (Hayat and Marty, 2014) or immunosensors (Gopinath et al., 2014) using nucleic acid aptamers or antibodies, respectively, specific for small organic molecules such as pollutants. In particular, methods involving immunochemical detection of small organic compounds are of interest due to their higher specificity and sensitivity compared with other methods (Kobayashi and Oyama, 2011). Antibodies with affinity constants over $10^8\,M^{-1}$ can now be produced easily, under automated procedures and with reduced use of laboratory animals (Brichta et al., 2005). Moreover, progresses in modern molecular biology and particularly antibody engineering have revolutionized the production of antibodies directed toward small organic molecules and not only large proteins. Examples of acetaminophen immunosensors have been published recently. Tertis et al. (2015) reported a label-free immunosensor for the selective detection of acetaminophen using graphite based screen-printed electrode and graphene oxide, by using square wave voltammetry, with a limit of detection of 0.17 μM. In another article, Hosu et al. (2015), reported an immunosensor for acetaminophen based on protein G-magnetic microparticles. The modified beads were used to capture the anti-acetaminophen antibody immediately followed by the immunological reaction between the antibody and its antigen, acetaminophen. The reported limit of detection is 1.76 μM using differential pulse voltammetry.

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A critical consideration in the preparation of an electrochemical immunosensor (E-immunosensor) is to design a bio-recognition interface able to reduce the loss of recognition efficiency of the antibody when immobilized (Sadik et al., 2009; Khor et al., 2011), for example by its convenient orientation on the electrode surface (Prieto-Simó et al., 2014). Another problem of E-immunosensors, in contrast to enzymatic sensors, is the absence of electrochemical events intrinsic to the complexation; therefore there is no straightforward means to measure it electrochemically. To overcome these problems, different strategies have been developed: bio-engineering of the antibodies to graft them from their non-specific heavy chains, to couple them with an enzyme, or the use of additional steps, e.g. labeling of a competitor of the target molecule (so-called competitive immunoassay) with an electroactive label. These strategies make the operations more complicated.

We propose in this work a versatile methodology for surface modification based on electropolymerization of an electroactive molecule (juglone, JUG) acting as a redox transducer and carrying an azide group, onto which a mimic of the target molecule (the hapten, here acetaminophen – APAP) modified with an alkynyl group was coupled using click chemistry. This methodology allows one to change the hapten easily depending on the pollutant to detect without having to develop a specific synthesis for each. The recognition and transduction steps were based on the use of a specific antibody (anti-APAP) that competitively binds either the immobilized hapten (JUG-APAP) or the pollutant alone (APAP) in solution. The current delivered by the device depends on this competitive equilibrium and therefore depends on APAP concentration. The determination of APAP was performed measuring a current change by square wave voltammetry (SWV), which was due to the change of steric hindrance upon anti-APAP binding to poly(JUG-APAP). Moreover, this strategy allowed the immobilization of the antibody in a non-covalent, non-denaturing way, which ensured the highest possible antigen-binding efficiency compared to traditional methods involving covalent and random grafting. The sensor's architecture is given in Fig. 1.

To the best of our knowledge, this is the first report describing a label-free and reagentless E-immunosensor for acetaminophen detection. This system presents a detection limit of ca. 1 pM ($S/N=3$), much lower than traditional systems involving electro-oxidation of pollutants (detection limit from the μM to the nM range). In addition, this E-immunosensor showed a very high selectivity towards selected structural analogs (aspirin, BPA, and piperazine), even in a mixture.

