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The pentaene macrolide antibiotic filipin prefers more rigid DPPC bilayers: a fluorescence pressure dependence study

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Abstract

Filipin is a pentaene macrolide antibiotic which was previously shown to incorporate more extensively into DPPC bilayers below the main phase transition temperature than above this temperature. This result was extremely unusual because drugs tend to be expelled from ordered gel phases. However, such results could not be safely attributed to the phase change of the bilayer itself because the temperature was changing concomitantly. In this work we changed the bilayer phase isothermally (53°C) by hydrostatic pressure variation and discovered that filipin has a slightly more extensive incorporation in the pure DPPC gel phase ($P >$ ca. 54.4 MPa): $K_{p,lc} \approx 3\times10^3$ vs. $K_{p,gel} \approx 6\times10^3$. The presence of sterols (45% molar ergosterol or cholesterol) caused an increase in the partition coefficients, regardless of pressure, ergosterol having a more pronounced effect ($K_p \approx 2\times10^4-6\times10^4$). K_p was pressure dependent in both cases, but mainly with cholesterol ($K_p \approx 2\times10^3-2\times10^4$). At variance with cholesterol, when ergosterol was used, no phase transition was detected. This difference cannot be due to a more extended uptake of filipin by cholesterol-containing membranes, and so must be due to specific interactions with cholesterol. In agreement with this finding, we discovered that filipin is more tightly packed (lower partial molar volume) in the cholesterol-rich phase than in the ergosterol-rich phase. Our results also point to a 2:1 DPPC:cholesterol stoichiometry in the cholesterol-rich phase (17% molar cholesterol). All partition coefficients were calculated from steady-state fluorescence anisotropy measurements.

Keywords: Partition constant; Fluorescence anisotropy; Filipin; Macrolide; Polyene; Pressure

1. Introduction

Filipin is a pentaene macrolide antibiotic extracted from *Streptomyces filipinensis* [1]. Several polyene macrolide antibiotics, such as nystatin and amphotericin B, have a very selective action against fungi (for reviews see [2,3]). This activity is believed to be related to the ability of the molecules to interact with plasmatic membranes' ergosterol, but not with cholesterol. Ergosterol is the major sterol in fungi mem-

Abbreviations: DPPC, L-α-1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; POPOP, *p*-bis-[2-(5-phenylox-azolyl)]benzene

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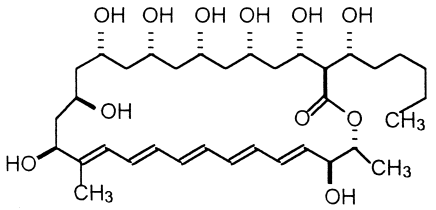

Fig. 1. Filipin III. Filipin is a mixture of molecules having minor differences between them, filipin III being the most abundant.

branes and cholesterol is the major sterol in mammalian membranes. Filipin, however, is an exception: it is toxic to mammals at low concentrations and provokes leakage of entrapped components in cholesterol-containing vesicles [2,3]. Filipin is a mixture of several molecules with minor differences between them [4]. Filipin III (Fig. 1) is the most common one. Recently, some progress has been made on the elucidation of the molecular structure of filipin III [5,6]. It was demonstrated that filipin can also incorporate into the hydrophobic region of sterol-free membranes and it was proposed that the two key issues that control the action of filipin are the aggregation state of filipin in the aqueous environment and the presence or absence of sterol-rich phases in the membrane (see [7] and references therein). The circumstances under which cholesterol-rich regions form in L-α-1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) are known and the phase diagram for this mixture is well established [8,9]. Detailed information on the organization and dimensions of these regions is, however, a matter of debate [10,11]. Among other open questions, the molar ratio of DPPC:cholesterol has attracted attention with some authors suggesting a 1:1 ratio, while others suggest 2:1.

In a previous work [12], we have calculated the partition coefficient of the antibiotic into the DPPC lipidic bilayer matrix and found that it is larger when the lipid is in the gel phase. A larger partition coefficient in the gel state than in the liquid crystal phase is extremely unusual. To our best knowledge, only *trans*-parinaric acid [13] and nystatin [14] present such singular behavior. This preference for the gel phase was also reported for the clinically used polyene antibiotic amphotericin B from exchange experiments between vesicles of lipids in different phases [15]. However, in most studies the partition coefficients were obtained at different temperatures. The temperature change was then used to change the phase of the membrane, from a gel to a liquid crystal. Whether the increase in the partition coefficient could be ascribed only to a different organization of the lipids or if it was also related to the change in temperature, was hence impossible to differentiate. This situation prompted us to carry out the present study, wherein the change in the membrane organization can be achieved isothermically by alteration of the hydrostatic pressure of the medium. Pressure dependence studies have been successfully applied to the study of membrane structure (e.g. [16–19]), and membrane interactions with foreign molecules (e.g. [20,21]). Pressure increases chain order, reducing the cross-section area occupied per hydrocarbon chain. Temperature has the opposite effect [17]. Depending on the temperature and pressure, DPPC multilayers may be in three different pure phases: liquid crystalline, gel or interdigitated [16,22]. Our goal was to relate filipin incorporation in the membrane with the membrane rigidity (i.e. packing density), in the presence and absence of sterols (cholesterol and ergosterol). Moreover, it was our purpose to elucidate whether the more powerful lytic action of the antibiotic, in the presence of cholesterol compared to the presence of ergosterol, was due to an increased uptake of the drug into the lipid matrix in the presence of cholesterol, thus gaining insight into its biochemical mode of action. We also addressed the DPPC:cholesterol stoichiometry in sterol-rich regions of the membrane.

2. Materials and methods

2.1. Chemicals

Filipin, DPPC, cholesterol and ergosterol were obtained from Sigma (USA) and used as received. Ergosterol purity was checked by thin layer chromatography and UV absorption (wavelength cutoff). Filipin is a mixture of macrolides with minor differences in their structures [4], having a pentaene chromophore as a common feature (Fig. 1). Chloroform was from Fisher Scientific (USA).

Filipin stock solutions in pH 7.9 Tris (ICN, USA) buffer (50 mM) with 1% v/v in ethanol were kept in the dark at 4°C.

2.2. Vesicle preparation

The appropriate volumes of DPPC and, when necessary, cholesterol or ergosterol stock solutions (30, 33 and 30 mM, respectively), all in chloroform, were mixed. Then, evaporation of the chloroform was achieved under air flow until a homogeneous film was deposited in the vessel. The vessels were kept in vacuum for 48 h, and finally the lipids with or without sterol were resuspended in buffer (blanks) or in a filipin solution with a desired concentration. Solubilization in the buffer was carried out by vortexing and warming (50°C) the solution above the phase transition temperature of the lipid. Sterol incorporation was considered quantitative up to 50 mol% (e.g. [23]).

