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Poly(brilliant cresyl blue) modified glassy carbon electrodes: Electrosynthesis, characterisation and application in biosensors

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ABSTRACT

The phenoxazine dye, brilliant cresyl blue (BCB) has been polymerised on glassy carbon electrodes by potential cycling, and the resulting poly(brilliant cresyl blue) (PBCB) film has been studied by cyclic voltammetry, differential pulse voltammetry and electrochemical impedance spectroscopy. A differential pulse voltammetry study in different supporting electrolytes with different pH values revealed that the current intensity of the oxidation peak of the resulting polymer increases with increase in pH up to pH 4.1 where the highest response is exhibited and then begins to decrease. It was also observed that the peak potential moves to more negative values with increase in pH with a slope of −51 mV/pH, indicating an equal number of electrons and protons in the redox process. The electrode modified with poly(brilliant cresyl blue) was successfully applied to the determination of hydrogen peroxide in amperometric mode at 0.0 V *vs.* SCE. Glucose oxidase enzyme was then immobilised by crosslinking with glutaraldehyde and bovine serum albumin (BSA): the polymer functions as a redox mediator in a glucose biosensor allowing determination of glucose at small negative potentials with low interferences.

1. Introduction

Since the discovery of the formation of polymer semiconductor films by the electrochemical oxidation of pyrrole [1] and aniline [2], the interest in electrogenerated polymers has been continuously growing. Conducting polymers are very attractive due to their potential use as electrochromic devices, capacitors, gas separation membranes, anti-static membranes, catalysts and electromagnetic shielding devices. However, when used together with proteins and enzymes the result is not always so encouraging due to the low catalytic current obtained [3]. In order to overcome this inconvenience, one can prepare conducting films by electropolymerisation from a monomer which is itself a redox-active compound. In this way azine dyes have proved to be very interesting compounds due to their ability to act as electron donors/acceptors in enzyme reactions and, simultaneously, such dyes are able to undergo electropolymerisation. Among these dyes, several phenazines, phenothiazines and phenoxazines have been polymerised on different electrodes and have been used as redox mediators mainly with NAD-dependent dehydrogenases [4,5] and also with oxidase enzymes [6,7].

Brilliant cresyl blue (BCB) is a cationic quinine-imide dye with a planar and rigid structure as shown in Scheme 1a and has been proven to posses promising properties as a redox catalyst. Brilliant cresyl blue has been used as a fluorescent indicator for measurements across biological membranes [8]. It can react with heparin which permits its determination by UV–Vis spectrophotometry and linear sweep voltammetry [9], and interacts with dsDNA by both electrostatic attraction and intercalation thus permitting detection of natural DNA damage [10]. BCB can adsorb strongly on electrode surfaces and these chemically modified electrodes have been used for the determination of NADH [11] and to study the redox behaviour of haemoglobin [12]. BCB was also used for the determination of oxalate [13], formaldehyde [14], nitrite [15], protein [16], hydrazine [17] and cyclodextrin [18] using spectrophotometric detection. Another application was as mediator in electrochemical biosensors [19,20]. However, the electrochemical behaviour of BCB has been investigated only by using polarography [21] or transparent thin layer spectroelectrochemistry [22] and reports concerning the electropolymerisation of BCB are just two to our knowledge [23,24] with only brief characterisation.

We have previously reported on the polymerisation of the phenazine dye neutral red (NR) [7,32] and the phenothiazines methylene blue (MB) and methylene green (MG) [29], the characterisation of the resulting polymers and their potential use as redox mediators. In this context of studying azine dyes, the present work focuses on the electrochemical synthesis of poly(brilliant cresyl blue), from the phenoxazine brilliant cresyl blue, optimisation of the polymerisation conditions and study of the electrochemical properties of the resulting redox polymer as well as possible application as redox mediator in enzymatic sensors. BCB was electropolymerised on glassy carbon electrodes. The PBCB polymer-modified electrode was found to possess high stability and was characterised by cyclic voltammetry, differential pulse voltamme-

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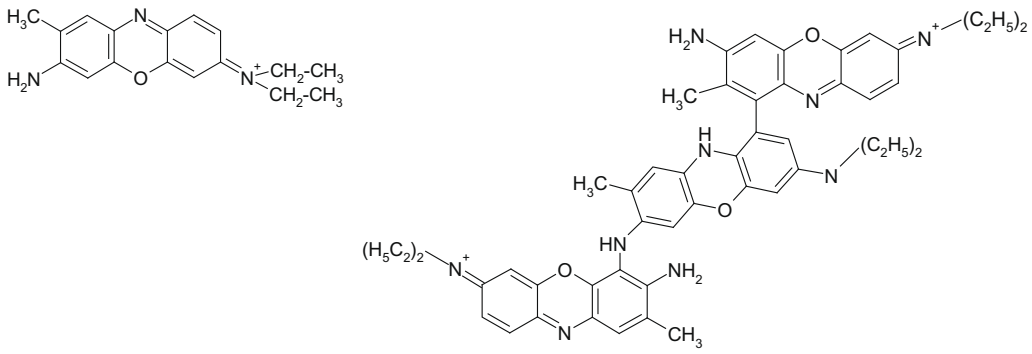


Scheme 1. (a) Chemical structure of brilliant cresyl blue monomer and (b) possible structure of a trimer.

try and electrochemical impedance spectroscopy. Glassy carbon electrodes modified with PBCB were then applied to the determination of hydrogen peroxide at 0.0 V *vs.* SCE and finally, after glucose oxidase immobilisation, determination of glucose was carried out in amperometric mode. The possible mechanism of PBCB as redox mediator in the presence and absence of dissolved oxygen was also investigated.

