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Phenylalanine Blocks Defects Induced in Gel Lipid Membranes by Osmotic Stress

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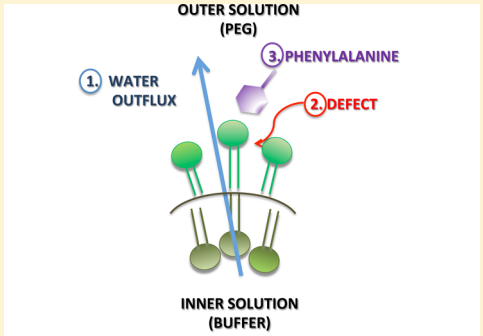
Supporting Information

ABSTRACT: We study the binding of phenylalanine (Phe) with dipalmitoylphosphocholine (DPPC) vesicles in gel (25 °C) and in liquid crystalline states (50 °C) and in gel large unilamellar vesicles (LUVs) subjected to osmotic dehydration with merocyanine (MC 540) as a fluorescent surface membrane marker. Phe does not produce significant changes in MC 540 monomer concentration in DPPC LUVs at 50 °C. In contrast, it significantly decreases the monomer adsorption in defects present in DPPC LUVs at 25 °C. When DPPC LUVs were subjected to hypertonic stress, dehydration caused more defects, and in this case phenylalanine is also able to block such defects.



Phenylalanine inserts in packing defects induced by osmotic shrinkage

INTRODUCTION

Phenylalanine (Phe) seems to play a significant role in biological systems. In particular, it has been shown to produce damage in thylakoid membranes at very low concentrations during freezing-inducing leakage and membrane fusion in liposomes of phosphatidylcholine, phosphatidylethanolamine, and galactolipids.[1] These phenolic compounds are synthesized as protective agents after mechanical injuries by activation of phenylalanine ammonium lyase (PAL) to reduce the leakage of water. Thus, interaction may be related to the hydration state of the lipid membrane.[2]

According to previous reports, Phe has a high interfacial hydrophobicity, which suggests that this molecule may stabilize at the interface of lipid membranes.[3] Phe is rather hydrophilic (log $P = -1.38$) and highly soluble in water (log $S = -0.788$) at 25 °C.[4] Thus, the damage may occur at relatively low concentrations of Phe in the membrane, probably due to modest partition and singular accommodation of the hydrophobic phenyl ring.[5,6] However, no details on the membrane condition at which this interaction may occur are available.

In this regard, it has been reported that amphiphilic compounds with a phenyl group, such as arbutin, protect membranes under conditions of low water availability.[7,8] This tyrosine analogue may protect membranes in conditions of hydric stress, inserting the phenolic ring in defects at the membrane surface.[9,10] Therefore, according to these results, it may be inferred that the phenolic group in Phe interaction might act by a similar mechanism.

The loss of water in lipid vesicles under hypertonic stress produces packing constraints resulting in defects in the periodic pattern of phospholipid arrangements.[10,11] They are likely to occur with an enhancement of the water−hydrocarbon contact area, favoring the hydrophobic interaction of the amino acid.

In this regard, it has been found that hydrophobic defects can be induced by osmotic shrinkage of vesicles and liposomes produced by impermeable osmolytes in the outer solution. The different exposure to water of nonpolar residues of the lipid molecules in shocked vesicles has been related to mismatches of water populations linked to the carbonyl groups.[12−14] Therefore, such mismatches can generate defects with an excess of surface energy, which can favor insertion of the aromatic ring of Phe (Figure 1B).[11]

A way to detect defects is measuring the partition of the monomer of merocyanine 540 (MC 540) as a membrane probe (Figure 1A). Other fluorophores such as anilinonaphthalene-1-sulfonic acid (ANS) can also be used with similar results.[11] MC monomer may insert in hydrophobic defects. In contrast, dimer stabilizes on the external surface of the lipid membrane in the gel state.[14,15] Thus, quantification of monomers and dimers at the membrane phase gives direct information on the relative amount of defects on the external membrane surface of vesicles and on the degree of packing of the membrane.[15]

Changes in the hydration of the interphase at different packing can be inferred from the changes in generalized polarization of laurdan inserted in the lipid membrane.[16,17] In these conditions, the appearance of defects was measured with MC 540 monomer in the presence of different Phe concentrations to determine whether this amino acid is able