2.3. Fluorescence measurements

Fluorescence anisotropy measurements were carried out with a SLM spectrofluorometer, in an L-format geometry, using Glan–Thompson polarizers and a Schott KV399 cut-on filter in the emission side which passed wavelengths greater than 380 nm. All measurements were performed at 366 nm excitation (Hg lamp) and the background intensities were taken into account. Fluorescence anisotropies were determined from Eq. 1 where I_{vv} and I_{vh} are fluorescence intensities (the two subscripts indicate the orientation of the excitation and emission polarizers, respectively: h for horizontal and v for vertical) and $G = I_{hv}/I_{hh}$ is the instrumental factor.

$$r = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}} \quad (1)$$

The pressure cell used in this work was previously described by Paladini and Weber [24]. The scrambling effect of pressure on anisotropy and total fluorescence intensity were taken into account by calculating the α correction factor with a solution of *p*-bis-[2-(5-phenylox-azolyl)]benzene (POPOP) in glycerol (see Section 3).

Fluorescence anisotropy measurements dependence on pressure and lipid concentration were carried out starting with the most concentrated lipidic suspension and measuring anisotropy in the whole range of pressure studied. Then, the desired dilution of the lipid was made with buffer (blanks) or filipin solution 3 μM. The first suspension was allowed to incubate for 2 h and the others for 15 min, following each dilution. After altering the pressure, the systems were allowed to equilibrate for at least 5 min. All these measurements were carried out at 53°C.

2.4. Partition constant calculation from fluorescence anisotropy

In cases wherein a lipophilic chromophore has a detectable fluorescence when present in the aqueous solvent, the anisotropy, r, measured for the entire system is an average between the anisotropy of the molecules incorporated in the membrane and in the aqueous phase. This average depends on the quantum yield, absorptivity and mole fractions of both species. The partition coefficient (K_p; Eq. 2) can be related to this average and can be calculated from non-linear regression analysis [12]. Eq. 3 was used in data treatment. γ is the molar volume of the lipid and r_l and r_w are the anisotropies in the lipid and aqueous environment, respectively. r_w is directly measured and r_l is also obtained from non-linear regression analysis. ϕ's are the quantum yields in the aqueous solution, ϕ_w, or in the lipid, ϕ_l, and ε's are the molar absorption coefficients in the aqueous medium, ε_w, or in the lipid, ε_l. L is the lipid molar concentration and K_p is the ratio between the effective concentration of fluorophore in the lipid and aqueous environment. The apparent partition coefficient, $K_{p,app}$, is defined in Eq. 4.

$$K_p = \frac{n_l/V_l}{n_w/V_w} \quad (2)$$

$$r = \frac{(1-\gamma L)r_w + \gamma L K_{p,app} r_l}{1-\gamma L + \gamma L K_{p,app}} \quad (3)$$

$$K_{p,app} = K_p \frac{\phi_l \varepsilon_l}{\phi_w \varepsilon_w} \quad (4)$$

It should be stressed that the use of Eq. 2 implies that we are regarding the aqueous and lipidic media as two non-miscible solvents, rather than the case of specific binding sites in the membrane, as the usage of equilibrium binding constants would imply. The values of γ used in our calculations involving pure lipids were taken from Tosh and Collings [25] with

minor simplifications: for pressures lower than 40.8 MPa, $\gamma = 0.720$ dm^3 mol^{-1}, for pressures of 40.8 or 47.6 MPa, $\gamma = 0.705$ dm^3 mol^{-1}, and for pressures higher than 47.6 MPa, $\gamma = 0.690$ dm^3 mol^{-1}. In fact, these are average values for each of the pressure ranges studied, but the total variation of γ in each case is non-significant. Whenever sterols were present, γ was considered constant and equal to 0.705 dm^3 mol^{-1}. Since the exact values are not available in the literature to our best knowledge, we considered the average value for the liquid crystal to gel transition because sterols often place the lipidic membrane properties in between these two states. It should be noted, however, that γ changes only slightly over the total pressure range studied, so that the choice of γ does not significantly affect the results. The changes in lipid concentration associated with water compressibility were not taken into account because for pressures up to 102.0 MPa, the water volume is still more than 95% of its initial value (e.g. [22]).

2.5. Fitting equations to the data

For the fitting of theoretical equations to experimental data we used Marquardt algorithm-based software in the 'simple-weighting' mode and the fitting criterion was the minimization of the χ^2 parameter (e.g. [26]). In this approach, the optimized function is proportional to the sum of the square of the residuals and all the residuals have the same weight.

3. Theoretical background

3.1. Window birefringency correction in measurements involving pressure

As discussed earlier by Paladini and Weber [24], the optical properties of the windows used in high pressure cell holders show a pressure-dependent scrambling of polarization. A correction factor, α, was proposed by these authors to calculate the true polarization. Taking into account the relationship (Eq. 5) between polarization, p, and anisotropy, r, Eq. 6 can be deduced without any assumption regarding the α's magnitude. Eq. 6 relates the uncorrected, r', and corrected anisotropies, r, relative to the birefringency of the cell windows.

$$p = \frac{3r}{r+2} \tag{5}$$

$$r = \frac{r'}{1-3\alpha+\alpha^2(2+r')} \tag{6}$$

Paladini and Weber [24] also proposed a method for the determination of α, namely the use of Eq. 6 with a pressure independent sample. r can be calculated at atmospheric pressure ($\alpha = 0$) and used at any given pressure.

Although not explicitly mentioned by Paladini and Weber [24], the total fluorescence intensity measurements calculated as $I_\parallel + 2I_\perp$, (where $I_\parallel$ and $I_\perp$ are the intensities of light polarized along the parallel and perpendicular orientations relative to the incident exciting radiation) need correction also. The uncorrected intensities, $S_\parallel$ and $S_\perp$, relate to the corrected intensities such that (assuming the pressure cell excitation and emission windows have identical properties):

$$S_\parallel + 2S_\perp = I_\parallel(1-2\alpha+\alpha^2) + I_\perp(2\alpha-\alpha^2) + 2I_\parallel(\alpha-\alpha^2) + 2I_\perp(1-\alpha+\alpha^2) \tag{7}$$

Eq. 7 can be rewritten as Eq. 8, which is equivalent to Eq. 9, where $S_\parallel + 2S_\perp = I'$, the uncorrected intensity, and $I_\parallel + 2I_\perp = I$, the corrected intensity.

$$(S_\parallel + 2S_\perp)/(I_\parallel + 2I_\perp) = 1-\alpha^2(I_\parallel - I_\perp)/(I_\parallel + 2I_\perp) \tag{8}$$

$$I = I'/(1-\alpha^2 r) \tag{9}$$

Eq. 9 shows that the intensity correction is anisotropy dependent. It should be stressed that the intensity correction is dependent on the squared α and α is very close to zero in a wide pressure range (see below). Therefore, intensity correction factors are often non-significant.

3.2. The 'reaction' volume

Most chemical reactions result in volume changes because the sum of the partial molar volumes of the products is generally not equal to that of the reac-

tants. This volume difference, ΔV, may be obtained from pressure response of the system at equilibrium, according to

$$\left(\frac{\partial \ln K}{\partial P}\right)_T = -\frac{\Delta V}{RT} \quad (10)$$

where K is the equilibrium constant, P is the pressure, R is the gas constant and T is the absolute temperature. The same equation is valid for the physical process of partitioning between two phases. In this case, ΔV is the difference between the partial molar volumes of the solute in the two phases involved.

