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Comparison Between Copper and Gold as Substrates for Sensing: an Electrochemical Evaluation.

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Electrochemical sensors based in the use of proteins have a wide range of applications. In order to adsorb the protein to get the modified electrode, heterocyclic thiols as thiobarbituric acid offers an interesting option, considering the short time to get an ordered monolayer and the electron transfer properties through this film. But further adsorption of the protein would play a relevant role on the electron transfer of the modified electrode, and the metal used as substrate could affect the whole behavior of the ensemble. The aim of this work was to evaluate the behavior towards electron transfer of a Au/thiobarbituric/myoglobin modified electrode, using Ru-hexaammine as probe molecule. The evaluation was carried out using cyclic voltammetry, impedance spectroscopy and quartz crystal microbalance measurements.

Keywords: QCM, impedance, thiobarbituric acid

1. INTRODUCTION

The need for sensing covers a wide range of applications. There is a continuing demand for fast and simple analytical methods for the determination of many clinical, biochemical and environmental analytes. From health to environmental purposes, and from antigen to pesticides detection, they are all based on a very simple requirement: the detection must be fast, reproducible and selective.

Many possibilities are found in the literature, and among them, electrochemical sensors are the focus of hundred of groups around the world. For such devices, the way of sensing and also the architecture of their design are also different and there are more than one accepted approximations.

Electrochemical detection overcomes problems associated with other modes of detection, as for example, concerns of health hazards and disposal with the use of radioactive agents, or limited

sensitivity in the analysis of colored or turbid samples when using optical detection. Electrochemical sensors enable fast, simple, and economical detection free of these problems. The relevant reactions take place at the electrode–solution interface rather than in bulk solution. But electron transfer is a main factor to consider and is the main problem to solve when constructing electrochemical devices for sensing and accessibility of a molecule to the electrode surface where electron transfer takes place will be involved.

When building an electrochemical device, the immobilization of molecules is accomplished by changes in the interfacial charge, capacitance, resistance, mass, and thickness at the surface [1-3].

Some methods combined with electrochemistry are useful, as piezoelectric ones, where the sensor surface is coated with a molecule and the mass variation induced by the target binding is correlated to the concentration of the target [4-6]. For quartz crystal microbalance (QCM) the sensitivity to the mass change in air on the transducer surface is about 1 Hz ng^{-1} for a bulk acoustic wave device with 9 MHz of fundamental frequency, as described by the Sauerbrey equation [7].

Some immobilization alternatives have been developed, and the use of self-assembled monolayers is an attractive method to trap the desired reagents. By taking advantage of the spontaneous chemisorption of alkanethiols to metals as gold, silver or copper, highly ordered monolayers can be assembled. This point is exploited to provide controlled orientation of the adsorbed biomolecules. Heterocyclic thiols have been used, mainly to as corrosion inhibitors [8, 9]. In particular, 2-thiobarbituric acid (TBA) has been proved by our group to develop ordered surfaces [10-12] on gold and copper. TBA showed exceptional properties, that is, a short immersion time to obtain a stable coverage, a high surface coverage, and excellent electron transfer properties, including good capability towards protein binding.

The aim of this work was the development of Au/TBA/myoglobin electrodes, and the evaluation of the electron transfer through such assembly using $[Ru(NH_3)_6]^{3+}$ as probe molecule. The whole process was followed using cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS) and quartz crystal microbalance (QCM) measurements. Results were compared with the observed behavior for a similar ensemble of Cu/TBA/myoglobin [10].

Myoglobin (myo) was chosen because it has some interesting characteristics to perform this study: is a small sized protein and would allow a suitable electron transfer when attached to an electrode surface, with a well adsorption behavior on thiol-modified surfaces, and many of its functional groups are negatively charged at a working pH of 7.5 [13-15].

