

Poly(Neutral Red)/Cholesterol Oxidase Modified Carbon Film Electrode for Cholesterol Biosensing

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Abstract

New amperometric cholesterol oxidase (ChOx) based enzyme biosensors for cholesterol have been developed. The enzyme was immobilised with and without glutaraldehyde cross-linking on top of carbon film electrodes modified with redox mediators. Mediators tested were: poly(neutral red) (PNR), Prussian blue and cobalt hexacyanoferrates. Amperometric detection of cholesterol showed that PNR/ChOx modified electrodes exhibited the best characteristics; under optimised conditions cholesterol was determined at -0.4 V vs. SCE with a detection limit of 1.9 μ M. The biosensors showed good reproducibility and stability and only a small influence from potential interferents in food. Analyses of cholesterol in egg yolk were successfully performed.

Keywords: Enzyme biosensors, Cholesterol oxidase, Carbon film electrodes, Poly(neutral red), Egg yolk

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1 Introduction

Nowadays the concern for a better and healthy life is increasing. The majority of cardiovascular diseases and atherosclerosis has its origin in high levels of cholesterol in blood serum, caused by an intake of high concentrations of cholesterol in foods.

Different methods have been used for the determination of cholesterol, such as colorimetric [1], fluorometric [2], HPLC [3], HPLC with electrochemical detection [4], gas-liquid chromatography [5], gas chromatography-mass spectrometry [6]. However, these methods need sample pre-treatment or component separation and they are time- and reagent-consuming. For this reason, it is very important to develop efficient and rapid analytical methods for cholesterol estimation in food and clinical samples. Currently, enzyme biosensors have practically replaced chemical methods, particularly for health care, as described in the review in [7]. To improve the selectivity, accuracy and precision of the assay, enzymes are often combined with electrochemical detection [8]. By immobilisation of the enzyme, reusable, stable and easy-to-handle systems can be developed. Biosensors present various advantages like simplicity, rapidness and cost effectiveness; amperometric biosensors are more attractive due to their high sensitivity and wide linear range, and for these reasons they hold a leading position among the presently available biosensor systems [9–11].

One of the important applications in foods is the measurement of cholesterol in egg yolks. Eggs are an essential part of the Mediterranean diet and so a knowledge of the

amount of cholesterol is a very important in the control of cholesterol consumption. Despite the complex composition of the egg yolk, analyses can be done without any special treatment of the egg yolk since the cholesterol in a yolk mostly exists as free cholesterol [12]. Non-enzymatic methods, such as gas chromatography or high-performance liquid chromatography have been preferred in studies in the literature, e.g. [5, 13–16].

Electrochemical detection systems for cholesterol assay are frequently based on monitoring the consumption of oxygen or the rate of production of hydrogen peroxide by an enzymatic reaction [17–19]. In order to improve enzyme substrate detection by lowering the overpotential at which the products of enzyme reaction are detected electrochemically, redox mediators were introduced. However, only a few studies report the use of redox mediators in cholesterol biosensors: potassium hexacyanoferrate(III) [20], ferrocene [21] or Prussian blue [22].

Phenazines have been used as redox mediators in biosensors [23]. Phenazine monomers contain a primary amino group as ring substituent which can release a proton upon oxidation, yielding a singly-charged cation-radical, which is responsible for the direct electrochemical polymerisation of monomers, forming the corresponding semiconducting polymer film [24]. Among them, electropolymerised neutral red (3-trimethylphenazine-2,8-diamine), poly(neutral red) (PNR) has already been used in a number of enzyme mediated sensors [24, 25].

Transition metal hexacyanoferrates (MHCF) are also widely used as redox mediators for biosensors because of

their mixed-valence cluster organization that can transfer electrons during reduction and oxidation processes [26]. Prussian blue (ferric ferrocyanide) (PB) has been the most widely used MHCF in redox-mediated enzyme biosensors [22,27]. Cobalt hexacyanoferrate (CoHCF) is another MHCF which found application as mediator in electrochemical biosensors [28,29].

