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Electrocatalytic activity of cobalt phthalocyanine CoPc adsorbed on a graphite electrode for the oxidation of reduced L-glutathione (GSH) and the reduction of its disulfide (GSSG) at physiological pH

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Abstract

Modified electrodes coated by adsorbed cobalt phthalocyanines are known to show substantial electrocatalytic activity for the electro-oxidation of several thiols in alkaline aqueous solution. In this context, we explore in this study the electrocatalytic activity of adsorbed cobalt phthalocyanine (CoPc) on ordinary pyrolytic graphite electrode for the oxidation of reduced L-glutathione GSH and the reduction of its disulfide GSSG at physiological pH. To do so, cyclic and rotating disk voltammetries were performed and the amperometric results show that a stable electrochemical sensing material, with good reproducibility and sensitivity (in accordance with the concentrations of GSH expected in biological media), can be easily achieved. This opens the way for the design of an electrochemical sensor able to detect these two analytes in biologically relevant experimental conditions (in terms of pH).

Keywords: Amperometry; Cobalt phthalocyanine; Electrochemical detection; L-glutathione; Modified electrode

1. Introduction

Reduced glutathione (GSH) (γ-L-glutamyl-L-cysteinyl-glycine, see Fig. 1) is the major non-protein thiol in living cells, with cellular concentrations ranging from 0.5 to 10 mmol L^{-1}. This tripeptide containing a sulfurhydryl group plays important biological functions in the organism, including protein and DNA synthesis, enzyme activity, metabolism and cell protection [1–5]. GSH is capable of scavenging oxygen-derived free radicals, which are thought to contribute to the development of many common diseases including cancer, heart attack, stroke, arthritis [6–8], and also helps in regenerating other antioxidants as vitamin E and ascorbic acid [9,10]. Therefore, this tripeptide was established to be a marker of oxidative stress [11] and its concentration in the organism can be relevant for the clinical diagnosis.

Several methods have been described in the literature for GSH analysis. In biological samples, the most commonly used assay is the Ellman's method [12]. This is based on the reaction between GSH and 5,5-dithiobis(2-nitrobenzoic acid) generating 2-nitro-5-mercapto-benzoic acid, which is monitored spectrophotometrically at 412 nm. Although this test is inexpensive and easy to use, it is not sensitive enough. Other methods such as high-performance liquid chromatography [13–16], spectrofluorimetry [17], spectrophotometry [18,19] and potentiometry [20] have been reported and successfully used. They are less susceptible to interference problems, but are not suitable in many cases where a rapid and accurate GSH determination is necessary, as they often require extraction and pre-concentration steps. Electrochemical sensors have been developed in the recent years [21–25]. However, the electrochemical detection of thiols, generally, is often hampered by slow rate of electron transfer at the electrode surface and the application of large over-potential is usually needed.

Chemically modified electrodes have been demonstrated to be able to improve the rate of electron transfer from substrates to

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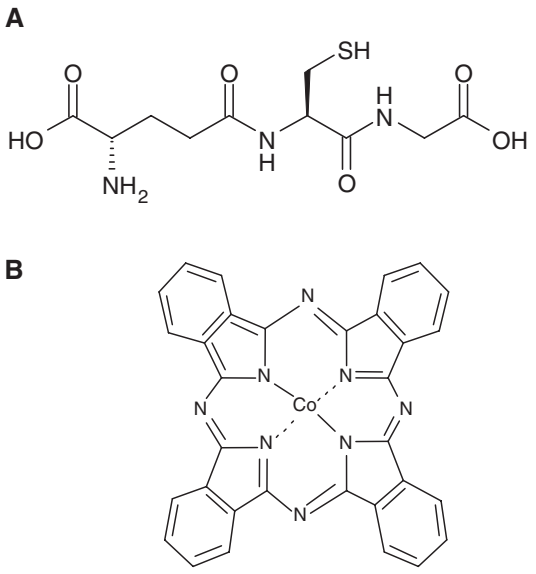

Fig. 1. Molecular Structure of (A) reduced L-glutathione (or *N*-(*N*-L-gamma-glutamyl-L-cysteinyl) glycine or γ-glutamyl-cysteinyl-glycine; GSH) and (B) cobalt phthalocyanine (CoPc).

the electrode. Hence, the development of such modified electrodes is a growing field of interest in analytical chemistry. Metallophthalocyanines have been reported to be efficient catalysts [26], especially concerning the electrochemical oxidation of thiols in acid or alkaline solutions [27–38]. In particular, it has been reported that modified electrodes coated by adsorbed cobalt phthalocyanines show substantial electrocatalytic activity towards the electro-oxidation of L-cysteine, 2-mercaptoethanol, reduced L-glutathione [27,28] and 2-aminoethanethiol [29] in alkaline aqueous solution.

