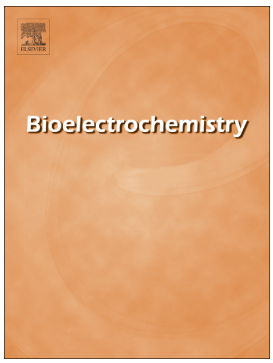

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Detection of choline in biological fluids from patients on haemodialysis by an amperometric biosensor based on a novel anti-interference bilayer

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Abstract

A new and highly selective amperometric biosensor able to analyse choline in clinical samples from patients suffering from renal diseases and receiving repetitive haemodialysis treatment is described. The proposed biosensor is based on choline oxidase immobilized by co-crosslinking onto a novel anti-fouling and anti-interferent membrane. Between the several polymeric films electrosynthesized on a Pt electrode whose permselective behaviours were here investigated, those based on overoxidized polypyrrole/poly(*o*-aminophenol) bilayer revealed the most effective in rejecting common interferents usually present in biological fluids. The so realised biosensor showed notably analytical performances, displaying linear choline responses up to 100 μM, a sensitivity of 156 nA $mM^{-1}mm^{-2}$ and a limit of detection, calculated at a signal-to-noise ratio equal to 3, of 1 μM; further, the within-a-day coefficients of variation for replicate (n = 3) were 2.7% and 1.2% at 100 μM and 10 μM choline levels, respectively. The remarkable performances and anti-interference behaviour allowed us the use of the proposed biosensor for the selective and fouling-free detection of choline in dialysate coming from patients on haemodialysis and even in their unpretreated human sera. Preliminary results gave choline levels in good agreement with the expected values.

Keywords: biosensor, choline oxidase, overoxidized polypyrrole/poly(*o*-aminophenol) bilayer, choline analysis, human serum and dialysate

1. Introduction

Choline is a vitamin-like essential nutrient necessary for several biological functions in human body: it plays an important role in the synthesis of some essential phospholipids, is a major source of methyl groups via its metabolite betaine and is a precursor of the neurotransmitter acetylcholine [1]. Choline-phospholipids are signal transducers mediating and modulating the transmembrane signalling: this mechanism, from the exterior of cells to the nucleus, is a powerful tool for gene transcription regulation and an essential process for cell growth and function [2]. Choline is present as well in blood and other biological fluids, such as cerebrospinal liquid, amniotic liquid and urine in specific ranges of concentrations. Changes in serum choline levels are associated with a variety of human diseases. Deficiency in circulating choline levels for example causes hepatic, renal, pancreatic, memory and growth abnormalities [3]. Dietary choline deficiency is associated with an increased incidence of spontaneous liver cancer [4] and aberration in choline metabolism is an emerging metabolic hallmark associated with oncogenesis and tumour progression [5]. Furthermore, recent studies revealed that choline exhibits beneficial effects on several cardiac diseases by activating Akt/mTORC pathway [6].

Choline deficiency often occurs in malnourished hospitalized patients, such as patients affected by cirrhosis or requiring total parenteral nutrition. Renal disease patients, on the contrary, show higher choline plasma level due to both reset of the choline homeostatic mechanism kidney-mediated and impaired excretion [7]. Indeed, abnormal level of free choline in serum could be considered a useful marker to detect chronic renal dysfunction. Nevertheless, a dialysis-induced choline deficiency in uremic patients is reported to occur after haemodialysis [8] and peritoneal dialysis [9]. Due to the acute decrease in plasma free choline, confusion and sleepiness could be observed in uremic patients during dialysis, related to a decreased acetylcholine production and to the role of choline in the regulation of cerebral metabolism [10,11]. Having a fast, sensitive, accurate and reproducible method for determining choline in such specimens could be useful for monitoring its level during the dialysis treatment and to implement a prompt pharmacological treatment aimed to restore the recommended levels in plasma thus preventing potential collateral effects.

Several analytical methods have been developed for the quantification of choline in biological samples, including enzymatic radiochemical and colorimetric assay [12], separative techniques coupled to mass spectrometry [13,14], infrared spectroscopy [15] and 1H-NMR [16]. Despite the reliable sensitivity, their practicability is restricted by the expensive equipment, time consuming sample pre-treatment and need of skilled operators. Amperometric biosensors based on the enzyme choline oxidase (ChO), at the same time, have gained increasing attention owing to the

related advantages such as low cost, appreciable sensitivity, rapid response and minimal sample treatment, thus representing a valid alternative to the above techniques.

In oxidase-based biosensors, measurement is mostly based on the amperometric detection of the enzyme produced H_2O_2 whose oxidation requires a high overpotential at Pt electrodes: endogenous electroactive substances in real samples, *i.e.* ascorbic acid, uric acid, etc., are also oxidized at that potentials, resulting in interfering signals with substrate detection; furthermore, several proteins and related compounds endogenously present in complex sample like blood are known to foul the electrode surface of amperometric biosensors so reducing their sensitivities or worse stopping their operations. Biosensors coupling ChO recognition to various redox mediators or horseradish peroxidase were developed in the attempt to lower the detection potential: unfortunately, these approaches may also be subject to a loss of selectivity due to readily oxidizable species, *e.g.* phenolic compounds, which interfere in the assay acting as electron donors for peroxidase [17] and mediators as well. During the last decade, a great number of studies involving transition metals hexacyanometallates as Prussian Blu have appeared, using different oxidase enzymes. This has led to an on-going replacement of the more common enzymatic detection method involving horseradish peroxidase. The high electrocatalytic activity and low operating overpotential have contributed to the diversification of the use of this class of compounds in enzyme-based biosensors and immunosensors [18, 19, 20].

Choline biosensors based on electrodes modified with various nanomaterials able to lower H_2O_2 overpotential and detect choline at potential values not higher than +0.2 V [21, 22] have been developed as well. Lowering of the detection potential by these approaches certainly produces an improvement of the selectivity of amperometric detection but their associated electrode modification steps complicate the biosensor assembly and, more importantly, the analytical performances (*e.g.* nonlinear responses and/or high response times). Finally, some peculiar expedients have been also adopted to cope with the interference problems. As an example, interference from ascorbic acid was solved by pre-treating the sample solution with MnO_2, enabling its chemical reduction [23]: of course, the skill to directly analyse samples, typical of biosensors, is lost in this way. Further, sample dilution was proposed to minimise interference effects coming mostly from paracetamol and uric acid, which were still present while operating at +0.3 V [24]: this approach, obviously, loss of usefulness when analysing real samples where choline content is at a low level.

An alternative and well-established approach to overcome faradic interference and fouling effects is represented by modifying the Pt detector with the electrosynthesis of non-conducting polymeric films with built-in permselectivity, where a notable selectivity is generally achieved even

operating at high overpotentials [25]. The relevance of this topic is confirmed by the increasing demand for more performing films in terms of both improved permselectivity and biocompatibility as it is well documented by recent literature in biosensor applications [26, 27].

