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The simpler the better: Highly sensitive 17α-ethinylestradiol sensor based on an unmodified carbon paper transducer

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ABSTRACT

The remarkable features of a carbon fiber paper sensor (CP) were employed for detection of the estrogenic hormone 17α-ethinylestradiol (EE2), considered a contaminant of emerging concern due to its potential ecotoxicity and widespread in the aquatic ecosystems. In this work, an unpreceded CP pre-treatment study was conducted with the (II)-hexacyanoferrate(III) ion pair, however a bare sensor without pre-treatment revealed higher efficiency on the oxidation of EE2 compared to a chemical and electrochemical pre-treated CP and a gold nanoparticles modified CP, being thus selected for EE2 determinations. The analytical conditions were thoroughly optimized in terms of electrolyte pH (pH 7), differential pulse voltammetry parameters (modulation time 0.003 s, amplitude 0.09 V, interval time 0.1 s and step potential 0.01 V), and analyte preconcentration potential (0.4 V) and time (180 s). The hormone can be determined by the CP in a wide linear range from 0.1 to 1000 nM, achieving a detection limit of 0.14 ± 0.005 nM and an outstanding sensitivity of 1636 ± 232 μA μM^{-1} cm^{-2} in the lowest linear zone (0.1–1 nM). The sensor was validated in river water and fish reaching good recoveries (91.2 ± 4.6 to 109.0 ± 7.1%), reproducibility and repeatability. Moreover, the sensor showed high selectivity to EE2 in the presence of several potential interfering compounds and frequently prescribed drugs, though it could not discriminate the similar hormone, 17β-estradiol, being the total concentration obtained in this case. CP-based sensors emerge as efficient electroanalytical tools, suggesting that modification of the surface may not always be beneficial in terms of sensitivity.

1. Introduction

The sustainability of aquatic species and consequently fisheries as well as the perception of food safety from the consumption of seafood are in part dependent on an effective management and control of pollution released into water bodies [1,2]. Substances designated as contaminants of emerging concern (CECs) are an environmental problem capable of negatively impact ecosystems and consequently human health, that need serious attention (from governmental agencies, the scientific community as well as the general public). In particular, pharmaceutical compounds are a relevant group of CECs [3–5] as they are increasingly consumed by an ever-growing world population [6,7]. Since about 80% of wastewaters are estimated to be released without any treatment [8] and allied to some relatively inefficiency of treatment plants [8–10], pharmaceuticals are ubiquitous in the aquatic environment, being thus qualified at least as pseudo-persistent compounds [4,5]. In addition, some type of pharmaceuticals present toxicological activity on the level of endocrine disruption or bacterial resistance as the case of hormones, some anti-inflammatories and antibiotic drugs [11,12]. In this regard, 17α-ethinylestradiol (EE2) is a synthetic hormone with endocrine disruptive properties capable of causing harm in aquatic ecosystems even at trace levels. It is a widely prescribed drug [13], which consequently leads to be frequently found in both fresh and marine waters [14], being thus bioavailable and detected in aquatic animal species as highlighted in recent reviews [14,15]. Due to insufficient information concerning the presence of EE2 in the aquatic environment, the European Commission included this hormone alongside 9 other potentially hazardous substances in the 1st watch list created in 2015 [16]. Besides the importance of environmental monitoring, analysis of EE2 can be also critical for industrial quality control processes and for the clinical field.

The environmental determination of hazardous compounds has been performed mostly by conventional analytical techniques due to their reliable and high throughput capacity. In turn, sensor technology has

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interesting particularities in comparison to these established techniques especially on the level of versatility, portability and sustainability principles that can result in potential alternative solutions in the near future [17–19]. Specifically regarding electrochemical sensors, several works have already been developed for the detection of this hormone exploiting the characteristics of different transducing materials (such as hanging mercury drop electrode (HMDE) [20,21], indium tin oxide (ITO) [22], fluorine doped tin oxide (FTO) [23]) including the widely used carbon-based electrodes (glassy carbon (GCE) [24–26], carbon paste (CPE) [27], boron-doped diamond (BDD) [28] and screen printed (SPCE) [21,29,30]). To achieve good analytical performance most of these EE2 sensors employ surface modification with diverse (nano) materials.

In the present work, we propose a simple but highly sensitive electrochemical sensor based on an unmodified and untreated carbon paper (CP) transducer for EE2 detection in aquatic ecosystems, i.e., water but also in complex samples such as fish. Carbon paper is a porous fiber matrix with high specific surface area and excellent mechanical and electronic properties that has recently been under the focus of the scientific community for electroanalysis [19]. However, the influence of surface pre-treatment (chemical and/or electrochemical) on CP performance has not yet been thoroughly studied. Moreover its application for pharmaceutical compounds screening is still very limited (progesterone [31] and ketoprofen [32]) leading to interesting research opportunities in the study of this type of contaminants and CP surface modification strategies. As far as we know, this is the first work concerning the detection of hormonal compounds by this interesting material and the effect of surface pre-treatments on the transducer performance.

2. Experimental methods

2.1. Materials, reagents and solutions

All chemicals were analytical grade and used as received without further purification.

