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Interaction of horseradish peroxidase with Langmuir monolayers of phospholipids

Thaís F. Schmidt [a], Luciano Caseli [a,*], Thatyane M. Nobre [b], Maria E.D. Zaniquelli [b], Osvaldo N. Oliveira Jr. [a]

[a] *Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, São Paulo, Brazil*
[b] *Departamento de Química, Faculdade de Filosofia, Ciência e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, São Paulo, Brazil*

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Abstract

The method employed to incorporate guest molecules onto phospholipid Langmuir monolayers plays an important role in the interaction between the monolayer and the guest molecules. In this paper, we show that for the interaction between horseradish peroxidase (HRP) and a monolayer of dipalmitoylphosphatidylglycerol (DPPG) does depend on the method of HRP incorporation. The surface pressure isotherms of the mixed DPPG/HRP monolayers, for instance, were less expanded when the two materials were co-spread than in the case where HRP was injected into the subphase. Therefore, the method for incorporation affected not only the penetration of HRP but also the changes in molecular packing caused to the DPPG monolayer. With experiments with the monolayer on a pendant drop, we observed that the incorporation of HRP affects the dynamic elasticity of the DPPG monolayer, on a way that varies with the surface pressure. At low pressures, HRP causes the monolayer to be more rigid, while the converse is true for surface pressures above 8 mN/m. Taken all the results together, we conclude that HRP is more efficiently incorporated if injected into the subphase on which a DPPG monolayer had been spread and that the interaction between HRP and DPPG is maintained even at high surface pressures. This is promising for the possible transfer of mixed films onto solid substrates and for applications in biosensors and drug delivery systems.
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1. Introduction

Molecular recognition is the most important process to build biological systems and to mimic cell membrane [1], which are made of proteins and lipids arranged in a way that their interaction maintains the selective permeability of the membrane. Protein–lipid interactions are also involved in recognizing specific molecules, which is useful for drug delivery [2] and biosensors [3]. As major building blocks of biological membranes, phospholipids have been extensively studied over the last decades using a variety of approaches. Langmuir monolayers at the air–water interface, in particular, exhibit properties that can be easily examined by many techniques, serving therefore as suitable models for biomembranes [4]. With Langmuir monolayers, interactions in two-dimensional arrangements of amphiphilic molecules can be investigated with the unique advantage that the density and composition of the lipids can be controlled. The energetics involved in the membrane at distinct stages of packing can be studied via surface tension measurements. Furthermore, the transfer of mixed phospholipid–enzyme monolayers onto solid supports using the Langmuir–Blodgett technique has been used to produce biosensors, in which one exploits the capability of phospholipids in preserving the enzyme conformation [3].

Among the proteins that may be studied in phospholipid Langmuir and LB films, we chose the horseradish peroxidase, for which there is no report in the literature—to the best of our knowledge. Peroxidases are oxide-reductase enzymes with hydrogen peroxide as electron acceptor [5]. Immobilization matrices for peroxidases include proteins, phospholipids, fatty acids and polysaccharides. The horseradish peroxidase is extracted from a hardy perennial herb, whose roots are

* Corresponding author. Tel.: +55 16 3373 8061; fax: +55 16 3371 5365.
E-mail address: lcaseli@usp.br (L. Caseli).

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particularly important for culinary [5]. Studies concerning the interaction of this enzyme with phospholipids are still scarce in the literature though [6].

With the example of horseradish peroxidase, in this work we address another relevant aspect of cell membrane models, namely the strategy of incorporation of proteins in lipid films at the air–water interface. Several methods of co-mixing may be used, which result in different surface behaviors and micro-heterogeneities [7]. Such differences are important not only to probe interactions of proteins in cell membranes, but also for the construction of artificial systems containing proteins. The latter is the case of as bio-devices, such as biosensors [3]. Although horseradish peroxidase (HRP) is not properly a membrane protein, organized proteo-lipid nanostructures can be obtained to allow accurate manipulation of the biomacromolecule, which can be immobilized in a layered lipid structure that is highly controlled in terms of composition, thickness and charge [3]. Because HRP is soluble in water and amphiphilic, mixtures with lipids may have an impact in the polypeptide structure. Here, we investigate the interaction between horseradish peroxidase (HRP) and a phospholipid at the air–water interface through quasi-equilibrium (Langmuir trough) and dynamic (pendant drop) conditions, using different methods of enzyme–lipid spreading conditions on the air–water interface.

