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Incorporation of arachidonic and palmitic acids in large unilamellar vesicles. A comparison of electrical surface parameters

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We determine the influence caused by the incorporation, of arachidonic and palmitic acids on the electrical surface parameters (potential, charge density, pK) of large, electrically charged and uncharged, unilamellar vesicles, utilizing fluorescence spectroscopy. The molecular relation of incorporated acid varies in the range of 8–24 lipid pairs per acid molecule. The surface potential and charge density vary in the range 12–19 mV and 1200–7000 esu cm^{-2} (1 esu cm^{-2} = 3.335 × 10^{-9} C m^{-2}). The ΔpK is one-tenth of the pK unit. We found in vesicles without charge (DMPC) that the saturation ratio for the incorporation of fatty acids is arachidonic : palmitic = 0.6; this relation is inverted in vesicles with charge (soy lecithin), arachidonic : palmitic = 8, showing an electrical influence on the arachidonic acid incorporation.

I. Introduction

The ionic environment in the neighbourhood of a biological membrane which normally bears a negative charge density is different from that of the bulk phase, mainly because the membrane surface contains ionizable groups that alter the mobility of ion distribution. The local physicochemical properties are quite sensitive to small changes not only in the bulk medium but also those caused by probes or molecules trying to interact with the membrane.

The ionization properties of some molecules in or near the membrane influence their own transport: for instance at pH 7.4 fatty acids stay in the neighbourhood of a phospholipid bilayer, resulting in a high population of the un-ionized form (≈50%), which diffuses across the lipid bilayer (flip-flops) rapidly ($t_{1/2} < 1$ s).[1]

An NMR study by Ptak *et al.*[2] on the ionization of fatty acids, fatty amines and *n*-acylamino acids incorporated in phosphatidylcholine vesicles found the apparent pK_a of fatty acids in phospatidylcholine bilayers to be 7.2–7.4 and those of fatty amines to be approximately 9.5. These pK_a values depend on many different parameters related to the structure of the lipid/solution interface, to the composition of the aqueous medium and to the localization of the ionizable groups. A special sensitivity to the ionic strength and to the surface charge has also been found. It was also found that the pK_a depends on the fatty acids length chain (8–26 carbons) and is slightly affected by the phospholipid composition and cholesterol content of the model membrane[3] of the fatty acid. Also, it has been established that significant modifications of the physicochemical properties of the membrane may be achieved by small changes in fatty acid composition within the lipid bilayer.[4,5]

Arachidonic acid is rapidly released during signal transduction by the activation of phospholipases A_2[6,7] in many types of eukaryotic cells. The release of arachidonic acid has been shown to cause localized changes in the unsaturated fatty acid composition.[5] It was shown in a study using fluorescence and NMR spectroscopy[8] that physiologically relevant amounts of arachidonic acid (∼5 mol%) can modify the dynamics of plasmenylcholine and phosphatidylcholine membrane bilayers and also that the dynamics of the bis-allytic protons in arachidonic acid are substantially different in plasmenylcholine as compared to phosphatidylcholine bilayers.

In another study[9] it was shown that the ratio of arachidonic to palmitic acid was significantly greater in cells from stroke-prone spontaneously hypertensive rats as compared to those cells isolated from Wistar–Kyoto reference strain ($p = 0.002$ for the difference in each ratio); the analyses were performed in three subcellular fractions: plasma membrane, mitochondrial-plus-lysosomal, and nuclear fractions. The results showed decreased lateral diffusion (decreased membrane fluidity) in vascular smooth muscle cells from stroke-prone spontaneously hypertensive rats which was associated with increased content of arachidonic acid, the major precursor of prostaglandins and other eicosanoids.

The present study makes use of the fluorescent properties of the lipid pH indicator heptadecyl-7-hydroxycoumarin (U-2) to investigate the influence of arachidonic (AA) and palmitic (PA) acids on the packing density of phospholipid molecules in large unilamellar vesicles (LUVs).

