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Suitability of Copper Based Electrodes for Assessing the Interaction Between Ru(III)-Hexaammine and Myoglobin

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The goal of this work was the evaluation of the use of copper as suitable substrate for assessing the trapping of a small metallic compound by immobilized myoglobin (myo). Our proposal was to demonstrate that disposable devices based in the use of this metal would be as interesting as more widely used gold ones and more suitable since the economical point of view, despite the well-known problem of copper dissolution and oxidation. For such reason copper electrodes covered with 2-thiobarbituric acid (TBA) were tested as a platform to obtain myo modified electrodes with aptitude to trap a Ru complex present in a solution. The assembly process was followed using cyclic voltammetry, electrochemical impedance spectroscopy and contact angle measurements. Our results were consistent with the obtaining of a stable electrode of copper covered with a protein adsorbed onto TBA with reproducible behavior, pointing that copper could be a suitable material to develop electrochemical devices for biosensing. This stable Cu/TBA/myo electrode had enough sensitivity to detect the capture of a compound with low molecular weight and with low affinity for the protein, and, when immobilizing other biomolecules, it would sense the trapping of their specific target.

Keywords: Metal-protein interaction, impedance, cyclic voltammetry, contact angle, copper electrodes

1. INTRODUCTION

Biosensors have been under development for many years and within them, electrochemical sensors have achieved widespread commercial success [1-5]. They are of particular interest because of their low detection limits (within the range 10^{-9} to 10^{-6} M), small volume samples, easy and fast signal transduction, fast analyses, inexpensive instrumentation and simple operation protocols.

Electrochemical sensors for the measurement of analytes are of interest in environmental, clinical and food chemistry.

Most of them are based on the high selectivity of biological molecules for the recognition, and they are mainly based on the detection of current or potential changes resulting from interactions occurring at the interface.

Among electrochemical methods, impedance based measurements are of particular interest in comparison with amperometric ones because the need for electroactive species is overcome [6-12]. Measurements are based on the application of periodic small amplitude AC signals over a wide frequency range, and the electrical response represents changes at the electrode-solution interface. EIS is a powerful technique and its use and applications have increased very fast, including the evaluation of devices for biosensing [13-15].

In particular, EIS and other electrochemical techniques based in the use of protein modified electrodes are used with great variability of purposes. As an example, myoglobin immobilized onto SAMs modified electrodes on gold was reported to trap selectively cationic metal compounds [16]. Moreover, the use of self-assembled monolayers (SAMs) is widely reported with the aim to prepare protein containing electrodes due to the characteristics of such thiolated films, as well defined and organized surface, tight packing and stability [17-23].

But most reported works based in the use of SAMs modified electrodes are ascribed to the use of gold and not to the use of copper as substrate; formation of SAMs on Cu are mainly mentioned in the literature as inhibidors or protective films in corrosion studies [24-29].

In this work, we propose the use of copper as substrate to immobilize myoglobin onto SAMs electrodes as a way to obtain a device that could be suitable for biosensing. The evaluation of such ensemble was performed by using EIS in combination with cyclic voltammetry and contact angle measurements. The selected thiol was 2-thiobarbituric acid, which exceptional properties were reported by our group [30, 31], namely, a short immersion time to obtain a stable coverage (ca. 30 minutes), a high surface coverage, and excellent electron transfer properties, including good capability towards protein binding.

Copper was selected because it has a low cost (price is around 3 times more expensive for a gold wire or rod with the same characteristics of a Cu one), and, as a consequence, it would be more interesting than gold to develop electrochemical disposable devices. Moreover, and as mentioned above, formation of SAMs on this metal is extensively reported and it has also good electron transfer property. However, copper as supporting material to prepare modified electrodes has some complexities, arising from the fact that its dissolution starts at low potential values (ca. 0.3 V vs. RHE) [32, 33] and shows a great tendency towards oxidation [29, 34].

The protein was chosen because it has some interesting characteristics to perform this study. Myoglobin is a small sized compound and, as a consequence, it would allow a suitable electron transfer when attached to an electrode surface [16, 35]. For this reason, once the protein is adsorbed to a modified SAMs electrode, the electrochemical detection of the capture of small molecular weight metal species present in a solution would be accurate with high sensitivity. In addition, myoglobin shows a well adsorption behavior on thiolated surfaces [36, 37]. Additionally, this biomolecule was preferred because many of its functional groups are negatively charged at the working pH of 7.5 and,

as a consequence, it would be able to capture and adsorb cationic species such as the selected Ru-complex.

As mentioned, the ability of the Cu/TBA/myo electrode to trap the cationic compound $[Ru(NH_3)_6]^{3+}$ was evaluated. This compound was chosen as a model because it involves simple outer-sphere electron transfer on most electrodes [30] with electrode kinetics that is relatively insensitive to the surface microstructure, surface oxides, adsorbed monolayers, and electrolyte composition. Moreover, the redox potential of this compound is located within the potential values of the stability window for the adsorbed monolayer of TBA (ca. -0.7 V to +0.5 V vs. Ag/AgCl, see Figure 1), a very important point taking into account the electrochemical methods used in this work to evaluate the modified electrodes.

