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An Optimal Surface Concentration of Pure Cardiolipin Deposited Onto Glassy Carbon Electrode Promoting the Direct Electron Transfer of Cytochrome-*c*

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Abstract

Pure cardiolipin deposit onto electrodes is optimized and shown to yield an efficient supported lipid film for promoting cytochrome-*c* immobilization and electroactivity. Cyclic voltammetry and electrochemical impedance spectroscopy measurements in an aqueous electrolyte with potassium ferri- and ferrocyanide as a redox probe evidence that an optimized pure cardiolipin film is reached for a 7 μg cm^{-2} deposit onto glassy carbon electrode. At this optimized surface concentration the pure cardiolipin deposit yields the most compact and less permeable supported lipid film on electrode surface. The thickness and the organization of the pure cardiolipin films were analyzed by atomic force microscopy (AFM) measurements. AFM imaging in aqueous buffer shows that the lipid deposit onto the surface forms a thick deposit of approximately 30 ± 10 nm of height with 4 nm average roughness and includes

defects. Cytochrome-*c* electroactivity was studied with the redox protein either in solution or immobilized onto the modified electrode. First, the optimized amount of pure cardiolipin was deposited onto glassy carbon electrodes to study the stable and electrochemically quasi-reversible redox system of cytochrome-*c* in solution. Then, the potential cycling of a pure cardiolipin-modified glassy carbon electrode in a cytochrome-*c* solution led to the immobilization of the protein in its native state keeping intact its electrochemical properties, and with a surface coverage of 8 pmol cm^{-2} corresponding to 50% of a monolayer.

Keywords. Cardiolipin, Cytochrome-*c*, Supported Lipid Deposit, Cyclic Voltammetry, Electrochemical Impedance Spectroscopy, Atomic Force Microscopy.

1. Introduction

Cardiolipin is a phospholipid first isolated from beef heart in 1942[1] and found to be present as a small fraction (ca. 10-20%) of the inner membrane of mitochondria.[2] Cardiolipin is closely associated to the bioenergetic processes.[3, 4] Its specific function arises from its unique dimeric structure containing four unsaturated alkyl chains and an overall net negative charge at neutral pH (Figure 1).[5] In addition to its key role for maintaining optimal activity of numerous mitochondrial processes such as electron transfer in the respiratory chain, cardiolipin is also involved in the initiation of the cell apoptosis machinery in particular by forming a peroxidase complex with cytochrome-*c*.[2, 4, 6-14] Indeed a direct relationship between cardiolipin loss and cytochrome-*c* release into the cytoplasm was identified as an initial step in the pathway to apoptosis.[2] The specific and selective interaction between cardiolipin and cytochrome-*c* is not well understood and has been assigned to a combination of electrostatic and hydrophobic effects, hydrogen bonding and/or the formation of a cardiolipin/cytochrome-*c* complex.[2, 6-12, 15-18]

The electrochemical behavior of redox proteins such as cytochrome-*c*, a water-soluble haemoprotein involved in the respiratory chain of mitochondria, has been extensively reported since 1977.[19-22] This model redox protein has often been studied at lipid-modified electrodes because the detection of its electroactivity is seldom possible at bare electrode surfaces even with large scanning potential range.[23-26] This is ascribed to adsorption, structural alteration (denaturation) of the protein at the electrode which prevents or slows down electron transfer.[25, 26] Several surface modification strategies have been used to provide a suitable environment for preserving the native protein structure and promoting electron transfer. In this context, the deposit of a mixed-lipids bilayer onto the electrode surface, mimicking the periplasmic interface of the inner membrane of mitochondria,[3] was demonstrated to be particularly relevant.[25-28]

Different methods can be successfully employed to form supported lipid bilayers including the Langmuir-Blodgett technique,[26] spin coating of lipid solution,[26, 29] solvent or droplet evaporation [30, 31] or fusion of lipid vesicles at the electrode surface.[32] In most cases, the presence of a fraction of cardiolipin in the supported mixed-lipids bilayer has been demonstrated to be an important factor for the efficient detection of cytochrome-*c* electroactivity at carbon electrodes.[26, 29, 31] Indeed most of the lipid films investigated to date were mainly composed of phosphatidylcholine associated with a small fraction of cardiolipin. We note that phosphatidylcholine alone is ineffective for the detection of the cytochrome-*c* electroactivity.[29, 33, 34] To the best of our knowledge however, the electrochemical characterization of a modified electrode with a pure cardiolipin film has never been reported so far except on one occasion with a very high loading of cardiolipin (700 μg cm^{-2}) in the absence and in the presence of a cholesterol under-layer.[26] From this literature overview, it is evident that the role of the cardiolipin/cytochrome-*c* interaction is crucial for electron transfer in the respiratory chain or at an electrode. Nevertheless no previous study focused on the relation between the structure and the organization of supported pure cardiolipin films and the electrochemical behavior of cytochrome-*c* at the corresponding modified electrodes. In addition, the deposition of a pure cardiolipin film by solvent evaporation is a much more straightforward experimental procedure compared with the preparation of a well-defined supported mixed-lipids bilayer.

