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Oxidation of Langmuir–Blodgett films of monounsaturated lipids studied by atomic force microscopy

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Abstract: In this work, we studied the stability in time of Langmuir–Blodgett films of POPC and OPPC, two unsaturated phospholipids with similar chains, differing by the relative position of these chains on the glycerol backbone. These films, transferred from the air–water interface onto freshly cleaved mica, were characterised by Atomic Force Microscopy (AFM) giving information on their topography at a lateral and perpendicular resolution in the nm range. AFM images (obtained in tapping mode) of freshly transferred films are homogenous, in agreement with the fact that these two lipids are in a liquid-expanded phase under our experimental conditions. After two days, small domains are observed, higher than the surrounding phase of about 0.8 nm in both types of samples. These domains are not observed if the samples are kept under vacuum, or if LB films are made of saturated phospholipids, suggesting that they are due to the local oxidation of POPC or OPPC, the oxidation being slightly more pronounced in the last case. Their dispersion in LB films suggests that oxidation occurs at different points at the same time, likely in areas presenting a loose packing or a defect. The local increase of thickness could be due to the reversal of the oxidised chain, raising the oxidised lipid above the surrounding phase.

Keywords: Langmuir–Blodgett film; unsaturated lipid; oxidation; atomic force microscopy.

Biographical notes: Ndeye Rokhaya Faye after a Master 1 degree of Structural Biochemistry at the Interface of Biology, Chemistry and Physics (University of Bordeaux 2, France) had a Master 2 degree in Cutaneous Physiology, Pharmacology and Bioavailability (University of Lyon 1, France) in 2010. She applied her complementary knowledge during a training of six months in the

Therapeutic Research Center on the University of Queensland in Brisbane (Australia) on the interaction of nanoparticles with human skin. She began a PhD on the nanoparticle-membrane topic at the University of Bordeaux 1 (France) in 2010.

Fabien Moroté was recruited in 2008 at the University of Bordeaux 1 (France) after a technical degree in Physics in 2007. He belongs to the technical staff of LOMA, where he is in charge of the Nano-Spectro-Imaging technical platform. He is particularly involved in the maintenance of AFM set-ups and all the experimentations made on them, concerning for instance the characterisation of various biological systems, such as Langmuir-Blodgett films of lipids, lipid bilayers, bacteria or algae.

Christine Grauby-Heywang received a degree in Biochemistry and Molecular Biophysics from the University of Paris 6 (France) and received her PhD in Molecular Biophysics from the same university in 1998. After a temporary Assistant Professor position at the University of Paris-Nord (France) and a Post-Doctoral position for one year in the group of E. Sackmann at the Technische Universität München (Germany), she became Assistant Professor at the University of Bordeaux 1 (France) in 1999, where she teaches mainly physics. Her research fields concern mainly Langmuir monolayers and supported models of cellular membranes obtained by Langmuir-Blodgett and Langmuir-Schaeffer methods or vesicle spreading, and made of lipids supposed to be present in rafts (glycolipids, sphingolipids, sterols). She studies also molecular planar systems made of organic molecules such as functionalised hemicyanines with particular optical properties, which can be applied for the specific detection of cations or light to electrical energy conversion, in collaboration with Russian colleagues from the Frumkin Institute in Moscow. She turned two years ago to the interaction of nanoparticles with membrane models, studied by fluorescence microscopy and atomic force microscopy.

Touria Cohen-Bouhacina received her PhD in Electronics from the Paul Sabatier University of Toulouse (France) in 1989. She became Assistant Professor of Physics at the University of Bordeaux 1 (France) in 1991 and Professor in the same university in 2005. In these last years, she developed different applications of atomic force microscopy, applying this method to various topics, such as the physicochemical treatments of surfaces and their nanomechanical properties or the hydrodynamics in the vicinity of surfaces. She also developed experiments on soft systems, such as polymers and biological systems (ATP synthase, cells, bacteria, etc.).