4. Results and discussion

4.1. Calculation of the window birefringency correction factor, α

The method proposed by Paladini and Weber [24] was followed, using POPOP in glycerol at 22°C. The sample was excited at 366 nm. The total fluorescence intensity recorded without polarizers was constant in the pressure ranges of this study. The dependence of α with pressure was calculated from Eq. 6 by setting $r'(P) = r'(P = 1 \text{ atm})$ at any pressure. The results are presented in Fig. 2 and were used to correct all the fluorescence anisotropy values used throughout this study.

4.2. Anisotropy measurements

The filipin fluorescence anisotropy was recorded according to pressure and DPPC concentration. The lipid concentration dependent data was fitted with Eq. 3 for each pressure (Fig. 3). The fitted curves were plotted together in Figs. 4–6. The lines at constant concentration only link the fitted curves and have no physical meaning. Apparent partition coefficients, $K_{p,app}(P)$, and the fluorescence anisotropy of the filipin located inside the lipid, $r_l(P)$, were obtained as fitting parameters for each pressure. As can be seen from Figs. 4 and 7 the anisotropy of filipin in the presence of sterol-free bilayers has a sudden increase on going from pressures below 47.6 MPa to pressures above 61.2 MPa. This increase results from the phase transition of pure DPPC. The phase transition at 53°C occurs at 44.2 MPa [16,25], but filipin probably induces local disorder in the membrane, causing an apparently slightly shifted phase transition towards higher pressures. It should be stressed that this feature is common to all the phase transitions detected with intramembrane fluorescent probes (e.g. [18,21,27]). When ergosterol was added (45% mole fraction) to DPPC, no phase

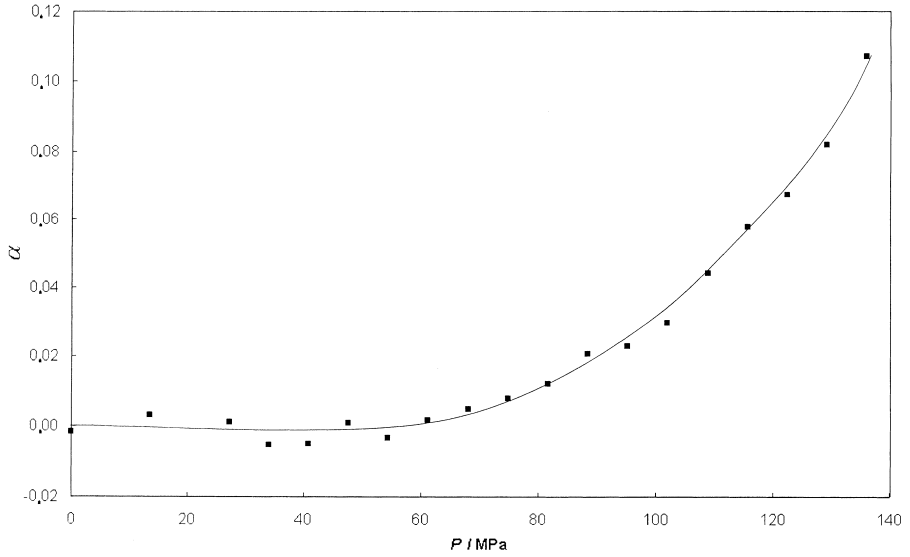

Fig. 2. Pressure dependence of the correction factor, α. This factor is used to correct the fluorescence anisotropy for the scrambling effect due to the birefringency of the cell window which increases as pressure increases. The squares represent the median from data sets having, at least five measurements. The line is merely a guide for the eye.

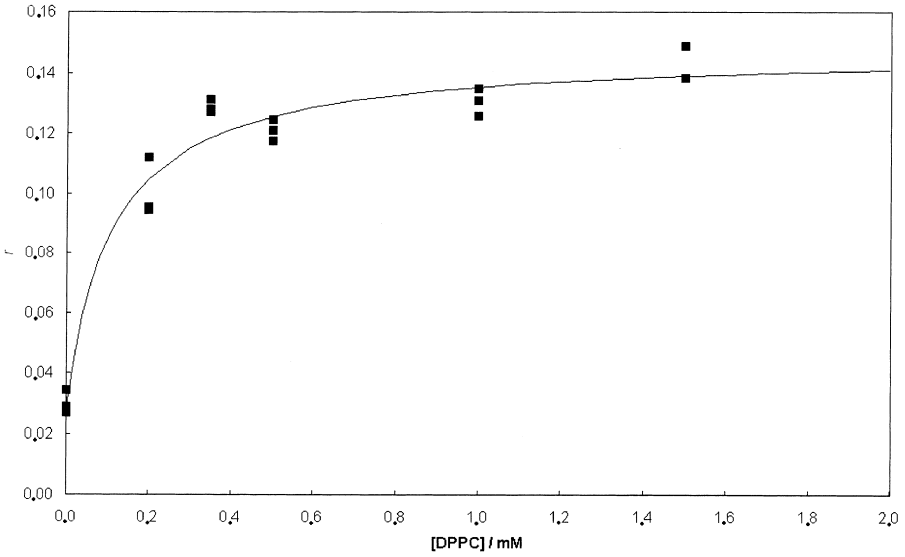

Fig. 3. Example of the dependence of fluorescence anisotropy on lipid (MLV of DPPC) concentration (53°C and 74.8 MPa). A series of data sets was recorded at other pressures. The squares represent experimental data and the line is the result of the fit of these data with Eq. 3. From this fit, the partition coefficient, K_p, and the fluorescence anisotropy inside the lipid, r_l, are calculated.

transition was detected. Filipin fluorescence anisotropy inside the bilayers was approximately constant over the entire pressure range, $r_l = 0.18$ (Fig. 7). Concentration–temperature phase diagrams for phospholid/sterol mixtures indicate that for high sterol mole fractions, temperature-induced phase transi-

