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Short Communication

Decreasing Hg(II) Toxicity on Cytosolic GSH-S Transferases from Rat Liver Using a PEDOT Modified Electrode

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3,4-ethylenedioxythiophene (EDOT) was electro-polymerized by potentiodynamic (scan potential range between -1.0 and 1.5 V *vs* SCE) and potentiostatic method (potential step of 1.4 V during 5 min) on stainless steel AISI 316 (SS) electrodes, using lithium perchlorate as support electrolyte in acetonitrile, to obtain the modified electrode SS|PEDOT, to exploit it n-doping/undoping propertie to remove mercury, as has been reported elsewhere. In this particular case, the objective of the study is to verify whether the removal of mercury allows decreasing Hg(II) toxicity on cytosolic GSH-S transferases from rat liver. The electrochemical responses were recorded in aqueous solution of phosphate buffer at physiological pH (PBS) containing mercury(II) diacetate, and/or enzyme with its substrates, as appropriate, to verify the effect of Hg^{2+} cations extraction using the method previously reported, which is based on the SS|PEDOT n-doping/undoping property. The results are very promising, because on the one hand shows that PEDOT does not affect the enzymatic activity of cGST from rat liver, which is very relevant when considering a possible application of this methodology in biological systems affected by mercury. By the other hand, it was shown that the method also allows reverse the inhibition of the enzyme produced by mercury, opening the door for future procedures aimed not only at removing mercury from biological systems, but also recover the biological properties of the proteins affected by this metal, using this type of modified electrodes, which are a simple and inexpensive alternative, as with all electrochemical techniques.

Keywords: 3,4-ethylenedioxythiophene, poly(3,4-ethylenedioxythiophene), polymer modified electrode, Hg toxicity, cytosolic GSH-S transferases.

1. INTRODUCTION

Mercury is a heavy metal associated to wide range of toxics effects on living organism [1]. There exist several forms of this metal, including inorganic mercury salts, metallic mercury (mercury vapor), and organic mercury [2]. By *in vivo* metabolism, the inorganic forms of mercury undergo methylations and/or oxidations, delaying its elimination and then, increasing its toxicity [3, 4].

The mechanism of action of mercury is related to interactions with macromolecules, in particular with thiols containing proteins, causing inactivation of the catalytic activity of these enzymes or altering the structural properties of proteins [5]. In addition, oxidative stress is related to mercury exposition [6].

In Minamata bay (Japan), and in Iraq, public health disasters have been associated to mercury exposition [7, 8]. In human, the toxic symptoms are related to the form of mercury, dose, and exposure rate [9]. In acute exposure to mercury vapor, the main target organ is the brain, because the inhalation of mercury vapor easily crosses the blood brain barrier, and then it is oxided to a stable and water-soluble form of mercury, being accumulated in the brain [10]. This condition is associated to severe neurological symptoms [9]. Also, large acute exposure to this form of mercury is related to severe and lethal pneumonitis [11].

Moreover, inorganic mercury is poorly absorbed and can induce gastrointestinal and renal damage, although such cases are rare [12]. In contrast, organic forms of mercury are completely and rapidly absorbed from the gastrointestinal tract and react with sulfhydryl groups throughout the body triggering structural, oxidative, catalytic and genomic damage [1, 9].

The treatments are based on the clinical use of BAL, EDTA, DMPS or penicillamine to remove mercury from the human body [13]. Clinical data suggest that chelating agent shows uncertain and limited efficacy in the treatment of mercury poisoning [14]. Thus, develop new strategies to remove mercury from biological systems is crucial to improve the currents treatments.

On the other hand, glutathione S-transferases are a superfamily of isoenzymes associated to drugs metabolism and lipids mediator synthesis [15]. The cytosolic family includes at least 16 subunits, mainly related to conjugate glutathione with lipophilic and electrophilic compounds [16], conferring an essential role in detoxification and maintenance of redox state [15]. This enzyme is as monomer unit in cytoplasm, which active form is generated by disulfide interaction between two monomeric units through thiols contained in each polypeptide [15]. Reports show that mercury decreases the catalytic properties of this thiol content protein by interaction with their sulfhydryl groups [17].

