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Short communication

Biosensor based on chemically-designed anchorable cytochrome *c* for the detection of H_2O_2 released by aquatic cells

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ABSTRACT

A novel third generation biosensor was developed based on one-shot adsorption of chemically-modified cytochrome *c* (cyt *c*) onto bare gold electrodes. In contrast to the classic approach which consists of attaching cyt *c* onto an active self-assembled monolayer (SAM) priory chemisorbed on gold, here short-chain thiol derivatives (mercaptopropionic acid, MPA) were chemically introduced on cyt *c* protein shell via its lysine residues enabling 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 heterogeneous ET rate constant was estimated to be $k_s = 1600\ s^{-1}$, confirming that short anchors facilitate the ET via an efficient orientation of the heme pocket. In comparison, no ET was observed in the case of native and long-anchor (mercaptoundecanoic acid, MUA) modified cyt *c* directly adsorbed on gold. However, in both cases the ET was efficiently restored upon in-bulk generation of gold nanoparticles which acted as electron shuttles. This observation emphasizes that the lack of electroactivity might be caused by either an inappropriate orientation of the protein (native cyt *c*) or a critical distance (MUA-cyt *c*). Finally, the sensitivity of the bare gold electrode directly modified with MPA-cyt *c* to hydrogen peroxide (H_2O_2) was evaluated by amperometry and the so-made amperometric biosensor was able to perform real-time and non-invasive detection of endogeneous H_2O_2 released by unicellular aquatic microorganisms, *Chlamydomonas reinhardtii*, upon cadmium exposure.

1. Introduction

Over the last two decades amperometric biosensors based on the enzymatically-catalyzed reduction of H_2O_2 have attracted a broad interest caused by their accurate sensitivity and specificity. In such an approach, the analytical performance of the biosensor is strongly determined by the electron transfer (ET) efficiency between the electrode material and the immobilized heme-containing protein that exhibits perodixase (or pseudo-) activity. Therefore, in the case of the so-called third generation biosensors which rely on direct ET the ability to achieve an efficient electrical communication resorts to orientation and distance issues between the electrode surface and the protein redox center (Gorton et al., 1999). Among the large hemoprotein family, cytochrome *c* (cyt *c*) is a globular redox protein with 12.4 kDa molecular weight that is involved in cellular electron transfer pathways. Thanks to its pseudo-peroxidase activity cyt *c* has been used to detect H_2O_2 in biochemical assays (Vandewalle and Petersen, 1987) or electrochemical biosensors (Zhao et al., 2005). However, it is also known that cyt *c* exhibits a very low ET once in contact with solid bare electrodes which results in a poor electrochemical behavior presumably caused by protein denaturing (Allen et al., 1997). Thus, a number of studies have focused on the modification of the gold electrode surface in order to generate a suitable molecular environment that prevents cyt *c* from denaturing and enhances the ET rate constant. One approach consists of modifying the gold electrode surface with a chemisorbed self-assembled monolayer (SAM) of alkanethiols that exhibit a carboxylic headgroup at the solution interface (Tanimura et al., 2002). In this case, the strategy relies on the attractive interaction taking place between the negatively charged carboxylates and the region at the vicinity of the heme pocket which contains five positively charged lysine residues (Nakano et al., 2007). The efficient orientation and immobilization of cyt *c* on the electrode via the promoter SAM results in an enhanced ET that is distance-independent for short chains and then decreases exponentially when the SAM length increases, as expected for a tunneling mechanism. The addition of mercaptoalcohol to form compact mixed SAM provides an even more favorable environment to display fast ET rate constants (Davis et al., 2008; Lisdat

