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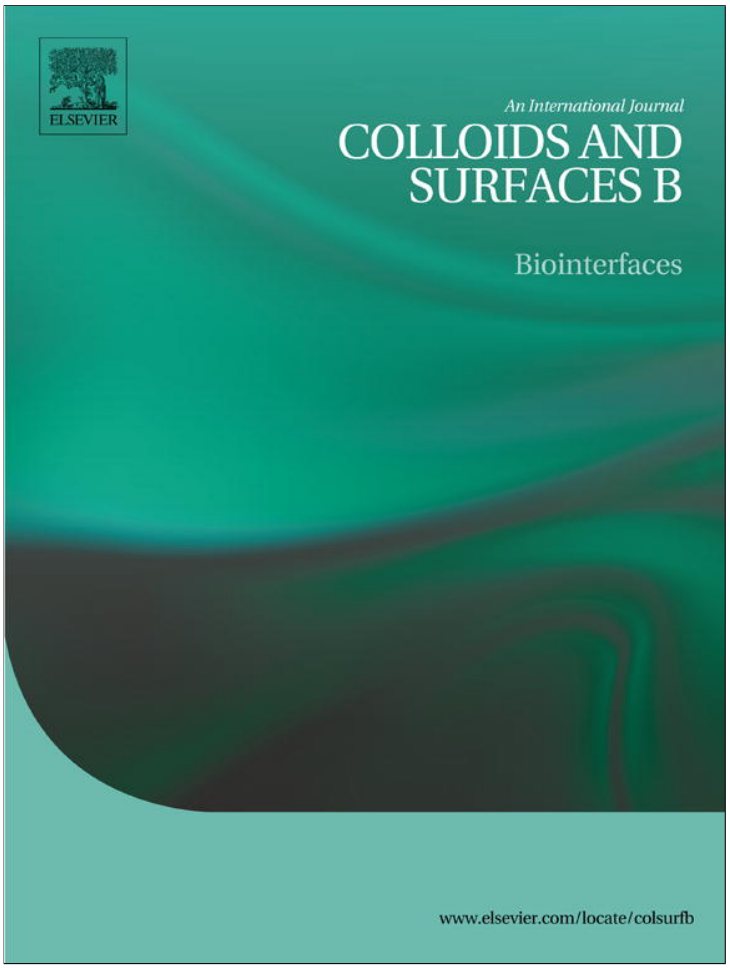

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Competition for space between a protein and lipid monolayers

Giovanna Fragneto [a,*], Stéphane Alexandre [b], Jean-Marc Valleton [b], Francis Rondelez [a]

[a] Institut Curie, Labo PSI, 11 rue Pierre et Marie Curie, 75231 Paris, France
[b] UMR 6270 CNRS, UFR des Sciences, Université de Rouen, 76821 Mont-Saint-Aignan, France

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ABSTRACT

Competitive adsorption is a general problem both in polymer and in biological systems. The equilibrium composition at a surface in contact either with polymer solutions or biological fluids depends on the competition between all the surface active material present in the medium. Such competition is particularly important in cell membranes where membrane proteins generated on ribosomes have to incorporate in the cell. Here we use fluovideo microscopy to study the competition for adsorption at the air/water interface between the enzyme glucose oxidase (GOx) and fluid monolayers of pentadecanoic acid (PDA). Although water soluble, GOx has a strong affinity for the air/water interface. We show that under certain conditions it inserts in the monolayer and causes a contraction of the Langmuir film and the formation of condensed domains. When exposed to a heterogeneous surface it is inserted in the less dense regions. Its crystallization leads to the deformation of the condensed domains followed by the destruction of their initial shape. By compressing the layer the protein is not removed from the interface where it eventually forms three-dimensional structures.