2. Experimental

2.1. Reagents and buffers

All reagents were of analytical grade and were used without further purification. Glucose oxidase (GOx, E.C. 1.1.3.4, from *Aspergillus Niger*, 24 U/mg) was acquired from Fluka, Switzerland. α-D(+)-glucose, glutaraldehyde (GA) (25% v/v in water) and bovine serum albumin (BSA) were purchased from Sigma, Germany. Hydrogen peroxide (H_2O_2) 35% was from José M. Vaz Pereira, Portugal. Brilliant Cresyl Blue was obtained from Fluka, Switzerland.

For electrochemical measurements the supporting electrolyte solutions were prepared with ultrapure water supplied by a Millipore Milli-Q nanopure system (resistivity > 18 MΩ cm) as shown in Table 1. All reagents used for the preparation of buffers were purchased from Riedel de Haën, Germany, except potassium chloride (Fluka, Switzerland), sodium hydrogen phosphate (Sigma, Germany) and sodium tetraborate (Merck, Germany).

Stock solutions of 0.10 mol L^{-1} glucose and 10 mmol L^{-1} hydrogen peroxide (calibrated by permanganate titration) were prepared in phosphate buffer supporting electrolyte and were kept in the refrigerator.

All experiments were performed at room temperature, 25 ± 1 °C.

2.2. Methods and instruments

Measurements were performed in a 15 mL, one-compartment cell containing a glassy carbon (GC) modified electrode (geometric area 0.28 cm^2) as working electrode, a platinum auxiliary electrode and a saturated calomel electrode (SCE) as reference. Voltammetric and amperometric experiments were carried out using a CV-50 W Voltammetric Analyser from Bioanalytical Systems, West Lafayette, IN, USA, controlled by BAS CV-2.1 software. Electrochemical impedance measurements were carried out with a PC-controlled Solartron 1250 Frequency Response Analyser coupled to a Solartron 1286 electrochemical interface using ZPlot 2.4 software (Solartron Analytical, UK). A sinusoidal voltage perturbation of amplitude 10 mV rms was applied in the frequency range between 65 kHz and 0.1 Hz with 10 frequency steps per decade.

The pH measurements were performed with a CRISON 2001 micro pH-meter.

Table 1
Composition of the solutions used for the preparation of the 0.2 M supporting electrolyte solutions and corresponding pH values.

Buffer electrolyte solutions	pH
0.2 M KCl + 0.2 M HCl	1.2
0.2 M KCl + 0.2 M HCl	1.8
0.2 M NaOAc + 0.2 M HOAc	3.4
0.2 M NaOAc + 0.2 M HOAc	4.1
0.2 M NaOAc + 0.2 M HOAc	5.2
0.2 M Na_2HPO_4 + 0.2 M NaH_2PO_4	5.8
0.2 M Na_2HPO_4 + 0.2 M NaH_2PO_4	7.0
0.025 M $Na_2B_4O_7$ + 0.1 M HCl	8.6
0.2 M KCl + 0.2 M NaOH	11.9

2.3. Modified electrode and biosensor preparation

The working electrode was a glassy carbon disk electrode, diameter d = 6 mm. Prior to use the electrode was cleaned by polishing with diamond spray (Kemet International Ltd., UK) of decreasing particle size down to 1 μm. This was followed by polishing with 0.3 μm alumina particles (Al_2O_3) and afterwards washed abundantly with double distilled water. The state of the surface was monitored by running different scans in supporting electrolyte.

Following this, poly(brilliant cresyl blue) was deposited on the glassy carbon electrode. In the optimised procedure, brilliant cresyl blue was polymerised by cycling the glassy carbon electrode 30 times between −0.6 V and 1.0 V *vs.* SCE at a scan rate of 50 mV s^{-1} in a freshly prepared solution containing 0.1 mM brilliant cresyl blue in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 M KNO_3.

Finally, glucose oxidase (GOx) was immobilised using the cross-linking method. Enzyme immobilisation was performed using the cross-linking reaction with glutaraldehyde and bovine serum albumin. A mixture containing 5 μL of GA (2.5% v/v in water) and 10 μL of enzyme solution was used. The latter was prepared by dissolving 40 mg BSA and 10 mg GOx in 1 mL of 0.1 M NaPBS (pH 7.0). For drop-coating, 10 μL of the mixture described before were placed onto the electrode previously modified with the polymer. The electrode assembly was left to dry at room temperature for at least 1 h after which the biosensor could be used immediately. When not in use, the electrode was kept at 4 °C in phosphate buffer electrolyte, pH 7.0.

3. Results and discussion

3.1. Polymerisation of brilliant cresyl blue

In order to optimise the polymerisation conditions, several parameters were studied, namely the influence of monomer con-

centration, pH of the supporting electrolyte and of different anions present in the supporting electrolyte. Three different monomer concentrations were investigated: 0.10, 0.25 and 0.50 mM brilliant cresyl blue. It was observed that with decreasing monomer concentration the polymer film growth was better; an increase of 97% in the growth rate was observed in the height of the polymer oxidation peak (at ~0.0 V *vs.* SCE in pH 7.0 solution) for 0.25 mM and of 141% for 0.10 mM monomer concentration compared with 0.5 mM monomer concentration. This behaviour might be explained by the fact that at high concentrations of monomer the rate of polymerisation initiation is faster and leads to the formation of a thicker, more porous polymer film. From this point of view, brilliant cresyl blue differs from its analogues which could be polymerised very well at a higher concentration of monomer of 1 mM [29]. It may also be that BCB monomer adsorbs more strongly on the electrode surface in such a way as to make polymer growth more difficult.