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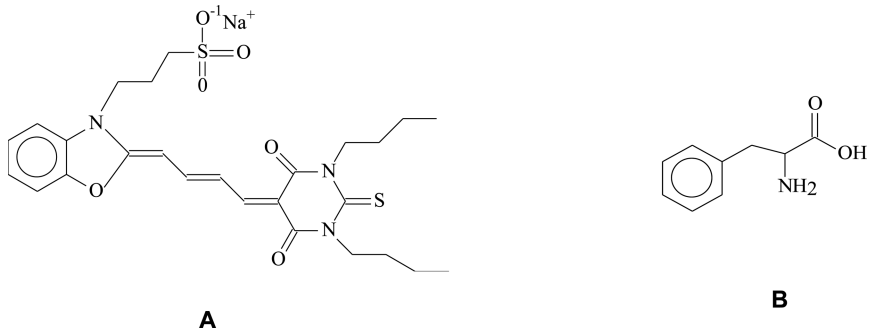

Figure 1. Molecular structures of (A) merocyanine 540 and (B) L-phenylalanine.

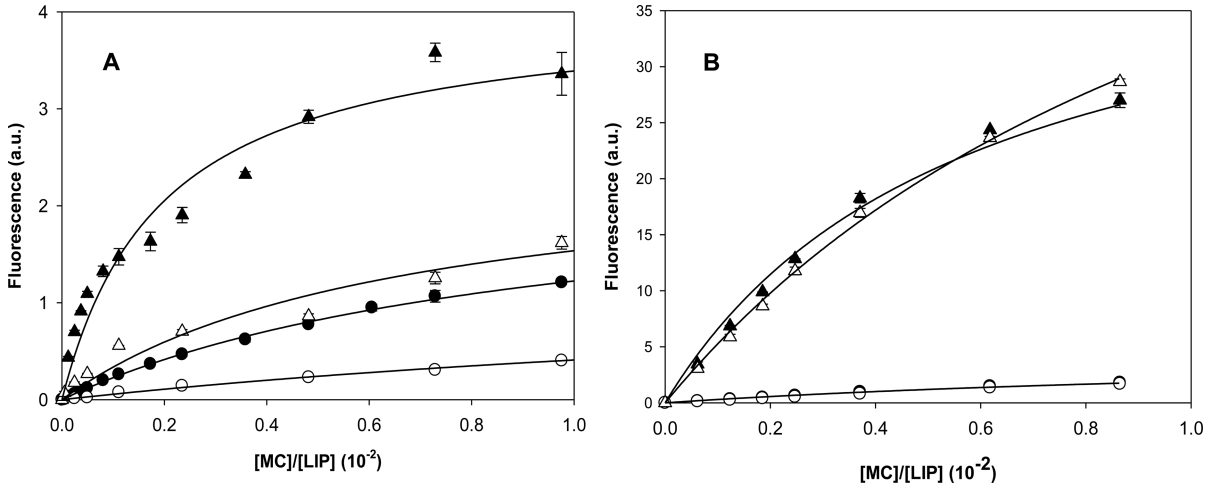

Figure 2. Interaction of MC 540 monomer DPPC LUVs in the presence of Phe. Intensity of fluorescence is plotted as a function of the merocyanine to dipalmitoylphosphatidylcholine ([MC]/[DPPC]) ratio. (A) LUVs at 25 °C; (B) LUVs at 50 °C. (▲) MC 540 monomer in the absence of Phe; (△) MC 540 monomer in the presence of 13 mM Phe; (●) MC dimer without Phe; (○) MC dimer in the presence of 13 mM Phe.

to block defects on DPPC unilamellar vesicles subjected to increasing hypertonic stress.

These results could be relevant to understand the mechanisms of interaction of peptides containing Phe residues that are important to evaluate the potential and toxicity of antimicrobial peptides in addition to cryoprotective processes.[18,19]

MATERIALS AND METHODS

Dipalmitoylphosphocholine (DPPC) was purchased from Avanti Polar Lipids, Inc. Purity was checked by thin-layer chromatography, and it was used without further purification. Merocyanine 540, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), poly(ethylene glycol) (PEG, MW = 10 000), and L-phenylalanine were obtained from Sigma–Aldrich. All chemicals were analytical grade, and water was ultrapure (Milli-Q quality). MC 540 stock solution was prepared by dissolving an appropriate quantity in 10 mM HEPES buffer, pH = 7.3. The same medium was used for liposome preparation in all the assays.