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1. Introduction

GOx is an enzyme that has been identified from various sources [1,2]. We used GOx from *Aspergillus niger* which is a homodimer of molecular weight 150–180 kDa containing two tightly bound Flavin Adenin Dinucleotide molecules (FAD). The amino acid sequence for the 583 residues protein has been derived from the DNA sequence [3,4]. Its crystal structure has been established by x-ray crystallography [5]. The monomer is a compact spheroid of dimensions $60 \times 52 \times 37$ Å^3. The native protein is acidic having an isoelectric point of 4.2. Its importance is related to the fact that it catalyses the reduction of β-D-glucose by molecular oxygen to δ-gluconolactone. Since it is surface active and can be incorporated in lipid films, efforts have been made to pursuit reliable biosensors for measuring glucose levels from mixed Langmuir–Blodgett films of GOx with fatty acids [6]. Such biosensors are interesting because the absence of covalent bonds between the enzyme and the support limits the problems of denaturation, and also because of their fast response due to the small thickness of the acid layer (order of 20 Å). The study described here is of interest for the realization of mixed Langmuir–Blodgett films but also for the understanding of the mechanism of incorporation of transmembrane proteins in biological membranes. In fact, the insertion of transmembrane proteins is usually driven by specialized proteins or other complex mechanisms. Here we show that also very general physico–chemical mechanisms may be efficient to position proteins within membranes. It has been suggested that the abundance of non-bilayer lipids in many biomembranes may be explained by the fact that they preserve the functional structure of membrane proteins by preserving their shape inside the layer [7]. Here we propose that the difference in chemical composition within a lipid membrane may create local concentration heterogeneities which in turn favour protein incorporation. Langmuir films are excellent model systems for the study of biomembranes [8,9]. In this work we investigated monolayers of pentadecanoic acid, PDA ($C_{14}H_{29}COOH$) and myristic acid, MYA ($C_{13}H_{27}COOH$) which behaviour at the air/water interface is well understood in terms of the images produced by fluorescence microscopy [10]. Langmuir films are formed by spreading on the surface of water amphiphilic molecules dissolved in a solvent which does not mix with water and that after evaporation leaves a monolayer of the amphiphile at the interface. The monolayer structural phase at a given temperature, depends on the mean area per molecule, *A*. For fatty acids for instance, at high values of *A* (>1000 Å^2), the surface density is low and the amphiphiles are in a disordered gas phase. By compressing, the monolayer density increases and the chains start to interact and to emerge out of the water surface. A so called liquid expanded (LE) phase appears in equilibrium with the gas. Further increase in pressure leads to a phase transition and a surface completely in the LE phase (starting from values of *A* of the order of 40–60 Å^2), then the chains assume increasingly the trans conformation and a region of

* Corresponding author. Current address: Institut Laue-Langevin, BP 156, F-38042 Grenoble, France.
E-mail address: fragneto@ill.eu (G. Fragneto).

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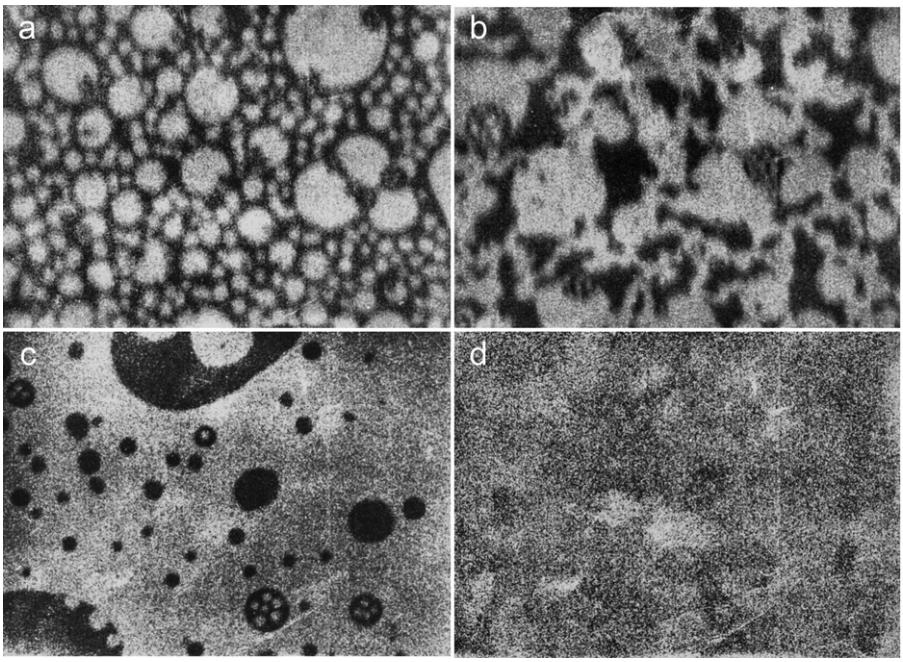

