

New redox and conducting polymer modified electrodes for cholesterol biosensing

Cite this: *Anal. Methods*, 2013, **5**, 1199

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New enzyme biosensors for cholesterol detection based on a recently developed transduction platform constituted of a GCE modified with polymer redox mediator poly(methylene blue) (PMB) and conducting polymer poly(3,4-ethylenedioxythiophene) (PEDOT) were for the first time prepared and evaluated. The enzyme cholesterol oxidase (ChOx) was immobilized by simple adsorption, the ChOx/PEDOT/PMB/GCE biosensor being applied for amperometric determination of cholesterol by monitoring the peroxide reduction produced by the enzymatic reaction at -0.4 V vs. SCE. Amperometric studies at fixed potential reveal that almost interference-free cholesterol determination can be achieved at the newly developed biosensor in a range between 10 and 220 μM with a sensitivity of $79.0 \mu\text{A cm}^{-2} \text{mM}^{-1}$ and a detection limit of 1.6 μM . The recovery and storage stability of the biosensor were evaluated and the biosensor was applied to cholesterol detection in whole cow milk and chicken egg yolk.

Received 15th November 2012

Accepted 10th January 2013

DOI: 10.1039/c3ay26409b

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1 Introduction

Cholesterol, an organic chemical substance classified as a waxy steroid of fat, is an essential structural component of mammalian cell membranes and is required to establish proper membrane permeability and fluidity. In addition, cholesterol is an important component for the manufacture of bile acids, steroid hormones, and vitamin D, being the principal sterol synthesized by animals, predominantly in the liver.^{1,2} Although cholesterol is important and necessary for the above-mentioned biological processes, its high levels in blood have been linked to damage to arteries and cardiovascular disease.³ Therefore, determination of cholesterol is of great importance for food and clinical applications.

Analytical methods used for cholesterol assays include colorimetric,⁴ high performance liquid chromatography (HPLC),⁵ spectrophotometric⁶ and electrochemical methods.^{7–11} Among these methods, electrochemical techniques offer advantages such as simplicity and relatively low cost, and their sensitivity and selectivity can be further improved by using biosensors.¹² A cholesterol biosensor often uses cholesterol oxidase (ChOx) as the biosensing element, which is a flavin adenine dinucleotide (FAD) containing flavoenzyme, that catalyses the oxidation of cholesterol to cholest-4-en-3-one and H_2O_2 in the presence of oxygen.¹³ For biosensor construction, the immobilization of enzyme on the electrode surface is a

critical step and substrate diffusion to the catalytic site of the enzyme plays an important role in biosensor performance. ChOx has been immobilized on different platforms, on top of metal nanoparticles (NP): AuNP,^{2,14,15} PtNP,^{16,17} Au and Pt NP alloy,¹⁸ NiNP,¹⁹ AgNP,²⁰ which are mostly dispersed together with carbon nanotube (CNT) in chitosan (Chit) solution. It was incorporated in a graphite–Teflon composite matrix, together with horseradish peroxidase (HRP) and ferrocyanide mediator²¹ and also immobilized on top of a highly hydrophilic multi-walled carbon nanotube (MWCNT)-containing layer.²² Various enzyme immobilization procedures have been used, mostly through physical adsorption or electrostatic interaction,^{14,23–25} covalent linkage^{26–28} and also sol-gel entrapment.^{29–31}

Conducting polymers, such as polypyrrole, polyaniline and poly(3,4-ethylenedioxythiophene) (PEDOT), have been used as a new material for enzyme immobilization.³² This is due to the fact that they have a high conductivity, good stability in the air and aqueous solutions, and the enzyme can be immobilized directly into the polymer film to form an enzyme electrode without using any additional agent.³³

This paper reports the use for the first time of a newly developed PEDOT/poly(methylene blue)(PMB) modified glassy carbon electrode (PEDOT/PMB/GCE)³⁴ as a platform for a cholesterol biosensor. MB is a water-soluble cationic dye molecule that has been extensively used as a mediator due to its high electron transfer efficiency and low cost.^{35–39} The poor stability of PMB-modified glassy carbon electrodes, due to the high solubility of PMB films formed by electropolymerisation, was improved by protecting the film with a hydrophobic PEDOT electropolymerised film. The PEDOT/PMB/GCE was already used as sensor for ascorbate and biosensor for glucose, as reported in

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ref. 40, the results being promising for its use in the construction of other enzyme biosensors, as presented here for a cholesterol biosensor. The analytical performance of the biosensor has been compared with those of recently reported cholesterol biosensors and the influence of interfering species present in real samples where cholesterol is present usually were assessed. The cholesterol concentration in whole cow milk and egg yolk was determined with the ChOx/PEDOT/PMB/GCE biosensor.

