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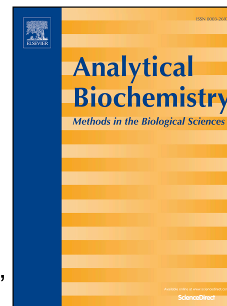

Ultrasensitive environmental assessment of xeno-estrogens in water samples using label-free graphene immunosensors

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samples using label-free graphene immunosensors

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and may not appear to share any structural similarity other than usually being low molecular mass (<1000 Daltons) 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.

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Keywords

Immunosensor; Screen-printed electrode; Graphene; Environmental estrogens; Endocrine disrupting chemicals.

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 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 [16,17,18]. 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

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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) functionalization 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 electropolymerisation 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].

2. Material and Methods

2.1. 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),

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2.2. 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.

2.3. 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 -0.7V to +0.7V at 50mV/s. Electrochemical Impedance Spectroscopy (EIS) assays were conducted with the same redox couple $[Fe(CN)6]^{3-/4-}$ at a standard potential of +0.10V, using a sinusoidal potential perturbation with amplitude of 100mV and a frequency equal to 50Hz, logarithmically distributed over a frequency range of 1000Hz-0.05Hz. All assays were conducted in triplicate.

2.4. 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 1mg/ml) was mixed with 25 mmol/L EDAC, and 50 mmol/L of NHS, for 2 hours at RT. A 10µL of this resulting solution was then placed on the PANI/Gr-SPE surface. After 2 hours, at room temperature, the electrode was rinsed away and replaced for BSA solution (0.5 mg/mL in PBS buffer) solution, for 30 minutes. The immunosensor was then washed 3 times with PBS buffer.

- *Please insert Figure 1 –*

2.5. Testing and Calibration

E1, E2 and EE2 binding to their respective antibodies already immobilised on the immunosensor was achieved by placing a 10µL 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

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measurements.

2.6. Sample Collection and Preparation

2.6.1. 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 50 ml samples were extracted from this domestic site. As a negative control, drinking water was collected from one tap in Swansea university (UK). At the time of sampling, each sample was tested for pH, temperature and conductivity, using pH-indicator paper Litmus (Merck Millipore), thermometer (Lo Tox Laboratory Thermometers, Brannan), and a Hanna Instruments HI8733 Conductivity Meter (RS Components Ltd) respectively.

2.6.2. Sample Preparation

Samples were kept at -80°C until use. 10 mL of the sample was then taken and centrifuged at 1000 rpm for 5 minutes to separate larger debris out of the sample. From this solution, 10 µL was taken from the supernatant and deposited onto the modified sensor surface.

2.6.3. ELISA test

Measurements of total estrogens for calibration were taken using commercial ELISA kits (Ecologiena® Estrogen (E1/E2/E3), Tokiwa Chemical Industries, Japan). For estrogen quantification using ELISA, 2ml of each water sample was acidified with 10% Methanol and filtered through GF/C glass filter papers (Whatman,1.2 um). In a clean microplate, the standard or sample (100 µL) and

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washing, coloring reagent (100 µL) was added to each well and the microplate was incubated (22–25°C) for 30 minutes before adding the stop solution (100 µL). To verify calibration accuracy, known concentration-standards were run in duplicate on each microplate for each ELISA test. To improve sample accuracy, each water sample replicate was run in triplicates. Blanks were run using 10% methanol solution. Absorbance at 450 nm was measured in a microplate reader within 15 minutes of the reaction stop time.

For each ELISA analysis, a set of 5 standards (0, 0.05, 0.15, 0.50, 3.0 µg/L E2) in duplicate was used generated a calibration curve, which was fitted within a three-parameter exponential equation. Using the average absorbance values for two scans of the each duplicate standards, the fitting parameters were determined for each standard using Excel®. The calibration curve used average values of each triplicate sample for the fitting parameters from all 5 standards. Estrogen concentration for samples was determined through interpolation and absorbance.

3. Results and discussion

3.1. Sensor validation

Chemical modifications at graphene surface were monitored using CV and EIS to validate each stage of the fabrication process (Figure 2), characterising changes in electron transfer properties against the redox probe [32]. The EIS data was analysed by Nyquist plots to show the frequency response of the electrode/electrolyte system and area plots of the imaginary component (Z``) of the impedance against the real component (Z`). The charge-transfer resistance (R_{ct}) at the electrode surface is given by the semicircle diameter obtained in EIS and can be used to define the interface properties of the electrode.

