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Myoglobin modified electrodes as anchors for *d* metal cationic complexes

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Abstract

The capability of adsorption of different electroactive cationic Re(V)–amine complexes onto myoglobin-containing electrodes has been investigated. The goal of this work was the development of an Au/thiol/myo electrode and, after incubation of such ensemble in the presence of three different Re(V)–amine complexes, the evaluation of the extent of surface coverage by the complexes (as a way to evaluate the interaction complex-protein) using electrochemical techniques. Our results showed that a protein-containing electrode could therefore be used for the detection of the interaction of small electroactive cationic complexes and the biomolecule. The extent of the coverage of the myoglobin electrode by the complex depends on the number of free tails from the ligands and the total charge of the complex.

Keywords: SAM; Myoglobin; Modified electrodes; Protein–metal interaction

1. Introduction

The interest in the uses and applications of metal-based drugs and imaging agents continues to expand. The approach to these fields in a systematic manner includes the study of the drug interaction with proteins because protein-drug binding plays an important role in pharmacology and pharmacodynamics [1]. It is well known that metallic complexes are transported attached to peptides and proteins to the intracellular media [2]. The bonding between proteins and complexes is the result of covalent, ionic and/or van der Waals forces. The nature of such binding has a great influence on how complexes reach the target organ, and its extent determines the in vivo activity, the absorption, metabolism and excretion of the metallic therapeutic agent [2–7].

On the other hand, the interaction between proteins and metal complexes can be used to immobilise metallic compounds, that is, once the protein is attached to a surface, the macromolecule can be a moiety able to trap the complexes. In this way, it is possible to obtain a protein-complex ensemble fixed onto a particular surface.

From our previous work, the electrochemical behaviour of Re(V) complexes have been thoroughly studied, and it was found that all of them display an anodic wave at ca. 0.8 V vs. Ag/AgCl [8–10]. The interaction of these compounds with albumin was proved to take place in solution, being possible to detect the signal at 0.8 V under diffusion controlled reaction conditions [11]. As previously discussed, the studied cationic Re(V) complexes are able to interact with the protein either through electrostatic forces, or through short-range interactions (hydrogen bonding and/or van der Waals interactions via their free tails) [11]. These studies indicated that proteins could be used to anchor cationic Re(V) compounds, and based on the above-mentioned oxidation signal, the anchorage process can be demonstrated.

The aim of this work is to obtain a protein-modified electrode to anchor cationic complexes. The methodology followed in this work is based on protein immobilisation onto self-assembled monolayers of thiols. Once the protein is attached to the Au/thiol electrode, the macromolecule is able to interact with the Re(V) complex and, in this way, the complex remains adsorbed to the modified electrode.

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Many physical and chemical methods for immobilising proteins and enzymes on solid surfaces, such as physisorption on the bare metal electrode and entrapment within membranes or gels, have been widely used [12–19]. Nevertheless, they have some problems such as adsorption with random orientation, detachment of the protein and low durability of the matrix resulting in a worse performance. Thus, there is an increasing demand for assembly techniques to immobilise macromolecular biological species onto a previously modified electrode substrate [17]. The self-assembled monolayer on metal electrodes approach has been widely used as a new strategy for the immobilisation, orientation and molecular organisation of biomolecules at interfaces [12,18] and such chemically modified electrodes have proved to have good stability. The films are densely packed, pseudo crystalline, and strongly adhered to the metal surface; the physicochemical properties can be adapted and the thickness can be controlled at the angstrom level. In addition, electron transport occurs via the heterocyclic bases and aromatic side chains of these biopolymers [20].

Myoglobin was selected due to several advantages, namely a small size to allow a suitable electron transfer to allow the detection of the electroactive Re-complexes, and a well adsorption behaviour on thiolated surfaces, as reported [21,22]. This protein was also selected because many of its functional groups are negatively charged at physiological pH. For this reason the biomolecule will be able to capture and adsorb cationic specie such as the Re-complexes [12]. For protein immobilisation, compact layers of short-chain alkanothiol were used [16,23,24].

As a consequence of the interaction between the protein and the complex, the metallic compound would remain attached to the protein immobilised onto the electrode, and the redox signal of the complex could therefore be detected even when electrochemical measurements are performed in the absence of a complex containing solution.

