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Towards Combined Electrochemistry and Surface-Enhanced Resonance Raman of Heme Proteins: Improvement of Diffusion Electrochemistry of Cytochrome *c* at Silver Electrodes Chemically Modified with 4-Mercaptopyridine

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To date, a successful combination of surface-enhanced resonance Raman spectroscopy (SERRS) and electrochemistry to study heme proteins is inhibited by the problems raised by the prerequisite to use silver as electrode metal. This paper indicates an approach to overcome these problems. It describes a quick and reproducible procedure to prepare silver electrodes chemically modified with 4-mercaptopyridine suitable to perform diffusion electrochemistry of cytochrome *c* (cyt *c*). The method involves the employment of a mechanical and a chemical treatment and avoids the use of alumina slurries and any electrochemical pretreatment. Cyclic voltammetry (CV) was used to test the electrochemical response of cyt *c*, and the CV signals were found identical with those obtained on gold electrodes under the same experimental conditions. Compared to previous literature, a significant improvement of the CV signal of cyt *c* at silver electrodes was achieved. Preliminary results show that this treatment can be also successfully employed for the preparation of SERRS-active electrodes.

Cytochrome *c* (cyt *c*) is an ubiquitous heme protein playing a crucial role in many biological processes in aerobic organisms such as photosynthesis, cell respiration, and apoptosis.[1] Even though this redox protein has been intensively studied through the last three decades,[2−4] many aspects of its behavior are still far from being completely understood. In particular, it is not clear how the mutual interplay between the metal ion and the protein matrix influences the redox potential of cyt *c*, which is of particular relevance in the comprehension of the folding and unfolding processes, the solvent influence, and the temperature and pH dependences.

For these reasons, cyt *c* has been studied with voltammetric techniques both in diffusion and in adsorption conditions at modified electrode surfaces. Several metals are suitable for such purpose, but at the present state of research, gold seems to be prevalent whereas silver is not the metal of choice. From the electrochemical point of view, there are several reasons to prefer gold instead of silver as electrode material: (a) the mechanical polishing, which plays a crucial role in the quality and the reproducibility of the cyclic voltammetry (CV) signal, is easily performed for gold electrodes but is well known to lead to inhomogeneous silver surface;[5] (b) the chemicals generally successfully used to treat gold surfaces, such as hot solutions of concentrated sulfuric acid, are too aggressive to silver; therefore, it is very difficult to renew the silver electrode surface by removing the monolayer and the contaminants without polishing;[6] (c) compared to gold, silver exhibits a smaller potential window, and consequently, electrochemical pretreatments commonly employed on gold are not applicable on silver;[7,8] (d) gold is generally considered a better substrate for self-assambled monolayers (SAMs);[9,10] (e) sulfur-containing SAMs built on gold surfaces have been widely characterized over the past decade, while not much work has been done on silver.[11]

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For these reasons, silver received only minor attention from electrochemists involved in protein studies. But nowadays, the relevance of spectroscopic techniques such as surface-enhanced resonance Raman spectroscopy (SERRS) as a powerful tool to investigate proteins and enzymes stimulated the electrochemists to spend much effort in developing electrochemical characterization of silver surfaces. This technique, which can be employed when silver is used as electrode material, probes solely the vibrational spectrum of the active site of the biomolecules providing structural information about the coordination, the spin, and the oxidation state of the central metal ion.[12−15] The reason silver is to be preferred as electrode material is that the crucial condition of resonance with the chromophore (the heme group) can be achieved in the range of the plasmon frequencies of the metal, while for gold, surface-enhanced Raman spectroscopy is usually more efficient at a wavelength far from resonance of the heme group. The intent to couple SERRS with voltammetric techniques is the main reason to focus on silver as electrode material.[16]

Despite the problems discussed above, the present work shows that it is possible to perform CV of cyt *c* at silver electrodes chemically modified with 4-mercaptopyridine (PySH) and obtain CV signals essentially identical to those obtained on gold electrodes under the same experimental conditions.