The reagents 17α-ethinylestradiol, 17β-estradiol, acetaminophen, acetone, acetonitrile, acetylsalicylic acid, ascorbic acid, diclofenac, disodium hydrogen phosphate, hydrochloric acid (37%), nitric acid (65%), sulfuric acid (98%), lactose, potassium hexacyanoferrate (III), potassium hexacyanoferrate (II) trihydrate, sodium dihydrogen phosphate and sulfuric acid (95%) were acquired from Sigma-Aldrich (Germany). Glutamic acid, phosphoric acid, tetrachloroauric acid and sodium sulphate were acquired from Merck (Germany) whereas boric acid and potassium chloride were from VWR (Belgium), acetic acid glacial and ethanol absolute anhydrous from Carlo Erba Reagents (France), sodium hydroxide from Labkem (Spain), calcium carbonate from Fluka (Switzerland), sulfuric acid from Honeywell (USA) and lactose from Riedel-de Haën (Germany).

All aqueous solutions and electrolytes were prepared with ultrapure water obtained from a Milliporewater purification system (18 MΩ, Milli-Q, Millipore, Molsheim, France).

Stock solutions of EE2 and 17β-estradiol were prepared in absolute ethanol and then diluted with PBS pH 7 when necessary.

2.2. Instrumentation and measurements

The electrochemical assays were run with a potentiostat Metrohm, model Autolab PGSTAT12, controlled by GPES version 4.9 software (Herisau, Switzerland). The electrochemical characterizations were performed by cyclic voltammetry (CV) and differential pulse voltammetry (DPV) techniques using a three-electrode cell configuration where the CP sensor, a platinum rod and an Ag/AgCl (KCl, 3 M) electrode were respectively the working, counter and reference electrodes.

The CP sensor was simply assembled by cutting a rectangular piece of Toray carbon paper (TGP-H-60, 0.19 mm thickness; Alfa Aesar, Germany) with dimensions of about 2.5 × 0.7 cm^2 and covering one end with aluminium foil for better connection with a crocodile clip. An image of the CP sensor is displayed on Fig. 1 with scanning electron microscopy (SEM) analysis being performed on the surface and side of the sensor. For current density assessment, the geometric area immersed in the electrolyte was accurately measured after each analysis and corresponded to about 0.7 cm^2. Phosphate buffer solution (PBS) 0.1 M, pH 7, was used as electrolyte in all experiments except for pH optimization studies where Britton-Robinson (BR) buffer 0.1 M was used.

Modification of the CP sensor with gold nanoparticles (AuNPs) was also evaluated. The procedure comprised the electrodeposition of AuNPs through chronoamperometry performed at −0.2 V during 150 s using an aqueous solution of $HAuCl_4$ at different concentrations (0.1, 0.5 and 1 mM).

The different pre-treatments performed on the CP including the modification with AuNPs were characterized through SEM analysis and energy-dispersive X-ray spectroscopy (EDS) using a High resolution (Schottky) Environmental Electron Microscope with X-Ray Microanalysis and Electron Backscattered Diffraction analysis (FEI Quanta 400 FEG ESEM/EDAX Genesis X4M). Also, the tested CP sensors were characterized by Fourier-transform infrared spectroscopy (FTIR) using a ALPHA-P spectrophotometer (Bruker Corporation, Billerica, MA, EUA). The spectra resulted from the mean of 64 scans at 4 cm^{-1} resolution, in the 4000-350 cm^{-1} spectral range. Contact angle measurements were performed using high resolution smartphone camera (12 megapixel) with data analysed by open-source image software processor, ImageJ, with *dropanalysis* plugin. The electrochemical active areas were determined from the plot between peak current and scan rate, assessed by cyclic voltammetry and through application of Randles-Sevcik equation: $I_{pa} = (2.69 \times 10^5)\ n^{2/3}\ A\ D^{1/2}\ \nu^{1/2}\ C_0$ [33]. The concentration of hexacyanoferrate was 5 mM and the considered diffusion coefficient corresponded to 7.2×10^{-6} $cm^2\ s^{-1}$ [34].

All calibrations curves were performed through DPV measurements using the same sensor in the assessment of the blank (buffer solution) and all EE2 (or 17β-estradiol) concentrations. At each analyte addition, the solution was slightly hand-stirred for better homogenization and between each measurement the electrochemical cell was set to be off in order to avoid unintended deposition.

2.3. Sensor surface pre-treatment

A pre-treatment study of the CP was performed. The procedures adopted were based on literature studies that commonly employ either a simple surface cleaning step through a chemical pre-treatment [35,36] or a electrochemical pre-treatment [37] or a conjugation of the two [38]. The chemical pre-treatment consisted in the use of organic solvents or acids. Briefly, the CP was immersed in acetone or ethanol absolute and subjected to ultrasounds for 30 min (JP Selecta Ultrasounds HD, Spain) and dried in the oven at 60 °C for 15 min. Different acids, namely HCl, H_2SO_4, or HNO_3 at different concentrations (without dilution, i.e. 37%, 98%, and 65%, respectively, and 1 M) were similarly employed as the organic solvents in the cleaning of the surface. The electrochemical pre-treatment involved scanning between −0.2 and 1 V by CV for 50 scans at 50 mV s^{-1} testing H_2SO_4 as electrolyte solution with 3 different concentrations (0.1, 0.5 and 1 M). Lastly, a combination of both chemical and electrochemical pre-treatments was performed by applying sonication for 10 min to the CP immersed in acetone, ethanol or acids (1 M) followed by CV in 1 M H_2SO_4 (or in the corresponded acid of the sonication process) electrolyte. For better clarity, a flowchart of the pre-treatment study is presented in the support information (Fig. S1). The various pre-treatments and combinations were assessed by CV at 50 mV s^{-1} in 5 mM $Fe(CN)_6^{3-/4-}$ solution with 0.1 M KCl.