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1 Introduction

Cellular membranes are complex structures, made of a lipid bilayer in interaction with various extrinsic and intrinsic proteins acting for instance as enzymes or carriers. The variety of lipids is also very large, lipids being composed of different neutral or charged polar head groups and hydrophobic chains of various lengths and unsaturations [1]. The

first coherent model of cellular membrane described as a fluid mosaic by Singer and Nicolson [2] has been modified for many years with the notion of distinct membrane domains called rafts [3, 4].

Under these conditions, the study of such molecular systems can be very difficult, since it is impossible to discriminate the role of one lipid species in a given phenomenon, such as an interaction with a specific protein, a virus.... Different simplified membrane models have been developed for many years, containing usually no more than three lipid species, interacting eventually with a protein. Vesicles and liposomes are one possible model, made of a closed bilayer containing an aqueous solution, which can be different from the external one. Planar monolayers and bilayers are another type of membrane model. These models are deposited onto a planar surface, such as freshly cleaved mica, silica or glass. Different methods are possible: Langmuir–Blodgett (LB) and Langmuir–Schaeffer methods, vesicle spreading or spin-coating [5–8]. Some methods can also be combined, the first monolayer being built by the LB technique and the second one by the vesicle spreading for instance. In some cases, it can be useful to build and to study only one monolayer, corresponding to half a membrane [9]. This is for instance the case when the polar head groups are only involved in an interaction with an extrinsic protein [10]. At last, planar membrane models are particularly well-adapted to surface analysis techniques, such as Atomic Force Microscopy (AFM) or fluorescence microscopy as imaging techniques [11–14] or other methods such as quartz crystal microbalance [6, 15, 16].

Another pertinent parameter concerning membrane models is lipid choice. Lipids and thus cellular membranes present different phases depending on various parameters such as the temperature, the composition of the lipid mixture, and the presence of ions [17].... The behaviour of pure lipids is relatively simple, main part of lipids being characterised by a melting temperature (T_m). At a temperature below T_m , lipids are in a condensed phase, whereas at higher temperature, they are in a fluid phase. In membranes, the situation is more complex, with the formation of a liquid ordered phase present in rafts. The choice of lipids (saturated or unsaturated lipids, presence of cholesterol or not...) and of experimental conditions is thus very important to understand the behaviour of the membrane model in a given situation.

At last, an important point concerns the conditions where the samples are kept before their examination. In the case of planar bilayers, the second monolayer must be in contact with water or buffer, in order to have a stable molecular system. In the case of planar monolayers deposited on a hydrophilic support, lipid chains remain in contact with air. This can be a problem in the case of some lipids. Chemically, lipids are usually rather stable, but some of them are sensitive to oxidation. This is for instance the case of cholesterol or of unsaturated lipids, and more particularly polyunsaturated ones [18–21]. It is thus necessary to study the stability in time of these systems, before using them as membrane models, lipid oxidation promoting important changes in the lipid packing or phase separation in biological membranes [22–24] and membrane models [25–30]. Moreover, it is interesting to better understand the behaviour of these oxidised lipids in membranes, since these molecules could be involved in important biological processes, acting for instance as molecular tag for the recognition of cells by macrophage receptors [31]. Under these conditions, planar monolayers are particularly well-adapted to study the effect of oxidation: monolayers could be more sensitive to oxidation than bilayers because of the absence of a ‘protecting’ water layer above the sample, even if this oxidation has been observed in the case of liposomes [19, 23, 28, 30].

In this work, we studied the stability of planar LB films made of two types of unsaturated lipids: 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) and 1-oleoyl-2-palmitoyl-phosphatidylcholine (OPPC). Both have a saturated palmitic chain (C16) and an unsaturated oleoyl one (C18:1), but their relative positions are inverted on the glycerol backbone. POPC and OPPC monolayers were transferred from the air–water interface onto freshly cleaved mica by the LB method and AFM was used to observe them just after their deposition and two and five days later. AFM is a high resolution scanning probe surface analysis technique, whose essential property is to measure the interaction forces acting between a fine tip and a sample, which depends on their distance. A key advantage of this technique is, on one hand, its ability to give morphological (or topographical) details of soft matter with less damage at the nanoscale. On the other hand, AFM provides information about tribology but also provides mechanical properties of samples [32–35]. At last, using monolayers instead of bilayers enables to use the tapping mode in AFM, this mode being less disturbing for the sample and giving consequently a better resolution.