Liposome Preparation. A stock solution of DPPC (4 mM) was prepared in chloroform. A thin lipid film was obtained by slow evaporation of appropriate aliquots of the stock solution under vacuum in a rotatory evaporator for at least 4 h. The dry film was then hydrated in a medium containing 10 mM HEPES, pH = 7.30, at 55 °C. The multilamellar vesicle suspension (MLV) obtained was extruded 10 times at 55 °C through a polycarbonate filter (pore diameter 100 nm) in order to prepare large unilamellar vesicle suspensions (LUV). According to previous determination by microscopy, a narrow distribution of unilamellar vesicles around 100 nm diameter was obtained with this procedure.

For the osmotic experiments vesicles prepared in buffer without PEG, as described above, were dispersed in a solution containing 14 mM PEG10000 (MW = 10 000). Thus, the difference in osmotic pressure caused a volume reduction with time by water diffusion driven by the osmotic gradient between the inner solution (without PEG) and the outer solution (with PEG). The water decrease in the vesicles was followed by measuring the generalized polarization (GP) of vesicles doped with Laurdan after equilibration for 1 and 24 h in 14 mM PEG. In this equilibrium condition, MC was added to the external solution in the presence and the absence of Phe as described in each figure.

Fluorescence Measurements. *Merocyanine 540.* Fluorescence spectra were recorded on a SLM 4800 spectrofluorometer (excitation and emission slits of 8 nm) with stirring and thermostating. Different aliquots of MC stock solution were added to the LUV suspensions in order to obtain different MC:lipid ratios. Final concentrations of MC in the cuvettes were between 5×10^{-3} and 2 μM, and final lipid concentration was 0.2 mM. Thus, the ratio of dye to lipid was on the order of 10^{-5}, at which no perturbation of the membrane by the dye was detected. Fluorescence emission spectra were collected in the region 400–700 nm with an increment of 1 nm, after excitation of the sample at 530 and 569 nm. MC monomer and dimer

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emission spectra were obtained in the presence of DPPC LUVs in the gel and liquid crystalline states with and without Phe. Data are the average of duplicate samples from three different batches of vesicles prepared under the same conditions. Standard deviations are reported as bars in the figures.

Laurdan. Laurdan was used to follow the water decrease at membrane level in hypertonic shocks. Once incorporated into membranes, the (nanosecond) fluorescent decay of this probe is strongly affected by changes in the local polarity and relaxation dynamics of restricted water molecules existing at the membrane/water interface. Laurdan generalized polarization (GP)[16,17] was calculated for each emission wavelength by

$$GP_{exc} = \frac{I_{440} - I_{490}}{I_{440} + I_{490}} \quad (1)$$

where I_{440} and I_{490} are the fluorescence intensities at each excitation wavelength, from 350 to 400 nm, measured with fixed emission wavelengths of 440 and 490 nm, respectively. Lateral distribution of Laurdan and its fluorescence response to membrane packing become a powerful experimental tool to gain information about membrane lateral heterogeneity and polarity of the interphase related to water ratio.[15,16]

RESULTS

The titration of DPPC vesicles prepared and equilibrated in buffer in isosmotic conditions produces a significant increase in monomer fluorescence at temperatures below the lipid phase transition (Figure 2A). In the same figure, it is observed that, in the presence of 13 mM Phe, the monomer relative fluorescence is 2-fold lower than in LUVs of pure DPPC. This indicates that Phe at that concentration inhibits the MC monomer fluorescence. Although to a lower extent, Phe also decreases the fluorescence of the dimer. In Figure 2B, data from a similar experiment done with DPPC LUVs in the liquid crystalline state show that Phe does not affect MC monomer fluorescence.

