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# Creation of a gold nanoparticle based electrochemical assay for the detection of inhibitors of bacterial cytochrome *bd* oxidases

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ABSTRACT

Cytochrome *bd* oxidases are membrane proteins expressed by bacteria including a number of pathogens, which make them an attractive target for the discovery of new antibiotics. An electrochemical assay is developed to study the activity of these proteins and inhibition by quinone binding site tool compounds. The setup relies on their immobilization at electrodes specifically modified with gold nanoparticles, which allows achieving a direct electron transfer to/from the heme cofactors of this large enzyme. After optimization of the protein coverages, the assay shows at pH 7 a good reproducibility and readout stability over time, and it is thus suitable for further screening of small molecule collections.



## 1. Introduction

Cytochromes *bd* oxidases are respiratory membrane proteins expressed by several pathogens such as *Escherichia coli*, *Mycobacterium tuberculosis* and *Klebsiella pneumonia* [1,2]. They seem to play a crucial role in the protection against oxidative stress [3,4] as well as in the virulence, adaptability and resistance of these bacteria. Therefore, they are a novel potential target for the discovery of novel inhibitors with different mode of action [5–7]. A better understanding of the common features and differences in the catalytic mechanisms of cytochrome *bd* oxidases and homolog terminal oxidases found in mammals will allow the selective inhibition of important bioenergetic pathways of the pathogens named above.

Unlike mitochondrial heme-copper terminal oxidases, cytochrome *bd* oxidases use quinols as substrates and they do not act as proton pumps. They contribute to the generation of a transmembrane potential by taking the four protons required for $O_2$ reduction from the cytoplasmic side of the membrane and transferring the protons released from quinol oxidation to the periplasmic side. Cytochrome *bd* oxidases are also characterized by their high affinity for $O_2$ and their relative insensitivity to inhibition by cyanide [8–10]. Their first X-ray crystal structure has just been solved [11]. They contain three heme centers, namely heme *d*, heme $b_{558}$ and heme $b_{595}$. The role of heme $b_{595}$ in substrate binding and catalysis is still largely debated [12–17]. A hydrophilic loop (known as the "Q-loop") connecting transmembrane helices 6 and 7 in subunit I is believed to be involved in quinone binding [18–21].

A reaction mechanism was suggested on the basis of kinetic analyses and detection of intermediates [22–25]. Under normal turnover conditions, the enzymes exist mainly in a one-electron reduced state $A_1$ and is bound to $O_2$. Upon transfer of electrons from a first molecule of quinol $QH_2$, a short-lived three electron reduced adduct $A_3$ is formed, which eventually evolves into an oxoferryl state F probably through a peroxy intermediate P [26]. The transfer of electrons from a second molecule of quinone regenerates a one-electron reduced form of the enzyme.

Cytochrome *bd* catalytic activity is measured by following oxygen consumption of either homogenized membrane samples containing the enzyme or pure protein samples solubilized with detergent [17,22,27]. For these measurements, a Clark electrode is often used. The reactions are initiated by adding ubiquinol-1 or *N*,*N*,*N*′,*N*′-Tetramethyl-*p*-Phenylenediamine (TMPD) substrates. Sacrificial donors such as dithiothreitol or ascorbate respectively are used to maintain the substrates in the reduced state. These studies in solution are somewhat complicated by the high dependence of cytochrome *bd* activity and substrate affinity on the detergent or lipids required for solubilization [27]. Immobilization of the protein on the surface of electrodes for direct electrochemical analysis of enzyme catalytic activity is thus suggested here as an alternative as long as direct electron transfer to/from the heme cofactors of

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this large enzyme can be achieved [28–32]. For this purpose, the use of metal nanoparticles (NPs) is of great interest [33–35]. In 2003 Xiao et al. have shown that gold NPs can act as an efficient relay in the long range electron transfer between the electrode and the flavine cofactor of glucose oxidase [36]. Since this pioneering work, gold NPs have been used to improve the electrochemical response of various soluble proteins such as galactose oxidase [37], laccase [38], myoglobin [39], hemoglobin [39], and cytochromes *c* [39–41]. Recently, we have shown that modified gold NPs also allow the study of the electron transfer processes and the catalytic reaction of membrane proteins, including various terminal heme-copper oxidases, like cytochrome $bo_3$ [42], cytochrome $ba_3$ and cytochrome $aa_3$ oxidases [43,44].

Due to its complex structure and function, several groups of inhibitors of cytochrome *bd* oxidases can be considered: inhibitors of the $O_2$ binding site, inhibitors of the quinone binding site, and lipid packing perturbators. It is noted that quinone binding sites, albeit found in many enzymes, are often specific to each type of enzymes [45,46]. They thus constitute interesting targets in drug research [47,48]. A few selective inhibitors of the quinone binding site of cytochrome *bd* oxidases have already been reported, such as aurachin D [5] and prenylphenols isolated from fungi [7].

We describe herein a setup based on three-dimensional gold NPs electrodes for the study of *E. coli* cytochrome *bd* oxidase catalytic activity. Optimization of the assay conditions, such as enzyme quantity, quinone concentration and pH is presented. This system is then applied to the detection of inhibition with a high affinity quinone-binding site inhibitor. To the best of our knowledge, it is the first use of direct electrochemical methods for the functional characterization and inhibition of these membrane proteins.