The pH of the buffer solution has an important influence on the formation of the polymer film. As was demonstrated previously for methylene blue [6] and neutral red [25] the formation of the polymer begins by irreversible oxidation of the monomer and formation of a radical cation which is unstable and binds with other radicals, through the amino group. However the possibility of ring-to-ring coupling in polymer azines have been suggested by Karyakin [23], so for the polymerisation both ring-to-ring and amino-to-aromatic ring bindings should be considered (see Scheme 1b for structure of a trimer). Using cyclic voltammetry it is necessary to cycle the electrode up to the potential where the monomer is oxidised in order to initiate the polymerisation. Fig. 1a shows the anodic peak potential values for the irreversible oxidation of the brilliant cresyl blue monomer ($E_{p,mon}$) as a function of pH. It was observed that there is strong pH dependence in the pH range 3–9 with a slope of −45 mV/pH unit. A similar behaviour was previously observed for other monomers containing primary amino groups as ring substituents such as neutral red [23], so that one can expect that these monomers can release a proton upon oxidation yielding a singly-charged cation-radical. In order not to overoxidise the monomer, higher pH values are preferable for PBCB growth since monomer oxidation occurs at lower potentials. On the other hand, the polymer oxidation–reduction peak potentials move to more negative values with increasing pH and the peak separation increases. Under these conditions, the better electrolytes of those studied are those with pH 5.2, 5.8 and 7.0 since the peak potentials of the polymer are close to 0.0 V *vs.* SCE which could be very important for using PBCB as a redox mediator in sensors, in order to minimise interferences. Estimating the polymer growth rate for these three buffers, an increase of 36% for pH 5.8 and of 79% for pH 7.0 was obtained compared with pH 5.2. It was therefore decided to perform the polymerisation of brilliant cresyl blue in pH 7.0 supporting electrolyte.

The final step was to investigate whether the anions in solution have any catalytic effect on the polymer growth. For this purpose BCB was polymerised from a solution containing 0.1 mM monomer in sodium phosphate buffer (pH 7.0) without and with addition of different anions: NO_3^-; SO_4^{2-}; ClO_4^- or Cl^-. The rate of polymer growth was estimated after 30 potential cycles and it was observed that in the case of buffer containing Cl^- or SO_4^{2-} the growth rate was even lower than in the case of simply phosphate buffer. However, it was found that nitrate ions accelerate the BCB polymer growth rate, an increase of 23% in both oxidation and reduction peak currents being obtained. NO_3^- is known to be a shielding agent and was also reported to increase the growth of poly(methylene blue) films [6]. An increase in polymer oxidation/reduction currents was also obtained when using ClO_4^- anions, but as seen previously for MB these ions are known to form tight ionic pairs with the charged monomer, precipitating it. To avoid this, NO_3^- was chosen for future experiments to improve polymer growth. Thus, the optimised conditions for brilliant cresyl blue polymerisation were 0.1 mM monomer concentration in 0.1 M sodium phosphate buffer pH 7.0 with addition of 0.1 M KNO_3. It should be pointed out that the optimised conditions for PBCB growth are different from those for PMB and PMG which are better polymerised from alkaline buffer solutions [29], whilst PNR grows better in weakly acidic media [32]. Before polymerisation, the electrode was cycled in phosphate buffer solution (pH 7.0) between −0.6 V and +1.0 V *vs.* SCE at a scan rate of 100 mV s^{-1}, until a stable voltammogram was obtained. Afterwards the potential was cycled between −0.6 V and +1.0 V *vs.* SCE at 50 mV s^{-1} during 30 scans.

Fig. 2a shows cyclic voltammograms of polymer growth: at about 0.86 V *vs.* SCE the irreversible oxidation of monomer occurs, at negative potentials two redox couples are visible, the more negative one with the formal potential (calculated as the mean value between the oxidation and reduction peak potentials) at about −0.28 V corresponds to oxidation/reduction of the monomer. This couple decreases in intensity until it almost disappears and the other couple with formal potential around −0.04 V increases in height. The latter couple is ascribed to the polymer. The formation of PBCB occurs in the same way as for PMB with the appearance of a new set of peaks corresponding to the polymer and differs from PNR for which monomer and polymer peaks overlap. An additional advantage of this polymer as redox mediator is that the formal potential of the polymer is closer to 0.0 V. The increasing currents of

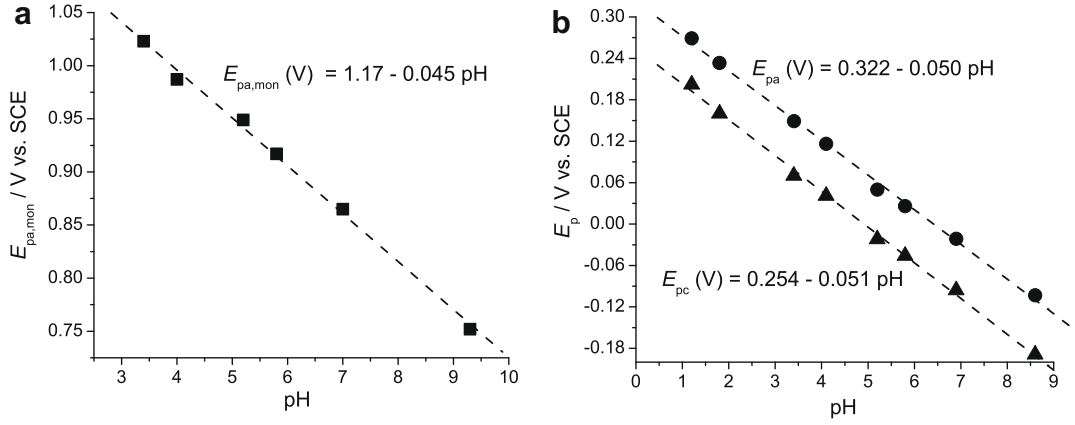


Fig. 1. The influence of pH on (a) anodic peak potential of the irreversible oxidation of BCB and (b) anodic and cathodic peak potential of PBCB. All values were taken from cyclic voltammograms recorded at 50 mV s^{-1}.