Recently, our research group has been showed that mercury ions are easily extracted from solution using a poly(3,4-ethylendioxythiophene) modified electrode (SS|PEDOT) based on the polymer n-doping process [18] , such as previously shown in extracting anions by polymer modified electrodes based on its p-doping/undoping process [19-21].

The extraction efficiency depends on the number of extraction cycles and the electrode area, showing that the methodology can be optimized to improve the metal extraction.

In this work, we studied the use of SS|PEDOT electrode in the extraction of mercury, with the aim to decrease the toxics effects of this metal on the catalytic properties of cytosolic S-transferases from rat liver.

2. EXPERIMENTAL

Adult male Sprague-Dawley rats (200-250 g) were used; all procedures were performed using approved protocols according to institutional ethical committee.

Cytosolic fraction was obtained according [22], briefly; rats were fasted for 15 h with water *ad libitum*, and sacrificed by decapitation. Livers were perfused *in situ* with 0.9% (w/v) NaCl, excised, and homogenized in a Dounce Wheaton B homogenizer. Homogenates were centrifuged at 9,000xg for 15 min in a Suprafuge 22 Heraeus centrifuge at 4 ºC. Supernatants were then centrifuged at 105,000xg for 60 min in a refrigerated Suprafuge 22 Heraeus centrifuge. Supernatants (cytosol fraction) were stored at -80 ºC until use. Protein determinations were performed according to Lowry *et al*. [23].

Cytosolic Glutathione S–transferase (cGST) activity was assayed by the method described by Habig *et al*. [24], using 10 μg of cytosolic protein, 1mmol·L^{-1} 1-chloro-2,4-dinitrobenzene as substrate, and 4mmol·L^{-1} GSH, in 100 mmol·L^{-1} sodium phosphate buffer, pH 7.4. The conjugate generation was continuously followed at 340 nm in an analytik jena SPECORD 40 spectrophotometer. All cGST activity assays were realized in conditions of linearity respect to incubation time and protein concentration.

All solutions were prepared with freshly deionized water in a Heal Force (Smart Series) water purification system. The measurements were performed in an anchor-type three-compartment cell at room temperature (20 °C) under high-purity argon atmosphere. An AISI 316 steel disc 0.07 cm^2 geometric area and a coil of Pt wire were used as working and counter electrode, respectively. Ag|AgCl in tetramethylammonium chloride, whose potential matches that of a saturated calomel electrode at room temperature, was used as reference electrode throughout the work [25].

EDOT electro-oxidation on steel (AISI 316) was optimized in order to obtain a PEDOT polymer deposit whose n-doping/undoping was suitable for the proposed process. EDOT (97%, Aldrich) electro-polymerization was accomplished on a VoltaLab PGZ 100 (Radiometer Analytical) potentiostat using cyclic voltammetry at various potential windows and scan rate 50 mV·s^{-1}, from solutions containing different concentrations of the monomer and diverse supporting electrolytes. The optima conditions for the achievement of the present work's goal were as follows: EDOT concentration 0.01 mol·L^{-1}, lithium perchlorate ($LiClO_4$, 99.99%, Aldrich) 0.1 mol·L^{-1} as supporting electrolyte in CH_3CN, and scan potential range between -1.0 and 1.5 V [18].

The potentiostatic method was next attempted. The best potential to prepare satisfactory modified electrodes of PEDOT on Stainless Steel (SS|PEDOT) was 1.4 V. In each case, to optimize the working conditions, SS|PEDOT stable electrochemical response was obtained in solution containing only supporting electrolyte. Also, the stable electrochemical response was recorded in aqueous solution of phosphate buffer at physiological pH (PBS) containing mercury(II) diacetate (all

high purity chemicals supplied by Aldrich), to verify the effect of Hg^{2+} cations in the PEDOT n-doping/undoping response.

Once the most suitable conditions to prepare SS|PEDOT to be utilized for Hg(II) extraction were stated, a BAS SP-2 potentiostat along with the abovementioned solutions, cells and electrodes, except the working electrode whose geometric area was increased to 6 cm^2, were employed to optimize the extraction method. A potential of 1.4 V was applied for 20 min. The prepared SS|PEDOT electrode was attempted for Hg(II) extraction employing extraction cycles, ExC. This approach consists in immersing the SS|PEDOT electrode in a Cell A, containing a 1 $mmol \cdot L^{-1}$ Hg(II) in PBS solution. Subsequently, the potential is stepped to -1.0 V (the polymer n-doping potential) for 5 min, after which the electrode is transferred to Cell B, containing only PBS, and potentiostatically disturbed at the n-undoping potential (0.3 V) for 5 min. This process (ExC), performed in succession, corresponds to the extraction approach proposed herein [18].