2. MATERIAL AND METHODS

2.1. Solutions

3 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 0.3 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 measurements (CV and EIS)

A polycrystalline Au-pc (99.999%, 4 mm diameter), 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 (0.195 V vs. SHE) reference electrode. Electrochemical measurements were carried out under Ar atmosphere in non-stirred solutions. Cyclic voltammetry profiles were obtained at potential scan rates within the range $0.002\ Vs^{-1} \leq \nu \leq 0.07\ Vs^{-1}$.

The EIS experiments were carried out under potentiostatic control in the range -0.35 to 0.10 V vs. Ag/AgCl potential ranges stepping each 0.15 V, and in the frequency range from 100 mHz to 1 MHz. There were performed in supporting electrolyte in the presence and in the absence of the Ru compound.

In order to work with a reproducible surface, prior to use gold electrodes were cleaned with piranha solution (H_2SO_4/H_2O_2 3:1 v/v) and after that were hand polished with 0.3 μm and 0.05 μm alumina suspensions.

For the assembly process, the Au electrode was immersed in the TBA-containing solution for 40 minutes, and thereafter thoroughly washed and sonicated in water.

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

To evaluate the adsorption of the probe molecule to the myoglobin, voltammetric profiles of the modified electrode were recorded in two different ways:

a) Immediately after immersion (t=0), CV profiles were recorded in the Ru containing solution

b) After the Au/TBA/myo electrode was kept in a 1 mM $[Ru(NH_3)_6]^{3+}$ solution during 20 hours. In this case CV profiles of the obtained Au/TBA/myo/Ru electrode were recorded in the supporting electrolyte.

2.3. Quartz Crystal Microbalance (QCM) measurements

A CHI 440 potentiostat/galvanostat time-resolved quartz crystal microbalance was employed for QCM measurements. The working electrode was a circularly shaped gold layer with a calculated surface exposed to the electrolyte of 0.215 cm^2. The system was completed with a Pt wire counter electrode, and a saturated Ag/AgCl reference electrode. All potentials in the text are referenced to this electrode. Measurements for QCM were made in time-resolved mode, thus the frequency difference of

the working crystal and the reference crystal was measured. The reference crystal had an oscillation frequency of 8.000 MHz. Adsorption measurements were carried out with no applied potential.

Before use, the gold electrode was rinsed with ethanol and dried under Ar flow.

In a first step, the gold disc was kept in contact with the TBA solution; once removed, the cell was washed with milliQ water (10 times x 1 mL). Then, buffer phosphate (pH = 7.5, 5 mM) was added to the cell, and the resonator was switched on to stabilize the system prior the addition of myoglobin. After that, the Au/TBA/myo electrode was washed with buffer solution. Finally, the solution of the Ru complex in phosphate buffer was added to the cell containing the myoglobin containing electrode.

3. RESULTS

3.1. CV

As showed in Figure 1, after formation of the TBA layer on the gold electrode, a slightly decrease in the intensity of the current peaks for the Ru couple was observed. Similar behavior is detected also after addition of the myoglobin. For the last step of the assembly, the binding of the Ru complex to the immobilized protein, it was possible to detect the cathodic counterpeak of the Ru couple at very low scan rates (0.002 to 0.005 Vs^{-1}).

