Talanta 147 (2016) 328–334

Contents lists available at ScienceDirect

Talanta

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

Electrochemical immunosensor for ethinylestradiol using diazonium salt grafting onto silver nanoparticles-silica–graphene oxide hybrids

Fernando H. Cincotto [a], Gonzalo Martínez-García [c], Paloma Yáñez-Sedeño [c,*], Thiago. C. Canevari [b], S.A.S. Machado [a], José M. Pingarrón [c]

[a] Institute of Chemistry, State University of São Paulo, São Carlos, Brazil
[b] Engineering School, Mackenzie Presbiterian University, São Paulo, Brazil
[c] Department of Analytical Chemistry, Faculty of Chemistry, University Complutense of Madrid, Madrid, Spain

ARTICLE INFO

Article history:
Received 10 August 2015
Received in revised form
18 September 2015
Accepted 24 September 2015
Available online 1 October 2015

Keywords:
Ethinylestradiol
EE2
Electrochemical immunosensor
Silver nanoparticles
Graphene oxide
Urine

ABSTRACT

This work describes the preparation of an electrochemical immunosensor for ethinylestradiol (EE2) based on grafting of diazonium salt of 4-aminobenzoic acid onto a glassy carbon electrode modified with silver nanoparticles/SiO_2/graphene oxide hybrid followed by covalent binding of anti-ethinylestradiol (anti-EE2) to activated carboxyl groups. A competitive immunoassay was developed for the determination of the hormone using peroxidase-labeled ethinylestradiol (HRP-EE2) and measurement of the amperometric response at −200 mV in the presence of hydroquinone (HQ) as redox mediator. The calibration curve for EE2 exhibited a linear range between 0.1 and 50 ng/mL (r^2=0.996), with a detection limit of 65 pg/mL. Interference studies with other hormones related with EE2 revealed the practical specificity of the developed method for the analyte. A good reproducibility, with RSD=4.5% (n=10) was also observed. The operating stability of a single bioelectrode modified with anti-EE2 was maintained at least for 15 days when it was stored at 4 °C under humid conditions between measurements. The developed immunosensor was applied to the analysis of spiked urine with good results.

1. Introduction

Ethinylestradiol (EE2) is one of the most potent synthetic estrogenic hormones. It is an essential constituent of oral contraceptives widely prescribed in women [1,2]. Adverse effects of EE2 include accelerated coagulation and fibrinolysis. Furthermore, use of combined hormonal contraceptives has shown to be associated with increased risk of venous thrombosis depending on estrogen concentration [3,4]. EE2 is rapidly absorbed orally, yielding a peak in plasma between 1 and 2 h after taking [5]. A major challenge is the presence of residues of EE2 and its derivatives in the environment coming from excretions, where negative impact on the reproductive system in wildlife and human can be produced [6].

The relevance of EE2 determination both in biological and environmental samples has made available a large number of analytical methods. In addition to chromatography using GC or HPLC coupled to at least one mass spectrometer, various immunoassay methods have been developed. RIA and ELISA methods were reported early to determine EE2 in body fluids [7,8]. Currently, various ELISA kits are available for the analysis of biological samples or water. Table 1 summarizes the analytical characteristics of some of these configurations. A typical assay is based on competitive interaction between EE2 and biotinylated EE2 for the binding sites of a pre-coated specific antibody. Colorimetric detection using a peroxidase conjugate, H_2O_2 and TMB, allows the EE2 determination to be performed in a non-linear dynamic range extending up to thousands of pg/mL, and with an analysis time lasting about 2–2.5 h [6]. Other immunoassay formats using fluorimetric [9] or chemiluminiscence measurements [10] were also described. A competitive microfluidic immunoassay based on the immobilization of anti-EE2 on 3-aminopropyl functionalized magnetic beads and amperometric detection, was also reported [11]. Regarding immunosensors, a configuration was proposed using magnetic beads functionalized with a synthetic estrogen derivative. Competitive immunoassay with anti-EE2, alkaline phosphatase-labeled IgG, and 1-naphthyl phosphate, allowed the determination of the estrogen with a limit of detection of 10 pg/mL using square wave voltammetric detection [12].