In this approach, the electrode modification is carried out under electrochemical control thus assuring a reproducible and uniform distribution of the immobilized layer, unlike the solvent casting method commonly adopted also for nanomaterials deposition [21]. For example, poly(*o*-phenylenediamine) (PPD) has been employed for the realization of a microelectrochemical biosensor to detect brain extracellular choline [28]: being choline concentration relatively low in brain, a dip absorption method was adopted for a total of 10 layers to maximize the enzyme loading, which rendered the biosensor assembly quite laborious; notably, the investigation on the anti-interference behaviour was not extended to endogenous species other than ascorbic acid. Indeed, when dealing with samples containing various electroactive interferents, the rejection behaviour of the electrosynthesized membrane could be not efficient for all of them so that to widen the spectrum of the rejected species, multi-layered structures have been proposed. As an example, a very efficient rejection of electroactive interferents was reported by employing a polymeric bilayer composed of overoxidized poly(pyrrole) (PPy_{ox}) and poly(2-naphtol) (P2NAP) films for acetylcholine and choline detection [29] and to detect biogenic amines in food products [30].

In this contest, the aim of the present work was to develop a reliable ChO-based biosensor for a fast and selective detection of choline in different biological fluids, without any sample pre-treatment. The research work has been firstly focused on the investigation of the rejection performances of several monolayer and combined films, towards common electroactive species present in clinical samples, to overcome the common fouling and interference problems occurring in biological matrix analysis. The most attractive rejection properties were achieved by a novel electrosynthesized bilayer, made of PPyox and poly(*o*-aminophenol) (PoAP), able to drastically flatten the interferent faradic response, while still assuring an appreciable analytic signal. ChO was then immobilized onto this bilayer by co-crosslinking to realize an interference-free biosensor showing appreciable sensitivity and stability. The biosensor performances were tested by determining choline concentration in unpretreated clinical samples, *i.e.* serum and dialysates from renal disease patient who is receiving repetitive haemodialysis treatment.

2. Experimental

2.1. Reagents

Choline chloride, choline oxidase (EC 1.1.3.17 from *Alcaligenes species*, 14 U mg^{-1} of solid), bovine albumin (fraction V), glutaraldehyde (grade II, 25% aqueous solution), ascorbic acid, uric

acid, L-cysteine and acetaminophen were obtained from Sigma (Sigma Chemical Co.; St. Louis, MO) and used without further purification. Before its use, choline chloride was dried under vacuum over P_2O_5 and stored in a vacuum desiccator. Pyrrole (Aldrich, Steinheim, Germany) was purified by distillation under vacuum at 52 °C and stored in the dark under nitrogen atmosphere at -20 °C. *o*-phenylenediamine and 2-naphtol (Aldrich, Steinheim, Germany) were purified by vacuum sublimation at 90 and 80 °C respectively and stored in the dark. *o*-aminophenol (Fluke Chemie, Switzerland) was purified by ethylacetate recrystallization. All other chemicals were of analytical reagent grade. Choline stock solutions were stored in the dark at 4°C. More diluted choline solutions, monomer and interferents solutions were prepared just before use. In flow injection experiments, the carrier solution was a supporting electrolyte at pH 8.0 with the following composition: sodium bicarbonate 35 mM, sodium acetate 8 mM, potassium chloride 2 mM, calcium chloride 1.75 mM, sodium chloride 100 mM and glucose 2 g/L.

2.2. Apparatus

A Gilson (Gilson Medical Electronics, Villiers-Le-Bel, France) Minipuls 3 peristaltic pump and a seven-port injection valve (Rheodyne mod. 7725, Cotati, CA, USA) equipped with a 20 microliters sample loop were used for the flow injection experiments. A PEEK tubing (0.25 mm ID, 150 cm length) was used to connect the sample injection valve to the electrochemical cell. An EG&G (Princeton Applied Research, Princenton, NJ, USA) Model 400 electrochemical detector was used. The detector included a thin-layer electrochemical cell with a Pt disk (3 mm diameter) working electrode and an Ag/AgCl, 3 M NaCl reference electrode. Two thin layer flow cell dual gaskets (Bioanalytical Systems, Inc., USA) with a thickness of 0.004 inch were used. A Kipp & Zonen (Delft BV, Holland) mod. BD 11 E Flatbed Yt recorder was used.

Polymers electrosynthesis was carried out using an EG&G model 263A potentiostat/galvanostat equipped with a M270 electrochemical research software (EG&G) version 4.23 for data control and acquisition.

Scanning electron microscope (SEM) studies were performed with an ESEMXL30 FEI (Philips Electron Optics, Eindhoven, The Netherlands).

2.3. Electrode modification and biosensor preparation

Before each modification, the Pt working electrode was cleaned by dipping in hot nitric acid and then by alumina (0.05 µm particles) mechanical abrasion, extensive washing and sonication in double-distilled water. The electrode was then immersed in 0.5 M sulfuric acid solution and its

potential cycled between −0.255 and +1.225 V vs. Ag/AgCl, in saturated KCl, at 100 mV s^{-1} until a steady-state cyclic voltammogram was obtained.

The electropolymerization of polypyrrole films (PPy) was performed at +0.7 V versus Ag/AgCl by using a 0.4 M pyrrole solution in 0.1 M KCl supporting electrolyte until a deposition charge of typically 300 mC/cm^2 was achieved. The Pt/PPy modified electrode was then overoxidized at +0.7 V versus Ag/AgCl in phosphate buffer (pH = 7.0, I = 0.1 M) for at least 7 h.

P2NAP films were electrochemically grown by cyclic voltammetry (20 scan cycles) using a 5 mM 2-naphtol solution in phosphate buffer (0.1 M, pH 7). The potential ranged from 0 to +0.8 V (vs. Ag/AgCl, KCl satd.) at a scan rate of 50 mV s^{-1}. PoAP was electrosynthesized by scanning the electrode potential for 20 cycles between -0.1 and +1.0 V (vs Ag/AgCl, KCl saturated) using a 5 mM *o*-aminophenol solution in phosphate buffer (0.1 M, pH 7) at a scan rate of 50 mV s^{-1}. PPD films were electrochemically grown by potential cycling at 50 mV s^{-1} in the range -0.1/+0.6 V (vs Ag/AgCl, KCl saturated) using a 5 mM monomer solution in phosphate buffer (0.1 M, pH 7).

Electrodes based on bilayer films were obtained by the deposition of P2NAP, PoAP or PPD (see above) at a conducting PPy film, followed by the overoxidation of the resulting Pt/PPy-P2NAP, Pt/PPy-PoAP or Pt/PPy-PPD modified electrodes.

The ChO enzyme layer was deposited on Pt/PPy_{ox}-PoAP electrodes as follows [31]: 300 µl of phosphate buffer (0.1 M, pH 6.5) containing 16 mg of BSA and 1 mg of ChO were mixed with 30 µl of a 2.5% glutaraldehyde solution; 3 µl of the resulting solution were carefully pipetted onto the working electrode surface and spread out to cover it completely. The modified electrode was then air-dried at room temperature for few minutes and successively soaked in the background electrolyte to remove weakly bound or adsorbed enzyme. When not in use, the enzyme electrode was stored in phosphate buffer, pH 6.5, I 0.1 M, at 4 °C in the dark.

2.4. Film morphology

SEM studies were performed on a modified Pt foil (10×15×0.127 mm) 99.99% pure (Aldrich) to illustrate film morphology. For this purpose, the Pt foil was modified by an PPy_{ox}-PoAP layer exactly as described in the "*Electrode modification and biosensor preparation*" section to produce a surface modification almost identical to that prepared for modified electrodes.