In this context, we explore in this study the electrocatalytic activity of adsorbed cobalt phthalocyanine (CoPc, see structure in Fig. 1) for the oxidation of GSH and the reduction of its disulfide GSSG at physiological pH, in order to conceptualize an electrochemical sensor able to detect these two analytes in biologically relevant experimental conditions (in terms of pH).

2. Experimental

Electrochemical measurements were performed using a BAS CV-50w voltammetric analyser. The working electrode was an ordinary pyrolytic graphite disk (OPG) exposing a geometrical area of 0.44 cm^2 mounted in Teflon. The OPG electrode was polished before each experiment with 1200 and 2400 grit emery paper followed by ultrasonic treatment in purified water for 2 min. As a reference, a saturated calomel electrode (SCE) was used and the auxiliary electrode was a platinum spiral wire (99.99%, Aldrich) exposing an area of 1.4 cm^2. A conventional Pyrex glass electrochemical cell was used. Electrolytic solutions were prepared from deionised and bidistilled water and deaerated with ultra pure N_2 gas. All electrochemical measurements were conducted in deaerated solutions.

Cobalt phthalocyanine (CoPc), reduced L-glutathione (GSH) and oxidized L-glutathione (GSSG) were obtained from Aldrich. GSH and GSSG were kept refrigerated at all times. Electrolytes ($KCl/KH_2PO_4/NaCl/Na_2HPO_4{\cdot}7H_20$) were analytical grade from Merck and were used without further purification.

Phosphate buffer solution (PBS) has the following composition: KCl (2.60 mmol L^{-1}), KH_2PO_4 (1.46 mmol L^{-1}), NaCl (137 mmol L^{-1}) and $Na_2HPO_4{\cdot}7H_2O$ (8 mmol L^{-1}). The pH of this solution is 7.4 and it was regulated with a solution of NaOH aqueous solution (0.1 mol L^{-1}) when high concentrations of GSH and GSSG were used.

CoPc was adsorbed on the OPG electrode by placing a drop of a 50 mmol L^{-1} solution of CoPc dissolved in DMF over the surface of the OPG electrode for 30 min. Then, the electrode was washed several times with pure DMF to remove any excess of CoPc. After drying, the electrode was rinsed with purified water. Then the modified electrode was ready to use and introduced in the electrochemical cell. The modified electrodes obtained are then denoted as CoPc/OPG.

3. Results and discussion

3.1. Electrochemistry of adsorbed CoPc at physiological pH = 7.4

The first step of this study is aimed at describing and validating the electrocatalytic properties of the CoPc/OPG modified electrode. Fig. 2a shows the typical cyclic voltammogram of an OPG electrode obtained in PBS (pH 7.4). It exhibits weak voltammetric peaks that can be related to the presence of possible reactive functional groups on the surface of the bare OPG. Fig. 2b shows the typical voltammogram of a CoPc/OPG modified electrode obtained in PBS (pH 7.4). The potential scan was started at −1.1 V/SCE and directed towards positive values up to 0.6 V/SCE. Under these experimental conditions, the obtained voltammogram exhibits a well-defined and intense reversible pair of peaks at ca. −0.60 V vs. SCE which can be assigned to the redox Co(II)/Co(I) reversible process of the adsorbed CoPc [34–38]. Three other extra weak peaks of low

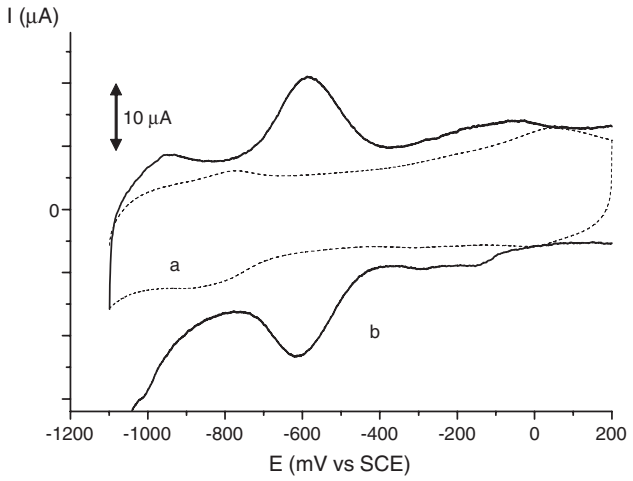

Fig. 2. Cyclic voltammogram of bare OPG electrode (curve a) and CoPc/OPG modified electrode (curve b) in PBS (pH = 7.4). Scan rate 0.1 V s^{-1}.