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and Karube, 2002). Another approach relies on the use of small diameter (<5 nm) gold nanoparticles (AuNPs) with immobilized on the electrode surface as efficient electron relays that facilitate long-range cyt *c* interfacial ET. In the best cyt *c*–AuNP hybrid system described by Jensen et al. (2007) the ET rate constant is enhanced by one order of magnitude compared to the AuNP-free configuration . A similar effect has also been demonstrated in self-assembled multilayer configurations where cyt *c* undergoes fast ET once immobilized into a hybrid matrix made of AuNP (13 nm diameter) coated with thiolated ssDNA (Zhao et al., 2008). In general, most of the strategies applied to enhance cyt *c* ET rely on engineering the electrode surface with functional SAMs or more complex hybrid/polymeric architectures (Beissenhirtz et al., 2004; Dronov et al., 2008; Jia et al., 2002). However, despite the well standardized approach based on gold modification with functional SAMs prior protein attachment, the modification engineering of proteins by introducing reactive groups such as thiols or other tags, represents an important research area in vivid development (Suárez et al., 2007; Della Pia et al., 2011; McMillan et al., 2002; Holland et al., 2011; So et al., 2009). So far, this protein engineering is mainly based on genetic engineering techniques like site-directed mutagenesis or transpeptidase *Y* that enable the direct chemisorption of recombinants on bare gold. In that context, the possibility to modify the cyt *c* protein shell with promoters which facilitate its chemisorption and enhance ET rate constant has been scarcely reported. By analogy to the yeast iso-1-cyt *c* (cyt *c*) which contains a cystein in position C102 (Bortolotti et al., 2006; Heering et al., 2004), Andolfi et al. (2007) succeeded in replacing the side chain T102 of cyt *c* with a cystein via site-directed mutagenesis but the resulting mutant remained electroinactive.

Considering this prior art, our strategy reports a chemical alternative that relies on a thiol-modified cyt *c* which spontaneously forms, within 5 min, a chemisorbed electroactive layer that exhibits a very high heterogeneous ET rate constant. The introduction of thiol derivatives—via chemical coupling on cyt *c* lysine residues—that act as anchors on gold prevents protein denaturing and enables controllable "tuning" of the ET rate with the chain length. 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.

2. Materials and methods

2.1. Chemicals

N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 3-mercaptopropionic acid (MPA), 11-mercaptoundecanoic acid (MUA), 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), phosphate buffered saline (PBS), hydrogen peroxide, gold(III) chloride hydrate, cadmium ion standard solution (Cd(II)) and bovine heart cytochrome *c* (cyt *c*) were all purchased from Sigma-Aldrich (Buchs; Switzerland).

2.2. Electrochemical measurements

Cyclic voltammetric and amperometric measurements were performed on a model μStat 200 Bipotentiostat (DropSens, LLanera, Spain) using a conventional three-electrode electrochemical cell equipped with Ag/AgCl pseudo-reference electrode and a Pt-disk counter electrode. Either PBS or 10 mM HEPES buffer at pH 7.4 were used as supporting electrolyte.

2.3. Chemical modification of cyt c

The chemical modification of cyt *c* consisted of reacting its lysine residues with carboxyl-terminated thiol derivatives–either mercaptopropionic acid (MPA) or mercaptoundecanoic (MUA)–via carbodiimide/N-hydroxysuccinimide (EDC/NHS) activation. Typically, 0.5 M solutions of MPA or MUA were prepared into phosphate buffer saline (PBS) pH 7.0 and an equimolar amount of EDC and NHS were added under stirring. Then, 50 μL of this solution was mixed with 0.5 mL of 500 μM cyt *c* in PBS and left to react while stirring at room temperature. After 2 h, the protein was reduced by addition of ascorbic acid and then purified from reagents excess with a separation column (Sephadex G25; GE Healthcare Life Sciences) before recollection into PBS. The final cyt *c* concentrations were estimated by absorption measurements; stock solutions of 200 μM were prepared and stored at 4 °C.

2.4. Preparation of Au/cyt c electrodes

Prior to modification, gold surfaces were treated with hot "Piranha" solution (7:3 mixture of concentrated H_2SO_4 and 30% H_2O_2; caution, piranha reacts violently with organic compounds) for 2 h. Freshly cleaned gold planar electrodes (0.1 cm^{-2} active surfaces) were dipped into PBS electrolyte containing 5 μM of either native cyt *c*, MPA-cyt *c* or MUA-cyt *c* for an incubation time ranging from 5 to 60 min. Finally, all cyt *c*-modified electrodes were washed in PBS in order to remove any loosely attached protein and kept at 4 °C until use.