2 Experimental

2.1 Reagents

All chemicals were analytical reagent grade and used as received. The monomers 2,3-dihydrothieno[3,4-*b*]-1,4-dioxin (EDOT), methylene blue (MB), cholesterol oxidase from *Streptomyces* species (ChOx, 54 U mg⁻¹), cholesterol, linoleic acid, retinal, ascorbic acid, α -D(+)-glucose, sodium phosphate monobasic monohydrate sodium and poly(styrene sulfonate) (NaPSS) were from Sigma-Aldrich (Steinheim, Germany). Potassium hydrogen phosphate, di-sodium hydrogen phosphate 2-hydrate, potassium nitrate and sodium chloride were from Riedel-deHaën (Seelze, Germany), potassium hydrogen phosphate 3-hydrate from Pan-reac (Barcelona, Spain) and sodium borate and sodium sulfate were from Merck (Darmstadt, Germany).

The solutions used for the polymerization of methylene blue (MB) and EDOT were those reported in ref. 34 and in ref. 41 being 1 mM MB in 0.025 M NaB₄O₇ + 0.1 M Na₂SO₄ pH 9.2 and 10 mM EDOT in 0.1 M NaPSS, respectively.

Amperometric detection of cholesterol was done in sodium phosphate buffer saline (NaPBS) (0.1 M phosphate buffer + 0.05 M NaCl, pH = 7.0), prepared from sodium di-hydrogen phosphate, di-sodium hydrogen phosphate and sodium chloride. A stock solution of 10 mM cholesterol was prepared by dissolving the appropriate amount of cholesterol in water, which was achieved by heating the cholesterol solution during 20 min in a water bath at 80 °C, followed by ultrasonication during 15 min in an ultrasound bath, at room temperature.

Millipore Milli-Q nanopure water (resistivity \geq 18 M Ω cm) and analytical reagents were used for the preparation of all solutions. Experiments were performed at room temperature (25 \pm 1 °C).

2.2 Apparatus

Electrochemical experiments were performed with a computer-controlled μ -Autolab type I potentiostat-galvanostat with GPES software (Metrohm-Autolab, Utrecht, Netherlands). A 10 mL cell was used containing the modified glassy carbon electrode as working electrode, a platinum wire as counter electrode and a saturated calomel electrode (SCE) as reference electrode.

The pH-measurements were carried out with a Crison 2001 micro pH-meter (Crison Instruments SA, Barcelona, Spain) at room temperature.

2.3 Biosensor preparation

Before use, the GC electrode was polished to a mirror finish using 6 micron diamond spray (Kemet, UK) followed by a

thorough rinse with Millipore Milli-Q nanopure water. The electrode was pretreated by cycling the potential between -1.0 and +1.0 V vs. SCE at 100 mV s⁻¹, until a stable voltammogram was recorded. The preparation of PEDOT/PMB/GCE was done by following the procedure described in ref. 34. First, MB was polymerized from a solution containing 1 mM MB in 0.025 M NaB₄O₇ + 0.1 M Na₂SO₄ pH 9.2 by potential cycling between -0.6 and 1.0 V vs. SCE, during 30 cycles. The PMB/GCE was then left to dry in the dark at room temperature for at least 12 h, and EDOT was polymerized on top, by potential cycling between -0.6 and 1.2 V vs. SCE in a solution of 10 mM EDOT in 0.1 M NaPSS, for 10 cycles. Following this, the PEDOT/PMB/GCE electrode was left to dry for another 12 h at room temperature. The enzyme ChOx was then immobilized on top of the PEDOT/PMB/GCE electrode by dropping 3 μ L of 1 U mL⁻¹ enzyme solution. Before use, the biosensor was left to dry for 4 h at room temperature and, in between uses, was stored at 4 °C, immersed in 0.1 M NaPBS pH 7.0.