Cholesterol biosensors in the literature usually use platinum as electrode substrate [30,31], although a recent report has used grafting on gold nanoparticles [32]. In this work, carbon film electrodes were used since, besides their availability in a variety of forms, carbon electrodes are generally inexpensive and the slow kinetics of carbon oxidation leads to a wide useful potential range. These characteristics are important advantages over platinum which can exhibit significant background currents and avoids the complex surface preparation procedures described in [32].

The present study concerns the development, evaluation and characterisation of a new cholesterol oxidase (ChOx) electrochemical biosensor on a carbon film electrode (CFE) support made from low-cost electrical resistors, which can be used as a short-term-use or disposable sensor. Three different redox mediators: poly(neutral red), Prussian blue and cobalt hexacyanoferrate have been tested and the best configuration was chosen. The novelty of this work resides in the comparison between these two families of mediators, phenazines and transition metal hexacyanoferrates none of which, to our knowledge, have been studied in the development of a cholesterol oxidase biosensor.

Optimisation included the influence of glutaraldehyde as enzyme cross-linking agent and of the surfactant Triton, as well as of the applied potential. The stability and selectivity of the biosensor was also evaluated and it was applied to the determination of cholesterol in egg yolks.

2 Experimental

2.1 Reagents and Solutions

Cholesterol oxidase (E.C. 232-842-1, from *Streptomyces* species 20 U/mg protein) was purchased from Sigma-Aldrich. Cholesterol (95%) and PNR (65% dye content) were from Aldrich. Glutaraldehyde (GA) (25% v/v) aqueous solution was purchased from Sigma. Potassium phosphate buffer solution (KPB), constituted by potassium dihydrogenphosphate (KH_2PO_4) and di-potassium hydrogenphosphate (K_2HPO_4) were purchased from Riedel-de-Haën. Potassium nitrate (KNO_3), iron(III) chloride-6-hydrate (FeCl_3) and hydrochloric acid (HCl) were purchased from Riedel-de-Haën. Potassium chloride (KCl) was from Fluka. Potassium hexacyanoferrate III ($\text{K}_3\text{Fe}(\text{CN})_6$) and cobalt(II) chloride (CoCl_2) were purchased from Merck. Linoleic acid and retinol were from Sigma.

2.2 Electrochemical Measurements and Apparatus

Measurements were made in a one-compartment cell containing a platinum auxiliary electrode and a saturated calomel electrode (SCE) as reference. The working electrodes were made from carbon film resistors (2Ω nominal resistance) of length 6 mm and 1.5 mm in diameter. The resistors were fabricated from ceramic cylinders by pyrolytic deposition of carbon from methane in a nitrogen atmosphere [33]. One of the two tight-fitting metal caps, linked to an external contact wire was removed and the other one covered in plastic and protected by normal epoxy resin. The geometric area of the electrodes is 0.20 cm^2 . Voltammetric and amperometric experiments were carried out using a PalmSens potentiostat (Palm Instruments BV).

2.3 Carbon Film Electrode Preparation

Since carbon film electrode surfaces cannot be renewed by polishing or other mechanical methods, electrochemical pre-treatment was chosen in order to obtain a reproducible electrode response. The electrochemical pre-treatment was always performed before use, by potential cycling between -1.0 and $+1.0\text{ V}$ versus SCE, at a scan rate of 100 mV s^{-1} , until a stable voltammogram was obtained.

2.3.1 NR Polymerisation

Electropolymerisation of the monomer dye NR was performed by potential cycling in a solution of 0.025 M potassium phosphate buffer (KPB) with 0.1 M potassium nitrate (KNO_3), pH 5.5, containing 1 mM monomer. The potential was cycled between -1.0 and $+1.0\text{ V}$ vs. SCE for 15 cycles at 50 mV s^{-1} [24].