2.5. Cell culture conditions

The wildtype unicellular freshwater algae *C. Reinhardtii* were provided by the Canadian Phycological Culture Center. Prior to all experiments algal cells contained in Erlenmeyer flasks were grown exponentially in the "TAP" culture medium to their mid-exponential growth phase, harvested by gentle filtration, washed and recollected in 10 mM HEPES at pH 7.4. Cell numbers and surface areas were determined by a Coulter Multisizer III particle counter (Beckman-Coulter, Inc.) for each experimental run.

3. Results and discussion

The electrochemical behavior of the different cyt *c* attached onto gold is illustrated with the corresponding cyclicvoltammograms (1) in Fig. 1. As expected for native cyt *c* no faradaic peak developed on the capacitive current whereas a pair of well-defined peak, corresponding to the electrochemical oxidation and reduction of heme group, is visible for cyt *c*-MPA. On the other hand, the cyt *c*-MUA chemisorbed onto gold does not show any sign of ET between the redox center and the electrode. In this first stage it is safe to deduce that the introduction of short anchors (three carbons chain) on the cyt *c* shell enables strong attachment to the bare gold surface and dramatically enhances its electrical communication.

However, the lack of electroactivity observed for native cyt *c* and MUA-cyt *c* might be caused by a combination of several critical factors which are: (i) weak attachment on gold leading to a low surface coverage; (ii) protein denaturing due to unfolding interactions on gold; (iii) high protein shielding caused by inappropriate orientation; and (iv) inefficient ET due to excessive distance between the heme group and the electrode surface.

Therefore, an experiment was carried out in order to elucidate which factors determine the electrochemical behavior of the different electrodes configurations. In other words, the main objective here was to connect efficiently all cyt *c* immobilized at