We have selected three different cationic Re(V) complexes to test this hypothesis: $[ReO_2(amine)_2]I$ (amine = dien and tren; dien = *N*-(2-aminoethyl)-*1*,*2*-ethanodiamine and tren = *N*, *N*-*bis* ((2-aminoethyl)-*1*,*2*-ethanodiamine) and [ReO(dien-H)val]I (val = valine). The used complexes contain iodide coming from their synthetic procedures [25,26]. The choice is based in two facts. First, their electrochemical behaviour is well known [8,9]. Second, the complexes contain free tails with amine or carboxylic groups that are able to interact with other species such as amino acids, peptides or macromolecules [25,26]. Moreover, the complexes under study are cationic at neutral pH values, being $[ReO_2(dien)_2]^+$, $[ReO_2(Htren)(tren)]^{2+}$, and $[ReO(dien\text{-}H)val]^+$ the predominant species. These cationic complexes are also expected to approach and interact with myoglobin at neutral pH, because many functional groups in the protein are negatively charged (Isoelectric point = 7.0) [12]. Besides the Re(V) complexes, their iodide counter ion is electroactive [8,9] and, as a consequence, it is possible to detect both of them by electrochemical procedures. The advantage of employing a protein-modified electrode is to detect the interaction between protein and cationic complexes without the interference of electroactive anions.

2. Experimental part

2.1. Solutions

Two different ethanol thiol-containing solutions were employed, namely 1 mM 6-mercapto-1-hexanol (6 C thiol), or 1 mM 6 C thiol + 1 mM 3-mercapto-1-propanol (3 C thiol). As supporting electrolyte, aqueous 0.02 M $NaClO_4$ + 0.01 M NaCl (pH = 7.5) was employed and as the probe solution, aqueous 1 mM 1,1′-ferrocene dicarboxylic acid (FDCA) was used.

A 0.03 mM myoglobin (FLUKA, horse heart, min. 90%, 17.6 KDa mol^{-1}) aqueous solution was used to prepare the protein-containing electrode. Finally, for the interaction assessment experiments, Re(V) complexes aqueous solutions namely $[ReO_2(amine)_2]I$ (amine = dien and tren) and [ReO(dien-H)val]I (val = valine) in the concentration range 0.1–100 mM, were employed.

2.2. Electrochemical set up

A polycrystalline Au-pc disc (99.999%, 4 mm diameter, shrouded in Teflon for mounting it to a RDE, M636, PAR), either bare or modified, and a Pt wire, 0.5 cm^2 geometric area, were used as working and counter electrodes, respectively. All potentials in the text are referred to a Ag/AgCl/3 M KCl (0.207 V vs. SHE) reference electrode. Electrochemical measurements were carried out under Ar atmosphere in non-stirred solutions (by means of a computer controlled electrochemical interface, IM6, Zahner Elektrik).

In order to work with a reproducible surface, the Au electrode was mechanically polished with 0.3 μm and 0.05 μm alumina suspension at a pressure of 8 N, at 80 rpm. Then, it was hand polished with 0.05 μm alumina mixed with solid KCN. Finally, the electrode was mounted to the RDE and electrochemically polished in 0.1 M $HClO_4$ at 4000 rpm applying a triple potential pulse cycle for 10 min: first at $E_1 = 1.2$ V, $t = 0.1$ s to oxidise organic impurities; then at $E_2 = 0$ V, $t = 0.1$ s to reduce the formed Au oxide, and finally at $E_3 = -0.8$ V, $t = 0.1$ s.

2.3. Preparation and characterization of modified electrodes

For the assembly process, the Au electrode was immersed in the thiol-containing solution for 48 h, and thereafter thoroughly washed with ethanol; the obtained electrode is named Au/thiol. Afterwards, the Au/thiol electrode was immersed in the protein containing solution for 3 days to give the Au/thiol/myo electrode; when removed from the protein containing solution it was thoroughly washed with the supporting electrolyte. After each step of the procedure, the electrochemical properties of the modified electrodes were determined as described below, and in both cases, immersion times correspond to the time needed to achieve a stable voltammetric profile in the probe solution.