Lipoid fluorescent pH indicators were used as probes of electrical potential and polarity in micelles, and shifts of the apparent pK_a values for the dyes were found for charged as well as for uncharged micelles.[10–13] In the case of neutral micelles the shift was attributed to a reduced polarity at the micelle surface for which a value of $\varepsilon \approx 32$ was estimated. With respect to charged micelles this polarity effect is responsible for part of the apparent pK_a shift, the remaining part being caused by the electrical potential at the surface of the charged micelles. The influence of AA and PA on the surface potential and charge density and membrane pK_{mem} is also analysed. In addition, the fluorescent probe

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diphenylhexatriene (DPH) was used to study the influence of the fatty acids on the microviscocity of the vesicles.

II. Materials and methods

A. Fluorescence measurement

The fluorescent probe 7-hydroxycoumarin (umbelliferone) and the alkylated derivative heptadecyl-7-hydroxycoumarin (U-2) were purchased from Molecular Probes Inc. (Eugene, OR, USA). Soybean lecithin, arachidonic acid (AA) and palmitic acid (PA) were obtained from Sigma Chem. Co. (St. Louis, MO, USA), and the phospholipid dimyristoylphosphatidylcholine (DMPC) was from Avanti Polar Lipids (Birmingham, AL, USA).

Optical absorption measurements were performed using an HP 8452 A diode array spectrophotometer. For steady state fluorescence experiments we employed a Fluorolog 3 Jobin Yvon-Spex spectrometer. Excitation and emission slits of 1 or 2 nm band pass were used, depending on the fluorescence intensity of the sample. In fluorescence anisotropy experiments Glan Thomson polarizers were employed in the excitation and emission optical path. The temperature was controlled using a Forma Scientific 2006 thermal bath, and pH control was performed in the same cuvette used in the fluorescence experiments, employing a Metler pH meter calibrated with standard buffer solutions.

Membrane microviscosity was measured as the fluorescence anisotropy of the fluorophore diphenylhexatriene (DPH; Molecular Probes Inc., Eugene, OR, USA) incorporated into the lipid vesicles (prepared as described in the next section). The molar ratio probe : lipid was approximately 1 : 1000 so that the probe did not perturb the membrane microviscosity. Excitation and emission wavelengths were set at 358 and 425 nm respectively.

B. Vesicles preparation

The vesicles were prepared from a chloroform solution containing the phospholipid and the fluorescent probe (U2) in a molar ratio of 1000 : 1. The solvent was then evaporated under a nitrogen gas flux and the films were held under vacuum (10^{-3} mmHg) for 3 h. Subsequently, the film was resuspended in HEPES buffer, 5 mM (pH 7.4) to give a final concentration of DMPC of 5.0 mM and of U2 of 5.0 μM. The suspension was extruded under pressure from nitrogen gas, passing several times through Nuclepore membranes of decreasing porosity (0.4, 0.2 and 0.1 nm). At the end of the process, vesicles were obtained having a mean diameter around 1000 Å.[14] A similar procedure was employed for the preparation of vesicles containing the fluorescent probe DPH. Stock solutions of fatty acids were prepared by dissolving the solid material in ethanol, to concentrations of 5×10^{-3} M.

C. Titration–p*K* values

The p*K* values were determined according to the fluorometric method developed by Fromherz,[10–13] based on the dependency of the umbelliferone spectroscopic properties with the pH of the medium. With excitation at 370 nm, the intensity of the emission band centred at 450 nm is dependent on the equilibrium between the acidic (AH) and the ionized (A^-) forms of the probe. The plot of the fluorescence intensity *vs.* the pH of the vesicle suspension is equivalent to the curve relating the degree of ionization α of the probe to the pH of the medium. The equilibrium p*K* between AH and A^- corresponds to a value of α equal to 0.5. In the titration experiments we obtained fluorescence emission spectra of the vesicle incorporated probe U2 at increasing bulk pH, starting from the value corresponding to the vesicle preparation and adding small aliquots of a stock NaOH 1.0 M solution. Fluorescence intensity data as a function of pH were fitted to sigmoid curves (using Origin software) for the obtention of the p*K* of the incorporated probe.