2 Materials and methods

2.1 Molecules and solvents

POPC and OPPC were purchased from Sigma (France). They were >99% pure and used without further purification. Ethanol and chloroform used to solubilise lipids were both HPLC grade and purchased from Fluka and Sigma, respectively. Millipore water (pH 5.5, resistivity > 18 M Ω .cm) was used as subphase in surface pressure measurements.

2.2 Surface pressure measurements and Langmuir–Blodgett transfer

Surface pressure experiments were carried out in air, by using a Nima Langmuir trough (approximately 30 cm $\times$ 10 cm $\times$ 0.5 cm) equipped with a Wilhelmy balance (Nima). Briefly, lipids were dissolved in a 1/1 (v/v) mixture of ethanol and chloroform at a concentration of 1 mM. After spreading of the solution at the air–water interface and evaporation of solvents (15 min), lipids were compressed continuously at a rate of 5 cm².min⁻¹. The temperature of the room was kept constant to 20 $\pm$ 1°C.

Monolayers were transferred from the air–water interface onto freshly cleaved mica by the Langmuir–Blodgett method, by using a dip coater mechanism from Nima. Mica was first immersed in water and the lipid monolayer was prepared as previously described and compressed until a surface pressure of 30 mNm⁻¹. This surface pressure was then kept constant, thanks to a control system maintaining the pressure by adjusting the surface occupied by the monolayer. After stabilisation of the lipid monolayer (a few minutes), mica was removed from water at a rate of 5 mm min⁻¹ and kept protected from dust until its imaging by AFM at different times after its transfer.

2.3 Atomic force microscopy

AFM images were recorded with a BioscopeII AFM set-up (Veeco-Brucker, Santa Barbara, CA) equipped with a G scanner (maximum XYZ scan range of 150 μ m $\times$ 150 μ m $\times$ 12 μ m). In this work, samples were scanned in tapping mode using

PPP-NCL silicon probes (NANOSENSORS™) with a spring constant of about 32 N/m and a corresponding measured resonance frequency of about 165 kHz. In contact mode, triangular SNL silicon nitride probes (Veeco) with a nominal spring constant of 0.58 N/m were used. All scans were done at air and room temperature ($20 \pm 1^\circ\text{C}$) with scan rates between 0.3 Hz and 1 Hz (according to the scan size and the scanning mode). Samples were scanned just after monolayer deposition and two and five days later, using the same probes for both tapping and contact modes. For each sample, three different LB films were taken to ensure reproducibility. For data processing, AFM data were processed using the Nanoscope (version 7.30, Veeco) and the Gwiddion softwares. Each time, six images were recorded at the same time: trace and retrace height images (topography), trace and retrace amplitude images (error signal) and trace and retrace phase images (mechanical properties) in tapping mode; trace and retrace height images (topography), trace and retrace deflection images (error signal) and trace and retrace friction images (mechanical properties) in contact mode. For more clarity flattened images are mainly shown in the paper.

3 Results and discussion

Figures 1 and 2 show the chemical structure of POPC and OPPC and their π -A isotherms at the air–water interface, respectively. These isotherms are on the whole similar, showing in particular that both lipids are in a liquid-expanded phase during all the compression under our experimental conditions. These results are on the whole in agreement with previously published isotherms, obtained under similar experimental conditions [36, 37], in particular at temperatures higher than the transition temperatures of these lipids, around -3°C and -9°C for POPC and OPPC, respectively [38–40].