We report herein the use of pure cardiolipin (without any other lipids) deposited in optimized surface concentrations by solvent evaporation at the glassy carbon electrode for promoting the electroactivity of cytochrome-*c* immobilized in its native state. We focus on the organization of an optimized amount of pure cardiolipin on a glassy carbon electrode rather than on the preparation of typical mixed-lipids bilayers. The properties of the optimized pure cardiolipin deposit is investigated with respect to cytochrome-*c* electroactivity and

immobilization. First the permeability and compactness of a supported pure cardiolipin film is studied by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) in the presence of a redox probe (ferri/ferrocyanide) as a function of the amount of lipid deposited. Then, the organization and the thickness of the optimized supported pure cardiolipin film is analyzed by atomic force microscopy (AFM) in liquid conditions and compared with a different pure lipid deposit (1,2-dipalmitoylphosphatidylcholine, DPPC). The optimized pure cardiolipin loading at a glassy carbon electrode is finally tested for cytochrome-*c* electroactivity with the protein in solution or immobilized onto the lipid deposit.

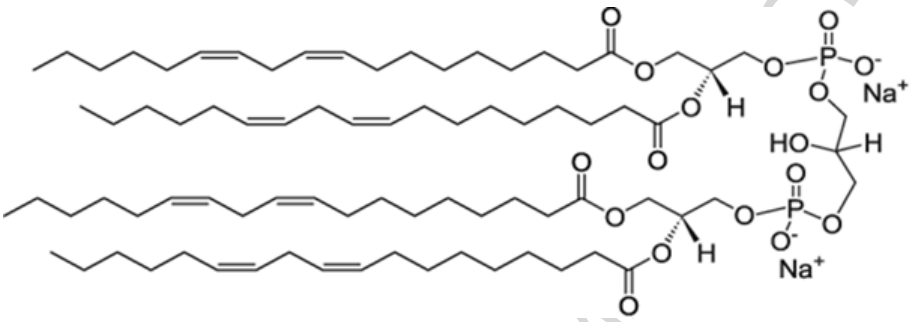

Figure 1. Chemical structure of cardiolipin.

2. Material and methods

2.1. Reagents

All solutions were prepared with Milli-Q water (18.2 MΩ cm^{-1}). Cardiolipin solution from bovine heart (~ 4.7-5.3 mg/mL in ethanol, ≥97%) containing >80% polyunsaturated fatty acid content, primarily linoleic acid, and cytochrome-*c* from equine heart (≥95%) were obtained from Sigma Aldrich and stored in a freezer (-18°C). 1,2-dipalmitoylphosphatidylcholine (DPPC) lipids were purchased in powder from Avanti Polar Lipids and stored in a freezer. Potassium ferrocyanide trihydrate (99+%) was purchased from Acros Organics and potassium hexacyanoferrate (III) (~99%) from Sigma Aldrich. Sodium hydrogen phosphate anhydrous ACS (99.0% min) and potassium dihydrogen phosphate ACS (99.0% min) from Alfa Aesar were used to prepare the 10 mM aqueous phosphate buffer pH

7. Anhydrous absolute ethanol from Carlo Erba Reagents was used to prepare a cardiolipin solution of 0.2 g/L by dilution of the commercial solution and a DPPC solution of 0.3 g/L.

2.2. Preparation of cardiolipin-modified electrodes

The cardiolipin film was obtained by solvent (droplet) evaporation on glassy carbon disk electrodes (3 mm diameter) obtained from BASi. Small volumes between 1 and 10 μL of cardiolipin solution diluted at 0.2 g/L were deposited on the electrode surface with a micropipette to obtain a total amount of deposited lipids between 0.2 and 2.0 μg. Ethanol was then left to evaporate under air. After the complete evaporation of ethanol the dry electrode was immediately dipped into the aqueous electrolyte.