Fig. 1. Fluorescence microscopy images of Texas red-labelled GOx adsorption at a water/air interface as a function of time (a) image taken after 15 min: mobile quasi circular GOx domains can be observed; (b) image taken after 60 min: GOx domains tend to coalesce; (c) image taken after 120 min: GOx-free domains (or LE domains in a LC phase) are visible in a continuous condensed GOx phase; (d) image taken after 240 min: for this time the GOx film becomes nearly homogeneous.

coexistence with a liquid condensed (LC) phase appears (*A* between 20 and 40 Å^2). At values of *A* around 20 Å^2 all the molecules are in the condensed phase. By further compressing heads and chains become completely ordered in a solid like phase. A small amount of fluorescent dye (<1%) in the monolayer, which shows preferential partitioning in the various phases, allows visualization at the interface with the technique of fluorescence microscopy [10].

Fluorescent imaging has been used recently [11] to study the binding and partial insertion of myelin basic protein (MBP) into a lipid bilayer. It was possible to conclude that the interaction with the protein leads to a dramatic rearrangement and morphological changes of the lipid domains via non-specific interactions.

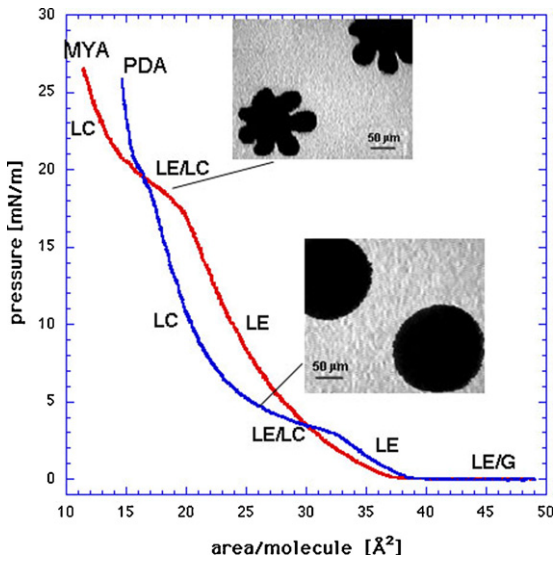

Fig. 2. Adsorption isotherm of PDA and MYA. The insets show the typical shapes of the LC domains (black areas) in the LC/LE coexistence regions of the two fatty acids.

2. Materials and methods

GOx from *A. niger* was purchased from Sigma and used as received. Langmuir isotherms were performed on a home-built (Institut Curie, Paris) Langmuir through equipped with two barriers. All measurements were done at 21 °C and the subphase was pure water. PDA and MYA were purchased from Aldrich (purity 99%) and used as received. The fatty acids were spread from stock solutions in chloroform at a concentration of 1.5 mM. They contained 0.1% of the dye Bodipy FL C16 (from Molecular Probes).