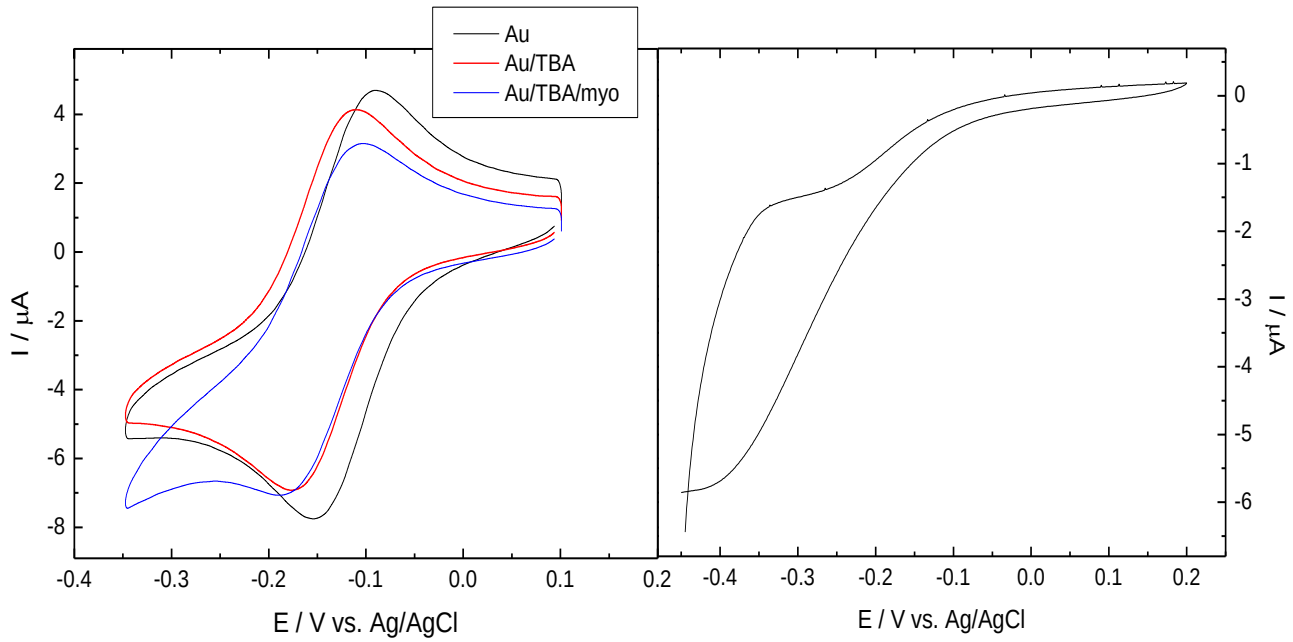


Figure 1 - Left: Voltammetric profiles for the electrodes in 1mM Ru-hexaammine in 0.1 M $NaClO_4$: naked gold, Au/TBA and Au/TBA/myo, v = 0.050 Vs^{-1} - Right: Voltammetric profile for the Au/TBA/myo electrode in 0.1 M $NaClO_4$, after keeping the electrode during 1 day immersed in a 1mM Ru-hexaammine solution, v = 0.002 Vs^{-1}

Moreover, dimensionally of the electrodes, calculated from the slopes of the ln I vs. ln *v* representations, changed from 2.01 (naked gold) to 1.91 (Au/TBA) to 2.45 (Au/TBA/myo). These showed the conversion from a 2-dimensional surface to a rough one when the protein is adsorbed, a point that could affect the accessibility of the probe molecule to the surface.

3.2. EIS

A Randles equivalent circuit was utilized to fit the impedance data for experiments carried out in the presence of the couple, while for those carried out in the supporting electrolyte a 3-elements equivalent circuit was chosen.

Changes in R, CPE and W values were observed during the assembly process, as can be observed in Table 1. In particular R and CPE values decreased after the TBA layer is formed onto the gold surface, in agreement with the electron transfer properties showed by this heterocyclic compound [10, 11, 16].

After formation of the ensemble Au/TBA/myo, R and CPE increased, accordingly to the addition of a compound where electron transfer is less favorable. When the electrode Au/TBA/myo/Ru is formed, R and CPE decreased showing enhance of the electron transfer with the presence of the complex: the trap of the cationic compound by the immobilized myoglobin is confirmed in this way.