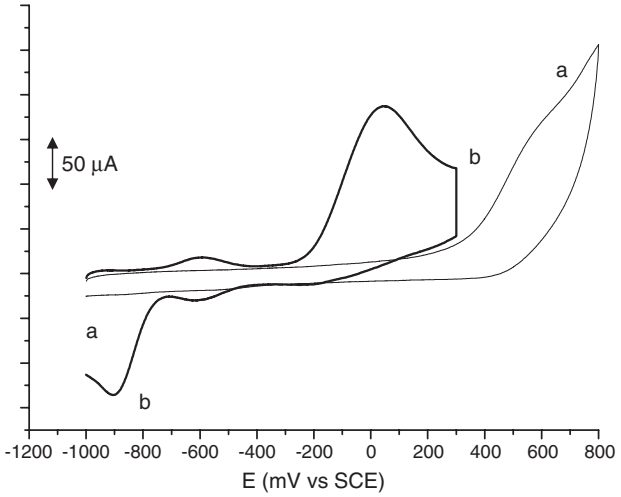

Fig. 3. Cyclic voltammograms of unmodified OPG electrode (curve a) and CoPc/OPG modified electrode (curve b) in the presence of 3 mmol L^{-1} of GSH in deaerated PBS buffer solution (pH = 7.4). Scan rate 0.1 V s^{-1}.

intensity are also observed. We suggest that these could be possibly attributed to the presence of the CoPc on the surface that affect the double layer capacity in this potential region. We do not believe that they might be due to faradaic processes linked to the complex. In general, these signals are not observed at high pH values. However further studies are required to clarify this point.

The amount of the apparent electroactive cobalt sites can be estimated from the electrical charge under the oxidative or reductive peak shown in Fig. 2b. The calculation gives a total amount of 3.36×10^{-10} mol cm^{-2} (result obtained from 7 samples), by considering the geometrical area of the electrode (without any surface roughness correction). By taking into account the size and the shape of the square-planar cobalt phthalocyanine complex (12×12 Å^{22}) and the average distance between the two staked complexes (4 Å) [39], one may estimate the thickness of the electroactive CoPc deposit to 1.2 nm or the equivalence of 3 monolayers of complex [34]. However, the real surface coverage is probably lower since it is likely that the roughness factor of the surface is higher than one. The adsorbed layer of CoPc was stable and the results obtained showed a good reproducibility.

3.2. Electro-oxidation of reduced L-glutathione GSH at physiological pH = 7.4

Fig. 3 shows a series of voltammograms obtained with unmodified OPG (curve a) and CoPc/OPG modified electrode (curve b) after adding 3 mmol L^{-1} of GSH in deaerated PBS solution (pH = 7.4). The potential scan was started at −1.0 V/SCE and directed towards positive values up to 0.8 V/SCE and 0.3 V/SCE, respectively. In the case of bare OPG electrode, an oxidation peak appears at 0.6 V/SCE which is indicative of the GSH oxidation process. It is noticeable that during the reverse cathodic scan, no reduction process is observed. This is indicative of the fact that the formed disulfide is not electroreducible in the investigated potential range (down to −1.0 V/SCE) in the absence of CoPc. In the case of the CoPc/OPG modified electrode, a new well-defined couple of peaks is observed, whereas no redox processes appeared with the unmodified electrode. Indeed, on the CoPc/OPG modified electrode, an intense oxidation peak appears at