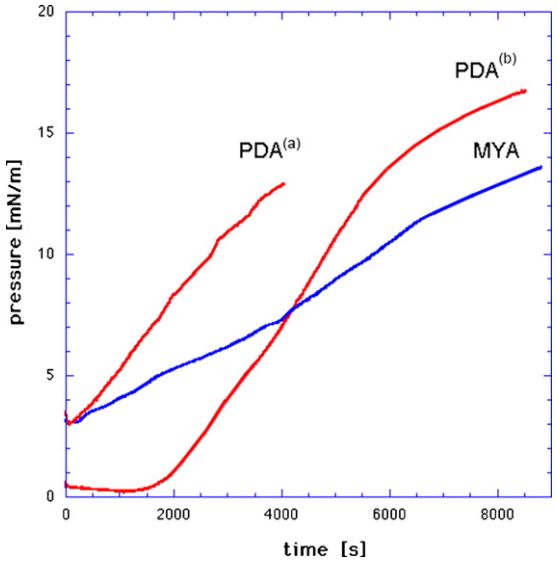

Fig. 3. Lateral pressure exerted at the interface with a solution of glucose oxidase 3.2 mg/l in contact with monolayers of PDA and MYA at 21 °C as a function of time. The curves marked MYA and PDA (a) the enzyme was inserted under the already formed fatty acid monolayers spread at an area/molecule of 50 Å^2. The curve marked PDA (b) was recorded when the fatty acid was spread at an area/molecule of 50 Å^2 from a stock solution in chloroform with the GOx already present in the substrate.

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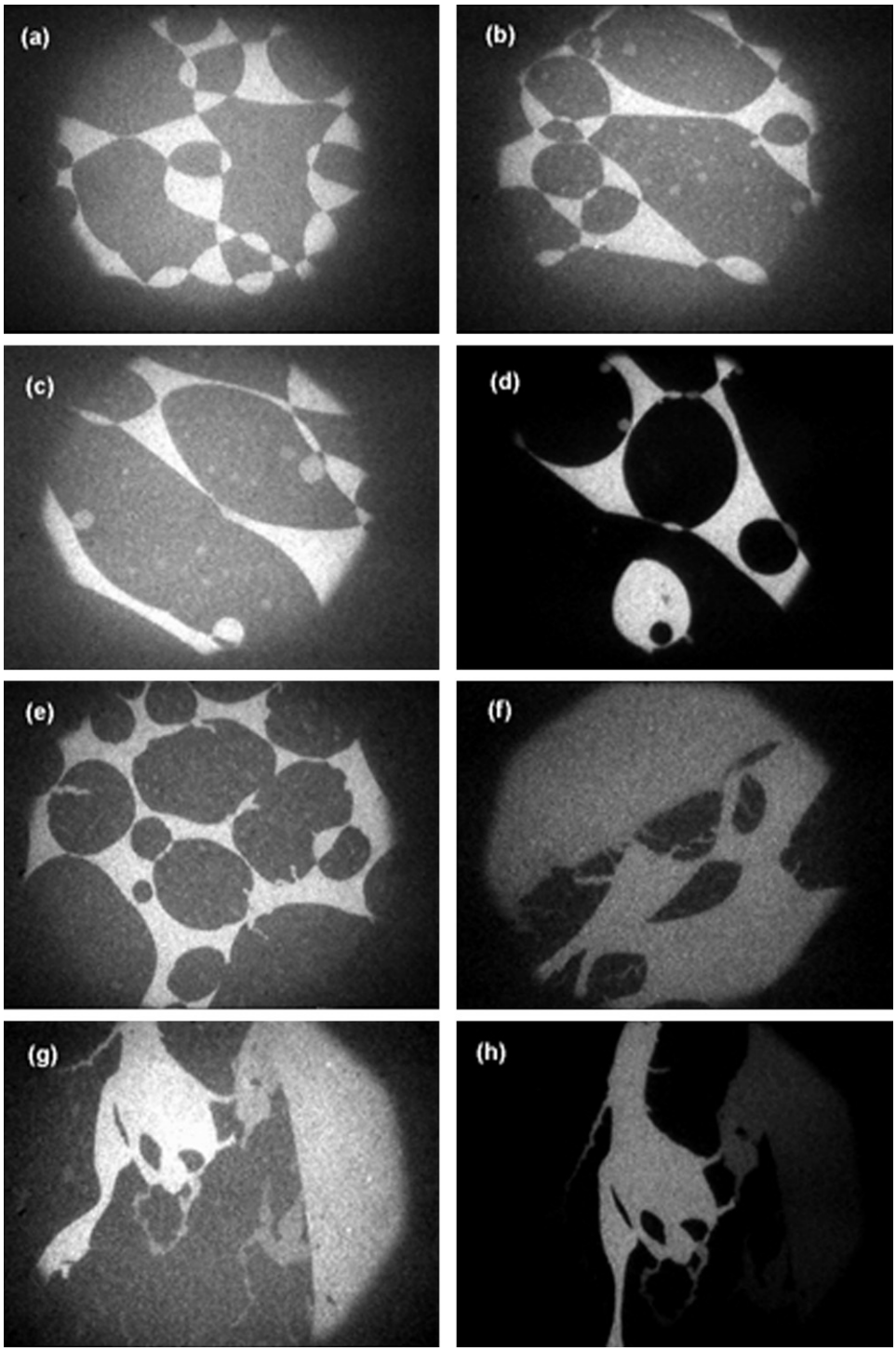