It is important to remark that such an interesting electroactive cationic compound would be very attractive to evaluate the aptitude of the protein containing copper electrode to trap soluble species. As a consequence, could be used as a model for other systems involving the interaction between similar modified electrodes with other soluble targets. This can be stated keeping in mind that the interaction between the Ru compound with the myoglobine is unspecific (i.e, there are not specific regions or binding sites at the protein available for such complex) and takes place involving low energy bonds as van der Waals forces [16, 38]. Therefore, if the Cu/TBA/myoglobin electrode is suitable to prove the capture of the Ru compound, would be more than appropriate to detect the interaction between two molecules that involves higher energy and affinity binding.

2. EXPERIMENTAL

2.1. *Solutions*

2 mM solutions of 2 thiobarbituric acid were prepared with Millipore-MilliQ water.

As supporting electrolyte, aqueous 0.1 M $NaClO_4$ pH = 7.5 (adjusted with 0.1 M NaOH) was employed, and aqueous 1 mM hexaammine ruthenium(III) chloride (SIGMA, 98%, 309.6) was used as the probe molecule.

A 0.03 mM myoglobin (FLUKA, horse heart, min. 90%, 17.6 KDa mol^{-1}) of the protein in phosphate buffer 7.5, 5 mM, was used to prepare the protein-containing electrode.

2.2. *Electrochemical set up*

A polycrystalline Cu-*pc* rod (Goodfellow, 99.99 + %, 0.48 cm diameter, 0.18 cm^2 geometric area, mounted into a plastic eppendorf tip), 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). Cyclic voltammetry profiles were obtained at five different potential scan rates within the range 0.01 $Vs^{-1} \leq \nu \leq 0.05$ Vs^{-1}.

In order to work with a reproducible surface, prior to use copper electrodes were cleaned with piranha (H_2O_2/H_2SO_4) solution and after that were hand polished with 0.3 μm and 0.05 μm alumina suspension. All experiments were performed under Ar atmosphere.

2.3. *Contact angle measurements*

Static contact angle measurements were carried out using homemade equipment [39]. A clean alumina-polished, N_2 dried copper disk, 0.48 cm in diameter (Goodfellow, 99.99+%), was placed in a closed acrylic chamber under controlled humidity (100% RH) and back-lighted with a high-intensity white LED. A 1 μL water drop was injected through a septum-covered hole in the chamber cover on the studied surface and imaged with a high-definition web camera. The image was analyzed with free ImageJ software (National Institutes of Health), which has an implemented module for contact angle measurements. All measurements were replicated five times.

Same procedure was followed in case of modified copper disks that is, after adding TBA to get a Cu/TBA disk, and after keeping such disk in contact with a myoglobin containing solution in order to obtain a Cu/TBA/myo electrode. In those cases, measurements were also replicated five times.

2.4. *Preparation and characterization of modified electrodes*

For the assembly process, the Cu electrode was immersed in the 2 mM thiol-containing aqueous solution for 30-40 minutes, and thereafter thoroughly washed and sonicated in water; the obtained electrode was named Cu/TBA.

Afterwards, the Cu/TBA electrode was immersed in the protein containing buffered solution for 20 h to give the Cu/TBA/myo electrode at room temperature; when removed from the protein containing solution it was thoroughly washed with buffer.

For each step of the ensemble procedure, immersion times corresponded to the time needed to achieve a stable voltammetric profile in the probe solution.

The electrochemical characteristics of the bare and modified Cu electrodes were evaluated in the probe solution by cyclic voltammetry (CV), electrochemical impedance spectroscopy measurements (EIS) and contact angle measurements (see section 2.3). Some EIS experiments were also performed in supporting electrolyte without the Ru compound. In the CV experiments, the peak potential differences between anodic and cathodic current peaks, ΔEp, which are related to the heterogeneous electron transfer rate through the interface [40], were measured. The EIS experiments were carried out under potentiostatic control in the -0.45 to 0.0 V vs. Ag/AgCl potential ranges stepping each 0.15 V, and in the frequency range from 100 mHz to 1 MHz.

2.5. Interaction studies

Immediately after immersion (t=0) in the Ru containing solution, the voltammetric profile of the Cu/TBA/myo modified electrode was recorded, and in order to assess the capture of the cationic

complex by the immobilized protein, the Cu/TBA/myo electrode was kept in a 1 mM $[Ru(NH_3)_6]^{3+}$ solution in buffer, during 20 hours. After such time, the obtained Cu/TBA/myo/Ru electrode was thoroughly rinsed with the supporting electrolyte, and the I-E profile and the impedance behavior were recorded in such solution. Perchlorate was chosen (considering that phosphate is electroactive and affect protein adsorption [41]) because our previous results involving the interaction of metal complexes and proteins showed that the use of $NaClO_4$ instead of phosphate buffer did not affect the binding between both species [16, 38].

