Mixed Monolayers of Phosphatidylethanolamine and (Laurylamido)-*N*,*N*′-dimethylpropylamine Oxide at the Air/Water Interface. Lateral Proton Conduction along a Mixed Structure of Conducting and Nonconducting Entities

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Abstract: A localized lateral proton pathway is present along the phospholipid polar heads and bound water molecules when the lipids are spread in monolayers at the air/water interface. Mixed films of (laurylamido)-*N*,*N*′-dimethylpropylamine oxide (LAPAO) and phosphatidylethanolamine (PE) are studied by surface pressure and fluorescence measurements. The polar head of this amphiphilic detergent shows no global electric charge but a $N^+ \leftarrow O^-$ dipole. The localization of this detergent at the air/water interface changes when the mixed monolayer is compressed. The mixed monomolecular film is metastable, and a leakage of the amphiphile is observed even at very low surface pressure. Whatever the initial LAPAO/PE molar ratio spread at the air/water interface, when the monolayer is compressed, all molecules of LAPAO are expelled from the film to the bulk at a surface pressure of 20 mN/m. Nevertheless, some of them remain adsorbed at the interface. For mixed monomolecular films with a surface pressure of 5 mN/m, a facilitated lateral proton conduction is present. An increase in the percentage of LAPAO in the spread LAPAO/PE mixture modulates the lateral proton conduction when larger than a critical value. Mixed LAPAO/PE monolayers at 5 mN/m, with a spread ratio LAPAO/PE larger than 19/1 (mol/mol), present a slowing down of the kinetics in proton conduction. No alteration of the structure or the dynamics of the film can be observed under these conditions. The effect of LAPAO on preferential lateral proton conduction must be explained by a subtle change in the organization of the membrane/water interface.

Introduction

Preferential lateral proton conduction at the phospholipid/water interface has been observed at a macroscopic scale by using different methodologies: measuring the local proton concentration change at the interface level by fluorescence,[1-3] measuring surface potential variations,[4] measuring changes in surface pressure for acidic lipids,[5] and indirectly measuring the increase in surface conductance[6-8] as previously described with multilamellar systems.[9-11]

This lateral proton conduction was proposed to be a diffusion process through a hydrogen-bond network involving the polar head groups of phospholipids and the hydration layer.[3] The phenomenon is supposed to occur in three steps. **Step 1**: adsorption of the protons from the bulk phase to the interface. This step is a limiting one because an unstirred layer exists along the lipid monolayer, as at any interface.[12-14] **Step 2**: fast diffusion of the H^+ along the interface by a "hop and turn" mechanism[15-17] along a hydrogen-bond network. **Step 3**: leakage of protons back to the bulk phase across the unstirred layer mentioned in step 1.

All previous studies were performed on pure phospholipids. A more complicated system than a single phospholipid monolayer was recently studied.[18] In this case, cationic charges were inserted at the interface using an amphiphilic detergent molecule, cetyltrimethylammonium bromide (CTAB). The detergent changed the properties of the interface and modulated the facilitated lateral proton conduction. The mixed film of detergent/phospholipid was shown to be metastable by surface pressure and fluorescence measurements: the detergent left the interface for the bulk phase. Nevertheless, a fraction of the spread cationic detergent remained in the lipid matrix, as shown by the binding of the anionic fluorescent probe 8-anilino-1-naphthalenesulfonate. Proton conduction was prevented only when the surface concentration of the detergent was larger than a critical value. Such an effect could be due either to the disruption in the continuity of the conducting hydrogen-bond network or to an electrostatic repulsion of the protons by the cationic interface.

