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β -Cyclodextrin carbon nanotube-enhanced sensor for ciprofloxacin detection

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ABSTRACT

A simple and expedite electrochemical methodology was developed for the determination of ciprofloxacin, based on a glassy carbon (GC) electrode modified by a combination of multi-walled carbon nanotubes (MWCNT) with β -cyclodextrin (β -CD) incorporated in a polyaniline film. The combined use of β -CD and MWCNT in the electrochemical sensor leads to a significant signal improvement. The β -CD/MWCNT modified GC electrode exhibited efficient electrocatalytic behavior in the oxidation of ciprofloxacin with relatively high sensitivity, stability and lifetime. Molecular modeling studies showed that ciprofloxacin binds preferably to β -CD rather than to CNT edges, leading to an improved sensitivity of the sensor. Under optimized conditions, a linear calibration curve was obtained for ciprofloxacin in the concentration range 10–80 μ M with a detection limit of 50 nM. The analytical performance of this sensor was evaluated for the detection of ciprofloxacin in a wastewater treatment plant effluent.

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electrochemical sensor;
 β -cyclodextrin; carbon
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Introduction

Pharmaceuticals are nowadays intimately associated with better public health and quality of life. Nevertheless, the growing use of pharmaceuticals is becoming a new environmental problem, both via human and animal urinary or faecal excretion and pharmaceutical manufacturing discharges, leading to increasing concentrations of pharmaceuticals reaching sewage treatment plants. The use of large amounts of antibiotics, hormones, analgesic and sedative drugs, and different disinfection preparations as well as the difficulty of achieving their complete inactivation in water treatment is a serious problem, since treatment plants were not originally designed for the elimination of xenobiotics.^[1–3] Recent reports have shown some evidence that substances of pharmaceutical origin are inefficiently eliminated during wastewater treatment and are not biodegraded in the environment.^[4–6] Among the wide variety of pharmaceutical compounds, antibiotics assume special significance due to their extensive use in human therapy, in veterinary medicine, as husbandry growth promoters, and to their ability to alter microbial community structure, facilitating the development of antibiotic-resistant human pathogens.^[1,7–10] High levels of broad-spectrum antibiotics are likely to promote the development of highly antibiotic-resistant microorganisms and possibly the horizontal transfer of resistance factors to human pathogens.^[11]

The volume of outpatient antibiotic use increased in most European countries between 1997 and 2009.^[12] Over this period, the relative use of quinolones significantly increased with respect to other major antibiotic groups such as macrolides and sulphoamides.^[12] The quinolone class of antimicrobial agents has

generated considerable interest since its discovery some 50 years ago.^[13] Since the mid-1980s, fluoroquinolones have gained broad acceptance in hospitalized and community patients since they have excellent in vitro activity against a wide range of both Gram-negative and Gram-positive organisms. They can be prescribed orally, with excellent bioavailability. Administered fluoroquinolones are mostly excreted as unchanged compounds in urine and discharged into hospital sewage or municipal wastewater.^[14] Sewage water treatment plants are not able to completely remove these compounds, and thus, significant quantities of the active compound are transported into environmental aquatic systems.^[14] Fluoroquinolones have been found in high concentrations in effluents from sewage treatment plants in several European countries, such as Portugal, and also in the United States and Canada.^[3,8,15–17] Monitoring studies are therefore needed at a local level to determine exactly how the antibiotics make their way into public waterways and to better understand their transport and environmental fate.

Most of the methods available for the determination of fluoroquinolones in environmental water samples involve the use of HPLC with fluorescence, mass spectrometric, or tandem mass spectrometric detection.^[18] Electrochemical methods have been found to be a highly sensitive and effective tool for the analysis of important pharmaceutical and environmental compounds owing to their simplicity, low cost and relatively short analysis time compared to other analytical techniques.^[19–21] Therefore, the development of sensitive sensors to monitor hazardous substances in the environment is an active area of research.