Fig. 4. Fluorescence microscopy image of LE/LC domains from PDA doped with Bodipy FL C16 and GOx partly labelled with Texas Red. The dark domains consist of PDA LC phase. (A) Image taken immediately after spreading the acid: GOx is present in LE phase which is the brighter in the image; (b) after 3 min: GOx starts to be visible inside the LC domains which edges are smoother than in the previous picture; images (c) and (d) were both taken after about 5 min: the fluorescent dots into the LC domains were visible with a double filter for Texas Red and Bodipy (c) but they were not visible with a filter only for Bodipy (d), which confirms that the fluorescence comes from Texas Red, that is from the protein (the two images do not show exactly the same domains because at this stage the domains still move quite freely on the surface and by the time the filter had been changed, few seconds, the domains shown in (b) had already moved away from the objective); (e) after 10 min: LC domains show cracks due to the pressure applied by the GOx in the LE phase and in the defects of the LC phase. (f) After 20 min: destruction of the initial shape of the domains. Here the border between a region containing only the protein (top left) and a region containing both the protein and domains of the acid (bottom right) is visible. Images (g) and (h) were both taken after 30 min, (g) with the double filter and (h) with the single one. The region on the left side, although visible in the two images, is much brighter when the double filter is used which means that it is very rich in protein. The dark areas show a region where the acid has segregated (note that here the two images have been taken on the same spot of surface since the domains move much more slowly than in the case of images (c) and (d). The aperture of the objective has a magnification of 40 for images (a)–(d) and of 50 for all the others. For corresponding pressures see Fig. 2.

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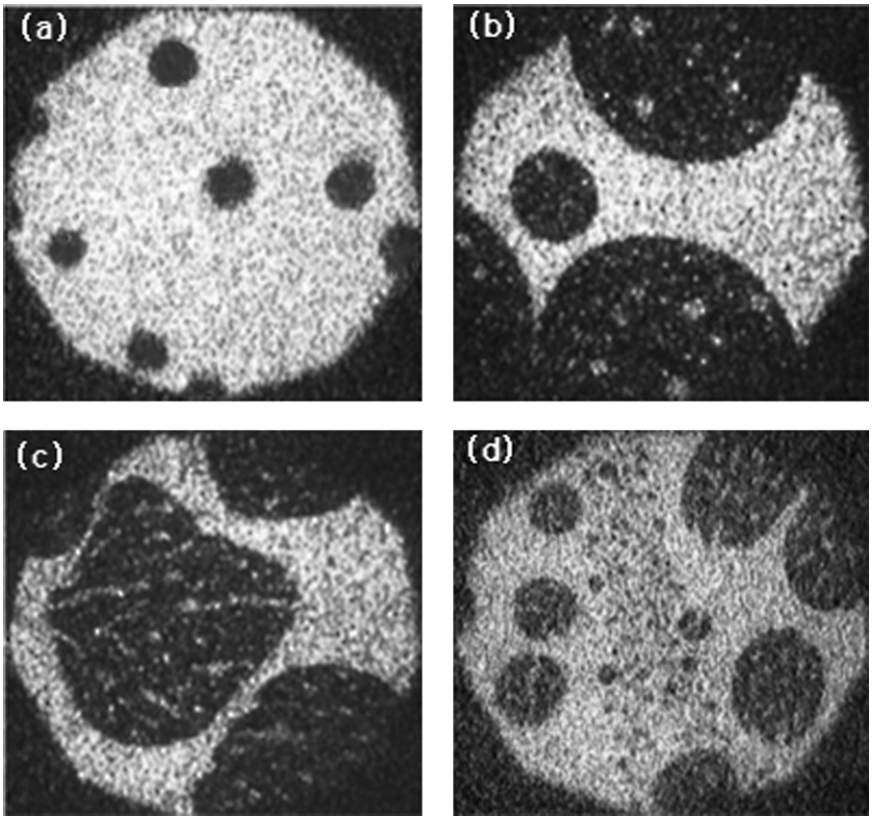