2.5. Electrochemical measurement

All the electrochemical measurements were performed using a detection potential of +0.7 V versus Ag/AgCl/NaCl (3M) in the supporting electrolyte already described in the "*Reagents*" section. The flow rate in flow injection experiments was 0.2 ml min^{-1}, the injection volume was 20

μl and the electrochemical detector time constant was set to 1 s. Solutions and carrier stream were air saturated and the temperature was ambient.

2.6. Samples collection, preparation and analysis.

Human serum and dialysate specimens from a male patient (on haemodialysis three times weekly for four hours) were collected and used by a local hospital (Azienda Ospedaliera San Carlo, Potenza, Italy) in accordance with its local ethics committee and informed consent was obtained from donor for experimentation and use of his samples; further, our Department provided us the relevant consent to use in our laboratory biological sample for research purposes in accordance with the ethics committee of our University.

Haemodialysis was carried out in bicarbonate employing a polysulfone dialyzer membrane (F8, Fresenius Medical Care AG, Hamburg, Germany). A constant blood flow of 300 ml min^{-1} was employed and the dialysate flow rate was maintained at about 500 ml min^{-1}. Whole blood was collected from patient's venous access before starting and at the end of dialysis; dialysate samples were collected from the fluid leaving the dialyzer from exit port. All blood samples were centrifuged at 10000 rpm for 10 min at 4 °C. The isolated serum and dialysate samples were stored at -20 °C until analysis. Choline analysis was performed on serum diluted 1:3 with supporting electrolyte (to minimize any undesired artefact during detection) and on undiluted dialysate.

3. Results and discussions

3.1. Anti-interference behaviour of electrosynthesized mono and bilayers membranes.

Quantitative determination of choline is important in biological and clinical analysis for its implication in the outbreak of several diseases, as mentioned in the introductive part. Nevertheless, choline detection in real biological samples, like body fluids, is limited by interference problems coming from endogenous compounds. Therefore, we focused our interest on interferent species commonly present in clinical samples, with the perspective to realize a device for choline detection in body fluids from renal diseases patients. The strategy we adopted to overcome interference problems, along with sensor fouling effects, is based on the employment of non-conducting films with built-in permselectivity. A deep investigation has been carried out by comparing the rejection performances of various insulating films, as monolayer or composite, already employed in the field of biosensors. The choice of polymers to be used in our research has been made taking into consideration both the recent trends in the field of neurotransmitter biosensors [32] and the biocompatibility of polymers, in the perspective to translate the biosensor technology from standard

conditions to real clinical application such as real time monitoring the loss of choline during haemodialysis session.

The rejection performances were evaluated by the flow injection (FI) amperometric responses of hydrogen peroxide, uric acid (UA), acetaminophen (AC), ascorbic acid (AA) and cysteine (CYS), acquired at a bare and at a Pt electrode modified by the electrosynthesized films, at concentration values near the upper limits of their physiological ranges. A supporting electrolyte with the composition specified in the experimental section was used to reproduce the dialysate medium. For each interferent, the degree of the faradic signal suppression exerted by the polymer was quantified by the ratio $R_{int} = r_{i,m}/r_{i,u}$, where $r_{i,m}$ and $r_{i,u}$ are the FI responses of interferent i at the modified and unmodified electrode, respectively. Since hydrogen peroxide response was depressed by the film as well, R_{H2O2} was also evaluated similarly to R_{int}. Then, the ratio R_{int}/ R_{H2O2} was computed as an estimation of the anti-interference characteristics of the polymers under studying. The relevant data are summarized in Table 1.

Our investigation started from P2NAP. In the past we have characterized this film proposing a possible structure [33] and use it for the realization of a glucose biosensor [25]. We showed that the repeat sequence of closely spaced polycyclic rings alternating by ether links confers a high compactness to the film, controlling the "exclusion" mechanism towards interferent molecules, irrespective of their charged state. Data reported in Table 1, indeed, reveal a significant ability of P2NAP to reject AU, AC and CYS and the highest interferent ratio was obtained for AA. When Pt electrode was modified by PPD, on the contrary, the degree of AA rejection was similar but a worse permselective behaviour towards other interferents was noticed; as previously described, PPD has already been used for the realization of a choline microelectrochemical biosensor for the in vivo detection of brain extracellular choline [28] so, under the light of their studies, limited only to ascorbic acid, and our results, the selectivity of the approach appears now questionable.

In the recent years we have carried out a deep investigation on insulating PoAP [34-38] and on its propensity to entrap enzyme for biosensor realization [39]. A constant repeat formula made of alternating quinoneimine and monomer units was suggested, with water molecules as an integral part of the polymer structure. The presence of tightly bound water molecules within the polymer structure could be responsible for poor film compactness, causing a scarce anti-interference behaviour, as it can be seen from data reported in Table 1. Coherently, also for the detected specie, hydrogen peroxide, a lower current attenuation was recorded if compared to that occurring in the presence of the other polymers (R_{H2O2} data shown in Table 1). Unexpectedly, an interferent ratio relatively low was evaluated for AA. Table 1 instead shows that PPy_{ox} revealed the most efficient monolayer film. The permselective characteristics of this polymer are well established, as well as its

use for the development of amperometric biosensor operating in real matrices [40-44]. Briefly, the overoxidation process of PPy generates on the polymer an increment of oxygenated carbon species and the formation of COOH groups particularly onto its outer surface; the overoxidation process is also followed by an iminic transformation of nitrogen groups influencing further the nature of the film [45]. Effectively, interferences evaluated in the present work from AU, PA and CYS were negligible; nevertheless, a higher interferent ratio toward AA was computed compared to that observed at Pt/PoAP electrode.

An improvement of PPy_{ox} permselectivity was then attempted by electrosynthesizing bilayer structures: the aim was to combine the rejecting properties of this polymer with the permeability performances of the other films here studied. P2NAP was shown to be promising for enhancing the permselective behaviour of PPy_{ox} in a bilayer membrane composed of both films [29, 30]: a quick comparison of the interferential ratios reported in Table 1 shows that the use of PPY_{ox}-P2NAP bilayer certainly improves the permselectivity of P2NAF but does not solve the interference from ascorbic acid; in other words, the presence of P2NAF does not enhance the rejection ability of PPy_{ox}. PPD also has been already coupled to PPy_{ox}, showing performances not as efficient as PPY_{ox}-P2NAP [30]: Table 1 indeed reveals a certain "cooperation" between the two polymers, allowing an improvement of the overall permselectivity; the presence of PPy_{ox} drastically reduces the interference from uric acid, paracetamol and cysteine which was notable in correspondence of the single PPD film and, at the same time, the presence of PPD slightly reduces the interference from ascorbic acid observed at PPy_{ox} modified electrodes.

Finally, we explored the possibility to combine PPy_{ox} with PoAP for the realization of a novel bilayer structure, never explored before. As Table 1 shows, PPY_{ox} and PoAP exerted a much more pronounced synergistic effect in rejecting interferents: the presence of PPy_{ox} drastically improves the poor anti-interferential properties of PoAP towards uric acid, paracetamol and cysteine; on the other hand, the presence of PoAP significantly reduces the anti-interference behaviour of PPy_{ox} towards ascorbic acid assuring, moreover, adequate H_2O_2 permeation through the membrane as already noticed for the single layer of PoAP (R_{H2O2} values in Table 1). In this respect, Figure 1 shows the FI profiles acquired for hydrogen peroxide and interferents at a bare Pt and at a Pt electrode modified by the PPy_{ox}-PoAP bilayer: a fast response time was assured as it is attested by the FI time profiles practically identical to those acquired at bare Pt, despite the presence of two electrosynthesized films.