Sensors using cyclodextrin-incorporated multi-walled carbon nanotubes (MWCNTs) on polyaniline (PANI) modified glassy carbon electrodes (GCE) have recently been successfully used to study and quantify many organic molecules because of the promising properties of both materials.^[22–24] The chemical recognition of guest molecules by the use of cyclodextrins (CDs) is combined with the added advantage of a faster electron transfer process due to the CNT present at the electrode interface. Based on this approach, a novel strategy based on the simultaneous modification of a glassy carbon electrode with a novel polyaniline–CNT cyclodextrin matrix (PANI– β -CD/fMWCNT) is proposed for the determination of the fluoroquinolone antibiotic ciprofloxacin (Scheme 1). Molecular dynamics calculations were carried out to gain insight at the molecular level into the β -CD/CNT/ciprofloxacin interactions. The modified electrode was used for the determination of ciprofloxacin in water samples by cyclic voltammetry.

Materials and methods

Reagents

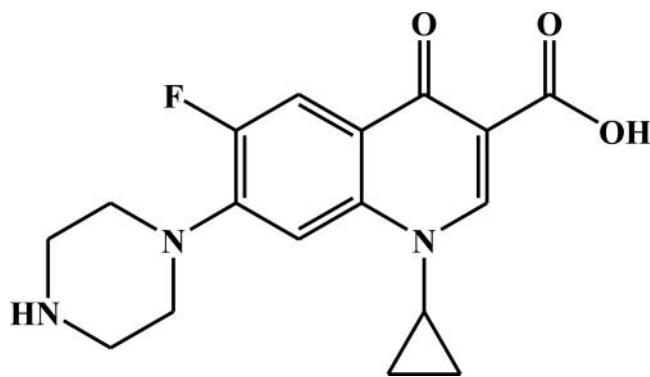
Multi-walled carbon nanotubes (MWCNT) were obtained from NanoLab (Waltham, MA, USA). Ciprofloxacin, aniline and β -cyclodextrin (β -CD) were supplied by Sigma-Aldrich Química (Sintra, Portugal). All other chemicals and reagents (Sigma-Aldrich Química) employed were of analytical grade and were used as received without any further purification.

All solutions were prepared with deionised water (Milli-Q–50 18 M Ω cm). Buffer solutions employed for voltammetric determinations were 0.1 mol L^{–1} in the pH range of 3–9.

Apparatus

Voltammetric experiments were performed using an Autolab PGSTAT 12 potentiostat/galvanostat (Metrohm Autolab, the Netherlands) in a one-compartment glass electrochemical cell equipped with a three-electrode system arrangement. The working electrode used was a bare or a modified glassy carbon electrode (GCE, $d = 2$ mm), the counter electrode was a platinum wire, with a saturated Ag/AgCl reference electrode completing the circuit. All measurements were carried out at room temperature ($25 \pm 1^\circ\text{C}$).

The pH measurements were done using a Crison pH-meter (Crison, Spain) equipped with a glass electrode.



Scheme 1. Molecular structure of ciprofloxacin.

Preparation of modified electrochemical sensors

The PANI– β -CD/fMWCNT modified GCE was prepared and characterized as previously described.^[23] Briefly, a mass of two milligrams of (–COOH) functionalised MWCNT (fMWCNT) was dispersed using ultrasonic agitation in 1-mL aqueous β -CD solution (2%) to give a 2 mg mL^{–1} black suspension. Before surface modification, the 2-mm bare GCE was carefully polished to a mirror finish with an aqueous slurry of alumina powder (BDH Chemicals, VWR, Radnor, PA, USA) on a microcloth pad and then ultrasonically cleaned in ultra-pure water followed by ethanol to remove traces of alumina and possible contaminants. Subsequently, a solution of aniline (0.011 mol L^{–1}) was electropolymerized on the cleaned GCE, in a sulphuric acid aqueous solution (0.025 mol L^{–1}), sweeping the potential between –0.1 V and 1.0 V vs. Ag/AgCl at a scan rate of 50 mV s^{–1} for 50 cycles. After preparation of the polyaniline film on the GC electrode surface, an aliquot of 6 μL (2 mg mL^{–1}) of the MWCNT or fMWCNT dispersion was drop cast onto the GCE surface and dried in air at ambient temperature. Finally, the surface of the PANI– β -CD/MWCNT modified GCE was gently washed with water to remove the loosely attached β -CD/MWCNT.

