Colloids and Surfaces B: Biointerfaces 126 (2015) 232–236

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Langmuir and Langmuir–Blodgett films of lipids and penicillinase: Studies on adsorption and enzymatic activity

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ARTICLE INFO

Article history:
Received 16 September 2014
Received in revised form 4 December 2014
Accepted 17 December 2014
Available online 31 December 2014

Keywords:
Penicillinase
Air–water interface
Langmuir monolayers
Langmuir–Blodgett
Enzyme activity

ABSTRACT

Bioelectronic devices, such as biosensors, can be constructed with enzymes immobilized in ultrathin solid films, for which preserving the enzymatic catalytic activity is fundamental for optimal performance. In this sense, nanostructured films in which molecular architectures can be controlled are of interest. In this present work, the adsorption of the enzyme penicillinase onto Langmuir monolayers of the phospholipid dimyristoylphosphatidic acid was investigated and characterized with surface pressure–area isotherms and polarization-modulated infrared reflection-absorption spectroscopy (PM-IRRAS). The incorporation of the enzyme in the lipid monolayer not only caused the film to expand, but also could be identified through amide bands in the PM-IRRAS spectra, with the C—N and C═O dipole moments being identified, lying parallel to monolayer plane. Structuring of the enzyme into α-helices was identified in the mixed enzyme–phospholipid monolayer and preserved when transferred to solid as a Langmuir–Blodgett (LB) film. The enzyme–lipid LB films were then characterized with PM-IRRAS, atomic force microscopy and fluorescence spectroscopy. Measurements of the catalytic activity showed that the enzyme accommodated in the LB films preserved 76% of the enzyme activity in relation to the homogeneous medium. The method presented here not only allows for enhanced catalytic activity toward penicillin, but also can be useful to explain why certain film architectures exhibit better enzyme activity.

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1. Introduction

The immobilization of enzymes on solid supports as a Langmuir–Blodgett (LB) film is a recent strategy for obtaining nanostructured films whose molecular architectures are controlled at the molecular level. For this purpose, stable monomolecular films must be first formed at the air–water interface to be afterwards transferred to solid supports by dipping or removing the support passing vertically through the interface. Pure enzymes can be spread on the air–water interface [1,2], but the use of protector molecules, such as phospholipids, may avoid the denaturation of the enzyme [3–6]. Phospholipids may provide a suitable orientation of the polypeptide moiety of the enzyme, enhancing the accessibility of the catalytic substrate. As most enzymes have specificity for some substances, films where enzymes and phospholipids are mixed can be useful for obtaining well-ordered nanostructured systems with a recognizing element, which is useful to be employed as a biosensor.

Penicillinase is an enzyme specific for penicillin and belongs to the class of β-lactamase enzymes, being capable of hydrolyzing β-lactam rings. This enzyme can therefore be used in penicillin sensing, but studies on this topic remain scarce [7–9]. Also, reports on this enzyme incorporated in phospholipid Langmuir monolayers and Langmuir–Blodgett (LB) films were not found in the literature.

The importance in detecting penicillin relies on the fact that it may be used as an antibiotic, being among the first drugs to be effective against a series of diseases, including those ones caused by bacterial infection. Their mechanism of action is related to the interference in the synthesis of the bacterial cell wall. In this sense, efforts are being made to reduce morbidity, mortality and costs of antibiotic-resistant infections treatments [10]. One of the causes that are responsible for antibiotic resistance is an overdose of medication. In some special situations, the real-time monitoring of the levels of antibiotics in the body is critical, thereby encouraging the use of biosensors. For this reason, the immobilization of this enzyme in ultrathin films could help construct a device capable of detecting penicillin in a controlled way by means of a nanostructured film, whose architecture could be assembled at the molecular level.

In this paper, the adsorption of penicillinase at the air–water interface was investigated with emphasis on its interaction with the

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http://dx.doi.org/10.1016/j.colsurfb.2014.12.033
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phospholipid dimyristoylphosphatidic acid (DMPA) and its transfer onto solid supports using the LB technique. As we want to consider further the ability of such structures to recognize penicillin, the enzymatic activity of penicillinase was also investigated.

2. Experimental

Water used in all experiments was purified using a Milli-Q® system (resistivity of 18.2 Ω cm^{-1}, pH ~ 6.0). DMPA solutions were obtained dissolving the lipid (Sigma–Aldrich, purity higher than 99%) in chloroform (Synth) to a concentration of 0.5 mg/mL. DMPA was chosen because of the ease to transfer this lipid to solid supports as demonstrated in previous studies [11].

Penicillinase solutions with a concentration of 0.5 mg/mL were obtained by dissolving the enzyme (Sigma–Aldrich, purity higher than 99%, obtained from *Bacillus cerus*) 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.