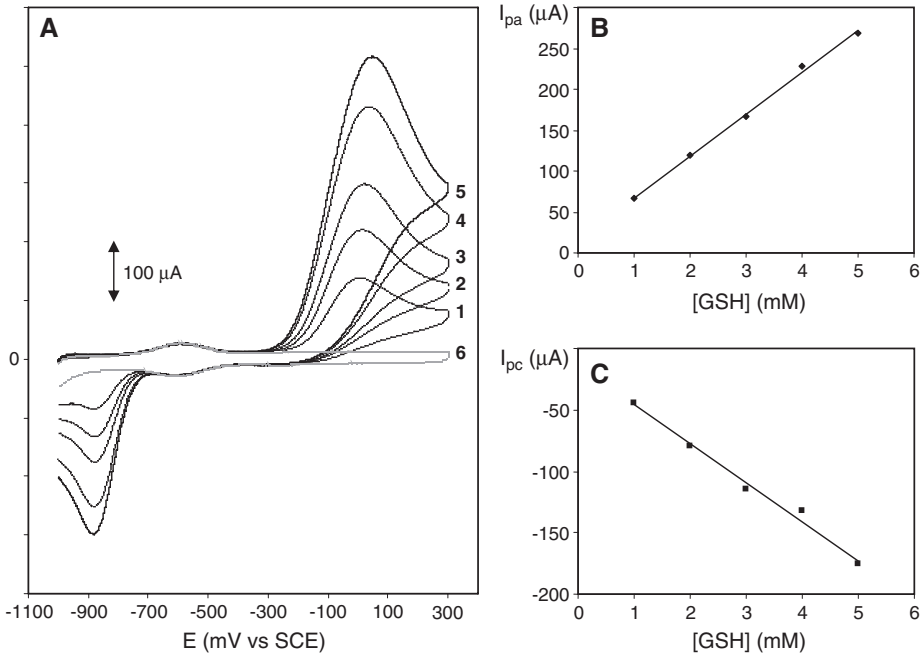

Fig. 4. (A) Cyclic voltammograms of GSH in PBS buffer solution (pH = 7.4) at various concentrations: 1 mmol L^{-1} (curve 1), 2 mmol L^{-1} (curve 2), 3 mmol L^{-1} (curve 3), 4 mmol L^{-1} (curve 4), 5 mmol L^{-1} (curve 5) at CoPc/OPG modified electrode. Scan rate 0.1 V s^{-1}. Curve 6 is obtained in the absence of GSH. (B) Linear variation of the intensity of the oxidation peak, Ip_a vs. GSH concentration. (C) Linear variation of the intensity of the reduction peak, Ip_c vs. GSH concentration.

ca. 0 V and it can be associated to the electrocatalytic oxidation of GSH. The appearance of the oxidation peak at ca. 0 V is concomitant with the appearance of the reduction peak at −0.92 V, during the reverse scan. According to the previously reported studies on the electrocatalytic oxidation of several other thiols by adsorbed and polymer-based metallophthalocyanines [28–32,34–38] in alkaline solutions, the large cathodic peak may be related to the reduction of the corresponding GSSG disulfide. Thus, this result clearly shows that CoPc not only acts as a real catalyst towards the oxidation of GSH but also acts as a catalyst for the reduction of the corresponding disulfide.

The voltammogram shown has the usual shape for an irreversible oxidation process of thiols in alkaline solution, through an adsorption-like and irreversible ligation of the thiolate anion (GS^-) to the central metal of the phthalocyanine, prior to electron-transfer [40]. Indeed, it has been previously proposed, for the oxidation of other thiols on monolayers of adsorbed cobalt phthalocyanines in alkaline solution, that the Co^{II}/Co^{I} redox process is involved [28–38] via an inner sphere mechanism: an adduct between GS^- and Co^{I} center is formed and its oxidation occurs at a potential which is higher than that of the Co^{II}/Co^{I} to process. A similar explanation can be provided for the reduction of the disulfide. Thus, the electrocatalytic mechanism involves Co^{II} and Co^{I} forms, as it was previously suggested by spectroelectrochemical experiments in the case of adsorbed cobalt phorphyrin in presence of 2-mercaptoethanol [34].

Although previous work demonstrated that the oxidation of GSH, as of other thiols, could be catalysed by CoPc adsorbed on graphite electrodes, most of this work was performed in alkaline solutions. Therefore, the remarkable result that should be emphasized here is that the electrocatalytic oxidation of GSH by adsorbed CoPc is clearly occurring at physiological pH.

Further experiments were carried out using different concentrations of GSH. The results obtained are shown in Fig. 4A. They were recorded by using the same CoPc/OPG modified electrode, confirming that no passivation of the electrode surface occurs upon GSH oxidation. They also show that there is a clear linear correlation between the intensity of both oxidation and reduction peaks (Ip_a and Ip_c, respectively) and GSH concentration ([GSH]) (Fig. 4B and C). These linear correlations illustrate the potential analytical application of this electrode. Also, this series of voltammograms show that the CoPc catalytic sites within the adsorbed layer are not poisoned by the product of the oxidation of GSH, since as the area of the peaks relative to Co(I)/Co(II) redox process does not change upon repeated scans and several experiments.