The PANI– β -CD/fMWCNT film coated GC sensor was activated in phosphate buffer (pH 6) by cyclic voltammetric sweeps between +0.5 and +1.1 V vs. Ag/AgCl until stable cyclic voltammograms were obtained.

For the cleaning of the PANI– β -CD/fMWCNT film coated GC sensor, successive cyclic voltammetric sweeps in 0.1 mol L^{–1} phosphate buffer (pH 6) solution were performed until unchanged cyclic voltammograms were obtained (six cycles).

Analytical procedure

The PANI– β -CD/fMWCNT film coated GC sensor was first activated in 0.1 mol L^{–1} phosphate buffer (pH 6) by cyclic voltammetric sweeps between +0.5 and +1.1 V until stable cyclic voltammograms were obtained. Accurate volumes of the stock standard solution of ciprofloxacin (10 mM) were then added to the voltammetric cell, and CVs were recorded from +0.5 to +1.1 V at a scan rate of 20 mV s^{–1}. The same procedure was followed for the analysis of natural water samples. After each measurement, the PANI– β -CD/fMWCNT film coated GC sensor was cleaned by successive cyclic voltammetric sweeps in 0.1 mol L^{–1} phosphate buffer (pH 6) solution until unchanged cyclic voltammograms were obtained.

Analysis of ciprofloxacin in water samples

A water sample from the effluent of a sewage treatment plant located in Porto, Northern Portugal, was collected in 2.5-L brown glass bottles (wastewater effluent characterization: pH 7.2, conductivity 563 $\mu\text{S cm}^{-1}$, total phosphorus 4.5 mg L^{–1} as P, total suspended solids 18 mg L^{–1}, total nitrogen 9.2 mg L^{–1} as N, BOD₅ 14 mg L^{–1} O₂ and COD 45 mg L^{–1} O₂). Immediately after arrival in the laboratory, the samples were filtered through 1- μm glass fibre filters and 0.45- μm cellulose acetate filters, sequentially, to remove suspended particles.

Standard stock solutions of ciprofloxacin (10 mM) were prepared in water. For calibration curves, standard solutions were

prepared in the voltammetric cell by adding accurate volumes of the stock standard solution of ciprofloxacin to the selected phosphate pH 6 supporting electrolyte in order to obtain concentrations between 10 and 80 μM . The calibration curve for CV analysis was constructed by plotting the peak current against the ciprofloxacin concentration (10, 20, 40, 60 and 80 μM).

The limit of detection (LOD) was calculated according to IUPAC recommendations, using an S/N ratio of three.^[25] Method precision was checked on different days, within day ($n = 5$) and between days ($n = 5$), for three different concentrations. Recovery assays were carried out at different concentrations by adding known amounts of standard solution of ciprofloxacin to the water samples. The same procedure was applied in the sample analysis as in the calibration experiments. The final concentrations of ciprofloxacin, in the voltammetric cell, were 10, 40 and 80 μM .

Computer modeling

Molecular modeling was used to study the binding of ciprofloxacin to β -CD and CNT edges. CNT edges were represented, for simplicity, by a molecular analogue, hexabenzocoronene (HBC).^[26] The MMFF94 force field was used with a set of modified parameters to improve the energetics and geometries of π - π stacking.^[27] Molecular dynamics (MD) simulations were performed at room temperature, and water was considered implicitly by the generalized Born model augmented with a hydrophobic solvent accessible surface area term. In order to improve conformational sampling, random collisions with solvent molecules were considered through the use of stochastic dynamics with a friction coefficient of 2 ps^{-1} . To study the ciprofloxacin β -CD complex formation, different MD runs were performed. In order to improve the exploration of favourable binding modes, energy constraints were applied to bias the sampling towards shorter intermolecular distances. These constraints work by adding an energy penalty with a harmonic potential for distances exceeding a set distance; the distances used were: 2, 3, 4, 5, 8, 10 and 15 Angstrom. Each MD simulation was run for 10 ns, and snapshots of the trajectory were selected at 10 ps intervals yielding 1000 snapshots per MD run. Geometries for all snapshots were optimized with the Newton-Raphson method, and the minima that showed strongest binding were selected. MMFF94 energies do not describe π - π stacking accurately;^[27] consequently, the binding energies reported were calculated with the semiempirical PM6 Hamiltonian augmented by the D3H4 function that improves the description of hydrogen bonding and dispersion.^[28] Molecular mechanics simulations were performed with a modified version of the Tinker software.^[29] Semiempirical simulations were performed with the implicit solvent method of the MOPAC2012 program.^[30]