In order to rule out the hypothesis of an electrical contribution in this alteration of the conduction and understand what happens at the interfacial molecular level, mixed films of (laurylamido)-*N*,*N*′-dimethylpropylamine oxide (LAPAO) and phosphatidylethanolamine (PE) were cospread at the air/water interface. This

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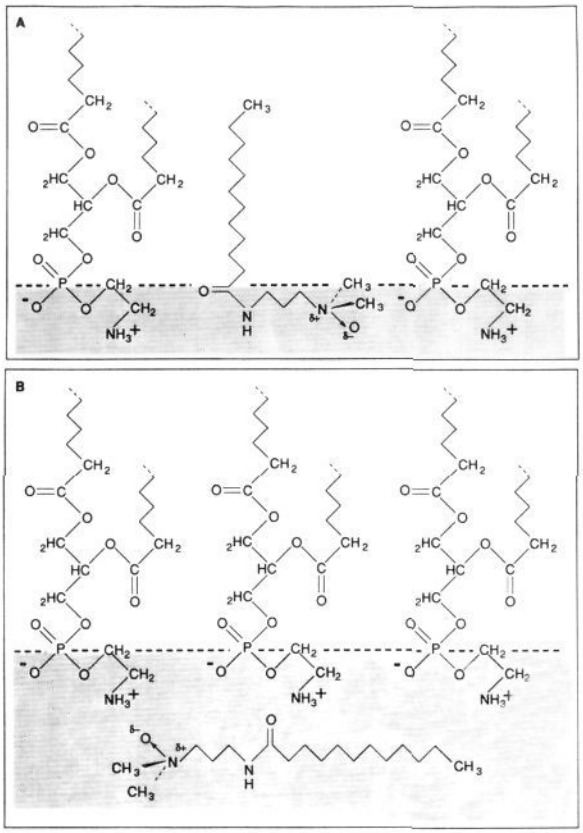

Figure 1. Chemical structure and schematic structuration of LAPAO at the phospholipid/water interface. (A) At low surface pressure, the detergent is partly embedded in the phosphatidylethanolamine film as shown by the expansion of the mixed film when compared to that of the pure PE monolayer. The polar group is presumed to be parallel to the interface. (B) At high surface pressure (larger than 20 mN/m), the detergent is expelled from the PE matrix as shown by the compression isotherms but it remains adsorbed at the interface and, as such, affects the binding of ANS.

detergent, synthetized by Brandolin et al.,[19] was used routinely for the membrane purification of adenine nucleotide carrier proteins.[20] Membrane protein purification required the breakage of hydrogen bonds which stabilized the protein–membrane interaction. This detergent molecule presents amphiphilic properties due to a $N^+ \leftarrow O^-$ dipole in its polar head and a slightly hydrophilic amido group in its hydrophobic core (Figure 1). Its hydrophobic tail consists of 10 CH_2 with a further 3 CH_2 separating the two hydrophilic parts $N^+ \leftarrow O^-$ and CO–NH. No net charge will be added when the amphiphile is present in the lipid matrix. It appeared as a good candidate for perturbation of hydrogen bonds at the lipid/water interface. The molecular structuration of mixed LAPAO/PE films at the air/water interface was tentatively studied and was shown to modulate the interfacial lateral proton conduction.

Materials and Methods

Chemicals. *Escherichia coli* phosphatidylethanolamine and 12-(9-anthroyloxy)stearic acid (AS) were purchased from Sigma. 8-Anilino-1-naphthalenesulfonate magnesium salt (ANS) was purchased from Kodak. (Laurylamido)-*N*,*N*'-dimethylpropylamine oxide was a generous gift from Dr. Block (CEA, Grenoble, France). Synthesis of the pH fluorescent indicator probe fluorescein phosphatidylethanolamine thiocarbamide (FPE) was previously described.[21] Salts were of analytical grade. Ultrapure water, free from surfactant, was prepared with a MilliQ system (Millipore, France).

Monolayer Preparation. Buffered saline solutions were prepared with ultrapure water. Lipid/detergent mixtures, from a chloroform/methanol solution (5/1, v/v) were spread on the aqueous phase, and a 5-min period was allowed for solvent evaporation. The film surface pressure was monitored by means of a platinum plate (Prolabo, France) connected to a force transducer constructed at the laboratory. The sensitivity of the surface pressure determination exceeded 0.2 mN/m. Temperature was 20 °C (± 0.5 °C). Compression isotherms were obtained by moving a Teflon barrier driven by an electric motor in order to change the total surface area of the monolayer.