3 Results and discussion

3.1 Influence of the applied potential on the amperometric response of ChOx/PEDOT/PMB biosensor

A study of the influence of applied potential was performed at the ChOx/PEDOT/PMB biosensor, by monitoring the change in current following injection of cholesterol at different applied potentials in fixed potential amperometric measurements, from -0.5 to 0.0 V vs. SCE (see Fig. 1). All experiments were performed in deoxygenated 0.1 M NaPBS pH 7.0 solution, after 20 min of nitrogen bubbling, since it was observed that the biosensor is not functional without following this procedure. In all cases a cathodic change in current was recorded after cholesterol injection, from -0.5 up to -0.2 V vs. SCE, above which the biosensor stopped giving a measurable response. Probably the biosensor did not respond to cholesterol when oxygen is not removed, due to the fact that dissolved oxygen reduction may mask the significantly lower reduction currents produced by the enzymatic reaction. On removing the dissolved

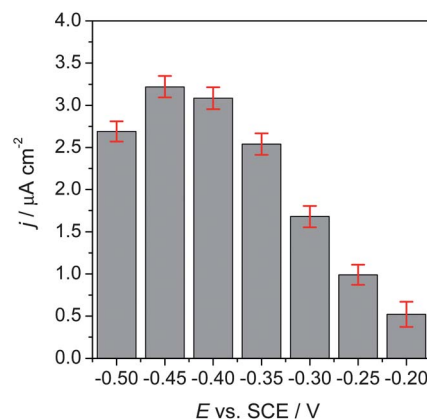


Fig. 1 Change in current recorded at ChOx/PEDOT/PMB/GCE biosensor subsequent to cholesterol injection at different potentials from -0.5 to -0.2 V vs. SCE in 0.1 M NaPBS pH 7.0; final cholesterol concentration 30 μ M cholesterol.

oxygen, the baseline current becomes less negative and approaches zero, and the cathodic change in current after cholesterol injection can be seen. All these experimental observations led us to consider a mechanism in which hydrogen peroxide is reduced to water at the PMB polymer, enough oxygen remaining in the solution and in the enzymatic layer, after nitrogen bubbling, in order to accept the electrons from the reduced FADH_2 . The same behaviour was observed in the case of a ChOx/poly(neutral red)(PNR) biosensor,⁴² which reported the mechanism described above and presented in Scheme 1. A different response was observed in the case of a biosensor containing glucose oxidase instead of ChOx immobilized on top of the PEDOT/PMB/GCE, where direct electronic communication between PMB and the enzyme cofactor happens, so that the mediator is oxidized at the electrode surface after receiving the electrons from the FADH_2 .⁴⁰

As observed in Fig. 1, the greatest change in current was recorded at -0.45 V vs. SCE, which coincides with the formal potential of the enzyme cofactor FAD, $E^{o'} = -0.45$ V vs. SCE,⁴³ being similar at -0.4 V and lower at -0.5 V, continuing to decrease at less negative applied potentials. Thus, a potential of -0.4 V vs. SCE was chosen to be employed in further experiments.

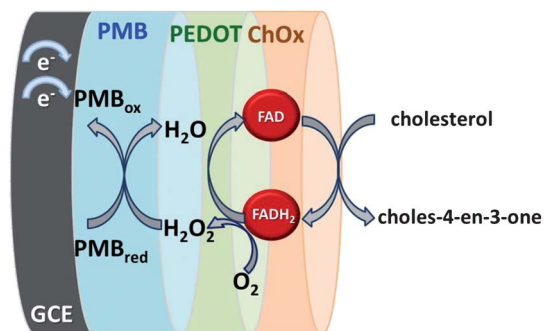
3.2 Analytical properties of the ChOx/PEDOT/PMB/GCE biosensor

Successive amperometric measurements at -0.4 V vs. SCE, for three different solutions containing 10 mM cholesterol, at the same biosensor, were done to estimate the relative standard deviation (RSD) of the procedure. These three different cholesterol solutions were prepared by dissolving the appropriate amount of cholesterol in water, followed by 20 min heating in a water bath at 80 °C and 15 min of ultrasonication in an ultrasound bath, at room temperature. The calculated RSD was 5.3%, indicating a good repeatability and that the cholesterol dissolved well by following the above mentioned preparation procedure. This was a very satisfactory result, since the addition of surfactant to ensure cholesterol dissolution was not needed, very often mentioned in the literature.^{12,15–19,47–50}