The electrochemical characteristics of the bare and modified Au electrodes were evaluated in the probe solution by cyclic voltammetry (CV) and electrochemical impedance spectroscopy measurements (EIS). Some EIS experiments were also performed

in supporting electrolyte without FDCA. In the CV experiments, the peak potential differences between anodic and cathodic current peaks, ΔE_p, which are related to the heterogeneous electron transfer rate through the interface [27], were measured. The EIS experiments were carried out under potentiostatic control in the −0.15 V to 0.20 V potential ranges stepping each 0.05 V, and in the frequency range from 100 mHz to 1 MHz.

In order to determine the adsorbed amount of myoglobin onto the SAM modified electrode, some experiments were performed using a CHI 440 Potentiostat/Galvanostat time-resolved Quartz Crystal Microbalance — QCM (reference crystal oscillation frequency = 8.000 MHz, gold electrodes with geometric area = 0.196 10^{-4} m^2).

2.4. Interaction studies

Immediately after immersion (t=0) in the Re(V)-containing solution, the voltammetric profile of the Au/thiol/myo modified electrode was recorded. After incubating the electrode in the complex solution for 1 day (t=24 h), the obtained Au/thiol/myo/Re electrode was thoroughly rinsed with the supporting electrolyte. Afterwards, the I-E profile, and the impedance behaviour at applied potentials varying between 0.0 V and 0.15 V in 0.05 V steps were recorded in the supporting electrolyte.

The time-stability of the Au/thiol/myo/Re electrode towards detachment of the Re(V) complex from the Au/thiol/myo was studied by storing the Re-containing electrode in supporting electrolyte for 1 or 2 days and the voltammetric profile and the impedance spectra of such electrode were subsequently recorded in the supporting electrolyte.

3. Results and discussion

3.1. Cyclic voltammetric measurements

In Fig. 1 the voltammetric profiles of the interfaces Au, Au/thiol and Au/thiol/myo in FDCA aqueous solution are shown.

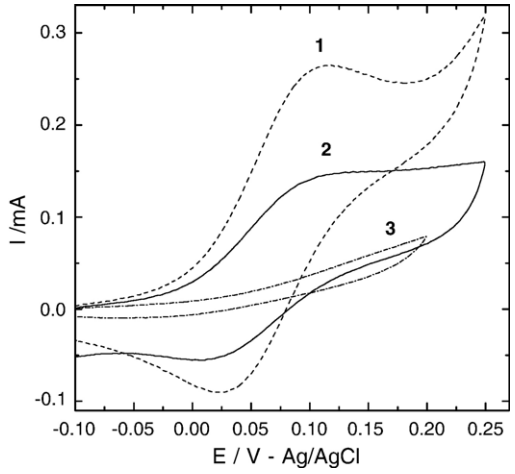


Fig. 1. Voltammetric profiles for different electrodes in 0.02 M $NaClO_4$ + 0.01 M NaCl containing 1 mM 1,1′-ferrocene dicarboxylic acid; Au-*pc* (line 1), Au/thiol (line 2) and Au/thiol/myo (line 3) (T=25 °C, pH=7.5, v=0.1 V s^{-1}).

For Au (line 1) the peak potential difference, ΔE, was 0.060 V, while for Au/thiol electrode the value of ΔE increases to 0.085 V. Bearing in mind the chain length of the employed thiols, it is highly probable that an electron tunnelling through the thiol layer will still allow the detection of the redox couples for the probe molecule [16,23,24]. However, for Au/thiol/myo electrode the FDCA redox peaks were hardly detected. These facts indicates that the electron transfer process is becoming more difficult as far as the Au surface is subsequently modified, specially after adsorption of myoglobin which acts as a more effective barrier (in comparison with the thiolated film) for the electron transfer process.

The FDCA undergoes electron transfer at an electrode by an outer-sphere mechanism, then, this electroactive soluble species needs to reach the interface electrolyte/electrode to exchange the electrons. For these reasons, the electron transfer ability for this species present in the solution is reduced when the thickness of the modified electrode is increased. Moreover, the voltammetric profiles were carried out a pH=7.5; at this pH functional groups in the myoglobin are mostly negative charged, as well as the ferrocene species. Thus, the arrival of the electroactive species to the interface is obstructed due to electrostatic repulsion.