Hybrid materials prepared from graphene and SiO_2 constitute excellent substrates for the development of electrochemical sensors. The huge conductivity, high surface area, biocompatibility and robustness of graphene, coupled with the physical and chemical resistance of silica, its hydrophylicity, chemical inertness, and the high surface area/volume ratio, all contribute to increase the electroactive surface, thus enhancing sensitivity [13]. On the

* Corresponding author.

http://dx.doi.org/10.1016/j.talanta.2015.09.061
0039-9140/

Table 1
Analytical performance of some methods based on immunoassay for the determination of ethinylestradiol (EE2)

Configuration and methodology	Dynamic range, pg/mL	LOD, pg/mL	Reproducibility, RSD,%	Total time, min	Application	Ref.
CEK510Ge ELISA kit (Uscn) Immobilization of monoclonal anti-EE2. Competitive immunoassay between biotin-EE2 and EE2. Addition of HRP-avidin. Colorimetric detection using H_2O_2 and TMB	24.6–2000	9.28	< 10% (intra-assay) < 12% (inter-assay)	≈ 120	Biological fluids	
ABIN1873485 (Abyntek) Immobilization of monoclonal anti-EE2. Competitive immunoassay between biotin-EE2 and EE2. Addition of HRP-avidin. Colorimetric detection using H_2O_2 and TMB	24.7–2000	9.28	< 10% (intra-assay) < 12% (inter-assay)	150	Biological fluids	
R-Biopharma™ Ridascreen ELISA Kit. Competitive immunoassay between EE2 and HRP-EE2. Colorimetric detection using H_2O_2 and TMB	up to 8100	370	< 10% (intra-assay) < 10% (inter-assay)	150	Bovine urine	
Ecologena™ EE2 ELISA Kit (Tokiwa Chemical Industries). Competitive immunoassay between EE2 and HRP-EE2. Colorimetric detection using H_2O_2 and TMB	50–3000	–	< 10%	150	Water	–
Competitive ELISA immunoassay using a biotinylated EE2 derivative and HRP-streptavidin. Colorimetric detection using H_2O_2 and TMB	22–1200	14 ($S/N=3$)	< 5%	120	Water	[6]
Competitive immunoassay using total internal reflection fluorescence (TIRF) with Cy5 labeled antibody, or energy transfer (ETIA) with Cy5 labeled EE2 or Cy5.5 labeled antibody	60–1.8×10^4 (TIRF); 40–2×10^5 (ETIA)	70 (TIRF) 10 (ETIA			Waste water	[9]
Competitive ELISA immunoassay using a HRP-EE2-6-CMO conjugate and chemiluminescence detection.	0.8–100	0.2 ± 0.1	< 10%	40	Water	[10]
Competitive microfluidic immunoassay using EE2 and HRP-EE2. Immobilization of anti-EE2 on 3-aminopropyl-MBs. Amperometric detection with H_2O_2 and catechol at a gold electrode.	0.01–60	0.09	4.1% (intra-assay) 5.8% (inter-assay)	30	River water	[11]
Electrochemical AP-IgG-anti-EE2-EE2-hexa-MBs/SPCE immunosensor. Competitive immunoassay using anti-EE2, AP-IgG and 1-NPP. SWV detection	$0.1–5\times10^4$	10	–	120	Waters	[12]
Electrochemical HRP-EE2-anti-EE2/AgNPs/SiO_2/GO/GCE immunosensor. Competitive immunoassay between EE2 and HRP-EE2. Amperometry with H_2O_2 using HQ as redox mediator.	$10^{-2}–5\times10^4$	65	4.5% (intra-assay) 5.4% (inter-assay)	120	Human urine	This work