Figure 1 Chemical structures of POPC and OPPC

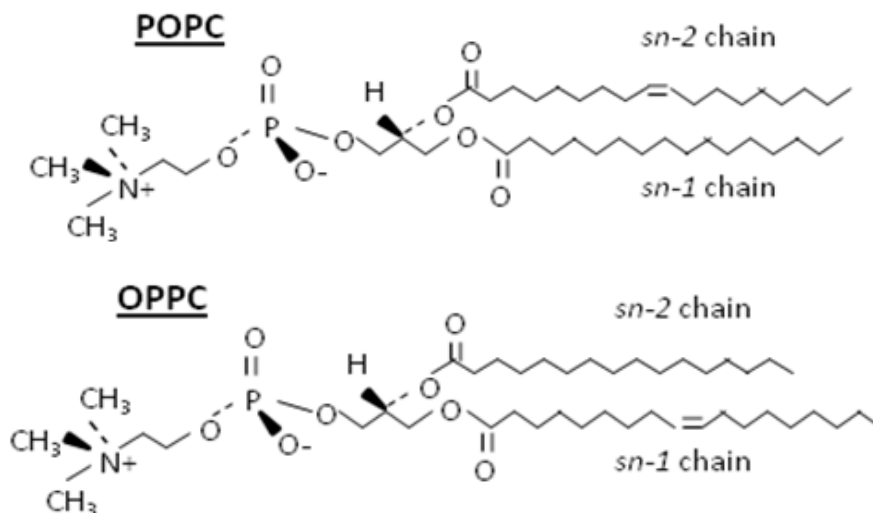


Figure 3 shows AFM phase images of POPC and OPPC LB films just after their transfer at 30 m.Nm^{-1} on freshly cleaved mica (J1). All these images are obtained in repulsive mode to get information on the topography of surfaces. In both cases, these very

homogenous images confirm that both lipids are in an expanded phase. The average surface roughnesses (taking into account the experimental noise) are 0.10 nm and 0.14 nm in the case of POPC and OPPC, respectively, at J1 (see Figure 4a), whereas their average thickness (see Figure 5), obtained after the local scratching of the films, is around 0.5 nm [41]. This value is low compared to the length of a lipid molecule and to previous results obtained in the case of POPC LB films [33]. However, it is necessary to use a stiff probe and a high force (in contact mode) in scratching experiments. This can explain the imprecise thickness obtained here, in combination with the incomplete removal of lipids from the surface. Indeed, as underlined in a previous study, the estimation of thickness by scratching is difficult in the case of LB films of fluid lipids, these lipids tending to re-spread immediately after scratching or being laid down onto mica because of their contact with the tip [33]. Under these conditions, another method is possible, using the width of the jump observed in the force-extension curves [33].

Figure 2 π -A compression isotherms of POPC and OPPC on pure water ($20 \pm 1^\circ\text{C}$, compression rate $5 \text{ cm}^2 \text{ min}^{-1}$)

