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Protocols

Controlling the molecular architecture of lactase immobilized in Langmuir-Blodgett films of phospholipids to modulate the enzyme activity

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ABSTRACT

In this present work, the adsorption of the enzyme lactase onto Langmuir monolayers of the phospholipid dimyristoylphosphatidic acid (DMPA) was investigated and characterized with surface pressure-area isotherms, surface potential-area isotherms and polarization-modulated infrared reflection-absorption spectroscopy (PM-IRRAS). The adsorption of the enzyme at the air-water interface expanded the lipid monolayer and increased the film compressibility at high surface pressures. Amide bands in the PM-IRRAS spectra were identified, with the C—N and C═O dipole moments lying parallel to the monolayer plane, revealing that the structuring of the enzyme into β-sheets was kept in the mixed monolayer. The enzyme-lipid films were transferred from the floating monolayer to solid supports as Langmuir-Blodgett (LB) films and characterized with fluorescence spectroscopy and atomic force microscopy. The catalytic activity of the films was measured and compared to the homogenous medium. The enzyme accommodated in the LB films preserved more than 80% of the enzyme activity after 20 days, in contrast for the homogeneous medium, which preserved less than 60% of the enzyme activity. The method presented in this present work not only allows for an enhanced catalytic activity toward lactose, but also can help explain why certain film architectures exhibit better performance.

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1. Introduction

Enzymes can be immobilized on solid surfaces in a nanostructured assembly, allowing for more efficient use as sensors and biocatalysts. In the last decades, some structures have been proposed in order to produce robust devices with improved enzyme activity properties. One of these structures includes the so-called Langmuir-Blodgett (LB) films [1], which are formed when amphiphilic monolayers at the air-water interfaces are transferred to solid supports that intercept vertically the interface at a given surface pressure. Enzymes, as amphiphilic compounds, can present surface activity at air-water interfaces [2,3], but do not form coherent Langmuir monolayers since they can be partly solubilized into the aqueous subphase. For improving the molecular attachment of enzymes at the air-water interface, mixtures with lipids [4,5] and other biological compounds such as polysaccharides [6,7] have been proposed, producing reliable and stable materials, whose architecture and catalytic properties could be easily manipulated.

Lactase or galactosidase is an enzyme produced in the cells of the epithelium of the small intestine and its function is to catalyze the hydrolysis of lactose to glucose and galactose, which are further metabolized. The production of this enzyme is reduced in adults in relation to children causing intolerance to lactose, although many children also suffer from this problem. Unmetabolized lactose makes intestinal bacteria splits lactose in short-chain fatty acids that can be absorbed by the intestine and used as metabolites. This process may involve by-products such as methane and carbon dioxide, causing nausea, abdominal cramps, diarrhea and gas [8–11].

Based on the facts cited above, it seems fundamental to detect lactose in biological fluids and in dairy products. Among the methods employed nowadays to estimate lactose, one can include spectrophotometry, titrimetry and chromatography [12]. These methods usually consume time because of the preparation of the sample, producing instable devices and with problems of reuse. On the other hand, when enzymes are molecularly accommodated in certain assemblies, they can maintain active for longer periods of time. Also, in some specific nanostructured systems, only the monomolecular layer attached to a solid support must be active [13,14], and then a lower quantity of enzyme is necessary.

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Particularly, the enzyme lactase has previously been attached to surfaces to reduce lactose in packaged dairy products [15,16]. However, the lowered surface area available for conjugation of active agents limits the enzyme activity. In this sense, organized molecular films produced by the layer-by-layer (LbL) and LB techniques have been proposed in order to immobilized lactase in ultrathin films, improving the enzyme efficiency. Wong et al. [17] produced an LbL assembly composed of lactase covalently bound to a low-density polyethylene. The quantity of enzyme could be controlled with the number of layers deposited on the solid support. However, the increase of the amount of lactase incorporated did not increase the enzyme activity, which may limit the application for a bioactive packaging use. Earlier, lactase was reported as an active component of lactose biosensors based on LB films [18]. Lactase and galactose oxidase were immobilized in LB films of poly(3-hexyl thiophene) and stearic acid, which was employed as a working electrode to determine lactose amperometrically, which proved the feasibility of this method. However, many questions still remain open, such as: (i) how the secondary structure of this enzyme can be changed when immobilized in these films? (ii) is it necessary a single molecular layer to perform a robust biosensor to lactose, or are multilayers necessary? Complex structures such as polymers are necessary or can we produce simple systems based on simple mixed lipid-enzyme films?