In the case of vesicles without charge the standard medium contained 2.5 mM DMPC in 5 mM HEPES and for charged vesicles the standard medium contained 2.5 mM of soy lecithin in 5 mM HEPES. The concentration of bound U2 was 8 nmol (mg of lipid)$^{-1}$. The mean error in the fitting of pK^{probe} was ± 0.02 with a SD of ± 0.01. The parameters α_{mem} and pK_{mem} were determined at pH = 7.4. We used the standard $pK_{ap}^{\circ} = 8.80$.[13]

D. Fatty acid addition

The effect of fatty acid on the bilayer was examined by adding small aliquots of the stock solutions to the vesicle suspension. The amount of stock added was controlled so that the fatty acid concentration present in the vesicle suspension ranged in the interval 5 to 150 μM. Fatty acid was added at temperatures above the phase transition of DMPC vesicles, corresponding to the liquid crystalline phase. Most experiments on the incorporation of fatty acids to model membranes were carried out at high temperatures,[1] as in our case. After addition of the controlled amount of stock, we waited 5 min for the system to reach equilibrium. A complete titration experiment was performed for each concentration of fatty acid added to the phospholipid suspension, following the procedure described above. The highest fatty acid concentration corresponded to an addition of 15 μl of ethanol. No effect due to ethanol solely was observed in control samples. As the U2 probe is located at the membrane surface,[15] the p*K* we obtained reflects local electrical surface properties dependent on the surface charge density.

III. Lipid bilayer surface

We consider a surface immersed in an electrolyte solution. The surface bears acidic ionizable groups at a density $1/S$, where S is the area of surface per acidic group. A fraction α (the degree of dissociation) of these groups will be dissociated so that the surface charge density is

$$\sigma = -\frac{e_0 \alpha}{S} \tag{1}$$

where $e_0 = 1.602 \times 10^{-19}$ C is the proton charge. From here we adopt the International System of units (SI). The degree of dissociation α is given by

$$\alpha = \frac{1}{1 + \exp[(pK_{ap} - pH)/0.43429]} \tag{2}$$

where pK_{ap} is the apparent p*K* and is related to the intrinsic pK_a by[16]

$$pK_{ap} = pK_{\alpha} - 0.434 y_s \tag{3}$$

where $y_s = (e_0 \psi_s)/kT$ is the dimensionless potential at the beginning of the diffuse double layer whose electric potential is ψ_s.

The surface charge density given by eqn. (1) has to be equal to that resulting from the ionic equilibrium of the charged surface immersed in the solution (governed by the Poisson–Boltzmann equation). For large vesicles and plane surfaces immersed in a symmetrical *z*–*z* electrolyte solution this is given by:[17]

$$\sigma = -(8kT\varepsilon\varepsilon_0 n)^{1/2} \sinh \frac{ze_0 \Psi_s}{2kT} \tag{4}$$

where n is the ionic density (number of ions per m^3), k is the Boltzmann constant (1.38×10^{-23} J K^{-1}), T is the absolute

temperature, ε is the dielectric constant of the medium ($\varepsilon = 80$ for water) and $\varepsilon_0 = 8.85 \times 10^{-12}$ F m^{-1} is the permittivity of a vacuum.

IV. Probes for electrical surface potential

To measure the electrical potential at the surface of a charged membrane or interface a probe of molecular size is required which does not alter the system. The interfacial ionization equilibria have been analysed in detail by Fernández and Fromherz.[18] The pK_a of any protonatable probe group at a membrane interface, pK_a^i, for the amphiphile in water because of thermodynamic differences in the ionization equilibria at the two locations is given by:[18–20]

$$\mathrm{p}K_a^i - \mathrm{p}K_a^\circ = \Delta\mathrm{p}K_a^{el} \pm |\Delta\mathrm{p}K_a^{pol}| \quad (5)$$

with the standard p$K_a^\circ = 8.80$ being the pK in a membrane of vanishing potential and $\Delta\mathrm{p}K_a^{el} = -y_s/\ln 10$ is the electrostatic shift which is determined by the surface potential, y_s, of the membrane. The polarity-induced shift, $\Delta\mathrm{p}K_a^{pol}$, accounts for the intrinsic difference in ionization equilibria and takes a positive sign for the dissociation of a molecular acid ($HA \rightleftharpoons H^+ + A^-$) and a negative sign for the dissociation of a cationic acid ($HB^+ \rightleftharpoons H^+ + B$).