- *Please insert Figure 2 -*

The formation of a PANI layer on graphene, via electropolymerization, yielded a decrease in current with an increasing number of cycles (Figure 2A). After 10 cycles, the current density of the oxidation peak was greatly reduced, thus conforming the formation of a PANI layer. Electrochemical data, obtained by using an iron redox probe with the newly formed PANI layer, showed Nyquist plots where the Rct increased compared to unmodified graphene which was in agreement with the CV data obtained at the electropolymerization stage. The CV for the modification of the sensor surface was as expected, showing a large increase in the magnitude of the redox peaks after the PANI modification followed by decreases with the addition of the Ab and BSA, resulting in an increased peak-to-peak potential separation (Figure 2B). These decreases tie in with the decreased conductivity of the Ab and BSA compared to PANI/Gr-SPE, resulting in a lower current flow.

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the $[Fe(CN)6]^{3-/4-}$ redox couple with ΔEp of 0.168 V and ΔIp of 0.247 mA. The modification of graphene-SPE surface with PANI results in a ΔIp increase of 0.129 mA and a ΔEp decrease of 0.865 V. This result may be attributed to the positively charged amino group of the PANI molecule which attracts the negative charge of $[Fe(CN)6]^{3-/4-}$, causing an easy electron transfer reaction on the electrode surface [33]. A cyclic voltammogram of the Ab/PANI/Gr-SPE electrode showed a decrease peak-to-peak potential separation (ΔEp of 0.083 V). Further, addition of the BSA blocking agent to the Ab/PANI/Gr-SPE electrode surface gave rise to a change on the electrochemical behaviour of the $[Fe(CN)6]^{3-/4-}$ couple, leading to ΔEp increase of 0.003 V and decreased ΔIp value of 0.019 mA. BSA molecules cause masking of the electrode surface for oxidation/reduction of the redox probe $[Fe(CN)6]^{3-/4-}$ [34]. The same procedure was repeated for E2 and EE2 and reported the same behaviour and trends (Supplementary Figure S1). The morphology, topography and structure of the SPE-graphene following each fabrication stage were also characterized using both SEM and AFM (Supplementary Figure S2 and S3).

The non-modified graphene-SPEs showed a very small semicircle at the Nyquist plot, indicating the presence of a very fast electron-transfer process on the graphene support (Figure 2C). Subsequent deposition of a PANI film contributed to an increase in the R_{ct}, thus acting as a barrier to the electron transfer of the redox probe. This increase is small but consistent with the presence of a polymeric material that has a small fraction of protonated amine functions (bearing the opposite charge to that of the redox probe).

Subsequent antibody and BSA binding, both proteins, generated an additional increase in the R_{ct}, indicating as an additional barrier to electron transfer processes. All CV assays supported the results of EIS studies (Figure 2B), with decreased current peaks after protein binding and an increase in the peak-to-peak potential separation.

3.2. Analytical performance of E1, E2, and EE2 sensors

To establish baseline measurements for the three antibodies, anti-E1, anti-E2 and anti-EE2, impedance measurements where conducted.

- Please insert Figure 3 -

When compared, the three different Nyquist plot of the antibodies (Figure 3) demonstrated very small R_{ct} difference between the three, E2 = 385.94 Ω, E1 = 441.14 Ω and EE2 = 500.77 Ω was observed.

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- *Please insert Figure 4* -

Following the basic characterization to monitor antibody functionalisation of the sensor, a range of concentrations of E1, E2 and EE2 solubilized in PBS (Figure 4) were used to simulate a simplified model of environmental estrogens to evaluate the sensors. The Mr of these compounds ranges from 270 – 296 Da, and as such are very small molecules compared to the larger proteins previously detected using this type of sensor [28,30].

Figure 5A shows the Nyquist plots of the immunosensor Ab/PANI/Gr-SPE for the anti-EE2 antibody against increasing concentrations, while Figure 5B shows the corresponding EIS calibration curve, plotting R_{ct} Ab/PANI/Gr-SPE against the logarithm of EE2 concentration. The concentration range used for calibration was 0.39 to 50 ng/L. No diffusion controlled effect was observed in the EIS spectrum, and the diameter of the semicircle increased with increasing EE2 concentrations. The same procedure was repeated for E1 and E2 and reported the same behaviour trends (Supplementary Figure S4).