2.3. Electrochemical measurements

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were performed in a three-electrode cell with a glassy carbon electrode as the working electrode. The working electrode was polished on silicon carbide paper (4000-grid SiC paper, Struers), rinsed with ultra-pure water and sonicated in ultra-pure water for 5 minutes before each cardiolipin deposition or experiment. All potentials are reported versus an Ag/AgCl, KCl 3 M reference electrode. A platinum wire was used as a counter electrode. Electrochemical experiments were performed at room temperature (21 ± 3 °C) with an Autolab PGSTAT302N potentiostat/galvanostat (Eco Chemie B.V., the Netherlands) using Nova as the electrochemical software (Metrohm). All solutions were deaerated by bubbling argon for 20 minutes before each measurement. EIS measurements were performed at open circuit potential (OCP: +0.21 V) in the frequency range from 100 kHz down to 50 mHz with a signal amplitude of 10 mV, using an equimolar mixture of $K_3Fe(CN)_6/K_4Fe(CN)_6$ as the redox probe (total concentration of 10 mM). To immobilize cytochrome-*c* on the deposited cardiolipin

film, the electrode was immersed in a 0.15 mM cytochrome-*c* solution and cycled (10 cycles) between +0.3 and -0.2 V at a scan rate of 20 mV s^{-1}. Then, the electrode was gently washed with phosphate buffer solution and transferred to a cytochrome-free phosphate buffer electrolyte for cyclic voltammetry experiments.

2.4. Atomic Force Microscopy

Pyrolyzed photoresist film (PPF) are used as substrates for imaging the lipid deposits. The procedure followed for PPF preparation is reported in the Supporting Information.[35] The lipid deposit was obtained by solvent evaporation. An appropriate volume of lipid solution diluted at 0.2 g/L or 0.3 g/L was deposited on the carbon surface with a micropipette to obtain a surface concentration of deposited lipids of approximately 5 nmol cm^{-2}. After the complete evaporation of ethanol, droplets of 10 mM phosphate buffer aqueous electrolyte at pH 7 were added onto the dry modified PPF to keep the lipid film in contact with the liquid phase during AFM measurements. Two types of pure lipid deposits were studied by AFM: cardiolipin (CL) and 1,2-dipalmitoylphosphatidylcholine (DPPC).

AFM height images in liquid were obtained using a Nanoscope 8 Multimode AFM (Bruker) with triangular Si_3N_4 cantilevers (ScanAsyst-Fluid+) with a nominal spring constant of 0.7 N m^{-1}. The instrument was equipped with a "J" scanner (100 µm × 100 µm). To minimize the applied force on the sample the set point was continuously adjusted during imaging. Images were acquired with a scan rate of 1 Hz. All images were processed using the Nanoscope 8 software with the ScanAsyst-Fluid mode and were recorded at room temperature (21 ± 3 °C).

3. Results and Discussion

3.1. Optimization of the pure cardiolipin deposit

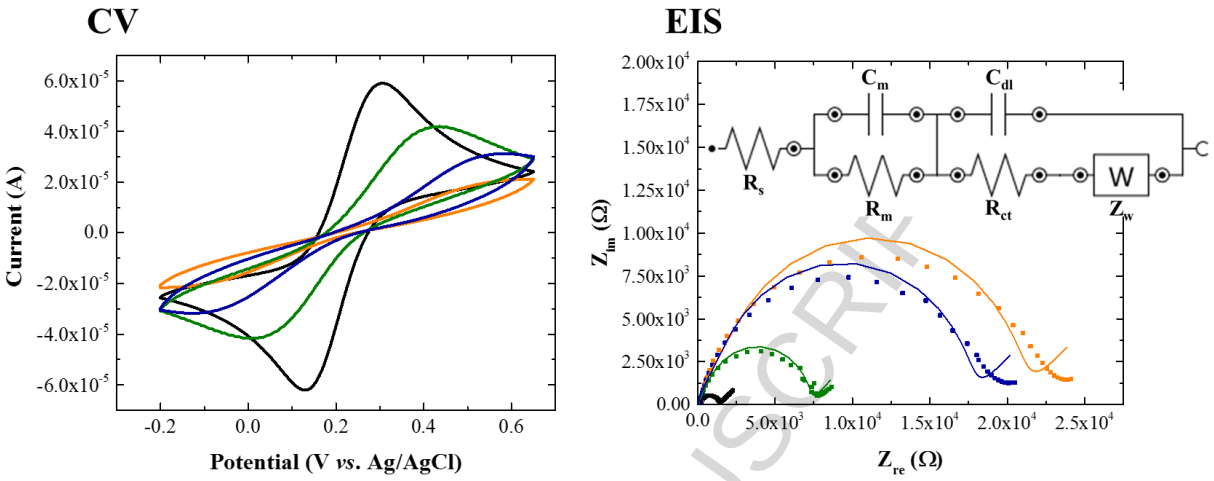

Figure 2. (***Left***) Representative CVs recorded at 50 mV s^{-1} and (***Right***) Nyquist plots with fit and simulation (equivalent circuit) from electrochemical impedance spectroscopy measurements recorded at OCP on glassy carbon electrodes unmodified (black) and modified by a 3 (green), 7 (orange) and 14 (blue) µg cm^{-2} cardiolipin deposit in $K_4Fe(CN)_6/K_3Fe(CN)_6$ (both at 5 mM) with 0.1 M phosphate buffer aqueous electrolyte at pH 7 under inert atmosphere (Ar).