From eqn. (5) we can write:

$$\Psi_s = -2.3\,\frac{kT}{e_0}\,(\mathrm{p}K_a^i - \mathrm{p}K_a^\circ \pm |\Delta\mathrm{p}K_a^{pol}|) \quad (6)$$

Fromherz showed,[10–13] that ψ_s can also be obtained from the shift of the "apparent" pK, pK_{ap}, of the pH indicator bound to the surface as referred to pK_{ap}° of a membrane of null electric potential, as is shown in the following equation:

$$\Psi_s = -2.3\,\frac{kT}{e_0}\,(\mathrm{p}K_{ap} - \mathrm{p}K_{ap}^\circ) \quad (7)$$

V. Titration evaluation

We obtained the degree of dissociation of the probe, α_m^{probe}, at a certain pH from the fluorescence intensity I by subtracting the signal I_0 in the limit of low pH as a background and by normalization to the signal I_M in the limit of high pH:

$$\alpha_m^{probe} = \frac{I - I_0}{I_M - I_0} \quad (8)$$

The α_m^{probe} data are plotted *vs.* pH and adjusted in accordance with eqn. (2), applying the non-linear fitting of the program ORIGIN5.0, using the Boltzmann equation with the parameter dx maintained constant ($dx = 0.434\,29$).

VI. Procedure for the evaluation of the theoretical–experimental parameters

(1) Measurement of pK_{ap}° for vesicles without electric charge, our case, DMPC vesicles.
(2) Measurement of pK_{ap} for vesicles after having incorporated the fatty acid.
(3) Estimation of the electric potential through eqn. (7), namely:

$$\Psi_s = -2.3\,\frac{kT}{e_0}\,(\mathrm{p}K_{ap} - \mathrm{p}K_{ap}^\circ)$$

(4) Determination of the surface charge density using eqn. (4), namely:

$$\sigma_{mem} = (8kT\varepsilon\varepsilon_0 n)^{1/2}\,\sinh\frac{ze_0\Psi_s}{2kT}$$

(5) Determination of the degree of dissociation α_{mem} on the membrane surface using eqn. (1):

$$\alpha_{mem} = \frac{\sigma_{mem} \times S}{e_0} \quad (9)$$

(6) Determination of pK_{mem} on the membrane surface using Tanford's equation:[21]

$$\mathrm{p}K_{mem} = \mathrm{pH} + 0.434y_s - \log_{10}\frac{\alpha_{mem}}{1 - \alpha_{mem}} \quad (10)$$

VII. Results and discussion

A. Microviscocity of the vesicles

In Fig. 1 we can observe the influence of the fatty acid addition on the anisotropy of the fluorescent probe DPH incorporated into the DMPC-LUVs membranes. The experiments were performed in the temperature range 15 to 30 °C that comprises the transition from gel to liquid crystalline phase.

In the low temperature region, corresponding to the gel phase of the DMPC vesicles, the presence of AA in a molar ratio fatty acid : phospholipid of 1 : 25 largely decreased the anisotropy of DPH. A similar decrease was produced by the addition of the saturated PA in the same molar ratio. However, the disordering effect in the hydrophobic core of the lipid bilayers sensed by the fluorescence is more accentuated in the case of the kinked structure of AA (Fig. 1).

Disordering effects were also reported[8] by studying the interaction of AA with small unilamellar vesicles (SUV) of dipalmitoylphosphatidylcholine (DPPC) and plasmenylcholine, in experiments performed over a range of temperatures corresponding to the gel phase. In our study, we extended the temperature range to examine the influence of fatty acids at the phase transition temperature (T_m) of DMPC vesicles. As observed in Fig. 1, pure DMPC vesicles give a T_m of 23.8 °C due to the incorporation of AA. The disordering effect promoted by this tetraenoic fatty acid is also revealed by the decrease of T_m, because less energy is required to change the DMPC vesicles from the ordered gel phase to the liquid crystalline phase.