2.3.2 Prussian Blue Deposition

For the deposition of Prussian Blue mediator films, potential cycling was carried out in a solution of 1 mM $\text{K}_3\text{Fe}(\text{CN})_6 + 1\text{ mM}$ FeCl_3 in 0.1 M KCl + 0.1 M HCl, scanning for 25 cycles between -0.1 and 0.5 V vs. SCE, at a scan rate of 10 mV s^{-1} [27]. The films obtained were stabilised by heating in a jet of hot air at 70°C during 5 min and were stored at room temperature in the dark.

2.3.3 Cobalt Hexacyanoferrate Deposition

Deposition of cobalt hexacyanoferrate was carried out by potential cycling between -0.2 and $+0.9\text{ V}$ vs. SCE, at a scan rate of 50 mV s^{-1} for 25 cycles. The solution used contained 5 mM $\text{CoCl}_2 + 2.5\text{ mM}$ $\text{K}_3\text{Fe}(\text{CN})_6$ in 0.05 M NaCl (pH 3.0) [29]. After deposition, the film was stabilised in 0.05 M NaCl (pH 3.0) for 1 h.

2.3.4 Enzyme Immobilisation

ChOx was immobilised by drop coating the electrode in one of two different ways. The first was by direct deposition of 3 μL of 0.1 M phosphate buffer, pH 6.9 containing 1 U of cholesterol oxidase and then drying during at least 1 h at room temperature. In the second, ChOx was immobilised together with glutaraldehyde (GA) cross-linking agent. A mixture containing 3 μL of enzyme solution + 1 μL of GA (2.5% v/v in water) was placed onto the electrode and then dried for at least 1 h.

When not in use, enzyme electrodes were kept at 4 °C in 0.1 M phosphate buffer electrolyte, pH 7.0.

3 Results and Discussion

3.1 Biosensors for Cholesterol with Different Redox Mediators – Optimisation

Carbon film electrodes were modified with one of the three different redox mediators: poly(neutral red) (PNR), Prussian blue (PB) or cobalt hexacyanoferrate (CoHCF). Figure 1 shows the polymerisation of NR and the deposition of PB and CoHCF, all carried out by potential cycling. For NR one irreversible peak, due to monomer oxidation, and two redox couples are observed: between -0.5 and -0.6 V oxidation/reduction of the monomer/polymer occurs and the positive peak (between 0.0 and 0.2 V) is due to doping/dedoping of the polymer [19]. For PB and CoHCF deposition, one redox couple was observed in the potential range studied. For PB, this couple is due to Prussian blue/Prussian white [34] and in the case of CoHCF the peaks can be ascribed to $\text{Na}_2\text{Co}_3^{II}[\text{Fe}^{II}(\text{CN})_6]_2$ oxidised to $\text{Na}_2\text{Co}_3^{III}[\text{Fe}^{III}(\text{CN})_6]_2$ [35]. It is clear in all cases that the current peaks increase in height with the increase in cycle number, consistent with film growth on the electrode surface.

Biosensors were prepared by immobilising the enzyme cholesterol oxidase on top of the three mediators, which will be designated PNR/ChOx, PB/ChOx and CoHCF/ChOx. The enzyme was immobilised in the same way on the three mediators, by simple drop coating, without glutaraldehyde. The performance of the resulting biosensors was evaluated by amperometry at fixed potential.

In order to maximise the response to cholesterol, an investigation of the best applied potential was performed, testing values between -0.5 V and $+0.3$ V. It was found necessary to remove dissolved O_2 because no response to cholesterol was seen in the presence of dissolved O_2 under natural conditions (N_2 was bubbled for 10 min before measurement and a flux of N_2 left on top of the solution during experiments). This can be partly because of the large cathodic current due to O_2 reduction that masks any biosensor response and also because, according to [36], O_2 (in high concentrations) can act as an inhibitor for cholesterol oxidase from *Streptomyces* species. In these conditions, and for all biosensor assemblies, no response was obtained between -0.3 and $+0.3$ V. Between