The reproducibility of the biosensor construction procedure was also evaluated by constructing a calibration curve at four different ChOx/PEDOT/PMB/GCE biosensors. In this case, the RSD value was 6.8%, a good achievement considering that the

enzyme was immobilized by simply dropping the ChOx solution on top of the electrodes, by hydrophobic interactions. A typical amperometric response of the cholesterol biosensor is presented in Fig. 2a with the corresponding calibration plot in Fig. 2b. As observed, reduction currents are recorded, following the reaction mechanism based on hydrogen peroxide detection presented in Scheme 1. In order to compare the performance of the newly developed biosensor, its main analytical parameters have been compared with those of other recently reported cholesterol biosensors and are summarized in Table 1. Bionzymatic biosensors, in which HRP is used together with ChOx for peroxide detection,^{44–46} were excluded from the table, although only one of them exhibited higher sensitivity.⁴⁴

The sensitivity of the biosensor was $79.0 \pm 5.4 \mu\text{A cm}^{-2} \text{mM}^{-1}$ (RSD = 6.8%, $n = 4$), amongst the highest values achieved by the other biosensors presented in Table 1, higher ones being only a {ChOx-MWCNTs}₅/PDDA/graphite biosensor, which monitored cholesterol by the decrease in the reduction current of oxygen consumed during the enzymatic reaction and ChOx/AuPt-Chit-IL/GCE which was slightly superior. This was a very good achievement, considering that all these biosensors present a more complex and costly architecture, the majority of which contain either CNTs or metal nanoparticles. The biosensor mechanism is mostly based on peroxide detection, either through its oxidation at positive potentials,^{16,17,24,47,51} or its reduction,^{15,18,19,42,50} as in the present case.



Scheme 1 Proposed mechanism at ChOx/PEDOT/PMB/GCE biosensor.

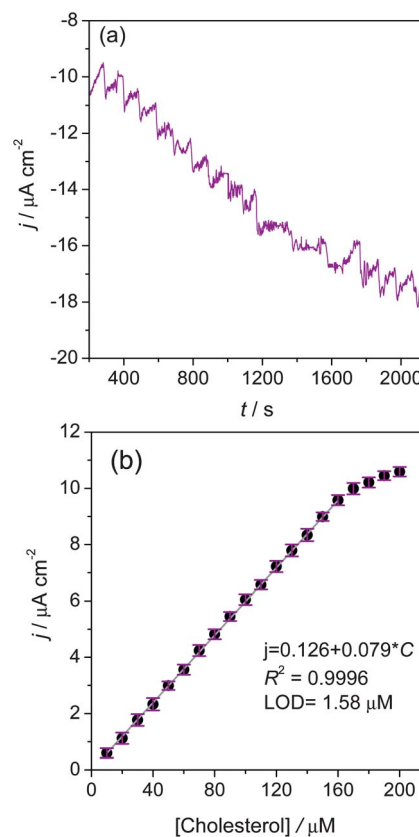


Fig. 2 (a) Typical chronoamperogram and (b) calibration curve recorded at GCE/PMB/PEDOT/ChOx for successive additions of cholesterol in 0.1 M NaPBS pH 7.0; applied potential -0.4 V vs. SCE.

Table 1 Comparison of ChOx/PEDOT/PMB/GCE analytical parameters with those of other recently reported amperometric cholesterol biosensors^a

Biosensor configuration	Applied potential	Linear range/mM	LOD/ μM	Sensitivity/ $\mu\text{A cm}^{-2} \text{mM}^{-1}$	Reference
ChOx-ChE/AuNPs/PTH/GCE	-0.2 V vs. SCE	0.002-1	0.6	2.5	15
ChOx/{PtNP-CNT-Chit/PSS} ₇ /MPS/Au	0.1 V vs. SCE	0.01-3.0	5	3.8	16
ChOx/PtNP-Chit-MWCNTs/GCE	0.4 V vs. Ag/AgCl	—	4.8	44	17
ChOx/AuNP-PtNP-Chit-IL/GCE	-0.1 V vs. Ag/AgCl	0.05-11.2	10	90.7	18
ChOx/Chit-NiNP-MWCNT/GCE	-0.2 V vs. SCE	0.005-3.0	1	6.4	19
ChOx/AgNPs/GCE	0.35 V vs. SCE	0.28-3.3	180	0.70	20
ChOx/PVF ⁺ ClO ⁴⁻ /Pt	0.7 V vs. Ag/AgCl	0.1-0.5	—	0.14	24
ChOx/PNR/CFE	-0.4 V vs. SCE	0.01-0.22	1.9	18	42
ChOx/AuNPs/PDDA/MWCNTs/GCE	0.6 V vs. Ag/AgCl	0.02-1.2	4.4	31.4	47
ChOx/PANI/CNTs/ITO	LSV	1.29-12.93	1290	6.8	48
{ChOx-MWCNTs} ₅ /PDDA/graphite	CV	0.2-1	30	482.3	49
ChOx/Chit-IL/AuNP-MWCNT(SH)/ITO	-0.05 V vs. Ag/AgCl	0.5-5.0	—	0.2	50
ChOx/LDHs-Chit/Pt	0.55 V vs. SCE	0.0005-0.8	0.1	10.4	51
ChOx/PEDOT/PMB/GCE	-0.4 V vs. SCE	0.01-0.16	1.6	79.0	This work