Results and discussion

Electrochemical behavior of ciprofloxacin

To evaluate and compare the voltammetric behavior of PANI, β -CD, MWCNT and fMWCNT in relation to ciprofloxacin oxidation, cyclic voltammograms were recorded using the modified electrodes. The potential was scanned from +0.5 to +1.1 V vs. Ag/AgCl in 0.1 mol L^{-1} phosphate buffer solution

(pH 6.4), containing 60 μM of ciprofloxacin. Figure 1 depicts the CV response obtained using a scan rate of 20 mV s^{-1} at the bare GC electrode (curve A), at the PANI/MWCNT (curve B), at the PANI- β -CD/MWCNT (curve C) and PANI- β -CD/fMWCNT (curve D). At the bare GC electrode, a weak and broad oxidation peak (curve A) is obtained for ciprofloxacin at 1.00 V, while the response was improved at the PANI/MWCNT GC electrode. The shift in peak potential to a lower value of ~ 0.95 V can be ascribed to an electrocatalytic effect and the higher peak current to the greater surface active area of the PANI/MWCNT film. Upon addition of β -CD to the film-modified GC electrodes, there was a further significant increase in the peak currents (curves C and D), occurring at a slightly less positive potential of 0.93 V. In all cases, the results showed no reduction peak in the reverse scan, suggesting that the electrochemical reaction is an irreversible process.

The increase in the current observed for PANI/MWCNT film-modified electrodes after the addition of β -CD can be a consequence of the occurrence of an interaction between cyclodextrin and ciprofloxacin, for instance, through the formation of an inclusion complex.^[31] The ability of β -CD to bind with ciprofloxacin, as shown by computer modelling (see later in the article), can lead to a signal improvement due to the increase of ciprofloxacin concentration at the PANI- β -CD/MWCNTs film electrode surface. The use of functionalised MWCNTs slightly enhanced the peak current, which can be related to the increase in the surface-active area of the carbon nanotubes (curve D).

Influence of pH and scan rate

Cyclic voltammograms of ciprofloxacin in different pH electrolytes were recorded at the PANI- β -CD/fMWCNT electrode in order to investigate the influence of the pH of the buffer solution on the electrode reaction, peak potential, E_p , and peak current, I_p (Fig. 2).

The plot of E_p vs. pH shows that the peak potential is dependent on pH in the interval from 3.1 to 8.0, shifting negatively with

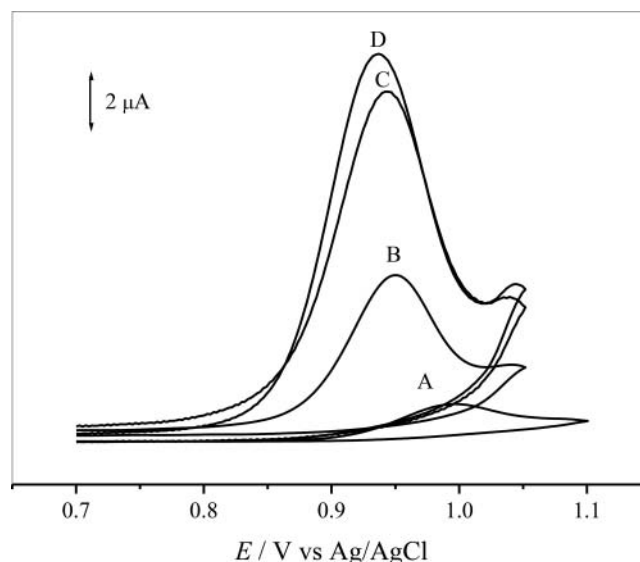


Figure 1. Cyclic voltammograms of 60- μM solutions of ciprofloxacin at (a) GCE, (b) PANI/MWCNT/GCE, (c) PANI- β -CD/MWCNT/GCE and (d) PANI- β -CD/fMWCNT/GCE in pH 6 phosphate buffer electrolyte. Scan rate: 20 mV s^{-1} .

