Biosensors and Bioelectronics 25 (2010) 1376–1381

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Modified paramagnetic beads in a microfluidic system for the determination of ethinylestradiol (EE2) in river water samples

Noelia A. Martínez[a], Rudolf J. Schneider[b], Germán A. Messina[a], Julio Raba[a,*]

[a] INQUISAL, Departamento de Química, Universidad Nacional de San Luis, CONICET. Chacabuco 917, D5700BWS, San Luis, Argentina
[b] BAM-Federal Institute for Materials Research and Testing, Department of Analytical Chemistry, Reference Materials, Division of BioAnalytics, Richard-Willstaetter-Str. 11, D-12489 Berlin, Germany

ARTICLE INFO

Article history:
Received 7 August 2009
Received in revised form 20 October 2009
Accepted 21 October 2009
Available online 30 October 2009

Keywords:
Enzyme immunoassays
Ethinylestradiol
Paramagnetic beads
Horseradish peroxidase
Microfluidic
Flow injection analysis

ABSTRACT

In this work, we have developed and characterized a novel microfluidic immunoassay methodology for rapid and sensitive quantification of ethinylestradiol (EE2) in river water samples. The detection of EE2 was carried out using a competitive direct immunoassay method based on the use of anti-EE2 polyclonal antibodies immobilized on magnetic microspheres 3-aminopropyl-modified manipulated for an external removable magnet. The EE2 present in the water sample was allowed to compete with EE2-horseradish peroxidase (HPR) conjugated for the immobilized anti-EE2 antibody. The HPR, in the presence of hydrogen peroxide (H_2O_2) catalyzes the oxidation of catechol (Q) whose back electrochemical reduction was detected on gold electrode at 0.0 V. The response current obtained from the product of enzymatic reaction is inversely proportional to the amount of EE2 in the water sample. The electrochemical detection can be done within 1 min and total assay time was 30 min. The calculated detection limits for electrochemical detection and the ELISA procedure are 0.09 and 0.32 ng L^{-1} respectively and the intra- and inter-assay coefficients of variation were below 5.8%. Our electrochemical immunosensor showed higher sensitivity and lower time consumed than the standard spectrophotometric detection ELISA method, which shows the potential for assessment of EE2 in river water samples.

1. Introduction

Emerging contaminants are previously unknown or unrecognized pollutants. Most of them have been present in the environment for a long time, but their significance and presence are only now being elucidated and, therefore, they are generally not included in the legislation. There is not a clear agreement about which compounds can be considered as emerging pollutants. They can be classified in various categories according to their chemical class (chemicals of totally new structure), type of use (new uses in industry or in consumer realms), type of effect (new discovered effects), source (new or previously unknown origins for existing chemicals), and exposure (pathways that had not been anticipated or had been previously discounted as not possible) (Daughton, 2004). Taking into account these criteria, compounds that can be considered as emerging contaminants are the so-called pharmaceuticals and personal care products (PPCPs), steroids, xenoestrogens, endocrine disrupting compounds (EDCs), and others (Lopez de Alda et al., 2003).

Between these chemical substances we can find: ethinylestradiol (EE2, 17-ethinyl-13-metil-7,8,9,11,12,13,14,15, 16,17-decahidro-6H-ciclopenta[a] fenantrene-3,17-diol) this is a synthetic estrogen, estradiol derivative for oral administration and it is largely utilized as part of oral contraceptive. On the other hand, EE2 was incorporated at environment and its considered an EDCs. Endocrine disrupting effects observed in the aquatic ecosystem have stimulated broad scientific and public interest. First studies already began in the 1970s when adverse effects of synthetic estrogens were discussed for the first time (Tabak and Bunch, 1970). Research was intensified in the early 1990s with the advent of reproductive problems in some freshwater fish populations (Sumpter et al., 1994; Bern, 1991).

Steroid hormones, both natural and synthetic, can be found in the environment as a result of human or animal excretion due to growing population concentration and intensive farming. Hormones, such as estradiol, estrone and EE2 have been found in water at ng L^{-1} levels (Larsson et al., 1999; Ternes et al., 1999; Belfroid et al., 1999) but, even at these low concentrations, some of them may induce estrogenic responses and cause adverse effects on aquatic and terrestrial organisms and on humans (Daughton, 2004; Lopez de Alda et al., 2001).

* Corresponding author at: Department of Chemistry, National University of San Luis, Chacabuco y Pedernera, 5700 San Luis, Argentina. Tel.: +54 2652 425385; fax: +54 2652 430224.
E-mail address: jraba@unsl.edu.ar (J. Raba).

0956-5663/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2009.10.031

The EE2 and other estrogenic hormones are usually quantified in aqueous matrices using standard instrumental methods such as gas chromatography–mass spectrometry (GC–MS/MS) or high performance liquid chromatography–mass spectrometry (LC–MS/MS). Current GC–MS/MS and LC–MS/MS methods achieve detection limits below 1 ng L^{-1} after sample enrichment. This enrichment might cause problems with recovery. In addition chromatographic methods require an extensive clean-up sample due to ionization suppression. Enrichment and clean-up steps are by themselves error-prone and can lead to decreased precision and accuracy of results (Zühlke et al., 2004; Aerni et al., 2004; Hohenblum et al., 2004; Tacey et al., 1994; Warren and Fotherby, 1974; Fotherby et al., 1981; Morvay et al., 1980; De la Pena et al., 1975; Dyas et al., 1981). Alternatively immunoassays can be used for monitoring of EE2 in environmental samples (Schneider et al., 2004, 2005; Coille et al., 2002; Goda et al., 2003). Immunoassay methods are rapid, sensitive, and cost effective analyses for a variety of environmental contaminants and they are a very efficient screening tool. These methods are also highly selective due to the extraordinary discriminatory capabilities of antibodies. Immunoassay methods are fast and relatively easy compared to conventional GC/MS and HPLC.

