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Direct Anchoring of Cytochrome *c* onto Bare Gold Electrode for Sensing Oxidative Stress in Aquatic Cells

G. Suárez,[1] Ch. Santschi,[1] V. I. Slaveykova[2] and O. J. F. Martin[1]

[1]*Nanophotonics and Metrology Laboratory, Swiss Federal Institute of Technology Lausanne (EPFL), CH-1015 Lausanne, Switzerland*

[2] *Institute F. A. Forel, University of Geneva, Route de Suisse 10, PoBox 416, CH-1290 Versoix, Switzerland*

Summary

A novel third generation biosensor was developed based on one-shot adsorption of chemically modified cytochrome *c* (cyt *c*) onto bare gold electrodes. The introduction of short-chain thiol derivatives (mercaptopropionic acid, MPA) on the lysine residues of cyt *c* enabled the very fast formation (<5 min) of an electroactive biological self-assembled monolayer (SAM) exhibiting a quasi-reversible electrochemical behavior and a fast direct electron transfer (ET). The high value estimated for the heterogeneous ET rate constant, k_s = 1600 s^{-1}, indicates that short anchors might facilitate the ET via an efficient orientation of the heme pocket. In comparison, no direct ET was observed in the case of native and long-anchor modified (mercaptoundecanoic acid, MUA) cyt *c* adsorbed on gold. The so-made amperometric biosensor enabled real-time and non-invasive detection of extracellular H_2O_2 released by unicellular aquatic microorganisms *Chlamydomonas reinhardtii* as a consequence of cadmium-induced oxidative stress.

Motivation and results

Over the last two decades amperometric biosensors based on the enzymatically-catalyzed reduction of H_2O_2 have attracted a wide interest due to their accurate sensitivity and specificity. In the case of the so-called third generation biosensors which rely on direct ET the ability to achieve an efficient electrical communication underlies resolving orientation and distance issues between the electrode surface and the protein redox center.[1] It is known that cyt *c* exhibits a very low ET once in contact to solid bare electrodes which results in a poor electrochemical behavior.[2] Thus, a number of studies have focused on modifying the gold electrode surface in order to generate a suitable molecular environment that prevents from cyt *c* denaturing and enhances the ET rate constant.

Here we report a chemically modified cyt *c* that forms adsorbed monolayer onto bare gold and exhibits very fast ET rate constant. The introduction of thiol derivatives -via chemical coupling on cyt *c* lysine residues - that act as anchor molecules on gold prevents protein denaturing and enables rational "tuning" of the ET rate with the chain length. As expected for native cyt *c* no faradaic peak developed on the capacitive current whereas well defined peaks are visible for cyt *c*-MPA which corresponds to the electrochemical oxidation and reduction of heme group (*Fig. 1*). However, cyt *c*-MUA that exhibits a longer anchor-spacer shows no direct ET albeit its electroactivity is restored as soon as small gold nanoparticles act as electron shuttle. In contrast, the cyt *c*-MPA layer shows a quasi-reversible electrochemical behavior indicating that fast interfacial ET mechanism is predominant due to a favorable protein orientation on gold.

The voltammetric monitoring of MPA-cyt *c* chemisorption on bare gold emphasizes the rapidity of the electroactive protein layer formation with the maximum peak intensity reached within 5 minutes (20 cycles at ν = 0.05 V s^{-1}), as shown in Figure 2A. Moreover, the analysis of the peaks intensity shows a reversible electrochemical behaviour with a value of Ia/Ic = 0.96. The calculation of the E_{FWHM} = 86 mV which is below the theoretical value of 90.6 mV defines a nerstian monoelectronic process strongly adsorbed on the electrode surface.[3] The surface coverage (Γ) of electroactive MPA-cyt *c* on gold was estimated to be $4x10^{-12}$ mol cm^{-2} which corresponds roughly to 25 % of the theoretical value reported by Nakano et al.[4] for a fully packed cyt *c* surface coverage. It is also observable that the formal potential E°' = -48 mV (vs Ag/AgCl) is negative-shifted as compared to native cyt *c* in solution, as expected for a covalently immobilized protein.[5] Furthermore, cyclicvoltammetry was performed at different scan rates in order to study the ET mechanism taking place at a MPA-cyt *c* modified electrode. The voltammograms in Figure 2B show the influence of the scan rate both on the peak current intensity and the peak-to-peak separation. As depicted in Figure 2C the anodic and cathodic peaks currents are linearly proportional to the scan rate in the range between 0.01 and 0.6 V s^{-1}, which is expected for a surface-controlled electrochemical process. An estimated value of the heterogeneous ET rate constant, k_s, has been calculated from the analysis of peak-to-peak separations fusing Laviron's method. Considering a charge transfer coefficient α = 0.5 a high value of k_s = 1600 s^{-1} was calculated, which is in good agreement with those reported for cyt *c* covalently attached onto a SAM of mercaptobutyric acid[6] or a mixed SAM of MPA/mercaptoethanol.[7]