^a LSV-linear sweep voltammetry; CV-cyclic voltammetry; PANI-polyaniline; IL-ionic liquid; LDHs-layered double hydroxides.

The limit of detection was $1.62 \pm 0.14 \mu\text{M}$ (RSD = 8.6%, $n = 4$), calculated as $(3 \times \text{SD})/\text{sensitivity}$,⁵² similar to that exhibited by ChOx/PNR/CFE and comparable with the best ones reported in the literature (see Table 1 – note that different criteria were used to calculate LODs).

The linear range was smaller than others reported; however, the very low detection limit brings advantages for the use of this biosensor for real sample detection since, if needed, dilution of the cholesterol-containing sample will decrease the matrix effect, improving the accuracy of its determination.

3.3 Interferences

The amperometric response at -0.4 V vs. SCE to cholesterol in the presence of linoleic and lactic acid, retinal, glucose and ascorbate, electroactive compounds which are possible interferents in the determination of cholesterol in real samples, was measured using the ChOx/PEDOT/PMB/GCE biosensor (see Fig. 3). A concentration of $80 \mu\text{M}$ cholesterol (chosen to be in the middle of the sensor linear range) was injected in 0.1 M NaPBS pH 7.0, before and after the addition of the interfering compounds, in a concentration half that of the interferents, and results are shown in Table 2. As observed, at -0.4 V vs. SCE applied potential, linoleic and lactic acid and retinal did not give any response, despite the high interferent-to-cholesterol concentration ratio. Small oxidation currents were observed after the injection of glucose and ascorbate, decreasing the response to cholesterol by only 2 and 6%, respectively. The same interference study was also carried out for linoleic and lactic acids and for retinal with concentration ratios 4 : 1 interferent : cholesterol, and no influence from these compounds continued to be observed. Furthermore, the response of the biosensor to cholesterol in the presence of the interfering compounds was also evaluated, being 94.8% of the initial response. All these results evidence the applicability of the developed biosensor for the determination of cholesterol in complex matrices.

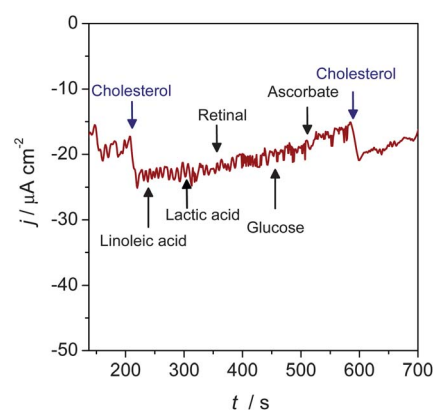


Fig. 3 Influence of electroactive interfering compounds on the response to cholesterol at GCE/PMB/PEDOT/ChOx biosensor in 0.1 M NaPBS pH 7.0; ratio of interfering compound to cholesterol 2 : 1; applied potential -0.4 V vs. SCE.

Table 2 Interferences at ChOx/PEDOT/PMB/GCE biosensor; ratio of interfering compound to cholesterol 2 : 1

Interfering compound	Biosensor response in the presence of interfering compound/%
Linoleic acid	100
Lactic acid	100
Retinal	100
Glucose	98
Ascorbate	94

3.4 Storage stability of the biosensor

Two ChOx/PEDOT/PMB/GCE biosensors were stored, one in buffer solution and the other in air at 4 °C and 5-point calibration curves were done each week during one month. When stored in buffer, the sensitivity decreased by 15% after one week and then only slightly decreased, the biosensor maintaining 80% of its initial sensitivity after one month. The other biosensor stored in air, only lost 4.4% of its sensitivity after the

first week, but in the second week the sensitivity drastically decreased by 80%, being lost afterwards. This clearly indicates that the biosensor must be stored in buffer for better enzyme activity preservation.