The amount of adsorbed myoglobin onto the Au/thiol electrode was determined through QCM measurements using the Sauerbrey equation.

The Sauerbrey equation relates the measured change in the frequency, Δf, produced by the adsorption of a foreign substance with mass Δm (g):

$$\Delta f = -(2f_0^2/A\mu^{1/2}\rho^{1/2})\Delta m \quad (1)$$

where f_0 is the resonant frequency of the fundamental mode of the quartz crystal (8.0 MHz), A is the piezoelectrically active area of the gold disk coated over a thin chromium adhesion mediator film (0.196 10^{-4} m^2), μ are the shear modules of quartz (2.947 10^{10} kg m^{-1} s^{-2}) and ρ is the quartz density (2.648 10^3 kg m^{-3}) [30]. Hence:

$$\Delta f = -0.145\,10^8(\Delta m/A)m^2kg^{-1}s^{-1}$$

where the frequency change is expressed in Hz, the area in m^2 and the foreign mass in kg. Taking into account the electrode surface area (0.196 10^{-4} m^2), the absolute mass sensitivity was 0.74 10^{12} Hz kg^{-1}, or 1.35 10^{-12} kg Hz^{-1}.

Although the Sauerbrey equation is valid for ad-layers which are sufficiently thin and rigid, it was demonstrated that it is possible to apply this equation to QCM studies involving myoglobin adsorption [31].

For the 6C thiol containing modified electrode, a value of 1.64 10^{-7} mol m^{-2} was obtained (Fig. 2). Using the geometrically random value of 1.5 10^{-7} mol m^{-2} as the monolayer surface coverage [32] (assuming that no spreading of the protein occurs due to adsorption induced conformational changes), it can be concluded that the myoglobin adsorption amount is equivalent to one monolayer.

Finally, to evaluate the interaction of the composite electrode with cationic Re-complexes, the voltammetric profiles for the electrode were recorded at t=0 and a t=24 h. In both cases the anodic peak at 0.8 V, related to the oxidation of the Re(V) core

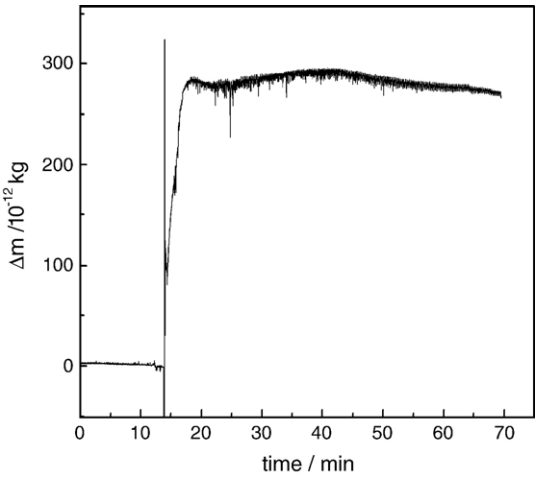


Fig. 2. QCM profile for myoglobin adsorption from a 0.03 mM aqueous solution onto the Au/6C-thiol electrode.

[8–11] was detected, and slightly shifted to more negative potentials after 24 h (Fig. 3). This fact is in line with our previous results [11], i.e., the potential of the Re(V)/Re(VI) couple shifts towards more negative potentials when the complex interacts with a protein. At $t=0$ it was also possible to observe a redox couple at ca. 0.5 V due to the electrochemical response of the counter ion I^- [8–10]. The anodic current peak related to the Re (V) oxidation followed a linear relationship with the potential scan rate (v), indicating that the Re complex is adsorbed onto the electrode [28,29].

Contrary to that observed for the FDCA, the approach of Re cationic complexes to the interface is favoured by the negative net charge of the myoglobin, allowing the clear detection of the redox couple Re(VI)/Re(V) even on the voltammetric profile for the myoglobin containing electrode. This could be explained because the metallic core involved in the redox process could find a path for the electron transfer, due to the adsorption of the complex onto the composite Au/thiol/myo electrode.