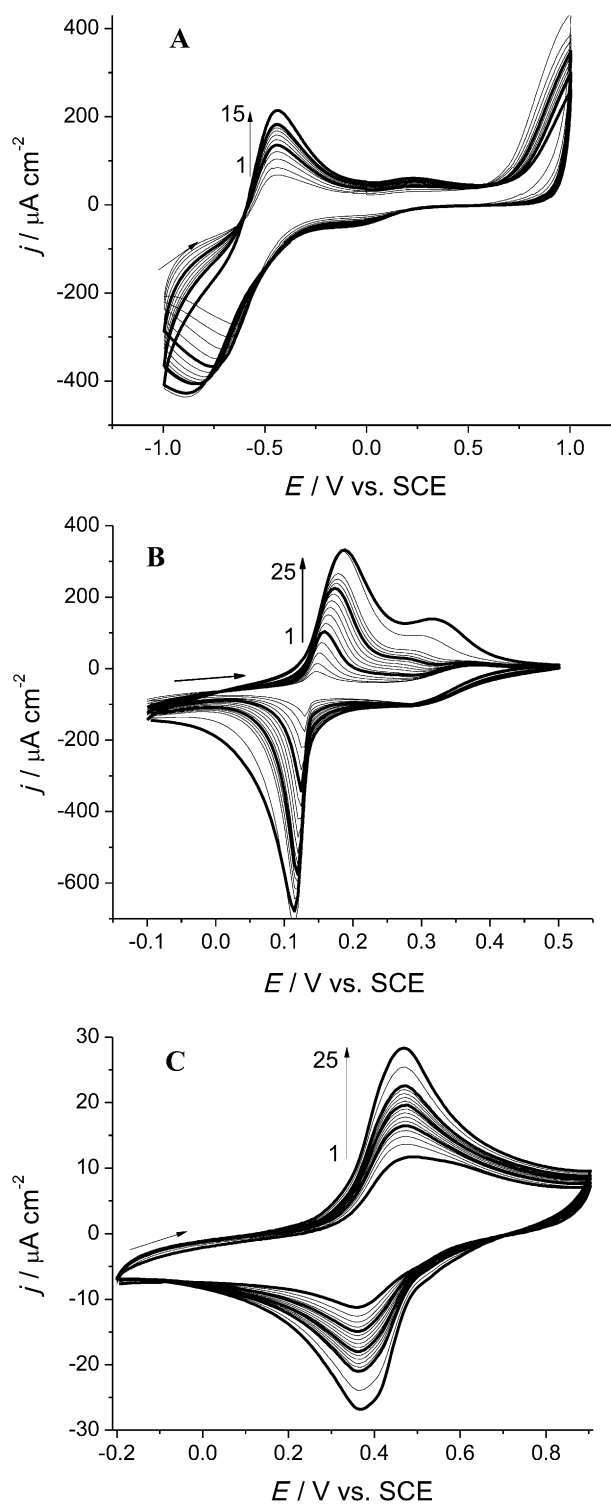


Fig. 1. (A) Polymerisation of NR: 1 mM monomer in 0.025 M KPB + 0.1 M KNO_3 , pH 5.5; (B) Deposition of PB: 1 mM FeCl_3 + 1 mM $\text{K}_3\text{Fe}(\text{CN})_6$ in 0.1 M HCl + 0.1 M KCl, pH 1.0; (C) Deposition of CoHCF: 5 mM CoCl_2 + 2.5 mM $\text{K}_3\text{Fe}(\text{CN})_6$ in 0.05 M NaCl, pH 3.0.