In this section, the optimization of the pure cardiolipin loading onto glassy carbon electrode was performed. In order to optimize and evaluate the permeability and the thickness of supported pure cardiolipin films, three different lipid amounts have been deposited onto glassy carbon corresponding to 3, 7 and 14 µg cm^{-2} surface concentrations. These lipid loadings are in the range commonly used for lipid mixture deposition in the literature.[29] These modified electrodes were studied by CV and EIS in an aqueous redox probe solution of equimolar ferri-/ferrocyanide as presented in Figure 2. This redox probe is particularly sensitive to electrode surface treatment and is especially well-suited for evaluating the effect

of supported lipid deposits on electron transfer.[28-30, 32, 35-40] CV experiments (Figure 2, Left) show that the electron transfer is partially blocked at the cardiolipin-modified glassy carbon electrodes in comparison to the electrochemically reversible redox system observed at the unmodified glassy carbon electrode. Interestingly, this blocking effect is the highest with the intermediary 7 μg cm^{-2} cardiolipin deposit. EIS measurements (Figure 2, Right) confirm that the charge-transfer resistance is the largest at this modified electrode. The Nyquist plots show a charge-transfer resistance maximized for a cardiolipin deposit of 7 μg cm^{-2} onto glassy carbon electrode while it is lower for the 3 or 14 μg cm^{-2} deposits. This reproducible result (over three independent experiments) suggests that the organization of the cardiolipin film onto glassy carbon is optimized around 7 μg cm^{-2}, with a lower permeability and a more compact structure of the lipid deposit. However, in all cases the lipid deposit does not fully block the redox probe electron transfer probably because of the presence of defects in the supported lipid films and the formation of thick heterogeneous layers onto electrode surface. Note that a similar behavior with the same trend was also observed on cardiolipin-modified gold electrodes (Figure S1) and that CV and EIS experiments with non-negatively charged redox probes such as ferrocenemethanol or ruthenium hexaammine do not show differences between bare and modified electrodes.

A Randles circuit (insert, Figure 2, Right) commonly applied to simulate the EIS data of supported lipid bilayers was used to assess the capacitance and resistance values of cardiolipin deposits.[28, 30, 41-43] In the equivalent circuit, R_s represents the solution resistance, R_m the cardiolipin film resistance, C_m the cardiolipin film capacitance, R_{ct} charge-transfer resistance, C_{dl} the double-layer capacitance and Z_w the Warburg element. According to the values of equivalent circuit elements obtained by fitting the experimental data for cardiolipin-modified glassy carbon electrodes with the Nova 2.1 software, the 7 μg cm^{-2} cardiolipin-modified electrode exhibits the highest R_m and R_{ct}. The C_m value is directly

related to the thickness/packing density of the lipid film and the estimated capacitance per surface unit (electrode area 0.071 cm^2) are lower than those usually reported for classical supported lipid bilayers.[28, 30, 42, 44, 45] The lowest C_m value obtained for the 7 µg cm^{-2} deposit suggests a higher thickness of the supported cardiolipin film coupled to a specific compact structural organization. The experimental EIS data and the simulation (Figure 2, Right) do not show a perfect fit, confirming that the supported lipid film is not organized as a bilayer.

To demonstrate that an optimal surface concentration of pure cardiolipin leads to a more compact and less permeable lipid film on carbon surface with a structure different from a lipid bilayer organization, the aspect and the thickness of lipid deposits in fluid were further probed by atomic force microscopy (AFM) as discussed next.