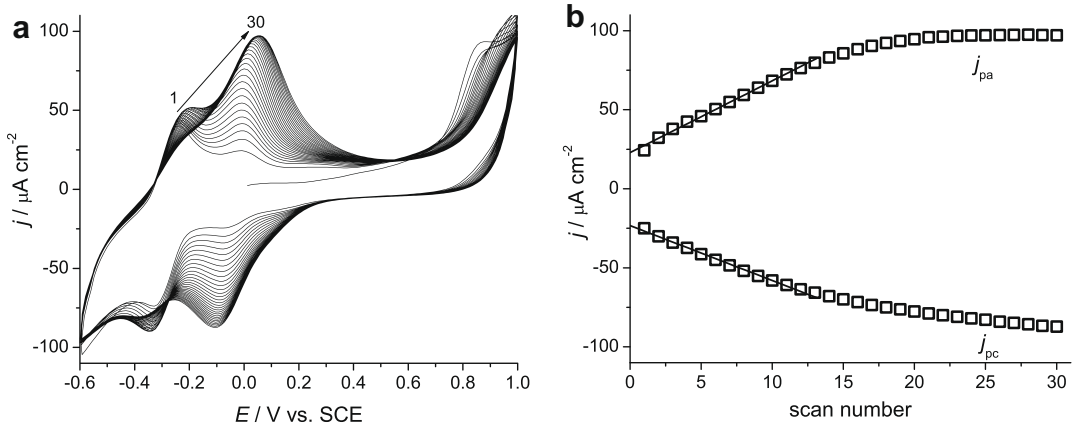


Fig. 2. (a) Cyclic voltammograms showing continuous growth of BCB polymer on glassy carbon electrode and (b) dependence of anodic and cathodic peak currents on the number of cycles; 0.1 mM BCB in 0.1 M NaPB (pH 7.0) + 0.1 M KNO_3. Scan rate 50 mV s^{-1}.

both oxidation and reduction peaks prove the polymer growth, which is more rapid during the first 12 cycles when the increase is linear with the scan number and then becomes slower almost reaching saturation after 22 cycles for the oxidation peak while the reduction peak still increases but very slowly. However, it was estimated that the 30th scan was the upper limit after which the polymer will not grow any more (Fig. 2b).

3.2. Electrochemical characterisation of the poly(brilliant cresyl blue) film

The poly(brilliant cresyl blue) film was characterised by using cyclic voltammetry, differential pulse voltammetry and electrochemical impedance spectroscopy in different supporting electrolytes.

3.2.1. Cyclic voltammetry

The poly(brilliant cresyl blue) synthesised polymer was investigated by cyclic voltammetry in supporting electrolyte solutions of different pH. The cyclic voltammograms exhibited both the monomer-type and polymer-type sets of peaks (data not shown) as was found previously for poly(methylene blue) [6] or poly(toluidine blue) [23], especially at low pH values, then disappeared.

As was reported before, the redox reaction in aqueous solution of phenazine, phenothiazine and phenoxazine derivatives occurs with participation of two electrons [11]. However, for BCB different results have been reported: at mercury electrodes a 2 electrons–2 protons redox reaction was found by linear sweep voltammetry [26] as well as by spectroelectrochemistry on platinum gauze optically transparent thin film electrodes [27]. On the other hand, a reversible one electron transfer process at a platinum gauze electrode has been suggested by other authors [22]. The total number of protons that participates in the redox process, as well as the formal potential $E^{\ominus'}$ of the compound may vary with the functional groups of the aromatic skeleton [11]. It was therefore necessary to investigate the variation of the formal potential of PBCB with pH since this is very important for choosing the applied potential at which it can be used as redox mediator. Over the whole pH range studied the PBCB electrode exhibited the typical behaviour of an close-to-electrochemically reversible reaction at low scan rates of 5 mV s^{-1} with anodic and cathodic peaks of almost the same height and peak potential separations of 70–80 mV, the smallest and biggest separation being obtained in acidic and alkaline media, respectively. These results are in agreement with a previous study reporting that the difference between the peak potentials of different polyazines is not significantly affected by the pH [23]. The peak separation increased with increasing scan rate. The values of the slopes of both E_{pa} and E_{pc} versus pH plots in the pH range from 1.2 to 11.9 show that in the polymer redox process equal number of protons and electrons are involved (Fig. 1b): the values of $E^{\ominus'}$ decrease linearly with increase in pH according to the equation: $E^{\ominus'}/V = 0.283 - 0.051$ pH. A similar behaviour has been reported for PNR, PMB and PMG [29]. However, in the case of PNR quite different slopes were obtained for the anodic and cathodic peak dependence suggesting that different numbers of protons are involved in the oxidation and reduction processes described by these peaks, whilst for PBCB the main oxidation and reduction processes occur with involvement of an equal number of protons.