In conclusion, the bilayer PPy_{ox}-PoAP was selected to realize the ChO biosensor as it represents the best compromise between an efficient abatement of the interfering signals and an appreciable analyte permeation.

3.2. Morphological characterization of the PPy_{ox}-PoAP bilayer

The morphology of PPy_{ox} films has been already investigated by environmental scanning electron microscope (ESEM) showing a substantial homogeneity of its surface without any structuration or holes [44]. A morphological investigation has been carried out also on PoAP by employing *ex situ* [34, 37] and *in situ* [38] Atomic Force Microscopy (AFM). It was evidenced that, because of its thinness, PoAP grows by 'retracing' the features of the underlying Pt substrate forming a homogeneous layer. The presence of aggregates irregularly and randomly protruding over the polymer zone were noticed, being the result of the kinetically induced deposition of suspended oligomers, favoured by repeat cycles [36]. The *in situ* analysis confirmed the sporadic presence of simultaneous precipitates [38].

Starting from these findings, the morphology of the resulting bilayer PPy_{ox}-PoAP was here investigated and the relevant SEM images are depicted in Figure 2. As can be seen, the polymer bilayer shows a high degree of compactness. The imperfections visible as "black points" are present also onto the Pt surface (see Fig. 2a) thus suggesting that the used Pt substrate was not perfectly flat at that its imperfections were translated to the polymers layer.

From the interface with the Pt substrate a thickness of 630 ± 60 nm was evaluated. This value is very close to the thickness of about 0.67 μm estimated for PPy grown as a single layer till reaching a charge density of 300 mC/cm^2. Indeed, a charge density of 45 mC/cm^2 during the electrodeposition process produced a film thickness of 0.1 μm [45]. As expected, the additional presence of PoAP did not involve a significant variation, being its thickness of about 10 nm [35] lower than the estimated uncertainty.

Interestingly, the SEM image of PPy_{ox}-PoAP reported in Figure 2.b shows some features superimposed on the underlaying compact structure which resemble the aggregates typically found on PoAP. Going down in details (see Figure 2.c), a globular structure of the entire membrane can be noticed. Indeed, a granular aspect was found for PoAP films electrosynthesized on oxidized Pt substrate [34]. Particularly in that study it was shown that PoAP grows by mirroring the initial substrate morphology and that insulating PoAP with high smoothness can be formed on less oxidized surfaces. The images acquired for the bilayer seem therefore to suggest that the outer part of the membrane has a morphology more like that of PoAP. The granular structure evidenced could be attributed to the growth of PoAP onto a PPy layer which probably exhibits some oxidized zones in the experimental conditions adopted. Anyway, the high compactness of PPy_{ox}-PoAP, with no holes or defects, justifies its notable rejection properties.

3.3. Analytical characterization of the ChO biosensor

A choline biosensor was realized by immobilizing choline oxidase through co-crosslinking on the permselective bilayer membrane previously described. The immobilization procedure and the enzyme loading used for the biosensor construction were already studied and optimised elsewhere [29 and references therein cited] so here we report the analytical performances of this novel composite structure used for choline sensing.

The FI peaks relevant to repeated injections of increasing concentrations of standard choline solutions at a Pt/PPy_{ox}-PoAP/ChO electrode are shown in Figure 3. The choline responses were almost identical at each choline level and the low response time of the biosensor of 5 seconds, evaluated as $t_{95\%}$, gave nicely responsive peaks permitting high throughput for sample injections. The flow injection system here implemented for choline analysis is cheap, simple to achieve while being flexible at the same time, showed a rapid start up time (less than five minutes), low carrier consumption (due to the low flow rate), no drift in signal measurements and allowed sample sizes as low as few microliters, which is particularly desirable for clinical analysis.

The relevant calibration graph shown in Figure 4 resembles the behaviour expected for an enzyme-catalysed reaction with linear and saturated response at low and high substrate concentrations, respectively. Fitting of the experimental data with the well-known Michaelis-Menten kinetic model gave an apparent K_M value of 0.46 ± 0.03 mM and an I_{max} value of 0.84 ± 0.01 μA.

From the linear part of the calibration graph (inset of Figure 4) a regression line with a slope of (1.10 ± 0.01) μA/mM and an intercept of (3.9 ± 7.4) $x10^{-4}$ μA (correlation coefficient better than 0.999) was obtained. Choline responses were linear up to 100 μM and the sensitivity for choline detection was 156 nA $mM^{-1}mm^{-2}$. The limit of detection calculated for signal to noise ratio of 3 was 1 μM, and hence well suitable to detect choline in biological fluids such as human serum where concentration values of about 10 - 13 μM are typically found for healthy persons; indeed, as it will be shown later, such values are substantially higher for persons suffering from renal diseases.

The within-day coefficients of variation for replicate (n = 5) injections of choline were 0.98% and 1.56% at 100 μM and 10 μM levels, respectively. The storage stability was studied by discontinuously monitoring the biosensor sensitivity stored, when not in use, in phosphate buffer, pH 6.5, I 0.1 M, at 4 °C in the dark: even if no particular efforts were made to avoid bacterial growth in the storage buffer, after more than 40 days the sensitivity was about the 80 % of the initial value.

Considering the fast response time, the ease of execution with no sample pre-treatment, and the low cost of the instrumental set up, the device developed in this work could be proposed as a valid

alternative to conventional techniques [13-16]. Also, in the scenario of amperometric choline biosensors, the method we developed offers significant advantages. For a quick comparison, the performances of several choline oxidase biosensors based on amperometric detection are reported in Table 2.

The proposed device assures a completely interferent- and fouling-free detection without the need to lower the detection potential using mediators [46] or samples pre-treatment [24]. Furthermore, the concentration levels of interferents we employed were near the upper limit of their physiological range whereas lower interferent contents have been often tested elsewhere [21,47]. The limit of detection for the here described choline sensor is not as low as elsewhere reported [19] but comparable if not even lower than those reported for those based on nanomaterials [21, 48-49]; even so, the limit of detection of the proposed device is fully appropriate for choline analysis in blood as it will be shown later; moreover, the electrodic modification we adopted ensures an upper limit for the linear range higher than that obtained with more sensitive devices [22]. Not less important, the enzyme immobilization technique here used assured performances in terms of long-term stability higher than those already reported [17,23,47,49].

3.4. Analysis of real samples

The promising results, as herein shown, encouraged us to evaluate the feasibility of detecting choline in real samples through the proposed method; accordingly, the content of choline in serum and dialysate specimen from a patient on haemodialysis was evaluated.

