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Effect of Phloretin on the Binding of 1-Anilino-8-naphtalene sulfonate (ANS) to 1,2-Dimyristoyl-*sn*-glycero-3-phosphocoline (DMPC) Vesicles in the Gel and Liquid-Crystalline State

Andrea C. Cutró · Guillermo Montich · Oscar A. Roveri

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Abstract Phloretin is a known modifier of the internal dipole potential of lipid membranes. We studied the interaction of phloretin with model lipid membranes and how it influences the membrane dipole organization using ANS as fluorescent probe. The fluorescence increase observed when ANS binds to DMPC liposomes in gel phase (13 °C) was 2.5 times larger in the presence of phloretin. This effect was due to an increase in ANS affinity, which can be related to the known capability of phloretin in decreasing the dipole potential. Conversely, when the experiments were carried out at 33 °C (liquid crystalline phase), phloretin completely inhibited the increase in ANS fluorescence. In addition, phloretin only affected the electrical properties of the membrane in the gel phase, whereas it modifies structural ones in the liquid-crystalline state. We postulate that phloretin was bound only to the DMPC interface in the gel phase decreasing the surface negative charge density without modifying the structural properties of the ANS binding sites. In the liquid-crystalline phase instead, it increased the accessibility of water to the ANS binding sites decreasing the intrinsic affinity and the fluorescence quantum yield of ANS.

Keywords Phloretin · ANS binding · Liposomes · Phase state · Surface potential

A. C. Cutró · O. A. Roveri (✉)
Área Biofísica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, S2002LRK Rosario, Argentina
e-mail: oroveri@fbioyf.unr.edu.ar

A. C. Cutró
e-mail: andreacutro@yahoo.com.ar

Present Address:
A. C. Cutró
Laboratorio de Biointerfases y Sistemas Biomiméticos, Laboratorios Centrales Centro de Investigación y Transferencia Santiago del Estero, CITSE-UNSE CONICET, El Zanjón, RN 9, km 1125, Santiago del Estero, Argentina

G. Montich
Centro de Investigaciones en Química Biológica de Córdoba (CIQUIBIC, UNC –CONICET), Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina
e-mail: gmontich@fcq.unc.edu.ar

Introduction

It is known that phloretin (Fig. 1a) binds to lipid monolayers and bilayers decreasing the dipole potential (Anderson et al. 1976; Melnik et al. 1977; Reyes et al. 1983). It has been shown that the decrease by phloretin of the dipole potential in phosphatidylcholine (PC) monolayers is more significant in the less hydrated gel state than in fluid state. This effect was attributed to neutralization or reorientation of dipole moieties at the interface others that the acyl chain carbonyl groups, since results obtained with ether-linked DMPC were similar to those obtained with esther-linked DMPC (Lairion and Disalvo 2004). More recently, Efimova and Ostroumova (2012) studied the effect of phloretin and other dipole modifiers on the magnitude of the dipole potential of planar bilayers composed by different phospholipid and sterols mixtures at room temperature. They evaluated the dipole potential by measuring changes in the steady-state conductance induced by cation-ionophore complexes. Phloretin (among other chalcones and flavonols) decreased the dipole potential of phospholipid- and sterol-containing membranes. The presence of cholesterol strongly influenced the magnitude of

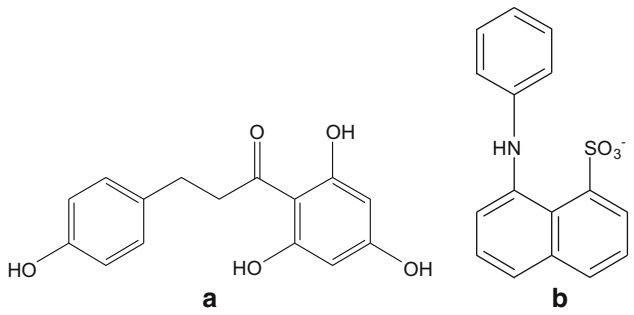


Fig. 1 Chemical structures: **a** phloretin (3-[4-hydroxyphenyl]-1-[2,4,6-tri-hydroxyphenyl]-1-propanona); **b** ANS (1-anilino-8-naphtalenesulfonate)

phloretin's effect (Lairion and Disalvo 2004; Efimova and Ostroumova 2012; Ostroumova et al. 2013).