Cyclic voltammograms, Fig. 3, were recorded at different scan rates in pH 4.1 acetate buffer and pH 7.0 phosphate buffer, chosen because in acetate buffer (pH 4.1) PBCB exhibited the highest response (see next section) and phosphate buffer (pH 7.0) is normally used in biosensor studies. In both cases the voltammogram shape is influenced by the scan rate and both anodic and cathodic peak currents depend linearly on the square root of the scan rate over the whole scan rate range examined, according to $j_{pa} = -10.2 + 5.7v^{1/2}$ and $j_{pc} = 10.8 - 5.3v^{1/2}$ at pH 4.1 and to $j_{pa} = -9.9 + 5.4v^{1/2}$ and $j_{pc} = 10.1 - 5.1v^{1/2}$ at pH 7.0, where current densities are expressed in μA cm^{-2} and scan rates in mV s^{-1}. These results imply diffusion control [28], namely by the electrolyte counter ions into and out of the polymer film, which play an important role in maintaining electroneutrality on the electrode surface [29]. In both cases the slope is slightly higher for the oxidation peak, meaning that expulsion of the positive counter ion is faster that its diffusion into the polymer film, a behaviour previously reported for PMB and PMG [29]. The difference between the slopes in the two buffers is not large, since the counter ion is the same, sodium; however, the protons also play a role since, as was observed; the slope is higher in more acidic media. In order to determine the diffusion coefficients through the polymer film the Randles–Sevcik equation was used. Considering the previously determined slopes (from Fig. 3), the charge of the counter ions $n = 1$, the geometric area of the electrode $A = 0.28$ cm^2 and concentration of the counter ions C = 0.1 mol L^{-1}, the diffusion coefficients can be estimated as of the order of 10^{-10} cm^2 s^{-1}, as observed with other polyazine dyes [29].

The quantity of electroactive polymer deposited by electropolymerisation after 30 scans was calculated using Faraday's law, considering that one electron is involved in the redox reaction (as will be demonstrated in the following section). The charge was calculated from the first cyclic voltammogram recorded after prepara-

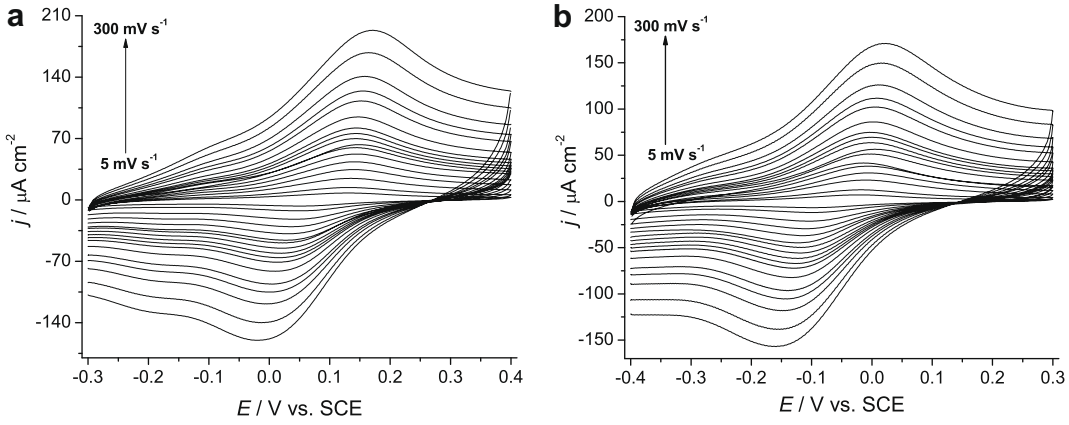


Fig. 3. Cyclic voltammograms at different scan rates at PBCB modified electrodes in (a) acetate buffer pH 4.1 and (b) phosphate buffer pH 7.0.

tion (this was in acetate buffer pH 4.1, in which it will be shown that PBCB has the better response) by integration under the oxidation peak and was found to be 5.2 μC. The total surface coverage was calculated to be approximately 19 nmol cm^{-2}.

The stability of the deposited polymer was also investigated by cyclic voltammetry, performing consecutive scans between −0.3 V and 0.4 V in acetate buffer pH 4.1 and between −0.4 V and 0.3 V in phosphate buffer pH 7.0 at 50 mV s^{-1}. The polymer film showed good stability in both supporting electrolyte used, after 50 successive scans a decrease of 11 ± 1% of the oxidation and reduction peaks was observed in acetate buffer and 7 ± 1% in phosphate buffer.

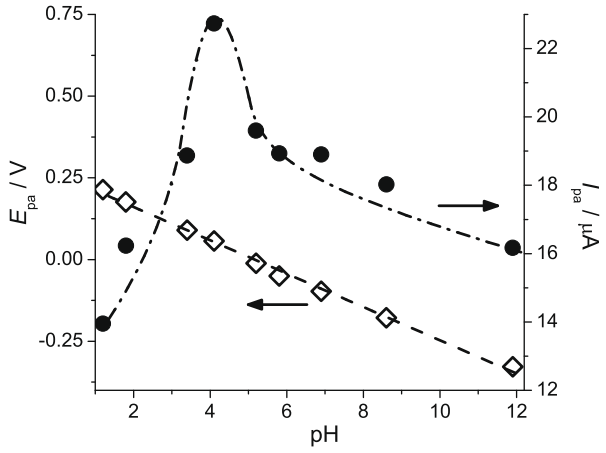
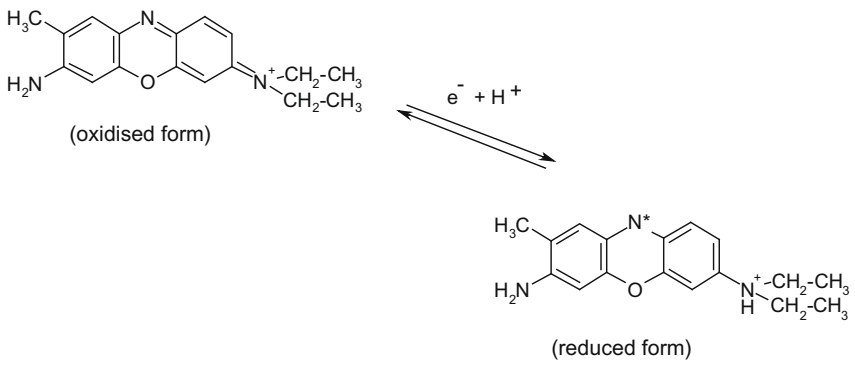


Fig. 4. Plot of current and potential of oxidation peak of PBCB *vs.* buffer pH. Dashed line corresponds to a slope of −51 mV per pH unit.