An opposite effect was observed with addition of PA in that T_m of the DMPC vesicles increased to 24.9 °C. Fernández *et*

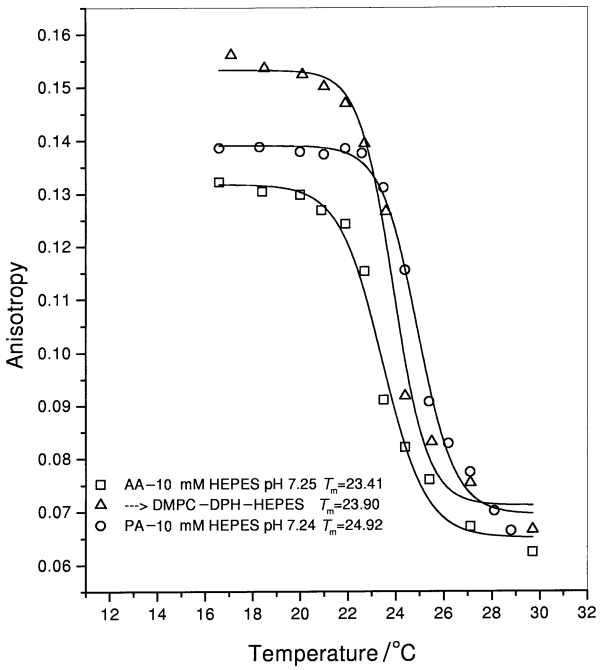

Fig. 1 Anisotropy (microviscosity) of DMPC-LUVs membranes, measured with the DPH probe (2.5 mM DMPC-LUVs in 100 μM acid).

al.,[22] measuring light scattering from DPPC liposomes containing 10% PA, verified that at low pH, below 7.0, the T_m was about 5 °C higher than that of pure liposomes. As the pH was raised, the differences in T_m between pure and PA containing liposomes gradually decreased, becoming similar at pH 9.0. A pK of 7.7 was then determined for the equilibrium between protonated and deprotonated forms of PA inserted into the lipid liposomes. Our experiments were performed at pH 7.2 and the increase in T_m of DMPC vesicles suggests a predominance of protonated species of PA in the lipid phase of the vesicles.

Experiments were also performed in soy lecithin vesicles. Without fatty acid addition, the DPH anisotropy continuously decreased with increasing temperature, from 15 to 30 °C, and the probe could not detect any phase transition. The result is not surprising because soybean membranes contain a large amount of lipids having unsaturated fatty acyl chains and therefore exist in the liquid crystalline state in the temperature range 15–30 °C. Addition of AA or PA had the effect of decreasing the anisotropy to values below those measured in pure vesicles over the whole range of temperatures, the disordering effect being higher for AA than for PA.

B. Variation of membrane parameters

The main results are shown in Fig. 2 and Table 1.

Results were obtained by considering that the area per vesicle (1000 Å radius for LUVs) is 3.14×10^6 Å^2 and a mean area per lipid molecule of 70 Å^2 in the liquid-crystalline phase,[23] the number of lipid molecules per vesicle is 4.48×10^4, corresponding to approximately to the same number of molecular lipid pairs (one pair corresponds to two lipids, from inner and outer leaflets). The number of vesicles in a 2 ml sample is 2.4×10^{13}.

In the calculation of the membrane surface charge density we considered the zwitterion HEPES as being a 1–1 symmetrical electrolyte.

C. Vesicles without charge (DMPC)

1. Arachidonic acid incorporation. The saturation in the aggregation of AA to vesicles happens at an acid concentra-

Table 1 Overall variation of membrane parameters

	DMPC		Soy	
	AA	PA	AA	PA
c_{sat} [a]/μM	60	100	150	50
%	4.8	8.0	12	4.0
$\Delta\psi_{2\,Å}$/mV	−12	−18	−19	−14
$\Delta\sigma_{mem}$/esu cm^{-2}	−1214	−1808	−7000	−4938
$10^{-2}\,\Delta\alpha_{mem}$	1.74	2.44	10	6.8
ΔpK_{mem}	−0.3	−0.5	−0.35	−0.35

[a] c_{sat} is the saturation concentration.