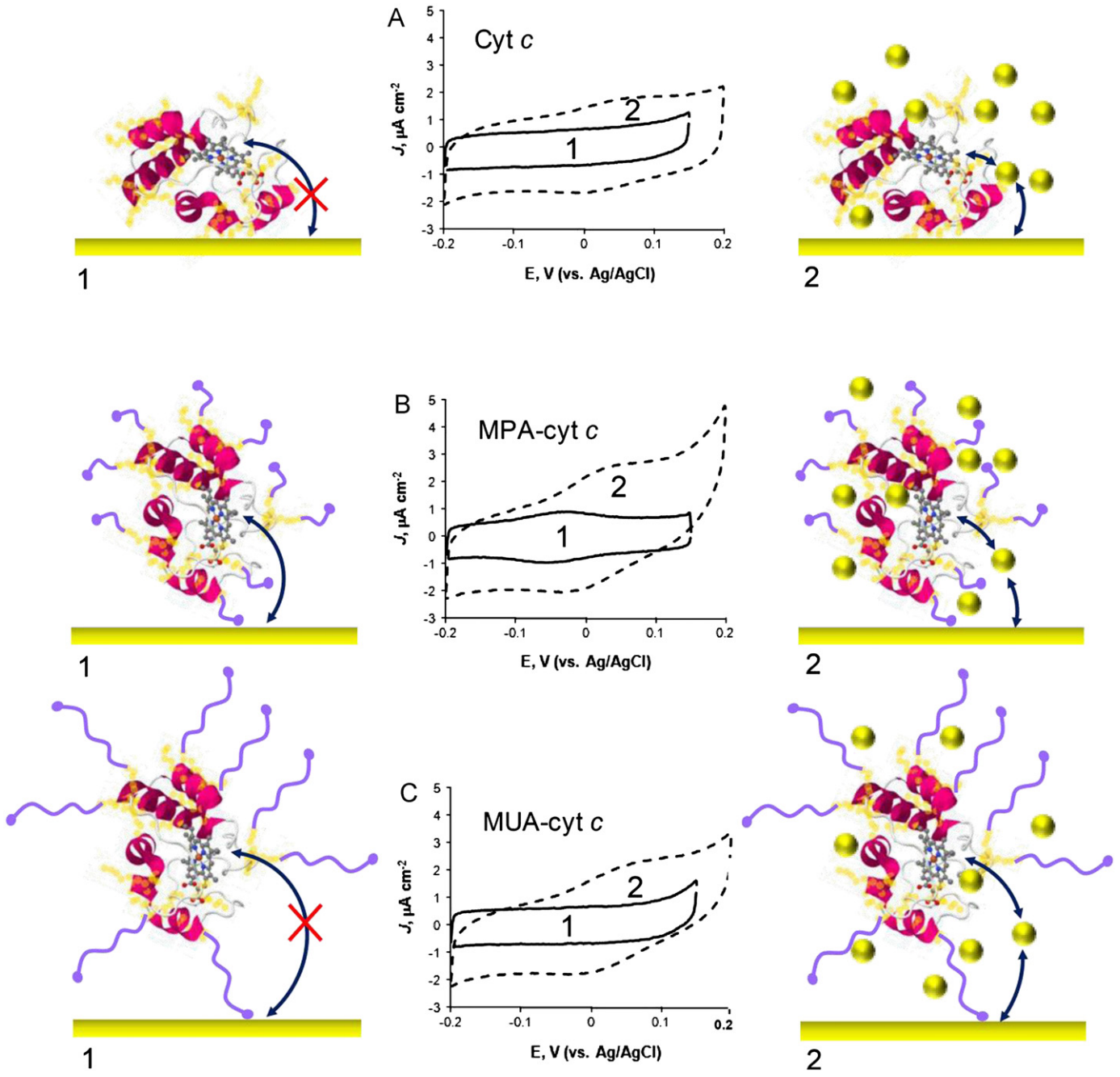

Fig. 1. (Center) Cyclicvoltammograms obtained for native cyt *c* (A), MPA-cyt *c* (B) and MUA-cyt *c* (C) adsorbed onto bare gold electrodes in the absence (1) or presence (2) of "in-situ" generated gold nanoparticles that act as electron shuttles. (Right and left) Corresponding schematic representation of cyt *c* adsorbed on gold (Lys residues in orange; secondary structure in red and white; MPA or MUA anchors in purple). Black arrows represent the electron transfer between heme group and electrode surface. Experimental conditions: scan rate: 0.05 V s^{-1}; supporting electrolyte: PBS; and reference electrode: Ag/AgCl.

the electrode surface independently from their orientation/distance via the addition of electron shuttles in the electrolyte. To that end "in-situ" synthesis of AuNPs was initiated directly in the electrochemical cell upon addition of 100 μM $HAuCl_4$ and 1 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and the cyclicvoltammetry was performed 15 min later. The HEPES-induced reduction of auric salt into AuNPs (Xie et al., 2007) was preferred to the more conventional ascorbic acid method since it has no effect on the redox state of the heme group. The ability of gold nanoparticles to shorten the distance between metalloproteins redox center and the electrode surface has been well reported elsewhere (Abad et al., 2009; Jensen et al., 2007). A critical parameter to achieve an efficient AuNP-assisted electron transfer (ET) is the dimension of the nanoparticle that should be abled to access the heme pocket with no major steric hindrance. Therefore, the "in-situ" generation of AuNPs represents a more opportunistic configuration where particles are formed from seeds to clusters, which then grow up into bigger nanoparticles. In that case, we ensured to generate particles with the appropriate size to make their way to the heme pocket and contribute to connect it to the gold surface. As expected, the presence of AuNPs acting as electron relays between cyt *c* and the electrode strongly modified the cyclicvoltammograms previously reported.