3.2. Imaging of the lipid films with liquid AFM measurements

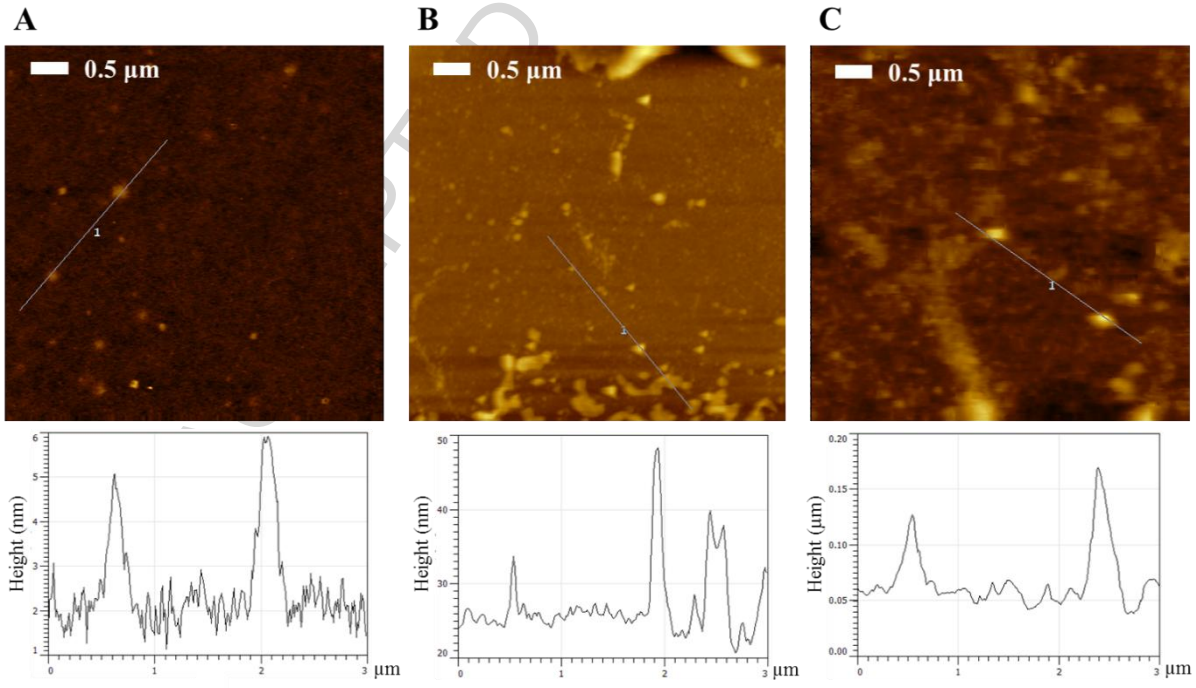

Figure 3. AFM height images (5 × 5 μm^2) recorded in 10 mM phosphate buffer aqueous electrolyte at pH 7 of a bare PPF (A), a PPF modified by 5 nmol cm^{-2} DPPC (B) and 5 nmol cm^{-2} CL (C) deposit.

For comparing the organization and the thickness of lipid deposits, three PPF samples were observed by atomic force microscopy in liquid (Figure 3): bare PPF surface and PPF surfaces modified by a 5 nmol cm^{-2} pure lipid deposit of either 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) or cardiolipin (CL). PPF samples are chosen because their structure is very close to that of glassy carbon, while exhibiting a very smooth surface suitable for AFM measurements. The lipid surface concentration deposited on PPF substrates corresponds to the optimized cardiolipin loading previously reached on glassy carbon electrode (~ 7 µg cm^{-2}). The bare PPF sample image is presented in Figure 3A where the approximate height is evaluated at 3 ± 1 nm and the roughness average is estimated at 0.3 nm, in agreement with this nearly atomically smooth carbon surface.[46] DPPC is a typical lipid used to form supported lipid bilayer, it bears two alkyl chains and is zwitterionic and neutral at pH 7. The main reason to use this lipid for comparison with cardiolipin is that DPPC phase transition temperature (40 °C) is comparable to that of cardiolipin (60 °C) and hence the lipids are in the same phase in our experimental conditions at room temperature. After the deposition of DPPC on PPF (Figure 3B), a heterogeneous film is observed with a height of 9 ± 4 nm and a roughness average of 1 nm. Several lipid agglomerates appear on PPF sample modified by the DPPC deposit with a random distribution. In addition the PPF surface seems only partially covered by the DPPC lipid deposit. This heterogeneous surface organization shows that the solvent evaporation method is not appropriate for the preparation of uniform supported lipid bilayers. Deposition of pure cardiolipin on PPF yields a lipid film with a higher thickness and surface coverage and the presence of defects (Figure 3C). Indeed, the approximate height of

cardiolipin deposit is estimated at 30 ± 10 nm for a roughness average at 4 nm. Despite the presence of defects and a high roughness, the cardiolipin deposit seems to completely cover the PPF surface. This huge difference between DPPC and cardiolipin deposits definitely confirms the absence of a lipid bilayer in both cases and suggests an organization of the cardiolipin film more voluminous and less even than classical supported lipid layers. Finally, these AFM images demonstrate that a small amount of cardiolipin deposited onto carbon surface leads to the formation of a thick and rough film able to partially block the electron transfer of a redox probe as discussed above. In order to evaluate the impact of the pure cardiolipin deposit toward the electrochemical activity of cytochrome-*c*, cyclic voltammetry measurements were recorded at a cardiolipin-modified glassy carbon electrode.