2.4. Application in spiked real samples

The CP sensor (without modification) was applied for EE2 detection in two different types of samples namely river water and fish. The water

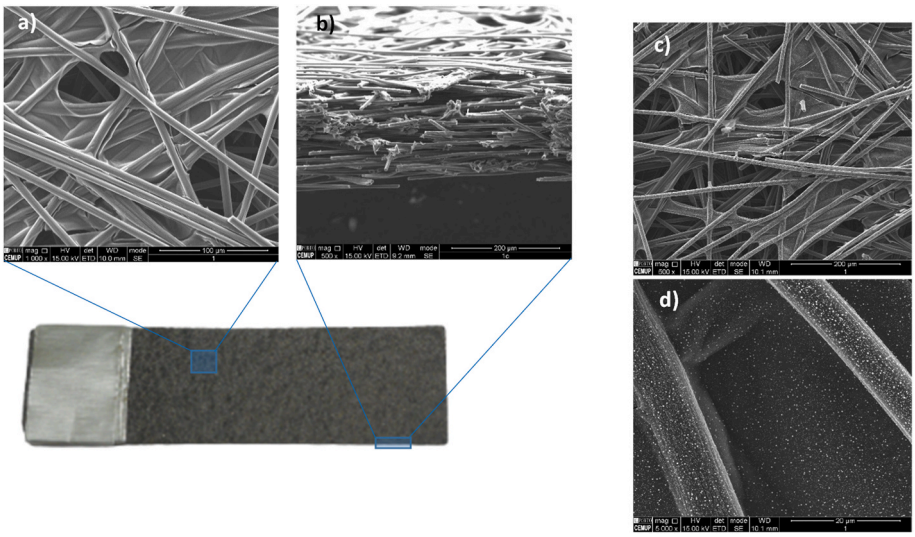

Fig. 1. Image of the carbon paper sensor displaying scanning electron microscopy analysis of the untreated surface a) at 1000 times magnification and b) of the side at 500 times magnification, and the gold nanoparticles modified surface with magnifications of c) 500 times, d) 5000 times. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

samples were collected in Lis river after a discharge from a wastewater treatment plant and was diluted in PBS 0.3 M pH 7 in the ratio 2:1 (sample/buffer). About 10 mL of this mixture was directly analysed for the presence of the hormone and then further spiked with EE2 stock solutions in order to obtain different concentrations, namely 0.001, 0.005, 0.01, 0.05 and 0.5 μM.

Regarding the solid samples, *Sardina pilchardus* fish specimens were obtained from a local market (Porto, Portugal), and were subjected to a validated extraction methodology [39]. The procedure consisted in weighting about 0.5 g portion of edible fish tissue into 50 mL falcon tube, spiked with EE2 stock solution and mixed in the vortex (VWR VV3, United Kingdom) for 30 s. Next, 5 mL of water were added, vortexed and followed by the addition of 10 mL of acetonitrile. After hand shacking for 30 s, QuEChERS salts (4 g magnesium sulphate and 1 g sodium chloride; Agilent, USA) were added and agitated for 1 min followed by centrifugation (Thermo Scientific Heraeus Megafuge 16R) at 4000 rpm during 5 min at 4 °C. The supernatant (6 mL) was then transferred to a 15 mL tube containing the dispersive solid phase extraction salts (composed of 150 mg C18 and 900 mg magnesium sulphate; Agilent USA), agitated in the vortex during 1 min and centrifuged at 13,000 rpm for 3 min at 4 °C. The obtained supernatant was finally evaporated under nitrogen stream and redissolved in a given volume of water:acetonitrile (4:1 v/v) accordingly to the desired final concentration. An appropriate aliquot of this extract was then added to 10 mL electrolyte in the electrochemical cell for the analysis by the standard addition method.

3. Results and discussion

3.1. Sensor surface pre-treatment

In electroanalysis, optimal performance is dependent on the electrode surface. Pre-treatment procedures aim to increase the electrochemical activity and achieve a reproducible response, through the formation of reactive oxygen functionalities or elimination of adsorbed impurities and/or removal of surface layers originating a more pristine surface. This way, and for the first time, a pre-treatment study of the CP sensor was conducted using chemical and/or electrochemical procedures by employing organic solvents (absolute ethanol and acetone) and acids (HCl, H_2SO_4 and HNO_3) (Fig. S1). The impact of each pre-treatment on the signal was characterized using the redox system $Fe(CN)_6^{3-/4-}$ by CV with the voltammograms being displayed in Fig. S2 and the respective peak heights and peak-to-peak separation presented in Table S1; these assays were complemented by morphological and physico-chemical characterizations. Generally, in terms of peak heights, the CP performed slightly better when chemically treated with acetone compared with ethanol (Fig. S2a) and the same is observed for H_2SO_4 used rather concentrated (Fig. S2b) or diluted to 1 M (Fig. S2c) when compared with the other acids. In the electrochemical pre-treatment, a higher electrolyte concentration resulted in higher voltammetric peaks (Fig. S2d), which was further selected for the combination between chemical and electrochemical pre-treatment (Fig. S2e and Fig. S2f). A summary of these results comparing the best of each pre-treatment is represented in Fig. 2 and the determined peak heights presented in Table 1. It seems evident that the electrochemical pre-treatment is more effective for the redox system in study compared to the chemical pre-treatments. The peaks intensity further increases by combining the chemical treatment in sulfuric acid (1 M) and the electrochemical pre-