No desorption of the Re complex took place in the time course duration of CV or EIS experiments. Moreover, no changes in the voltammetric profile were observed after maintaining the electrode immersed for 1 and 2 days in the supporting electrolyte solution.

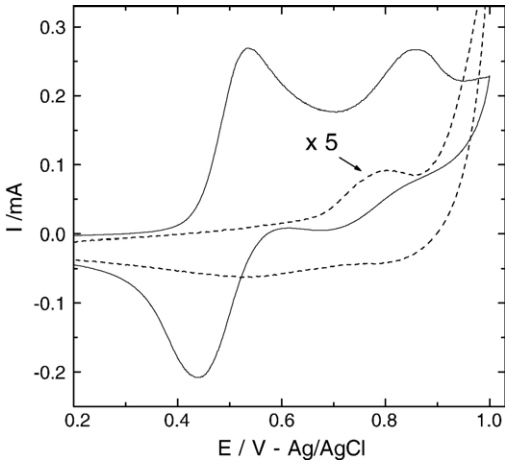


Fig. 3. Modified electrode of Au/thiol/myo in 1 mM $[ReO_2(tren)_2]I$ in 0.02 M $NaClO_4$+0.01 M NaCl at $t=0$ (solid line). After 24 h of soaking in the Re(V) complex solution (dashed line) ($T=25$ °C, pH=7.5, $v=0.050$ V s^{-1}).

The extent of the coverage of the Au/thiol/myo electrode after interaction with the complex was evaluated in two different ways:

I) by comparison of the integrated area under the peak located at ca. 0.8 V in the voltammetric profile of the electrode obtained with and without the Re compound in solution, i.e. for $t=0$ and $t=24$ h.
II) by comparing the slopes of the anodic current peak (I_p) vs. v graphs, for $t=0$ and $t=24$ h.

According to the relationship between I_p and v [27]:

$$I_p = kvA\Gamma^* \quad (2)$$

in the presence of the Re-complex solution, where k is a constant, v is the scan rate, and A is the geometric area of the electrode. The value of Γ^* represents the surface amount of the adsorbed electroactive complex obtained when the Au/thiol/myo electrode is immersed into concentrated solutions (0.1– 100 mM) of the complex to give a total constant surface concentration ($t=0$). The Γ^* value resulted independent of the Re-solution concentration, indicating that the current peak is only due to the adsorbed species without any diffusion contribution. After 24 h in the Re-containing solution, only a fraction of the electroactive complex remains attached to Au/thiol/myo electrode; this amount is labelled Γ^w. At $t=0$, the Au/thiol/myo electrode is exposed to a Re containing solution and, in this situation, the process is very fast and the highest amount of the complex (Γ^*) is adsorbed onto the electrode surface; that is, the process is under kinetic control. After 24 h of immersion time in the complex containing solution, the amount of Re-compound up taken by the immobilised protein corresponds to the equilibrium value (Γ^w) resulting from the thermodynamic control. For this reason, Γ^w reflects the efficiency of the anchoring process.

The ratio (Γ^w/Γ^*) represents the fraction of electroactive Re(V)-complex effectively attached onto the surface. These values, expressed as percentages, are resumed in Table 1.

It can be concluded that:

— the Re–tren complex is the only one with four tails per mol containing a-NH_2 terminal group each [11]. It is then a compound with the ability to use these tails to establish interactions with the immobilised protein. As a

Table 1
Calculated percent of surface coverage with adsorbed myoglobin for the studied complexes

Complex	Surface coverage %	Absolute error
$[ReO_2(tren)_2]^+$	15	(±) 1
$[ReO_2(dien)_2]^+$	6	(±) 1
$[ReO(dien\text{-}H)(val)]^+$	3	(±) 0.5

Results are based on a minimum of five independent experiments.

consequence, it showed the highest percentage of surface coverage.

— the Re–dien complex has only two tails per mol also with a-NH_2 terminal group each [11]. Therefore, its ability to interact with myoglobin is lower than in the case of the Re–tren, and the amount of remaining complex attached to the protein is lower.

— finally, the Re–dienval complex contains a carboxylic group that would behave as a free tail [26]. However, this group is weakly bounded to the $[ReO]^{3+}$ core, diminishing its capability to interact with proteins. It showed the lowest surface coverage.