Already in 1976, Rennick et al. [7] reported that plasma choline concentrations increase in uremic patients and that, during haemodialysis, a significant amount of free choline is lost from plasma into the dialysate; by an enzymatic radiochemical assay, a mean concentration value of 32.5 ± 1.8 μM was evaluated for free choline in plasma of uremic patients on haemodialysis, whereas the mean value in healthy subjects was 13.4 ± 2.0 μM. Changes in plasma free choline of chronic haemodialysis patients were evaluated also by Buchman et al. [50], by using a gas chromatography-mass spectrometry approach: they showed that plasma free choline in patients at end stage renal disease (ESRD) at baseline was higher than in normal controls (11.7 ± 3.7 nmol/ml); further, a progressive decrease in venous plasma free choline concentration was observed after each hour of dialysis, approaching the normal range at its conclusion. Ilcol et al. [8] extended the study to a larger group of patients and showed, by an enzymatic radiochemical method, that the concentration of free choline was 12.9 ± 0.6 μM in the plasma of normal healthy subjects, rising to 37.3 ± 0.9 μM in ESRD patients; a marked decrease in plasma free choline occurred during haemodialysis, not directly correlated to the magnitude or rate of choline loss during dialysis since the observed

decrease in plasma free choline was considerably less than the amount of choline found in the dialysate. A cautious interpretation was that a highly effective homeostatic mechanism is activated during the haemodialysis in response to acute depletion. The possibility of a transient phase of choline deficiency induced during dyalisis has however been suggested

Accordingly, a deeper understanding of the mechanisms involved during haemodialysis is strictly connected to a more general consideration of the changes occurring in various serum metabolites. Considering the risk that the loss of choline and subsequent changes in choline metabolism may contribute to the metabolic and neuroendocrine alterations, which occur in ESRD patients, the possibility of additional choline requirements in these patients and the use of choline supplements during haemodialysis should be addressed.

Preliminary experiments carried out with the proposed biosensor move in this direction. Firstly, we wanted to show the feasibility of our device to detect choline in real samples as complex as serum and dialysate. In Figure 5, the FI peaks relevant to injections of dialysate and serum samples at the Pt/PPy_{ox}-PoAP/ChO biosensor are depicted. Particularly, dialysate specimen after one hour of haemodialysis, serum sample from the patient before and after four hours of dialysis and serum sample from a healthy volunteer were analysed.

The triplicate injections of real samples showed an almost constant signal thus indicating that the biosensor here developed is free from fouling limitations. After one hour of dialysis a choline content of 7.8 ± 0.7 μM was computed in dialysate. This content showed light oscillations during the haemodialysis, attesting to a mean value of 7.7 μM. This evidence is in accordance with Ilcol [8] where a significant variation of choline in dialysate was not observed. Rennick [7] also showed a levelling of the choline content in dialysate from the second hour of haemodialysis. Interestingly, the serum choline values for the healthy person and for the patient before commencing the haemodialysis session were 9.6 ± 2.0 μM and 28.5 ± 2.0 μM, respectively. These values are in good agreement with data reported in literature [7,8] showing an effective increase in choline concentration for ESRD patient. After four hours of dialysis, choline content in serum decreased to 19.0 ± 2.0 μM. Again, the loss of choline in serum (9.5 ± 2.8 μM) was of the same order of that computed by Ilcol et al. (8.1 ± 0.6 μmol/L) [8].

To the best of our knowledge this is the first study that report the possibility to measure choline levels in different biological fluids from a patient, with none interference due to the notable anti-interference properties of the developed bilayer membrane. To further confirm the improvement in the faradic response selectivity achieved by combining PPy_{ox} and PoAP, the following control experiment was carried out. Undiluted human serum from a healthy volunteer was injected at a Pt electrode modified by PoAP alone, then by PPy_{ox} alone and finally by the bilayer PPy_{ox}-PoAP. The

relevant current signals are depicted in Figure S1 of Supplementary Material. The mean current response of triplicate injections at each electrode was converted to choline concentration by employing the regression line equation above reported. At Pt/PoAP electrode, the non-enzymatic current signal coming from electroactive substances present in serum simulates a dramatically high choline concentration of 35 μM. A fouling effect was also noticed testified by the decreasing peak height upon repeated injections. Passing to Pt/PPy_{ox} modified electrode, the interference quantified as choline concentration was substantially lower, attesting to 3.8 μM. Considering that choline content in healthy persons is about 10 - 13 μM, an interference of 29-38% is to be expected. When the electrode was modified by the bilayer PPyox-PoAP, current response was so low that it was no possible to estimate a choline concentration, being below the estimated detection limit. The proposed bilayer revealed surely promising for the realization of amperometric biosensors based on hydrogen peroxide detection.

Work is in progress in our laboratory to validate the method with the final goal of integrating the device in the haemodialysis system for continuous “on line” monitoring of choline during all the haemodialysis session. At this aim in the supplementary material we show our early results simulating the “on line” analysis (see Figure S2 in Supplementary Data). Current signals from standard choline solutions showed excellent stability: the absence of hydrodynamic noise and artefacts due to injection system would allow detection limits below the values obtained with the "flow injection" technique.

4. Conclusions

In this work, a choline biosensor, based on ChO immobilized by co-crosslinking on a Pt electrode previously modified by a novel electrosynthesized PPy_{ox}-PoAP bilayer, has been successfully developed. Much effort has been devoted to the appropriate choice of the non-conducting permselective membrane, able to assure the best interferent rejection characteristics towards major electroactive compounds, to develop a stable and versatile device for clinical applications. This feature, combined with the notable analytical performances of the technique adopted for enzyme immobilization, resulted in a biosensor, for choline detection, which appears promising for real sample analysis with low risk of interferences.

Even though the methodology must be further implemented, validated and engineered, preliminary results showed that the proposed biosensor might be successful for choline detection in two matrices, different in terms of composition and interferences, from ESRD patients: serum, a body fluid, and dialysate specimen, a complex fluid. Indeed, we found an evident increase in serum choline level for persons suffering from renal disease compared with apparently healthy persons as

measured by the current biosensor. A decrease in serum choline concentration was observed at the end of the dialysis treatment. These results are in good agreement with literature data, encouraging a possible employment of the developed device for the "on line" monitoring of choline during haemodialysis or for a novel recent application like the therapeutic drug monitoring (TDM).

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Competing interests

The authors declare that they have no conflict of interest.

Appendix A. Supplementary Data

Supplementary data of this article can be found online at _____

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Tables and figures captions

Table 1 Interferent ratios relevant to different electrosynthesized mono- and bilayers-membranes.

Table 2 Comparison of ChO amperometric biosensor.

Fig. 1 Flow injection responses for triplicate injections of 0.5 mM H_2O_2, 0.5 mM uric acid (UA), 0.2 mM acetaminophen (AC), 0.1 mM ascorbic acid (AA) and 0.1 mM cysteine (CYS) at a) bare and b) PPy_{ox}-PoAP modified platinum electrode. Injection volume: 20 μL. Carrier solution as specified in the experimental section. Flow rate: 0.2 ml min^{-1}.

Fig. 2 Typical SEM images of the boundary Pt/PPy_{ox}-PoAP (a) and of PPy_{ox}-PoAP layer at different magnifications (b) and (c). Experimental conditions as described in the Experimental section.

Fig. 3 Typical flow injection peaks for triplicate injections respectively of (a) 0.010, (b) 0.025, (c) 0.050 and (d) 0.075 mM choline standard solutions at Pt/PPy_{ox}-PoAP/ChO biosensor. Injection volume: 20 μL. Carrier solution as specified in the experimental section. Flow rate: 0.2 ml min^{-1}.