Fig. 5. Fluorescence microscopy images of the PDA/GOx system with the enzyme introduced in the subphase after having spread the PDA at an area/molecule of 50 Å^2. Here the acid is undoped and the enzyme is labelled with FITC (from molecular probes). (a) Small round domains of acid started to be visible 20 min after introducing the enzyme in the subphase; (b) image taken after 30 min: dots of protein are visible in the grown domains; (c) image taken after 35 min: domains are cracked and deformed; (d) image taken after 45 min: the small dark dots at the centre of the image are what remains of a previously bigger LC domain destroyed by the GOx. For corresponding pressures see Fig. 3. The aperture of the objective is 50×.

Proteins were labelled with 1% Texas-Red (from Molecular Probes). Lateral pressures were recorded with a Riegler and Kirstein film balance.

All images were taken from a Leitz-Leica microscope with aperture of objective with a magnification of 40 and 50 and recorded with a CCD camera (Lhesa). A high pressure mercury lamp was used to excite the fluorescence.

3. Results and discussion

Fluorescence images of the interface with solutions of the Texas red-labelled protein confirmed that GOx adsorbs immediately and tends to aggregate at the interface and to form domains (Fig. 1). By adsorbing it causes an increase in the surface pressure that at the concentration investigated (3.2 mg/l) reaches a plateau at 15.5 ± 0.5 mN/m in a time span of about 11 h. The use of fluorescent GOx allows the localization of the protein and the organization of the structure of the layer. The addition of GOx to the fatty acid system changes both the pressure area isotherm behaviour and the fluorescence microscopy images, thus indicating strong interaction. Surface pressure/area isotherms of PDA and MYA are shown in Fig. 2. Fig. 3 shows the rise of pressure with time of the fatty acids at the interface in the presence of protein. Differences arise by varying the procedure of formation of the mixed layer. Here the fatty acids were both spread on the water already containing the protein and also before protein insertion in the subphase. When a monolayer of PDA was spread on a subphase containing GOx (3.2 mg/l) in a quantity corresponding to an area per molecule of 50 Å^2 on the free surface, the pressure rose and LC domains were immediately formed. Since in the absence of protein the monolayer should be in a homogeneous LE phase at such PDA surface density (see Fig. 2), the area available for the formation of the fatty acid monolayer is reduced by the presence of the protein. By using both protein and acid labelled with fluorescent dyes adsorbing and emitting in different regions of the spectrum, it was possible to localize the protein and the distribution of the PDA molecules at the interface. Big areas of protein alone and protein mixed with the LE phase of the fatty acids were detected. Initially the LE phase was fluid and the PDA LC domains were observed to drift quickly on the surface. However, its viscosity increased with time and after about 1 h the LC domains were nearly still. This viscosity increase is consistent with the fact that the surface pressure is now much higher (21 mN/m). It is also interesting to note that the LC domains here are not of the usual round shape [10] but they present edges. This may be related to a different density state of the LC domains in the presence of the protein (or possible formation of a solid phase). After invading the LE phase (Fig. 4a), the presence of the protein started to be evident also in the LC domains: this insertion took place through localized defects of the condensed phase (Fig. 4b and c). As the pressure continued to rise, fractures starting from the edges of the domains were generated (Fig. 4d). Eventually, the domains lost their shape and became fragmented (Fig. 4e and f).