3.3. Cytochrome-*c* electroactivity in solution and immobilized onto the electrode

In this section, two efficient methods easy to perform are presented for studying the cytochrome-*c* electroactivity at a glassy carbon electrode modified by the optimized supported cardiolipin deposit. The protein is first detected in solution (Figure 4A). Then, by potential cycling in the cytochrome *c* solution, the redox protein is immobilized on the cardiolipin-modified glassy carbon electrode. Finally the electrochemical response of the immobilized cytochrome *c* can be observed in an aqueous protein-free electrolyte (Figure 4B).

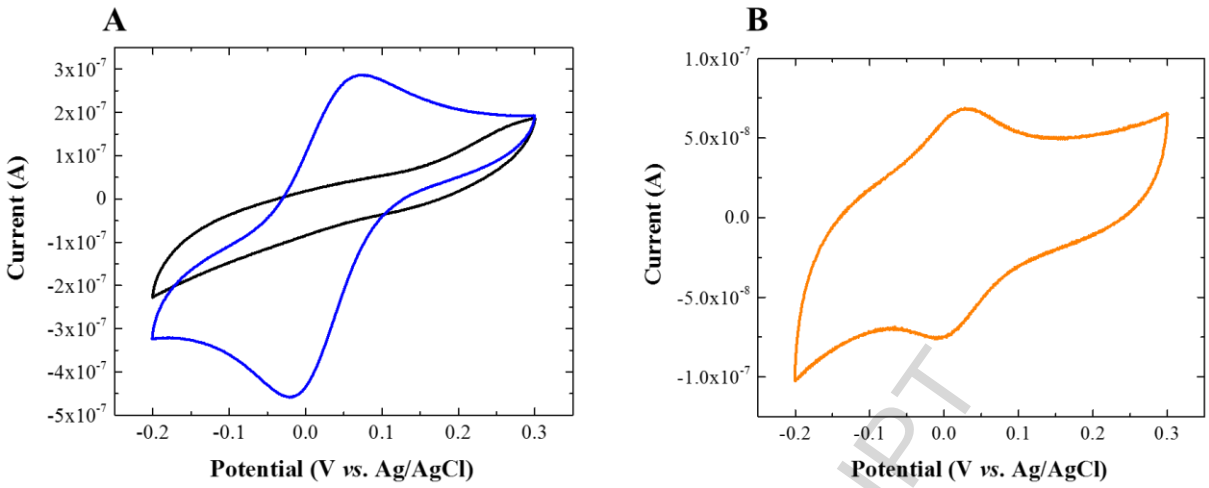

Figure 4. CV recorded at 20 mV s^{-1} on glassy carbon electrodes in 10 mM phosphate buffer aqueous electrolyte at pH 7 under Ar. **A.** Cytochrome-*c* freely diffusing: unmodified electrode (black) and electrode modified with a 7 µg cm^{-2} cardiolipin deposit (blue) in a 0.15 mM cytochrome-*c* solution. **B.** Immobilized cytochrome-*c* (orange): electrode modified with a 7 µg cm^{-2} cardiolipin deposit, first cycled ten times in a 0.15 mM cytochrome-*c* solution for protein immobilization and then transferred in a cytochrome-free electrolyte.

In Figure 4A, no redox system is detected on an unmodified glassy carbon electrode in a cytochrome-*c* solution in the scanned potential range because of the protein denaturation or unfavorable orientation.[26, 27] As expected, the same result is obtained at the phosphatidylcholine-modified glassy carbon electrode (Figure S2). In contrast, at the 7 µg cm^{-2} cardiolipin modified glassy carbon electrode, cytochrome-*c* in solution displays a well-defined quasi-reversible (ΔE_p = 90 ± 4 mV) redox system at 0.025 V. From the linear relationship of the oxidative peak currents versus the square root of the scan rate, the diffusion coefficient was estimated to 6.4×10^{-7} cm^2 s^{-1}, by applying the analytical equation for a quasi-reversible monoelectronic electron transfer (*vide infra*) (Figure S3A, see SI).[47] This value is in good agreement with those reported in literature for cytochrome-*c* in solution.[20, 48-50] The apparent kinetic rate constant has been estimated through numerical simulations of the

cyclic voltammograms using the KISSA software.[51, 52] The kinetic rate constant k_s of cytochrome-*c* in solution at a cardiolipin-modified glassy carbon electrode was determined as 1.0 (± 0.5) × 10^{-3} cm s^{-1}. This value is consistent with the one reported previously (2.0 × 10^{-3} cm s^{-1}) at a glassy carbon electrode modified with a high loading of pure cardiolipin (700 μg cm^{-2}).[24] The estimated kinetic rate constant also falls in the range reported for other redox proteins in solution.[53] This result shows that only a small amount of pure cardiolipin (7 μg cm^{-2}) simply deposited by solvent evaporation is sufficient for promoting the direct electron transfer of cytochrome-*c* in solution as a stable and reproducible redox signal at a modified glassy carbon electrode.