Fig. 5A shows a series of voltammograms 1 mmol L^{-1} GSH in PBS at pH 7.4 obtained at different potential scan rates (varying from 50 to 500 mV s^{-1}). The intensities of the oxidation and reduction peaks related to the thiol redox processes vary linearly with the square root of potential sweep rate (Fig 5B), with a slope close to 0.5 (while that of the Co(II)/Co(I) redox processes of the adsorbed catalyst vary with a slope close to 1, as expected for surface confined species). This reveals that the rate of the electro-oxidation of GSH at the surface of CoPc-modified electrode is controlled by the diffusion of the thiol in solution to the electrode surface (within the range of the investigated scan rates).

The electrocatalytic oxidation of GSH is now analyzed in more detail further on, using the rotating disk electrode technique, RDE. Fig. 6 shows the voltammograms for the oxidation of GSH on CoPc/OPG electrode, obtained with different electrode rotation rates. A typical diffusional plateau is observed at potential values higher than 0.2 V. However, at overpotentials higher than 0.25 V, a slight decrease of the current was observed probably due to a possible passivation of

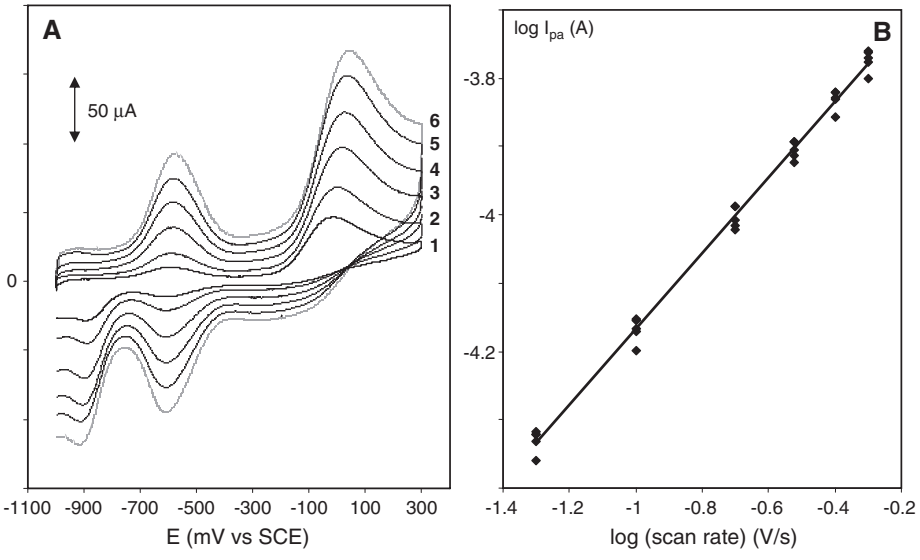

Fig. 5. (A) Cyclic voltammograms of 1 mmol L^{-1} GSH in PBS buffer solution (pH = 7.4) on CoPc/OPG electrode, at different scan rates: 50 mV s^{-1} (curve 1), 100 mV s^{-1} (curve 2), 200 mV s^{-1} (curve 3), 300 mV s^{-1} (curve 4), 400 mV s^{-1} (curve 5) and 500 mV s^{-1} (curve 6). (B) Linear variation of log(Ip_a) vs. log(scan rate).

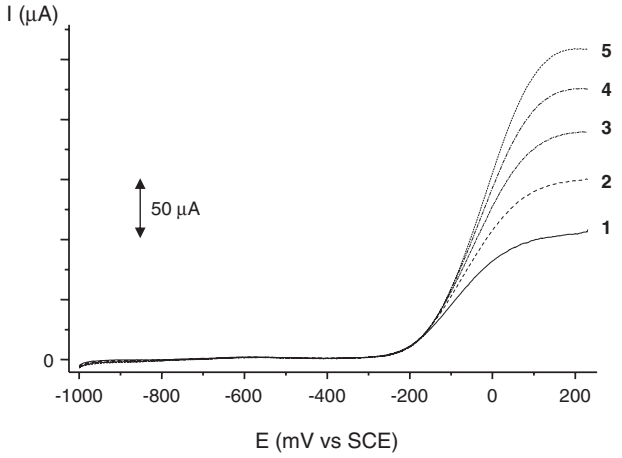

Fig. 6. Rotating disk electrode voltammograms of 1 mmol L^{-1} GSH in PBS buffer solution (pH=7.4) on CoPc/OPG modified electrode for different electrode rotation rates: 400 rpm (curve 1), 900 rpm (curve 2), 1600 rpm (curve 3), 2500 rpm (curve 4) and 3600 rpm (curve 5). Scan rate=10 mV s^{-1}.