3.2.2. Differential pulse voltammetry

Since by cyclic voltammetry it was more difficult to evaluate in which electrolyte PBCB exhibited the best response, the oxidation of PBCB was examined by differential pulse voltammetry (DPV) in the pH range 1.2–11.9. To our knowledge this technique was not previously reported for characterisation of azine-derived polymer modified electrodes, but has the advantage of being more sensitive than cyclic voltammetry and permits the estimation of the number of electrons transferred in the redox reaction. It was possible to see two peaks in all buffers, one at more negative potentials corresponding to the monomer and one at more positive potential values which was ascribed to the polymer. From cyclic voltammetry, at many of the values of pH studied, it was not possible to see the monomer oxidation peak. DPV also demonstrated the dependence on pH of PBCB oxidation: the peak current increases with increase in pH up to pH 4.1, where the highest response is exhibited, and then decreases again. The plot of peak potential *vs.* pH (Fig. 4) showed a linear dependence with a slope of −51 mV/pH, showing, as did CV, that an equal number of electrons and protons is involved. The peak width at half height in pH 4.1 acetate buffer, scan rate 10 mV s^{-1} was estimated as $W_{1/2}$ = 90 mV for the second oxidation peak corresponding to polymer, this peak becoming narrower at lower scan rates. This suggests that the total number of electrons transferred in the oxidation of each electroactive centre in the polymer (second peak) is 1, together with one proton. Based on these results, the mechanism



Scheme 2. Possible mechanism of the BCB redox reaction.

of reaction of BCB is proposed in Scheme 2, in a similar way as in [22] where the reduced form of BCB is a radical cation.

3.2.3. Impedance spectroscopy

Electrochemical impedance spectroscopy was used to examine the interfacial properties of the poly(brilliant cresyl blue) modified electrode. The experiments were performed at potentials in the range from −0.4 to +0.4 V *vs*. SCE in 0.1 M acetate buffer pH 4.1 and phosphate buffer pH 7.0, the same used for characterisation by cyclic voltammetry, because the best response of the polymer was in pH 4.1 acetate buffer solution and for applications in biosensors phosphate buffer pH 7.0 is normally used. This potential range was also chosen to encompass the redox reaction of the polymer in both electrolytes. Complex plane impedance spectra are illustrated in Fig. 5. In both electrolytes studied the behaviour was similar, and indicate a single time-constant process at the polymer modified electrode; Bode plots show that there are no hidden high frequency features. Since a diffusion-controlled process was observed by cyclic voltammetry, which may be ascribed to the fact that oxygen can diffuse through the polymer layer and be reduced, experiments were also carried out in the absence of oxygen (removed by bubbling nitrogen during 15 min).

The spectra were fitted to two equivalent circuits: from +0.4 V to −0.2 V in both phosphate and acetate buffer the spectra are straight lines because no faradaic reaction occurs; in these cases the fitting circuit comprises a cell resistance R_Ω, in series with a constant phase element CPE, modelled as non-ideal capacitor, given by CPE = $-1/(i\omega C)^n$, where C is the capacitance, ω is the frequency in rad s^{-1} and the exponent n reflects the surface non-uniformity and porosity of the polymer film. The second circuit was used for spectra at −0.3 and −0.4 V and is the first circuit plus an additional resistance R_1 in parallel with the constant phase element, expressing the charge transfer resistance occurring at the polymer/solution interface. This circuit has been previously used for polymer-modified electrodes [30] and also in [7,32] for PNR-modified electrodes.

The cell resistance was ~75 Ω cm^2 in acetate buffer and 15 Ω cm^2 in phosphate buffer electrolyte solution. Table 2 gives the values of C and R; in all cases the error in the parameter values was less than 5%. In both electrolytes the tendency was the same, with the values of C_1 increasing from +0.4 V down to a certain value of potential (0.0 V in acetate buffer and −0.1 V in phosphate buffer) and then decreasing again, the values of the exponent n being between 0.80 and 0.86 (error ± 0.02). The higher values of C_1 correspond to the potentials at which the oxidation/reduction reaction of the polymer occurs. The charge transfer resistance values decrease from −0.3 V to −0.4 V which is attributed partly to oxygen reduction – see below.

Spectra in the absence of oxygen are similar to those in its presence, with just some small differences in the values of the equiva-