- *Please insert Figure 5* -

As expected due to the surface bound structures acting as barriers to electrical transfer, the R_{ct} in the Nyquist plot exhibited a linear increase relevant to the logarithmic scale of EE2 concentrations. The average slope of the R_{ct} versus log[EE2] was 0.28 KΩ/[EE2, ng/L] with an R2 correlation coefficient of 0.94. The limit of detection (LOD) was determined to be 0.043 pg/L for E1, and 0.07 pg/L for EE2, both polyclonal antibodies. A five fold reduced LOD (0.19 ng/L) was observed for E2, probably due to the increased selectivity of a monoclonal antibody.

3.3. Evaluation of E1 levels in environmental water samples

Standard ELISA tests were used to detect the environmental estrogen levels from three sources in the South Wales (UK) area. Three of these, river Taff/Tawe and Cilfynydd are typical urbanised areas served by piped water supply and drainage. Using the E1 ELISA, no detectable levels of E1 were assayed for in each of the river samples, due to the limit of detection or limitations of ELISA assay sensitivity. In order to prove this, ELISA sensitivity was calibrated using a sample of Cilfynydd WWTW - Site 1 water, initially concentrated with a known E1 concentration (3 μg/L). A serial

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graphene sensor (0.043pg/L).

In order to evaluate the application of the sensor, water samples were collected from the river Taff/Tawe and Cilfynydd, typical urbanised areas served by piped water supply and drainage. Existing information for E1 concentration in such water sources [35,36] allowed to use this chemical for multi-sample evaluation at each site. We measured water pH and temperature (directly related to estrogen degradation [37,38]) as well as electric conductivity, which has been related to estrogentic activity in rivers (Table 1) [39].

- *Please insert Table 1 -*

The concentrations of E1 in water samples were determined by EIS measurements and fitting to calibration curves previous prepared (Table 2).

- *Please insert Table 2 -*

Samples 1, 2, 3 and 4 from the WWTW effluent had E1 concentrations of 2.90 ng/L, 7.48 ng/L, 3.40 ng/L and 4.43 ng/L respectively. These levels are in agreement with values previously reported in the literature for this site (Table 2) and meet the EQS according to Baronti et al [40]. In contrast, river samples suggested concentrations over two times greater than the previously reported values [41], which could be due to seasonal variations in river estrogen. For example E1 varied from <2.5 ng/L to 21.7 ng/L between July/ August and February in the Llobregat River in Spain [42] whilst found variations in E1 concentrations from 2-17 ng/L were found in the River Thames in England [43]. E1 concentrations were higher in the more densely populated urban area of Cardiff (4,328 persons/km2) compared to Swansea area (3,389 persons/km2) (Office for National Statistics, 2001). Drinking water had the lowest E1 measurement recorded (2.5 ng/L), as previously reported in other studies (Table 2). Both Tawe and Taff rivers had higher concentrations of E1 than the WWTW effluent and drinking water. Both catchments were potentially under low risk of fish endocrine disruption according to the Environment Agency [14]. As E1 is estimated to have 85% of the efficiency of E2 in endocrine disruption [44], the concentrations measured in these rivers can be well above those recommended by the European Commission [14] for E2 and are higher than some independent predictions for no-effect-exposure level, which are as low as 6 ng/L for E1 [45]. Estrogens affect the physiological responses of some molluscs, resulting in feminisation and reproductive disruption [3,46], as well as the reproductive behaviour of crustaceans [47]. Moreover, the effects of EDCs in the aquatic environment

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estrogen exposure [6].

4. Conclusion

Estrogens that have accumulated in the environment have the potential to act as potent endocrine disruptors, negatively impacting on animal and human health. Accurate and simple methods to routinely monitor EDCs in the water are paramount to avoid such unwanted adverse effects in wildlife. Here we detailed the application of an anti-estrogen/PANI-GrSPEs sensor that demonstrates selective and sensitive detection of field collected water samples at significantly reduced detection limits compared to the commercially available ELISA alternatives. A wide linear range from 0.0975 ng/L to 200 ng/L was observed, reporting values for E1, E2 and EE2. The study highlights the benefits of using semiconducting polymers and graphene electrodes for the detection of a number of estrogenic substances that are known to accumulate in the environment. Due to their potential for wide geographical deployment, as well as inline monitoring at static measurement sites, the sensors are suitable for a wide range of environmental monitoring applications.

