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A glutathione amperometric biosensor based on an amphiphilic fullerene redox mediator immobilised within an amphiphilic polypyrrole film

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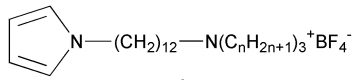
A novel biosensor for the amperometric detection of glutathione is proposed in which glutathione reductase is coupled to a pyrrolofullerene bis-adduct playing the role of redox mediator between electrode and enzyme. The adoption of a simple procedure for realizing the immobilization of the redox mediator, together with the enzyme, within a polypyrrole film cast onto a glassy carbon electrode, was made possible by the amphiphilic properties of the fullerene derivative. The biosensor showed a fast and reproducible response to glutathione and, because of its lower sensitivity, a wider linear range with respect to a similar device based on a diffusional redox mediator.

Introduction

The concept of electrical wiring of enzymes for the development of electrochemical biosensors and bioelectronic devices couples the recognition properties typical of the biological systems with the high sensitivity proper to electrochemical transducers, and has seen ever-increasing research effort over the last decades.[1] Various approaches have been investigated in order to realize the immobilization of the biomaterial on either conducting or semiconducting electrodes, comprising adsorption, covalent bonding, physical entrapment in sol–gel materials, carbon-paste electrodes, membranes and either conducting or redox organic polymers.[1–3] In the latter case, the application of a suitable potential to the electrode, immersed in the solution containing both the monomer and the biomaterial, allows the incorporation of the latter into the growing polymeric film. However, denaturation of the biomaterial may occur during the electropolymerization, due to the rapid pH decrease in the vicinity of the electrode surface. Moreover, while the amount of polymer that is deposited onto the electrode surface, *i.e.* the film thickness, may be monitored by the electrodeposition charge, the quantity of entrapped biomaterial cannot be easily controlled.[1c] In this regard, a convenient alternative strategy has been developed based on the co-immobilization of amphiphilic monomers **1** (Chart 1) and biomaterial by adsorption on the electrode surface before the electropolymerization step.[1c,2] The biomolecule is solubilized in an aqueous dispersion of the amphiphilic pyrrole derivative, obtained by ultrasonication, and subsequently the mixture is spread onto the electrode surface and vacuum-dried. The electropolymerization of the adsorbed monomer film, carried out in an aqueous electrolyte, thus realizes the irreversible entrapment of a controlled amount of enzyme in the polypyrrole film. Such a procedure has been largely used for the construction of amperometric enzymatic and multienzymatic biosensors.[2]

Since the direct electrical communication between the prosthetic group of the enzyme and the electrode surface is often kinetically prohibited,[4] artificial redox mediators are usually employed to shuttle electrons between the redox protein and the conducting support, comprising ferrocene derivatives, quinones and bipyridinium salts.[1–3] However, the resulting biosensors are not stable and tend to release the incorporated mediator, making such devices unsuitable for *in vivo* measurements. Covalent immobilization of the redox mediator within the polymer matrix may prevent its leakage but requires additional preparative efforts for the synthesis of suitably functionalised monomers or the chemical modification of the protein.[1] The use of redox mediators that may be confined within the polymeric films by virtue of their poor solubility in aqueous solutions or for steric reasons represents therefore a promising strategy for the development of novel biosensors. In the present work, the potential of such an approach is



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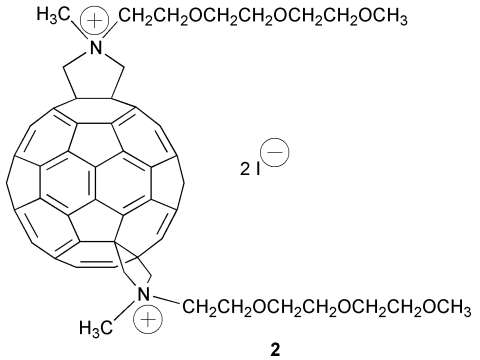

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Chart 1

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investigated by realizing the incorporation of the dipyrrolofullerene iodide salt **2** shown in Chart 1 in a poly-**1** ($n = 2$)–glutathione reductase (GR) film.