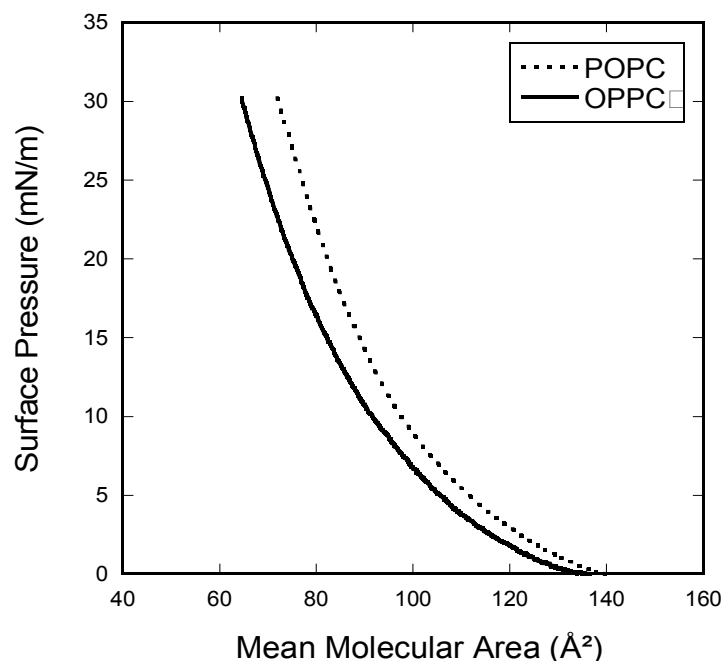
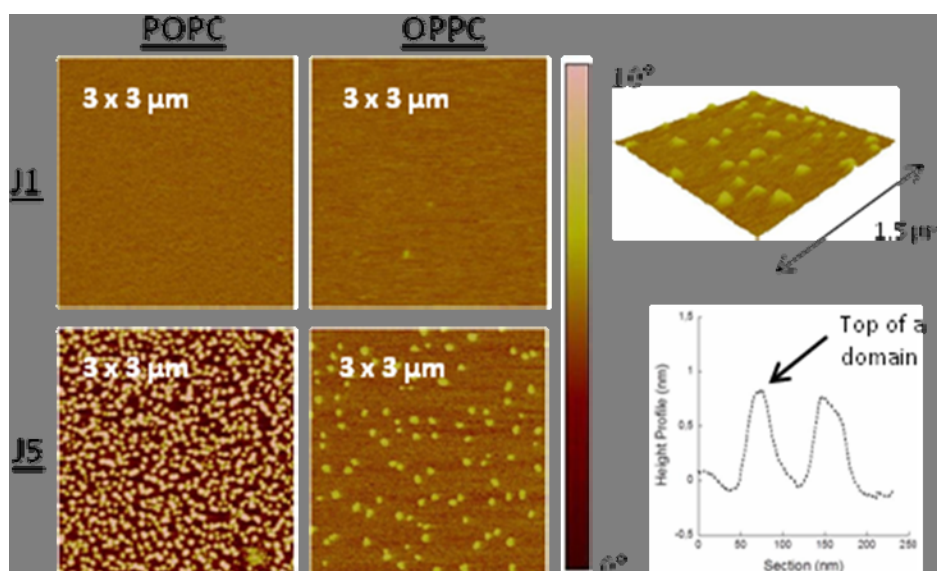


Figure 3 also shows AFM images of the same POPC or OPPC films after five days (J5). These images are clearly different from the previous ones: both films are not homogenous anymore, their surface being spotted with small domains regularly distributed and higher than the surrounding liquid-expanded phase of 0.8 nm in average (see Figure 3). The study of frictions in contact mode shows that the surfaces covered by domains are denser in both cases (data not shown). However, some discrepancies are observed between the two lipids in the evolution of the average surface roughness and the average surface of domains, OPPC appearing more prone to ageing than POPC (see Figure 4). This is corroborated by the estimation of the fraction of the surface occupied by oxidised lipids, increasing more rapidly in the case of OPPC, and representing 1% and 0.4% after five days in the case of OPPC and POPC, respectively (data not shown).

These results are reproducible with other similar films transferred and studied under the same experimental conditions. Moreover, if samples are kept under vacuum during the days following their transfer, domains are not observed (data not shown). They are not observed either with saturated lipids, such as sphingomyelin or dimiristoyl-phosphatidylcholine (DMPC) (data not shown). At last, in the case of phase-separating POPC-DMPC mixed LB films, just after the transfer, AFM images show the presence of domains of condensed DMPC in a fluid phase made of a mixture of POPC and DMPC (see Figure 6a), these domains being higher than the surrounding phase of about 0.65 nm. Two days later, small domains appear only in the areas containing POPC (see Figure 6b).

Figure 3 On the left: AFM phase images (tapping repulsive mode) of POPC and OPCC LB films, freshly prepared (J1) and after five days in contact with air (J5). On the right: 3D height image ($1.5 \times 1.5 \mu\text{m}$) of an oxidised POPC monolayer and corresponding height profile (section of 250 nm, difference of height between the non-oxidised monolayer and the top of the domain around 0.8 nm) (see online version for colours)



Taken together, these results suggest that the appearance of domains in POPC and OPCC ageing monolayers are due to the oxidation of these unsaturated lipids. To our knowledge, it is the first time that in situ oxidation is observed by AFM, whereas this method is particularly pertinent to study the organisation of lipids in planar membrane models. Oxidation can occur by different ways, such as enzymatic mechanisms or non-enzymatic ones. In this last case, they can be mediated by reactive oxygen species such as hydroxyl radicals. Under these conditions, oxidation of lipids leads to the formation of various compounds such as truncated phospholipids containing terminal polar groups (carbonyl, hydroxyl...), lysolipids... [21]. Polyunsaturated phospholipids are more sensitive to oxidation than monounsaturated ones [21]. It has been shown for instance that the infrared H-C=C-H stretching mode of olefinic groups in 1-stearoyl-2-

arachidonyl-phosphatidylcholine has a lifetime of less than 15 min at the air–water interface, even in the presence of argon [42]. On the contrary, previous studies showed that POPC is not sensitive to the oxidation induced by air oxygen at the air–water interface [36] or in aqueous solution [43]. However, POPC monolayers are highly sensitive to ozone [36].