As summary, the studied complexes can use their tails to interact with the protein through hydrogen bonding and van der Waals interactions. Because they are positive charged, it is also possible for them to interact through coulombic forces. The importance of the coulombic attraction was evaluated by changes in the ionic strength, that is, using a more concentrated supporting electrolyte. In particular, some experiments were carried out for 1 mM solutions of Re–tren and Re–dien compounds with 0.1 M $NaClO_4$ as supporting electrolyte. In both cases, the calculated surface coverage was lower than 2%, showing that the electrostatic attraction between the cationic compound and the immobilized protein is affected for a higher concentration of ions in solution. In order to prove that Re(V) cations (and not I^- counter ions) are able to interact only with the protein-modified electrode, the following blank experiments were performed:

— a Au/thiol electrode was immersed for 24 h in a 1 mM $[ReO_2(dien)_2]I$ solution

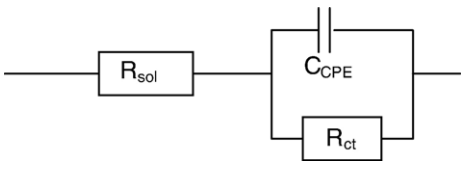
— a Au/thiol electrode was immersed for 24 h in a 1 mM KI solution

— an Au/thiol/myo electrode was immersed for 24 h in a 1 mM KI solution

In all cases, no signals coming from the electroactive species were detected.

3.2. Electrochemical impedance spectroscopy

For EIS measurements performed in the presence of FDCA, a three elements equivalent circuit was used to fit the experimental data. It consists of a capacitance (constant phase element, C_{CPE}) for the interfacial double layer, a charge transfer resistance element (Rct) and a resistive element associated to the ohmic resistance of the solution (Rsol) (Scheme 1).



Scheme 1. Equivalent elements circuit for fitting of EIS measurements data obtained in the presence of FDCA; C_{CPE} is the constant phase element for the interfacial capacitance, Rct is the charge transfer resistance and Rsol is the resistive element associated to the ohmic resistance of the solution.

Table 2
Results obtained by EIS measurements in the presence or the absence of 1 mM 1,1'-ferrocenedicarboxylic acid. Applied potential=0.10 V vs. Ag/AgCl

Electrode	R_{CT} (MΩ)	C_{CPE} (nF)
Au+FDCA	0.93	2100
Au/T+FDCA	1.05	1030
Au/T/M+FDCA	1.5	900
Electrode	**R (MΩ)**	**C_{CPE} (nF)**
Au	1.7	2900
Au/T	2.7	930
Au/T/M	1.8	850
Au/T/M/Re	1.3	580

For the whole assembly process to get the Au/thiol/myo/Re electrode, no great differences were found when using the six-chain carbon or the mixture three/six chain carbon alkyl thiols by EIS or CV measurements. Nevertheless, some solubility of the 3C-thiol in water was detected by EIS. In addition, QCM