3. RESULTS AND DISCUSSION

3.1. *CV measurements*

Prior to use, and after performing the cleaning procedure described above, voltammetric profiles of naked Cu electrodes were recorded in the supporting electrolyte and also in 0.5 M KOH solution (as shown in Figure 1).

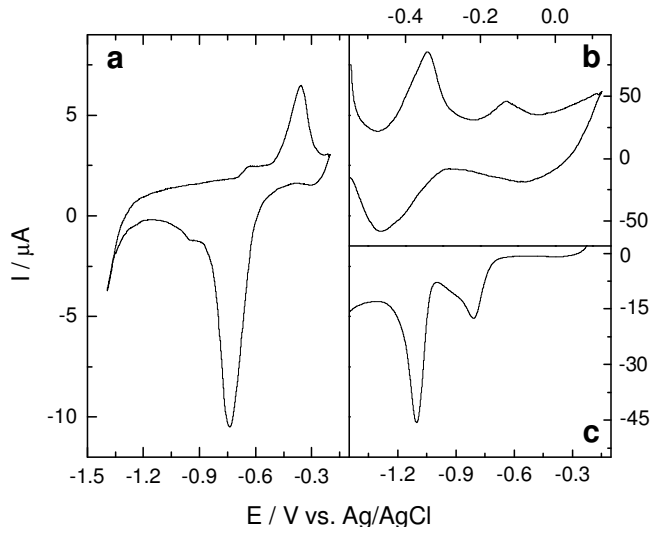


Figure 1. a) Voltammetric profile for naked copper in 0.5 M KOH solution; b) Voltammetric profile for naked copper in 0.1 M $NaClO_4$; c) Desorption profile for the Cu/TBA electrode in 0.1 M $NaClO_4$ solution, after performing a potential scan from $E_{initial}$ = -0.20 V vs. Ag/AgCl to E_{final} = -1.5 V vs. the reference. In all cases v = 0.050 Vs^{-1}.

The presence of similar profiles dominated by the copper dissolution and deposition intensity current peaks was always the desired result.

After corroboration of the surface cleanness, the modification process was carried out according to the steps detailed at Experimental. To evaluate the modification process CV measurements were performed in the presence of the Ru compound, and detected changes were clearly

associated to the assembly procedure. Showed results came from values obtained from completed modification processes (from naked to myoglobin modified ones) carried out for six different electrodes. At this point it is important to clarify that profiles of modified electrodes after each step were identically; otherwise, they were cleaned and the assembly procedure started once again.

For the six electrodes, some features in common were observed.

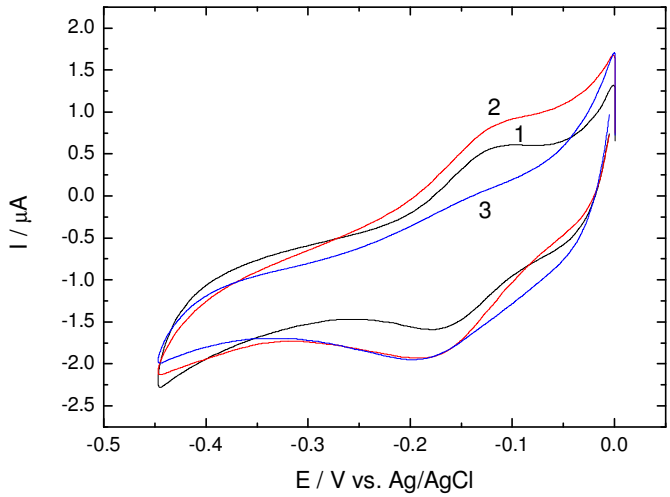


Figure 2. Voltammetric profile of the electrode in a 1 mM $[Ru(NH_3)_6]^{3+}$ in $NaClO_4$ 0.1 M, v = 0.05 Vs^{-1}. (a) For a naked copper electrode (line 1), (b) a modified Cu/TBA electrode (line 2) and (c) a modified Cu/TBA/myoglobin electrode (line 3).

Figure 2 shows the stable voltammetric profiles after multiple cycling for the electrodes in 1 mM Ru-solutions in the supporting electrolyte. After formation of the TBA layer on Cu, the main effect was the growth of the capacity of the interfacial double layer. On the following step, when the myoglobin electrode was obtained, intensity current peaks associated to the Ru(III)/Ru(II) couple decreased and the peak potential difference increased.

Figure 3 shows a comparison between the behavior of the Cu/TBA/myo electrode before (recorded in a recently prepared 1 mM metal complex solution in the supporting electrolyte) and after keeping this device during 20 h in the Ru-hexaammine solution (recorded only in supporting electrolyte). For this step, the Ru-complex capture by the myoglobin electrode, the CV profile was recorded only in supporting electrolyte to assess the presence or not of the Ru(III)/Ru(II) couple: the presence of the peak potentials ascribed to this couple, but with lower intensity than in case of the profile for the same electrode in the 1 mM Ru solution corroborated the protein-complex binding.