Fluorescence Measurements. The front-face fluorescence was monitored using an interface fluorimeter constructed in the laboratory.[22,23] The emission from a small illuminated area (about 2 mm in radius) was measured for different compression states of the monolayer. The trough was milled in Plexiglas in order to obtain a low degree of light scattering. Excitation wavelengths were selected by means of optical filters. The fluorescence intensity was measured using a photomultiplier tube (EMI 9558, England) connected to a data acquisition unit. Photobleaching experiments were carried out as previously described.[24] Lateral proton-diffusion experiments were run with the proton "window" jump technique using a trough and the experimental procedure previously described.[1] Monolayers were obtained by spreading a mixture of phospholipid/detergent and FPE (PE/FPE molar ratio 98/2) in a solution of $CHCl_3$/MeOH (5/1, v/v) onto an aqueous subphase (1 mM phosphate buffer, pH 6.8). The movement of protons from the injection compartment to the fluorescence observation area was observed by a change in the fluorescence emission of the pH-sensitive fluorescent chromophore FPE at the lipid/water interface. This proton lateral diffusion can be described by two parameters: T_{H^+}, the time between the acid injection and the beginning of the decrease in fluorescence, and ΔF, the magnitude of this decrease.

Determination of the Apparent p*K* (pK_{app}). pK_{app} was taken as the subphase pH at which the fluorescence F, emitted by the film for a given surface pressure Π, obeyed the relationship

$$\frac{F(pK_{app},\Pi) - F(pH = 4,\Pi)}{F(pH = 7.5,\Pi) - F(pK_{app},\Pi)} = 1$$

(the pH values being those of the subphase), since no change in the fluorescence intensity was observed for subphase pH values lower than 4 or greater than 7.5 regardless of film pressure.

ANS Binding Isotherms. 8-Anilino-1-naphthalenesulfonate is known to display a strong increase in the fluorescence yield and a large shift in the emission spectrum when bound to a structured and/or hydrophobic environment.[25] The binding is mainly due to hydrophobic forces but can be modulated by the electrostatic force.[26-28] From the changes in emission from the bound probe upon compression, which were evaluated for different concentrations, the dissociation constant K_{diss} and the saturating emission I_∞ were computed for different film packings.

Photobleaching Experiments. The experiments were carried out as previously described[22,24] by using the loss of fluorescence due to the photodimerization of the probe. The dimerization reaction of 12-(9-anthroyloxy)stearic acid is second order with respect to the probe. As the dimer is nonfluorescent and the fluorescence intensity of the monomer is linearly related to its concentration, the kinetics of the reaction is obtained from the decay in the fluorescence emission during illumination. The rate constant of dimerization K_d is obtained by plotting the reciprocal of the fluorescence intensity against the time of illumination, during the early steps of the photoreaction. K_d was shown to depend on spectroscopic and structural contributions (K_{DS}).[29] The reduced fluorescence intensity If_{AS} and K_{DS} reflect the influence of both the structure and the dynamics of the probe environment. After statistical analysis, taking into account the nonlinear relationship between the extent of the recovery and its duration,[30] recovery experiments were analyzed by a mathematical approach adapted for uniform disk illumination.[24]

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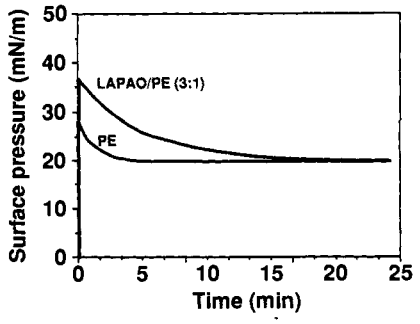

Figure 2. Kinetics of the surface pressure changes following the spreading. The lipid mixture in the solvent was spread and the surface pressure recorded during the stabilization step.