EXPERIMENTAL SECTION

Chemicals. Bovine heart cyt *c* was purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Phosphate buffer solutions (pH 7.0, 10 mM) were prepared from Na_2HPO_4 and NaH_2PO_4 (J.T. Baker, Deventer, The Netherlands). $NaClO_4$, KCl, and PySH were purchased from Sigma-Aldrich. Purified Milli-Q water (Millipore) was used for all preparations and procedures. All chemicals were of reagent grade.

Electrochemical Measurements. Polycrystalline silver and gold disks (IJ Cambria Scientific, Carms, UK) of 2-mm diameter were used as working electrodes; a platinum wire (IJ Cambria Scientific) and a saturated calomel electrode (Amel Instruments, Milano, Italy) were used as counter and reference electrodes (RE), respectively. The RE was kept in a glass tube and separated from the working solution by a porous frit. The chemical composition of the solution in the glass tube was identical to that of the working solution except for the protein. The RE and the cell (a conventional glass cell containing ~1 mL of protein solution) were kept at a constant temperature of 20 ± 0.1 °C during all the experiments. The three electrodes system was controlled with a μAutolab potentiostat (Eco Chemie, Utrecht, The Netherlands). All potentials are referred versus the saturated calomel electrode (244 mV vs SHE). Potentials were calibrated against the methyl viologen (MV) MV^{2+}/MV^{+} couple. The electrochemical measurements were repeated several times, and the potentials were found reproducible within ±2 mV. Protein solutions were freshly prepared before use.

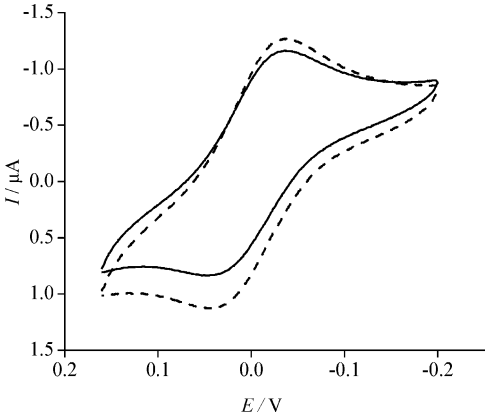

Figure 1. Comparison of cyclic voltammograms of 0.5 mM bovine heart cyt *c* in 10 mM phosphate buffer solution (pH 7.00) with 0.05 M $NaClO_4$ obtained at Ag (solid line) and Au (dashed line) disk electrodes chemically modified with 4-mercaptopyridine. CVs were performed from +0.16 to −0.2V at a scan rate of 100 mV/s.

The silver and the gold electrodes were ground with borcarbid water slurry (grit, 600; Tetrabor) on a glass surface, then prepolished with borcarbid water slurry (grit, 1200) on cloth, and finally polished on a borcarbid water slurry (grit, 1500) on silk. After each stage, the electrodes were kept in an ultrasonic bath for 10 min to remove excess powder. This treatment was performed only before using the electrodes for the first time. Final polishing was performed with water on aluminum oxide lapping film sheets (261× and 262×, 3M) from 5- to 1-μm grain size until a mirrorlike appearance of the surfaces was obtained. For this purpose, the electrodes were kept within a holder specifically built to maintain the electrode surfaces flat.

Immediately after the polishing procedure, the electrodes were put in aqueous HNO_3 (15%) for 1 min,[17] rinsed with abundant water, and then put into a freshly prepared 0.1 mM aqueous solution of PySH for 1 min. After the polishing procedure, the gold working electrode was chemically modified by dipping in 1 mM aqueous solution of PySH for 1 min.

RESULTS AND DISCUSSION

In Figure 1, the voltammetric responses of cyt *c* on silver and gold surfaces chemically modified with PySH are compared. In both cases, the CV signals show two well-defined current peaks, which can be ascribed to the monoelectronic reduction and oxidation of cyt *c* due to the Fe^{3+}/Fe^{2+} equilibrium of the heme iron. Anodic and cathodic peak currents were found to be identical, and both were proportional to the protein concentration and the square root of scan rates $v^{1/2}$, as expected for a diffusion-controlled electrochemical process (Figure 2). Given the quasi-reversibility of the electrochemical process (peak separation in CV experiments varied from 60 to 90 mV in the range of scan rates investigated), the half-wave potential $E_{1/2} = (E_{p,c} + E_{p,a})/2$ was assumed to represent the formal reduction potential $E^{\circ\prime}$. The associated formal reduction potential thus obtained, i.e., $E^{\circ\prime} = 10$ mV, was found to be constant over the whole scan rate range and in agreement with the reported values for solution cyt *c*.[18]