2. Materials and methods

Dipalmitoylphosphatidylglycerol (DPPG) and horseradish peroxidase (HRP) were purchased from Sigma, and the other chemicals were of the highest purity available. DPPG solutions (0.5–1.0 g/L) in chloroform:methanol (4:1 v/v) were spread on a 0.05 g/L phosphate buffer (with ionic strength controlled by 0.01 mol/L NaCl and pH 6.3) in a Nima Langmuir trough (model 601M, subphase volume: 500 mL). DPPG was chosen the abundance of lipids with glycerol polar heads in natural cell membranes and because it has opposite charge to HRP, which is positively charged at the conditions used in this paper. For producing mixed DPPG–HRP Langmuir monolayers, several methods were tested. In all cases a total mass of 40 μg of HRP and 75 μg of DPPG was used, corresponding to a lipid:enzyme ratio of 99:1 (mol:mol) in the trough. The surface pressure was measured with the Wilhelmy method, with monolayer compression performed using two barriers at a 10 cm^2/min rate to obtain surface pressure–area isotherms. All measurements were carried out at 23 ± 1 °C with water purified by a Milli-Q® system (18.2 Ωm and pH 6.3).

The methods for HRP incorporation are referred to as: (i) *co-spreading*: DPPG and HRP were dissolved together in a solution of chloroform, methanol and water (2:1:0.3 v/v/v); (ii) *subphase formation*: an aqueous HRP solution (0.15 μg/mL) filled the trough as subphase before DPPG spreading; (iii) *injection*: HRP solution was injected in the subphase after DPPG spreading; (iv) *on the top*: after DPPG spreading on the buffer solution, HRP was spread on the interface. This indicates that HRP and DPPG were both spread on the buffer solution, similarly to the method (i), but in separated steps.

Dynamic dilatational elasticity measurements were performed with the pendant drop technique (OCA-20 from Dataphysics Instruments GmvH, Germany, with oscillating drop accessory ODG-20). The HRP–DPPG mixed films were formed by gently touching a 15 μL drop of the aqueous enzyme solution (0.05 g/L) with 1 μL 10^{-4} mol/L chloroform/methanol DPPG solution. A new surface was formed by expanding the drop, and then the surface pressure was monitored with time. After adsorption equilibrium was reached, periodic oscillations with amplitude of 0.1 mm and 1 Hz frequency were imposed to the drop. The elasticity values were estimated as described elsewhere [8], determining the relation between the area deformation and the change in surface pressure.

3. Results and discussion

Two important parameters in the interaction between lipid Langmuir monolayers and guest biomolecules are the location of the guest molecule in the monomolecular film, and the changes in film fluidity and permeability due to the incorporation. The surface pressure–area isotherm of a DPPG monolayer was affected to different extents depending on the method to incorporate HRP, as depicted in Fig. 1. The isotherm for the pure DPPG is consistent with results from the literature obtained under similar conditions [9]. When the enzyme and lipid were co-spread with the same solution, the mixed monolayer displayed an expansion effect at low surface pressures and a condensing effect at high surface pressures, when compared to a pure DPPG monolayer. This indicates that the permanence of a certain amount of enzyme and the molecular arrangement at the interface helped to stabilize the molecular packing during compression. The isoelectric point of HRP is 7.5 [10], and therefore HRP molecules carry a net positive charge in a Langmuir monolayer with pH 6.3 in the subphase. Hence, interactions between the enzyme and DPPG (a negatively charged lipid) can be a combination of hydrophobic, dipole and electrostatic interactions. With compression, HRP can be expelled

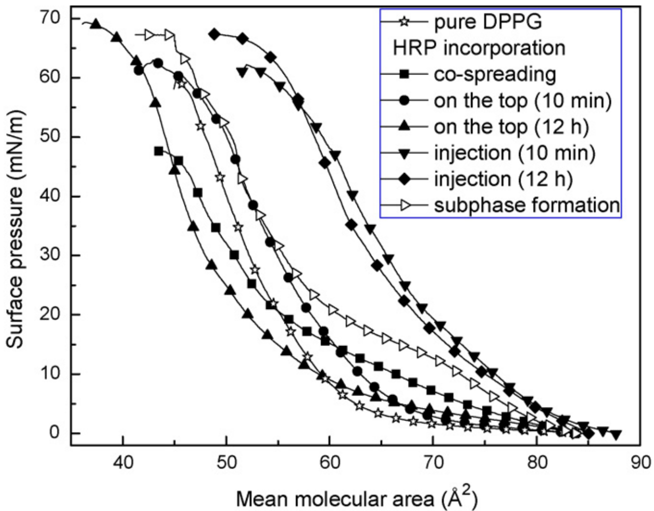

Fig. 1. Surface pressure–area (π–A) isotherms for pure DPPG and mixed HRP–DPPG monolayers with four methods to incorporate HRP (see inset).

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