Results and discussion

The choice of **2** as redox mediator in a biosensor for the enzymatic detection of oxidised glutathione was justified for several reasons: (i) **2** possesses suitable redox properties to be used in conjunction with redox enzymes operating in reductive pathways and, in particular, with GR.[5] The active site of GR consists of a disulfide bond formed by two cysteine residues and its standard reduction potential has been estimated to be $E = -0.25$ V at pH 7.0.[6] The first reduction of **2** is both chemically and electrochemically reversible and occurs, in tetrahydrofuran solution, with $E_{1/2} = -0.34$ V.[7a] From the thermodynamic point of view, therefore, reduced **2** can sustain the electrocatalytic reduction of glutathione by transferring an electron to oxidised GR (*vide infra*). In this respect, the redox properties of **2** resemble those of *N*,*N*′-dialkyl-4,4′-bipyridinium salts (viologens) that, for their favorable redox properties, have often been used as redox mediators in GR-based biosensors.[3a–e] Viologens are however very soluble in aqueous solutions and their confinement within the sensor was accomplished by covalent attachment.[1a,d,2c,3] Moreover, viologens are highly toxic.[3f] By contrast, (ii) **2** is nearly insoluble in aqueous solutions and additional stability with respect to leakage from the polymeric film is expected for steric reasons. Furthermore, most fullerene derivatives show very low toxicity compared with typical drugs.[8] At the same time, (iii) the presence of the two positive charges and of the triethylene glycol (TEG) chains gives **2** an amphiphilic character. This allows the adoption of the two-step procedure outlined above for realizing the entrapment of **2** in the poly-**1** matrix: an aqueous suspension of both **1** and **2** can in fact be obtained by ultrasonication, to which the enzyme is added before performing the *in situ* electropolymerization. Furthermore, **2** is a dication and is therefore expected to interact electrostatically with the redox protein (a polyanion at the operating pH), and this may in turn allow a better electronic contact of the redox mediator with the prosthetic group in the protein. Finally, (iv) it has been shown that films of poly-**1** incorporating C_{60} or C_{70} possess good electronic properties: charge-compensating tetrabutylammonium cations may easily permeate in the films and this allowed the observation of the first four reversible reductions of the fullerene materials in acetonitrile solutions.[9]

Films of poly-**1** ($n = 2$) containing the GR enzyme and the redox mediator **2** were prepared onto a glassy carbon disc electrode ($r = 1.5$ mm), following the simple procedure outlined above and described in the Experimental section. The polymerization was carried out by controlled potential electrolysis at 0.75 V in a 0.1 M $LiClO_4$ aqueous solution. Electrodes were also prepared, following the same procedure, that did not contain either the redox mediator or the enzyme, and, in the following, the three types of electrodes will be denoted as poly-**1**/**2**/GR, poly-**1**/GR and poly-**1**/**2**, respectively.