Table 1. Fitted values for the equivalent circuit elements at E = -0.20 V vs. Ag/AgCl for the electrodes in the supporting electrolyte, 0.1 M $NaClO_4$ (left side) and for the electrodes in a 1 mM Ru-hexa ammine in the supporting electrolyte (right side). R expressed in Ω; T(CPE) in μF; R (W) in Ω and T (W) in s.

electrode	**R_3**	**CPE_2: T P**	**R_1**	**electrode**	**R_3**	**CPE_2: T P**	**R_1**	**W_1: R T P**
naked Au	101	5.43 0.880	347700	naked Au	95	29.3 0.730	24350	58550 5.57 0.5
Au/TBA	101	4.75 0.915	115200	Au/TBA	95	21.0 0.774	1950	81050 13.8 0.429
Au/TBA/myo	101	8.16 0.876	269000	Au/TBA/myo	95	8.7 0.842	1050	140600 57.7 0.502
Au/TBA/myo/ + Ru	101	7.47 0.878	228470					

For measurements carried out in the 1 mM Ru solution, the effective diffusion thickness (deduced from the T term of the Warburg element) increased near 4 times from naked Au to Au/TBA/myo, growing from 2.5 10^{-3} cm to 8 10^{-3} cm.

Adsorption of Ru-hexaammine to myoglobin is also observed at Figure 2, where Bode and Nyquist diagrams confirm this behavior (phase angle of 45º at 0.1 Hz at Bode diagram, and curve shape line at Nyquist).

Then, the Ru compound is able to transfer electrons through the protein containing film, and probably this behavior is favored by the adsorption of at least a percent of the complex to the protein surface.