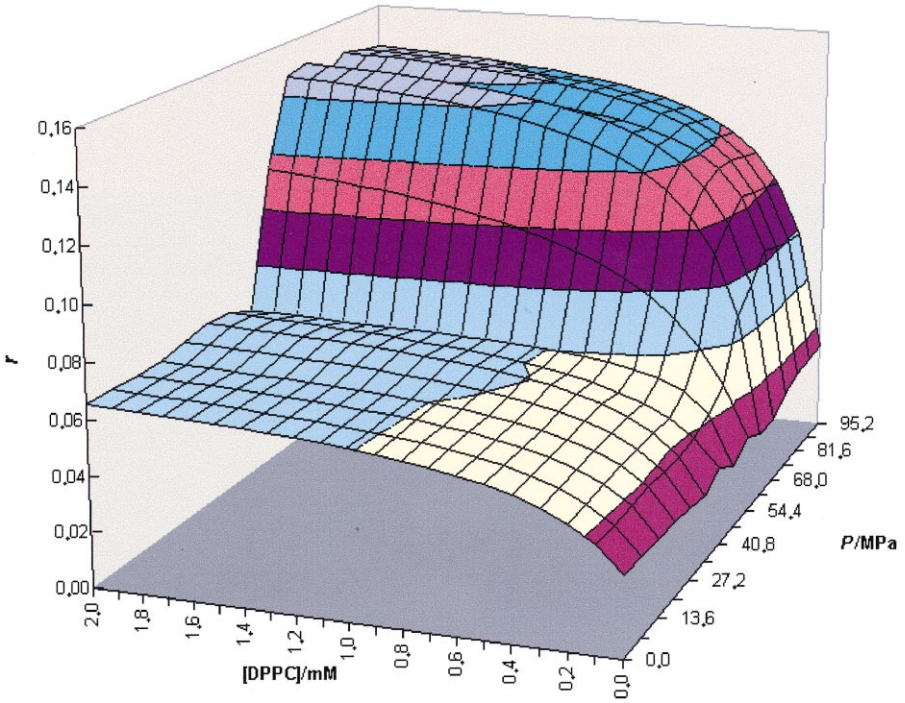

Fig. 4. Filipin fluorescence anisotropy dependence on pressure and lipid (MLV of DPPC) concentration. For the sake of simplicity, experimental data were not plotted. The fitted lines (Eq. 3) obtained at constant pressure were plotted and interpolated. The lines at constant lipid concentration only link the fitted curves and have no physical meaning. A sudden phase transition upon pressure increase is clear.

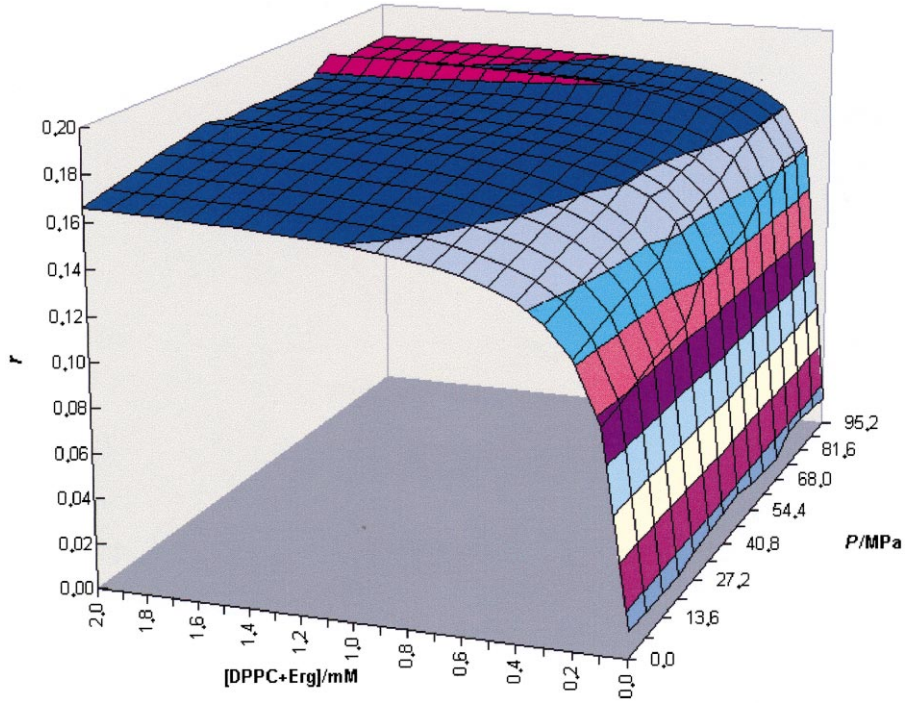

Fig. 5. Filipin fluorescence anisotropy dependence on pressure and lipid (MLV of DPPC and ergosterol; 45% molar in ergosterol) concentration. For the sake of simplicity, experimental data were not plotted. The fitted lines (Eq. 3) obtained at constant pressure were plotted and interpolated. The lines at constant lipid concentration only link the fitted curves and have no physical meaning. No phase transition is detected.

tions do not occur for a large variety of lipids and sterols [28–30]. Effects due to pressure changes may resemble effects due to temperature changes [17], thus no phase transitions upon pressure changes are expected either. Accordingly, Bernsdorff et al. [18] did not detect any phase transitions when DPH (1,6-diphenyl-1,3,5-hexatriene) related probes were incorporated in DPPC vesicles with 30% (molar) cholesterol content or more. This observation is in agreement with the data shown in Fig. 5, where a phase transition is not observed, and with a constant value of r_l. However, when cholesterol is added to DPPC instead of ergosterol, in the same concentration (45% mole fraction), the results are quite different: r_l increases smoothly with pressure (Fig. 7), as can be anticipated from the data in Fig. 6. The results suggest that the phase transition is partially recovered. This observation is in agreement with DSC results previously reported [31] and is probably related to filipin induced vesicle content leakage in cholesterol-rich membranes, which is larger than observed for other sterols [32], i.e. the regulator effect of cholesterol was reversed by filipin. In principle, the lack of information on the filipin-excited state lifetime variation with pressure prevents a precise rationalization of the differences in r_l based on the rigidity of the bilayers. Nevertheless, the quantum yield of filipin in solution is invariant with pressure, suggesting constant fluorescence lifetimes. Thus, a correlation between r_l and the rigidity of the bilayers is concluded and the data of Fig. 7 can be interpreted on this basis.

4.3. Partition coefficient, Kp

The partition coefficient, K_p, is related to the apparent partition coefficient, $K_{p,app}$, by Eq. 4. Thus, the ratio $\varepsilon_l\phi_l/(\varepsilon_w\phi_w)$ needs to be evaluated so that K_p can be calculated. Evaluation of this ratio was carried out by means of Eq. 11, where β represents a variable that decreases with the increase in lipid concentration ($\beta=1$ in pure aqueous solvents and $\beta=0$ would be the value obtained in the limit where all the fluorophore is located inside the membrane) [14,33]. I_{wm} is the fluorescence intensity in the presence of the lipidic bilayer and I_w is the fluorescence