The current transients relative to the early stages of the electropolymerization process were recorded. In all cases, after an initial decreasing portion, originating from capacitive charging processes,[10] the current went through a maximum and then decayed to a quasi-stationary value. Such behavior is typical of electrochemical nucleation phenomena, such as those associated with electrocrystallization and electrodeposition processes, in which the charge transfer step is fast and the rate of growth of crystallization nuclei is controlled by mass-transfer of electrodepositing ions to the growing centres.[11] Such a model can effectively apply to the present case where however the charge-balancing counterion (ClO_4^-) rather than the pyrrole derivative is the diffusing species: since the polymer film is grown in its oxidised, conductive state, the rate of the polymerization process is expected to be controlled by the diffusion of the ClO_4^- ions to the growing polypyrrole chains, rooted at the film/electrode interface and progressively extending through the monomer coating.[12] The analysis of the current transients may provide valuable information on both the nucleation and growth processes; in this respect, a convenient representation of the *I*–*t* transients is obtained in dimensionless coordinates by plotting $(I/I_m)^2$ *vs.* t/t_m, where I_m and t_m are the maximum current attained and the corresponding time. The experimental results are compared in Fig. 1 with the theoretical curves corresponding to the two different models of instantaneous (curve a) and progressive (curve b) nucleation.[11] The comparison would show that both the bulky fullerene derivative and the enzyme molecule greatly influence the kinetics of the polymerization process. In the case of pure **1**, the polymerization process is adequately described by the instantaneous nucleation model, *i.e.* the instantaneous formation of a discrete number of polymerization nuclei followed by their mass transfer-controlled growth. In contrast, the presence of either **2** or especially the protein slows down the nucleation step, which is now better described by the progressive nucleation model in which nuclei are produced, during the whole polymerization process, at a rate comparable to their growth. The protein is also responsible for a significant decrease of the polymer growth rate, as evidenced by a large increase of t_m with respect to **1** and **1** + **2** coatings, and this may be associated with a large decrease of the diffusion coefficient of ClO_4^- ions within the monomer coating due to the presence of the protein. According to the theoretical model,[11] in fact, the diffusion coefficient $D(ClO_4^-)$ can be obtained from the experimental value of the product $I_m^2 t_m$: while almost the same value of $D(ClO_4^-)$ was obtained in the **1** + **2** and pure **1** films, it was found to be more than one order of magnitude lower in the presence of the enzyme.[13]

After the polymerization, the films were characterized by cyclic voltammetry (CV), also performed in the 0.1 M $LiClO_4$ aqueous solution used for the polymerization step. The CV curves relative to a poly-**1**/**2** film, obtained at various scan rates, are shown in Fig. 2. The reversible oxidation peak with $E_{1/2} = 0.50$ V is typical of *N*-substituted polypyrrole films:[14] its height increases linearly with scan rate (Fig. 2, inset), and, from the slope of peak current, I_p,*vs.* scan rate, *v*, assuming that one in three pyrrole units is oxidised in the film,[14] a polymer

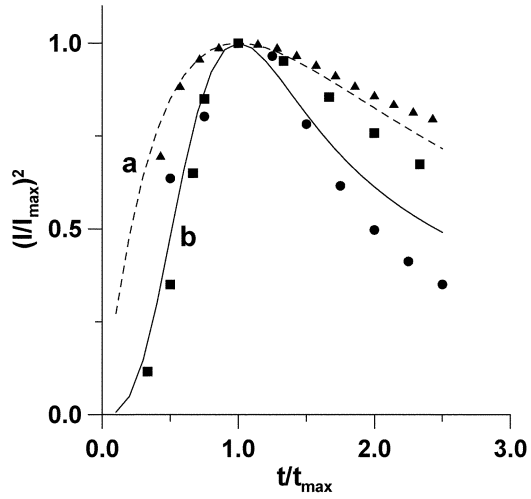


Fig. 1 Comparison of the experimental *I*–*t* transients recorded during the controlled-potential ($E = 0.75$ V) polymerization experiments with the theoretical transients corresponding to instantaneous (a) and progressive (b) nucleation. The sets of data refer to films of pure **1** (▲); **1** + **2** (■) and **1** + **2** + GR (●) adsorbed onto glassy carbon electrodes ($r = 1.5$ mm). $T = 25$ °C.