As shown in Fig. 1(2), the presence of AuNPs induces a broadening of the capacitive current that reveals an increase of the electrode effective dimensions. Furthermore, for each

electrode configuration, a quasi-reversible pair of peaks is now clearly visible which evidences the presence of active cyt *c* at the gold surface. Those results bring unexpected information on the state of the different cyt *c* adsorbed on the electrode. Contrary to the common belief the lack of electroactive behavior of native cyt *c* cannot be exclusively attributed to the irreversible denaturing effect of physisorption on bare gold, since ET is recovered when free-diffusing AuNPs shuttle the electrons through the system. It is more likely that the direct physisorption favours an inappropriate orientation of the protein where the amino acid shell acts as a shielding structure that prohibits ET. An alternative explanation has to be found in the case of MUA-cyt *c* where the distance between the redox center and the electrode surface is increased with longer anchors. Here the heme-surface distance is certainly the limiting factor for an interfacial ET mechanism to take place. However, diffusing AuNPs shortcut the critical distance and the ET rate constant becomes a combination of interfacial ET mechanisms between cyt *c* and AuNP and a diffusion-limited ET enabled by the AuNP/electrode system (Abad et al., 2009; Jensen et al., 2007). In the case of MPA-cyt *c* chemisorbed on gold the more noticeable change observed in the voltammetric response after generation of AuNPs is an increase of the peak separation indicative of a slower ET rate constant. This result confirms that fast interfacial ET mechanisms are predominant for electroactive MPA-cyt *c* as a result of a favorable orientation on gold. Once AuNPs are present in the system the badly oriented MPA-cyt *c* also contributes to the electrical response, which then enters a diffusion-limited ET regime.

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 min (20 cycles at $\upsilon = 0.05\ V\ s^{-1}$), as shown in Fig. 2A. Moreover, the analysis of the peaks intensity shows a reversible electrochemical behavior with a value of $I_a/I_c = 0.96$. 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 (Laviron, 1982). The surface coverage of electroactive MPA-cyt *c* on gold was estimated to be $\Gamma = 4 \times 10^{-12}\ mol\ cm^{-2}$ which corresponds roughly to 25% of the theoretical value reported by Nakano et al. (2007) for a fully packed cyt *c* surface coverage. It is also observable that the formal potential $E^{0\prime} = -48$ mV (vs. Ag/AgCl) is negative-shifted as compared to native cyt *c* in solution, as expected for a covalently immobilized protein (Yue and Waldeck, 2005). 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 Fig. 2B show the influence of the scan rate both on the peak current intensity and the peak-to-peak separation. As depicted in Fig. 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 (Laviron, 1979). Considering a charge transfer coefficient $\alpha = 0.5$ 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 (de Groot et al., 2007) or a mixed SAM of MPA/mercaptoethanol (Davis et al., 2008).

The characterization of MPA-cyt *c* modified electrode was further extended in order to evaluate its analytical performances for H_2O_2 detection. The electrocatalytic current generated at the poised electrode surface ($E_{app} = -0.15$ V vs. Ag/AgCl) upon addition of increasing H_2O_2 concentration was measured via standard amperometric technique. The corresponding calibration curve (Supplementary information, Fig. S1) exhibits a linear dynamic range from 0 to 250 μM of H_2O_2 with a limit-of-detection evaluated at 1 μM ($S/N = 3$). Finally, the apparent Michaelis–Menten constant was determined using Hanes–Woolf plot and found to be $K_{app} = 33$ μM. This low value for K_{app} reflects the high H_2O_2 affinity exhibited by the immobilized MPA-cyt *c*.

