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Rhodanese incorporated in Langmuir and Langmuir–Blodgett films of dimyristoylphosphatidic acid: Physical chemical properties and improvement of the enzyme activity

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ABSTRACT

Preserving the catalytic activity of enzymes immobilized in bioelectronics devices is essential for optimal performance in biosensors. Therefore, ultrathin films in which the architecture can be controlled at the molecular level are of interest. In this work, the enzyme rhodanese was adsorbed onto Langmuir monolayers of the phospholipid dimyristoylphosphatidic acid and characterized by surface pressure-area isotherms, polarization-modulated infrared reflection-absorption spectroscopy (PM-IRRAS), and Brewster angle microscopy (BAM). The incorporation of the enzyme (5% in mol) in the lipid monolayer expanded the film, providing small surface domains, as visualized by BAM. Also, amide bands could be identified in the PM-IRRAS spectra, confirming the presence of the enzyme at the air–water interface. Structuring of the enzyme into α-helices was identified in the mixed monolayer and was preserved when the film was transferred from the liquid interface to solids supports as Langmuir–Blodgett (LB) films. The enzyme-lipid LB films were then characterized by fluorescence spectroscopy, PM-IRRAS, and atomic force microscopy. Measurements of the catalytic activity towards cyanide showed that the enzyme accommodated in the LB films preserved more than 87% of the enzyme activity in relation to the homogeneous medium. After 1 month, the enzyme in the LB film maintained 85% of the activity in contrast to the homogeneous medium, which 24% of the enzyme activity was kept. The method presented in this work not only points to an enhanced catalytic activity toward cyanide, but also may explain why certain film architectures exhibit an improved performance.

1. Introduction

The immobilization of enzymes on solid supports as Langmuir–Blodgett (LB) films is a recent approach to obtain devices whose architectures can be controlled at the molecular level. For that, monomolecular films are first formed at the air–water interface and transferred to solid supports during their vertical passage through the floating monolayer. Although pure enzymes can be spread on the air–water interface [1,2], their incorporation in pre-formed phospholipid monolayers may preserve the conformation of the enzyme [3–5]. As a result, a well-ordered nanostructured system can be constructed and the enzyme may serve as a recognizing element. If an optical or electrical signal is generated due to a recognizing chemical reaction, the film is a potential candidate for a biosensor.

Particularly, rhodanese is a mitochondrial enzyme that detoxifies cyanide, converting it into thiocyanate [6]. This reaction takes place in two steps: in the first step, thiosulfate reacts with the thiol group to form a disulfide. In the second step, the disulfide reacts with cyanide to produce thiocyanate, and is converted again to thiol. This reaction is environmentally important because it leads to a reduced exposure of cyanide since the less toxic thiocyanate is formed. The use of a thiosulfate solution as an antidote for cyanide poisoning is based on the activation of the enzymatic cycle. Thus, the immobilization of rhodanese in ultrathin films is interesting since it can provide a device capable of remedying the amount of cyanide in a controlled manner. LB films seem to be a suitable and innovative strategy for the construction of nanostructured films since monomolecular films can be deposited on solid supports with control over the chemical composition and surface density. This approach has already been performed for some enzymes [5,7,8], but not found in the literature for rhodanese.

In this paper, emphasis on the interaction of the enzyme with the phospholipid dimyristoylphosphatidic acid (DMPA) was placed. As

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we want to consider further the ability of such architecture to recognize cyanide, the enzymatic activity was investigated for mixed lipid-enzyme films supported as LB films.

2. Materials and methods

DMPA was purchased from Sigma–Aldrich (purity higher than 99%) and dissolved in chloroform (Sytnh) to a concentration of 0.5 mg/mL. This lipid was chosen considering the ease to transfer it to solid supports as shown in previous studies [7,8]. Water used in all experiments was purified using a Milli-Q® system (resistivity of 18.2 Ω cm^{-1}, pH 6.0). Rhodanese from Sigma–Aldrich (purity higher than 99% obtained from bovine liver) was dissolved in an aqueous buffer solution of K_2HPO_4 (Sigma–Aldrich) and KH_2PO_4 (Sigma–Aldrich) with a salt concentration of 0.01 mol/L and pH 7.0. The final concentration of the enzyme solution was 1 mg/mL.

A Langmuir trough (KSV Instruments, Helsinki—Finland, model: Mini, 36.5 × 7.5 cm) was employed for the preparation of the Langmuir and LB films. It was filled with water and then 45–65 μL of the DMPA solution was spread on the air–water interface to obtain an area per molecule of ~90–120 $Å^2$. After the evaporation of chloroform for 15–20 min, the interface was compressed with two movable barriers at a rate of 10 cm min^{-1} (5 $Å^2$ $molecule^{-1}$ s^{-1}), and the surface pressure values were monitored with a Wilhelmy plate made of filter paper that intercepted the air–water interface. For mixed enzyme-lipid monolayers, pre-determined aliquots of the enzyme solution (16–80 μL) were carefully injected below a pre-formed DMPA monolayer into the aqueous subphase. After allowing the surface pressure to stabilize during 30 min., the interface was compressed and the surface pressure monitored.