1-Anilino-8-naphtalenesulfonate (ANS) is a hydrophobic anion widely used as fluorescent probe (Fig. 1b). When it binds to phospholipid membranes, a large enhancement of its fluorescence is observed. It has been suggested that the "binding site" is composed of four polar head groups and it is relatively inaccessible to water and also shielded from cations present in the aqueous phase (Haynes and Staerk 1974). The lipid membrane-bound ANS has a high quantum yield, which is responsible for the fluorescence enhancement. It has been shown by Haynes (1974) that the apparent affinity of ANS for its binding site is influenced by the surface potential. Then, the measurement of the affinity of ANS for the bilayer is a useful tool to determine the surface potential of natural and artificial membranes (Haynes 1974; Gibrat et al. 1983; Chiu et al. 1980; Robertson and Rottenberg 1983).

It has been reported by Lairion and Disalvo (2009) that the decrease in the dipole potential observed with increasing phloretin concentrations is paralleled by shifts of the zeta potential toward less negative values, likely as a consequence of a decrease in the negative surface charge density of liposomes. Hence, the surface potential, which is related to the surface charge density (Robertson and Rottenberg 1983; Njus 2000), would depend not only on the fixed net charges of the phospholipid headgroups but also on the contribution of partial charges due to the dipole moieties responsible of the internal dipole potential (see Maget-Dana 1999).

Preliminary studies have shown that phloretin inhibits ATP synthesis and decreased the apparent affinity of submitochondrial particles (SMPs) for ANS (Castelli, Fabregas and Roveri, unpublished results). This last result does not agree with the expectation that phloretin would increase the apparent affinity of ANS to membranes if its unique effect would be to decrease the dipole potential and consequently the density of negative charges at the membrane surface. SMPs are rather complex systems constituted by phospholipids and proteins. In order to understand the reason of the apparent discrepancy between the effect of phloretin on the binding of ANS to SMP and its known effect on the dipole potential, we studied the effect of phloretin on the binding of ANS to DMPC large unilamellar vesicles (LUVs) as model membranes.

The results reported here clearly indicate that phloretin interacted with DMPC in the gel phase differently than with DMPC in the liquid-crystalline state. We show that it increases the affinity of ANS for the gel phase simply by decreasing the internal dipole potential and consequently making less negative the surface potential. Instead, phloretin decreases the ANS affinity for the liquid crystalline phase, by distorting the lipid packing, modifying the accessibility of water to the ANS "binding sites," and consequently increasing the intrinsic dissociation constant and the fluorescence quantum yield.

Materials and Methods

Chemicals

Phloretin and ANS were obtained from SIGMA. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocoline (DMPC), 1,2-dioleyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and polycarbonate filter (pore diameter 100 nm) were purchased from Avanti Polar Lipids (Alabaster, USA). Solvents (pro analysis quality) were obtained from Merck. All other compounds were of analytical reagent grade.

General

Phloretin was dissolved in dimethyl sulfoxide (DMSO). Experiments were performed using 25–400 μM phloretin. Controls with the solvents (less than 2 %) were carried out for all the determinations reported.

Preparation of Large Unilamellar Liposomes (LUVs)

A stock solution in chloroform/methanol (2:1 V/V) of DMPC (20 mg/mL) was prepared. A thin lipid film was obtained by slow evaporation of appropriate aliquots of the stock solution by means of a nitrogen stream in a glass tube with conical base. The residual organic solvents were removed under vacuum for 4 h. Once dried, the film was hydrated in a medium containing 250 mM sucrose and 30 mM Tris–HCl (pH 8.0). The multilamellar vesicle suspension obtained was disrupted by five freeze–thaw cycles. Afterward, the lipid suspension was extruded ten times at 55 °C through a polycarbonate filter (pore diameter 100 nm) as described (Avanti Polar Lipids 2013).

Liposome Quantification

Phospholipid concentration was determined by quantifying inorganic phosphorus in samples mineralized essentially as described by Hess and Derr (1975). Inorganic phosphate was colorimetrically determined according to Chen et al. (1956) as described by Ames (1966).

ANS Fluorescence Measurements

Steady-state fluorescence measurements were carried out in a Cary Eclipse spectrofluorometer. The sample was excited at 380 nm and the emission fluorescence intensity at 480 nm was continuously recorded. The medium contained 250 mM sucrose and 30 mM Tris–HCl (pH 8.0). The reaction was started by the addition of ANS (15–130 μM final concentration).

Lifetime Fluorescence Measurements

Fluorescence lifetimes were measured in a time-correlated single photon counting (TCSPC) fluorometer. The sample was excited with a PLS340LED driven by a PDL 800-B unit. The excitation wavelength was 340 nm and the frequency was 5 MHz. The emission was detected with a PMA182 - NM photomultiplier. Single photon counting was performed with a PicoHarp 300E TCSPC unit. The analysis of the fluorescence decay data was done with the Global FluoFit Fluorescence Decay Data Software. PDL 800-B, PicoHarp 300, and the software were from PicoQuant GmbH, Berlin.