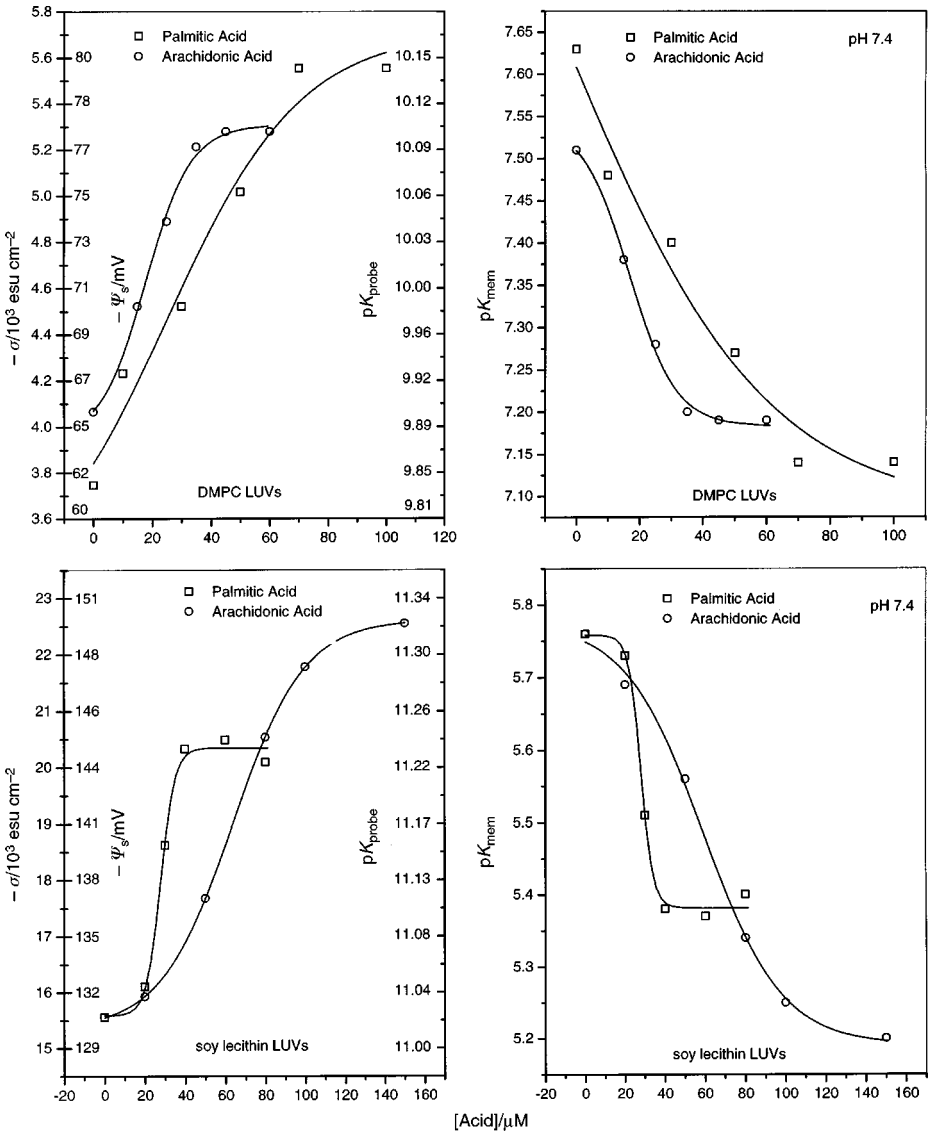

Fig. 2 Membrane and probe parameters for the incorporation of arachidonic and palmitic acids in LUVs (1 esu cm^{-2} = 3.335×10^{-9} C m^{-2}).

tion of 60 μM (Fig. 2), this means that there are 7.2×10^{16} molecules of AA in the sample. Thus, we conclude that 3000 molecules of AA were incorporated per vesicle, corresponding to ~15 pairs of lipid molecules per molecule of AA. This is equivalent to a fraction of 6.3% of molecules of AA per vesicle considering that the area per molecule of AA is approximately the same as the lipid molecule in the gel phase[23] (50 Å^2) due to its kinked structure; consequently, we obtain for the mean molecular area, assuming the additivity of areas[22] $A = 0.94 \times 70 + 0.06 \times 50 = 69$ Å^2. As the influence of AA in the available area per lipid is small, we conclude that the variation of the membrane surface charge density, $\Delta\sigma_{mem}$, is due to a variation of the surface charge, in this way we can write and use eqn. (9) with S being constant at each stage of the AA incorporation, and as a consequence we can write:

$$\Delta\alpha_{mem} = -\frac{S}{e_0}\Delta\sigma_{mem} \quad (11)$$

Considering the overall variations of the parameters in the interval of concentrations of AA added (0, c_{sat}), we observe from Table 1 that the variation in the surface charge density, $\Delta\sigma_{mem}$, is -1214 esu cm^{-2} corresponding to $\Delta\alpha_{mem} = 1.74 \times 10^{-2}$. The variation of the surface electric potential is $\Delta\psi_{2\,Å} \sim -12$ mV and $\Delta pK_{mem} \sim -0.3$.