Fig. 4 Choline calibration plot and relevant linear interval (inset) obtained at the Pt/PPy_{ox}-PoAP/ChO modified electrode. Experimental conditions as in Figure 3.

Fig. 5 Flow injection peaks for triplicate injections respectively of (a) dialysate after 1 hour of haemodialysis, (b) serum from patient before starting the haemodialysis session, (c) serum from patient at the end of the haemodialysis session and (d) serum from a healthy volunteer at Pt/PPy_{ox}-PoAP/ChO biosensor. Injection volume: 20 μL. Carrier solution as specified in the experimental section. Flow rate: 0.2 ml min^{-1}.

Table 1 Interferent ratios relevant to different electrosynthesized mono- and bilayers-membranes

Interferent (concentration)	Interferent ratio[a]						
	Single layer membrane				Bilayer membrane		
	Pt/P2NAP	Pt/PPD	Pt/PoAP	Pt/PPY$_{ox}$	Pt/PPY$_{ox}$/P2NAP	Pt/PPY$_{ox}$/PPD	Pt/PPY$_{ox}$/PoAP
Uric acid (0.5 mM)	0.023	0.155	0.127	-[c]	-	-	-
Acetaminophen (0.2 mM)	0.035	0.731	0.494	-	-	-	-
Ascorbic acid (0.1 mM)	0.136	0.129	0.078	0.180	0.230	0.098	0.033
L-cysteine (0.1 mM)	0.022	0.110	0.442	-	-	0.0004	-
H_2O_2 (0.5 mM)[b]	0.043	0.271	0.658	0.072	0.014	0.070	0.082

[a] The interferent ratio was evaluated as R_{int}/R_{H2O2}, where $R_{int} = r_{i,m}/r_{i,u}$, $r_{i,m}$ is the interferent response at the modified electrode, $r_{i,u}$ is the interferent response at the unmodified electrode, R_{H2O2} = response of H_2O_2 at the modified electrode/response of H_2O_2 at bare Pt

[b] R_{H2O2}

[c] where ratio was not reported, the interferent response was so low to be undetectable and any ratio can be evaluated.

Table 2 Comparison of ChO amperometric biosensors

Sensor type Detection Potential	Upper linear limit (µM)	LOD (µM)	Operational stability	Interferences	Application to real sample	Ref.
ChO/HRP-PHZ-CPE 0 mV vs Ag/AgCl	70	0.1	Fluctuating behaviour	Endogenous catechol and phenol on HRP; ascorbic acid at the biosensor	Rat submandibular gland acinar cells	17
ChO/(GNP)$_4$/MWCNT/GCE -0.3 V vs Ag/AgCl	120	0.6	Drop of 7% after one month	No interference from 10 µM AA, UA, dopamine and AC [a]	Milk samples	18
ChO-AChE/MWCNT/ZrO2NP/GCE +0.2 V vs Ag/AgCl	1	0.01	Drop of 50% after 60 days	No interference from 1 mM AA, UA, lactic acid, dopamine, heparin	Huma serum	19

				sodium [a]		
Nafion/ChO/MnO2NP/GCE +0.7 V vs SCE	1000	5.0	Drop of 15% after one month	Interference from 1mM Glucose, AC, serine, dopamine, 0.1 mM AA,0.5 mM UA below 4.5%	Milk and feedstuff	45
ChO/PPy-PVS/Pt +0.3 V vs Ag/AgCl	1 - 1000	0.1	Drop of 65% after 42 days	10.36% interference from 0.3 mM UA and 7.92% interference from 0.1 mM AC	—	21
$(PEI/GA/BSA/ChO)_{10}$ on MMA/CellAce/MMA/PPD/Pt +0.7 V vs SCE	100	—	—	AA sensitivity of 0.00210 ± 0.0002 nA/µM over a choline sensitivity of 0.54 ± 0.03 nA/µM	Living tissue striatum	25
ChO-CTAB-SiO2-AuSPE +0.7 V vs silver pseudoreference	600	6	Drop of 50% after 3 weeks	Severe interference from 0.1 mM ascorbic acid solved by pre-treating with MnO_2	Baby milk	20
ChO/ZnO/Chit/GCE -0.76 V vs Ag/AgCl	5100	647	Significant decrease after 11 days	Less than 8% interference from 0.1 mM AA, UA and citric acid	Breast cancer cell	46
ChO/Ni-PB/BG/GC -0.05 V vs Ag/AgCl	100	0.45	Drop of 10% after 30 days	No interference from 0.1 mM AA, UA, glucose, glycine and AC	—	43
ChO-poly(aniline-co-o-aminophenol)/Pt +0.4 V vs Ag/AgCl	100	—	Drop of 49 % after 40 days	Interference of 10% and 4.5 % from 50 µM AA and UA	—	44
ChO/PPyox-PoAP/Pt +0.7 V vs Ag/AgCl	100	1	Drop of less than 20 % after 40 days	No interference from 0.5 mM UA, 0.2 mM AC, 0.1 mM AA and 0.1 mM cysteine	Human serum and dialysate	This work

[a] no data or figures of merit provided

ChO: choline oxidase; HRP: horse radish peroxidase; PHZ: phenotiazine; CPE: carbon paste electrode; GNP: gold nanoparticles; MWCNT: multi walled carbon nanotube; GCE: glassy carbon electrode; AChE: acetylcholinesterase; NP: nanoparticles; PPy-PVS : polypyrrole-polyvinylsulfonate; PEI: polyethyleneimine; GA: glutaraldehyde; BSA: bovine serum albumine; MMA: methyl methacrylate; CellAce: cellulose acetate; PPD: poly-phenylenediamine CTAB: cetyltrimethylammonium bromide, AuSPE: gold screen printed electrode; chit: chitosan, AA: ascorbic acid; UA: uric acid, AC: acetaminophen