Differential Scanning Calorimetry (DSC)

DSC was performed in a MicroCal DSC-VP micro calorimeter (Northampton, MA). The reference cell was filled with a solution containing 250 mM sucrose and 30 mM Tris–HCl (pH 8.0). The scan rate was 30 °C/h.

Data Analysis

Experimental data were analyzed by a non-linear regression procedure based on the Marquardt–Levenberg algorithm. Fitting parameters were expressed as the expectation value ± standard deviation. Multiple comparisons were performed using one-way ANOVA with the Student–Newman–Keuls as a post-test. A value of $p < 0.05$ was considered statistically significant.

Results and Discussion

To understand how phloretin influences on the dipolar organization of the lipid membrane, we studied the binding and fluorescence properties of ANS in the interface. We measured the binding and quantum yield of ANS in the presence of phloretin both in the gel and in the liquid-crystalline phases.

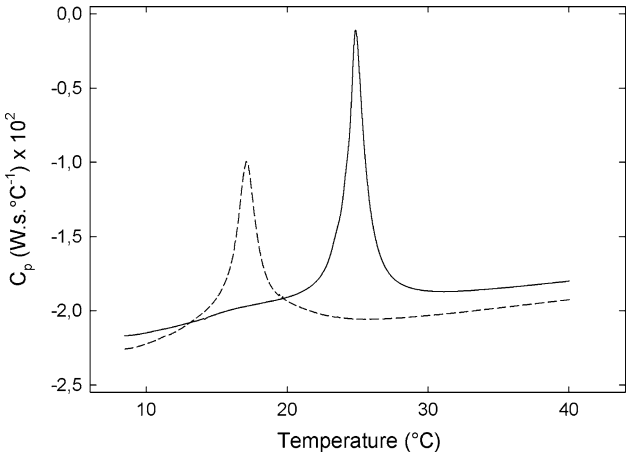


Fig. 2 DSC thermograms of DMPC liposomes. The DSC profiles were originated from heating scans using 1 mg/mL DMPC (the cell volume was 0.8 mL). The *continuous line* corresponds to the control (DMSO, 8 μL/mL), and the *broken line* is the thermogram obtained in the presence of phloretin (400 μM)

Effect of Phloretin on the Phase Behavior of DMPC Liposomes

In order to establish the conditions for working with liposomes in the gel phase or liquid-crystalline phase, we measured the transition temperature for DMPC LUVs. We observed a main phase transition with $T_m = 24.8$ °C and $\Delta H = 6.4$ kcal/mol. A not well-defined pre-transition was also observed at around 18 °C. Phloretin reduced the T_m of the transition to 17.1 °C and the transition enthalpy to 4.7 kcal/mol (Fig. 2). Hence, for studying the effect of phloretin on the binding of ANS to LUVs in the gel phase, a temperature of 13 °C was chosen.

ANS Fluorescence Enhancement Upon Binding to DMPC Liposomes

The fluorescence intensity of ANS strongly increases when it binds to DMPC liposomes showing a complex kinetic profile (Fig. 3, closed symbols), whose time dependence could be fitted by the sum of two exponentials, according to:

$$\Delta F = \Delta F_1(1 - \exp(-k_1 t)) + \Delta F_2(1 - \exp(-k_2 t)) \qquad (1)$$

ΔF_1 and ΔF_2 are the amplitudes of a fast and a slow kinetic components; k_1 and k_2 are their respective kinetic constants. The rate constant for the fast phase could not be measured since it was faster than the instrumental response, but its

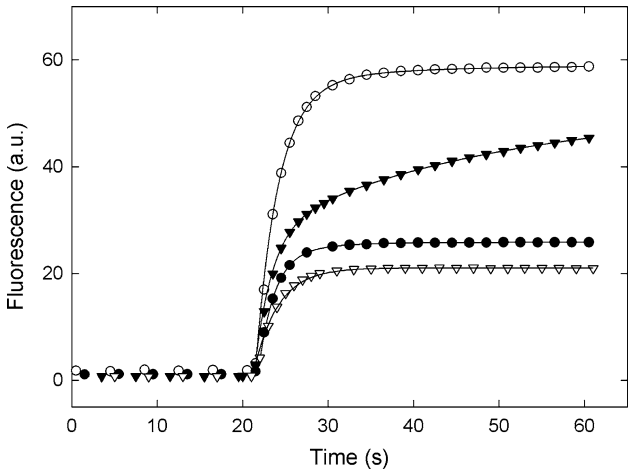


Fig. 3 Effect of phloretin on the kinetic profile of the ANS fluorescence increase upon binding to DMPC liposomes. 0.1 mg/mL DMPC LUVs were pre-incubated in sucrose 250 mM, Tris–HCl 30 mM (pH = 8.0). After 20 s ANS was added and the fluorescence was recorded as indicated under "Materials and Methods" section. The *circles* indicate the values obtained at 13 °C and the *triangles* those obtained at 33 °C in the absence (*closed circle*, *closed inverted triangle*) and in the presence (*open cicle*, *open inverted triangle*) of phloretin. The *lines* indicate the best fit of Eq. 1 to the experimentally determined data. [ANS] and [phloretin] were 80 and 200 μM at 13 °C and 16 and 125 μM at 33 °C