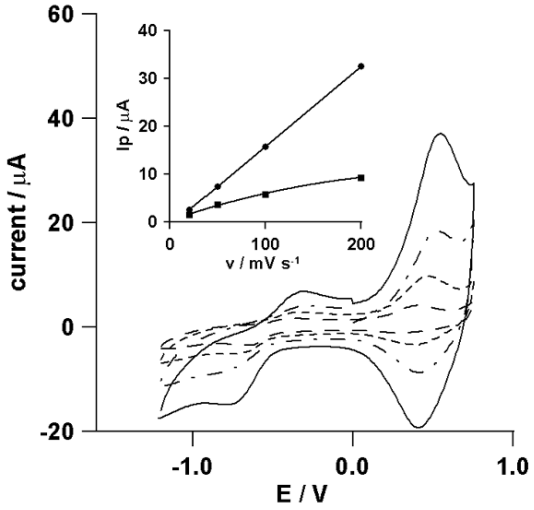


Fig. 2 Cyclic voltammetric curves relative to a poly-**1/2** film electrogenerated onto a glassy carbon electrode (r = 1.5 mm) in 0.1 M aqueous $LiClO_4$ solution. Scan rate: 0.02 (— — —), 0.05 (- - -), 0.1 (. – .) and 0.2 V/s (——). Inset: dependence of peak current on scan rate, for the polypyrrole oxidation (■) and **2**-centred reduction (●).

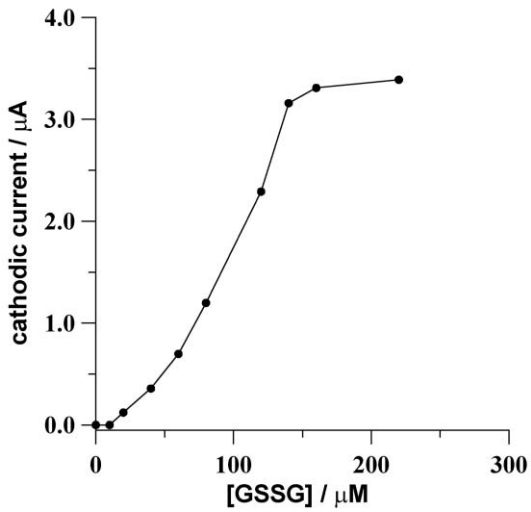


Fig. 3 Calibration curve corresponding to the chronoamperometric responses of the poly-**1**/GR film, at different concentrations of glutathione, in a 1 mM MV^{2+}, 0.1 M HEPES aqueous solution. Rotating disc glassy carbon electrode (r = 1.5 mm), Ω = 1000 rpm. Working potential: 0.75 V. T = 25 °C.

surface excess $\Gamma_{N+} = 7 \times 10^{-9}$ mol cm^{-2} was obtained.[15] In the region of negative potentials, a reversible reduction process, with $E_{1/2} = -0.55$ V, is observed showing the typical morphology of diffusion-controlled processes: in fact, the peak height varies, as expected in such a case, linearly with the square root of v (as shown by the curvature of the corresponding I_p *vs.* v Fig. 2, inset). Such a process, which is absent in the case of pure poly-**1** films, was attributed to the reversible one-electron reduction of **2**, in agreement with its CV behavior in aprotic solvents.[7a] Since diffusion of **2** within the polymer film is highly improbable, the redox process is likely to occur *via* vectorial electron self-exchange reactions involving neighbouring neutral and reduced **2** molecules, coupled to the diffusion of charge-compensating counterions (Li^+). Such a mechanism has been proposed in the case of other polymer matrices containing immobilized redox centres,[16] and, analogously, vectorial electron hopping between C_{60} molecules entrapped in a polymer film is at the basis of the functioning of solid-state photoconducting devices capable of performing light-to-electricity conversion.[17] Finally, the much larger cathodic-to-anodic peak separation in the CV curve of **2** in the poly-**1/2** film, compared to that measured in the homogeneous case,[7a] is in line with the low mobility of solvated Li^+ ions in the hydrophobic polymer matrix.