A simple and efficient strategy to immobilize cytochrome-*c* on the lipid modified electrode consists in performing recurrent potential cycles (here ten cycles, until a steady state current is reached, Figure S4A) in a cytochrome-*c* solution and then to transfer the modified electrode in a protein-free phosphate buffer where the electroactivity of the immobilized protein can be studied.[26, 29] In Figure 4B, the cyclic voltammogram recorded in a protein-free aqueous electrolyte on cardiolipin-modified glassy carbon electrode and previously cycled in cytochrome-*c* solution displays a redox system at +0.012 V assigned to cytochrome-*c* adsorbed or aggregated to the supported cardiolipin film. The peak-to-peak potential separation (ΔE_p = 30 ± 2 mV at 0.02 V s^{-1}, 45 ± 2 mV at 0.05 V.s^{-1}) is consistent with the immobilization of cytochrome-*c* at the lipid-modified electrode as reported in other works involving cardiolipin based films on carbon surface.[26, 41] For instance, the electrochemical signal of cytochrome-*c* immobilized at a glassy carbon electrode coated with a mixture of cholesterol/cardiolipin exhibits a peak-to-peak potential, ΔE_p = 80 mV, at 0.05 V s^{-1}.[26] This corresponds to an electrochemically quasi-reversible electron transfer reaction. In contrast, at a well-organized monolayer of 11-mercaptoundecanoic acid on a gold the immobilization of cytochrome-*c* led to an electrochemically reversible system as shown by a peak-to-peak

separation equal to 18 mV at 0.2 V s^{-1}.[54] The oxidative peak currents are linearly proportional to the scan rates up to 100 mV s^{-1}, consistent with an adsorption-controlled process (Figure S3B).[32] We also note that the redox potential of cytochrome-*c* in solution and immobilized onto modified glassy carbon electrode are close (+25 and +12 mV vs. Ag/AgCl, KCl 3 M). This suggests that the protein is present in its native state onto the surface and not as a cardiolipin/cytochrome-*c* complex that would yield a significant potential cathodic shift of the protein redox potential (> 0.3 V).[16, 29, 55] Indeed, cyclic voltammograms recorded at different scan rates and in a wider potential window (from -0.6 to +0.7 V) showed that the immobilized cytochrome-*c* is not denatured because of the absence of an additional reduction peak at more negative potential (Figure S3).[16-18, 22] A slight desorption of the immobilized protein could be observed after ten potential cycles between +0.3 V and -0.2 V, which led to about a 10% decrease of the redox signal (Figure S4B).[16-18] This indicates a better stability of the immobilized cytochrome-*c* at the glassy carbon electrode modified with a small amount of cardiolipin (7 μg cm^{-2}) in comparison with a previous study with a high cardiolipin lipid loading at the electrode (~700 μg cm^{-2}).[26] Indeed, in this latter work, the decrease of the protein redox signal was approximately 30% after three cyclic voltammograms. Thus, the immobilization of cytochrome-*c* onto the modified glassy carbon electrode surface is assigned to specific and selective interactions between cytochrome-*c* and cardiolipin as documented in the literature.[2, 6-9, 15] The electron transfer rate constant k_{ET} of cytochrome-*c* immobilized on cardiolipin-modified glassy carbon electrode has been estimated through numerical simulations[51, 52] of the cyclic voltammograms and amounts to 1.5 ± 0.5 s^{-1}. This value is slightly lower than those reported previously for immobilized cytochrome-*c* at cardiolipin/phosphatidylcholine-modified glassy carbon electrodes (8 to 30 s^{-1}) where the lipid deposit is characteristic to a bilayer of 2 nm thick,[29] probably because of the higher thickness of the pure cardiolipin