2. Palmitic acid incorporation. With respect to the incorporation of PA we observe similar behaviour, although the saturation happens at around 100 μM of added acid, showing greater incorporation of PA molecules, ~5000 molecules, equivalent to ~10% per vesicle or ~9 pairs of lipid molecules per PA. PA is smaller than AA and an area per molecule of 20 Å^2 is reported.[24] Using the same procedure as outlined above, we obtain an area per molecule equal to 65 Å^2 for the vesicles containing PA. The surface charge density variation is $\Delta\sigma_{mem} = -1808$ esu cm^{-2}, considering also here S as being constant we obtain $\Delta\alpha_{mem} = 2.44 \times 10^{-2}$, with $\Delta\psi_{2\,Å} \sim -18$ mV and $\Delta pK_{mem} \sim -0.5$.

D. Charged vesicles (soy lecithin)

1. Arachidonic acid incorporation. Saturation in the incorporation of AA happens at a concentration of 150 μM added acid, equivalent to 7500 molecules or ~14% per vesicle ~6 pairs of lipid molecules per acid molecule. The surface charge density variation is $\Delta\sigma_{mem} \sim 7000$ esu cm^{-2}, considering also here S as being constant ($S = 69$ Å^2) we obtain $\Delta\alpha_{mem} = 0.1$, with $\Delta\psi_{2\,Å} \sim -19$ mV and $\Delta pK_{mem} \sim -0.35$.

2. Palmitic acid incorporation. With respect to the incorporation of PA we can observe a sharp variation of the studied parameters with a saturation at around 50 μM, corresponding to ~2500 molecules of PA, equivalent to ~5% per vesicle or ~18 pairs of lipid molecules per PA. The variation in the surface charge density is $\Delta\sigma_{mem} = 4938$ esu cm^{-2}, with S constant, we obtain $\Delta\alpha_{mem} = 6.8 \times 10^{-2}$, with $\Delta\psi_{2\,Å} \sim -14$ mV and $\Delta pK_{mem} \sim -0.35$.

VIII. Conclusions

(1) In vesicles without charge (DMPC) the saturated ratio AA : PA is 0.6; this relation is inverted in charged heterogeneous vesicles of soy lecithin (AA : PA = 3). NMR monitoring of the bis-allylic protons of AA indicated a different degree of restriction to the fatty acid movement when inserted in different vesicles.[8] The composition of DMPC and soybean lecithin are not the same and the kinked structure of AA is not similar to the extended conformation of PA. It is possible that the combination of the two effects, leading to different restraints imposed on the fatty acids, can originate the inversion that we observed.

(2) In both acids the degree of incorporation is low and can be considered as impurities in the host bilayer matrices. In vesicles without charge (DMPC) we have 15 lipid pairs per AA molecule and 9 lipid pairs per PA molecule. In charged vesicles we have 6 lipid pairs per AA molecule and 18 lipid pairs per PA molecule.

(3) As a consequence of the low degree of incorporation, the influence on the electrical surface membrane parameters is not accentuated although differences exist between the two acids and between the lipid vesicles see Fig. 2 and Table 1. For instance a variation in the incorporation of AA in soy lecithin vesicles caused a $\Delta\sigma_{mem} \sim 7000$ esu cm^{-2} which is equivalent to ~0.1 electrons/head group area, while in DMPC vesicles $\Delta\sigma_{mem} \sim 1200$ esu cm^{-2} or $\sim 1.7 \times 10^{-2}$ electrons/head group area. The results suggest that only a fraction of the incorporated acid is in the anionic form.

(4) We can also observe from Fig. 2 and Table 1 the small influence on the degree of dissociation α and on the membrane pK_{mem}; this is consistent with the previous finding that the apparent pK of the FA (~7.5) is close to physiological,[19] due to the fact that the FA carboxyl group lies at the aqueous interface, and the large shift to higher pK is predicted from its proximity to the negative charges of the phospholipid.

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