Results

Apparent p*K* of the Fluorescent Probe FPE Embedded in a Monolayer of LAPAO/PE 3/1 (Spreading Ratio, mol/mol). The apparent p*K* of the fluorescent dye FPE, in mixed monolayers spread at a LAPAO/PE ratio of 3/1 (mol/mol), was evaluated to be 5.6 ± 0.4 at low surface pressure (5 mN/m). No shift was obtained as compared to that with the same fluorescent probe embedded in a monolayer of pure phosphatidylethanolamine.[21] FPE is therefore a reliable probe to monitor the local proton concentration changes at the lipid/water interface even in mixed films.

Surface Pressure Changes for Mixed Films of LAPAO/PE. The surface pressure of mixed monolayers LAPAO/PE was seen to decrease greatly during the stabilization time which was allowed for a good evaporation of the solvent (Figure 2). This decrease was much larger than that normally observed with pure phospholipid (more than two times in Figure 2). Its magnitude was very large when films with a high content of LAPAO were spread.

If a film spread with an initial low surface pressure (less than 5 mN/m) was compressed, the associated compression isotherms were observed to be dependent on the initial detergent to phospholipid molar ratio (Figure 3). The higher the initial ratio LAPAO/PE (mol/mol), the more expanded the film was. Nevertheless, at high packing densities ($\Pi > 22$ mN/m), the isotherms were identical to each other regardless of the initial composition of the film. There was no physical evidence that LAPAO remained embedded in the PE matrix in tightly packed films.

ANS Binding. The binding of the fluorescent amphiphile to the lipid assembly was greatly affected by the presence of the detergent in the phospholipid monolayer. When the LAPAO/PE mixture was spread at a molar ratio of 3/1, the dissociation constant K_d was decreased from 30 μM for compressed films to 5 μM for loose films. For a pure PE film, the same behavior was observed (Figure 4A). The results for the pure PE film correspond to our previous analysis of the interaction with phospholipids of the fluorescent amphiphilic probe *N*-phenylnaphthylamine (NPN).[31] The dissociation rate was observed to be constant for loosely packed films but greatly increased when the film pressure was larger than 10 mN/m (0.64 nm^2/mol). The ratio I_{476nm}/I_{498nm} increased upon compression of mixtures of LAPAO/PE spread at a molar ratio of 3/1 (mol/mol) up to surface pressures greater than 20 mN/m (0.58 nm^2/mol) (Figure 4B). This indicated a blue shift in the spectrum of the fluorescent probe.[22] For surface pressures higher than 20 mN/m, this ratio was constant and greater than that for a pure PE monolayer.

Lateral Proton Conduction by Mixed Monolayers of LAPAO/PE. A preferential lateral proton conduction along mixed films of LAPAO/PE was observed when the spreading molar ratio of LAPAO was kept smaller than 4/1 (mol/mol) as shown in Figure 5. This observation was similar to that observed for other pure phospholipid films in the liquid-expanded state. The surface pressures of the films were 5 mN/m after stabilization, in order to be sure that LAPAO molecules were present at the interface as shown by the compression isotherms. The T_{H^+} was of the same order as that observed for pure PE monolayers, and the ΔF remained constant. A dramatic change was only observed when

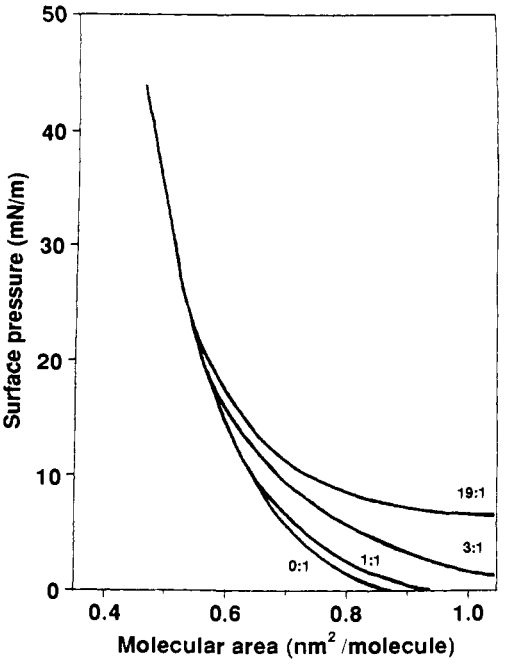

Figure 3. Compression isotherms of mixed films LAPAO/PE at various initial ratios of LAPAO to PE. Each compression isotherm has been reproduced three times. The isotherms were recorded at a compression speed of 0.1 nm^2/mol/min. The molecular area is relative to PE.