Figure 1

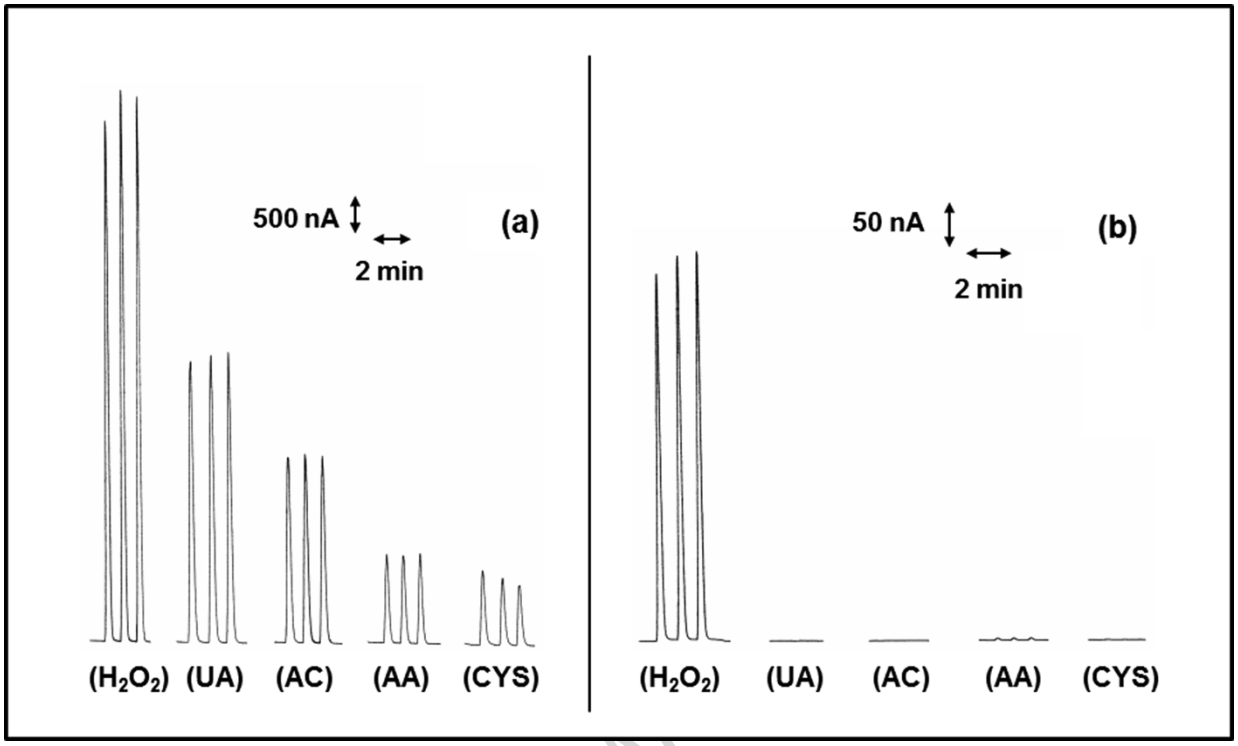


Figure 2

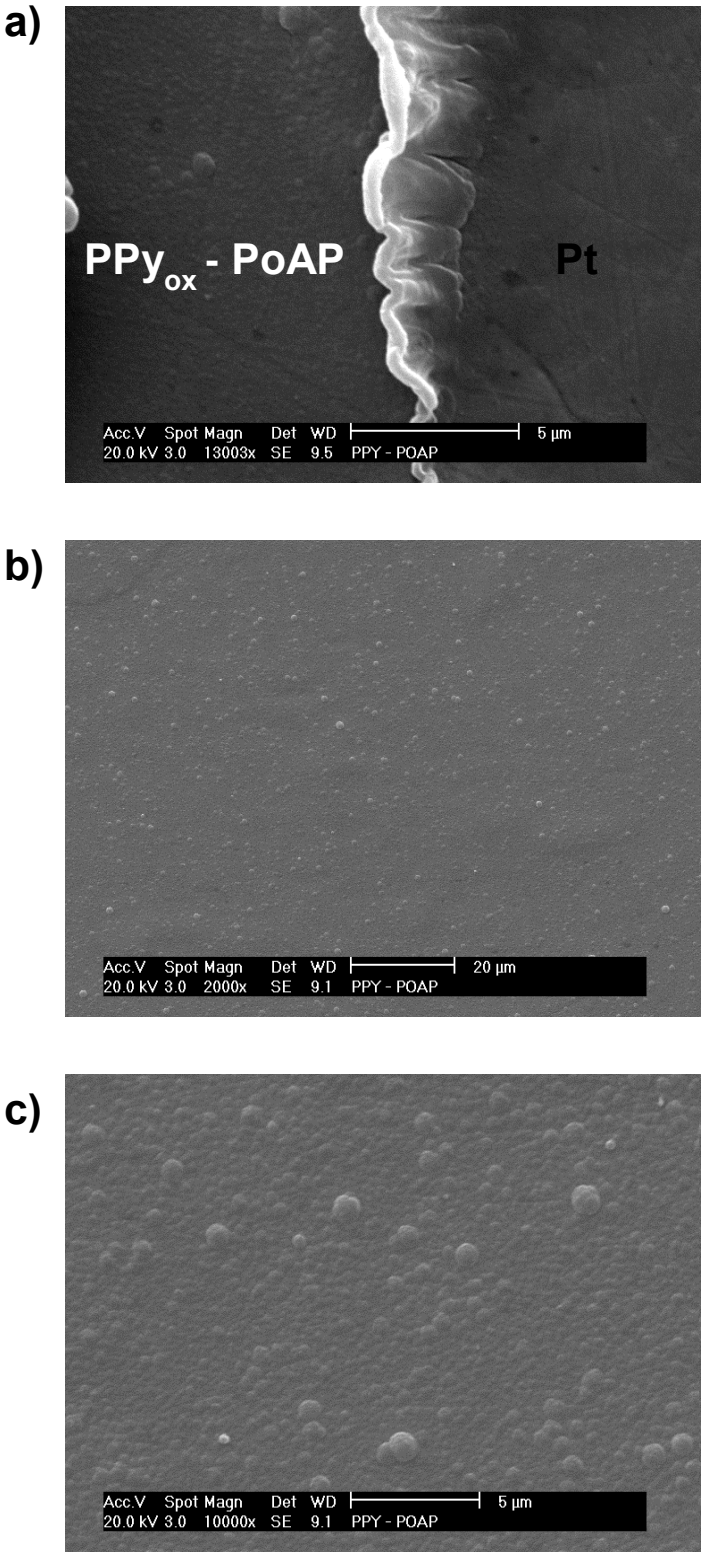


Figure 3

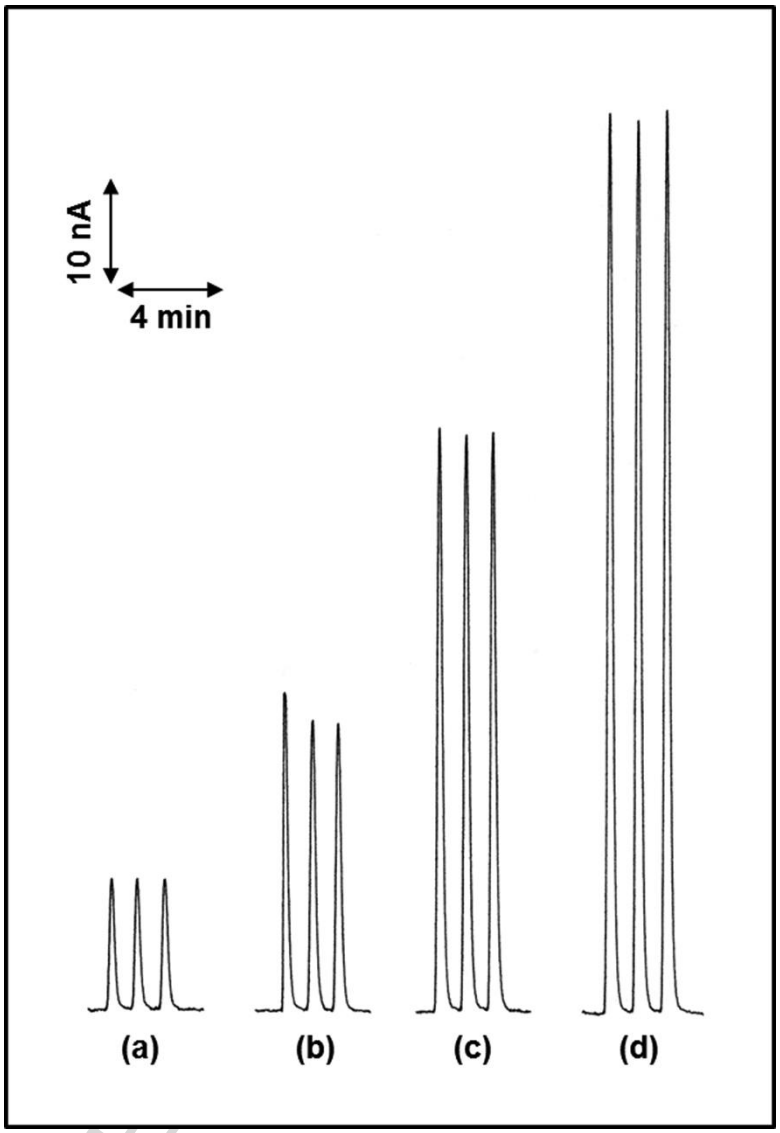


Figure 4

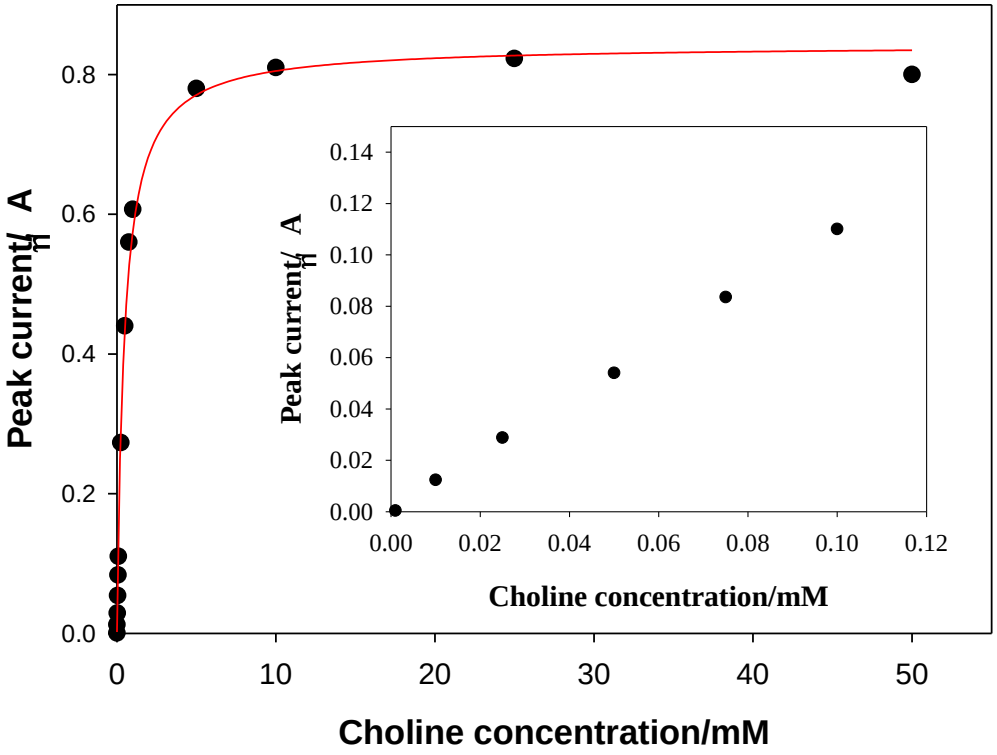
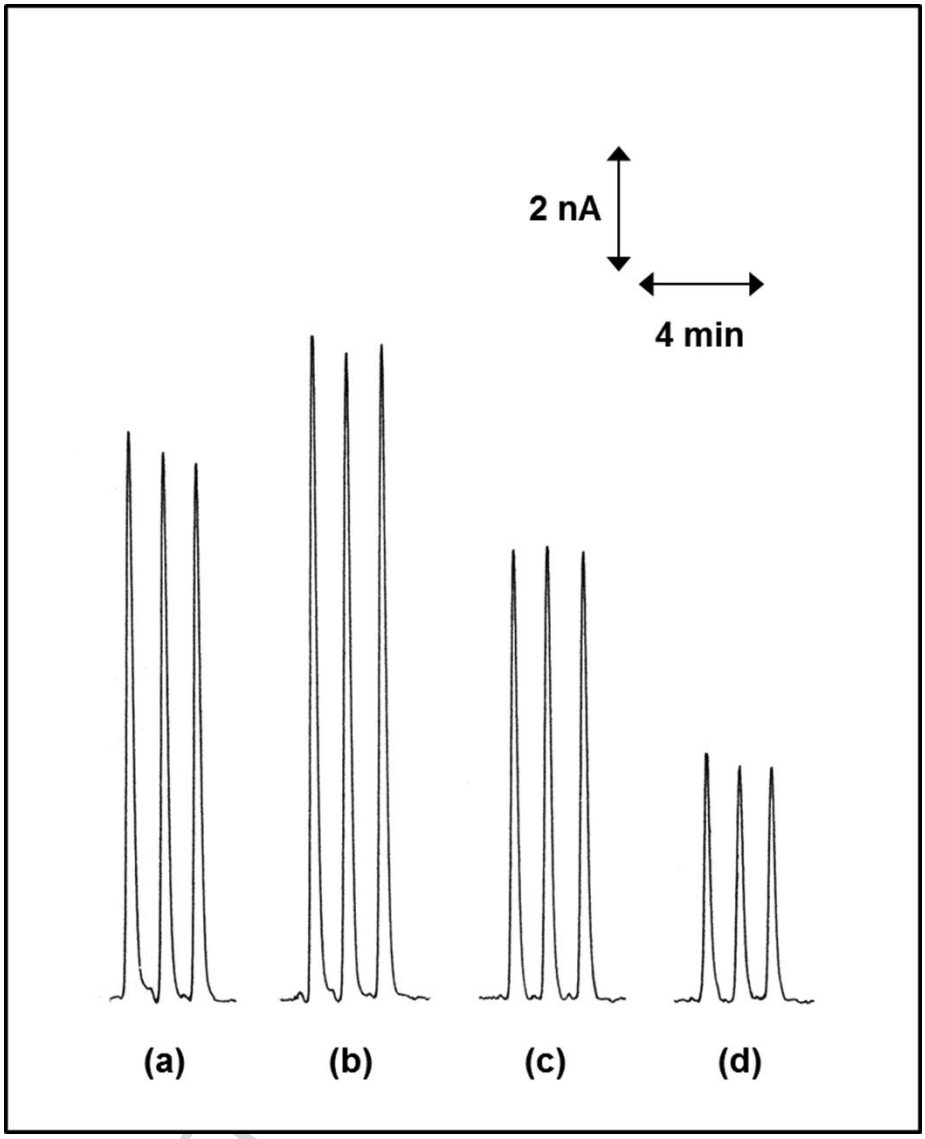


Figure 5



Highlights

- A biosensor for choline analysis in untreated human serum and dialysate is reported.
- A novel bilayer made of overoxidized poly(pyrrole)/poly(o-aminophenol) was realized.
- The morphology and permselective behaviour of the bilayer were studied.
- Choline biosensing was performed with high sensitivity and selectivity.
- Biological fluids from renal diseases patients on haemodialysis were analysed.