It is supposed that, at long times of incubation, an equilibrium of monomer in the membrane and in the solution can be achieved. When it is considered that a maximum of fluorescence intensity given by the monomer in the membrane (IF_{max}) is achieved at high MC concentration and that fluorescence intensity is null in the absence of MC, the degree of coverage (θ) can be calculated as

$$\theta = \frac{IF - IF_0}{IF_{max} - IF_0} \quad (2)$$

where IF_0 is the residual fluorescence.

At equilibrium, the rate of adsorption of monomer is equal to the rate of desorption according to the following equilibrium:

$$k_1(1 - \theta)[MC] = k_2\theta \quad (3)$$

where k_1 and k_2 represent the kinetic constants of adsorption and desorption, respectively. From eqs 2 and 3, the relationship between θ and monomer concentration can be expressed by the following equation:

$$\theta = \frac{IF - IF_0}{IF_{max} - IF_0} = \frac{[MC]}{K_d + [MC]} \quad (4)$$

where K_d is the apparent dissociation constant of the monomer ($K_d = k_2/k_1$). Indirect inferences of the MC monomer association have been reported previously when the dimerization of the dye in the membrane was calculated from spectroscopic data.[14] However, to our knowledge, this is the first time that the dissociation constant of the monomer is directly calculated from the fluorescence intensity.

A similar equation can be written for the dimer. Plots of θ versus MC concentration according to eq 4 are shown in Figure 3. From these, the values of the dissociation constant of MC 540 monomer and dimer in the presence and absence of Phe at 25 and 50 °C can be calculated (Table 1).

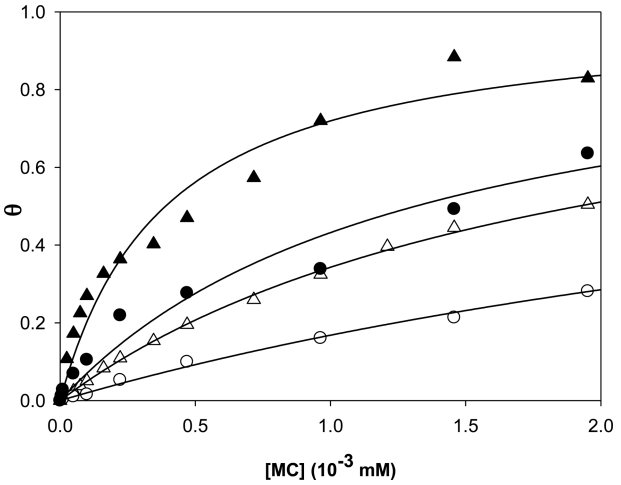

Figure 3. Degree of coverage of LUV DPPC vesicles in the gel state with and without Phe calculated from Figure 2A (same symbols as in Figure 2).

Table 1. Dissociation Constants of MC 540 Monomer and Dimer in the Presence and Absence of Phe at 25 and 50 °C

	monomer		dimer	
	T (°C)	K_d (mM)	T (°C)	K_d (mM)
DPPC LUVs	25	$0.4 \times 10^{-3} \pm 0.9 \times 10^{-4}$	25	$1.9 \times 10^{-3} \pm 0.1 \times 10^{-3}$
DPPC LUVs + Phe	25	$1.3 \times 10^{-3} \pm 0.4 \times 10^{-3}$	25	$4.6 \times 10^{-3} \pm 0.9 \times 10^{-3}$
DPPC LUVs	50	$1.4 \times 10^{-3} \pm 0.2 \times 10^{-3}$	50	$3.1 \times 10^{-3} \pm 0.5 \times 10^{-3}$
DPPC LUVs + Phe	50	$2.0 \times 10^{-3} \pm 0.2 \times 10^{-3}$	50	$6.7 \times 10^{-3} \pm 0.9 \times 10^{-3}$

The dissociation constant of the monomer is much lower in gel than in liquid crystalline state (0.4×10^{-3} versus 1.4×10^{-3} mM). In the presence of Phe, the dissociation constant of MC monomer in the gel state increases to 1.3×10^{-3} mM, a value very similar to that in liquid crystalline membranes. This suggests that discrete regions in the liquid crystalline state are present in the gel state, thus reducing the affinity of the dye. In addition, Phe reduces the degree of coverage for MC monomer from 80% to 50% (Figure 3). In contrast, in the gel and liquid crystalline states the affinity of the dimer decreases in the presence of Phe.