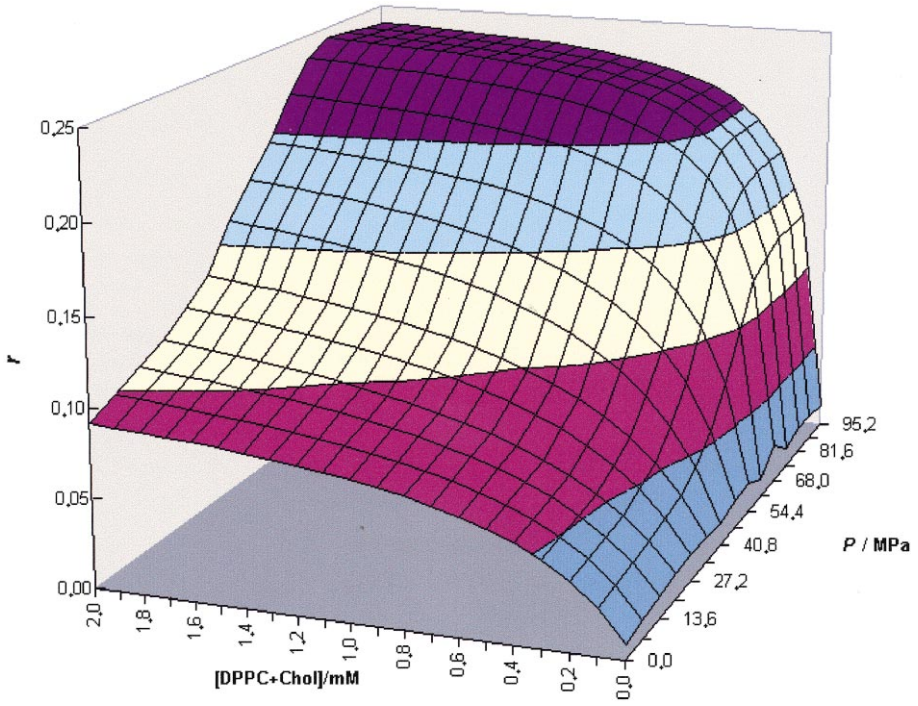

Fig. 6. Filipin fluorescence anisotropy dependence on pressure and lipid (MLV of DPPC and cholesterol; 45% molar in cholesterol) concentration. For the sake of simplicity, experimental data were not plotted. The fitted lines (Eq. 3) obtained at constant pressure were plotted and interpolated. The lines at constant lipid concentration only link the fitted curves and have no physical meaning. A smooth phase transition is detected.

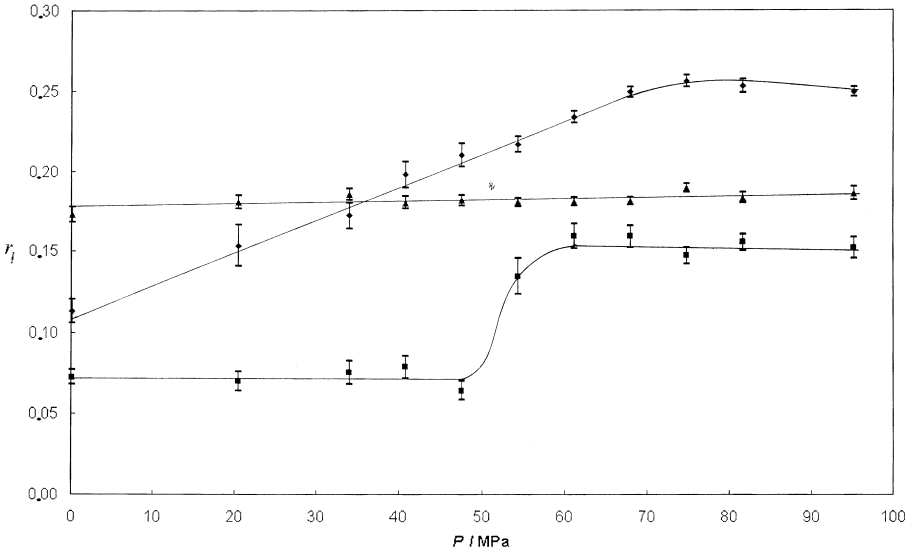

Fig. 7. Fluorescence anisotropy of filipin located inside the lipid matrix, r_l, of sterol-free (■) DPPC bilayers and sterol-containing (◆, cholesterol; ▲, ergosterol; 45% molar) bilayers. The data are represented between error bars. The lines are guides for the eye and have no physical meaning. A clear phase transition is detected in the pure DPPC bilayers. The effect is not so pronounced when cholesterol is used and is not detected when ergosterol is used.

intensity of a solution with identical concentration in the absence of bilayers. In the limit where $\beta = 0$, $I_{wm}/I_w = \varepsilon_l\phi_l/(\varepsilon_w\phi_w)$. Moreover, β is related to the lipid concentration, L, by Eq. 12. So, $\varepsilon_l\phi_l/(\varepsilon_w\phi_w)$ can be obtained from non-linear regression analysis of the data I_{wm}/I_w vs. DPPC concentration. It may appear that this method could be applied to the calculation of K_p itself. However, in practice $\phi_l\varepsilon_l/(\phi_w\varepsilon_w)$ is so close to one that the values of K_p calculated from Eqs. 11 and 12 are affected by extremely large errors, preventing any meaningful conclusion.

$$\frac{I_{wm}}{I_w} = \beta + (1-\beta)\frac{\phi_l\varepsilon_l}{\phi_w\varepsilon_w} \quad (11)$$

$$\beta = \frac{1}{1 + K_p L\gamma} \quad (12)$$

The partition coefficient dependence on the pressure is depicted in Fig. 8. For pure DPPC, there is only a slight increase of K_p with pressure upon phase transition. This observation is in agreement with a very small increase in the main transition temperature of phospholipid bilayers when filipin is present [31]. Solutes which prefer the gel-phase environment increase the transition temperature [34]. For sterol-rich vesicles, the K_p increases markedly with pressure. Filipin clearly incorporates more extensively in more rigid media. This observation indicates that filipin cannot be regarded as a common drug. Most drugs incorporate more extensively in fluid bilayers because they cannot fit into the highly ordered packing of a gel phase membrane (for a series of examples see e.g. [35]). Like sterols, filipin molecules do not at all resemble phospholipids; however, they probably have the ability to pack with them in an ordered manner. Other exceptions, which prefer more rigid media are *t*-parinaric acid, a fatty acid with a tetraene chromophore [13,36] and the polyene macrolide antibiotics nystatin [14] and amphotericin B [3]. Molecules having long alkyl chains interdigitate in the opposing leaflet of the bilayer and become more immobile [37]. Beck et al. [38] proposed that this interdigitation would lead such molecules to prefer the gel domains in binary lipid mixtures, where the shorter chain phospholipids form the liquid crystalline domains (see also e.g. [39]). However, their reasoning is only valid for molecules with long acyl chains 'backbones', so it cannot be applied to filipin. Moreover, in single lipid membranes, acyl chains interdigitation does not favor partitioning in the gel phase [40]. *t*-Parinaric acid also prefers the gel phase, even in membranes having longer chain phospholipids. Although filipin is located in the core of the membrane [7], interdigitation

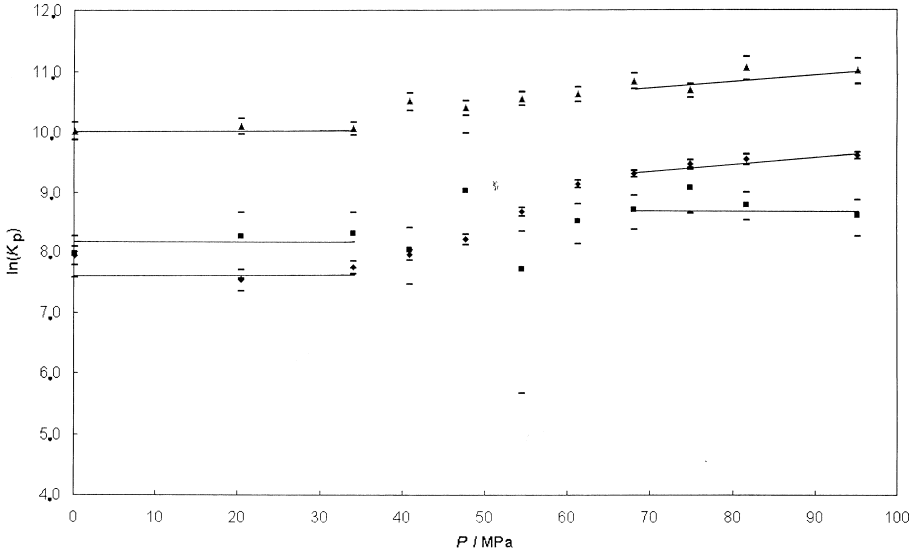

Fig. 8. Partition coefficient of filipin between the aqueous phase and sterol-free (■) DPPC bilayers or sterol-containing (◆, cholesterol; ▲, ergosterol; 45% molar) bilayers, K_p, dependence on pressure. The data are represented between the ln of the extrema of error bars. Application of Eq. 10 to the data enables the calculation of the partial molar volume change of filipin upon incorporation in the membranes. The lines are linear approximations to data variation above 68 MPa.

probably does not account for its preference for the gel phase.