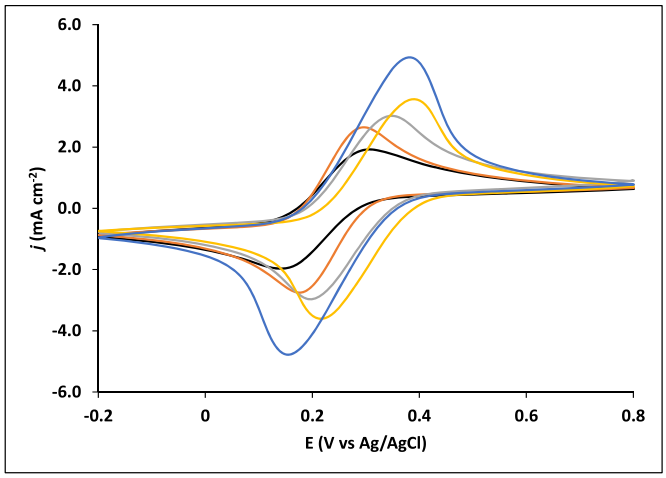

Fig. 2. CV analysis of 5 mM $Fe(CN)_6^{3-/4-}$ (in 0.1 M KCl) comparing the different types of CP pre-treatments: without pre-treatment (black line), chemical with acetone (orange line), chemical with H_2SO_4 1 M (grey line), electrochemical with 1 M H_2SO_4 as electrolyte (yellow line) and combination of chemical with H_2SO_4 1 M and electrochemical with 1 M H_2SO_4 as electrolyte (dark blue line). *S*can rate 50 mV s^{-1}. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Peak heights and peak-to-peak separation obtained from CV analysis of $Fe(CN)_6^{3-/4}$ for the optimum conditions for each type of pre-treatment applied to the CP sensor. Chemical pre-treatment: sonication in organic solvents or acids; electrochemical pre-treatment: cyclic voltammetry in acids as electrolyte.

Type of pre-treatment	i_{pa} (mA cm^{-2})	i_{pc} (mA cm^{-2})	ΔEp (V)
Without pre-treatment	2.40	2.40	0.165
Chemical (acetone)	2.90	2.90	0.122
Chemical (H_2SO_4 1 M)	3.25	3.30	0.152
Electrochemical (H_2SO_4 1 M)	3.90	3.90	0.180
Chemical (H_2SO_4 1 M) + electrochemical (H_2SO_4 1 M)	5.10	5.00	0.228

treatment using the same acid and concentration as electrolyte. The rise in peak heights is attributed to an increase of the hydrophilicity of the CP material, which is confirmed by the contact angle measurements presented on Fig. S3. Consequently, a better diffusion of species in the carbon fiber matrix is achieved with this assumption being also confirmed by the determination of the electrochemical active surface area that doubles for the CP treated with both ultrasounds and CV in H_2SO_4 (2.8 cm^2; Fig. S4) compared with the untreated CP (1.4 cm^2; Fig. S4). Regarding the efficiency of the pre-treatments on the electron transfer rate (peak-to-peak separation) of the probe, it seems that the chemical pre-treatment with acetone is the most effective (Table 1) as it lowers the peak separation compared with the untreated CP. This is probably due to the cleaning of the CP surface by removing impurities or debris that appear to be present in the untreated surface as seen in the SEM images with higher magnifications (Fig. S5). A similar effect was achieved with the other pre-treatments although no significant differences between them could be detected by SEM (Fig. S5) as well as EDS analysis (Fig. S6), which shows similar elemental composition of carbon and oxygen between pre-treatments. Besides cleaning the surface, treatment with acids, especially those involving electrochemical methods are known to generate an oxide layer capable of positively affecting the electron transfer kinetics [40,41]. An alteration of surface chemistry using acidic pre-treatments was detected through FTIR characterization (Fig. S7). The CP without pre-treatment displays characteristic C–H bonds at approximately 2800–2900 cm^{-1} and 1470 cm^{-1} but also the presence of some oxygen functionalities at 1700 cm^{-1} (C═O) and 1440 cm^{-1} (O–H). While the pre-treatment with organic solvents (acetone) results in practically unchanged spectra, the CP pre-treatment in acid using both sonication and CV leads to appearance of a broad peak around 3250 cm^{-1} attributed to O–H, which is characteristic of carboxylic acid groups besides the peak at 1700 cm^{-1} [42]. However, the likely presence of interstitial moisture in the CP matrix may contributed to some overlapping, due to H_2O characteristic groups transmitting in the region 3300-3450 cm^{-1} and 1650 cm^{-1} [42]. The effects of oxygen functionalities on the electron transfer rate of $Fe(CN)_6^{3-/4-}$ are not entirely consensual [43] with some authors affirming to be affected more by electrode surface state than the presence of surface oxides [41]. In this study, we observe a slight increase on peak separation for the CP treated with electrochemical methods (Table 1), which might suggest participation of oxygen functional groups. Conversely, yet to be explained is the nature of the resin that is used to bond the carbon fibers and if it contributes to surface changes when subjected to electrochemical pre-treatments. Nevertheless, from an analyte determination perspective, the attainment of higher peaks would be advantageously over kinetics, and so pre-treatment using both sonication and CV was performed on CP being tested in the analysis of the hormone drug, EE2.