the electrode surface upon GSH oxidation, under these hydrodynamic conditions (data not shown). Fig. 7 shows the expected linear Tafel plot constructed from data obtained with the rotating disk electrode. The slope is close to 0.12 V per decade (the slope is equal to 2.3RT/αF where α is a transfer coefficient). This indicates that the first-one electron transfer is the rate-determining step for GSH oxidation, according to the following possible mechanism previously reported for other thiols in alkaline solution [28,29,31]:

$$G-SH \leftrightarrows GS^- + H^+ (pK_a = 9.2) \quad (1)$$

$$GS^- + PcCo(II) \rightarrow [PcCo(I)-(GS)]^- \quad (2)$$

$$[PcCo(I)-(GS)]^- \xrightarrow{r.d.s.} [PcCo(II)-(GS)] + e^- \quad (3)$$

$$[PcCo(II)-(GS)] \rightarrow PcCo(II) + GS^\bullet \quad (4)$$

$$GS^\bullet + GS^\bullet \xrightarrow{fast} GSSG \quad (5)$$

Even though at the physiological pH of 7.4 the amount of dissociated GS^- is low, there seems to be enough of thiolate ion on the electrode to sustain the catalytic current. The remarkable result is that the electrocatalytic effect of adsorbed CoPc towards the oxidation of GSH at physiological pH is effective. It occurs following a mechanism that is similar to that reported previously [28,29,31] in the case of alkaline solution, where step 5 is extremely fast.

3.3. Electro-reduction of oxidized L-glutathione GSSG at physiological pH=7.4

As reported above for the oxidation of GSH, the investigation of the anodic process by using cyclic voltammetry allows the observation of a cathodic process confirming the formation of GSSG at the electrode. In order to further investigate GSSG electrochemical activation, experiments were performed with GSSG in solution, at physiological pH. Fig. 8A shows a series of cyclic voltammograms obtained on CoPc/OPG electrode in the presence of different concentrations of GSSG in PBS (pH 7.4). The potential scan was first started at −0.4 V/SCE, directed towards negative values down to −1.1 V/SCE, then reversed to 0.3 V/SCE and finally stopped at −1.1 V/SCE. The results obtained with the same modified electrode show that there is a linear correlation between the intensity of the reduction peak intensity (Ip_c) and GSSG concentration in solution ([GSSG]). This confirms the possible use of the Ip as an analytical parameter to determine GSSG concentration in solution, without any surface passivation of the electrode (in the range of the concentrations examined in Fig. 8B).

Fig. 9A shows a series of cyclic voltammograms of GSSG solution at pH 7.4, at different potential scan rates (varying from 50 to 500 mV s^{-1}). The obtained results show that the intensity of the GSSG reduction peak varies linearly with the square root of potential sweep rate (Fig. 9B), with a slope close to 0.5. These results confirm that the reduction of GSSG at the CoPc/OPG modified electrode is diffusion controlled in the range of the examined scan rates. Finally, the rotating disk electrode voltammograms of GSSG at the CoPc/OPG electrode, recorded with different electrode rotation rates exhibit the typical diffusion plateaus for all a rotation rates studied, although they are ill defined at high rotation speeds (data not shown).

3.4. Chronoamperometry analysis of reduced and oxidized L-glutathione at physiological pH=7.4

In order to test our modified electrode as a sensor for measuring GSH and GSSG concentrations in biological conditions, a series of chronoamperograms were performed. This was achieved by using a rotating disk electrode at a controlled rotation rate of 1600 rpm. The CoPc/OPG modified electrode was polarized at two different potentials: E=0.2 V/SCE for the detection of GSH measuring its electrocatalytic

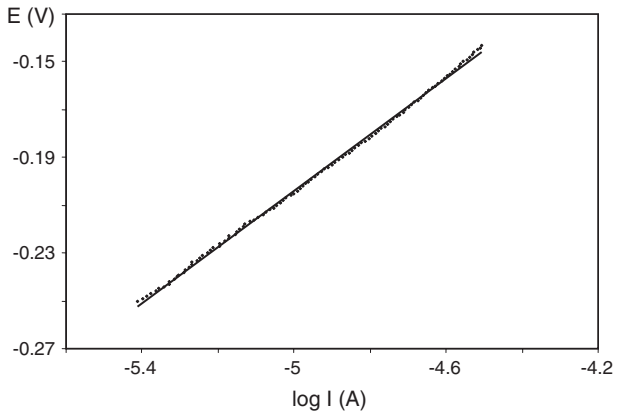

Fig. 7. Tafel plot for the oxidation of 1 mmol L^{-1} GSH in PBS buffer solution (pH=7.4) on CoPc/OPG modified electrode (data from Fig. 6).