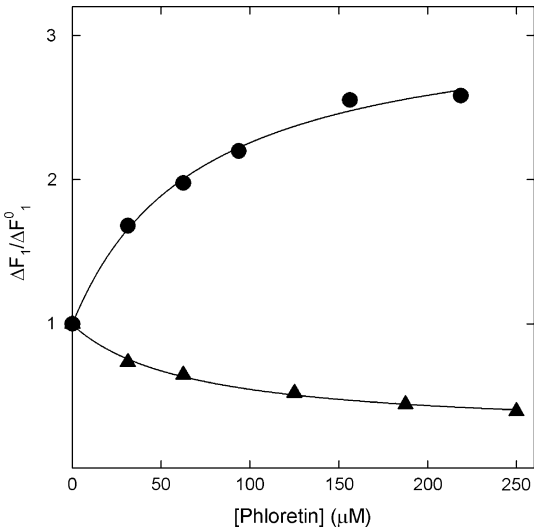


Fig. 4 Differential effect of phloretin on the binding of ANS to DMPC liposomes in gel and liquid crystalline phases. DMPC LUVs (0.1 mg/mL) were incubated as indicated in Fig. 2. ANS (80 μM) was added after 20 s pre-incubation and the fluorescence increase was continuously followed. Equation 1 was fitted to the kinetic profile and the amplitude of the fast kinetic phase was estimated. *Symbols* represent the amplitude estimated in the presence of different phloretin concentrations in the reaction media at 13 °C (*closed circle*) and at 33 °C (*closed triangle*) relative to that obtained in the absence of the compound. The *lines* indicate the best fit of Eq. 2 to the experimentally determined data

amplitude could be accurately determined. It has been suggested that the fast phase represents the interaction of ANS at the surface of the liposomes (Gains and Dawson 1975). Therefore, its amplitude is a good estimate of the binding of ANS at the external face of the bilayer.

When the lipids were in the gel phase (13 °C), the amplitude of the first fast exponential accounted for most of the total fluorescence increase ($\Delta F_1/(\Delta F_1 + \Delta F_2) = 0.91$, whereas at 33 °C (liquid crystalline phase) the contribution of the slow phase became much more important ($\Delta F_2/(\Delta F_1 + \Delta F_2) = 0.46$) (see Fig. 3). This observation is consistent with previous proposals that the slow phase of the fluorescence increase is due to migration of ANS through the bilayer towards the inner leaflet (Gains and Dawson 1975; Haynes and Simkowitz 1977). No significant changes were observed between the fluorescence decay measured immediately after mixing and up to 5 min incubation of ANS with liposomes: at any incubation time, the fluorescence decay could be fitted by only two exponential decays and no change in the fluorescence lifetime was observed. Therefore, the environment of the sites responsible for the fast phase and for the slow phase has similar polarities.

Effect of Phloretin on ANS Binding to DMPC Liposomes

At 13 °C, DMPC LUVs were in the gel phase both in the absence and in the presence of phloretin (Fig. 2). Phloretin produced a significant increase of the amplitude of both kinetic phases (Fig. 3, open circles). Contrarily, in the liquid-crystalline phase 125 μM phloretin decreased 50 % the amplitude of the fast kinetic phase and almost completely that of the slow phase (Fig. 3, open triangles).

We measured the effect of different phloretin concentrations on the amplitude of the fast kinetic phase at a fixed concentration of ANS as shown in Fig. 4. In both phase states, the changes in the amplitude showed a hyperbolic behavior that was described by the following equation:

$$\Delta F_1/\Delta F_1^0 = \left(\Delta F_1/\Delta F_1^0\right)^{\infty} + C_{0.5}\left(1 - \left(\Delta F_1/\Delta F_1^0\right)^{\infty}\right) / (C_{0.5} + [\text{phloretin}]) \quad (2)$$

where ΔF_1^0 is the amplitude of the first phase obtained in the absence of phloretin and ΔF_1 in its presence; $\left(\Delta F_1/\Delta F_1^0\right)^{\infty}$ is the maximal effect attainable at infinite concentration of phloretin and $C_{0.5}$ is the concentration that exerted half of the maximal effect. Phloretin increases more than three times ($\left(\Delta F_1/\Delta F_1^0\right)^{\infty} = 3.1 \pm 0.1$) the amplitude of the fast phase at 13 °C (Fig. 4, circles). Half of the maximal effect was achieved with 71 ± 12 μM.