A question to resolve is that the decrease in MC monomer fluorescence can be due to quenching of the dye by Phe in the membrane. In Figure 4, it is observed that the monomer fluorescence decrease faster with Phe in the presence of LUVs than when MC and Phe were codissolved in ethanol in the absence of lipids, suggesting that the fluorescence decrease is not due to interaction of Phe with MC in a nonpolar solvent. Thus, the variation of MC 540 fluorescence monomer in DPPC LUVs with increasing concentrations of Phe could be ascribed to displacement of the monomer from the membrane phase.

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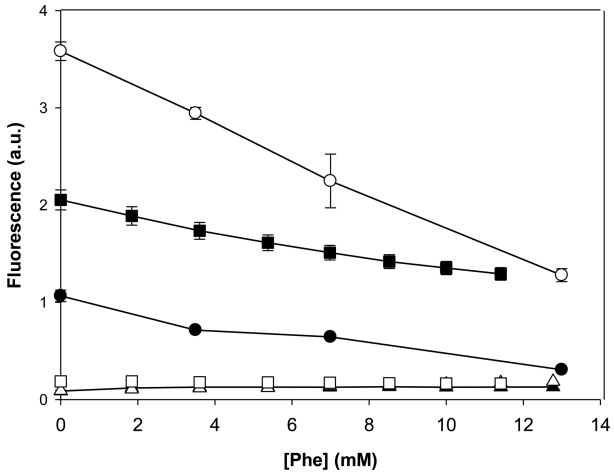

Figure 4. Fluorescence intensity of MC monomer at different Phe concentrations: (○) in the presence of DPPC LUVs at 25 °C, (●) in ethanol without DPPC, and (△) in buffer without DPPC. Behavior of dimer, (■) in the presence of DPPC and (□) in buffer, is also shown for comparison.

Upon comparing the fluorescence increase in Figure 2 panels A and B, it is concluded that the monomer adsorption is lower in gel LUVs than in fluid LUVs. This indicates that MC monomer adsorbs at discrete sites in the gel membrane and dissolves in all extension in fluid membranes. In addition, in this last case, Phe does not affect the partition.

A way to promote defects in the external face of vesicles is by producing osmotic shrinkage by increasing osmolyte concentration in the external medium. DPPC LUVs doped with Laurdan were dispersed in increasing osmolarities and incubated for 1 h (Figure 5). The increase in osmolarity in the external medium produces an increase in the generalized polarization of Laurdan (GP). Thus, Laurdan can be useful to follow water extrusion with and without Phe (data not shown).

In conditions in which water is extruded from the vesicles, MC assays performed in LUV DPPC at 25 °C previously shrunken in PEG, according to the protocol described in Materials and Methods, show noticeable effects. Monomer adsorption increases noticeably with respect to unshrunken vesicles after an hour of equilibration (Figure 6). In this condition, the fluorescence of MC monomer is severely attenuated when Phe is added.

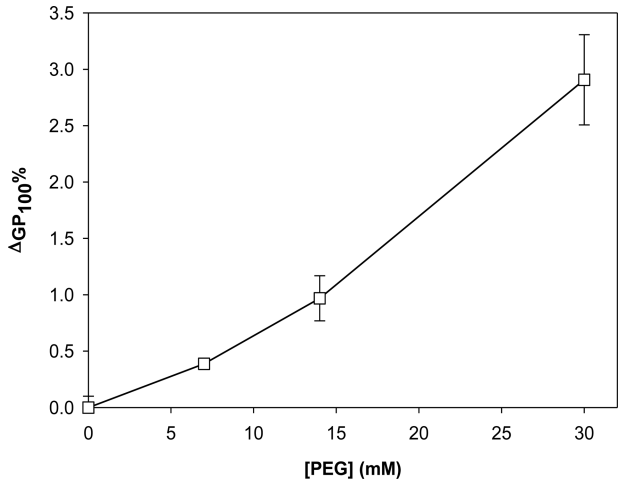

Figure 5. Effect of osmolarity on generalized polarization of Laurdan after (□) 1 h of incubation. The ΔGP% values are represented as a function of [PEG], where ΔGP (%) = $[(GP_{exc} - GP_{exc0})/GP_{exc0}] \times 100$, and GP_{exc} and GP_{exc0} are GP values in the presence and absence of PEG, respectively.