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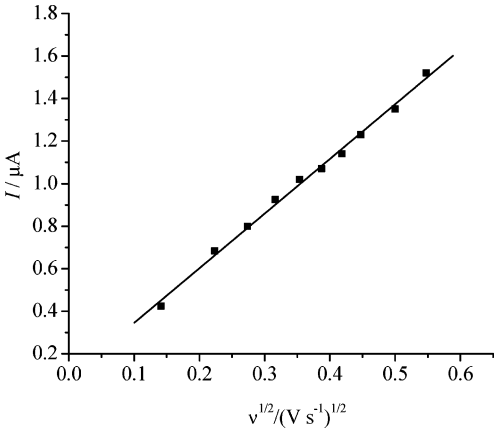

Figure 2. Plot of i_p vs $\nu^{1/2}$ of 0.5 mM bovine heart cyt *c* in 10 mM phosphate buffer solution (pH 7.00) with 0.05 M $NaClO_4$ at a Ag electrode chemically modified 4-mercaptopyridine.

The diffusion coefficient was determined using the Randles−Sevcik equation plotting the peak current versus $v^{1/2}$. The value obtained was equal to $5.05 \pm 0.40\ 10^{-7}$ cm^2 s^{-1}. The diffusion coefficient was used to determine the heterogeneous electron-transfer kinetic constant of the process k_s as previously described by Nicholson,[19] assuming the charge-transfer coefficient $\alpha = 0{,}5$ and the number of electrons $n = 1$ for a monoelectronic process at $T = 20$ °C. The value of $1.12 \pm 0.10\ 10^{-2}$ cm s^{-1} determined for k_s is similar to that found at the same temperature for horse heart cyt *c* on gold electrodes chemically modified with various promoters. This value is higher than the one obtained by Reed and Hawkridge on bare silver electrodes[18] and comparable to the one obtained by Magner et al. on Au/PySH.[20,21] This behavior can be rationalized by considering that the factor controlling the electron-transfer process (and thus the k_s value) is related to the conformation of the protein. This conformation is independent of the nature of the electrode material itself, but it is influenced by the surface preparation, the employment of the promoter, and the history of the electrode. Also, the diffusion coefficient value of $(5.05 \pm 0.40) \times 10^{-7}$ cm^2 s^{-1}, being in agreement with that found by other authors,[21,22] indicates that our Ag/PySH system performs properly from an electrochemical point of view.

As is obvious from Figure 1, the CV signal of cyt *c* obtained at Ag/PySH is essentially identical to the one obtained on Au/PySH, which indicates a significant improvement for the silver electrode performance in comparison to the literature.[18,23] We attribute this improvement to the particular treatment of the surface (see Experimental Section), a treatment procedure resulting from empirical optimization of several parameters (mechanical procedures, chemical treatments, electrochemical pretreatments, dipping conditions) directed at the increase of reproducibility and quality of the voltammetric signal.

As mentioned before, it is very difficult to perform chemical and electrochemical treatments on silver electrodes without damaging the metal surface or dramatically changing its chemical properties. In this scenario, the mechanical treatment assumes a decisive role in determining the quality of the voltammetric response.

The mechanical treatment adopted in the present study was found to be quicker to perform compared with the polishing procedures commonly used in electrochemistry (alumina slurries on different textures)[24] and additionally led to more stable and reproducible surfaces as well. Lapping film sheets enable us to obtain a mirrorlike surface in less than 5 min, avoiding the use of the ultrasonic bath to remove the powder in excess, and therefore, this approach is highly recommended.

Chemical treatment of the electrode with diluted HNO_3 was performed as suggested by Untereker and co-workers.[17] Immediately after the mechanical treatment, the electrode was dipped in HNO_3 15% for 1 min. Such treatment did not improve the CV signals significantly, but provided higher reproducibility in terms of overall shape and peak separation. Similar treatments with NH_3 and mixtures of NH_3 and H_2O_2 were also tested, but were abandoned since they worsened the CV signals considerably.