At 33 °C (fluid phase) instead, phloretin decreased 75 % ($\left(\Delta F_1/\Delta F_1^0\right)^{\infty} = 0.25 \pm 0.02$) the amplitude of the first phase. A $C_{0.5}$ value equal to 66 ± 6 μM could be estimated by

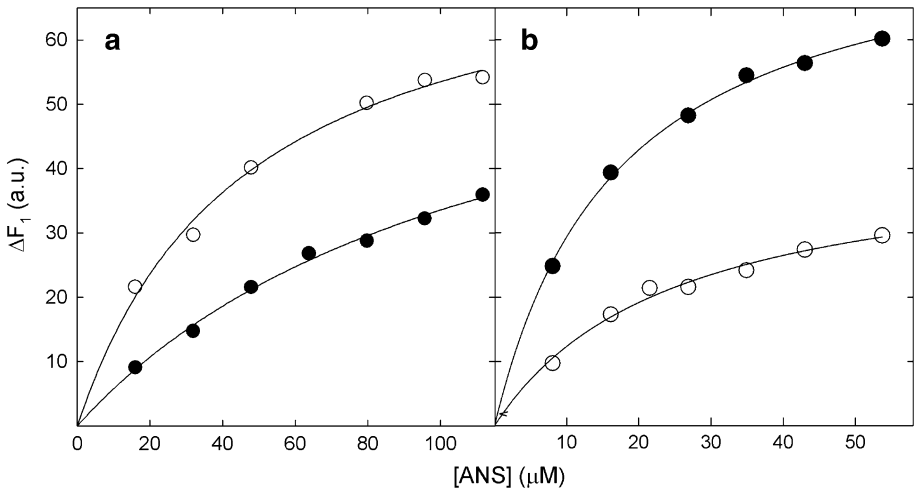


Fig. 5 Effect of phloretin on the ANS binding parameters. The amplitude of the first phase of the ANS fluorescence enhancement is presented as a function of ANS concentration at 13 (**a**) and 33 °C (**b**). The experimental conditions are similar to those detailed in the legend to Fig. 2. *Closed circles* represent the values obtained in the absence and *open circles* those estimated in the presence of 94 (13 °C) and 125 μM phloretin (33 °C). The *lines* indicate the best fit of the Eq. 3 to the experimental data

fitting Eq. 2 to the experimentally determined data (Fig. 4, triangles). Hence, phloretin exerted an opposite effect on the binding of ANS to DMPC liquid crystalline bilayers than the observed on the binding to liposomes in gel phase.

Effect of Phloretin on the Affinity of ANS for DMPC Liposomes

The amplitude of the fast kinetic phase depended on ANS concentration as shown in Fig. 5. Considering that the amplitude of the fast phase is a measure of the amount of bound ANS, we can evaluate the ANS dissociation constant according to:

$$\Delta F_1 = (\Delta F_1)_{\max}[\mathrm{ANS}]^n/\left(k_d^n + [\mathrm{ANS}]^n\right) \quad (3)$$

$(\Delta F_1)_{\max}$ is the amplitude of the fast phase at infinite ANS concentration and n is the parameter of heterogeneity (Disalvo and Bouchet 2014). Statistical analysis has shown that n did not significantly differ from 1 either in the gel or in the liquid lipid phase and either in the presence or in the absence of phloretin. Therefore, Eq. 3 simplifies to a simple hyperbola. We determined the effect of a fixed phloretin concentration on the binding constant (K_d) of ANS and on the $(\Delta F_1)_{\max}$. The former depends on the intrinsic dissociation constant and the surface potential and the latter on the fluorescence quantum yield of the bound ANS and the number of ANS binding sites. Phloretin decreased K_d, whereas no significant differences were observed either in $(\Delta F_1)_{\max}$ (Fig. 5a and Table 1) or in the fluorescence lifetime of ANS bound to liposomes at 13 °C (Fig. 6, closed circles). Therefore, phloretin interacts with DMPC LUVs in the gel phase increasing the affinity of the bilayer for ANS without modifying the polarity and the number of ANS binding sites. Similar effect, despite less notorious, was obtained with DPPC liposomes in the gel state (see Table 1). The increase in affinity is consistent with the expected decrease of the surface potential associated with the known ability of phloretin to decrease the dipole potential (Lairion and Disalvo 2009)

On the other hand, when experiments at variable concentrations of ANS maintaining fixed the phloretin concentration, were carried out with liposomes in the liquid-crystalline phase, a clearly different result was obtained: K_d and $(\Delta F_1)_{\max}$ values (Table 1) estimated from the data shown in Fig. 5b indicate that phloretin significantly decreased not only the affinity of the bilayer for ANS but also the value of the maximal amplitude of the fast phase ($(\Delta F_1)_{\max}$). Phloretin affected similarly the binding of ANS to DOPC and DPPC liposomes in the liquid state (see Table 1).