It should be stressed that although small unilamellar vesicles and multilamellar vesicles have remarkable differences in their structure, mainly in curvature, K_p is similar in both cases (Fig. 8 and [8]).

Filipin also incorporates more extensively in sterol-rich bilayers than in pure DPPC bilayers, ergosterol being the most effective. However, filipin causes an increased leakage in cholesterol-containing vesicles compared to ergosterol-containing vesicles (e.g. [32]). This fact means that the lytic action of filipin is mainly qualitative rather than quantitative: it is not related to the extent of incorporation of the antibiotic, but to its specific interaction with the sterol and the effect it may have on the bilayers. This conclusion is in agreement with the partial recovery of the phase transition (Fig. 7) detected in the cholesterol-containing bilayers. Whether filipin is removing cholesterol from the bilayers cannot be known with certainty from this study, but the increase in r_l with pressure strongly suggests that this is not the case. Filipin in aqueous medium, even in the presence of cholesterol microcrystals, has a low anisotropy [41]. If the membranes were being depleted of cholesterol, due to its subtraction by filipin, the anisotropy would decrease.

4.4. Phospholipid:cholesterol stoichiometry and filipin partition into sterol-rich regions

The phospholipid:cholesterol stoichiometry in cholesterol-rich regions has been a matter of debate in the literature. We have derived Eq. 13 (see Appendix) to predict filipin anisotropy changes with pressure in bilayers having cholesterol-rich regions placed in the lipidic matrix. The expected values for a bilayer containing 17% (molar) cholesterol are plotted along with experimental results (Fig. 9). Two possible DPPC:cholesterol stoichiometries were considered: 1:1 and 2:1. The agreement between calculated and experimental data is better in the case of 2:1 DPPC:cholesterol.

$$r = \frac{r_w + \frac{k}{3}(L+C)\gamma_D\left(K_{p,app,D}r_D + K_{p,app,DC}\left(\frac{7}{2}-\frac{3}{2}k\right)r_{DC}\right)}{1+\frac{k}{3}(L+C)\gamma_D\left(K_{p,app,D} + K_{p,app,DC}\left(\frac{7}{2}-\frac{3}{2}k\right)\right)} \quad (13)$$

(where the subscripts refer to either homogeneous bilayers of DPPC and cholesterol, DC, or bilayers

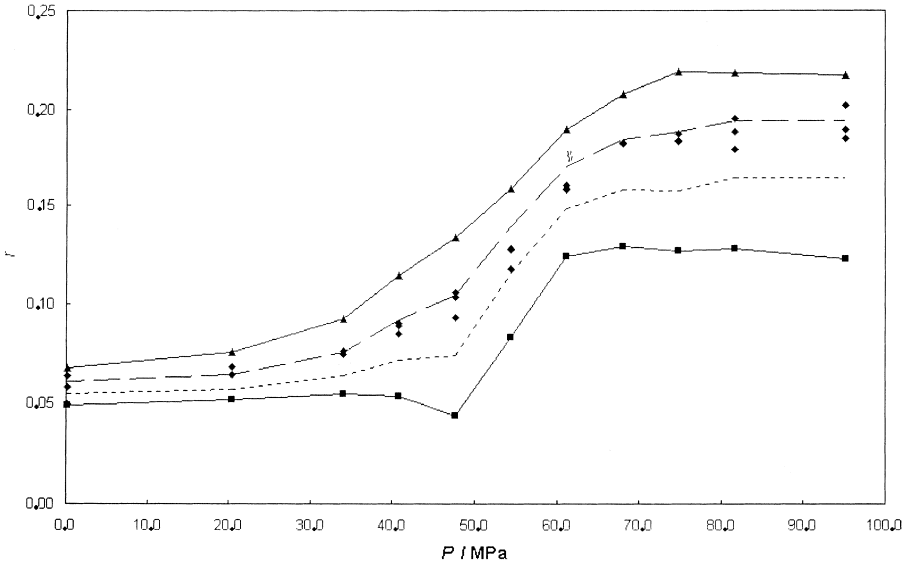

Fig. 9. Filipin fluorescence anisotropy variation in sterol-free DPPC bilayers (■) and, cholesterol-containing DPPC bilayers (47% (▲) and 17% (♦) molar). The solid lines are guides for the eye and have no physical meaning. The dashed lines were obtained with Eq. 13 ($\gamma = 0.705$ dm^3 mol^{-1}), and result from averaging the data of sterol-free (■) and 47% molar cholesterol-containing DPPC (▲) considering 17% molar sterol. This data treatment assumes the existence of sterol-rich regions in the DPPC bilayer matrix, having 2:1 (long dash) or 1:1 (short dash) DPPC:sterol molar stoichiometry. A good agreement is obtained between the experimental data (♦) and the theoretical expectation for a 2:1 stoichiometry (long dash).

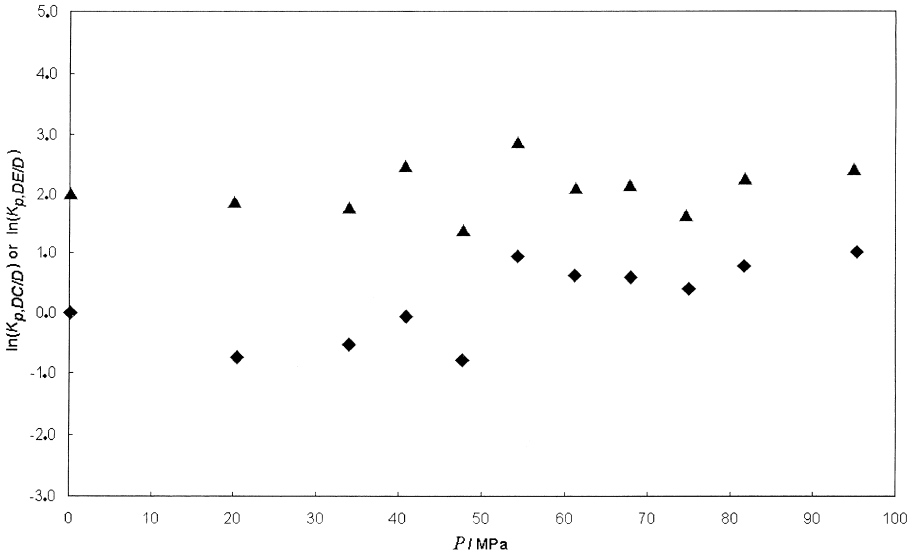

Fig. 10. The partition coefficient between the sterol-rich phase and the sterol-free phase in the bilayers, $K_{p,DC/D}$ for cholesterol (♦) and $K_{p,DE/D}$ for ergosterol (▲), as calculated from Eqs. 14 and 15. The more rigid the membrane becomes, the more filipin is segregated in the sterol-rich phase, this effect being more pronounced in cholesterol-containing membranes.

of pure DPPC, D; k is a constant which depends on the stoichiometry of DPPC:cholesterol in sterol-rich areas of the membrane that coexist with sterol-free areas in the lipid matrix: $k=2$ for a 1:1 stoichiometry and $k=1$ for a 2:1 DPPC:cholesterol mole ratio).