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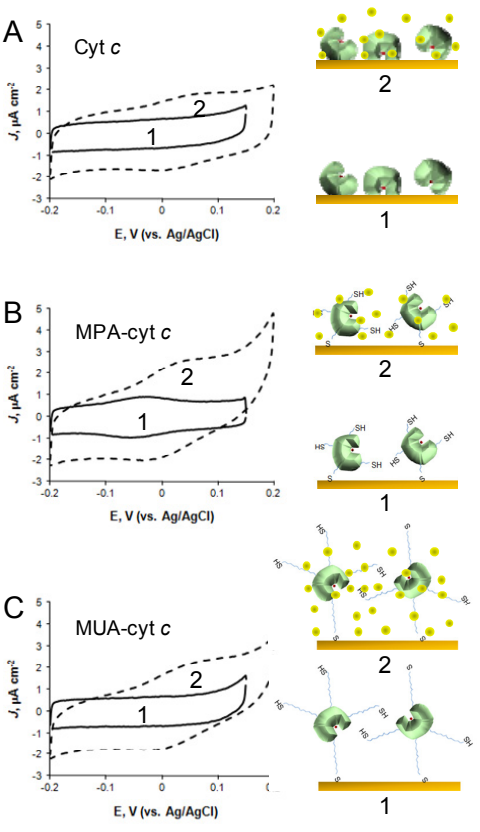

Fig. 1: Cyclicvoltammograms obtained for native cyt *c* (A), MPA-cyt *c* (B) and MUA-cyt *c* (C) adsorbed onto bare gold electrodes in the absence (1) or presence (2) of "in situ" generated gold nanoparticules. (Right) Schematic representation of cyt *c* adsorbed on gold. Experimental conditions: Scan rate 0.05 V s^{-1}; PBS as supporting electrolyte; reference electrode: Ag/AgCl.

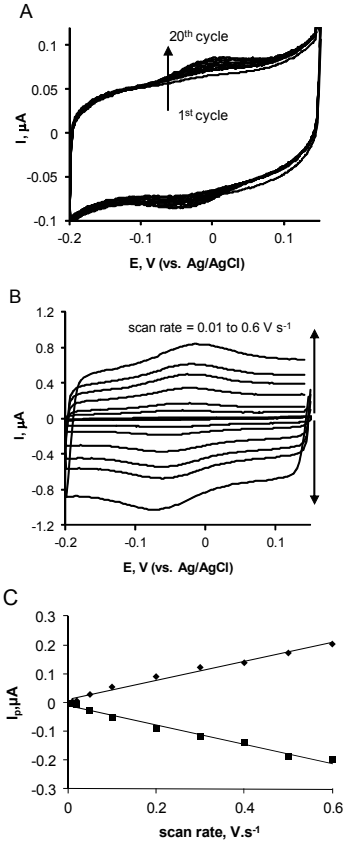

Fig. 2: (A) Cyclicvoltammograms obtained with gold electrodes in contact with MPA-cyt *c* solution (5 µM) for 20 cycles at υ = 0.05 V s^{-1}; B) Cyclicvoltammograms of MPA-cyt *c*/Au electrode at different scan rates ranging from υ = 0.01 to 0.6 V s^{-1}; C) Typical peak intensity dependence on the scan rate plot, from the experimental data in panel of B). Experimental conditions: PBS as supporting electrolyte; reference electrode: Ag/AgCl.

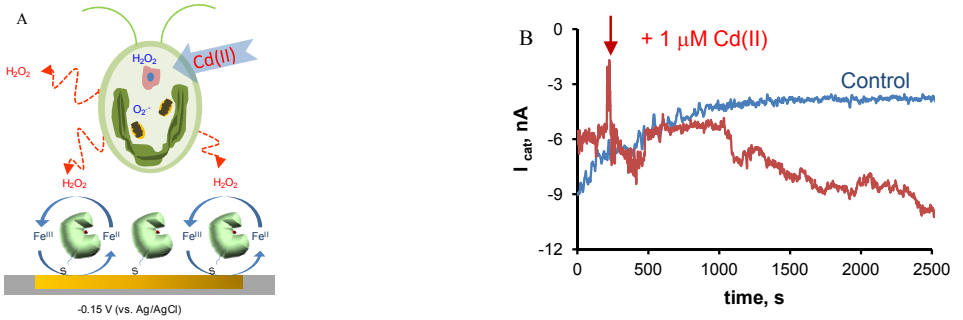

Fig 3: A) Schematic representation of extracellular detection of H_2O_2 released by *Chlamydomonas reinhardtii* upon Cd(II)-induced oxidative stress using the MPA-cyt *c*/Au biosensor configuration. B) Amperometric real-time detection of H_2O_2 as a response to Cd(II) addition into the sensing chamber (indicated by arrow). Experimental conditions: E_{app} = -0.15 V (vs. Ag/AgCl); *C. reinhardtii*: 10^6 cells mL^{-1} in HEPES 10 mM pH 7.4.

Finally, the as-prepared electrode poised at E_{app} = -0.15 V (vs. Ag/AgCl) was used to detect endogeneous H_2O_2 produced and released by phytoplanktonic cells, *Chlamydomonas reinhardtii*, upon addition of 1 µM of Cd(II). The progressive increase of the cathodic current provides evidence of the presence of H_2O_2 that oxidized MPA-cyt *c* at the electrode surface (*Fig. 3*). Such an easy-to-make biosensor is an adapted tool for non-invasive and real-time detection of extracellular H_2O_2 that can be used as a measure of the oxidative status in aquatic microorganisms.

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Corresponding author: Guillaume Suárez, EPFl – STI – IMT – NAM / ELG 237 / Station 11 / CH-1015 Lausanne / Switzerland, Tél: +41 21 693 68 71, Fax: +41 21 693 26 14, guillaume.suarez@epfl.ch