Effect of Phloretin on the Fluorescence Quantum Yield of ANS Bound to DMPC Liposomes

To further understand the effect of phloretin on the environment of the ANS binding sites and the quality of the interface we measured the fluorescence lifetime of membrane-bound ANS at increasing phloretin concentrations (Fig. 6).

While phloretin did not affect the fluorescence lifetime of ANS bound to DMPC liposomes in gel state (Fig. 6, circles) it decreased hyperbolically that of ANS bound to DMPC LUVs in liquid-crystalline phase (Fig. 6, triangles) accordingly to the following equation:

$$\tau = \tau_\infty + C_{0.5}(\tau_0 - \tau_\infty)/(C_{0.5} + [\mathrm{phloretin}]) \quad (4)$$

τ_0 (7.5 ± 0.2 ns) was the lifetime in the absence of phloretin and τ_∞ (6.2 ± 0.1 ns) that estimated at infinite phloretin concentration. Half of the maximal effect was obtained with 83 ± 24 μM phloretin. Since phloretin decreased 75 % the amplitude of the fast phase and only 18 % the fluorescence lifetime, it cannot be excluded the

Table 1 Effect of phloretin on the binding parameters of ANS to PC liposomes

Temperature	Physical state	K_d (μM)			$(\Delta F_1)_{max}$ (a.u.)		
		Control	+ phloretin	*p* value	Control	+ phloretin	*p* value
DMPC							
13 °C	Gel	110 ± 12	47 ± 6	$p < 0.0008$	70 ± 4	78 ± 4	ns
33 °C	Liquid crystal	17 ± 1	25 ± 4	$p < 0.0001$	79 ± 2	43 ± 4	$p < 0.04$
DOPC							
25 °C	Liquid crystal	18 ± 1	40 ± 7	$p = 0.0001$	93 ± 2	51 ± 3	$p = 0.0007$
DPPC							
25 °C	Gel	59 ± 7	44 ± 7	ns	35 ± 2	30 ± 2	ns
50 °C	Liquid crystal	30 ± 2	47 ± 3	$p = 0.0009$	52 ± 1	34 ± 1	$p = 0.0009$

The parameters shown are the best estimates obtained by fitting Eq. 3 to experimental data obtained as shown in Fig. 5. Phloretin concentrations were: 94 μM (DMPC 13 °C), 125 μM (DMPC 33 °C), 200 μM phloretin (DOPC and DPPC)

ns statistically not significant

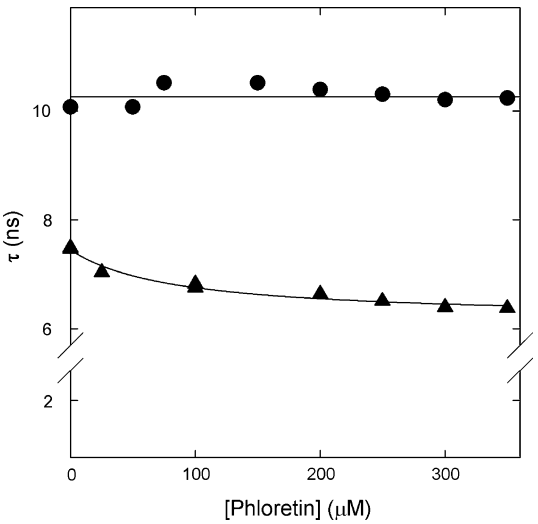


Fig. 6 Effect of phloretin on the fluorescence lifetime of ANS bound to DMPC liposomes. The fluorescence lifetime was determined as indicated under "Materials and Methods" section in a media containing 250 mM sucrose and 30 mM Tris–HCl (pH 8.0). Other conditions were: [DMPC] = 0.1 mg/mL and [ANS] = 25 μM. The *symbols* represent the lifetime values obtained at 13 (*closed circle*) and 33 °C (*closed triangle*). The *curve line* represents the best fit of Eq. 4 to the values estimated at 33 °C. The *straight line* is the mean of the values determined at 13 °C

possibility that phloretin also diminished the number of ANS "binding sites."