In a first step, the hapten-transducer JUG-APAP (11-(4-(3-(4-acetamidophenoxy)-3-oxopropyl)-1H-1,2,3-triazol-1-yl)undecyl-3-(8-hydroxy-1,4-dioxo-1,4-dihydronaphthalen-2(3)-yl)propanoate) was prepared using click chemistry (Lahann, 2009). Because alkyne and azide are orthogonal (i.e. do not react) to almost all other chemical functions, this coupling strategy is more specific than peptidic or isothiocyanate ones which lead to multiple coupling sites and random orientation. In a second step, JUG-APAP and JUG were electrooxidized to form a copolymer film poly (JUG-co-JUG-APAP). In a third step, α-APAP was added to bind to the copolymer surface, to afford the α-APAP/poly(JUG-co-JUG-APAP)-modified electrode. Upon addition of a sample containing APAP, a disassociation of the antibody occurs. This displacement is due to the ability of the combining site of the antibody to react with more than one antigenic determinant, but with different affinities. In particular, because α-APAP has a better affinity for APAP than for the immobilized hapten, it is removed from the surface if APAP is present in solution. Because the redox switching current of poly(JUG-co-JUG-APAP) is sensitive to changes in diffusion of ions at the polymer/electrolyte interface (Reisberg et al., 2010; Piro et al., 2007; Rubin et al., 2010), the current delivered by the device depends on the competitive equilibrium and therefore depends on the APAP concentration. This transduction scheme produces a current that increases with APAP concentration (signal-on), which is more satisfactory than traditional signal-off (current decrease) sensors which are usually sensitive to non-specific adsorption. In terms of sensitivity, the small size of the immobilized APAP (a few hundreds of g mol^{-1}) allows a thorough reorganization of the polymer/electrolyte interface upon competitive removal of α-APAP (typically 150,000*g* mol^{-1} for an IgG antibody), which strongly decreases steric hindrance, increases the apparent diffusion coefficient of counterions and increases the redox current of the polymer. The use of quinone as redox transducer, which presents very low redox potentials (-1 V/0 V vs. SCE in neutral aqueous medium), avoids the side-oxidation of interfering species or even oxidation of the analyte itself (ca. $+0.7$ V vs. SCE for APAP on GC).

2. Experimental

2.1. Chemicals

4-Dimethylaminopyridine (DMAP), 4-pentynoic acid ($C\equiv CCH_2CH_2CO_2H$), sodium azide (NaN_3), 5-hydroxy-1,4-naphthoquinone (juglone), napht-1-ol, lithium perchlorate and phosphate buffered saline (PBS) were purchased from Sigma Aldrich; silver nitrate ($AgNO_3$), succinic acid ($(CH_2–COOH)_2$) and L-ascorbic acid sodium salt ($C_6H_7NaO_6$) were from Alfa Aesar; ammonium persulfate $(NH_4)_2S_2O_8$, *tert*-butanol (*t*-BuOH), 4-acetamidophenol (APAP), bisphenol A (BPA), aspirin and 1-acetyl-4-(4-hydroxyphenyl)piperazine (AHPP) were provided by Acros Organics. Acetonitrile (MeCN) and dichloromethane (DCM) were HPLC grade, distilled over calcium hydride before use. All other reagents used (CaH_2, $CuSO_4\cdot 5H_2O$) and solvents (methanol (MeOH), ethanol (EtOH), petroleum ether (PE), ethyl acetate (EA)) were analytical grade. Thin-layer chromatography was performed on TLC plates of silica gel (layer thickness 0.2 mm, Merck). Column chromatography was carried out on silica gel 60 (Merck). Alumina slurry is from ESCIL, Chassieu, France. Aqueous solutions were made with MilliQ water. α-APAP, a sheep IgG antibody, was from American Research Products, Inc. Peroxidase-labeled purified secondary antibody to sheep IgG was from Jackson Immunoresearch. APAP ELISA Kit was provided by Neogen Corp.

2.2. Methods

A three-electrode cell was used with a VMP3 potentiostat (Biologic) driven by EC-lab software. Glassy carbon (GC) electrodes (0.07 cm^2) were from BASi Analytical Instruments. A platinum grid (1 cm^2) was used as auxiliary electrode, and a commercial saturated calomel electrode (SCE, Metrohm) as reference. Square wave voltammetry (SWV) was used instead of classical cyclic voltammetry to reduce the capacitive component of the current; the following parameters were used: pulse height 50 mV, pulse width 50 ms, scan increment 2 mV, frequency 12.5 Hz. All electrochemical experiments were conducted at room temperature, in argon-deaerated solutions. High-resolution mass spectroscopy (HRMS) was made on an LCT Premier XE (Waters). NMR spectra were recorded in $CDCl_3$ at 298 K on a Bruker Avance III 400 MHz spectrometer, and processed using TOP-SPIN software. ELISA experiments were performed using an Envision 2014 multilabel plate reader (Perkin Elmer).

To prepare the polymer-modified electrodes, bare GC electrodes were polished with 0.3 μm alumina slurry on a microfiber pad for 2–3 min, washed under sonication sequentially in distilled water, EtOH and MeCN for 1 min, and then air-dried. The electropolymerized mixture was 1×10^{-2} M JUG $+\,1\times10^{-3}$ M JUG-APAP $+\,1\times10^{-3}$ M napht-1-ol $+$ 0.1 M $LiClO_4$ in anhydrous MeCN.