3. RESULTS AND DISCUSSION

Figure 1 shows the effect of Hg(II) on catalytic activity of cGST from rat liver. Cytosolic solution (10 μg cytosolic protein$\cdot mL^{-1}$) was incubated during 30 minutes with increasing concentrations of Hg(II) (to 300 $\mu mol \cdot L^{-1}$) before measuring enzymatic activity. The conjugate formation was followed spectroscopically at 340 nm. Hg(II) showed decreased the conjugate formation in a manner that is concentration-dependent, and this result is in agreement with previous reports [17]. Furthermore, the concentration of Hg(II) required to reduce by 50% the enzyme's catalytic activity (IC_{50}) was 33.07 ± 3$\mu mol \cdot L^{-1}$ (Fig. 1, inset). Following experiments were performed using 100 $\mu mol \cdot L^{-1}$ Hg(II), because a significant inhibitory effect on cGST activity was observed (Fig. 1, inset).

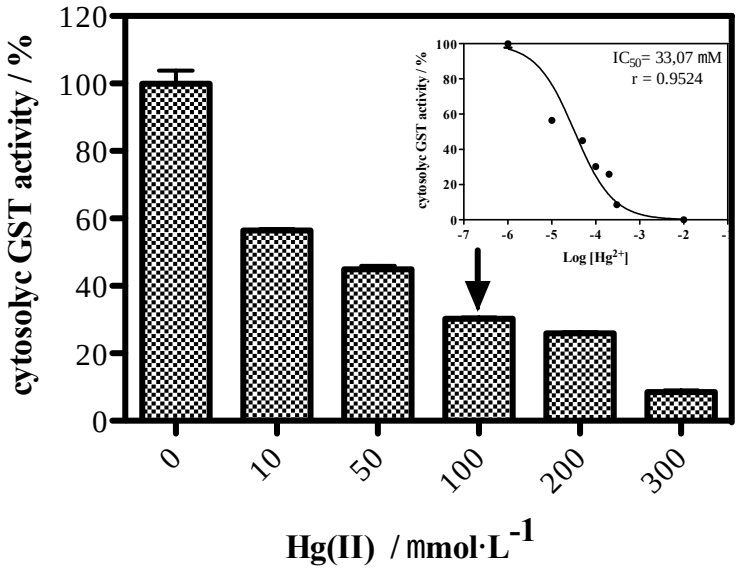

Figure 1. Effect of Hg(II) solutions on catalytic activity of cGST from rat liver.

In order to determine the effect of the extracting system on cGST activity, the effect of ExC on cGST activity in the absence of Hg(II) was assayed. Figure 2 shows that the catalytic activity decreased by less than 5% compared with the control, after 20 ExC.

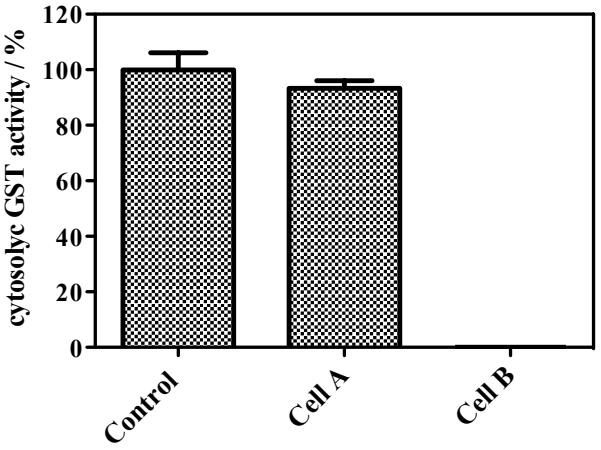

Figure 2. Effect of ExC on cGST activity in the absence of Hg(II).

This result suggest that the extraction process do not affect significantly the whole enzymatic activity, nevertheless, others parameters such as number of cycles of extraction or time of n-doping attending to optimization of the process, must be evaluated.

