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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.

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 (Fig. 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

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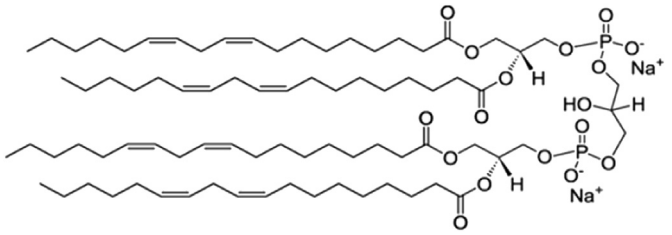

Fig. 1. Chemical structure of cardiolipin.

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.

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 min 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 min 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) is 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).

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