3.2. Electrochemical behaviour of 17α-ethinylestradiol

EE2 was first electrochemically characterized through CV in buffered conditions (pH 7), showing an oxidation peak at around +0.5 V with no reduction peak being observed in the studied potential window. Three different sensors, namely, the pre-treated CP (in H_2SO_4 1 M by sonication followed by CV in the same reagent), CP without pre-treatment and a AuNPs modified CP (CP/AuNPs – electrosynthesized AuNPs on CP confirmed by SEM, Fig. 1c and d, and EDS analysis, Fig. S8) were tested to assess the electrochemical behaviour of EE2 through CV (Fig. 3a). Although all three CP sensors presented a defined peak, a slightly better (25–35%) signal was achieved by the untreated and unmodified CP sensor compared with the treated CP and the CP/AuNPs. In fact, the CP/AuNPs presented the lowest peak height, attributed to a partial overlap with the characteristic peaks of Au appearing at a more positive potential (Fig. 3a). These results became more evident using DPV at a concentration of 0.5 μM (Fig. 3b). Surprisingly, the untreated bare CP gave a much higher (22.8 μA cm^{-2}) and better-defined peak, explained by the low background current, when compared with the pre-treated CP (10.3 μA cm^{-2}) or the CP/AuNPs (14.2 μA cm^{-2}). Accordingly to McCreery and Cline [40], electrochemical pre-treatments can originate higher background currents, which can help to justify the poorer performance of the pre-treated CP. In addition, this type of pre-treatment can enhance the selectivity towards certain charged species [40,41]. The creation of oxygen functionalities at the CP surface with negative charge leads to electrostatic repulsion of anionic species [44], which seems to be the case of EE2 molecule, lowering its signal. Henceforth, a CP sensor without pre-treatment and modification was used in the subsequent assays.

In order to determine the type of electron transfer mechanism involved on the oxidation of EE2 at the surface of the CP sensor, the anodic peak was evaluated at different scan rates (10–200 mV s^{-1}), with the results being displayed in Fig. S9. A linear relationship between peak intensity and scan rate (Fig. S9b) is more evident when compared to the plot between peak intensity and the square-root of scan rate (Fig. S9c) pointing to an electrochemical reaction controlled by adsorption at the CP surface. These results are in line with other literature studies [26–28, 45].

3.3. Optimization of the 17α-ethinylestradiol detection

Preliminary results on the detection of EE2 by the CP sensor showed higher sensitivity using DPV technique in comparison with square-wave voltammetry (results not presented) probably due to the reaction kinetics, being DPV thus selected for EE2 detection assays. The parameters of DPV were thoroughly optimized as well as the electrolyte pH in order to achieve maximum sensitivity of the sensor towards EE2. The influence of pH on EE2 oxidation was first assessed by varying the pH from 2 to 12 (Fig. 4a), reaching a maximum signal at pH 7, which then decreased constantly with further augmentation of pH (Fig. 4b). The peak potential also shifted significantly towards less positive values with the pH increase, with a linearity proportion of −0.057 V/pH which is close to the theoretical value of 59 mV/pH of the Nernstian equation, indicating the involvement of one proton for each electron transferred in the oxidation reaction of EE2 [25]. An electrolyte pH of 7 was selected for further optimizations and EE2 analysis, based on the peak intensity achieved.

Regarding optimization of the DPV technique, each parameter was varied by maintaining the others constant. Initially, modulation amplitude was varied from 10 to 200 mV, reaching a maximum peak intensity at 90 mV and then slightly decreasing onward (Fig. S10a). This value was selected for the assessment of modulation time (3–30 ms) that presented a maximum peak height at 3 ms (Fig. S10b). The ratio between step potential and interval time defines the scan rate of the technique. Variation of the interval time between 2 and 0.1 s resulted in the highest peak at 0.1 s (Fig. S10c), whereas in step potential a value of 10 mV was selected as optimum despite further peak increase at higher step potentials (Fig. S10d) due to measurement repeatability purposes.

A pre-concentration step of the analyte at the CP surface was also evaluated with respective optimization of deposition time (0–240 s) and

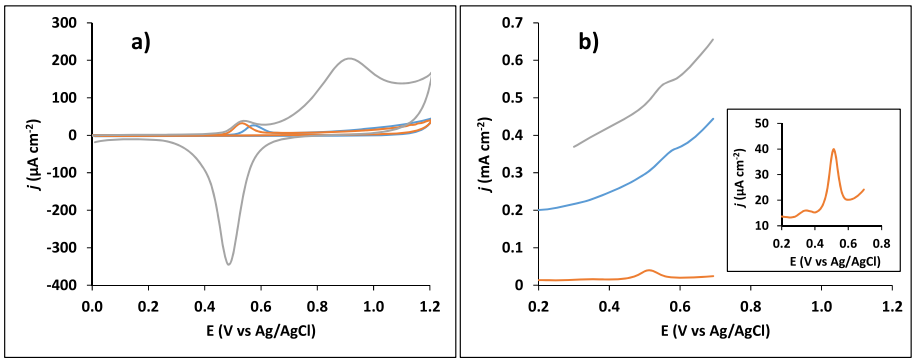

Fig. 3. Voltammetric behaviour of 17α-ethinylestradiol by pre-treated CP (blue line), CP without pre-treatment (orange line) and a CP/AuNPs sensor (grey line). a) Cyclic voltammetry of 10 μM 17α-ethinylestradiol in 0.1 M PBS pH 7 (scan rate 100 mV s^{-1}, first scan). b) Differential pulse voltammetry of 0.50 μM 17α-ethinylestradiol in 0.1 M PBS pH 7 (modulation time 0.005 s, modulation amplitude 0.07 V, interval time 0.2 s, step potential 0.01 V). Inset: Response of the CP without pre-treatment in a different scale of units of current density. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