The poly-**1/2** film was examined for its catalytic properties with respect to the reduction of oxidised glutathione (GSSG) to reduced glutathione (GSH). The modified electrode was transferred to a deaerated 0.1 M HEPES aqueous solution, and a potential of −0.75 V was applied, corresponding to the reduction of **2**. The stationary current passing at the poly-**1/2** electrode under controlled hydrodynamic conditions (rotating disc electrode, RDE, Ω = 1000 rpm) was then monitored, following the successive additions (by 10 μM steps) of a GSSG deaerated solution. No change of the stationary current was obtained thus proving that the fullerene–polymer system cannot by itself promote the reduction of GSSG. Analogously, the poly-**1**/GR electrode also did not show any biocatalytic property, and this was attributed to the lack of electrical contact between enzyme and electrode. The addition of a suitable diffusional redox mediator, namely, *N*,*N*′-dimethyl-4,4′-bipyridinium, methylviologen (MV^{2+}), to the buffer solution, in fact gave rise to a catalytic current corresponding to the reduction of GSSG to GSH. This demonstrates that enzymatically active GR molecules can be efficiently entrapped within poly-**1** films. Fig. 3 shows the poly-**1**/GR electrode amperometric response obtained, under the conditions given above, for successive additions of GSSG to the buffer solution also containing MV^{2+} (1 mM). The calibration curve shows a sigmoidal shape that is typical of cooperative enzymatic kinetics, which may be expected for GR which presents two identical active sites in its structure.[18] As a consequence, the calibration curve exhibits a very short linear range: 30–120 μM while the maximum current, corresponding to saturating substrate conditions, is obtained with [GSSG] ⩾ 220 μM.

The catalytic properties of the poly-**1/2**/GR electrodes were finally examined, under similar enzyme loading conditions as in the previous case. Cathodic stationary current steps were obtained, under the conditions reported above, corresponding to the successive additions of GSSG, as shown in Fig. 4 (inset). The steady-state current response is reproducible, and the response time, *i.e.* the time required, after GSSG injection, for the current to reach a steady-state value, was within 2–3 s, illustrating the fast electrical wiring of GR by the incorporated **2**. The poly-**1/2**/GR electrode is therefore able to promote the biocatalytic reduction of GSSG and the whole process giving rise to the catalytic current is the following:

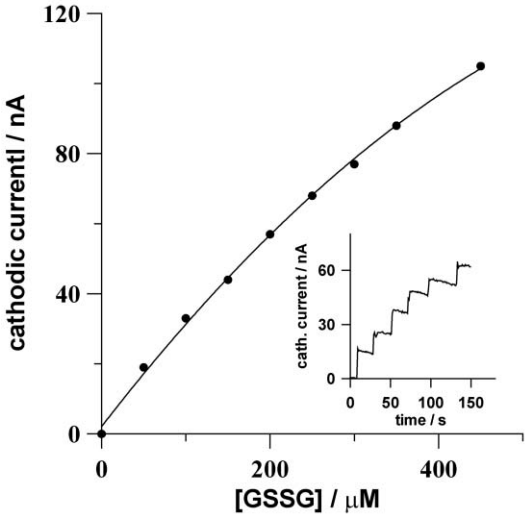
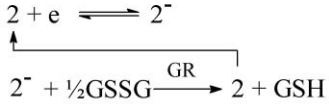


Fig. 4 Calibration curve for glutathione at a poly-**1/2**/GR film; inset: current responses as a function of time at a poly-**1/2**/GR film, with successive additions of glutathione (50 μM) in a 0.1 M HEPES aqueous solution. Experimental conditions as in Fig. 3.