Since electrochemical pretreatment of electrode surfaces is generally recommended in the electrochemical literature,[25,26] we have tried to improve the CV performance of our silver electrode by testing two procedures: (a) 60 voltammetric scans at 20 mV/s in 0.05 M $NaSO_4$ from 0.125 to −0.175 V, as previously performed by Reed and Hawkridge on Ag electrodes;[18] (b) 10 voltammetric scans at 100 mV/s in 0.1 M $NaClO_4$ from 0.3 to −0.3 V. In both cases, the silver electrode was mechanically treated as described in the Experimental Section and then put in an electrochemical cell to undergo the pretreatment. After completing this procedure, it was rinsed with water and dipped into the promoter solution. In both cases, the electrochemical pretreatment did not improve but even worsened the voltammetric response of cyt *c* at Ag/PySH electrode considerably (see Figure 3). The CV curve featured smaller current peaks and low signal-to-background level compared with that obtained in the absence of electrochemical pretreatment. An analogous pretreatment performed on gold electrodes, as previously described by Borsari et al. (data not shown), did not affect the quality of the CV measurements and thus was not employed.[27]

The dipping time, defined as the span the electrode is exposed to the promoter solution, is known to be an important parameter in SAM formation.[9] In his pioneering studies on solution cyt *c* at Ag/PySH electrodes, Taniguchi proposed a dipping time of 10 min.[23] In the present work, dipping times from seconds to hours scale were tested. Favorable signal-to-background levels were found for short time exposure of silver electrode (1 min). Degassing of the promoter solution or keeping it away from external light sources did not influence the voltammetric response at all.

As mentioned above, the electrochemistry is the main point of concern in combining SERRS and CV experiments on cyt *c*. To illustrate this point, experiments performed on electrochemi-

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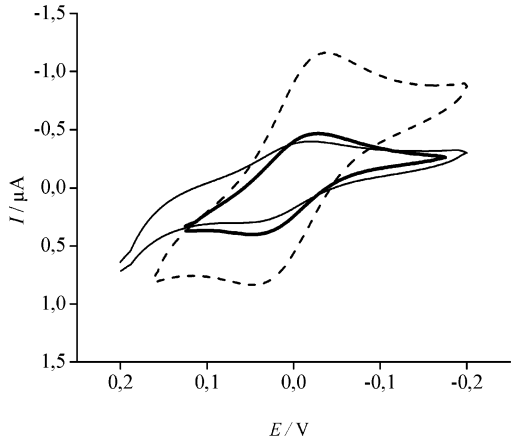

Figure 3. Comparison of the effect of electrochemical pretreatment on cyclic voltammograms of 0.5 mM bovine heart cytochrome *c* in 10 mM phosphate buffer solution (pH = 7.00) with 0.05 M $NaClO_4$ obtained at 4-mercaptopyridine-modified Ag disk electrode: without pretreatment (dotted line, shown for comparison), after 60 voltammetric scans at 0.02 V/s in 0.05 M $NaSO_4$ from 0.125 to −0.175 V (lighter solid curve), and after 10 voltammetric scans at 0.1 V/s in 0.1 M $NaClO_4$ from 0.3 to −0.3 V (darkest solid curve). Scan rates were 100 mV/s.

cally roughened silver surfaces coated with a monolayer of PySH indicated that such treatment can be successfully employed to prepare SERRS-active electrodes as well and, thus. can be useful for future applications in combining these SERRS and CV (manuscript in preparation).

CONCLUSIONS

Silver electrodes were found suitable to perform direct electrochemistry of solution cyt *c*, giving voltammetric responses essentially identical to those obtained on gold electrodes. We attribute this result to the particular treatment of the electrode, mainly focused on the employment of a novel polishing procedure performed with the lapping film (strongly recommended), the absence of any electrochemical pretreatment, and a short dipping time of the electrode in the promoter solution. This treatment was found to be rapid and led to stable and reproducible surfaces.

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