It is important to remark that for CV profiles obtained for the Cu/TBA/myo electrode the intensity of the current contributions coming from the Ru(III)/Ru(II) couple was very low and contributions were not well defined, especially for those registered in supporting electrolyte and after keeping the mentioned electrode during 20 h in the Ru-containing solution.

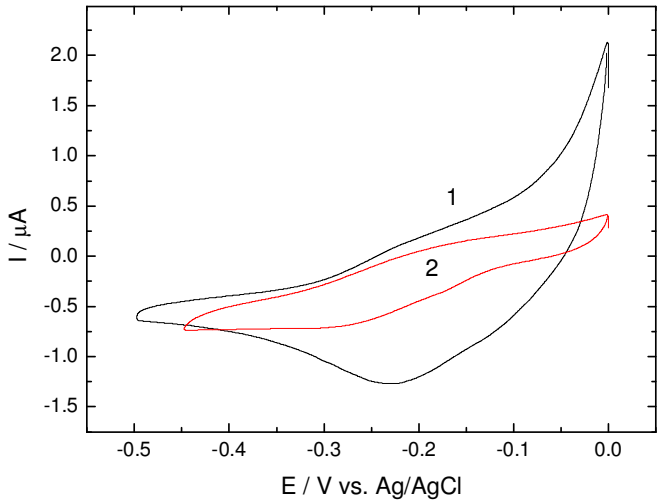


Figure 3. Voltammetric profile of a modified Cu/TBA/myoglobin electrode in a 1 mM $[Ru(NH_3)_6]^{3+}$ in $NaClO_4$ 0.1 M, v = 0.03 Vs^{-1} (line 1) compared with the Cu/TBA/myo/Ru modified electrode recorded in $NaClO_4$ 0.1 M, after incubation of Cu/TBA/myo during 20 hours in the hexaammine ruthenium(III) solution (line 2).

For this reason, and in order to confirm if the presence of the redox contributions related to the couple is due to the presence of the adsorbed Ru compound onto the myoglobin containing electrode, profiles for Cu/TBA and Cu/TBA/myo were also recorded in supporting electrolyte, as shown in Figure 4.

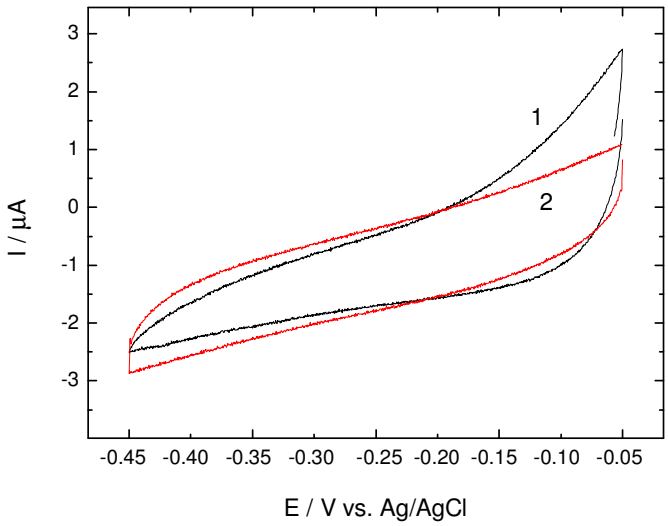


Figure 4. Voltammetric profile of the electrode in $NaClO_4$ 0.1 M, v = 0.05 Vs^{-1}. (a) Modified Cu/TBA electrode (line 1) and (b) a modified Cu/TBA/myoglobin electrode (line 2).

Additionally, and after analyzing the voltammetric profiles for the Ru containing electrode in the supporting electrolyte, obtained at five different potential scan rates in the range 0.01 $Vs^{-1} \leq v \leq$ 0.05 Vs^{-1}, some other data also support the capture of the complex by the protein modified electrode. That differed from the mixed behavior that is diffusion and adsoption, detected for the myoglobin containing electrode in the presence of the Ru couple.

For measurements carried out using the Cu/TBA/myo electrode immersed in the 1 mM Ru solution, the anodic and the cathodic intensity current peaks adjust linearly with the square root of the potential scan rate *v*, in accordance with diffusion controlled behavior, and in some cases were observed an Ip_a and Ip_c vs. *v* linear adjust, showing the adsorption of the electroactive Ru onto the surface of the myoglobin modified electrode. On the contrary, after keeping the electrode during 20 h in the Ru containing solution, the adsorption of the Ru complex was verified by the lineal adjust between Ip_a and Ip_c and *v*, when the profiles of the Cu/TBA/myo/Ru electrode were recorded in supporting electrolyte (see examples at Figure 5).

