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Ultrasensitive environmental assessment of xeno-estrogens in water samples using label-free graphene immunosensors

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ABSTRACT

There is a growing interest in the possible environmental health impact posed by endocrine-disrupting chemicals (EDCs). A challenge to the field of endocrine disruption is that these substances are diverse and may not appear to share any structural similarity other than usually being low molecular mass (< 1000 Da) compounds. Here we demonstrate the effectiveness of sensor device for the detection of low molecular weight, poorly water soluble, estrogenic compounds E1, E2 and EE2, fabricated by electropolymerization over graphene screen printed electrode (SPE).

The PANI/Gr-SPE-devices displayed linear responses to estrogenic substances, in EIS assays, from 0.0975 ng/L to 200 ng/L in water samples, with a detection limit of 0.043 pg/L for E1, 0.19 ng/L for E2 and 0.070 pg/L for EE2 which is lower than other current biosensing techniques. This portable, disposable immunosensor offers a solution for immediate measurement at sample collection sites, due to its excellent sensitivity and selectivity when testing water samples obtained directly from rivers and waste water treatment facilities. The simple screen printing production method will enable the low cost, high volume production required for this type of environmental analysis.

Introduction

Endocrine disruptive chemicals (EDCs) have been the focus of increasing attention over the last 20 years as they can have severe impacts on individual reproduction, behaviour [1] and ultimately on the long term survival of natural populations [2], even at concentrations as low as nanograms per litre (i.e. environmentally relevant concentrations) [3]. Environmental estrogens, also referred to as xeno-estrogens, are known (EDCs) that disrupt gonadal steroid signalling by interacting with vertebrate oestrogen receptors [4], and can be either naturally or synthetically produced. Estrogens produced by the metabolic pathways of organisms, such as phyto-estrogens produced by plants, are naturally released into the environment but synthetic xeno-estrogens and estrogen-like hormones are also abundant as a result of the use of pesticides (hexachlorocyclohexane- HCH), components of plastics (bisphenol A, BPA) and commonly used drugs (17α-ethinyl estradiol, EE2, a widely used as an active ingredient of contraceptive pills). Estrone (E1) and 17β-estradiol (E2) are naturally produced by humans and animals [5]. Concentrations of these estrogens in the environment have been rising as a result of increased population and intensive animal farming [6].

Negative environmental effects linked to xeno-estrogenic substance exposure are predominantly associated with fish reproductive function, having major environmental and economic impacts. Chemicals such as E2 and EE2 are commonly found at low ng/L concentrations in treated sewage effluents and highly populated downstream areas [7]. Critical aquatic population consequences of exposure include male fish feminisation [8], increased production of vitellogenin [9,10], reproductive disruption [11] and/or transgenerational effects affecting embryo development in exposed mothers [12]. Additionally, recent studies suggest that exposure to EDCs, and to xeno-estrogens in particular, has the potential to disrupt sexual selection [13] and affect the reproductive behaviour of several fish species. As a consequence, a new proposal by the European Commission suggested that the annual average environmental quality standard (EQS) for EE2 and E2 should be of 0.035 ng/L and 0.4 ng/L respectively [14], highlighting the importance of accurate

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quantification of exposure to estrogens in the aquatic environment [15].

Furthermore there are reported effects on human health including breast cancer, and reduction in sperm count [16–18]. Exposure to estrogenic substances through contaminated potable supplies is a daily occurrence in the western world. There is evidence supporting the combined interactions of cosmetic chemicals with environmental, pharmacological and physiological estrogens which could contribute negatively to human health.

Whilst a series of methods have been developed for the rapid, sensitive and accurate detection of estrogenic substances in water systems [19,20], there remains a requirement for a measurement platform allowing rapid, highly accurate on-site detection of low levels of environmental estrogens. A LOD ≤ 0.1 ng/L is desirable for estrogens in waste water and surface water samples, and a linear range of detection from near the LOD to ≥ 100 ng/L is necessary for accurate sample measurement.

Graphene offers many attractive qualities for an estrogen electrochemical sensor system, including a high surface area to volume ratio [21] and the required electron mobility [22]. Furthermore combining graphene with semiconducting polymers can improve the electronic/conductive properties of the base material [24]. Polyaniline (PANI) functionalisation harnesses specific physico-chemical characteristics such as a high specific capacitance, good conductivity and good environmental stability to enhance sensor sensitivity [25,26]. In addition, PANI acts as a surface to immobilise biological sensing material via chemical bonding of functional groups. Amine, thiol and carboxylic acid groups offer a variety of methods, where covalently linking the sensing molecule to the polymer provides selectivity, dependent on the antibody quality [29].