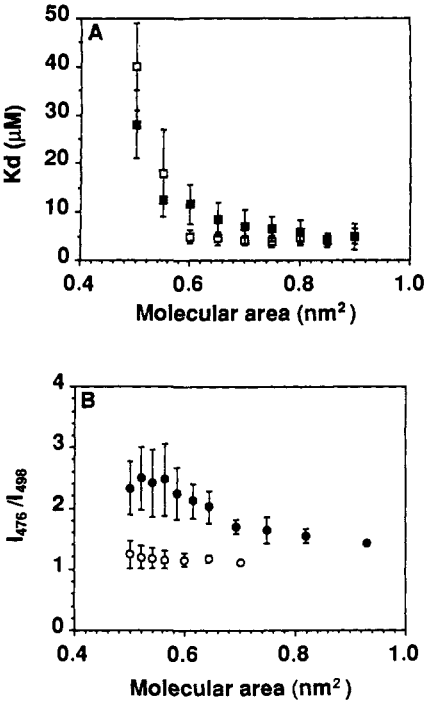

Figure 4. Binding of ANS to the mixed film. Results are shown for a pure PE monolayer (□, ○) and a mixed film LAPAO/PE 3/1 (mol/mol) (■, ●). Graph A shows the dissociation constant of ANS binding. The subphase was 1 mM phosphate–10 mM NaCl, pH = 6.80. Each experimental point is the mean value of three different determinations (±SD). Graph B shows the variation of the ratio I_{476nm}/I_{498nm}. The subphase was 1 mM phosphate–10 mM NaCl, pH = 6.80. Each experimental point is the mean value of three different determinations (±SD).

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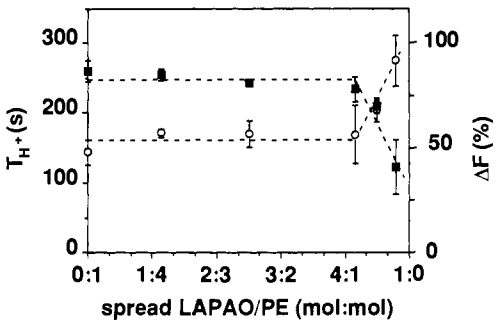

Figure 5. Lateral proton conduction along a mixed film LAPAO/PE. T_{H^+} and ΔF changes are shown when varying the percentage of LAPAO in the mixed film at the air/water interface. The subphase was 1 mM phosphate buffer, pH = 6.80. The surface pressure for each experiment was set at 5 mN/m: (○) time lag T_{H^+} and (■) relative amplitude of the fluorescence change ΔF. Each point is the mean value of three different determinations (±SD).

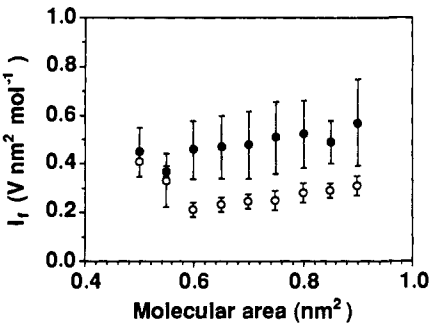

Figure 6. Variation of the reduced fluorescence If_{AS} with the host lipid molecular area. Results are shown for a pure PE monolayer (○) and a mixed film LAPAO/PE 3/1 (mol/mol) (●). The subphase was 1 mM phosphate, pH = 6.80. Each point is the mean value of three different determinations (±SD).

the LAPAO/PE ratio in the spread mixture was larger than 4/1. T_{H^+} was then observed to increase and ΔF to decrease with an increase in detergent content.