The calibration curve, Fig. 4, exhibits a linear response extending to GSSG concentrations of 350 μM, *i.e.* well above the saturating substrate limit observed in the case of the diffusional redox mediator. Furthermore the current steps corresponding to the addition of equal amounts of GSSG are significantly lower than in the case of poly-**1**/GR + MV^{2+}. Possible explanations of such a lower sensitivity of the poly-**1**/**2**/GR electrode may be related either to the varied mechanism of the charge-mediation step or to the structural modification of the poly-**1** matrix induced by the presence of **2**, or even to a combination of the two effects: (i) electron-hopping between fullerene centres is likely less effective than diffusion to propagate the charge across the polymeric film; (ii) the average minimum distance between the prosthetic group of GR and the redox mediator is likely much greater in the case of the sterically-hindered species **2** than for MV^{2+}.[19] Notice that, when alkyl-substituted viologens were covalently attached to GR in a cysteine-modified Au biosensor, thus decreasing the mobility of the redox mediator, the catalytic process was too slow to give rise to a measurable current and the occurrence of the electrocatalytic process was proved by GSH accumulation.[3a–e] Additionally, (iii) the driving force for the electron transfer process involving GR is lower in the case of **2** than with MV^{2+} ($E_{1/2}$(**2**/**2**$^-$) = −0.55 V *vs.* $E_{1/2}(MV^{2+}/MV^+)$ = −0.60 V, under the conditions of Fig. 3 and 4). Finally, (iv) the expectedly increased hydrophobic character of the poly-**1**/**2**/GR film with respect to the poly-**1**/GR one may lead to a significant decrease of the GSSG repartition coefficient and hence to a much lower effective substrate concentration in the fullerene-containing film.

Conclusions

A novel biosensor for the amperometric detection of glutathione was obtained by a simple procedure in which the co-immobilization of a redox enzyme (GR) with a redox mediator (dipyrrolofullerene **2**) is realized at the glassy carbon electrode surface by the use of the amphiphilic pyrrole derivative **1** (n = **2**). The reversible reduction of **2** entrapped within the poly-**1** film was observed in the CV curve, the electron transfer likely occurring *via* a vectorial charge-hopping mechanism involving neighbouring dipyrrolofullerene molecules, coupled to the diffusion of counterions. The poly-**1**/**2**/GR film, investigated for its electrocatalytic properties, proved to be able to promote the fast and reproducible reduction of GSSG to GSH; the modified electrode is less sensitive to the substrate than a corresponding system based on a diffusional redox mediator, thus widening the linear response range of the biosensor.

Experimental

Materials

Glutathione reductase (GR) (EC 1.6.4.2., from Bakers' Yeast, 131 Sigma units per mg) was purchased from Sigma. L-Glutathione oxidized form was purchased from Fluka. Compound **1** was prepared as described elsewhere.[20] Compound **2** was prepared from the corresponding neutral bisadduct[7b] which was alkylated in neat methyl iodide at room temperature for 48 h. Its electrospray mass spectrum showed a peak at m/z 546, which corresponds to $M^+/2$. $LiClO_4$ (pro analysi from Merck) or *N*-(2-hydroxyethyl)piperazine-*N*'-(ethane-2-sulfonic acid) (HEPES, from Sigma) were used as received. All other materials were reagent grade chemicals.

Enzyme electrode preparation

The poly-**1** electrodes containing either **2** or GR or both, were prepared following the procedure reported previously.[2c] The amphiphilic pyrrole **1** was dispersed ultrasonically in pure water to give a stable, optically transparent solution (3 mM). **2** was added to such a solution and dispersed ultrasonically. GR was dissolved in the resulting solution to provide 4 mg ml^{-1} enzyme concentration. The mixture was spread on the GC electrode and the solvent was evaporated to dryness under reduced pressure. The resulting modified electrode was transferred into a cell containing aqueous $LiClO_4$ solution. Then, the polymerization of the adsorbed monomer film was carried out by controlled-potential electrolysis at 0.75 V for 15–20 min.

Electrochemical instrumentation and measurements

The electrochemical experiments were carried out with an Autolab Model PGSTAT30. Potentials were measured with respect to a saturated calomel electrode (SCE). $E_{1/2}$ values correspond to (E_{pc} + E_{pa})/2 from CV. A disc glassy carbon (GC) electrode, 3 mm diameter, was used as working electrode, mounted on a Tacussel EDI rotating disc electrode. A platinum spiral was used as counter electrode and experiments were carried out in a three-compartment electrochemical cell.

Acknowledgement

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