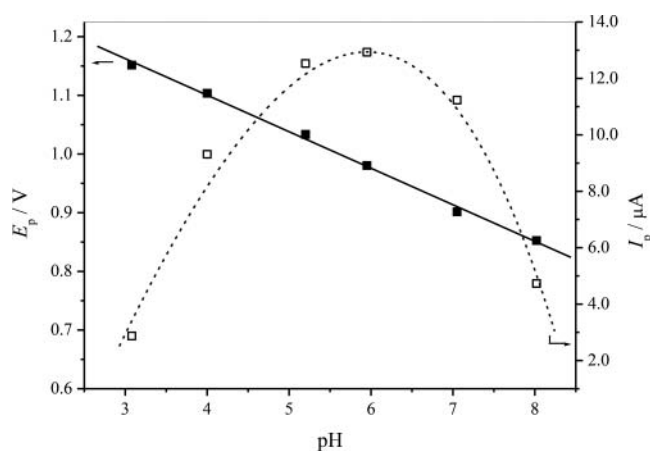


Figure 2. Plots of E_p (filled symbols) and I_p (open symbols) vs. pH from cyclic voltammograms of 0.1-mM solutions of ciprofloxacin at PANI- β -CD/fMWCNT film electrode in different buffer electrolytes as a function of pH. Scan rate: 20 mV s⁻¹.

increase in pH, indicating that protons participate in the electrode reaction. The slope of 62 mV per pH unit shows that the mechanism of the oxidation process in aqueous media involves the participation of the same number of electrons and protons. A plot of E_p vs. logarithm of scan rate (ν) (not shown), in the range 20–100 mV s⁻¹, gives a straight line with slope equal to $2.303RT/\alpha nF$. The value of αn was calculated to be 1.6, which indicates that two electrons are involved in the oxidation process of ciprofloxacin on the film electrode with the second electron-transfer being the rate-determining step. The oxidation mechanism for ciprofloxacin can be expected to involve the abstraction of electrons from the nitrogen of the piperazine moiety, see Scheme 2, as suggested in the literature for similar compounds.^[32,33]

The effect of electrolyte pH on the current response at the PANI- β -CD/fMWCNT film was also investigated. The plot of I_p vs. pH indicates that the peak current reaches a maximum around pH 6 (Fig. 2). Therefore, this buffer was chosen for the subsequent analytical experiments.

The effect of scan rate on the oxidation of ciprofloxacin at the modified electrode was studied in pH 6 phosphate buffer solution. The oxidation peak potential shifts with increasing scan rate towards a more positive value. A linear relationship was found between the peak current (I) and the square root of scan rate ($\nu^{1/2}$), in the range 20–100 mV s⁻¹, showing that the electrode reaction mechanism of ciprofloxacin is a diffusion-controlled process.

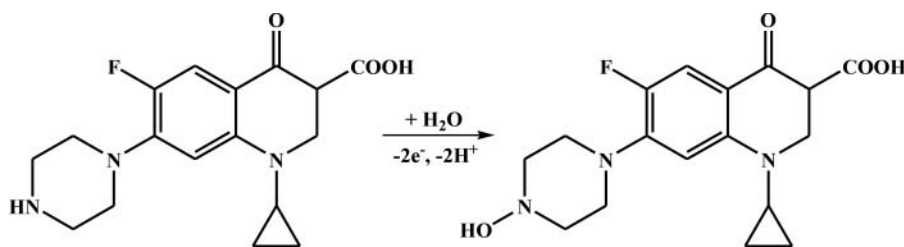
The results obtained using the PANI- β -CD/fMWCNT film were different from those described in the literature using a MWCNT film-modified glassy carbon electrode.^[32,34] Cyclic voltammograms recorded at a MWCNT/GCE, at several scan rates, revealed that the oxidation of ciprofloxacin was an adsorption-