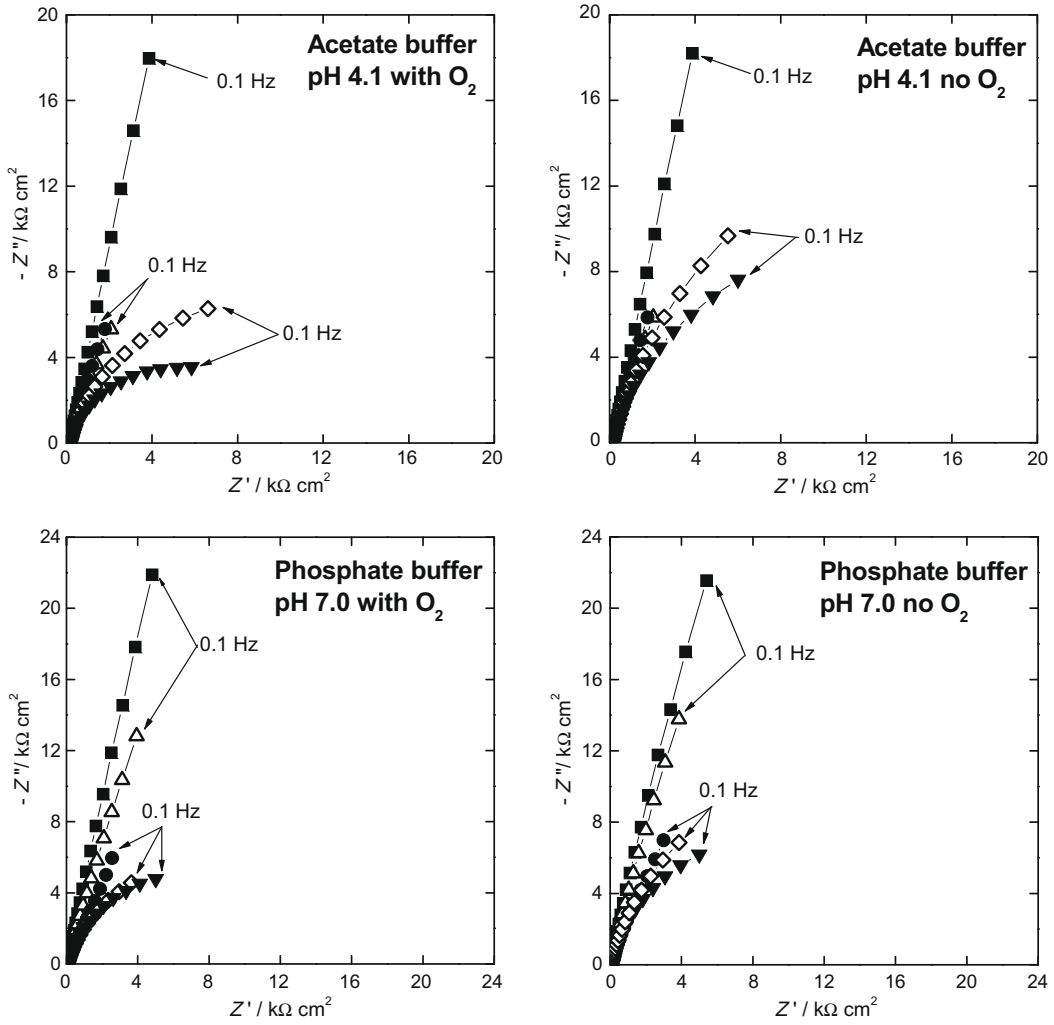


Fig. 5. Complex plane impedance spectra recorded in 0.1 M acetate buffer pH 4.1 and 0.1 M phosphate buffer pH 7.0 in the presence and in the absence of dissolved oxygen at different potentials: ■ +0.4 V, △ +0.1 V, ● 0.0 V ◇ −0.3 V, ▼ −0.4 V *vs*. SCE.

Table 2
Equivalent circuit fitting from impedance spectra for PBCB modified electrodes in 0.1 M acetate buffer pH 4.1 and phosphate buffer pH 7.0 at different potentials.

E/V *vs.* SCE	Presence of O_2		Absence of O_2	
	C_1/μF cm^{-2} s^{n-1}	R_1/kΩ cm^2	C_1/μF cm^{-2} s^{n-1}	R_1/kΩ cm^2
Acetate buffer				
0.4	84	–	82	–
0.3	95	–	95	–
0.2	139	–	133	–
0.1	258	–	240	–
0.0	269	–	248	–
−0.1	206	–	198	–
−0.2	179	–	185	–
−0.3	126	18.2	127	49.4
−0.4	124	9.8	129	25.7
Phosphate buffer				
0.4	69	–	68	–
0.3	68	–	69	–
0.2	80	–	77	–
0.1	109	–	103	–
0.0	200	–	174	–
−0.1	304	–	288	–
−0.2	229	–	220	–
−0.3	208	15.7	176	33.9
−0.4	154	12.5	152	18.6

lent circuit components (see Table 2), one being the increase of the value of the charge transfer resistance at negative potentials. The decrease in the capacitance values suggests that in the absence of oxygen the polymer is less polarised, as was observed in [29]. However, these differences are small indicating that the presence of oxygen is not very important.

To our knowledge no previous impedance studies have been carried out at poly(brilliant cresyl blue) modified electrodes. Our spectra can be modelled in the same way as those obtained for other polyazine-modified electrodes, such as PNR [3,29,32]. The results show that the conductivity of the polymer depends on the applied potential, as was already reported [3], at positive potentials the film being less conductive and at negative potentials oxygen diffusing through the polymer film and and being reduced.

3.3. Application as redox mediator in biosensors

3.3.1. Determination of hydrogen peroxide

After characterisation of the polymer film, application of the polymer-modified electrode was investigated. Amperometric measurement of hydrogen peroxide was carried out in 0.1 M phosphate buffer solution pH 7.0 at 0.0 V *vs.* SCE; the response was an increase in cathodic current, due to reduction of hydrogen peroxide, as observed for poly(neutral red) modified carbon film electrodes [32]. The response to H_2O_2 was linear up to 65 μM with a sensitivity of 963 nA cm^{-2} mM^{-1} and a detection limit (three times the signal to noise ratio) of 4.2 μM (data not shown). Successive calibration curves recorded over the full linear range at the same electrode led to a decrease of 33%, caused most probably by peroxide damaging the polymer film. However, this loss of sensitivity should not affect the operation of the enzyme biosensor since the mediator will be more protected under the enzyme layer and the quantity of hydrogen peroxide is much less.