Here we report the fabrication of a graphene sensor modified by electropolymerization capable of detecting different estrogenic substances at a range of concentrations relevant to environmental assessment in both purified and environmental samples. The immunosensor was produced via modification of a disposable graphene screen printed electrode (SPE) with an amine layer that preserves the chemical structure of graphene and allows subsequent surface functionalisation with antibodies for the detection of either E1, E2 or EE2. The sensors showed a wide linear range from 0.0975 ng/L to 200 ng/L. An LOD of 0.043 pg/L for E1, 0.19 ng/L for E2 and 0.070 pg/L for EE2 was achieved which is lower than other current biosensing techniques, and delivers the level sensitivity much greater than that required to evaluate and monitor EQS for EE2 and E2, and making the sensors suitable as early warning systems for environmental pollution [14].

Material and methods

Reagents and solutions

All chemicals used were of analytical grade and water was mostly ultrapure grade. Estrone, 17β-estradiol and 17α-ethinylestradiol, aniline solution, sulphuric acid (H_2SO_4), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC), N-hydroxysuccinimide (NHS), potassium hexacyanoferrate III ($K_3[Fe(CN)_6]$), potassium hexacyanoferrate II ($K_4[Fe(CN)_6]$) trihydrate, phosphate buffered saline solution (PBS) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (UK). Polyclonal rabbit Anti-Estrone antibody was purchased from Thermo Fisher, UK #PA1-24903. Monoclonal mouse Anti-17β Estradiol antibody #ab20626 and polyclonal sheep Anti-Ethinylestradiol antibody #ab59670 were purchased from Abcam (UK). E1, E2 and EE2 solutions were supplied by the Chemical and Environmental Engineering group at Swansea University. PBS was prepared in a ratio of 1 PBS sachet per 1000 mL DI water. EDAC (25 mM), NHS (50 mM), BSA and the electrolyte solutions were all prepared in PBS buffer. E1, E2, and EE2 solutions were prepared in water, diluted to a final concentration of 200 μg/L.

Apparatus

Electrochemical measurements were conducted with a potentiostat/galvanostat (Autolab). The potentiostat/galvanostat was controlled with NOVA software and possessed a Frequency Response Analysis module. A switch box was used to provide an interface to connect the graphene screen printed electrode (Gr-SPE) to the potentiostat/galvanostat. Gr-SPEs were purchased from DropSens/Metrohm (DRP110-GPH). Ultra-high resolution SEM measurements were performed using a Hitachi High-Technologies S-4800 and AFM measurements were performed using a BioScope Catalyst™ BioAFM.

Electrochemical assays

Cyclic Voltammetry (CV) measurements were conducted in 5.0 mmol/L of $[Fe(CN)6]^{3-}$ and 5.0 mmol/L of $[Fe(CN)6]^{4-}$ prepared in PBS buffer (pH 7.4). A voltametric potential sweep was induced, from −0.7 V to +0.7 V at 50 mV/s. Electrochemical Impedance Spectroscopy (EIS) assays were conducted with the same redox couple $[Fe(CN)6]^{3-/4-}$ at a standard potential of +0.10 V, using a sinusoidal potential perturbation with amplitude of 100 mV and a frequency equal to 50 Hz, logarithmically distributed over a frequency range of 1000Hz-0.05 Hz. All assays were conducted in triplicate.

Surface modification

The polyaniline film was obtained according to our previous work [28,30]. In parallel, each antibody solution (E1, E2 and EE2 each at 1 mg/ml) was mixed with 25 mmol/L EDAC, and 50 mmol/L of NHS, for 2 h at RT. A 10 μL of this resulting solution was then placed on the PANI/Gr-SPE surface. After 2 h, at room temperature, the electrode was rinsed away and replaced for BSA solution (0.5 mg/mL in PBS buffer) solution, for 30 min. The immunosensor was then washed 3 times with PBS buffer.

Testing and calibration

E1, E2 and EE2 binding to their respective antibodies already immobilised on the immunosensor was achieved by placing a 10 μL of the E1, E2 and EE2 solution on the sensor surface. Different concentrations of E1, E2 and EE2 solutions, ranging from 1.56 to 200 ng/L, were prepared by dilution of the 200 ng/L standard E1, E2 and EE2 solution in PBS. E1 was also detected in river samples from three different locations. Samples were incubated at room temperature for 15 min for equilibration of antigen/antibody binding, and then washed 3 times with PBS prior to redox probe EIS measurements (see Fig. 1).

Sample collection and preparation

Environmental water samples collection sites

Samples were collected from three locations in South Wales, UK, comprising of a mix of domestic urbanised and semi-rural areas representative of typical western infrastructure. According to a recent survey by the Environment Agency, the proportion of catchments classified as at risk of endocrine disruption for fish in Wales is low, due to a combination of low population density and high surface water runoff [31]. The EA classification of risk of endocrine disruption for fish is based on concentrations ranging from < 1 ng/L E2 equivalent (low risk) to > 1 ng/L E2 equivalent (at risk). The River Tawe, Swansea and the River Taff, Cardiff were selected as sample sites representative of low and medium risk urban areas respectively, both supplied by piped water and drainage.

Nine 50 mL samples were extracted from both the effluent flow of Cilfynydd Wastewater Treatment Works (WWTW) and the River Taff in the Cardiff city centre, representative of domestic sites. Due to the industrial heritage, and high risk status of the Swansea River Tawe, two