However, it should be stressed that the values of $K_{p,app,DC}$ used in Eq. 13 were obtained for approximately 1:1 DPPC:cholesterol bilayers. The resulting stoichiometry from Eq. 13 can only be admitted as valid if it is assumed that $K_{p,app,DC}$ is not significantly affected by DPPC:cholesterol in the range 2:1 to 1:1. Moreover, the model used to derive Eq. 13 considers the existence of sterol-free areas while, in fact, bilayers have sterol-poor, but not sterol-free, areas.

The partition coefficient between sterol-rich regions and sterol-free regions in the bilayers, $K_{p,DC/D}$ for cholesterol and $K_{p,DE/D}$ for ergosterol, can be calculated from Eqs. 14 and 15,

$$K_{p,DC/D} = (n_{DC}/V_{DC})/(n_D/V_D) = K_{p,DC}/K_{p,D} \quad (14)$$

$$K_{p,DE/D} = (n_{DE}/V_{DE})/(n_D/V_D) = K_{p,DE}/K_{p,D} \quad (15)$$

where, for the sake of simplicity, the parameters are labeled with subscripts having the following meaning: DC/D, coexistence of pure DPPC and DPPC/cholesterol regions; DE/D, coexistence of pure DPPC and DPPC/ergosterol regions; DC, homogeneous bilayers of DPPC and cholesterol; DE, homogeneous bilayer of DPPC and ergosterol; and D, bilayer of pure DPPC. The results are depicted in Fig. 10 and show that filipin incorporates slightly more extensively in sterol-rich phases as the pressure increases. The more rigid the membrane becomes, the more filipin is segregated in sterol-rich phases, this effect being more pronounced in cholesterol-containing membranes. The more rigid the membrane, the more extensive is the filipin uptake, mainly to sterol-rich phases of the bilayer (i.e. the preference of filipin to sterol-rich phases is increased in more rigid membranes).

4.5. Filipin packing

K_p is approximately constant at low pressures (up to 30 MPa; Fig. 8). Thus, the partial molar volumes of filipin inside the lipidic matrix and in the aqueous environment are approximately equal (Eq. 15) in this pressure range. At higher pressures (70 MPa and up), the situation is different: K_p increases with pressure if sterols are present. The partial molar volume inside the membrane is lower than in the aqueous environment, pointing to a tight packing of the filipin in the bilayer ($\Delta V_{colest} = -28$ cm^3 mol^{-1} and $\Delta V_{ergost} = -26$ cm^3 mol^{-1}) in this pressure range. In contrast,

for pure DPPC in the gel phase, the partial molar volumes are approximately constant. Thus, the sterols role is to improve filipin packing in the membrane.

4.6. Conclusions and concluding remarks

(1) Filipin incorporates more extensively in more rigid DPPC bilayers, with or without sterols. Pressure does not cause a removal of the antibiotic from the membrane, rather the contrary occurs. This behavior is quite unusual.

(2) The more rigid the bilayer, the more filipin incorporates in sterol-rich phases relative to sterol-poor phases, this effect being more pronounced in cholesterol than in ergosterol-containing membranes.

(3) If the membrane contains cholesterol, filipin partially reverts the 'fluidity regulator' effect of the sterol. If the membrane contains ergosterol, the antibiotic incorporates more extensively into the lipidic matrix, but does not revert the effect of the sterol over the fluidity of the membrane. This fact means that the more powerful lytic action of filipin when cholesterol is present [32] is qualitative, rather than quantitative, i.e. it is also related to the kind of the sterol which is present in the membrane, not only on the extent of the uptake of the drug to the membrane.

(4) Filipin is more tightly packed (lower partial molar volume) in the cholesterol-rich phase than it is in sterol-poor and ergosterol-rich phases.

(5) Our data present evidence that cholesterol-rich phases in DPPC bilayers have 2:1 molar ratios of DPPC:cholesterol.

(6) Only very few molecules have more extensive incorporation in gel phase membranes than in liquid crystal ones. Among them, there are at least three polyene macrolide antibiotics (filipin, nystatin and amphotericin B). This peculiarity may open a new insight into the biochemical mode of action of this class of drugs.

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Appendix

Considering that filipin can be located in three different environments (aqueous medium, w, pure – or, at least, sterol-poor – DPPC areas, D, and DPPC/cholesterol regions, DC), then the total fluorescence anisotropy is:

$$r = f_w r_w + f_D r_D + f_{DC} r_{DC} \quad \text{(A1)}$$

where f denotes the fractional fluorescence intensities and r_i the anisotropy of the filipin molecules located in environment i. Eq. A1 can be replaced by:

$$r = \frac{1}{V_t \sum_{i=w,DC,D} A_i \phi_{i\,j=w,DC,D}} \sum \varepsilon_j n_{F,j} \phi_j r_j \quad \text{(A2)}$$

(V_t is the total volume of the system, A is the absorbance, ε is the molar absorptivity, n_F are the moles of filipin, ϕ is the fluorescence quantum yield). Assuming that the molar volume of the sterol-rich and sterol-free areas of the bilayer is the same (γ_D), the total volume of the system is

$$V_t = V_w \left(1 + \frac{3}{k} L_f \gamma_D\right) \quad \text{(A3)}$$

(L_f is the molar concentration of lipid involved in pure lipid areas and k is a constant that depends on the molar ratio of DPPC:cholesterol at the sterol-rich regions: $k=2$ for a 1:1 ratio and $k=1$ for a 2:1 ratio). It results directly from the definition of $K_{p,D}$ that

$$\frac{n_{F,D}}{V_w} = K_{p,D} F_w L_f \gamma_D \quad \text{(A4)}$$

(F_w is the filipin concentration in the aqueous environment). Similarly,

$$\frac{n_{F,DC}}{V_w} = K_{p,DC} F_w \left(\frac{7}{2} - \frac{3}{2}k\right) L_f \gamma_D \quad \text{(A5)}$$

Replacing Eq. A3 and then Eq. A4 and A5 in Eq. A2, Eq. A6 is obtained.