Nevertheless, classical values of T_{H^+} and ΔF were obtained for a spreading ratio of 24/1 using compressed films of up to 20 mN/m before inducing the proton conduction (data not shown). It should be noted that there was no difference at this pressure between the mixed film and a pure PE monolayer as shown by the compression isotherms (Figure 3).

Structural and Dynamic Investigations of Mixed Monolayers. Lateral proton conduction along phospholipid monolayers is known to be abolished when the film is brought to the solid-condensed state.[3,32] It is of major importance to check that modifications to the film do not create a gel-like state. Results are shown in Figure 6 and Table I. A nonsignificant increase in If_{AS} was detected when LAPAO was present at the interface. No difference was observed when the concentration of the detergent was varied. The decrease in the diffusion coefficient D by 1 order of magnitude was not indicative of a change of state in regard to PE phospholipids, when detergent molecules were present at the interface. These observations indicated an increase in the matrix order when LAPAO was inserted in the monolayer. Nevertheless, the mobility of the probe was still very high, showing that the matrix remained fluid.

Discussion

The detergent/phospholipid LAPAO/PE mixed films presented a metastability after spreading and upon compression (Figure 2). One should assume that the detergent left the interface by a desorption mechanism:

$$({}_nPL,{}_mDet)_{interf} \rightarrow ({}_nPL,{}_pDet)_{interf} + {}_{m-p}Det_{bulk}$$

with PL = phospholipids, Det = detergent, and $p < m$.

This reaction is irreversible. Increasing the percentage of LAPAO in the spread mixture increased the apparent molecular area of phospholipids at low surface pressures (p is larger when m is larger). At 5 mN/m, a molecule of PE occupied a molecular area of 0.71 nm^2/mol; when a mixture of LAPAO/PE 1/1 (mol/mol) was analyzed at this pressure, the average molecular area occupied by a phospholipid was 0.73 nm^2/mol; when the LAPAO/PE ratio was raised to 3/1 (mol/mol), the molecular area increased to 0.83 nm^2/mol; and finally, for a spread LAPAO/PE ratio = 19/1 (mol/mol), the molecular area increased up to approximately 1.4 nm^2/mol. Under the assumption that for a given surface pressure the molecular areas of the amphiphile and of the phospholipid add, it is clear that only a minute fraction of the amphiphile spread remains at the interface. First, the surface pressure decreased greatly after spreading (Figure 2). Second, by considering the 19/1 mixture and assuming that all molecules remain at the interface and that the LAPAO molecular area is 0.5 nm^2, from theory we should observe a molecular area of:

$$0.71 + (19 \times 0.5) = 10.21 \text{ nm}^2$$

Experimentally, we observed only 1.4 nm^2/mol (Figure 3). In fact, only 1.5 amphiphile molecules out of 19 remained at the interface (under the obvious assumption that the phospholipid was not solubilized due to its two fatty acid chains). Under the same assumptions, 0.46 instead of 1 and 0.66 instead of 3 LAPAO molecules were present at the interface for the two other spread mixtures (1/1 and 3/1).

As no film expansion was observed, whatever the spread ratio, when the film pressure was larger than 20 mN/m, we should conclude that all detergent molecules were expelled from the phospholipid film, but some of them remained adsorbed at the interface as shown by the ANS fluorescence study. The observation that the nature of the ANS binding sites was altered allows us to conclude that a fraction of the expelled LAPAO molecules were adsorbed at the film interface. This site modification was indicated by the shift in the emission of the bound probe. Upon compression, the system can therefore be described as:

$$({}_nPL,{}_mDet)_{interf} \rightarrow {}_nPL_{interf} + {}_qDet_{adsorb} + {}_rDet_{bulk}$$

with $(q + r = m)$.