3.5 Analysis of cholesterol in real samples

3.5.1 Determination of cholesterol in milk and egg yolk.

The developed ChOx/PEDOT/PMB/GCE biosensor was employed for the detection of cholesterol in milk and egg yolk, using the standard addition method. Measurements were made in the same buffer solution, 0.1 M NaPBS pH 7.0, and the results are displayed in Fig. 4a and b (three measurements for each sample). The whole cow milk was injected without any other treatment, while the egg yolk was first liquefied in water and ultrasonicated until a homogeneous solution was obtained (16 mL egg yolk was brought up to a 30 mL volume). The amount of cholesterol found in whole milk was 0.35 ± 0.04 mM, corresponding to 27.1 ± 3.9 mg of cholesterol per 250 mL of whole milk, comparable with the contents declared in the literature (24–33 mg per 250 mL of whole milk). In egg yolk, the amount of cholesterol was $26.3 \pm$

Table 3 Recovery studies using the ChOx/PEDOT/PMB/GCE cholesterol biosensor in 0.1 M NaPBS pH 7.0

Sample	Added cholesterol/ μM	Found cholesterol/ μM	Recovery/%
Standard	—	15.0	—
Milk	20	35.6 ± 0.5	$101.7 \pm 1.4\%$
Egg yolk	40	55.7 ± 0.7	$101.3 \pm 1.3\%$

0.5 mM, which corresponds to 162.8 ± 3.1 mg per egg yolk, in agreement with the values found in literature, between 160 for small eggs to 180 mg for medium ones.

3.5.2 Precision and recovery. Repeated analyses of cholesterol detection in three samples of whole cow milk and 3 types of egg yolk solutions were performed to evaluate the precision of the developed biosensor. The precision found was between 3.4 and 5.7% for within-day analyses, increasing slightly to 4.7 and 6.3% for day-to-day analyses.

The accuracy of the analytical method using the cholesterol biosensor was tested using recovery studies. Aliquots of standard were spiked into the milk and egg yolk solutions and the change in current recorded after the injection of both spiked and unspiked solutions was recorded. The concentrations of cholesterol found in the samples were determined from interpolation on the calibration curve and results are presented in Table 3. Excellent recoveries very close to 100% were found.

4 Conclusions

This work showed successful application of a cholesterol oxidase enzyme biosensor for cholesterol determination, a second example of PEDOT/PMB/GCE platforms for the construction of enzyme biosensors. The immobilization of the enzyme cholesterol oxidase by adsorption enabled the construction of very reproducible biosensors, which exhibited very good analytical properties compared with much more complex-architecture biosensors recently reported in the literature. The precision of the biosensor is very good, and recovery was close to 100%. The results obtained in the determination of cholesterol in whole cow milk and egg yolk illustrate well the usefulness of the present biosensor design architecture for addressing cholesterol determination in foods.

Acknowledgements

SK thanks the Ministry of Research Science and Technology of Iran for financial support; Financial support from Fundação para a Ciência e a Tecnologia (FCT), PTDC/QUI-QUI/116091/2009, POCH, POFC-QREN (co-financed by FSE and European Community Fund FEDER/COMPETE) and CEMUC® (Research Unit 285), Portugal, is gratefully acknowledged. MMB thanks FCT for postdoctoral grant SFRH/BPD/72656/2010.

References

- 1 S. Aravamudhan, A. Kumar, S. Mohapatra and S. Bhansali, *Biosens. Bioelectron.*, 2007, **22**, 2289–2294.