$$r = \frac{F_w}{\left(1+\frac{3}{k}L_f\gamma_D\right)\sum_{i=w,DC,D} A_i\phi_i}\left(\varepsilon_w\phi_w r_w + \varepsilon_D K_{p,D} L_f\gamma_D\phi_D r_D + \varepsilon_{DC}K_{p,DC}\left(\frac{7}{2}-\frac{3}{2}k\right)L_f\gamma_D\phi_{DC}r_{DC}\right) \quad \text{(A6)}$$

F_w is not a practical variable and must be replaced. Since

$$f_w + f_D + f_{DC} = 1 \quad \text{(A7)}$$

replacing each fractional intensity in Eq. A7 by its counterpart in Eq. A6, leads to:

$$F_w = \frac{\left(1+\frac{3}{k}L_f\gamma_D\right)\sum_{i=w,DC,D} A_i\phi_i}{\left(\varepsilon_w\phi_w + \varepsilon_D K_{p,D} L_f\gamma_D\phi_D + \varepsilon_{DC}K_{p,DC}\left(\frac{7}{2}-\frac{3}{2}k\right)L_f\gamma_D\phi_{DC}\right)} \quad \text{(A8)}$$

L_f is also a difficult variable to deal with, but it can be related to the total concentration of lipid, L, and sterols, C, by:

$$L_f = (k/3)(L+C) \quad \text{(A9)}$$

Replacing Eq. A8 and A9 in Eq. A6

$$r = \frac{r_w + \frac{k}{3}(L+C)\gamma_D\left(K_{p,app,D}r_D + K_{p,app,DC}\left(\frac{7}{2}-\frac{3}{2}k\right)r_{DC}\right)}{1+\frac{k}{3}(L+C)\gamma_D\left(K_{p,app,D} + K_{p,app,DC}\left(\frac{7}{2}-\frac{3}{2}k\right)\right)} \quad \text{(A10)}$$

References

[1] G.B. Whitfield, T.D. Brock, A. Ammann, D. Gottlieb, H.E. Carter, J. Am. Chem. Soc. 77 (1955) 4799–4800.
[2] A.W. Norman, A.M. Spielvogel, R.G. Wong, Adv. Lipid Res. 14 (1976) 127–171.
[3] J. Bolard, Biochim. Biophys. Acta 864 (1986) 257–304.
[4] M. Bergy, T.D. Eble, Biochemistry 7 (1968) 653–659.
[5] A.R. Balakrishnan, K.R.K. Easwaran, J. Biomol. Struct. Dyn. 11 (1993) 417–428.
[6] S.D. Rychnovsky, T.I. Richardson, Angew. Chem. Int. Ed. Engl. 34 (1995) 653–659.
[7] M.A.R.B. Castanho, M. Prieto, Biophys. J 71 (1995) 1641–1650.
[8] J.H. Ipsen, G. Karlstrom, O.G. Mouritsen, H. Wennerstrom, M.J. Zuckermann, Biochim. Biophys. Acta 905 (1987) 162–172.
[9] M.R. Vist, J.H. Davis, Biochemistry 29 (1990) 451–464.
[10] F. Schroeder, J.R. Jefferson, A.B. Kier, J. Knittel, T.J. Scallen, W.G. Wood, I. Hapala, Proc. Soc. Exp. Biol. Med. 196 (1991) 235–252.
[11] P.L. Yeagle, Biochim. Biophys. Acta 822 (1985) 267–287.
[12] M.A.R.B. Castanho, M. Prieto, Eur. J. Biochem. 207 (1992) 125–134.
[13] A. Sklar, G.P. Miljanich, E.A. Dratz, Biochemistry 18 (1979) 1707–1716.
[14] A. Coutinho, M. Prieto, Biophys. J. 69 (1995) 2541–2557.
[15] J. Bolard, A. Vertut-Croquin, B.E. Cybulska, C.M. Gary-Bobo, Biochim. Biophys. Acta 647 (1981) 241–248.
[16] L.F. Braganza, D.L. Worcester, Biochemistry 25 (1986) 2591–2596.
[17] L.F. Braganza, D.L. Worcester, Biochemistry 25 (1986) 7484–7488.
[18] C. Bernsdorff, A. Wolf, R. Winter, E. Gratton, Biophys. J. 72 (1997) 1264–1277.
[19] P. Tauc, C.R. Mateo, J.C. Brochon, Biophys. J. 74 (1998) 1864–1870.
[20] P.L.-G. Chong, Biochemistry 27 (1988) 399–404.
[21] P.L.-G. Chong, G. Weber, Biochemistry 22 (1983) 5544–5550.
[22] L. Beney, J.-M. Perrier-Cornet, M. Hayert, P. Gervais, Biophys. J. 72 (1997) 1258–1263.
[23] J. Urbina, S. Pekerar, H. Le, J. Patterson, B. Montez, E. Oldfield, Biochim. Biophys. Acta 1238 (1995) 163–176.
[24] A.A. Paladini, G. Weber, Rev. Sci. Instrum. 52 (1981) 419–427.
[25] R.E. Tosh, P.J. Collings, Biochim. Biophys. Acta 859 (1986) 10–14.
[26] P.R. Bevington, D.K. Robinson, Data Reduction and Error Analysis for the Physical Sciences, 2nd edn., McGraw-Hill, New York, 1992.
[27] M. Sassaroli, M. Vauhkonen, P. Somerharju, S. Scarlata, Biophys. J. 64 (1993) 137–149.
[28] Y.L. Kao, P.L.-G. Chong, C.-H. Huang, Biochemistry 29 (1990) 1315–1322.
[29] T.P.W. McMullen, R.N.A.H. Lewis, R.N. McElhaney, Biophys. J. 66 (1994) 741–752.
[30] J.P. Slotte, M. Jungner, C. Vilchèze, R. Bittman, Biochim. Biophys. Acta 1190 (1994) 435–443.
[31] A.W. Norman, R.A. Demel, B. de Kruyff, L.L.M. van Deenen, J. Biol. Chem. 247 (1972) 1918–1929.
[32] D.B. Archer, Biochim. Biophys. Acta 76 (1976) 68–76.
[33] N.C. Santos, M.J.E. Prieto, M.A.R.B. Castanho, Biochemistry 37 (1998) 8674–8682.

[34] J.M. Sturtevant, Proc. Natl. Acad. Sci. USA 79 (1982) 3963–3967.
[35] L. Davenport, Methods Enzymol. 278 (1997) 487–512.
[36] C.R. Mateo, J.-C. Brochon, M.P. Lillo, U. Acuña, Biophys. J. 65 (1993) 2237–2247.
[37] I.E. Melhorn, E. Florio, K.R. Barber, C. Lordo, C.W.M. Grant, Biochim. Biophys. Acta 939 (1988) 151–159.
[38] A. Beck, D. Heissler, G. Duportail, Chem. Phys. Lipids 66 (1993) 135–142.
[39] C.H. Spink, M.D. Yeager, G.W. Feigenson, Biochim. Biophys. Acta 1023 (1990) 25–33.
[40] C.H. Spink, D. Clouser, J. O'Neil, Biochim. Biophys. Acta 1191 (1994) 164–172.
[41] M.A.R.B. Castanho, A. Coutinho, M.J.E. Prieto, J. Biol. Chem. 267 (1992) 204–209.