As PE is purified from *E. coli*, different lengths of the hydrophobic tail were present,[33] as were different levels of saturation. On the contrary, the hydrophobic tail of the LAPAO molecule has only 10 carbons. The metastability of the film could be explained by the molecular structure of the LAPAO detergent: the presence of only one short hydrophobic tail in the molecule supports the observation that the LAPAO molecules were expelled when the monolayer was compressed.

Concerning structural and dynamic investigations at the interface, the data using 12-9 AS for the changes appearing at the level of the interface permit us to conclude that the presence of the LAPAO molecules in the lipid matrix did not bring about dramatic changes at the level of the hydrocarbon layer, except for the expansion of the film at low surface pressures. The binding of ANS was indicative of an alteration of the interface induced by the insertion of the LAPAO molecule. A modification of the properties of the binding sites was observed through the blue shift of the emission of the bound probe. The AS photobleaching experiments were indicative of more order present in the mixed film than in the pure PE matrix as shown by the increase in the K_{DS} constant.[30] From the diffusion coefficient obtained from the FRAP experiments, the main effect of the insertion of the LAPAO

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Table I. Photobleaching Parameters of 12-9 AS for Different Mixtures of LAPAO/PE Spread as Monolayers[a]

	If_{AS} (10^{13} mV cm^2 mol^{-1})	D (cm^2/s)	K_D (10^{14} cm^2 mol^{-1} min^{-1})	K_{DS} (mV^{-1} min^{-1})
PE	4.87 ± 0.12	(1.5 ± 0.2) × 10^{-4}	7.32 ± 0.47	16.7 ± 1.4
LAPAO/PE 1/1 (mol/mol)	4.61 ± 0.17	(1.3 ± 0.6) × 10^{-5}	9 ± 0.1	18.2 ± 0.3
LAPAO/PE 5/1 (mol/mol)	4.3 ± 0.55	(1.1 ± 0.8) × 10^{-5}	12.5 ± 2.7	29.6 ± 6.5

[a] The film pressure was always set at 5 mN/m. The different parameters were obtained by photodimerization and FRAP experiments. Each value is the mean of three different determinations (±SD).

detergent in the phospholipid matrix can be seen to be either a decrease in the free volume of PE, if freely dispersed in the monolayer, or a hindrance to the diffusion by an archipelago phenomenon, if a molecular segregation was present.[34] This second possibility can be envisaged if at high concentrations of LAPAO, the detergent molecules formed aggregates which had the possibility of cancelling out the lateral proton conduction, operating as "spacing-out" units.

The lateral movement of protons, along a lipid monolayer, is driven by the proton concentration difference between the acid injection compartment and the neutral bulk volume.[2] A competition is present between the lateral movement and the leakages which may take place between the interface and the bulk phase through the interfacial unstirred layer.[1] As a first approach, we may consider that the leakage is negligible when compared to the lateral conduction and that the buffering capacity of the conducting pathway is negligible. A mathematical description of the local change in proton concentration is then given by

$$c(x,t) = c(0,0)[1 - \mathrm{erf}(x/2(D_{H^+}t)^{1/2})]$$

where $c(x,t)$ is the local proton interfacial concentration at distance x from the injection compartment and at time t after the proton injection, erf is the error function, and D_{H^+} is the diffusion coefficient of protons in the conducting network at the interface.[35] Our observation that T_{H^+} increases when LAPAO is present at the interface in the PE monolayer, at a concentration larger than a critical ratio, is described by a decrease in D_{H^+} if one assumes that the mixed film is an homogeneous system. Under such an assumption, LAPAO is a local breaker of the conducting pathway. This can be explained by postulating the inability of this molecule to build hydrogen bonds with the interfacial water molecules (Figure 7B). The LAPAO molecule is affecting the phospholipid molecules around it as pictured in Figure 7B. If the number of inserted detergent molecules is increased, the number of poorly conducting defects will increase, supporting our experimental observation that T_{H^+} increases with an increase in the LAPAO content in the film.