In order to investigate the kinetics of the adsorption, GOx was also introduced under a monolayer of PDA preformed on the surface at an area/molecule of 50 Å^2. The protein started to adsorb in the LE phase causing a reduction of the total area available to the PDA molecules. As a consequence, condensed domains formed within 15 min (Fig. 5a). The domains were initially round and grew in size with time. They were penetrated by the protein deformed

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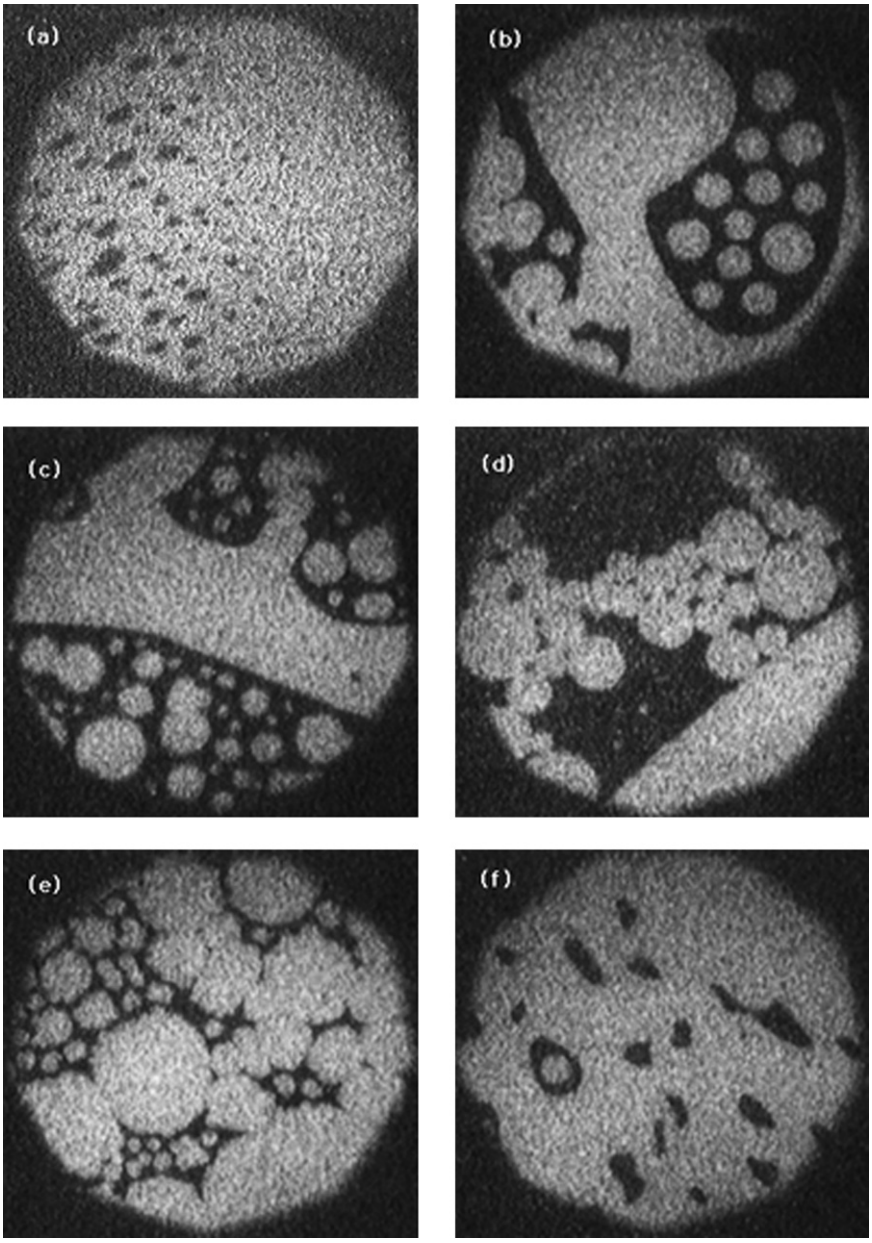