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Figures Caption:

Figure 1. Schematic diagram for the immunosensor assembly**.**

Figure 2. (A) Synthesis of polyaniline on Gr-SPE electrode (10 cycles performed with a scan rate of 50 mV/s); **(B)** CV records after modification of PANI/Gr-SPE with anti E1 antibody and BSA; **(C)** Nyquist plots of BSA/anti-E1/PANI/Gr-SPE sensor, obtained in 5.0 mM $[Fe(CN)6]^{3-/4-}$ PBS buffer pH 7.4.

Figure 3. Nyquist plots of anti-E1/PANI/Gr-SPE (red curve), anti-E2/PANI/Gr-SPE (blue curve) and anti-EE2/PANI/Gr-SPE (green curve) sensor.

Figure 4. Chemical structure of (A) Estrone (E1); (B) Estradiol (E2) and (C) Ethinylestradiol (EE2).

Figure 5. (A) Nyquist plot of BSA/anti-EE2/PANI/Gr-SPE sensor, incubated in increasing concentrations of EE2 (0.39-50 ng/L). **(B)** The R_{ct} values of the previous calibration plotted against EE2 concentration, with a standard deviation of 9.08%.

Table 1. Parameters of the water samples collected.

Table 2. Sensor performance with relevant water samples.

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	Sample	**pH**	**(°C)**	**Conductivity (µS/cm)**
Cilfynydd WWTW	1	6	12	216
	2	6	12	267
	3	6	12	215
	4	7	13	264
River Taff (Cardiff)	1	6	12	254
	2	6	13	178
	3	6	14	175
	4	7	13	450
	5	7	13	430
River Tawe (Swansea)	1	7	13	470
	2	7	13	480
Drinking Water	1	7	13	134

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Sample Source	Measured concentration (ng/L)	R^2 value	Values reported in literature (ng/L)
Cilfynydd WWTW - Site 1	2.90	0.9629	2.5 – 82.1 36
Cilfynydd WWTW – Site 2	7.48	0.9629	2.5 – 82.1 36
Cilfynydd WWTW – Site 3	3.40	0.9629	2.5 – 82.1 36
Cilfynydd WWTW – Site 4	4.43	0.9629	2.5 – 82.1 36
River Taff (Cardiff) – Site 1	15.4	0.8469	0.1 – 4.1 (±0.4) 37
River Taff (Cardiff) – Site 2	52.0	0.8469	0.1 – 4.1 (±0.4) 37
River Taff (Cardiff) – Site 3	37.0	0.8469	0.1 – 4.1 (±0.4) 37
River Taff (Cardiff) - Site 4	11.8	0.8469	0.1 – 4.1 (±0.4) 37
River Taff (Cardiff) – Site 5	34.8	0.8469	0.1 – 4.1 (±0.4) 37
River Tawe (Swansea) – Site 1	14	0.9805	0.1 – 4.1 (±0.4) 37
River Tawe (Swansea) – Site 2	17.2	0.9805	0.1 – 4.1 (±0.4) 37
Drinking Water	2.5	0.9382	0.4 37