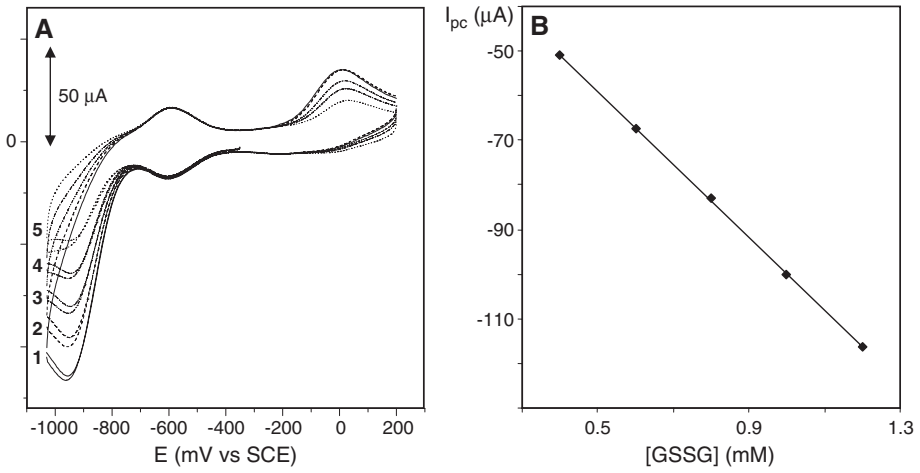

Fig. 8. (A) Cyclic voltammograms for the reduction of GSSG in PBS (pH=7.4) at various concentrations: 1.2 mmol L^{-1} (curve 1), 1 mmol L^{-1} (curve 2), 0.8 mmol L^{-1} (curve 3), 0.6 mmol L^{-1} (curve 4), 0.4 mmol L^{-1} (curve 5) on CoPc/OPG modified electrode. Scan rate=0.1 V s^{-1}. (B) Linear variation of the intensity of the reduction peak Ip_c vs. GSSG concentration.

oxidation, and at $E=-1$ V/SCE for the detection of GSSG measuring its electrocatalytic reduction. The results presented in Fig. 10 show very well-defined amperometric responses of the CoPc/OPG modified electrode for both GSSG and GSH (Fig. 10A and C, respectively). Indeed, an increase of the electrocatalytic currents for GSSG reduction and GSH oxidation as a function of GSSG and GSH concentrations are clearly observed. Fig. 10B and D shows the linear variation of the electrocatalytic current plateau with GSSG and GSH concentrations, in the range of 0.08–1 mM, with a linear regression coefficient of 0.998 in both cases. The calculated sensitivities are 23 µA µmol^{-1} L for GSSG and 11.5 µA µmol^{-1} L for GSH, which are highly acceptable. No passivation of the electrode was observed for the range of concentrations used, which are biologically relevant.

These preliminary results are remarkable for several reasons. First, they demonstrate that both GSH and GSSG can be detected in parallel by using the same modified electrode. Second, this can be simply done by changing the operating potential without having to deal with passivation complications.

4. Conclusion

These preliminary studies demonstrate the advantages of using a CoPc/OPG modified electrode for the electrochemical detection of GSH and GSSG at physiological pH. The voltammetric studies at pH 7.4 show that a similar electrocatalytic behaviour of both reduced and oxidized GSH and GSSG is observed. The amperometric results performed showed that a stable electrochemical sensing material, with good reproducibility and sensitivity can be easily obtained (in accordance with the concentrations of GSH expected in biological media). It opens the way for other important applications such as the study of nitrosothiols, in particular GSNO. Indeed, such a sensor could be used for