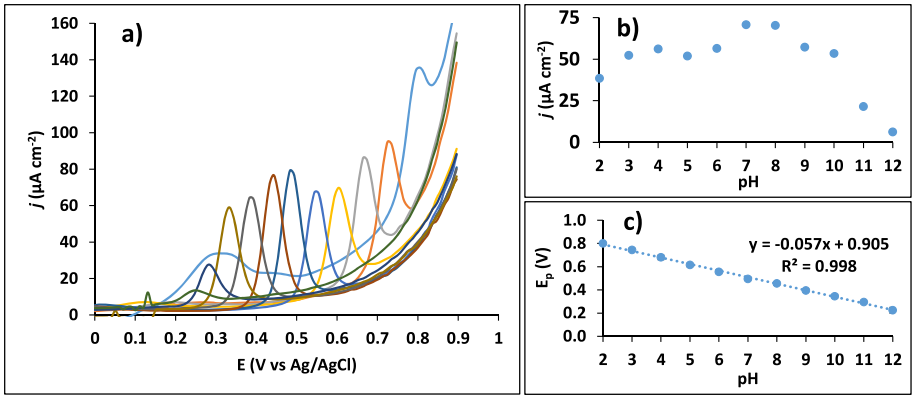

Fig. 4. Optimization of pH on 17α-ethinylestradiol oxidation. a) DPV analysis of 1 μM 17α-ethinylestradiol in 0.1 M BR buffer at different pH values (modulation time 0.005 s, modulation amplitude 0.07 V, interval time 0.2 s, step potential 0.01 V). b) Influence of pH in peak current intensity. c) Influence of pH on peak potential. Note: errors bars of standard deviation presented though hidden by bullet points.

deposition potential (−0.2, 0, 0.2, 0.4, 0.5, 0.6 and 0.8 V). In the first case, the peak intensity increased linearly with the deposition time, however, a duration of 180 s was selected to prevent extended analysis time as the difference to the deposition time of 240 s was not significant (Fig. S11a). Regarding the deposition potential, the applied value of 0.4 V showed a significantly higher peak (about 25–27% more) compared with −0.2, 0 and 0.2 V, being thus considered as optimum (Fig. S11b). When the applied potential was equal (about 0.5 V) or higher (0.6 and 0.8 V) than the oxidation potential of EE2, the signal significantly decreased since the drug reacted at the CP surface prior to the voltammetric scan.

3.4. 17α-ethinylestradiol electroanalytical features

The analysis of EE2 at different concentrations was then characterized by the DPV technique through addition of known volumes of EE2 stock solution to an initial 10 mL of 0.1 M PBS pH 7 (Fig. 5). After each addition the electrochemical cell was stirred for better homogenization of the solution, followed by the deposition step at +0.4 V during 180 s. From the calibration data displayed on Fig. 5a (mean data from 3 independent calibration curves), a wide linear range from 1.00×10^{-4} to 1.00 μM (Fig. 5a–b) can be observed, with four distinct linear zones, specifically from 1.00×10^{-4} to 1.00×10^{-3} μM (Fig. S12a), 1.00×10^{-3} to 1.00×10^{-2} μM (Fig. S12b), 1.00×10^{-2} to 1.00×10^{-1} μM (Fig. S12c) and from 1.00×10^{-1} to 1 μM (Fig. S12d). The figures of merit regarding sensitivity (obtained from the slope of the respective calibration curve) and the limit of detection (LOD) (calculated based on the standard deviation of the response and slope; $LOD = 3.3\sigma_{residual\text{-}CC}/slope$) [46] for each linear zone are presented in Table 2. The CP sensor presented a remarkable sensitivity of 1636 ± 232 μA μM^{-1} cm^{-2}, which

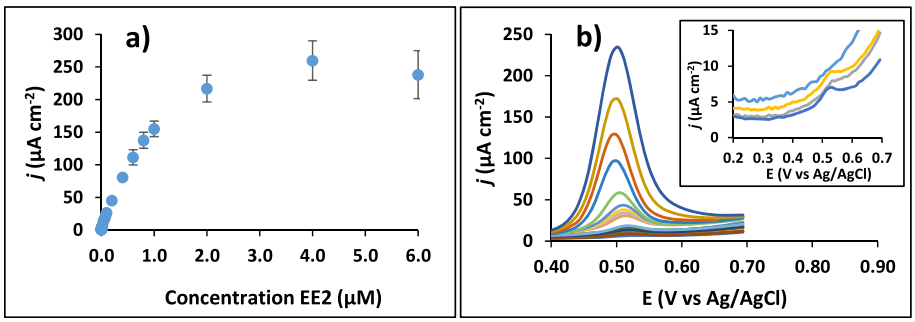

Fig. 5. Analysis of 17α-ethinylestradiol at different concentrations with CP sensor in 0.1 M PBS pH 7. a) Calibration curve from 0.1 to 6000 nM; b) DPV curves of 17α-ethinylestradiol at 0.1 nM to 1 μM using the optimized parameters (modulation amplitude 90 mV, modulation time 3 ms, interval time 0.1 s, step potential 0.01 V, deposition potential 0.4 V, deposition time 180 s). Insert: DPV curves for the blank and lowest determined concentrations of 0.1, 0.2 and 0.4 nM.