For the formation of monolayers at the air–water interface, a Langmuir trough (KSV Instruments, model: Mini) was initially filled with water. A DMPA solution in chloroform was then 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 monolayers were compressed with two movable barriers at a rate of 5 Å^2 molecule^{-1} s^{-1}. The surface pressure values monitored with a filter paper Wilhelmy plate that intercepted the air–water interface. Compression was stopped when the monolayer collapsed.

For the formation of mixed enzyme–lipid monolayers, after DMPA monolayer formation, pre-determined aliquots of the enzyme solution were carefully injected below the interface into the aqueous subphase. After allowing the surface pressure to stabilize after 30 min, the monolayer was compressed. The surface pressure was monitored as a function of the molecular area while the interface was compressed.

Polarization-modulation infrared reflection absorption spectroscopy (PM-IRRAS) measurements were performed using a KSV PMI 550 instrument (KSV Instrument Ltd., Helsinki, Finland) for the monolayers at the air–water interface with an incidence angle of 80° and recorded for a minimum of 600 scans.

The Langmuir films were transferred onto solid glass supports, previously cleaned with KOH and ethanol, by vertically withdrawing the support across the air–water interface with a speed of 5 mm min^{-1} and at a constant surface pressure of 30 mN/m, with transfer ratio of about 0.9–1.0 for further analysis. For analysis of the LB films and examination of co-transfer of the enzyme, fluorescence spectroscopy (Spectrophotometer model RF-5301PC, Shimadzu) was employed using 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. The LB film was also characterized by nanogravimmetry through a quartz crystal microbalance (SRS – Stanford Research Systems model QCM200). The mass of the film deposited on a surface bounded by gold electrodes in the thin disk of quartz as substrate was determined according to the Sauerbrey equation [12].

The catalytic activity of the enzyme was measured according to a method previously described in the literature [13]. For this purpose, the LB film was inserted in a solution containing penicillin, and the enzyme activity was a result of the discoloration of the starch-I_3 in consequence of the reduction of iodine by penicilloic acid, which was accompanied by measurements of absorption spectra at 620 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 25.0 ± 0.2 °C.

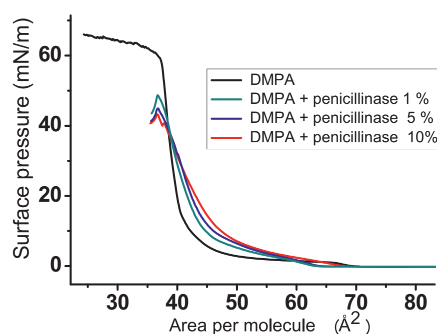

Fig. 1. Surface pressure-area isotherms for DMPA and penicillinase (relative proportions in mol of the enzyme injected in the subphase are indicated in the inset).

3. Results and discussion

3.1. Langmuir monolayers

For DMPA monolayers (Fig. 1), a typical isotherm is shown, being in agreement with the literature [14]. With increasing concentrations of penicillinase in the subphase from 1 to 10% in mol, a continuous shift of the isotherm to a higher molecular area is observed, which may be due to the incorporation of the enzyme and lipid monolayer expansion. However, as the molecular area is almost the same for pure DMPA at 30 mN/m, there is no evidence enough to affirm that the enzyme has been successfully adsorbed. The adsorption of the enzyme was then confirmed by PM-IRRAS measurements.

With higher concentrations of penicillinase, the isotherm is no longer shifted to higher areas, indicating saturation. This effect can be attributed to a probable enzyme aggregation in the aqueous subphase. Similar effects have previously been reported in the literature for other macromolecules adsorbing in lipid monolayers [15–17]. As the isoelectric point of this enzyme is 5.9 [18], it can be considered as neutral under the experimental conditions (pH around 6.0). Considering that DMPA is negatively charged at this pH, dipole-ion attractions between the lipid and the enzyme must be a key point for the adsorption of the enzyme. Therefore, since monolayer expansion was observed, it is probable that adsorption of the enzyme may occur. This fact may prevent enzyme from denaturation by maintaining its molecular conformation.

The maximum value of surface pressure is also worth mentioning. When the monolayer is compressed beyond its surface pressure of equilibrium, it attains a metastable state resulting in the collapse of the monolayer [19–21], which is characterized by the formation of multilayers. For pure DMPA, this surface pressure of collapse was of approximately 62 mN/m. With the presence of the enzyme in the monolayer, the pressure of collapse decreased to values in the range of 40–45 mN/m, which indicates a decrease in the ability of the monolayer to attain higher surface pressures owing to a new molecular structuring at the air–water interface.

In this sense, these isotherms show that the enzyme has surface activity increased by the presence of lipids at the air–water interface. The adsorption of soluble compounds at interfaces containing lipids is reported in the literature for chitosan [22] and for other enzymes [23].

Information on other changes in the monolayer caused by the enzyme can be obtained analyzing its rheological

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