deposit (~30 nm). It is also significantly lower than those reported for cytochrome-*c* covalently attached to modified gold electrodes via a mercaptoheptanoic acid self-assembled monolayer (200 to 250 s^{-1})[16] but close to those reported for cytochrome-*c* covalently attached to modified gold electrodes via a mercaptohexadecanoic acid self-assembled monolayer (3 s^{-1}).[56] In addition, the surface coverage of the immobilized electroactive cytochrome-*c* estimated from faradic charge integration of the cyclic voltammogram is ca. 8 pmol cm^{-2} and corresponds to ca. 50% of a theoretical protein monolayer. This is in agreement with previous studies using various modified glassy carbon or gold electrodes (self-assembled monolayers, lipid mono/bilayers).[16, 29, 57] Moreover, the estimated electron transfer rate k_{ET} for cytochrome-*c* immobilized at the rather thick cardiolipin deposit (1.5 ± 0.5 s^{-1}) is close to that reported for cytochrome-*c* covalently bound to a self-assembled monolayer of 16-mercaptohexadecanoic acid onto gold surfaces (1 to 3 s^{-1}).[56] This work demonstrates that a supported bilayer based on mixed-lipids and mimicking the inner membrane of mitochondria is not strictly required for immobilizing the redox protein in its native state and for promoting direct electron transfer between cytochrome-*c* and an electrode. The deposition of pure cardiolipin by solvent evaporation onto glassy carbon surface is an easy, low cost and efficient method for promoting and keeping intact the electrochemical properties of cytochrome-*c* both in solution and adsorbed on surface.

4. Conclusions

In summary, pure cardiolipin deposited onto glassy carbon electrode surface has been optimized for maximum compactness at low lipid loading. Cyclic voltammetry and electrochemical impedance spectroscopy measurements recorded in ferri/ferrocyanide solution have demonstrated that the most compact and the less permeable cardiolipin film was reached for a 7 μg cm^{-2} deposit on glassy carbon. AFM experiments in liquid phase have been

performed and have shown the formation of a thick and rough cardiolipin film with a height of the order of 30 ± 10 nm on the PPF sample. The optimized supported cardiolipin deposit at glassy carbon was used to investigate cytochrome-*c* electroactivity either in solution or immobilized at the modified surface. With cytochrome-*c* in solution, cyclic voltammetry experiments showed that the pure cardiolipin deposit made by simple solvent evaporation is necessary and sufficient for promoting the electron-transfer between the redox protein and the modified electrode. In addition, recurrent cycles in the cytochrome-*c* solution led to the immobilization of the protein in its native state into the lipid film with retention of its electroactivity. The pure cardiolipin deposit discussed here is not an authentic biomimetic system because cardiolipin is usually only a minor component of biological membranes and the deposit studied in this work is not organized as a bilayer. This work nevertheless demonstrates that cytochrome-*c* can be anchored to pure cardiolipin-modified electrode surface and detected electrochemically in its native state. It also suggests that other enzymes could be immobilized in this matrix for sensing or biofuel cell applications.

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Competing Financial Interest

The authors declare no competing financial interest.

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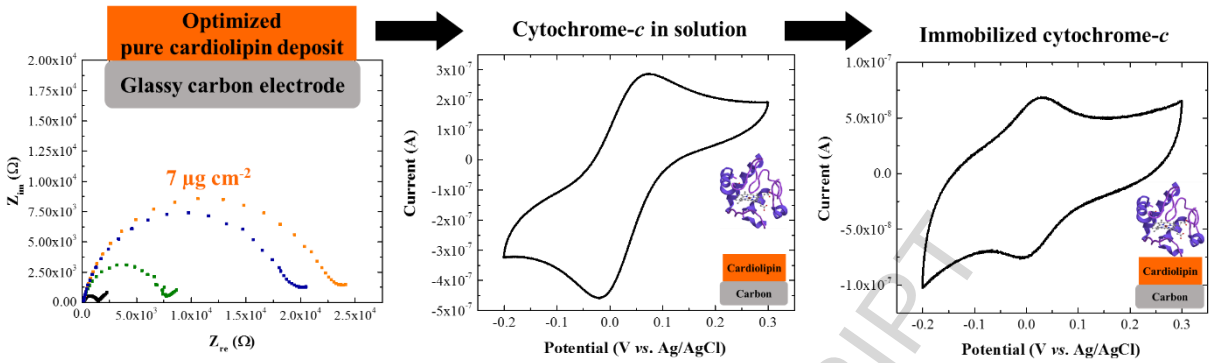
Table of Content Graphical Abstract



Highlights

- Cardiolipin-modified glassy carbon electrode is optimized for a 7 μg cm^{-2} deposit.
- Thick surface lipid film of 30 ± 10 nm is obtained for cardiolipin deposit on PPF.
- Cardiolipin deposit is sufficient for promoting the cytochrome-*c* electroactivity.
- Cardiolipin deposit is efficient for immobilizing cytochrome-*c* in its native state.
- Cardiolipin film is suited to detect cytochrome-*c* both in solution and on surface.