controlled process.^[34] The ability of organic molecules, namely ciprofloxacin, to adsorb on MWCNT has been the basis of their recent application in water purification and in environmental pollution control.^[35,36] Nevertheless, surface chemical functionalization of MWCNT can have an impact on the adsorption of the organic molecules. It has been shown that oxygen-containing functional groups including -COOH groups could reduce the hydrophobicity of CNT, significantly decreasing the adsorption of ciprofloxacin.^[35] Moreover, ciprofloxacin adsorption strongly depends on the pH of the solution. Ciprofloxacin can exist as a cationic, zwitterionic or anionic form in three regions divided by the two pK_a values (6.1 and 8.7).^[36] The zwitterionic form of ciprofloxacin is dominant at pH > 6.1, as a result, the negatively charged -COO⁻ groups existing at the ends or at the sidewall defects of the nanotube structure of functionalised carbon nanotubes can repel the ciprofloxacin anionic groups, thereby reducing its adsorption capacity onto the fMWCNT surface. These findings are consistent with the results of computer simulations, described in the next section.

Computer modeling

Computer simulations are widely used to rationally explain the experimental findings concerning CDs inclusion and recognition.^[37] Computer simulations were carried out to simulate the dynamic behavior of CDs in the presence of organic molecules and to get a deeper insight into their complexation mechanism, which is particularly relevant to interpret experimental results.

Molecular modeling was applied to study the binding of ciprofloxacin to β -CD and CNT edges. Figure 3 shows the most likely conformations for ciprofloxacin with β -CD and HBC from MD simulations. Notably, in these conformations, ciprofloxacin sits atop β -CD, which contrasts with the commonly found cavity binding.^[31,38,39] Furthermore, no cavity binding of neutral or zwitterionic ciprofloxacin and β -CD was found during the MD simulations. In addition, MD simulations starting from cavity binding geometries, for neutral and zwitterionic ciprofloxacin, also indicated that cavity binding is not energetically favoured in these systems. The binding energies for the complexes of β -CD at the PM6-D3H4 level in water are 21 and 20 kcal mol⁻¹ for neutral and zwitterionic ciprofloxacin, respectively. In contrast, ciprofloxacin binds to CNT edges with energies 16 and 9 kcal mol⁻¹ for the neutral and zwitterionic form, respectively. Thus, ciprofloxacin binds better to β -CD than to CNT edges, which is consistent with the improved sensitivity towards ciprofloxacin observed for electrodes containing β -CD. The difference in binding is stronger for zwitterionic ciprofloxacin, since the greater polarity of the ionized molecule causes weaker binding to hydrophobic CNTs.



Scheme 2. Oxidation mechanism proposed for ciprofloxacin.