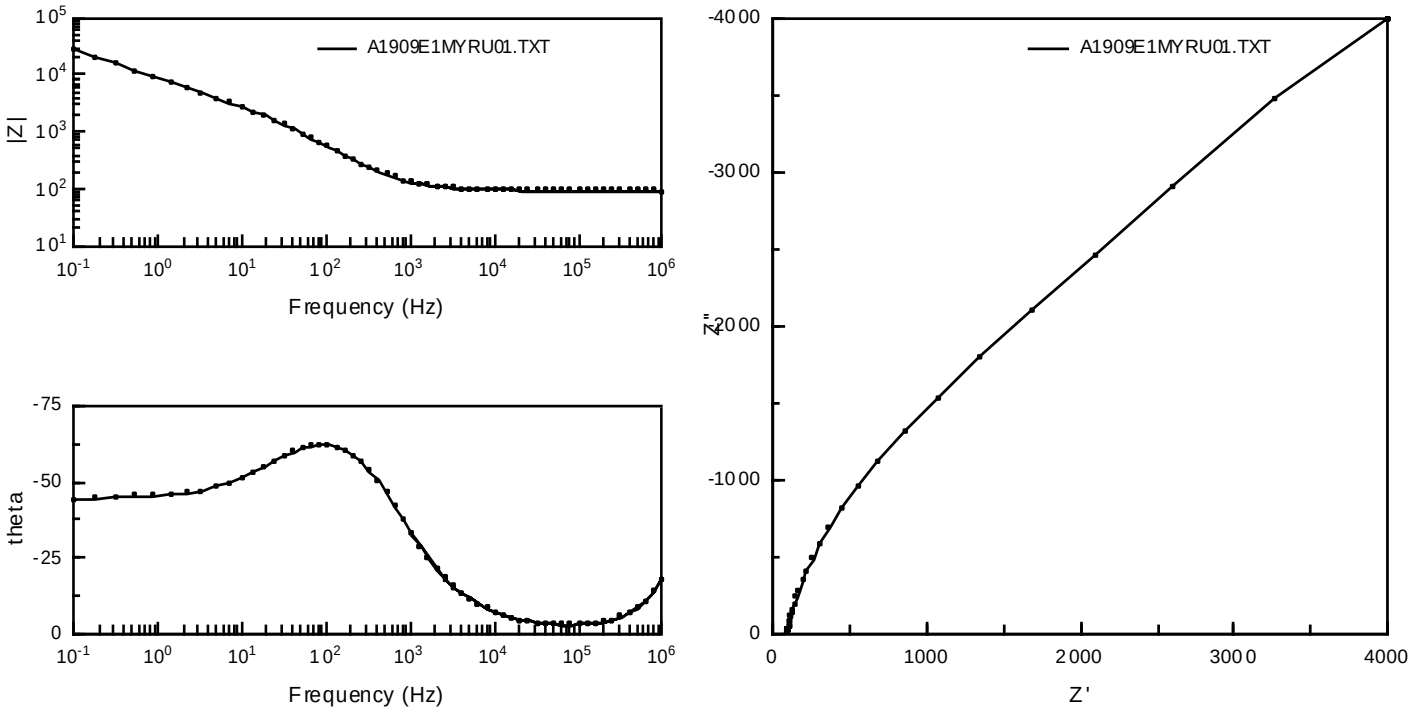


Figure 2. Bode (left) and Nyquist (right) representation for the measured data at E = -0.2 V vs. Ag/AgCl, for a modified Au/TBA/myo electrode in 1 mM Ru-hexaammine solution.

3.3 QCM

As can be observed in Figure 3, the assembly process was followed by use of this technique. After adsorption of TBA onto the naked Au surface a main change of 83 Hz is observed, with a loss of mass after some minutes, probably due to the formation of a disordered multilayer at the initial steps followed by the loss of TBA molecules to get an ordered monolayer.

In the following step, myoglobin was added to the Au/TBA electrode and a frequency change of 60 Hz was observed, with a little loss of mass after some minutes. This fact could be in agreement with the diffusion of trapped ions that could be retaining when the protein is adsorbed onto the

electrode surface. The last step, observed after addition of the Ru-complex solution to the Au/TBA/myo electrode, corresponded to a frequency change of ca. 20 Hz. The union of this compound with the immobilized protein would mainly take place through van der Waals forces (after the initial approach of the cationic compound to a negative charge place at the protein surface), and therefore, the union between the complex and the protein can be explained as a dynamic equilibrium. Cycles of adsorption and desorption of at least a percent of the complex could be establish, and this could be the explanation for what is observed using QCM: after the initial diminution in frequency, waves of little changes are detected.

If Sauerbrey equation is applied, only to make an estimative calculation, the amount of deposited myoglobin onto Au/TBA is 5 10^{-12} mol/cm^2, around one third of the reported value 1.5 10^{-11} mol/cm^2 to get a monolayer [13].