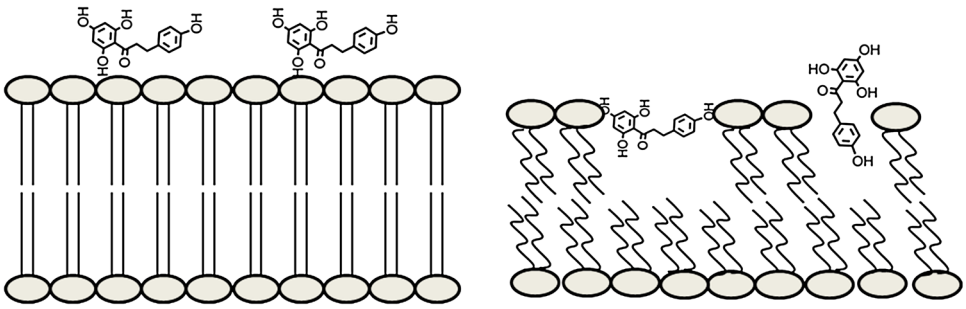
Conclusions

Phloretin affected ANS binding to DMPC liposomes in liquid-crystalline phase differently that it did on the gel phase: it decreased the affinity of ANS for the liquid-crystalline phase and it increased that for liposomes in the gel phase. This last effect can simply be related to the reported effect of phloretin on the dipole potential whereas the decrease in the apparent affinity of the DMPC liquid crystalline bilayer for ANS must be attributed to a decrease in the intrinsic affinity of the ANS "binding sites."

The fact that it decreased $(\Delta F_1)_{max}$ and the fluorescence lifetime of ANS bound to liquid crystalline bilayers, clearly suggests that phloretin distorts the bilayer lattice increasing the accessibility of water to the ANS "binding sites," which in addition would render them less hydrophobic and hence with lower affinity for the fluorescent probe.

Cseh and Benz (1999) reported that the interaction of phloretin with lipid monolayers depended on the surface pressure. At high pressures, phloretin would adsorb to the surface of the lipid monolayer with its dipole moment aligned in opposite direction to the lipid dipole, decreasing the surface potential without affecting the lipid packing. At lower pressures, phloretin would integrate in the monolayer modifying the lipid packing and the contribution of the dipole moment of the polar head group to the surface potential. More recently, Cseh et al. (2000) studied the interaction of phloretin with spherical supported unilamellar DMPC vesicles by NMR. From semi empirical conformational analysis, these authors have also shown that a wide distribution of the dipole moment of phloretin stable conformers can be calculated. Based on these studies, these authors postulated that phloretin localizes within the polar head groups and that they could change its conformation upon integrating in the bilayer and consequently the contribution of its dipole moment to the electrical properties of the membrane–water interface (Cseh et al. 2000).

The effectiveness of phloretin to reduce the dipole potential is strongly affected by the sterol content of the membrane and depends non-monotonically on the sterol concentrations (Ostroumova et al. 2013). Such complex behavior was attributed to the condensing and ordering effects of cholesterol at low concentrations that would result in modification of the area per lipid molecule increasing the effectiveness of phloretin to reduce the dipole potential. At higher cholesterol concentrations, the



Scheme 1 Interaction of phloretin with a lipid bilayer in gel and liquid-crystalline states

capability of the sterol of promoting a liquid-ordered phase would decrease the effectiveness of phloretin as dipole potential modifier as a consequence of a reduced possibility of formation of hydrogen bonds between hydroxyls of phloretin and the phospholipids' P=O group (Ostroumova et al. 2013). It has also been reported (Lairion and Disalvo 2004) that phloretin is more effective in reducing the dipole potential of DMPC monolayers in gel phase (from 515 to 270 mV) than in the liquid phase (from 449 to 297 mV).

The increase in the affinity of DMPC and DPPC LUVs in the gel phase for ANS reported here can be explained by postulating that phloretin simply adsorbs to bilayers in the gel phase as it does to monolayers at high surface pressures (Cseh and Benz 1999). Such an adsorption would not modify the bilayer structure, whereas the dipole moment oriented perpendicular to the membrane surface of the adsorbed phloretin molecules would contribute with partial positive charges to the charge density of the membrane surface, making less negative the surface potential and consequently increasing the apparent affinity of the bilayer for ANS (see Scheme 1). Conversely, phloretin would integrate in liposomes in the liquid-crystalline phase, modifying the lipid packing and the orientation of the polar head groups similarly as it does on monolayers at low pressures (Cseh and Benz 1999; Cseh et al. 2000). Such structural changes would result in: (i) decrease in the number of ANS "binding sites;" (ii) increase of the polarity of the ANS "binding sites" with the concomitant decrease in the fluorescence lifetime of the bound ANS and increase of the intrinsic ANS K_d. Phloretin also reduce the dipole potential in liquid-crystalline phases (Lairion and Disalvo 2004). Therefore, two opposite effects of phloretin regarding the apparent ANS dissociation constant must be considered: (i) an increase of the intrinsic dissociation constant; and (ii) a reduction of the dipole potential and consequently the density of negative surface charges. Since phloretin is less effective in reducing the dipole potential in liquid phases, such effect would not counterbalance that on the intrinsic K_d resulting in an increase in the apparent K_d.