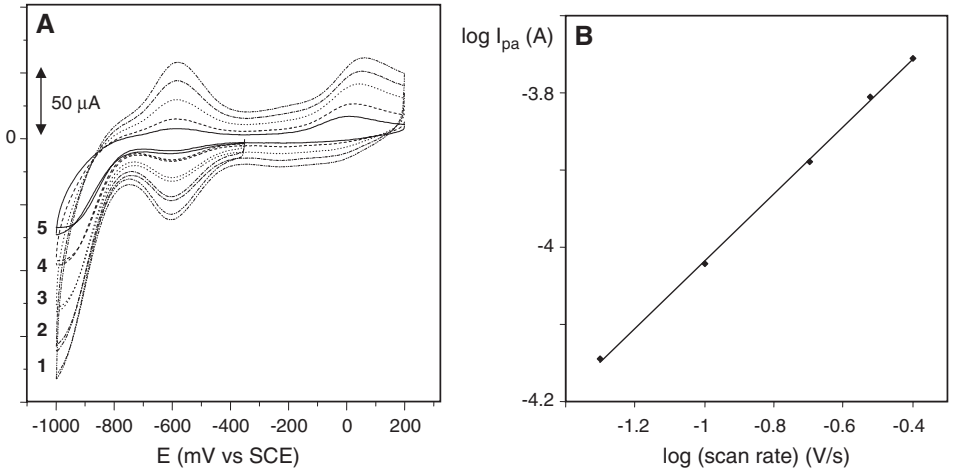

Fig. 9. (A) Cyclic voltammograms of 1 mmol L^{-1} GSSG in PBS buffer solution (pH=7.4) on CoPc/OPG modified electrode at different scan rates: 100 mV s^{-1} (curve 1), 200 mV s^{-1} (curve 2), 300 mV s^{-1} (curve 3), 400 mV s^{-1} (curve 4) and 500 mV s^{-1} (curve 5). (B) Linear variation of log(Ip_c) vs. log(scan rate).

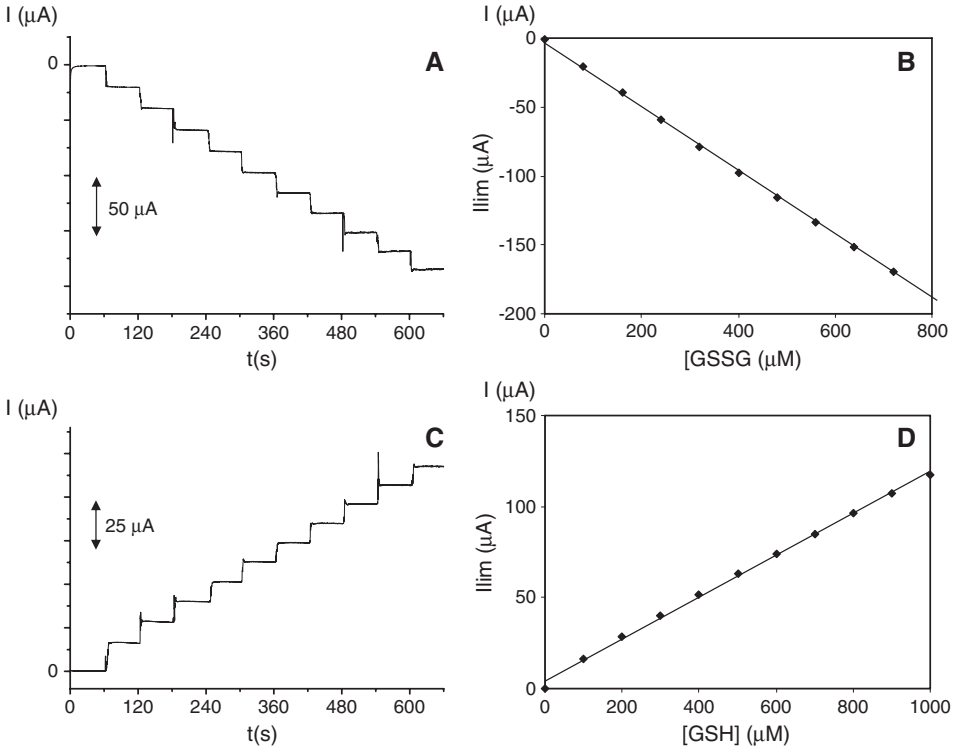

Fig. 10. Chronoamperometric measurement of successive additions of 80 μmol L^{-1} of GSSG (A) and 100 μmol L^{-1} GSH (C) on CoPc/OPG modified electrode in PBS (pH 7.4) poised at −1 V/SCE and 0.2 V/SCE, respectively, and linear correlation between the amperometric current and the concentration of GSSG (B) and GSH (D). Electrode rotation rate = 1600 rpm.

the detection of the released GSSG upon NO donation and provide accurate information on the GSH/GSSG balance ratio.

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