Figure 3 shows the recovery of the catalytic activity of cGST previously-inhibited by Hg(II). Incubation of the enzyme with mercury showed a decrease of its catalytic activity to 40% compared to control. When this solution (cell A) was submitted to 20 ExC, resulting enzyme activity was 83 % compared with the control value.

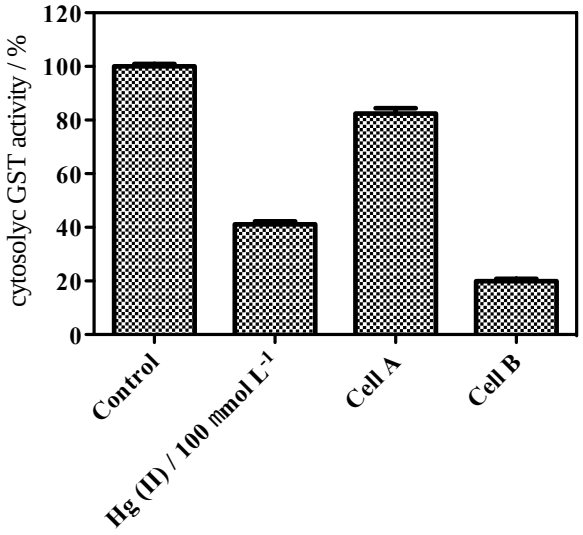

Figure 3. Recovery of catalytic activity of cGST previously-inhibited by Hg(II).

The later correlates with the observed value when enzyme was submitted to 20 ExC in the absence of Hg(II) (Figure 2, cell A). This result indicates that the modified electrode is not only capable of removing the mercury remains in the solution (which does not interact with the enzyme), but is also capable to reverse the inhibitory effect of this metal on the catalytic activity of the enzyme.

Surprisingly, when catalytic activity was measured in cell B, a value close to 20 % compared with the control was obtained, indicating that the procedure, in its entirety, completely recovers the enzyme activity. This result could mean that part of the enzyme remains bound to the metal, migrates bind to the electrode, into the cell B and when applying the n-undoping potential, the enzyme is released both from the electrode and the metal, recovering its catalytic ability.

Several reports shows that the inhibitory mechanism of Hg(II) on cGST proceeds through interaction between protein thiols and metal, and this interaction avoid the formation of catalytically active form of the enzyme (a dimmer generated by disulfide interaction between cysteines on each monomer). The latter imply an irreversible inhibition of the enzyme, given the high stability of sulfur-metal bind. However, Hg(II) may exert others inhibitory mechanisms on the enzyme, related to interactions with GSH or substrate binding domains, therefore, kinetics studies aimed to identify other competitive or allosteric inhibitory mechanisms of mercury must be performed.

In this paper we show the utility of PEDOT modified electrodes to reverse the inhibitory effect of mercury on catalytic activity of cGST from rat liver. This suggest that the procedure is able to remove Hg(II) from enzyme thiols, allowing the formation of the active form of the enzyme, or eventually from others sites able to bind Hg(II) into the enzyme. Moreover, we show that through the n-undoping process, Hg(II) and Hg(II)-mediated cGST releasing from the electrode was observed (Figure 3, cell B), and this phenomenon was not observed in the absence of Hg(II) (Figure 2, cell B). Moreover, our data suggest that the enzyme was free-Hg(II) released from the electrode to cell B, recovery its catalytic capability, and our results show that the whole extraction procedure was able to recover 100 % the enzymatic activity of cGST from rat liver.

Considering the low clinical efficacy of currents treatments aimed to remove mercury from the body, our results are extremely promising in the context of functional recovery of protein structures affected by mercury. Furthermore, since the procedure allows to remove Hg(II)-protein adducts toward the n-undoping cell (Figure 3, cell B), the use of this electrode would allow to remove only protein-interacting Hg(II) from biological systems affected by mercury, without altering the rest of the system (Figure 2, cell B). Actually, we try to determine the magnitude of this statement.

4. CONCLUSIONS

PEDOT do not affect the enzymatic activity of cGST from rat liver, even after 20 ExC. This result is significant considering a possible application of this methodology in biological systems affected by mercury.

Furthermore, it is demonstrated that the proposed method is able to reverse the enzyme inhibition mediated by mercury, opening the door for future procedures aimed not only at removing mercury from biological systems, but also recover the biological properties of the proteins affected by this or other metals, using this type of modified electrodes.

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