Figure 4 Averaged surface roughness (a) and averaged domain surface (b) in POPC and OPPC LB films freshly prepared, and after two and five days in contact with air. Error bars are calculated from the standard deviation of both parameters measured in three different samples

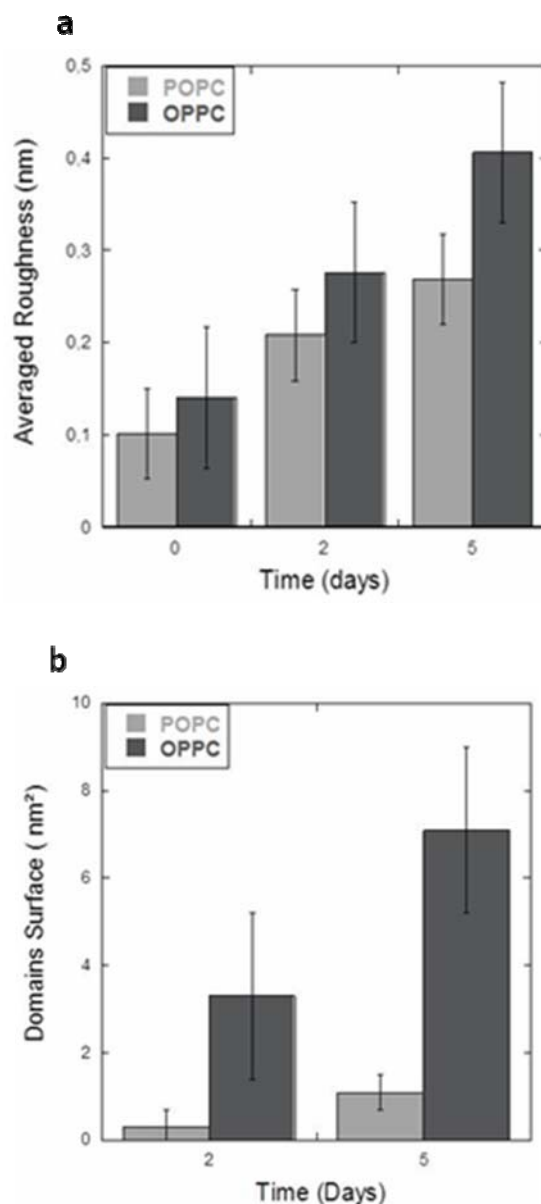
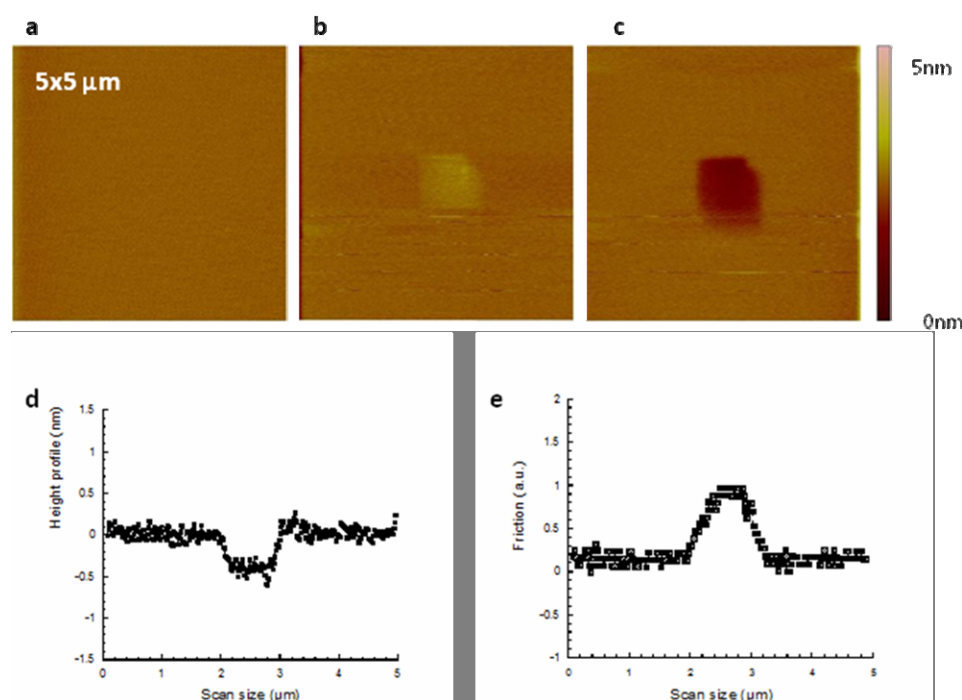