Table 2
Literature studies on electrochemical sensors for 17α-ethinylestradiol detection.

Sensor	Sensitivity (uA uM^{-1}/μA μM cm^{-2})	Linear range (μM)	LOD (nM)	Deposition (V/s)	Real sample	Reference
CP	-/1636 ± 232	$1.00 \times 10^{-4} - 1.00 \times 10^{-3}$	0.14 ± 0.005	+0.40/180	River water Fish	This work
	-/340 ± 57	$1.00 \times 10^{-3} - 1.00 \times 10^{-2}$	1.39 ± 0.14			
	-/237 ± 19	$1.00 \times 10^{-2} - 1.00 \times 10^{-1}$	13.5 ± 3.1			
	-/145 ± 19	$1.00 \times 10^{-1} - 1.00$	157.7 ± 23.7			
CP/AuNPs	-/127	0.1–0.8	66	–	–	This work
GCE/MWCNT-nafion/NiTPPS	0.12/15.3	0.2–60	120	–	Lake water Tap water	[24]
GCE/Ni	0.0027/0.039	0.5–80	130	–	River water	[50]
GCE/rGO-RuNPs	1.86/-	0.055–1.2	2	–	Urine	[51]
GCE/CB-Nafion	-/-	0.25–3	130	−0.35/240	Pharmaceuticals	[52]
GCE/MWCNT/CoPc	0.18/-	2.5–90	2200	–	Milk River water	[45]
GCE/Pd–C_3N_4-MWCNT	0.072/-	2–150	500	–	Feedstuff	[25]
GCE/MWCNT/TA-Fe_3O_4-AuNPs	0.676/9.52	0.01–120	3.3	–	Serum Urine Wastewater	[26]
HMDE	102/-	0.0019–0.600	0.59	−0.80/60	Plasma	[20]
HMDE	0.037/11.6	0.0068–0.20	1.7	−0.70/150	River water	[48]
HMDE	-/-	0.068–0.6	12	−0.05/60	Pharmaceuticals	[49]
HMDE	0.114/-	up to 1.4	33	−0.60/30	Pharmaceuticals	[21]
SPCE	0.374/-	up to 4	615	0/90	Urine	
SPCE(CNT)	0.329/-	up to 5.4	649	–		
SPE/rGO-Nafion-GQD	4.4/62.0	0.01–2.5	2.6	0/75	River water Serum	[29]
SPCE/Ni	0.0209/0.166	0.23–30	52	–	Struvite Urine	[30]
CPE	0.125/15.9	0.05–20	30	+0.30/120	Pharmaceuticals	[27]
FTO/Chit/CNT	0.216/-	0.05–20	90	–	Urine	[23]
BDD	0.106/0.43	0.99–5.2	300	–	Dam water	[28]
ITO/FeNPs/NiTsPc	-/0.308	0.07–30	7.8	–	Tap water Wastewater	[22]

AgNPs – silver nanoparticles, AuNPs – Gold nanoparticles, BDD – Boron-doped diamond, CB – Carbon black, Chit – Chitosan, CoPc – Cobalt phthalocyanine, CP – Carbon paper, CPE – Carbon paste electrode, FeNPs – Iron nanoparticles, FTO – Fluorine-doped tin oxide GCE – Glassy carbon electrode, GQD – Graphene quantum dots, ITO – Indium tin oxide, HMDE – Hanging mercury drop electrode, MWCNT – Multi-walled carbon nanotubes, NiTPPS - Ni(II)tetrakis(4-sulfonatophenyl) porphyrin, NiTsPc – Nickel phthalocyanine, rGO – Reduced graphene oxide, RuNPs – Ruthenium nanoparticles, SPCE – Screen-printed carbon electrode, SPE – Screen-printed electrode, TA – Tannic acid.

resulted in a low LOD value corresponding to 0.14 ± 0.005 nM. This sensitivity was expected for this transducing material since it is characterized by a high porosity, translating thus in a high specific surface area [19,47], which is confirmed by the high (about 2 times higher) electrochemical active area determined (Fig. S4) in comparison to the considered geometrical area. This is evident from the SEM images (Fig. 1 and Fig. S5) that show a porous carbon fiber matrix. Also on the SEM image displayed in Fig. 1b, it is observable that the sides and limits of the CP sensor are full of carbon fibre ends, which are normally rich in edge plane sites and functional groups that improve the electron transfer rate of the electrode [40], which is also confirmed by the presence of some oxygen functionalities on the untreated CP (light blue line FTIR spectrum in Fig. S7). Comparing with the available literature studies on electrochemical sensors for EE2 detection (Table 2), the developed CP sensor showed the best analytical performance in terms of LOD and sensitivity. From these previous sensors, the ones based on mercury transducers showed also good analytical performance [20,48,49], though their contemporary application is controversial due to the high toxicity of mercury. Sensors based on GCE have also been widely employed for EE2 quantification [24–26,45,50–52]. However, to attain good sensitivity they require some level of modification, increasing the complexity of the sensor. The same can be observed for SPCE-type sensors since an unmodified SPCE or simply modified with carbon nanotubes (SPCE(CNT)) obtained high LOD values for EE2, in the order of 600 nM [21]. Hence, the proposed sensor is advantageously simple and efficient for EE2 detection. Also, as comparison purposes, the electroanalytical features of the CP/AuNPs sensor were assessed with respective results displayed in Fig. S13. The CP/AuNPs achieved a sensitivity of 127 μA μM^{-1} cm^{-2} and a LOD of 66 nM corresponding to about 8% of the sensitivity value and a LOD 500 times higher when compared to the unmodified and untreated CP sensor. To the best of our knowledge, only one literature study employed AuNPs in sensor configuration alongside other nanomaterials such as multi-walled carbon nanotubes (MWCNT) and Fe_3O_4 magnetic particles, with this conjugation resulting in a lower LOD of 3.3 nM but also lower sensitivity (9.5 μA μM^{-1} cm^{-2}) [26]. Overall and accordingly to Table 2, the CP/AuNPs sensor performed better (in terms of LOD) than most GCE based sensors [24,25,45,50,52], the unmodified SPCE [21] and the BDD sensor [28].