## 2. Materials and methods

### 2.1. Chemicals

Sodium citrate, hydrogen tetrachloroaurate trihydrate, 6-mercaptohexan-1-ol, 1-hexanethiol, potassium phosphate dibasic trihydrate and ubiquinone-10 were purchased from Sigma and *N*-oxo-2-heptyl-4-Hydroxyquinoline (HQNO) from Enzo Life Sciences. These chemicals were used without further purifications.

### 2.2. Protein samples preparation

Wild type cytochrome *bd* oxidase from *E. coli* was purified as previously described [49]. Before immobilization on electrodes, the concentration of *n*-dodecyl β-D-maltoside in the sample solution was decreased by washing 30 μL of the protein stock solution with 250 μL of 100 mM phosphate buffer (pH 7) devoid of *n*-dodecyl β-D-maltoside with a membrane filter of 50 kDa (AMICON). The protein concentration was determined by UV/visible spectroscopy, from the heme $b_{558}$ band at 561 nm ($\Delta\varepsilon_{561-580} = 21\ mM^{-1}\cdot cm^{-1}$) [50] in the dithionite-reduced minus air-oxidized spectrum. The residual quinol content was estimated by redox-induced FTIR difference spectroscopy, on the basis of the C-$OCH_3$ mode previously identified at 1264 $cm^{-1}$ ($\Delta\varepsilon_{1264} = 0.4\ mM^{-1}\cdot cm^{-1}$) [51,52]. For studies in the presence of ubiquinone, the protein solution was further incubated for 4 h at 4 °C with 20 μM of ubiquinone-10.

### 2.3. Electrode preparation

Gold NPs of 15 nm average diameter were obtained by the procedure of Turkevich et al. [53] and Frens [54]. Briefly, a sodium citrate solution (39 mM, 13 mL) was added to a boiling solution of $HAuCl_4$ in water (1 mM, 125 mL). After appearance of the red color, the solution was maintained under boiling for 10 min and then cooled down to room temperature. Three-dimensional gold NPs electrodes were prepared by drop casting on a gold disk electrode as previously described [41,43] and were modified with a (1/1) mixture of 6-mercaptohexan-1-ol and 1-hexanethiol. Then 5 μL of protein solution were deposited on the modified electrode surface and left overnight under argon at 4 °C. The voltammetric measurements were performed in a standard three electrode cell connected to a Princeton Applied Research VERSASTAT 4 potentiostat. A Ag/AgCl (3 M NaCl) electrode was used as reference and a platinum wire as counter electrode. All the potentials mentioned here are referred to a standard hydrogen electrode (SHE). For noncatalytic experiments, the cell was flushed with argon for 20 min prior to measurements.

### 2.4. Inhibition studies

Inhibition of the protein was carried out by adding 1–70 μL aliquots of a 20 mM solution of HQNO in dimethyl sulfoxide (DMSO) to the 20 mL initial phosphate buffer solution. An equilibration time of 10 min was required before the measurement. In control experiments the same volume of DMSO only was added. The $IC_{50}$ value was determined from the plots of the residual catalytic current vs HQNO concentration.

### 2.5. Surface-enhanced infrared absorption spectroscopy (SEIRAS) studies

The SEIRAS characterization was performed using a Bruker Vertex 70 spectrometer equipped with a Harrick ATR cell. The silicon internal reflection crystal was modified with gold by electroless deposition [55]. Then 5 μL of gold NPs solution were dropcasted on the surface and allowed to dry. The gold surface was modified with a 1 mM solution of 1-hexanethiol and 6-mercaptohexan-1-ol in EtOH. After 60 min, the solution was removed and the surface was rinsed with fresh EtOH. Finally, 2 μL of cytochrome *bd* oxidase solution were deposited on the surface. 256 scans with 4 $cm^{-1}$ resolution were averaged for each spectrum. Residual absorption of water gas was subtracted and the spectra were smoothed with nine points.

## 3. Results and discussion

### 3.1. Assay development and characterization

The optical properties of gold NPs allow characterizing the successive electrode modification steps by SEIRAS. This technique probes the molecules directly attached to the gold interface that are as close as 10 nm [56,57]. The gold NPs exhibit bands at 1407, 1601 and 1702, (see Fig. 1) which were attributed to the carboxylate groups of the

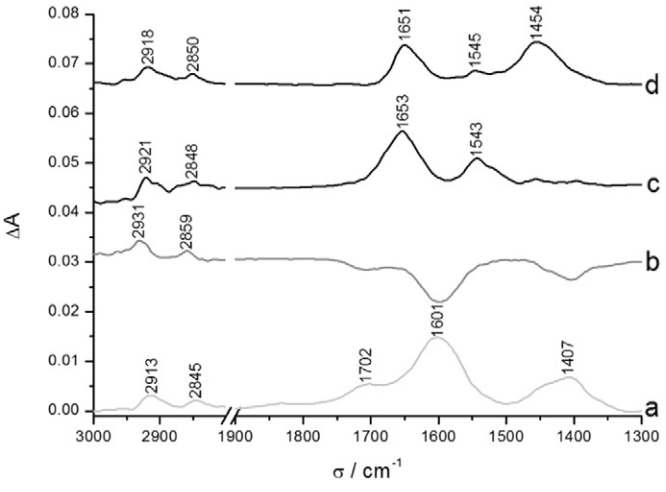


**Fig. 1.** SEIRAS difference spectra obtained after deposition of gold NPs (spectrum a), incubation with a (1/1) mixture of 6-mercaptohexan-1-ol and 1-hexanethiol (spectrum b), and then immobilization of cytochrome *bd* oxidase. Spectrum c was recorded in $H_2O$ (pH 7) and spectrum d in $D_2O$ (pD 7).