Key: HRP, horseradish peroxidase; TMB, tetramethylbenzidine, EE2-6-CMO, 1,3,5(10)-estratrien-17-ethinyl-3,17-diol-6-one-6-carboxymethyloxime, 1-NPP, 1-naphthyl phosphate.

other hand, metallic nanoparticles are characterized by their electrocatalytic ability together with the capacity for adsorption of biomolecules, biocompatibility and high conductivity. In this context, it has been claimed that functionalization of graphene with SiO_2 allows anchoring metal nanoparticles securely onto graphene support with a high dispersion thus enhancing the catalytic performance [14]. Despite their properties, only few examples of the use of metallic nanoparticles /silica/graphene hybrids can be found in the literature in connection to the preparation of electrochemical (bio)sensors. A hybrid material prepared with gold nanoparticles (AuNPs) immobilized onto mesoporous silica-coated reduced graphene oxide (rGO) was reported for cancer cell detection through hydrogen peroxide sensing [15]. Moreover, an interleukin-6 (IL-6) electrochemical immunosensor was prepared making use of AuNPs-graphene-silica sol–gel as immobilization biointerface and AuNP-poly-dopamine (PDA) @carbon nanotubes as the label of HRP-bound antibodies [16]. Recently, Cincotto et al. [17] reported the synthesis and characterization of AgNPs/SiO_2/GO hybrid and the preparation of a voltammetric sensor for the simultaneous determination of epinephrine and dopamine in urine. A good distribution of silver nanoparticles in the SiO_2/GO material was found with a synergistic effect among the hybrid components producing electrocatalytic activity toward the electrochemical responses thus leading to a high sensitivity and selectivity.

The work described in this manuscript faces the double objective of addressing the lack of immunosensors for the determination of EE2, and explores for the first time the ability of AgNPs/SiO_2/GO hybrids for the preparation of electrochemical immunosensors. The designed strategy for capture antibodies immobilization involved 4-aminobenzoic acid (ABA) grafting onto AgNPs/SiO_2/GO glassy carbon (GCE) modified electrodes by electrochemical reduction of the corresponding diazonium salt. This strategy provided a suitable surface for covalent attachment of the capture antibody allowing the development of a competitive immunoassay for the determination of the hormone using peroxidase-labeled ethinylestradiol (HRP-EE2), and measuring the amperometric response at −200 mV upon addition of H_2O_2 in the presence of hydroquinone (HQ) as redox mediator.

2. Experimental

2.1. Reagents and solutions

Graphite (Aldrich), tetraethylorthosilicate (TEOS, Sigma-Aldrich, 98%) and silver nitrate (Sigma-Aldrich, 99%) were used for the synthesis of the hybrid material. 4-aminobenzoic acid (ABA, Across), 1-ethyl-3-[3–dimethylaminopropyl]carbodiimide hydrochloride (EDC, Sigma) and N-hydroxysulfo-succinimide (NHSS, Sigma), were also used. Ethinylestradiol (EE2, Aldrich), anti-ethinyl-estradiol (anti-EE2), and HRP-labeled ethinylestradiol (HRP-EE2), both from Fitzgerald, were the reagents used for the immunosensor preparation. 0.1 M phosphate buffer solutions of pH 7.2 (PBS) and pH 6.0 were prepared from NaH_2PO_4 and Na_2HPO_4 (Scharlau, 99%). Blocker casein in PBS (Thermo), hydroquinone (HQ, Sigma), and H_2O_2 (Scharlau, 35%) were also employed.

Cortisol, β-estradiol (E2), estriol (E3), progesterone (Prog) and testosterone (Test), all from Sigma, were tested as potential interfering compounds. Solutions of each compound at a 10^{-3} μg/mL concentration in PBS were prepared. All other chemicals and solvents used were of analytical-reagent grade and distilled water was obtained from a Milli-Q purification system (Millipore, Bedford, NA, USA).