The reproducibility and repeatability of the CP sensor were also evaluated for two different concentrations (0.01 and 0.5 μM). For five different sensors, the reproducibility corresponded to a relative standard deviation (RSD) of 9.3% for 0.01 μM and 5.8% for 0.5 μM whereas repeatability for 5 sequent measurements using the same sensor gave a mean (n = 5) RSD of 5.5% for 0.01 μM and 5.3% for 0.5 μM.

3.5. Real samples analysis

The CP sensor was finally applied for detection of EE2 in river water and fish samples. No peak was obtained when the samples were directly analysed, therefore validation of the sensor was performed through spiking with known concentrations of EE2. For river water samples, seven different spiking levels were assessed from 0.001 to 0.5 μM with recoveries ranging from 91.2 to 107.3% and RSD varying from 1.3 to 4.6% (Table 3). Regarding fish samples, the standard addition method was employed given the complexity of the matrix, with the CP sensor achieving acceptable recoveries of 109.0, 104.5 and 102.2% for spiking levels of 0.002 0.02 and 0.2 μM, respectively (Table 3). To the best of our knowledge and as seen in Table 2, no other literature studies have considered such challenging matrices in EE2 detection.

Table 3
Analysis of real samples spiked with 17α-ethinylestradiol.

Sample	Spiking concentration (μM)	Recovery (%)	RSD (%)
River water	0.001	91.2	4.6
	0.005	94.5	3.2
	0.01	94.0	2.9
	0.05	97.4	1.3
	0.5	107.3	4.3
Fish	0.002	109.0	7.1
	0.02	104.5	1.4
	0.2	102.2	2.7

The selectivity of the CP sensor towards EE2 was assessed using different compounds that may be usually present as excipients in pharmaceutical formulations or in samples from environmental and physiological origin (Fig. S14). The widely used pharmaceutical drugs diclofenac, acetaminophen and aspirin were individually tested in a 1:1 proportion with EE2 (0.5 μM) showing practically no influence on the voltammetric peak, with an interference level (%current ratio = $i_{EE2-+interferent}/i_{EE2}$ x 100) of 102, 110 and 101% respectively (Table S2). The mixture of these three drugs with EE2 exerted also negligible interference (108%). Several other compounds were analysed as possible interferents namely ascorbic acid, glutamic acid, glucose, lactose, sodium nitrite, calcium carbonate and sodium sulphate. From these, only ascorbic acid affected EE2 signal which increased 25%, however the simultaneous analysis of all compounds mixed with EE2 showed no interference (Fig. S14, Table S2). Conversely, in the presence of an equal concentration of 17β-estradiol the peak height doubled which demonstrates, as expected, no discriminatory capacity of the CP sensor between these two similar estrogenic drugs. In fact, the detection of 17β-estradiol at different concentrations using the same analytical conditions led to similar results as EE2 detection through attainment of a wide linear range from 0.0002 to 4 μM (Fig. S15), corresponding to highest sensitivity and lowest LOD values of 1447 μA μM^{-1} cm^{-2} and 0.09 nM respectively. Although not tested, discrimination of both hormones could probably be achieved by performing a new comprehensive optimization study seeking significantly different experimental conditions (reduction vs. oxidation of the hormones, pH, *etc.*). Consequently, the CP sensor is suitable for total quantification of these two hormones when applied for environmental samples since its composition is normally unknown.

4. Conclusions

In the present work a very simple and efficient electrochemical sensor for 17α-ethinylestradiol was effectively developed, based on an unmodified and untreated carbon paper. Surprisingly this sensor showed better performance when compared to a pre-treated carbon paper or modified with gold nanoparticles, simplifying the sensing system. A remarkable high sensitivity and a low LOD was achieved for 17α-ethinylestradiol, which can be attributed to the structural and electrocatalytic properties inherent to this type of material. The optimization of the elctroanalytical conditions namely electrolyte pH and differential pulse voltammetry parameters enabled a selectivity enhancement towards potential interferents, however no discrimination was observed when the similar hormone 17β-estradiol was present. The carbon paper sensor was validated in spiked real samples of river water and complex fish matrices resulting in acceptable recoveries. The characteristics of this sensor in terms of dimensions, simplicity and performance envisage its successful application in both environmental and clinical analytical fields.

Credit author statement

Álvaro Torrinha: Methodology, Data curation, Writing- Original draft preparation. Pedro Carneiro: Methodology. Diana Dias: Methodology. Miguel Tavares: Methodology. Cristina Delerue-Matos: Funding acquisition. Simone Morais: Writing- Reviewing and Editing, Supervision, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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