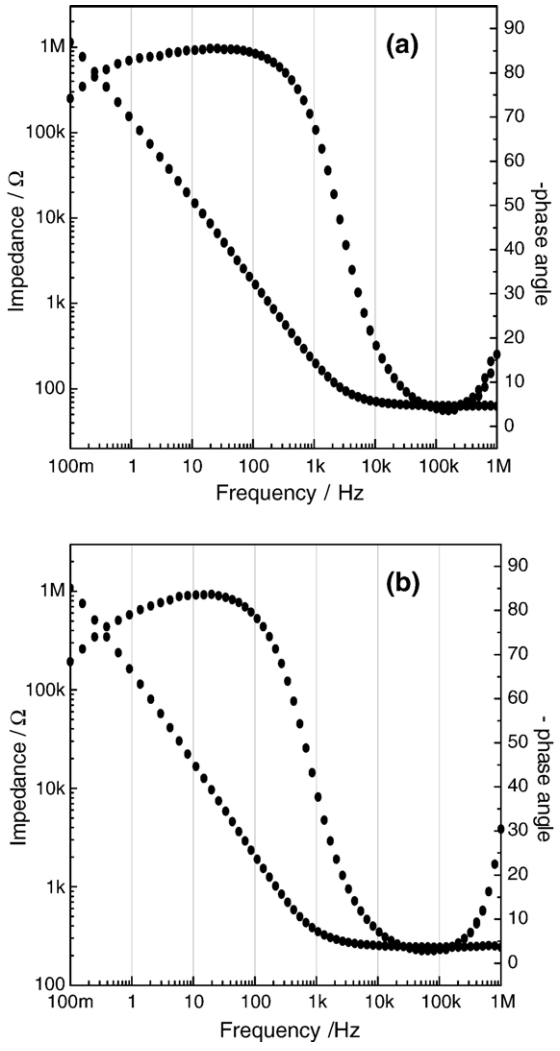


Fig. 4. Impedance and phase angle Bode plots for: (a) the Au/thiol electrode in 0.02 M $NaClO_4$+0.01 M NaCl containing 1 mM 1,1′-ferrocene dicarboxylic acid, and (b) the Au/thiol/myo modified electrode in 0.02 M $NaClO_4$+0.01 M NaCl, after 24 h soaking in a 1 mM solution of the $[ReO_2(tren)_2]^+$(E= 0.10 V vs. Ag/AgCl. T=25 °C, pH=7.5).

measurements showed a stable 6C-thiol film when working with aqueous solutions; on the contrary, 3C-thiol films were unstable due to its solubility in water. Therefore, when using the mixture, the adsorbed amount of 6C-thiol on the film would be higher than the amount of 3C-thiol.

Changes in the resistance of the element associated to the interfacial charge transfer (Rct) and also changes in the capacitance of the constant phase element were detected after incubation of the Au electrode in the thiol-containing solution (Table 2)(Fig. 4). These changes are related to the chemisorption of thiol to the gold surface; the wider the thickness of the modified electrode, the harder the electronic transference through the thiolated film; consequently the charge transfer resistance increases. When the results for the Au/thiol electrode measured in the supporting electrolyte are compared with the results obtained using FDCA, there is a significant decrease for Rct in the EIS measurements obtained using the redox probe molecule. This could be attributed to the presence of a redox active molecule adsorbed to the gold surface.

After incubation of the Au/thiol electrode in the myoglobin solution, once again the Rct and the C_{CPE} change, indicating that the adsorption process takes place. In particular, the C_{CPE} is lower in the presence of the protein; the protein repels water from the interface electrolyte/SAM and therefore the dielectric constant of a myoglobin film is lower than that of an aqueous electrolyte film of the same thickness.

Finally, after incubation of the Au/thiol/myo electrode in the presence of the Re–tren complex the Rct and the C_{CPE} changes markedly (Fig. 4). It is also important to remark that after keeping the Au/thiol/myo/Re electrode in supporting electrolyte for 1 day, the Rct and C_{CPE} values remain unchanged, and after 2 days only a slightly change was observed. These results showed that the complex is strongly attached to the protein and the situation remains almost unchanged when the electrode is immersed in an electrolyte-containing solution.

4. Conclusions

- A composite Au/thiol/myo electrode is capable to adsorb Re(V) complexes. The Re(V) compound is able to interact with the immobilised protein in a degree related to the presence of polar groups belonging to the ligands of the complex. Moreover, the amount of immobilised electroactive complex is affected by the ionic strength.
- The thiol/myo/Re-assembly sequence on Au can be established by both voltammetry and impedance spectra measurements, and it is also possible to demonstrate that a stable Au/thiol/myo/Re electrode can be obtained.
- By cyclic voltammetry, the detection of the presence of Re(V) complexes bounded to a protein-modified electrode is based on the electroactive behaviour of the metallic core, i.e. an anodic wave at ca. 0.8 V associated with the oxidation to Re(VI) maintaining the coordination sphere of the compound unchanged.
- Summarising, the electrode modifications proposed in this work have been successfully used to anchor and detect Re(V) cationic complexes without the interference of iodide electroactive counter anions. Such a protein-containing electrode can find application in selective separations of species from a solution. Taking into account the isoelectric point of the protein, the charge of the macromolecule can be regulated and, in this way, the capability to capture in a selective form negative or positive charged compounds.

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