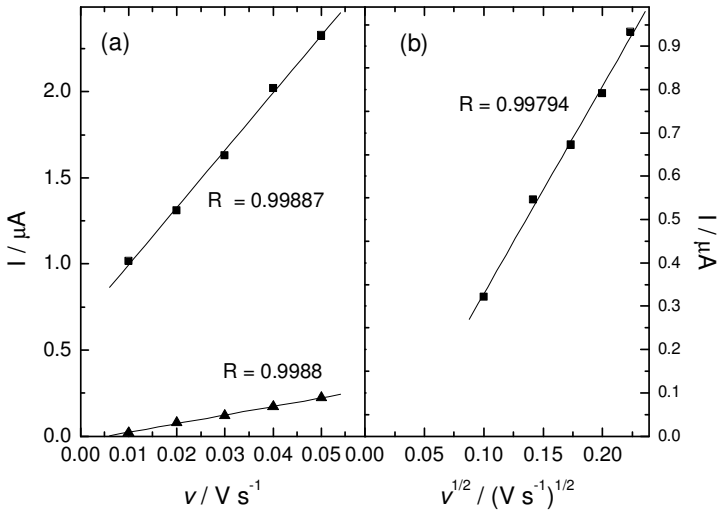


Figure 5. Linear relationship between the cathodic current peak at ca. -0.25 V and (a) potential scan rate for the Cu/TBA/myo electrode in 1 mM Ru-hexaammine solution (■) and Cu/TBA/myo/Ru in the supporting electrolyte (▲), (b) square root of the *v* for the Cu/TBA/myo electrode in 1 mM Ru-hexaammine solution, using another copper electrode for the evaluation.

Two other facts supported our assumption of the binding between the Ru compound to the immobilized myoglobin: a change in the values of the formal potential E'^0 and the peak potential difference ($\Delta Ep = Ep_a - Ep_c$), from E'^0 = -0.14 V and ΔEp = 0.07 V (Cu/TBA/myo recorded in 1 mM Ru solution) to E'^0 = -0.15 V and ΔEp = 0.09 V (Cu/TBA/myo/Ru recorded in the supporting electrolyte). Also, it was observed that the potential peak for the cathodic contribution varied linearly with ln *v*, and the slope changed from 0.015 V dec^{-1} (Cu/TBA/myo electrode) to 0.030 V dec^{-1}

(Cu/TBA/myo/Ru assembly), showing an increase in the difficulty to transfer the electron when the Ru compound is adsorbed to the surface.

Something important to remark is the stability of the union between the Ru compound and the protein, as deduced from the fact that the amount of the adsorbed cationic complex onto the myoglobin modified electrode did not change significantly with the time when recording the CV profiles in the supporting electrolyte, and considering the relationship between Ip and *v* [40]:

$$Ip = k\, v\, A\, \Gamma^*$$

where k is a constant, A is the geometric area of the electrode, and the value of Γ^* represents the surface amount of the adsorbed electroactive complex onto the surface of the Cu/TBA/myo electrode.

Finally, and in order to confirm the ability of the protein containing electrode to trap selectively only the Ru-cationic complex, some blank experiments were performed:

- a Cu/TBA electrode was immersed for 20 h in a 1 mM $[Ru(NH_3)_6]^{3+}$ solution

- a Cu/TBA/myo electrode was immersed for 20 h in a 1 mM KCl solution.

In all cases, no signals coming from the electroactive species were detected, and voltammetric profiles resembled the original ones shown at Figure 2.

3.2. *EIS measurements*

Measured data using this technique were analyzed using an equivalent circuit in agreement with the Bode and Nyquist representation of such data. Many obtained Bode diagrams showed the presence of two maxima raised at the medium frequency range (0.1 mHz to 100 Hz) pointing to the presence of at least two CPE – R in parallel combination, as could be expected if two zones with different behavior are present at the same electrode surface. The Bode representation of the data for the Cu/TBA electrode in a 1 mM $[Ru(NH_3)_6]^{3+}$ solution shown in Figure 6, is a good example of this observation.

Obtained data were then consistent with the use of an equivalent circuit composed of five elements (Tables 1 and 2), where R5 denoted the behavior of the solution, and R1-CPE2 and R3-CPE4 represented the behavior of these two different zones. For the naked metal, one zone behaved as naked metallic copper (represented by R1 and CPE2), and as a consequence could dissolve to give place to copper ion, and another one behaved as copper oxide (R3 and CPE4). The same circuit was used for the following steps of the modification process, and results were also in accordance with the existence of two different zones: one related to the modified electrode itself, and the other showed “holes” or zones where the copper remained uncover or even oxidized, but without showing the presence of the thiolated or the protein film (see scheme 1.).