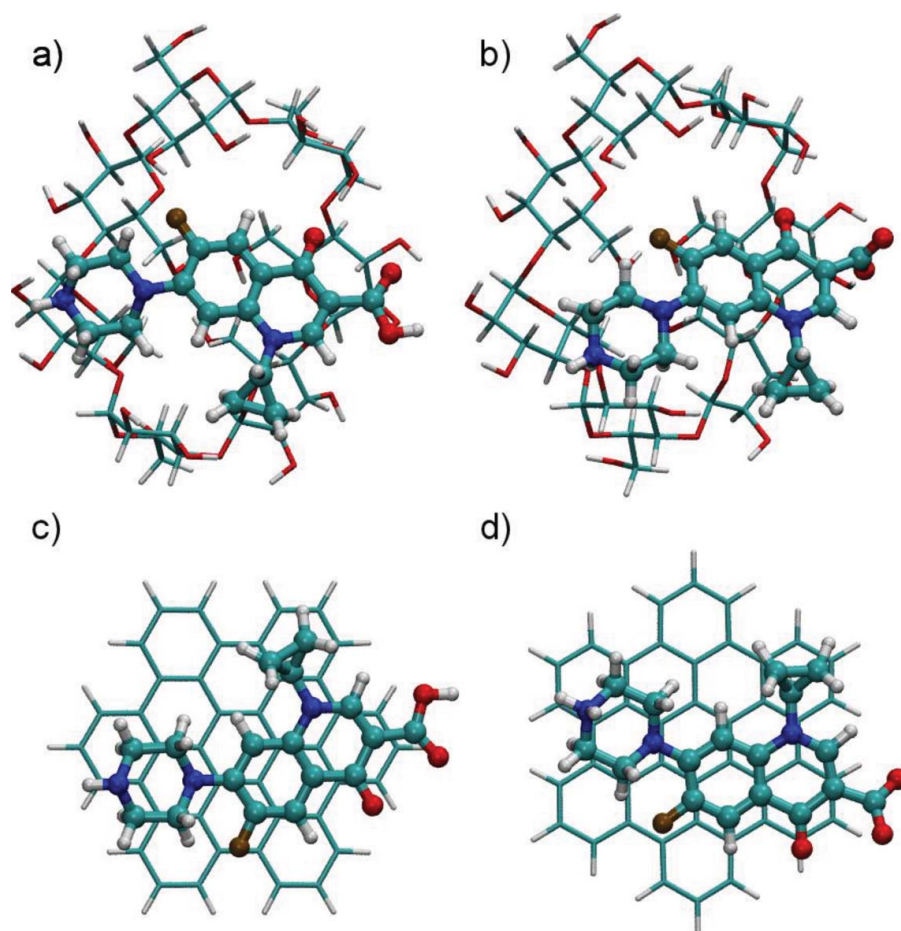


Figure 3. Molecular models of ciprofloxacin (ball–stick model) complexes with β -CD (stick model) in neutral (a) and zwitterionic form (b) and with HBC (stick model) in neutral (c) and zwitterionic form (d) from MD simulations. The colours are white for H, light blue for C, red for O, dark blue for N and brown for F.

This effect might also play a role in the electrochemical response at different pH values.

Analytical application

CV experiments, using the developed PANI- β -CD/fMWCNT film electrode, were carried out in triplicate, with the optimized experimental parameters, in order to obtain an analytical curve for the determination of ciprofloxacin.

Linear calibration plots with a sensitivity of $0.257 \pm 0.004 \text{ A M}^{-1}$ were obtained for ciprofloxacin concentrations ranging from 10 to $80 \mu\text{M}$ (Fig. 4). A limit of detection (LOD) of 50 nM was calculated using the 3S/N ratio, as recommended by IUPAC.^[25]

The PANI- β -CD/fMWCNT film electrode proposed in the present paper showed a significant improvement in terms of limit of detection and sensitivity compared with the use of multi-walled nanotube composite film–glassy carbon electrode, 50 nM vs. $6 \mu\text{M}$, respectively.^[34] The analytical sensitivity described is more than two times lower than that found here using the PANI- β -CD/fMWCNT film electrode.

The precision of the method was evaluated by repeatedly ($n = 5$) measuring ciprofloxacin, at three concentrations (10, 40 and $80 \mu\text{M}$) on the same day and over five consecutive days. The maximum values of the intra-day and inter-day precision obtained, expressed as coefficient of variation, were approximately 1.9% and 2.8%, respectively.

The proposed method was applied to the analysis of the antimicrobial agent in the effluent of a sewage treatment plant spiked with ciprofloxacin, using the standard addition method,