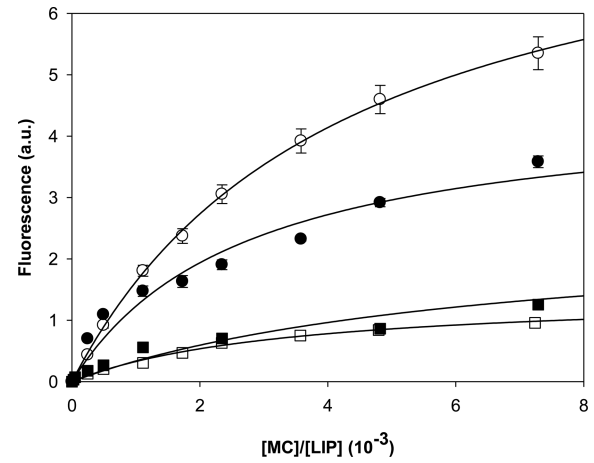

Figure 6. Interaction of monomer of MC 540 with DPPC LUVs subjected to osmotic stress in the presence and absence of 13 mM Phe: (●) DPPC in isotonic medium, (○) DPPC dispersed in hypertonic medium containing PEG, (□) DPPC in the presence of Phe in hypertonic medium, and (■) DPPC in the presence of Phe in isotonic medium.

The dimer affinity is also affected by PEG and modified by Phe (Figure 7). Table 2 summarizes the affinity values in each case.

DISCUSSION

Comparison of the control experiments in Figure 2 denotes that the affinity of the MC monomer is larger in the liquid crystalline state than in the gel state (see control data in Table 1). However, to a lower extent, a significant increase in

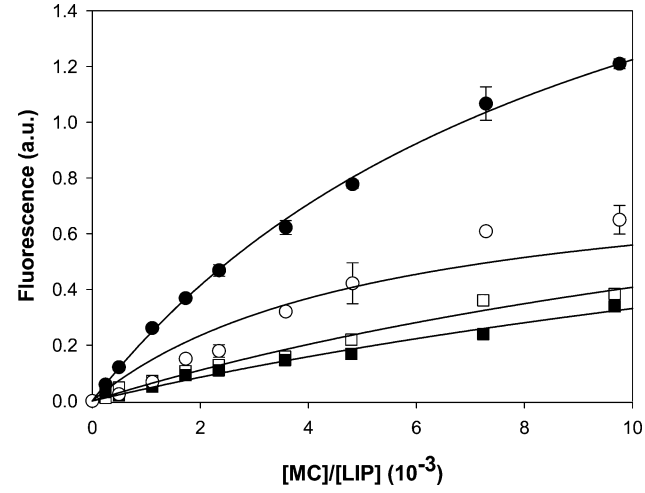

Figure 7. Interaction of dimer of MC 540 with DPPC LUVs subjected to osmotic stress in the presence and the absence of 13 mM Phe: (●) DPPC in isotonic medium, (○) DPPC dispersed in hypertonic medium containing PEG, (□) DPPC in the presence of Phe in hypertonic medium, and (■) DPPC in the presence of Phe in isotonic medium.

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Table 2. Dissociation Constant of MC 540 Monomer and Dimer in the Presence and Absence of Phe in DPPC GEL LUVs Shocked with PEG

	K_d (mM)	
	monomer	dimer
DPPC LUVs	$0.4 \times 10^{-3} \pm 0.9 \times 10^{-4}$	$1.9 \times 10^{-3} \pm 0.1 \times 10^{-3}$
DPPC LUVs + PEG	$0.8 \times 10^{-3} \pm 0.3 \times 10^{-4}$	$5.3 \times 10^{-3} \pm 0.9 \times 10^{-3}$
DPPC LUVs + Phe	$1.3 \times 10^{-3} \pm 0.4 \times 10^{-3}$	$4.6 \times 10^{-3} \pm 0.9 \times 10^{-3}$
DPPC LUVs + Phe + PEG	$6.9 \times 10^{-3} \pm 0.1 \times 10^{-4}$	$5.3 \times 10^{-3} \pm 2.0 \times 10^{-3}$

fluorescence is also observed in gel DPPC vesicles, indicating that discrete regions of similar properties to the liquid crystalline state are present. The novel finding is that the fluorescence increase observed in gel DPPC LUVs decreases drastically in the presence of Phe but is not affected in vesicles in the liquid crystalline state.