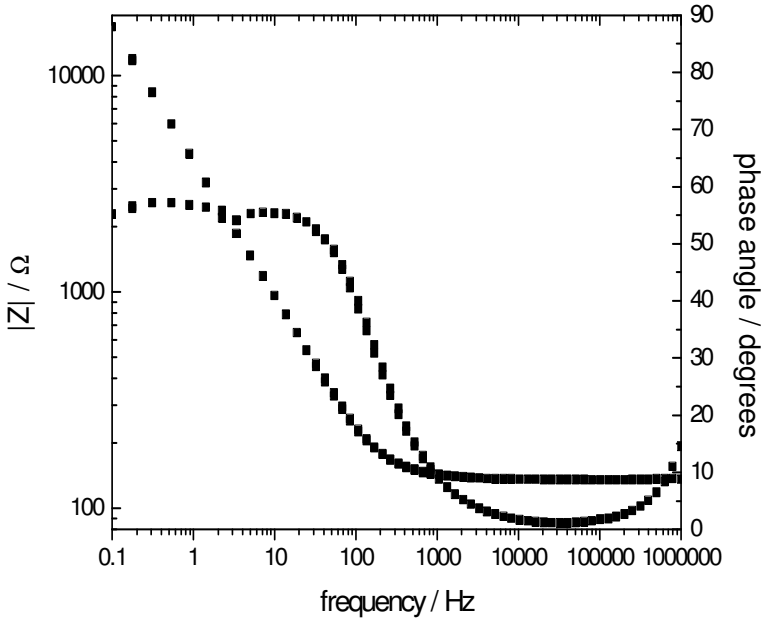
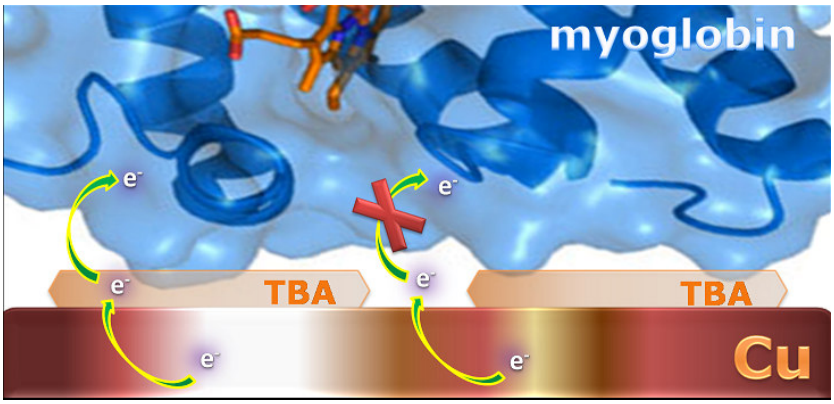


Figure 6. Bode representation for the obtained data measured for Cu/TBA electrode at E =-0.30 V vs. Ag/AgCl, in a 1 mM $[Ru(NH_3)_6]^{3+}$ solution in the supporting electrolyte.



Scheme 1. The diagram shows a representation of the desired ensemble. In particular the existence of two zones with different electrochemical behavior is symbolized: one where the Cu is covered with a TBA layer and furthermore with myoglobin, and other where holes or defects remained, and, as a consequence, charge transfer becomes more difficult.

Showed results came from values obtained from six different electrodes, and as can be deduced from Tables 1 and 2, changes in the calculated values of the elements of the equivalent circuit used to fit the experimental data were in accordance with the development of the modified electrode, from the naked metal to the Cu/TBA/myo one. The last step, the trapping of the Ru complex by the myoglobin containing electrode, was also detected by EIS measurements.

Figure 7 shows the Nyquist diagram obtained for the electrodes following the assembly process, measured in the supporting electrolyte.

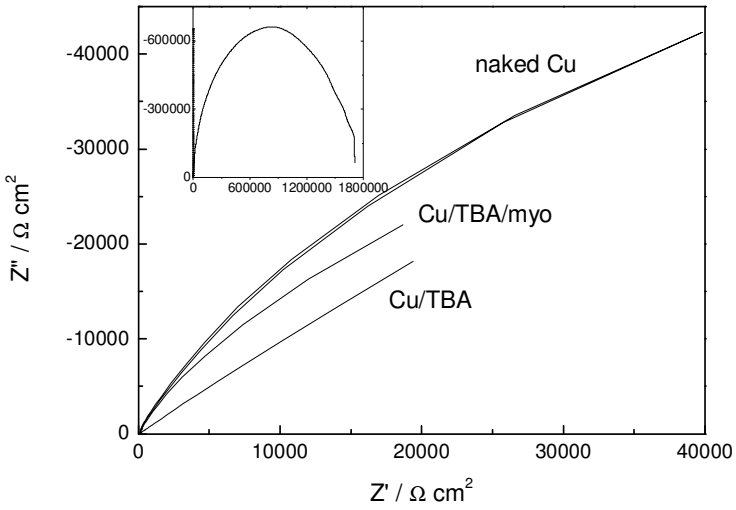


Figure 7. Nyquist diagram for the measured electrodes at E = -0.15 V in the supporting electrolyte. Inset: diagram for the measured data obtained for the Cu/TBA/myo modified electrode in the supporting electrolyte at E = -0.15 V after incubation in the Ru containing solution, and fitted data for the experimental points (dotted line).