Finally, the results described here clearly indicate that the enhancement of ANS fluorescence that follows its binding to bilayers is an useful tool for the study of the effect of biologically interesting compounds not only on surface electrical properties (changes in apparent ANS dissociation constant), but also on structural properties of membranes (changes in intrinsic dissociation constant, number of sites and/or fluorescence lifetime of bound ANS).

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References

Ames BN (1966) Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol 8:115–118

Anderson OS, Finkelstein A, Katz I, Cass A (1976) Effect of phloretin on the permeability of lipid membranes. J Gen Physiol 67:749–771

Avanti Polar Lipids (2013) http://www.avantilipids.com/index.php?option=com_content&view=article&id=185&Itemid=193

Chen PS, Toribara TY, Warner H (1956) Micro determination of phosphorus. Anal Chem 28:1756–1758

Chiu VCK, Mouring D, Watson BD, Haynes DH (1980) Measurement of surface potential and surface charge densities of sarcoplasmic reticulum membranes. J Memb Biol 56:121–132

Cseh R, Benz R (1999) Interaction of phloretin with lipid monolayers: relationship between structural changes and dipole potential change. Biophys J 77:1477–1488

Cseh R, Hetzer M, Wolf K, Kraus J, Bringmann G, Benz R (2000) Interaction of phloretin with membranes: on the mode of action of phloretin at the water-lipid interface. Eur Biophys J 29:172–183

Disalvo EA, Bouchet AM (2014) Electrophoretic mobility and zeta potential of liposomes due to arginine and polyarginine adsorption. Colloids Surf A 444:170–174

Efimova SS, Ostroumova OS (2012) Effect of dipole modifiers on the magnitude of the dipole potential of sterol-containing bilayers. Langmuir 28:9908–9914

Gains N, Dawson AP (1975) Transmembrane electrophoresis of 8-Anilino-1-naphtalene sulfonate through egg lecithin liposome membranes. J Membr Biol 24:237–248

Gibrat R, Romieu C, Grignon C (1983) A procedure for estimating the surface potential of charged and neutral membranes with 8-Anilino-1-naphtalenesulphonate probe. Adequacy of the Guoy-Chapman model. Biochim Biophys Acta 736:196–202

Haynes DH (1974) 1-Anilino-8-naphtalenesulfonate: a fluorescent indicator of ion binding and electrostatic potential on the membrane surface. J Membr Biol 17:341–366

Haynes DH, Simkowitz P (1977) 1-Anilino-8-Naphtalenesulfonate: a fluorescent probe of ion and ionophore transport kinetics and trans-membrane asymmetry. J Membr Biol 33:63–108

Haynes DH, Staerk H (1974) 1-Anilino-8-Naphtalenesulfonate: a Fluorescent Probe of Membrane Surface Structure, Composition and Mobility. J Membr Biol 16:313–340

Hess HH, Derr JE (1975) Assay of inorganic and organic phosphorus in the 0.1-5 nanomole range. Anal Biochem 63:607–613

Lairion F, Disalvo EA (2004) Effect of phloretin on the dipole potential of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylglycerol monolayers. Langmuir 20:9151–9155

Lairion F, Disalvo EA (2009) Effect of dipole potential variations on the surface charge potential of lipid membranes. J Phys Chem 113:1607–1614

Maget-Dana R (1999) The monolayer technique: a potent tool for studying the interfacial properties of antimicrobial and membrane-lytic peptides and their interaction with lipid membranes. Biochim Biophys Acta 1462:109–140

Melnik E, Latorre R, Hall JE, Tosteson DC (1977) Phloretin-induced changes in ion transport across lipid membranes. J Gen Physiol 69:243–247

Njus D. (2000) Membrane electrostatics, in "Fundamental principles of membrane biophysics", chapter 4, Department of Biological Sciences, Wayne State University. http://sun.science.wayne.edu/~bio669/Chap04.pdf

Ostroumova OS, Efimova SS, Schagina LV (2013) Phloretin-induced reduction in dipole potential of sterol-containing bilayers. J Membr Biol 246:985–991

Reyes J, Greco F, Motais R, Latorre R (1983) Phloretin and phloretin analogs: mode of action in planar lipid bilayers and monolayers. J Membr Biol 72:93–103

Robertson DE, Rottenberg H (1983) Membrane potential and surface potential in mitochondria. J Biol Chem 258:11039–11048