But another interpretation may take into account the possibility of a phase separation between the detergent molecules and the phospholipid molecules. We may then describe the film as an ocean of proton-conducting PE molecules where an archipelago of LAPAO molecules is present as described for lateral diffusion in heterologous systems.[34] Diffusion of protons is then hindered by the cluster of LAPAO molecules (Figure 7C). The effective length of the diffusion pathway is then larger than that for a pure PE layer (Figure 7A), and T_{H^+} increases at a constant diffusion coefficient, D_{H^+}. Such a model is in agreement with our experimental observations. When the percentage of LAPAO in the spread mixture is increased, the number of LAPAO molecules present at the interface is larger (expansion of the film). As a consequence, either the number of clusters would be larger or their size would increase. Both processes, by increasing the effective diffusion length, give an increase in T_{H^+}.

Due to the poor definition of the organization of the mixed layer that we obtained through the surface pressure and fluorescence measurements, it is impossible to discriminate between the two proposed interpretations. Nevertheless, the present observation provides experimental support for the occurrence of a lipid-supported interfacial proton-facilitated diffusion which is controlled by the matrix organization. Another conclusion from the present studies is that LAPAO must remain embedded in the PE film in order to alter the proton conduction. When LAPAO is only adsorbed on the PE surface as shown by the ANS fluorescence experiments, with no film expansion, T_{H^+} remains unaffected.

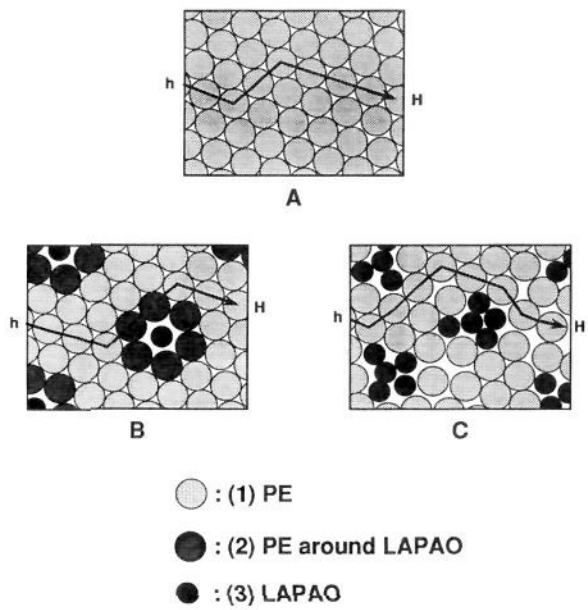

Figure 7. Interfacial lateral proton conduction modulation when increasing the interfacial percentage of LAPAO in the PE film. (A) Pure PE monolayer: the H^+ diffusion takes place with no barrier. A continuous wave of protons is flowing along the monolayer coming from the acidic compartment. (B) Mixture LAPAO/PE > 6/1 (mol/mol): "induction of local defects". The inserted detergent molecule perturbs the PE molecules which are in local contact with it. The conducting network is affected (dotted line) and less effective in the transfer of protons. Experimentally, T_{H^+} increases and ΔF decreases when a critical number of defects are present. (C) Mixture LAPAO/PE > 6/1 (mol/mol): "formation of clusters". A segregation is taking place and the detergent molecules form aggregates in the lipid matrix when they are present at a superficial concentration larger than a critical value. No proton conduction is possible in the LAPAO molecule clusters. They prevent the free running of the interfacial protons. The proton path is still present at the interface, but it becomes very long. An increase in T_{H^+} occurs as detected. One should notice that the effects described in B and C can be present simultaneously.

In a previous study on the proton lateral conduction along mixed films, conduction was prevented for critical film compositions and surface pressures.[32] But as the amphiphile present in the lipid matrix CTAB was positively charged, the contribution of an electrostatic repulsion, due to the interfacial CTAB density being larger than a critical value, was taken into account. In the present study, such an electrostatic contribution is not present and the slowing down of the conduction process must be explained only by a structural alteration of the interface where it occurs.

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