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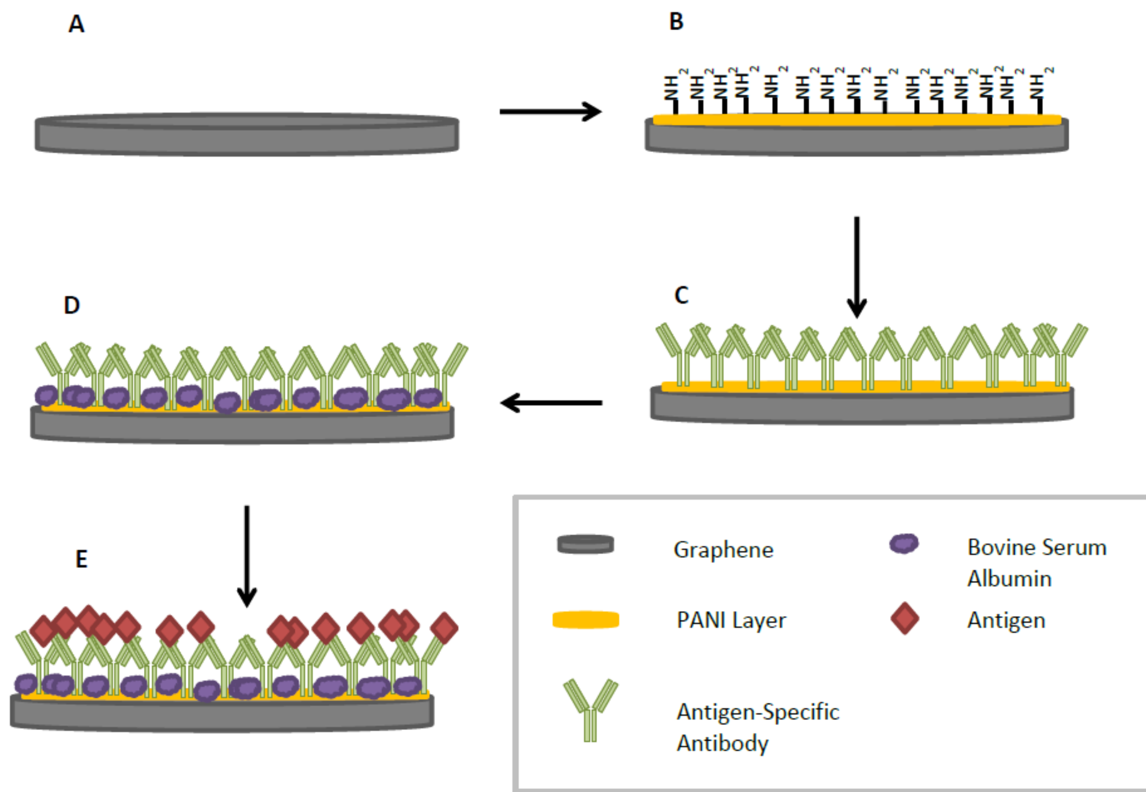

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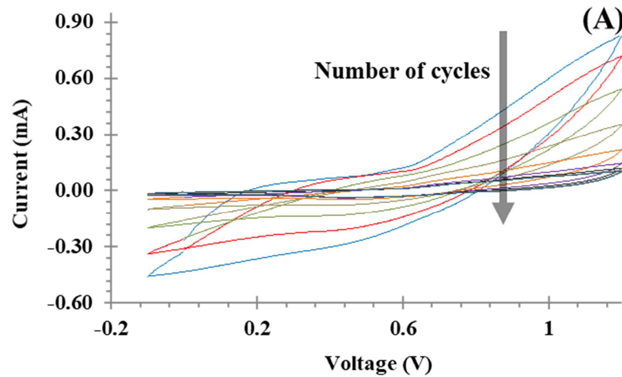