-0.4 and -0.5 V, a small increase in cathodic response is observed with increase in negative potential. In the case of hexacyanoferrates, this behaviour was unexpected

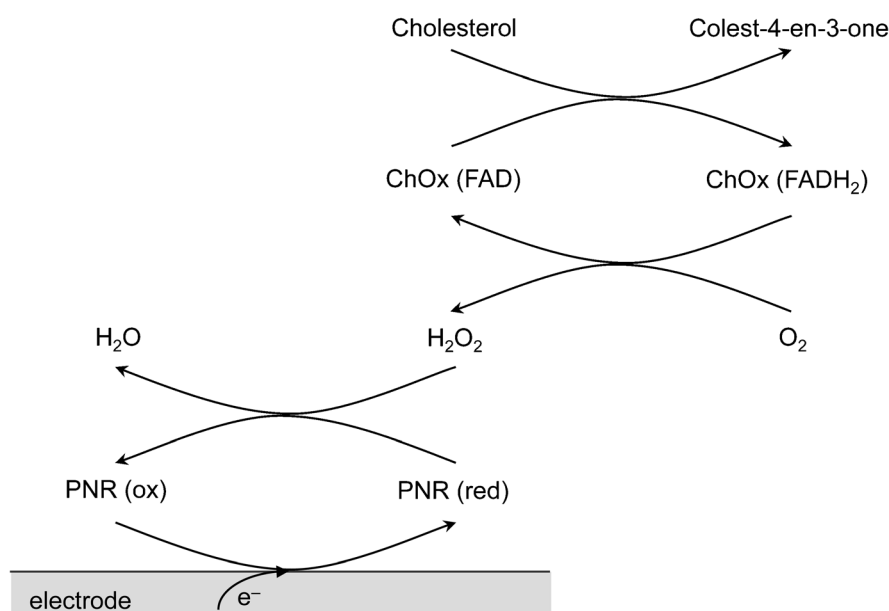


Fig. 2. Possible reaction mechanism at the PNR-based biosensor.

since, normally, mediators of the hexacyanoferrate family act by reducing the hydrogen peroxide generated from the enzyme-catalysed oxidation reaction and the working potential necessary is around 0.0 V [34]. Thus a more complex mechanism has to be invoked.

A possible mechanism, exemplified involving PNR, is shown in Figure 2, in agreement with the fact that a cathodic change in current was observed on addition of cholesterol to the solution and also involving the regeneration of FAD cofactor of the enzyme, which normally occurs around -0.45 V in phosphate buffer pH 7.0 [37]. Deoxygenation never removes all oxygen and there is certainly sufficient left within the structure of the modified electrode for this mechanism to be able to occur. It is similar to the mechanism proposed in [25] for PNR with glucose oxidase.

Owing to the fact that the increase of response at -0.5 V compared with -0.4 V is just 2.6% (data not shown), and in attempt to reduce interferences, a compromise was made and the potential chosen was -0.4 V, which gives sufficient overlap with the FAD/FADH₂ redox couple.

The determination of cholesterol with the three types of mediators was thus performed at -0.4 V and in phosphate buffer pH 7.0, the medium in which most of the studies were performed [11,20] because it was found to be optimum for cholesterol oxidase activity.

Figure 3 shows calibration curves for cholesterol obtained at the three different biosensors. The responses were compared regarding linear range, sensitivity and detection limit. At the hexacyanoferrate-based biosensor the response for PB and CoHCF mediators is similar: linear ranges up to $370 \mu\text{M}$, sensitivities of 1.2 and $0.8 \text{ nA } \mu\text{M}^{-1} \text{ cm}^{-2}$ and limits of detection 6 and $5 \mu\text{M}$, respectively. At PNR/ChOx electrodes, the linear range was