Measurements through polarization-modulation infrared reflection absorption spectroscopy (PM-IRRAS) were done with a KSV PMI 550 instrument (KSV Instruments Ltd., Helsinki—Finland). The monolayers were compressed to reach the surface pressure of 30 mN/m, and this value was kept constant by using mobile barriers while the spectra were obtained. The incidence angle to the normal was 80° and a minimum of 600 scans were obtained for each spectrum. The incoming light was continuously modulated between the p and s polarization, allowing simultaneous measurements of the spectra for both polarizations. The difference between the two absorbance signals gives surface-specific information and the sum provides the reference spectrum. Since the spectra are measured simultaneously, the effect of isotropic vibrations (water vapor and carbon dioxide) is largely reduced. Brewster angle microscopy (KSV-Nima Instruments, model: micro BAM3) was employed in order to obtain images of the monolayer at desired values of surface pressure.

Solid glass supports were inserted in the aqueous subphase of the Langmuir films. The films were then spread on the air–water interface and compressed to reach the surface pressure of 30 mN/m. The supports were vertically withdrawn across the air–water interface with a speed of 5 mm min^{-1} keeping the surface pressure of 30 mN/m constant during the passage of the support. Transfer ratio values of about 0.95–1.05 were required for further analysis. LB films with 1 single layer were characterized by PM-IRRAS and fluorescence spectroscopy (Spectrophotometer model RF-5301PC, Shimadzu) with an excitation wavelength of 285 nm with the glass directly placed in the fluorimeter holder. Atomic force microscopy (AFM) was also employed for further characterization, and the images were obtained in the tapping mode, employing a resonance frequency of approximately 300 kHz, a scan rate of 1.0 Hz, and scanned areas of 5.0 × 5.0 μm on films deposited on mica. Nanogravimmetry through a quartz crystal microbalance (SRS—Stanford Research Systems model QCM200) was employed to estimate the mass of film deposited on a surface bounded by gold electrodes in the thin disk of quartz as substrate. The mass of the deposited film was determined according to the Sauerbrey equation [9].

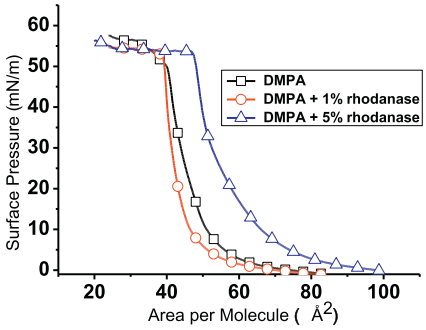

Fig. 1. Surface pressure-area isotherms for DMPA and rhodanese (relative proportions in mol of the enzyme injected in the subphase are indicated in the inset).

The catalytic activity of the enzyme was estimated according to a method previously described in the literature [10]. For this purpose, the LB film was inserted in a solution containing cyanide, and the enzyme activity measured due to the evolution of the optical density of the solution measured at 470 nm (UV–vis Hitashi, model U2001). The enzymatic activity was also measured in a homogeneous environment (enzyme dissolved in aqueous solution) for comparison. All enzymatic activities were measured again after 30 days with the sample kept at low temperatures (5–10 °C).

All experiments were performed at a temperature of 25 ± 1 °C.

3. Results and discussion

3.1. Langmuir monolayers

Firstly, it is important to emphasize that DMPA was chosen because of its easiness to be transferred to solid supports as a LB film and also because this lipid is reported as a suitable matrix for immobilizing enzymes [7,8].

DMPA monolayers present a typical surface pressure-area isotherm (Fig. 1), in agreement with the literature [11]. Inserting 1% in mol of rhodanese, a shift of the curve to smaller lipid areas is observed as a consequence of the condensation of the monolayer. With 5% in mol of the enzyme, the isotherm is shifted to higher areas, which may be a consequence of the incorporation of the enzyme inside the lipid chains, causing the lipid monolayer expansion. With higher concentrations of rhodanese, the isotherm is no longer shifted to higher areas, indicating saturation. This saturation effect can be attributed to a probable enzyme aggregation in the aqueous subphase as previously reported for other macromolecules adsorbing on lipid monolayers [12,13]. As the isoelectric point of this enzyme varies between 5.6 and 7.15 [14], rhodanese is zwitterionic under the experimental conditions (pH around 6.0). Being DMPA negatively charged at this pH, dipole-ion attractions between the lipid and the enzyme must influence the adsorption of the biomacromolecule and the subsequent monolayer expansion.

Rheological properties of the monolayer can be accessed by means of its compressional modulus (E), which is defined as $-A(\partial\pi/\partial A)_T$ [15], where A is the molecular area, π is the surface pressure of the monolayer, and T is the temperature. The highest values of E for DMPA were of ca. 300 mN/m, featuring the liquid-condensed state (Fig. 2A and B). DMPA mixed with 1% of rhodanese presents higher values of E (550 mN/m) as a consequence of the condensation of the monolayer caused by small amounts of the enzyme. With 5% of rhodanese, the maximum values of E are similar to those obtained for pure DMPA. It is probable that the penetration of the enzyme into the lipid monolayer makes the film more