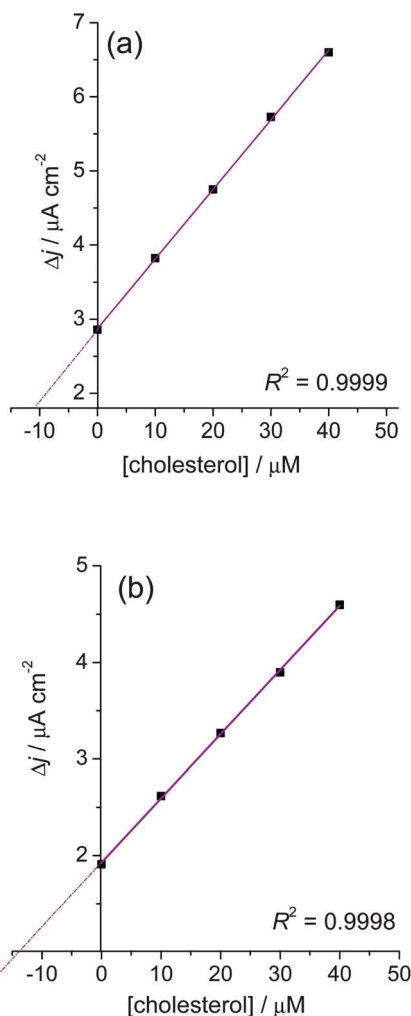


Fig. 4 Calibration plots recorded at GCE/PMB/PEDOT/ChOx biosensor in 0.1 M NaPBS pH 7.0, corresponding to standard addition method used to determine cholesterol concentration in (a) whole milk and (b) egg yolk.