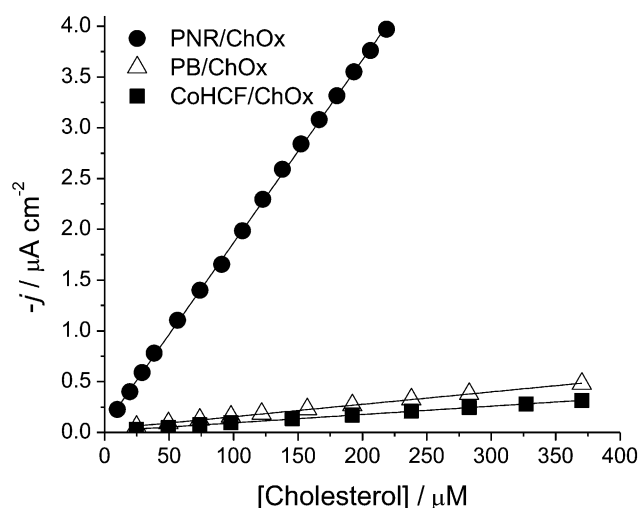


Fig. 3. Calibration curves for cholesterol obtained at -0.4 V in 0.1 M KPB for PNR/ChOx (\bullet); PB/ChOx (Δ) and CoHCF/ChOx (\blacksquare) biosensor.

smaller, up to $220 \mu\text{M}$, the sensitivity 15 times higher ($18 \text{ nA } \mu\text{M}^{-1} \text{ cm}^{-2}$), compared with hexacyanoferrates, and the detection limit was $1.9 \mu\text{M}$ (Table 1). Due to its higher sensitivity, the PNR-based biosensor was used for further experiments in this work.

3.2 Biosensor with PNR Redox Mediator: Optimisation, Stability and Reproducibility

Since cholesterol is not very soluble in aqueous solution (0.026 mg L^{-1} [38]), normally a surfactant is added, and Triton X-100 is the most commonly employed. When testing cholesterol solutions made with Triton the response of the biosensor was inhibited immediately after the first in-

Table 1. Comparison of analytical parameters of cholesterol biosensors using different redox mediators.

Electrode type	Linear range (μM)	Sensitivity ($\text{nA } \mu\text{M}^{-1} \text{cm}^{-2}$)	Detection limit (μM)
PNR/ChOx	220	18	1.9
PB/ChOx	375	1.2	6
CoHCF/ChOx	375	0.8	5

jection. Triton was already reported to reduce the activity of cholesterol oxidase [39], especially with repeated use of the biosensor. For this reason, the influence of Triton was investigated further, Triton being added to the buffer solution for each measurement. Control experiments without Triton in the buffer were also performed and the responses were compared. In the presence of Triton the biosensor response was smaller by 39% (see Table 2). Hence, the option was not to use Triton at all, and achieve solubilisation by heating and sonication.

Two types of cholesterol biosensor were prepared with PNR mediator, using different enzyme immobilisation methods, one without and one with cross-linking by glutaraldehyde (GA). Comparing the responses to cholesterol for the two biosensors (Figure 4) it was observed that without GA the response was 28% higher; however, the linear range and detection limit were the same (Table 2).

Since cross-linking can be expected to increase the robustness of the biosensor the long term stability at the two enzyme electrodes was also examined. Biosensor electrodes were kept in phosphate buffer at 4°C and tested once per week, see Figure 5. During the first 2 weeks, an increase in response was observed in both cases, as has been reported previously [10], that can be attributed to some reorganisation of the enzyme in the film. After 3 weeks the response began to decrease, fol-

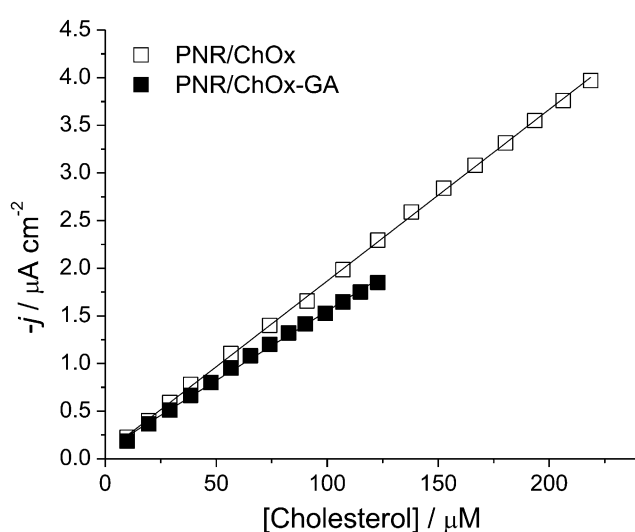
Fig. 4. Calibration curves for cholesterol at -0.4 V in 0.1 M KPB at (A) PNR/ChOx biosensor with (■) and without (□) GA.