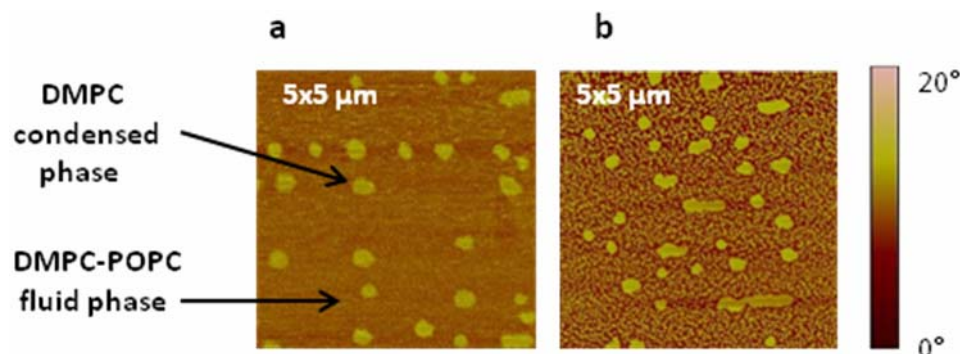
Figure 5 Scratching of a LB film of POPC (height image): the tip is used to scratch locally the surface of the sample (square in the middle of the images (b) and (c): $1 \times 1 \mu\text{m}$) and the apparent thickness of the film is determined from the height profile (d) obtained as follows. Generally, the contrast of a height image in contact mode includes topography information and mechanical properties due to mechanical friction forces, depending on scanning direction (trace or retrace) and adding to or subtracting from the true topography. To extract separately both information, two images of the same area (trace: (b); retrace: (c)) are recorded. Their sum provides true topographical information and the hole depth corresponding to the monolayer thickness, that is the difference between the hole bottom (hard substrate mica) and the top of the lipid monolayer (d). The half difference of the two images gives the evolution of friction on the probed surface (e). From this last curve, it is possible to deduce that the two areas, hard substrate mica and LB film, have different tribological behaviour, the friction forces being lower on the film than on mica (see online version for colours)



It is well accepted now that oxidation consists in three steps, initiation (formation of a lipid radical), propagation (addition of molecular oxygen on the radical) and termination [18]. The dispersed domains observed in LB films suggest that oxidation occurs at different points at the same time, likely in areas presenting a loose packing or a defect, oxidation propagating from these areas. The rather long time necessary to observe domains of oxidised lipids confirms also the relative resistance of POPC and OPPC to oxidation by air oxygen. Monolayers can be roughly compared to a highly viscous solvent incorporating less air oxygen than a more fluid one [18]. However, the rather regular organisation of lipid molecules into LB films (even if these lipids are in a liquid-expanded phase) could also contribute to an efficient propagation of oxidation: thus, the

rate of oxidation of arachidonic acid in liposomes of DMPC increases at a temperature below the phase transition temperature of DMPC [44]. In a same way, the presence of cholesterol in a lipid bilayer facilitates the propagation of free radicals through the bilayer, by increasing the lateral packing of lipids [43].