- 2 R. Manjunatha, D. H. Nagavaji, G. S. Suresh, J. S. Melo, S. F. D'souza and T. V. Venkatesha, *J. Electroanal. Chem.*, 2011, **651**, 24–29.
- 3 S. Aravamudhan, N. S. Ramgir and S. Bhansali, *Sens. Actuators, B*, 2007, **127**, 29–35.
- 4 R. N. Qureshi, W. T. Kok and D. J. Schoenmakers, *Anal. Chim. Acta*, 2009, **654**, 85–91.
- 5 B. Canabate-Diaz, C. A. Segura, A. Fernandez-Gutierrez, V. A. Belmonte, F. A. Garrido, V. J. L. Martinez and M. J. Duran, *Food Chem.*, 2007, **102**, 593–598.
- 6 C. K. Pires, B. F. Reis, C. X. Galhardo and P. B. Martelli, *Anal. Lett.*, 2003, **36**, 3011–3024.
- 7 Y. Y. Hosokawa, H. Hakamata, T. Murakami, S. Aoyagi, M. Kuroda, Y. Mimaki, A. Ito, S. Morosawa and F. Kusu, *Electrochim. Acta*, 2009, **54**, 6212–6416.
- 8 M. Barhadillo, E. Casero, M. D. Petit-Dominguez, L. Vazquez, F. Pariente and E. Lorento, *Talanta*, 2009, **80**, 797–802.
- 9 R. Khan, A. Kaushik and A. P. Mishra, *Mater. Sci. Eng.*, 2009, **29**, 1399–1403.
- 10 C. Fang, J. He and Z. Chen, *Sens. Actuators, B*, 2011, **155**, 545–550.
- 11 X.-R. Li, J.-J. Xu and H.-J. Chen, *Electrochim. Acta*, 2011, **56**, 9378–9385.
- 12 C. Zhao, L. Wan, L. Jiang, Q. Wang and K. Jiao, *Anal. Biochem.*, 2008, **383**, 25–30.
- 13 K. W. Ahn and N. S. Sampson, *Biochemistry*, 2004, **43**, 827–836.
- 14 N. Zhou, J. Wang, T. Chen, Z. Yu and G. Li, *Anal. Chem.*, 2006, **78**, 5227–5230.
- 15 Q. Huang, Y. An, L. Tang, X. Jiang, H. Chen, W. Bi, Z. Wang and W. Zhag, *Anal. Chim. Acta*, 2011, **707**, 135–141.
- 16 M. Yang, Y. Yang, H. Yang, G. Shen and R. Yu, *Biomaterials*, 2006, **27**, 246–255.
- 17 Y. C. Tsai, S. Y. Chen and C. A. Lee, *Sens. Actuators, B*, 2008, **135**, 96–101.
- 18 A. Safavi and F. Farjami, *Biosens. Bioelectron.*, 2011, **26**, 2547–2552.
- 19 M. Yang, Y. Yang, F. Qu, Y. Lu, G. Shen and R. Yu, *Anal. Chim. Acta*, 2006, **571**, 211–217.
- 20 Y. Li, H. Bai, Q. Liu, J. Bao, M. Han and Z. Dai, *Biosens. Bioelectron.*, 2010, **25**, 2356–2360.
- 21 N. Pena, G. Ruiz, A. J. Reviejo and J. M. Pingarron, *Anal. Chem.*, 2001, **73**, 1190–1195.
- 22 G. Li, J. M. Liao, G. Q. Hu, N. Z. Ma and P. J. Wu, *Biosens. Bioelectron.*, 2005, **20**, 2140–2144.
- 23 Ö. Türkarşlan, S. K. Kayahan and L. Toppare, *Sens. Actuators, B*, 2009, **136**, 484–488.
- 24 B. C. Özer, H. Özyörük, S. S. Çelebi and A. Yildiz, *Enzyme Microb. Technol.*, 2007, **40**, 262–265.
- 25 P. R. Solanki, S. K. Arya, S. P. Singh and B. D. Malhotra, *Sens. Actuators, B*, 2007, **123**, 829–839.
- 26 J. Shen and C. C. Liu, *Sens. Actuators, B*, 2007, **120**, 417–425.
- 27 S. Suman and C. S. Pundir, *Curr. Appl. Phys.*, 2003, **3**, 129–133.
- 28 A. Parra, E. Casero, F. Pariente, L. Vázquez and E. Lorenzo, *Anal. Bioanal. Chem.*, 2007, **388**, 1059–1067.
- 29 X. Tan, M. Li, P. Cai, L. Luo and X. Zou, *Anal. Biochem.*, 2005, **337**, 111–120.
- 30 S. Singh, R. Singhal and B. D. Malhotra, *Anal. Chim. Acta*, 2007, **582**, 335–343.
- 31 T. Yao and K. Takashima, *Biosens. Bioelectron.*, 1998, **13**, 67–73.
- 32 A. Rahman, P. Kumar, D. S. Park and Y. B. Shim, *Sensors*, 2008, **8**, 118–141.
- 33 H. Wang and S. Mu, *Sens. Actuators, B*, 1999, **56**, 22–30.
- 34 S. Kakhki, M. M. Barsan, E. Shams and C. M. A. Brett, *Synth. Met.*, 2012, **161**, 2718–2726.
- 35 J. Argüello, V. L. Leidens, H. A. Mayosso, R. R. Ramos and Y. Gushikem, *Electrochim. Acta*, 2008, **54**, 560–565.
- 36 C. M. A. Brett, G. Inzelt and V. Kertesz, *Anal. Chim. Acta*, 1999, **385**, 119–123.
- 37 U. Yogeswaran and S. M. Chen, *Sens. Actuators, B*, 2008, **130**, 739–749.
- 38 S. B. Khoo and F. Chen, *Anal. Chem.*, 2002, **74**, 5734.
- 39 M. Arvand, Sh. Sohrabnezhad, M. F. Mousavi, M. Shamsipur and M. A. Zanjanchi, *Anal. Chim. Acta*, 2003, **491**, 193–201.
- 40 S. Kakhki, M. M. Barsan, E. Shams and C. M. A. Brett, *Electroanalysis*, DOI: 10.1002/elan.201200402.
- 41 M. M. Barsan, E. M. Pinto and C. M. A. Brett, *Electrochim. Acta*, 2008, **53**, 3973–3982.
- 42 A. C. Torres, M. E. Ghica and C. M. A. Brett, *Electroanalysis*, 2012, **24**, 1547–1553.
- 43 A. Guiseppi-Ellie, C. Lei and R. H. Baughman, *Nanotechnology*, 2002, **13**, 559–564.
- 44 U. Saxena, M. Chakraborty and P. Goswami, *Biosens. Bioelectron.*, 2011, **26**, 3037–3043.
- 45 S. K. Arya, M. Datta, S. P. Singh and B. D. Malhotra, *Anal. Bioanal. Chem.*, 2007, **389**, 2235–2242.
- 46 A. Ahmadalinezhad and A. Chen, *Biosens. Bioelectron.*, 2011, **26**, 4508–4513.
- 47 M. Eguílaz, R. Villalonga, L. Agüí, P. Yáñez-sedeno and J. M. Pingarron, *J. Electroanal. Chem.*, 2011, **661**, 171–178.
- 48 C. Dhand, S. K. Arya, M. Datta and B. D. Malhotra, *Anal. Biochem.*, 2008, **383**, 194–199.
- 49 R. Manjuntha, D. H. Nagaraju, G. S. Suresh, J. S. Melo, S. F. D'Souza and T. V. Venkatesha, *J. Electroanal. Chem.*, 2011, **651**, 24–29.
- 50 A. I. Gopalan, K.-P. Lee and D. Ragupathy, *Biosens. Bioelectron.*, 2009, **24**, 2211–2217.
- 51 S.-N. Ding, D. Shan, T. Zhang and Y.-Z. Dou, *J. Electroanal. Chem.*, 2011, **659**, 1–5.
- 52 Analytical Methods Committee, *Analyst*, 1987, **112**, 199–204.