Table 2. Comparison of analytical parameters for PNR/ChOx and PNR/ChOx-GA biosensors and PNR/ChOx in the presence and absence of Triton.

Electrode type	Linear range (μM)	Sensitivity ($\text{nA } \mu\text{M}^{-1} \text{cm}^{-2}$)	Detection limit (μM)
PNR/ ChOx-GA	123	14.5	3.2
PNR/ChOx (no Triton)	220	18	1.9
PNR/ChOx (with Triton)	193	11.2	3.4

lowing a similar profile for the two biosensors, but more slowly for the biosensor without glutaraldehyde. After 1 month, 84% of the initial response is maintained for the PNR/ChOx biosensor and the PNR/ChOx-GA electrode has 57% of the initial response. After 2 months the responses were 27% and 22% for the PNR/ChOx and PNR/ChOx-GA, respectively, and after 3 months both electrodes gave a close-to-zero response. In another test, an electrode without GA was kept dry at room temperature and its stability profile was measured. In this case the response decreased faster: after 17 days, the electrode already lost 83% of the initial response. Thus, the modified electrodes must be kept in buffer solution at 4°C .

The repeatability of the PNR/ChOx electrode was evaluated by measuring the response to $100 \mu\text{M}$ cholesterol for 6 successive additions and comparing the results. The relative standard deviation (*RSD*) between measurements was 3%. The reproducibility of the PNR-based biosensors, evaluated by measuring the sensitivity for 3 different electrodes prepared in the same way, without GA, gave an *RSD* of 4.2%.

Comparison of the results with relevant articles in the literature is shown in Table 3. The linear range of the PNR/ChOx biosensor developed here ($200 \mu\text{M}$) is lower

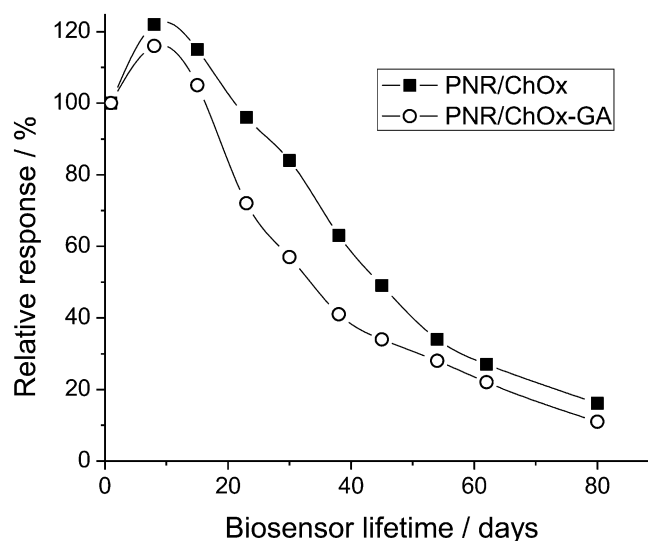


Fig. 5. Stability of cholesterol biosensors: PNR/ChOx (■) and PNR/ChOx-GA (○).

Table 3. Comparison of analytical parameters of various cholesterol biosensors with different surface modifications [a].