3.3.2. Determination of glucose

Glucose oxidase was immobilised on PBCB-modified glassy carbon electrodes and the resulting sensor was tested for glucose measurement. In order to determine the best working potential for glucose sensing, a plot of chronoamperometric current versus working potential was made (data not shown). The study was performed in 0.1 M phosphate buffer pH 7.0 applying different potentials in the range from −0.5 V to +0.4 V and analysing the response for the same glucose concentration, 0.5 mM. At positive applied potentials no response was observed and at 0.0 V and −0.1 V a slight increase in cathodic response was observed. Furthermore, the current increased at more negative potential values (data not shown), but this time an oxidation process occurs. Although the optimal conditions for using as redox mediator would be at an applied potential around 0.0 V in order to minimise response to potential interferents, the response was so small at 0.0 V and a good response was exhibited at negative potential values, so a compromise was made between avoiding interferences and having a convenient response to glucose. Thus, −0.3 V *vs.* SCE was chosen to perform future experiments.

The response to glucose measured in 0.1 M NaPBS pH 7.0 at −0.3 V *vs.* SCE was found to be linear up to 1.3 mM with a sensitivity of 917 nA cm^{-2} mM^{-1} and detection limit of 31 μM. The apparent Michaelis–Menten constant K_M^{app} determined from the Eadie–Hofstee plot (data not shown) was 1.2 mM.

The analytical parameters obtained compare well with those in [7] for glucose biosensors with GOx and poly(neutral red) redox mediator having a linear range up to 1.8 mM, detection limit of 22 μM and K_M = 2.4 mM and in [31] for a glucose biosensor with glucose dehydrogenase and poly(toluidine blue) redox mediator with linear range up to 3 mM and K_M = 2 mM. In the case of the PNR/GOx biosensor, the sensitivity is higher – however, a different potential was applied and a larger amount of enzyme was used in the enzyme layer.

The biosensor proved to have good stability under continuous measurements, after recording four consecutive full calibration curves there was a decrease in sensitivity of 7%, the sensitivity being partially recovered on storing in buffer solution. The storage stability was investigated by keeping the electrode in supporting electrolyte at 4 °C when not in use and performing one calibration curve per week and after 7 weeks the sensitivity only decreased slightly by 6% to 94% of the initial value.

Since it was observed by cyclic voltammetry at slow scan rates that oxygen reduction can occur at the poly(brilliant cresyl blue) modified electrode around −0.3 V *vs.* SCE and impedance spectra also showed some changes on removing oxygen, it is possible that oxygen could have an influence on biosensor performance. Thus, the response of the biosensor to glucose in the presence and in the absence of oxygen was compared (Fig. 6). A decrease in sensitivity of 11% was observed in the absence of dissolved oxygen, which is not significant for the amperometric determination of glucose. Taking into account the results obtained, a possible mechanism of PBCB mediation in glucose biosensors is as follows. Since the response at negative potentials −0.2 V to −0.5 V was anodic and at 0.0 and at −0.1 V cathodic it appears that the mechanism

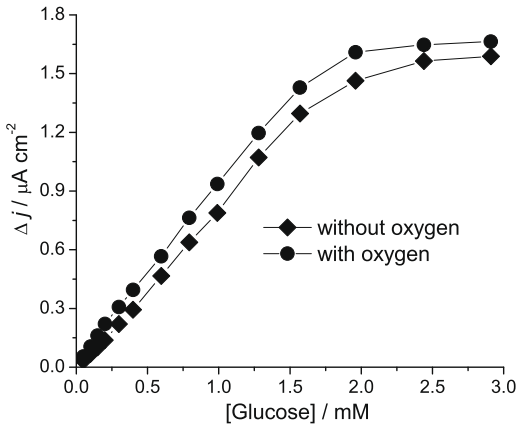


Fig. 6. Calibration curves for glucose at PBCB/GOx biosensor (●) with and (♦) without oxygen in 0.1 M NaPBS pH 7.0. Applied potential −0.3 V *vs.* SCE.

is similar to that observed for PNR when used in glucose biosensors [32], namely a competition between oxidative regeneration of the FAD cofactor of GOx (reduced by reaction with glucose) and reduction of H_2O_2. The total current obtained is the sum of the two processes: at more negative potentials the regeneration of FAD is the main process occurring which makes its contribution determinant to the total current and at less negative potentials closer to 0.0 V hydrogen peroxide reduction predominates. The results obtained, with good stability and storage capability, augur well for the use of PBCB as redox mediator in various types of electrochemical enzyme biosensor. Additionally, the combination of this mediator with carbon nanotubes may lead to a better analytical response in biosensors and possible use at lower potential values, closer to 0.0 V.

4. Conclusions

Brilliant cresyl blue has been polymerised onto glassy carbon electrode and the electrochemical properties of the resulting polymer have been investigated and compared with other polyazine-derived polymers. Differential pulse voltammetry revealed that poly(brilliant cresyl blue) exhibits a higher response at pH 4.1. By cyclic voltammetry the same behaviour was observed and the current intensity of both oxidation and reduction peaks was linearly dependent on the square root of scan rate. This behaviour is in agreement with diffusion controlled processes. The stability of the polymer film was good as was observed by performing continuous cycling in various supporting electrolytes. The electrode modified with poly(brilliant cresyl blue) was applied successfully to the determination of hydrogen peroxide at 0.0 V *vs.* SCE. The mechanism of functioning of the polymer as a redox mediator in glucose biosensors at −0.3 V *vs.* SCE led to a linear response up to 1.3 mM and a detection limit of 31 μM, which is very promising for future applications in electrochemical enzyme biosensors.

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