As can be observed, behavior changed after each step, i.e, it was different for the naked copper, the Cu/TBA and the Cu/TBA/myo modified electrode. For the naked metal, and taking into account that the potential of zero charge for polycrystalline copper measured in the same electrolyte and at the same pH is around -0.8 V vs. Ag/AgCl [42], the curve could showed some contribution of the weak adsorption of the anion perchlorate on the surface. A similar conduct was found for the myoglobin modified electrode, and that could be explained because even when at the working pH of 7.5 the protein is mostly negative charged, some positive charges still remains on the surface. On the contrary, for the Cu/TBA case, a fitted line with a slope equal to 0.959 ± 0.003 was found, showing almost a perfect diffusion behavior for the ions in such interface. This could be in agreement with the obtain of a better surface in the presence of the thiol, that is, smoother than in case of naked copper. Similar tendencies were observed for naked and thiol modified electrodes in a 1 mM Ru solution, showing that in particular the Ru(III) complex is not able to be adsorbed onto the Cu/TBA electrode surface.

But, as it can be observed at the inset of Figure 7, after keeping the myoglobin containing electrode during 20 h in the Ru solution, the Nyquist diagram for the Cu/TBA/myo/Ru electrode measured in supporting electrolyte showed the presence of an adsorbed species onto the electrode surface.

Comparing the observations from the Nyquist diagram with results showed in Table 1, where the tabulated values referred to measurements carried out in the supporting electrolyte at E = -0.30 V,

it was possible to arrive to similar conclusions. As can be observed at the Table, the value of R1 near duplicated from naked copper to the Cu/TBA electrode and increased ca. 6 times when the myoglobin modified electrode was obtained. On the contrary, after incubation in the Ru containing solution, the formation of the Cu/TBA/myo/Ru electrode could be assessed by the decrease of about 4 times in the value of this resistance, in accordance with the presence of the adsorbed +3 charge species.

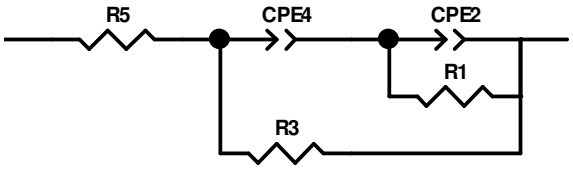
On a similar way, the behavior of CPE2 was in agreement with that observed for R1. This circuit element can be better described by:

$Z_{CPE} = 1 / [Q(j\omega)^{\alpha}]$

where Q is a constant in Ω^{-1} cm^{-2} s^{α} and α is related to the angle of rotation of a purely capacitive line on the complex plane plots [43].

CPE2 showed smooth changes in the values of coefficient Q, but α increased when Cu/TBA was obtained and approximated to the value of 1when Cu/TBA/myo was assembled, showing an uniformed surface and as a consequence in the electronic transfer through it when the protein modified electrode is formed. A remarkable decreased is detected in the value of α when the Ru-complex is trapped by the protein adsorbed onto the electrode surface.

Table 1. Fitted values for the equivalent circuit elements at E= -0.30 V and E = -0.15 V (vs. Ag/AgCl) for the electrodes in the supporting electrolyte (0.1 M $NaClO_4$).
R expressed in kΩ cm^2 ; Q in μΩ^{-1} cm^{-2} s^{α}



E / V	Element	naked Cu	Cu/TBA	Cu/TBA/myo	+ Ru
-0.30 V	R1	4.5 ± 0.4	11.5 ± 0.9	70.2 ± 1.4	18.6 ± 0.4
	CPE2(Q) α	58.3 ± 2.8 0.905	70 ± 1 0.925	57.8 ± 3.3 0.969	63.9 ± 5.6 0.645
	R3	55 ± 9	9.0 ± 0.7	88 ± 18	2.40 ± 0.09
	CPE4 (Q) α	37.2 ± 5.6 0.813	68 ± 11 0.850	13.3 ± 5.6 0.985	63.9 ± 5.6 0.810
	R5	0.0207 ± 5E-4	0.019 ± 1E-3	0.018 ± 2E-3	0.021 ± 1E-3
-0.15 V	R1	3.8 ± 0.2	10.8 ± 0.9	9 ± 2	300
	CPE2 (Q) α	60.5 ± 0.5 0.876	70.5 ± 0.5 0.880	63.9 ± 1.7 0.860	0.011 ± 0.004 0.956
	R3	41 ± 5	3.2 ± 0.4	4.1 ± 0.7	0.5 ± 0.2
	CPE4 (Q) α	25.5 ± 1.7 0.785	23.9 ± 2.8 0.740	75 ± 8 0.815	39.4 ± 5.6 0.794
	R5	0.0207 ± 5E-4	0.018 ± 5E-3	0.018 ± 2E-3	0.024 ± 4E-3

As already mentioned, R3 and CPE4 were assigned to sites or "holes" of the surface that did not follow the assembly process mentioned above, showing small changes when TBA or even myoglobin were added to the solution. It is remarkable that for naked copper the value of R3 is bigger than R1, maybe due to presence of some oxide.