At 25 °C, DPPC bilayers are in the planar gel state and a packed low-hydrated interphase is apparent.[20] The packing produced by condensation of the acyl chains and loose of water may affect the organization of polar head groups in the surface lattice. The increase in monomer fluorescence observed by means of the titration of gel DPPC LUVs with MC 540 can be interpreted as a consequence of the insertion of monomer in such hydrophobic defects. Phe inhibits this insertion; as observed in Figure 3, the degree of coverage decreases 30%, indicating that Phe blocks 70% of the sites for MC monomer.

The affinity constant of MC monomer in the presence of Phe is comparable to that in liquid crystalline membranes (Table 1), suggesting that at least some regions with similar features to a liquid crystalline phase are present in membranes at 25 °C. This observation can be explained by the presence of packing defects in the gel phase that expose hydrophobic regions to the water phase.

As the fluorescence of MC monomer is much less quenched by Phe in ethanol than in the presence of membranes (Figure 4), it is reasonable to think that the fluorescence decrease is not due to an association of MC with Phe in the membrane phase. It is likely that Phe blocks a number of sites for MC monomer. In fact, data in Figure 4 would indicate displacement of MC monomer from the membrane sites by Phe.

The polarity of the interphase can be sensed with Laurdan, which may be interpreted as a consequence of partial dehydration of the interphase. A parallel increase in Laurdan GP in the presence of solutes has been related to an increase in surface tension, suggesting changes in the lateral packing of phospholipids.[16,17] Water extrusion induced by PEG increases GP, denoting a dehydration of the interphase. However, the presence of Phe does not affect the change induced by osmosis (see Supporting Information).

The effect of Phe on shrunken vesicles can be observed with MC 540. Taken together, the dehydration caused by shrinkage can give place to the exposure of hydrophobic regions of the bilayer interphase, which would enhance partition of the monomer. If that is so, water organized near a hydrophobic region facilitates the adsorption of compounds such as MC and Phe.

Relative fluorescence decreases with increasing Phe concentration previous to the addition of MC. This can be interpreted as a consequence that Phe concentration blocks the sites of insertion of the MC monomer. This can explained by a phenol/lipid interaction at the defect or by a displacement of MC monomer from the membrane to the aqueous phase by Phe. The assays in ethanol show a much lower decrease in monomer fluorescence, suggesting that codissolution of MC and Phe in the hydrophobic sites would not be the case. Therefore, these results indicate that Phe mainly interacts in defects produced in the gel state of lipids.

In previous works, it was demonstrated that, under conditions where spontaneous or induced curvature is present, the relative populations of hydrated and nonhydrated carbonyl groups of the phospholipids increase.[12,13,21] This modification is found under the same conditions at which the adsorption of MC monomer increases with osmosis. Thus, it is likely that carbonyl groups rearrangement is related to the formation of defects where MC adsorbs.

It may be possible that this organization may promote hydrophobic interaction of the dye. In this case, the inhibition of MC adsorption by phenylalanine would be due to insertion of the nonpolar phenyl group of the amino acid into the hydrophobic defect. Thus, it might be possible that exposure of hydrophobic regions in defects may induce reorganization of water in terms of hydrogen bonding with an excess of surface free energy that favors Phe insertion. In this process, the hydrophobic interaction would displace polarized water from the defect.[22]

CONCLUSIONS

Under osmotic shrinkage, MC monomer partition is noticeably increased in DPPC LUV in the gel state. However, under the same conditions and in the presence of Phe, it is strongly attenuated. This is explained by considering that Phe blocks defects into which MC inserts. Water exclusion favors Phe interaction, since shrinkage produces more defects.

ASSOCIATED CONTENT

Supporting Information

Plot of GP_{exc} versus the Phe concentration. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcb.5b05590.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

DPPC, 1,2-dipalmitoylphosphatidylcholine; MC, merocyanine 540; Phe, L-phenylalanine; LUVs, large unilamellar vesicles

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