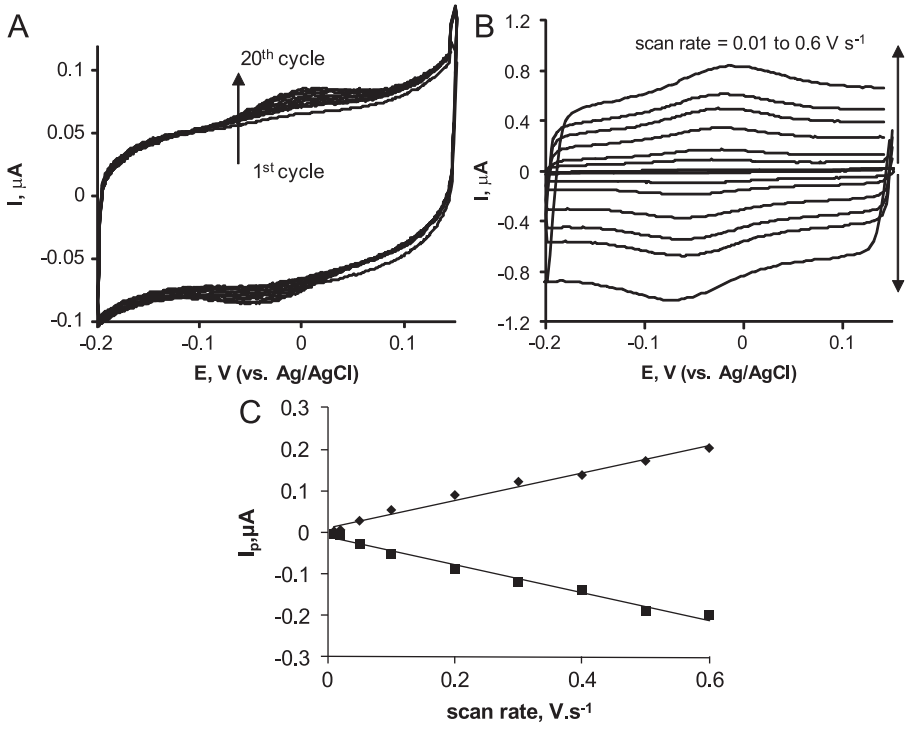

Fig. 2. (A) Cyclicvoltammograms obtained with gold electrodes in contact with MPA-cyt *c* solution (5 μM) for 20 cycles at $\upsilon = 0.05\ V\ s^{-1}$; (B) cyclicvoltammograms of MPA-cyt *c*/Au electrode at different scan rates ranging from $\upsilon = 0.01$ to $0.6\ V\ s^{-1}$; (C) Typical peak intensity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

McNeil et al. have demonstrated in several works that cyt *c*-based amperometric biosensors can efficiently perform real-time detection of free radical superoxide produced by stimulated cell lines (Henderson et al., 2009; Manning et al., 1998). However, despite being fast, specific, and sensitive, this technique remained largely under-use in the field of ecotoxicology. Here, the developed biosensor was further applied for real-time detection of H_2O_2 overproduction in aquatic microorganisms upon cadmium addition. *C. reinhardtii* was chosen as a model green microalga representative of the freshwater phytoplankton. The effect of Cd(II) on *C. reinhardtii* has already been studied with techniques involving standard ROS-specific probes (Szivak et al., 2009) or proteomic/genomic analysis (Hutchins et al., 2010; Simon et al., 2008). The presence of sub-lethal concentrations of Cd(II) induces an overproduction of ROS and, as a consequence, the level of antioxidants becomes up-regulated (Hanikenne, 2003; Simon et al., 2008). In the case of phytoplanktonic cells such as *C. reinhardtii*, the protective role played by the additional cell-wall prevents the most reactive ROS that are the oxygen and nitrogen radicals to reach the extracellular environment. Consequently, only H_2O_2 excess is able to diffuse through the cell-wall into the extracellular environment. To explore the capabilities of this novel biosensor to detect the changes of H_2O_2 concentrations in the extracellular environment, bare gold electrodes were modified with MPA-cyt *c* via a fast incubation step (15 min) and then rinsed. Suspension containing 10^6 cells mL^{-1} *C. reinhardtii* in the mid-exponential growth phase in 10 mM HEPES at pH 7.4 were prepared. Aliquots of 30 μL of algal suspensions were placed into the sensing chamber. The amperometric measurements were performed at $E_{app} = -0.15$ V (vs. Ag/AgCl) and at room temperature. In Fig. 3B, the current response profile in absence of Cd(II) is stable and caused by the capacitive current. Addition of 1 μM of Cd(II) results in a rapid and a progressive increase of the cathodic current which provides evidence for the presence of H_2O_2 that oxidizes MPA-cyt *c* at the electrode surface. This result clearly demonstrates that the developed biosensor is a well suited tool for non-invasive and real-time detection of endogeneous H_2O_2 in the extracellular medium; it can be used to measure the oxidative status of phytoplankton cells non-invasively.