Modified electrode	Linear range	Detection limit (μM)	Sensitivity ($\text{nA } \mu\text{M}^{-1} \text{ cm}^{-2}$)	Reference
Pt/PPy-ChOx	0–300 μM	5.7	0.62	[10]
Pt/poly(DAN)-ChOx	0–300 μM	15	0.50	[10]
ITO/DBS-PPy-ChOx	2–8 mM	–	5	[20]
Gr/CNT-Pt/sol gel-ChOx	4–100 μM	1.4	14	[40]
Pt/poly(3-TPAA)-ChOx	0–8 mM	420	4.49	[41]
Pt/Pt/PPy-ChOx/o-PPD	0–350 μM	12.6	0.88	[42]
Au/SAM-PQQ-BA-FAD/ChOx	0.07–1.25 mM	69	1.3	[43]
CFE/PNR/ChOx	0–220 μM	1.9	18	This work

[a] See text for explanation of abbreviations

than that of ChOx entrapped in poly(pyrrole) (PPy) or poly(diaminonaphthalene) (DAN) [10] of 300 μM , but the sensitivity is much higher and the detection of 1.9 μM limit lower. A slightly lower detection limit of 1.4 μM was obtained with CNT-Pt/sol gel-ChOx on a graphite (Gr) electrode substrate at -0.18 V vs. Ag/AgCl in [40], but the linear range was only up to 100 μM ; all the others have higher detection limits. The sensitivity of the PNR/ChOx biosensor is significantly higher than in all other sensors reported in Table 3, the closest being in [40] (14 $\text{nA } \mu\text{M}^{-1} \text{ cm}^{-2}$) in which ChOx was immobilised by physical adsorption on a dodecylbenzene sulfonate (DBS) doped PPy and in [41] based on covalent linkage with poly(3-thiopheneacetic acid) (3-TPAA).

More complex constructions are used in [42,43] without better analytical parameters, except for an extended linear range. In [42], a biosensor based on a polymer bilayer with PPy and poly(o-phenylenediamine) (PPD) in which ChOx was entrapped on previously platinized Pt was used. The highly complex sensor in [43] involves a self-assembled monolayer of cysteamine on gold to anchor PQQ and 3-aminophenylboronic acid (BA) on an in situ reconstituted cholesterol oxidase on a monolayer of FAD cofactor.

3.3 Interference Studies and Measurements in Natural Samples

Several compounds were tested as possible interferents for cholesterol: linoleic acid, retinol and glucose, which are normally found in foods containing cholesterol. Two different experiments were conducted. In the first experiment, after stabilisation of the baseline, cholesterol was added to the buffer solution containing the interferents. Comparing the response in the presence and in the absence of interferents a decrease of 8% was observed. In the second experiment, cholesterol was injected into a mixture containing all the other compounds, maintaining the ratio 1:1 of cholesterol to interferents; a decrease of 5% was obtained. These results were encouraging for applying the developed biosensor for measurements in natural samples using standard addition.

The sample chosen to measure cholesterol was egg yolk, in which the cholesterol exists mainly in the free form, so that no complicated sample pretreatment is nec-

essary. The yolk was separated from the white, the mass and volume (in a graduated cylinder) measured; then it was diluted two times with water. The determination of cholesterol in egg yolk was carried out using the standard addition method. A known aliquot of egg solution was injected into buffer electrolyte followed by known amounts of cholesterol. The value obtained from three determinations was $11.9 \pm 0.5 \text{ mg/g}$ cholesterol in egg yolk. This value compares well with values found in the literature (e.g. 12.4 mg/g with a relative standard deviation of 4% [44]).

4 Conclusions

A new cholesterol electrochemical biosensor has been developed at carbon film electrode substrates. Three different mediators were used in combination with cholesterol oxidase: poly(neutral red), Prussian blue and cobalt hexacyanoferrate and the first of these was demonstrated to be the most appropriate for the cholesterol biosensor. The measurement of cholesterol was performed by amperometry at fixed potential with high sensitivity and low detection limit, comparable or better than cholesterol biosensors in the literature and with very good stability. Only small interferences from components present in cholesterol-containing foods were observed, and the determination of cholesterol in egg yolk was successful, showing good correlation with the tabulated value. This augurs well for future applications of this sensor.

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