Microfluidic devices consist of microchannels for transporting fluids, with part or all of the necessary components to an immunoassay procedure integrated (Becker and Locascio, 2002; Sia and Whitesides, 2003; Erickson and Li, 2004). These devices offer many potential advantages including reduced reagent consumption, smaller analysis volumes, faster analysis times, and increased instrument portability (Park et al., 2006). Microfluidic biosensors based on electrochemical detection have also been investigated using amperometric (Kwakye et al., 2006; Lammertyn et al., 2006) and potentiometric (Suzuki and Matsugi, 2005) measurement. Conductometric biosensors are a very promising class of analytical devices characterized by their high sensitivity (Watson et al., 1987; Hnaiein et al., 2008).

The use of magnetic nanoparticles as labels in biosensing has become a very interesting topic in research (Jaffrezic-Renault et al., 2007), they are particularly suitable for integration in microfluidic devices. Magnetic nanoparticles are designed for concentration, separation, purification and identification of molecules and specific cells (Pankhurst et al., 2003; Gijs, 2004; Shinkai, 2002). Bead-based microfluidic immunoassays have an edge over normal fluidic systems, as it employs microbeads as a solid support. There are three main advantages in the use of these microbeads. Firstly, the surface to volume ratio is greatly increased even in a microfluidic device (Verpoorte, 2003). As a result, the sensitivity of assays is increased due to higher efficiency of interactions between samples and reagents. Secondly, the analytes attached onto the beads can be easily transported in a fluidic system using pressure-driven flow. Finally, there are a variety of surface modifications available on these microbeads, which will introduce multiple functionalities to a single microfluidic design. Therefore, antibodies, antigens, DNA, RNA and a vast number of other biological molecules can be easily attached to the microbeads for transport and analysis in a fluidic system. The benefit of incorporating microbeads in microfluidics systems has led researchers to seek different strategies to immobilize microbeads in channels of detection and reaction (Peterson, 2005).

In this article we developed a microfludic system with magnetic nanoparticles incorporated into the central microchannel, which were retained there for the action of the external magnet. This device was coupled with flow injection system and electrochemical detection for sentitive quantification of EE2 present in environmental samples. EE2 detection in these samples was carried out using a competitive immunoassay. The EE2 in the water sample is allowed to compete immunologically with EE2-HRP for the immobilized anti-EE2 antibodies. HRP in the presence of hydrogen peroxide (H_2O_2) catalyzed the oxidation of catechol (Q) to o-benzoquinone (P). The electrochemical reduction back to Q was detected on gold electrode at 0.0 V. The response current obtained from the product of enzymatic reaction is proportional to the activity of the enzyme and, consequently, to the amount of the EE2 in the water sample. Compared with the conventional immunoassay techniques, our microfluidic immunosensor showed enough sensitivity to determine very low levels of EE2 in unknown samples, with the advantage of the use of smaller volumes of reagents and samples and the improvement efficiency with regard to time analysis.

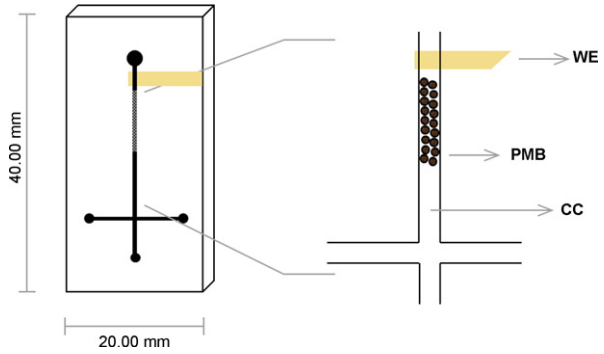

Fig. 1. Schematic representation of microfluidic immunosensor. WE: gold working electrode; PMB: paramagnetic beads; CC: central channel. All measurements are given in millimeters.

2. Materials and methods

2.1. Reagents and solutions

All reagents were of analytical or biochemical grade. Polyclonal rabbit anti-EE2 serum label (SA 2150) and EE2-HRP conjugate were supplied by Dr. Rudolf J. Schneider BAM Federal Institute for Materials Research and Testing Department I Analytical Chemistry, Reference Materials Division I.5 BioAnalytics Working Group I.51 Immunochemical Methods Berlin, Germany. For the ELISA assays, reagents were used in according with Hintemann et al. (2006). The development of the polyclonal antibody has been described in Schneider et al. (2004) and the preparation of enzyme conjugate has been described in Schneider et al. (2005). Glutaraldehyde (25% aqueous solution) and H_2O_2 were purchased from Merck, Darmstadt. Micro particles, magnetic, amino funtionalized (53572) were purchased by Fluka, Buchs/Schweiz, USA. Ethinylestradiol and Catechol was purchased from Sigma Chemical Co., St. Louis. Aqueous solutions were prepared using purified water from a Milli-Q system. The river water samples were collected from rivers of San Luis State, Argentina.

2.2. Sample preparation

Environmental water samples were collected from six rivers of San Luis State, Argentina. Tap water and ultrapure water were used as controls. For the electrochemical measurements the water samples (500 ml) were passed subsequently through folded filter papers (Whatman) and by vacuum through 934-AH^{TM} RTU Glass Microfiber Filters glass (Whatman), in according with Schneider et al. (2005) and adjusted to pH 7.0 using 0.1 M phosphate buffer.

2.3. Flow-through reactor/detector unit

The main body of the sensor was made of Plexiglas. Fig. 1 illustrates the design of the flow-through chamber containing the microfluidic immunosensor and the detector system. The gold layer