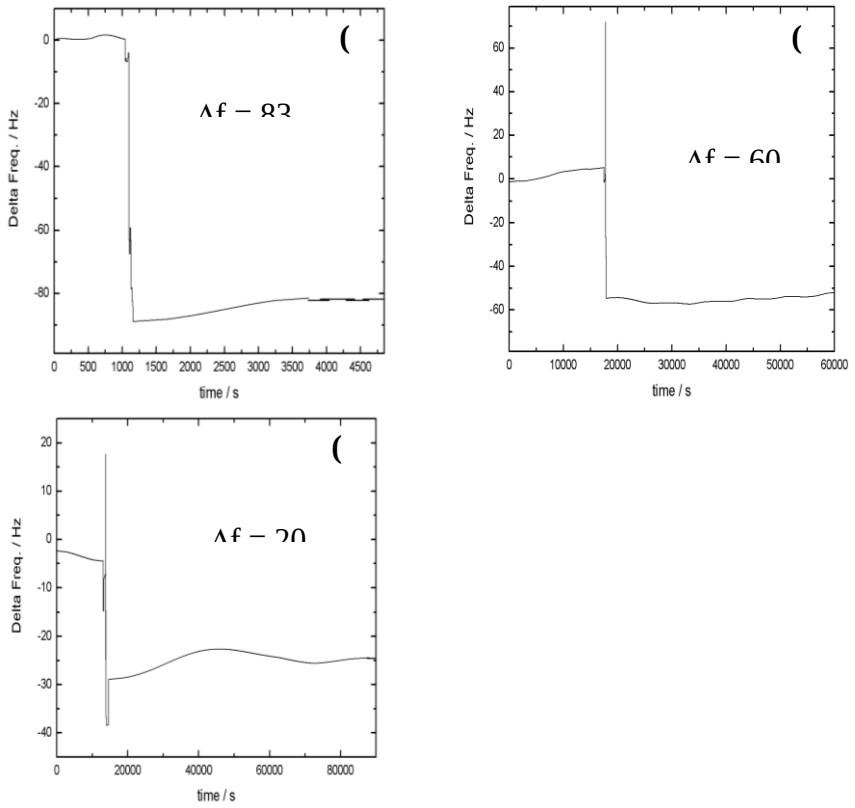


Figure 3. Measured frequency changes with time for: a) naked Au after addition of TBA; b) Au/TBA after addition of myoglobin; c) Au/TBA/myo after addition of Ru-hexaammine.

4. DISCUSSION

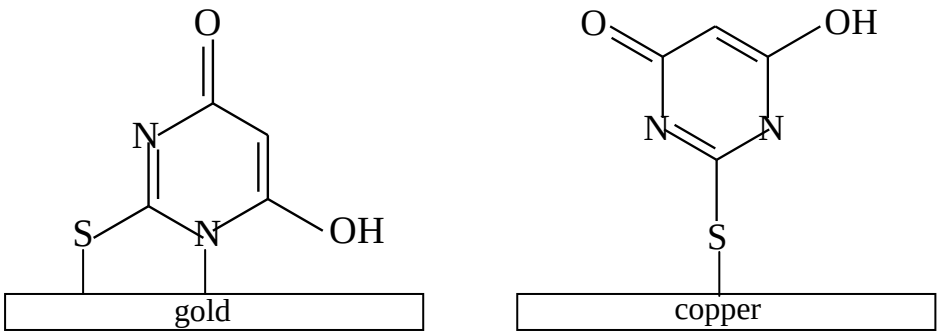
It is interesting to compare the obtained results with those obtained when working with copper as substrate [10].

The assembly process from naked copper to the Cu/TBA/myo could be clearly assessed from observed changes at CV profiles in shape, intensity or potential peaks values for the probe molecule. On the contrary, changes associated to the formation of the Au/TBA and to the Au/TBA/myo ensemble were not so noticeable. For the M/TBA/myo electrode, dimensionally was equal to1.70 for Cu and 2.45 for Au. EIS measurements were in line with CV results.

Electron transfer was excellent through TBA layers for both metals, but through the adsorbed protein seemed to be better for copper than in case of gold. To explain this point, dimensionally and the way adsorption of the TBA molecule takes place must be considered.

CV, EIS and QCM showed adsorption of the complex but not in a great extent. As a consequence, diffusion of the compound plays an important role on the electron exchange at the electrode surface.

For gold the surface roughness is high, whereas in case of copper non conductive zones are present on the electrode surface. When adsorbed onto the metal surface, on gold TBA is able to establish bounds using two atoms (leaving a carboxylic oxygen available for the protein), whereas on copper would probably use only one (leaving two sites available for the protein to generate bounds) [12, 16].



Scheme 1. TBA could adsorb onto Au surface probably through 2 of the atoms of the molecule (left side), whereas onto Cu would probably use one of the atoms (right side).

On gold, surface roughness is high and as a consequence would be hard for the probe molecule to reach the electrode surface. On copper the structure of the myoglobin containing surface would be less rigid than in gold, and could generate channels used by the probe molecule to reach the surface.

5. CONCLUSIONS

Electron transfer through TBA layers was excellent for gold, as observed previously for copper electrodes. Differences appeared when the myoglobin modified electrode is formed: in case of gold a

probe molecule as Ru-hexaammine would hardly find a path to transfer electron with the electrode surface, whereas working with copper and despite the existence of non conductive areas, the probe molecule would find a channel for the electron exchange.

In summary, if development of a protein modified electrode based in the use of a thiol layer with excellent electron transfers characteristics as TBA is the goal, then copper will certainly be a better choice than gold.

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