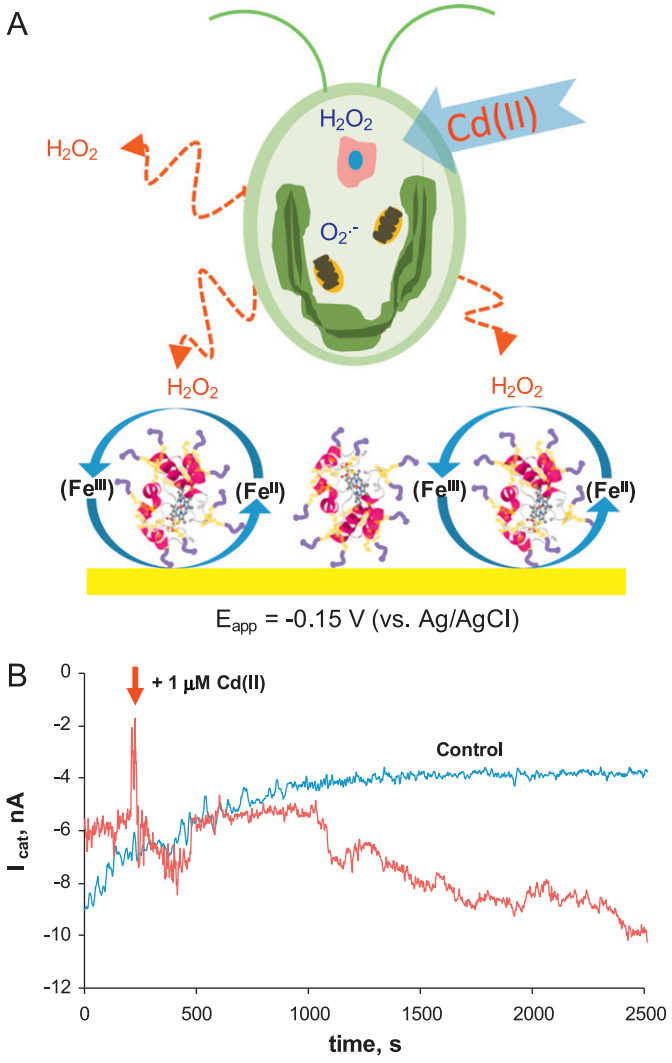

Fig. 3. (A) Schematic representation of extracellular detection of H_2O_2 released by *C. reinhardtii* upon Cd(II)-induced oxidative stress using the MPA-cyt *c*/Au biosensor. (B) Amperometric real-time detection of H_2O_2 as a response to Cd(II) addition into the sensing chamber (indicated by the arrow).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

4. Conclusion

The chemical introduction of thiol derivatives in the cyt *c* structure enables its fast chemisorption (< 5 min) onto bare gold electrodes. Most importantly, the self-adsorbed cyt *c* shows a quasi-reversible electrochemical behavior with an estimated heterogeneous ET rate constant particularly high ($k_s = 1600\ s^{-1}$). This novel third generation biosensor displays reliable analytical performances for H_2O_2 detection and exhibits a linear response up to 250 μM. Using the developed biosensor it has been possible to detect in real-time and non-invasively the H_2O_2 overproduced by phytoplanktonic cells in response to Cd(II) exposure. Furthermore, the one-shot biofunctionalization procedure demonstrated here might also be useful for a broad variety of gold transducers, as well as for many biosensing applications.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2012.10.083.

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