Once dissolution of copper surface gives place to the formation of Cu(II) at pH=7.5, this specie could be responsible for the formation of oxide or the formation of a coordination compound with the TBA present on the electrode surface. At working pH Cu(II) could not remain as aquo complex and competition with the Ru compound could not happen under this chemical form. But complexation of copper with thiols coming from the electrode surface is reported even for compounds structurally similar to TBA [44] so the existence of such complexes could be proposed at very low concentration, because the dissolution of the copper is not the main process when the surface is covered with a thiol. The existence of the Cu-TBA complex could explain some observed details in the CV profile (Figure 4, the rise in the anodic current from ca. -0.15 V). In this case, competition between Cu-complex and Ru-complex for similar anchorage places at the protein surface could take place, but the amount of Ru is certainly higher than the amount of Cu-complex and could be neglected.

With respect to values showed in Table 2, *i.e.* obtained in the presence of the Ru-hexaammine couple at E = -0.30 V, the main difference with those shown at Table 1 was that the value of R3 is much higher than R1, pointing to the presence of the oxide at the surface. This was also confirmed because the value of R1 increased when comparing the obtained value for Cu/TBA with respect to the naked Cu, whereas the value of R3 decreased after the formation of the thiolated film. The presence of the thiol would assure a homogenous surface for the electroactive Ru compound and the electron transfer is enhanced under this situation, and that would explain why the value of R3 decreased in comparison with the value for the oxidized copper surface.

For measurements carried out at E = -0.15 V, coinciding with the potential peak value for the Ru couple, it is noticeable that the value of R1 is higher than 100 MΩ when the myoglobin was adsorbed onto the electrode surface, but R3 did not change significantly after addition of the protein, confirming once again the existence of zones or "holes" on the surface that did not follow the assembly process as expected.

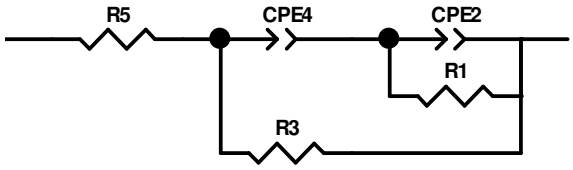
3.3. *Contact angle measurements*

The assembly process was also controlled using this technique. The obtained value was 50° ± 1° (n = 74, n denotes the number of different evaluated pictures) for naked copper, whereas for the modified Cu/TBA the value was 60° ± 3° (n = 27). Finally, after addition of myoglobin and adsorption of the protein on the surface, the angle was 75° ± 3° (n = 20).

The highly hydrophilic copper surface did not show changes in the behavior after formation of the thiol film, due to the hydrophilic characteristics of the used thiobarbituric acid. Nevertheless, the presence of the more hydrophobic protein adsorbed to the surface was showed by the rise in the measured contact angle value.

Table 2. Fitted values for the equivalent circuit elements at E = -0.30 V and E = -0.15 V (vs. Ag/AgCl), for the electrodes in a 1 mM Ru-hexaammine solution in the supporting electrolyte (0.1 M $NaClO_4$).

R expressed in Ω cm^2 ; Q in $\mu\Omega^{-1}$ cm^{-2} s^{α}



E / V	Element	naked Cu	Cu/TBA	Cu/TBA/myo
-0.30 V	R1	4.00 ± 0.02	46 ± 4	50.0 ± 0.4
	CPE2 (Q)	58.9 ± 0.6	167 ± 22	222 ± 61
	α	0.890	0.825	0.590
	R3	99 ± 9	0.19 ± 0.09	0.56 ± 0.36
	CPE4 (Q)	39.4 ± 5.5	257 ± 111	294 ± 50
	α	0.875	0.809	0.740
	R5	0.019 ± 0.001	0.0200 ± 4E-4	0.0190 ± 7E-4
-0.15 V	R1	5.4 ± 0.2	120 ± 36	R > 18000
	CPE2 (Q)	77.8 ± 1.1	88.3 ± 3.3	61 ± 22
	α	0.825	0.755	0.615
	R3	0.31 ± 0.07	0.36 ± 0.11	1.10 ± 0.11
	CPE4 (Q)	25 ± 2	41 ± 6	30.5 ± 1.1
	α	0.680	0.725	0.685
	R5	0.019 ± 1E-3	0.020 ± 1E-3	0.0190 ± 5E-4

4. CONCLUSIONS

The assembly process was evaluated using the combination of electrochemical and physical chemistry techniques. Results showed the formation of a reproducible Cu/TBA electrode followed by the obtaining of a Cu/TBA/myo electrode, which was stable towards protein desorption and also sensitive enough to detect the unspecific and in low extent binding between the protein and the small cationic complex, with good reproducibility. These points are very important when thinking in the use of copper as substrate to manufacture protein modified sensors involving enough selective interactions with their specific targets, and specially taking into account properties related to copper itself, as low dissolution potential values and great tendency towards oxidation.

With respect to the behavior of the electrode, copper devices showed two different zones: one that follows the assembly process, and other showing the presence of defects or holes. This situation did not affect the assembly process, and also did not affect the stability of the electrode itself or the ability of the myoglobin modified electrode to trap a cationic compound.

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