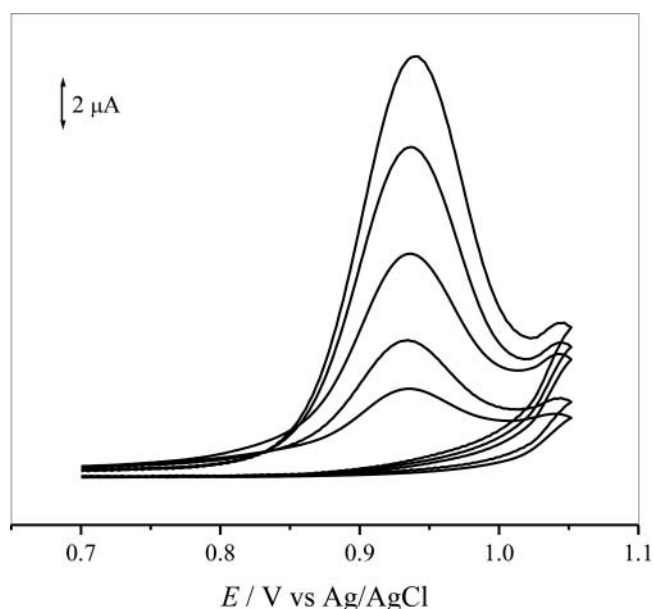


Figure 4. Cyclic voltammograms of ciprofloxacin standard solutions with concentrations of: (a) 10, (b) 20, (c) 40, (d) 60 and (e) $80 \mu\text{M}$ at PANI- β -CD/fMWCNT film electrode in pH 6 phosphate buffer electrolyte. Scan rate: 20 mV s^{-1} .

Table 1. Results obtained for the analysis of ciprofloxacin in spiked wastewater treatment plant effluent samples using the proposed cyclic voltammetric method.

Ciprofloxacin		
Added (μM)	Found ^a (μM)	Recovery (%)
10.2	10.1 \pm 0.2	99.1
39.9	42.7 \pm 0.7	107.0
78.6	77.2 \pm 0.6	98.2

^aAverage of five replicate measurements.

in order to eliminate any matrix effects. For this purpose, the effluent sample was spiked with ciprofloxacin in order to achieve final concentrations of 10, 40 and 80 μM . Recoveries between 98.2% and 107.0% were achieved (Table 1).

Operational lifetime and selectivity

The stability of the PANI- β -CD/fMWCNT modified electrode was tested over a 5-day period. Cyclic voltammetry of ciprofloxacin at the modified electrode showed that the oxidation peak potential remained unchanged, and the initial anodic peak current was maintained during this period, with a relative standard deviation lower than 5%.

The influence of various substances as compounds that can potentially interfere with the determination of ciprofloxacin was studied. As interfering species, some ions (e.g. Na^+ , Ca^{2+} , Mg^{2+} , NO_3^- , Cl^- , SO_4^{2-}) and other pharmaceuticals commonly found in Porto wastewater treatment plant effluents, azithromycin (antibiotic), furosemide (diuretic), lorazepam (anxiolytic), ketoprofen, naproxen and ibuprofen (anti-inflammatories), bezafibrate, gemfibrozil and simvastatin (lipid regulators), were selected.^[40] Ratios of 200-fold higher concentrations of ions and 50-fold higher concentrations of pharmaceuticals had almost no influence on the current response of ciprofloxacin (signal change less than 5%).

Conclusion

Attention has recently been devoted to the life and behaviour of pharmaceuticals in the water cycle. The wide spectrum of substances detected in receiving river waters indicates that wastewater treatment plants outlets are major contributors of pharmaceuticals in the aquatic environment. The absence of validated analytical methods and proper monitoring information of the pharmaceutical compounds, renders it difficult to make correct risk assessment.

Recently, a novel approach, based on the use of a PANI- β -CD/fMWCNT/GC modified electrode, has been successfully used to study and quantify many organic molecules because of the promising properties of both materials.

The PANI- β -CD/fMWCNT/GC film showed good electroanalytical performance for quantification of the fluoroquinolone antibiotic ciprofloxacin, with high sensitivity, linearity, repeatability, reproducibility and stability. The incorporation of β -CD in the PANI/MWCNT sensor leads to a significant signal improvement due to the increase of ciprofloxacin concentration at the electrode surface. Molecular modeling studies indicated that ciprofloxacin possesses a higher binding affinity to β -CD than to CNT, accounting

for the sensitivity enhancement. Since the PANI- β -CD/fMWCNT/GC film is easy to prepare and is low cost, it represents an interesting alternative, with improved analytical parameters compared to previous reports, for screening pharmaceuticals in wastewater treatment plant outlets.

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