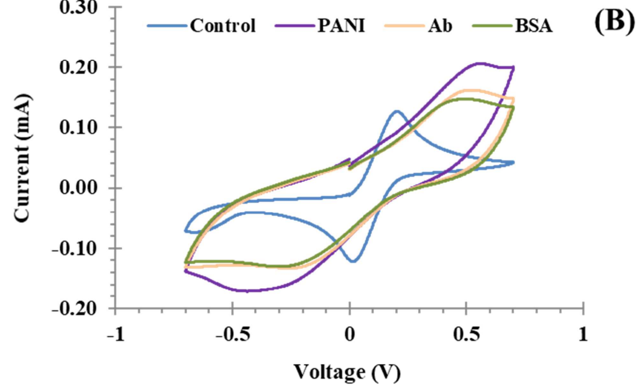

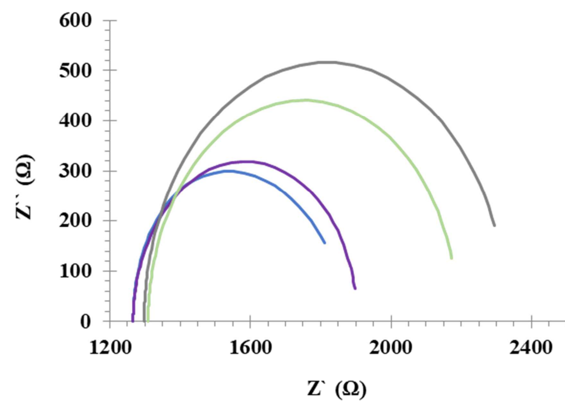

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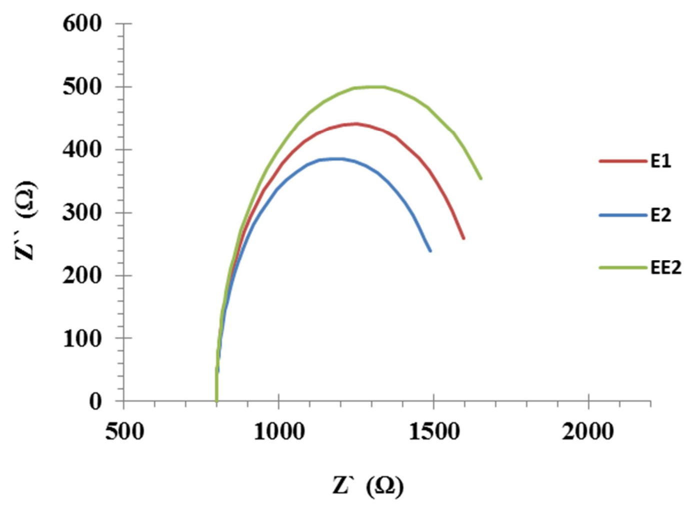

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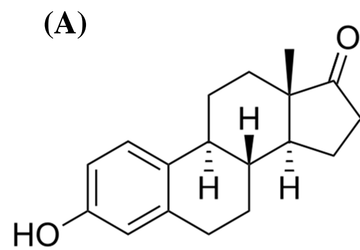

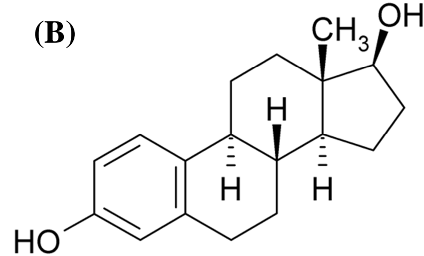

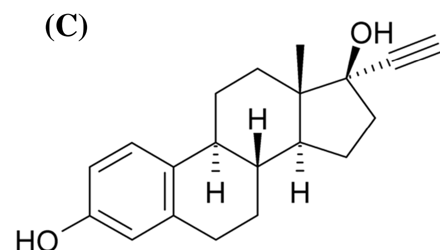

Estrone (E1)		17β-Estradiol (E2)		17α-Ethinylestradiol (EE2)	
Molecular weight (g /mol)	270.4	Molecular weight (g /mol)	272.4	Molecular weight (g /mol)	296.4
Water Solubility (mg /L at 20°C)	13	Water Solubility (mg /L at 20°C)	13	Water Solubility (mg /L at 20°C)	4.83

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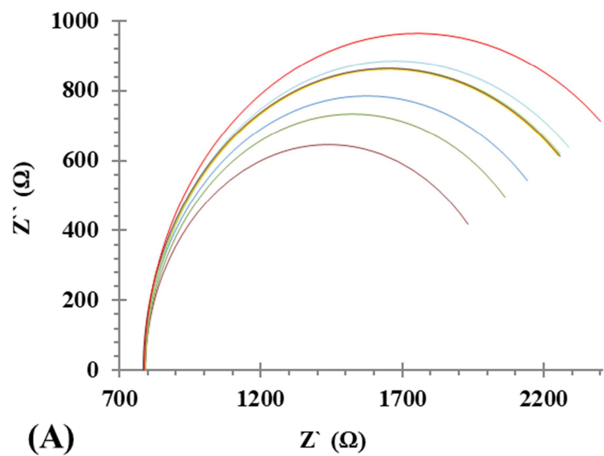

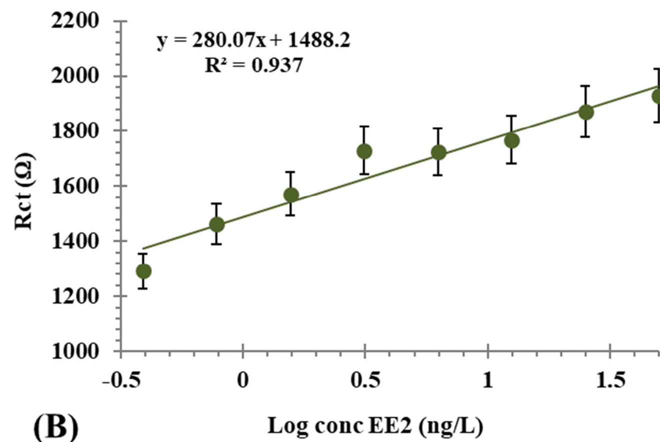

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