Fig. 6. Fluorescence microscopy images of the MYA/GOx system with the enzyme introduced in the subphase after having spread the MYA at an area/molecule of 50 Å^2. Here the acid is undoped and the enzyme is labelled with FITC (from molecular probes). (a) Image taken 1 hour after introducing GOx: the bright regions are MYA LE phase and GOx; the dark regions are MYA LC phase; (b) after 1 h and 20 min: MYA LC domains have grown in size and are surrounded by areas containing the protein (bright regions); (c) after one and half hour: more protein goes into the LC phase and forms small dots; (d) LE domains fuse; (e) LE domains 'eat off' the LC phase; (f) after two and half hours: fragments of LC phase. For corresponding pressures see Fig. 3. The aperture of the objective is 50×.

and eventually destroyed (Fig. 5b–d) within 1 h from its insertion. By compressing the layer, the domains were reduced to the shape of sticks all parallel to each other, while the protein clustered at their edges (see bright dots in Fig. 5d).

The differences in the images obtained with the two methods of inserting the protein are related to the fact that in the first case, although the surface of the solution containing the protein had been cleaned immediately before spreading the fatty acid, patches of proteins were already on the surface causing the fatty acid molecules to arrange in a condensed phase instantly. In the second case there is a delay due to the fact that the protein has to displace the fatty acid already covering the surface. The area available for the formation of the lipid domains is different in the two cases and therefore, although the amount of lipid and protein in the subphase is the same in the two systems, the lipid phase behaviour changes and is influenced by the irreversibly adsorbed protein. The fact that GOx inserts into defects of the condensed phase was confirmed by spreading the enzyme under a monolayer of PDA at an area of 21 Å^2. Domains were visible after some time with very bright edges when an undoped monolayer was coupled to doped protein in the subphase. The surface pressure remained stable around 21 mN/m.

Similar mechanisms were observed in the case of MYA, although with different timescales (see Fig. 6). Both when the acid was spread on a substrate containing the protein and when the protein was inserted under a preformed monolayer of acid at a value A of 50 Å^2, only big areas of protein were detected and no domains formed for nearly 2 h, the system being in the LE region of the phase diagram. Round domains then appeared and were eventually destroyed by the protein. The domains in this case were

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somewhat more 'fluid' than in the PDA/GOx system and their state was more difficult to interpret since the long time waited before the appearance of the domains does not allow a precise definition of the position on the pressure/area isotherm. When the enzyme was introduced in the substrate under a monolayer of fatty acid spread at 21 Å^2, the surface pressure decreased and no protein was visible on the surface for long times. After two and half hours some protein was detected which became more evident when the layer was further compressed. In fact, at 21 Å^2 the pressure of the MYA monolayer is higher than the pressure exerted by GOx.

In all the cases investigated the protein is incorporated inside the monolayer through the less dense LE phase and invades the LC phase afterwards. Once incorporated, it acts as a 'piston' exerting a continuous pressure on the monolayer. It is this pressure which changes the phase diagram of the fatty acid monolayer and causes the break up of the LC domains when a sufficient amount of protein has been incorporated to exert a sufficient force.

In all the systems studied the adsorption of the protein is totally irreversible even at the highest compressions: it remains at the air/water interface, eventually forming three-dimensional structures. An example is showed in Fig. 6. The protein generally crystallizes along lines and also during experiments at low lateral pressures it was often detected in domains where one of the dimensions was much bigger than the other.

Undoubtedly, the occupancy of GOx distorts the monolayer structure. The driving force for adsorption has to compensate for the increase in lateral pressure in the pure LE phase.

This driving force is the gain in enthalpy since the protein wants to aggregate and form two-dimensional crystals.

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