With these questions in mind, the objective of this work is to investigate the adsorption of lactase in bioinspired systems composed of Langmuir and Langmuir-Blodgett films of phospholipids. To start with the simplest phospholipid, we chose dimyristoylphosphatidic acid (DMPA), which has been employed successfully to immobilize other enzymes as LB film [6,19], and can be deposited as single or multilayer films [20]. The floating monolayers were characterized with surface pressure-area isotherms to obtain information about thermodynamic parameters. Polarization-modulation reflection-absorption infrared spectroscopy (PM-IRRAS) was employed to infer about molecular interactions, and Brewster Angle Microscopy (BAM) to analyze the morphology of the films. Particularly important, PM-IRRAS is able to detect vibrational transitions, including those related to amide groups in polypeptides since wavelengths and relative intensities of these bands are sensitive to changes in the secondary structure of proteins [21], being therefore a reliable method to investigate conformational changes in lactase. Mixed enzyme-lipid Langmuir monolayers were transferred to solid supports, characterized by fluorescence spectroscopy and atomic force microscopy. The enzyme activity was then investigated as a proof-of-concept experiment.

2. Materials and methods

Dimyristoylphosphatidic acid (DMPA) was purchased from Sigma-Aldrich (purity higher than 99%) and dissolved in chloroform (Synth) to render a final concentration of 0.5 mg/mL. Lactase (or β-galactosidase) from *Aspergillus oryzae* was obtained from Sigma-Aldrich (purity higher than 99%) and 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. Water used in all experiments was purified using a Milli-Q ® system (resistivity of 18.2 Ω cm^{-1}, pH ~ 6.0).

For the preparation of the Langmuir and LB films, a Langmuir trough (KSV Instruments, Helsinki-Finland, model: Mini, 36.5 × 7.5 cm) was employed. After filled with water, aliquots of 45–65 μL of the DMPA solution was spread on the air-water interface. An elapsed time of 15–20 min was waited for chloroform evaporation and the interface was compressed with two movable barriers at a rate of 10 cm min^{-1} (5 Å^2 $molecule^{-1}$ s^{-1}), with the surface pressure monitored with a Wilhelmy plate made of filter paper that intercepted the air-water interface, while the surface potential was mesured using a Kelvin probe. For mixed enzyme-lipid monolayers, pre-determined aliquots of the enzyme solution (30–80 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 then compressed and the surface pressure and surface potential were measured as long as the surface area decreased.

Polarization-modulation infrared reflection absorption spectroscopy (PM-IRRAS) measurements were employed with a KSV PMI 550 instrument (KSV Instruments Ltd, Helsinki- Finland). The monolayers were previously compressed to reach the surface pressure of 30 mN/m. This value was kept constant with mobile barriers during the obtainment of the spectra. The incidence angle to the normal was 75° and a minimum of 6000 scans were obtained for each spectrum. At this angle, the reflectivity is the highest and signal/noise ratio is minimum [22]. The incoming light was continuously modulated between the p and s polarizations, 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. As a result, the effect of isotropic vibrations (water vapor and carbon dioxide) was largely reduced and then the PM-IRRAS signal was related to surface-specific information. In order to overcome possible effects related to fluctuations in the spectra due to the compression of the monolayer during the acquisition of the spectra, the air-water interface was compressed until the desired surface pressure, and at least 15 min were waited to let the film to be as stable as possible, and then the spectra were taken. Also the high number of minimum scans (6000 at least) should minimize this effect. Images of the monolayer at desired values of surface pressure were obtained with a Brewster angle microscopy (KSV-Nima Instruments, model: micro BAM3).

For the construction of LB films, firstly solid glass supports were inserted in the aqueous subphase of the Langmuir films and the lipid monolayers were then formed the air-water interface to be further compressed until the surface pressure of 30 mN/m. The supports were then 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 should present values in the range of 0.95-1.05 for further analysis. 1-layer and 7-layer Y-type LB films were then characterized by 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 employed to obtain information about the morphology of the films. 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. To estimate the mass of film, quartz crystal microbalance (SRS – Stanford Research Systems model QCM200) was employed. For that, the solid supports were composed of 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 [23].

The catalytic activity of the enzyme was estimated according to a method described in the literature [24]. For this purpose, the LB film was inserted in a solution containing 1 mg/mL of nitrophenyl-β-D galactoside, and the enzyme activity measured due to the evolution of the optical density of the solution measured at 450 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

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