Figure 6 Phase images of mixed LB films of DMPC and POPC (1/1 in mol) transferred on mica at 30 m.Nm⁻¹: freshly prepared LB film (a) and two days later (b) (see online version for colours)



At last, we observe differences between POPC and OPPC monolayers, average surface roughness and average surface of domains being more affected by time in the case of OPPC (see Figure 4). This comparison suggests that the double bond is not similarly sensitive to oxidation on the *sn*-1 or *sn*-2 chain (see Figure 1). It has been reported that the acyl chains do not have the same orientation near the glycerol part. Consequently, one chain is shifted as compared to the other one from about 1.5 carbon-carbon bonds, this distance being estimated after projection on the bilayer normal [38]. It implies that the double bond is more deeply embedded in a bilayer of about 1.8–1.9 Å, when it is on the *sn*-1 chain or closer from the air in the case of our planar monolayers. This difference, even very weak, seems to influence the sensitivity of the double bond to oxidation between the two lipids, even if previous NMR measurements did not show any difference of packing between the two lipids in vesicles, this packing being sufficiently tight to prevent water molecules to penetrate deeply into the bilayer [45].

Reversed-phase liquid chromatography-mass spectrometry revealed the nature of oxidised products of POPC after oxidative treatments using Fe(II) and H₂O₂ [19]. Hydroperoxide, keto and hydroxyl derivatives were mainly found. Other derivatives such as lipids with a *sn*-2 truncated chain bearing a terminal carboxylic or aldehyde function are obtained with POPC [25]. We can suppose that similar derivatives can also be present in POPC and OPPC LB films. Numerous studies showed that oxidised lipid derivatives are able to perturb the bilayer organisation, even if contradictory results were sometimes found. For instance, Sabatini and co-workers showed that increasing amounts of carboxylic or aldehyde derivatives of POPC in Langmuir monolayers induce an expansion of the DPPC monolayer at a surface pressure lower than 20 m.Nm⁻¹ [25]. They assigned this expansion to the fact that the *sn*-2 chain of POPC would reverse its direction in order to accommodate the polar carboxyl or aldehyde group into the vicinity

of the lipid head group. The hypothesis of reversed chain is confirmed by NMR [31] and molecular dynamics simulations [26, 29, 30]. On the whole, these studies are coherent and show that the orientation of the unmodified chain does not change, whereas the orientation of the oxidised one is modified, the polar group getting closer to the interface. It can be more or less parallel to the interface or able to stick out of the membrane surface. A decrease of the bilayer thickness is also observed, assigned to the orientation changes. In our case, we observed, on the contrary, an increase of the monolayer thickness around 0.8 nm in oxidised domains. This difference is likely related to the different nature of the samples. Molecular dynamics simulations were performed on lipid bilayers in contact with water on both sides. Similarly, monolayers at the air–water interface are in contact with a high water volume. In our case, monolayers are transferred on a planar support. Even if a thin water layer is transferred with the monolayer, it is likely not sufficient to enable the oxidised chain to stay parallel to the support. We can suppose that the reversal is more important, this reversal raising oxidised lipids above the surface of the expanded surrounding phase.

4 Conclusion

POPC and OPPC LB films are both sensitive to oxidation induced by air oxygen, even if this process is rather slow. This oxidation, not observed if samples are kept under vacuum, is responsible for the formation of domains higher than the surrounding liquid-expanded phase of 0.8 nm on average. This increase of thickness is probably due to a particular orientation of oxidised lipids, leading to their raising above the surrounding phase. At last, our results show that AFM is a suitable method to study the oxidation of lipids in planar LB films, even if its applications in this field are surprisingly rare. Perspectives of this work could concern two aspects: first, inducing an oxidation of lipids by another more efficient method (such as ozone) and second, studying ‘artificial’ oxidised monolayers of POPC or OPPC with a variable and controlled amount of known oxidised lipids. However, in this last case different results could be observed as compared to the results shown here, since it is thermodynamically different to induce locally the oxidation or to let oxidised and not oxidised lipids organise freely.

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