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Filipin Orientation Revealed by Linear Dichroism. Implication for a Model of Action

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Abstract: The organization of the polyene antibiotic filipin in membranes containing cholesterol is a controversial matter of debate. Two contradictory models exist, one suggesting a parallel and the other perpendicular organization of filipin with respect to the plane of the membrane. UV−vis linear dichroism, ATR-FTIR, and fluorescence anisotropy decay techniques were combined to study the orientation of filipin in model systems of membranes composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) or 1,2-palmitoyl-sn-glycero-3-phosphocholine (DPPC) with and without cholesterol. Filipin's orientation is determined by the presence/absence of cholesterol when it is inserted in gel crystalline phase model membranes. When cholesterol (33%) is present in DPPC bilayers, filipin stands perpendicular to the membrane surface as expected in "pore-forming" models. At variance, absence of cholesterol leaves filipin in an essentially random organization in the lipidic matrix. In liquid crystalline phase bilayers (POPC) filipin's orientation is perpendicular to the membrane surface even in absence of cholesterol. Thus filipin's activity/organization depends not only on cholesterol presence but also in the lipid phase domain it is inserted in. These findings were combined with spectroscopy and microscopy data in the literature, solving controversial matters of debate.

Introduction

Filipin is a pentaene macrolide antibiotic with antifungal properties that cannot be used as a therapeutic agent, at variance with other polyene antibiotics, such as Nystatin and Amphotericin B.[1] Filipin is toxic to mammals, even at low concentrations, and promotes the leakage of components from vesicles containing cholesterol.[1]

For other polyenes, such as Nystatin and Amphotericin B, the pore formation in lipid bilayers in the presence of sterol is consensual,[2,3] although recent alternatives were proposed.[4] Linear dichroism studies[5] confirmed the orientation needed for pore formation. However, until now filipin's organization in membranes and the role of cholesterol in such organization remains an open question. There are two "long-lasting" models proposed for the organization of filipin in membranes: (1) filipin and cholesterol are packed at the bilayer core in a 1:1 ratio, parallel to each other, as well as parallel to the membrane's surface forming large aggregates[3] (Figure 1A; hereafter referred to as the de Kruijff's model), and (2) formation of two groups of four associations filipin:cholesterol at the bilayer surface, in a 1:1 ratio and perpendicularly oriented with respect to membrane's plane[6] (Figure 1B; Elias' model). Both models differ in filipin and cholesterol orientation, as well as in antibiotic location in the lipidic bilayer. Although filipin is inserted in the bilayer core as suggested in De Kruijff's model,[3] orientation remains an open question. Orientation can, in principle, be used to confirm, or not, the validity of these models. In 1986, Bolard wrote in his comprehensive review on polyene antibiotics action[1] that "further studies are therefore needed which would indicate the orientation of filipin". This need stands up-to-date.

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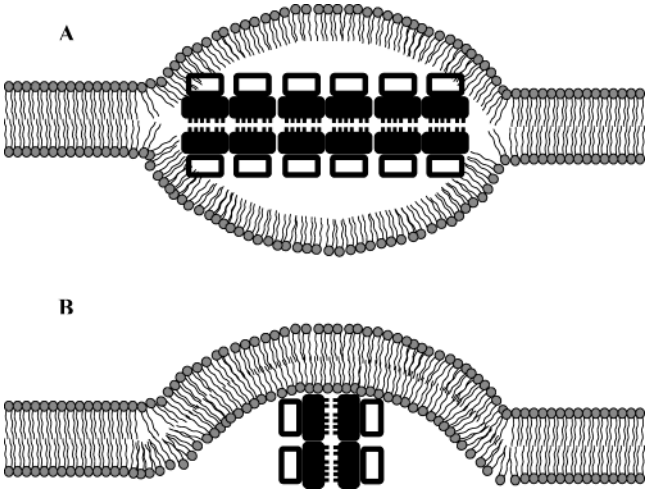

Figure 1. Schematic representation of the models proposed by De Kruijff and Demel (A) and Elias et al. (B) for filipin's organization in membranes, where black rectangles represent filipin molecules and open rectangles represent cholesterol molecules.

On the other hand Dufourc and Smith[7] concluded that some molecules of cholesterol were perpendicular orientated with respect to the lipid membrane's plane and speculated that filipin would lye parallel to sterols. Later work rendered the filipin mode of action more controversial when they revealed that filipin interacts with membranes without sterols.[8,9] Moreover, partition coefficient to the gel crystalline phase is bigger than to liquid crystalline phase.[9]

It is our goal to determine the orientation of Filipin in model system of membranes and how it is influenced by cholesterol. For that, we used a combination of UV−vis Linear dichroism (UV−vis LD), time-resolved fluorescence anisotropy, and attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR). This combination allowed us to obtain orientational probability density functions for different lipidic systems (with and without cholesterol).

Experimental Section

Filipin Complex and cholesterol were from Sigma Co. (ST. Louis, MO) and used with no further purification. NBD-Cholesterol was from Molecular Probes (Eugene, OR). All lipids were from Avanti Polar Lipids (Alabaster, AL) and the solvents were from Merck (Darmstadt, Germany) with spectroscopic grade.

UV−Vis Linear Dichroism Studies. Samples of aligned lipid multilayers (with or without cholesterol) were obtained as described in ref 10. The final molar ratios of lipid to filipin and lipid/cholesterol/filipin was 5:1 and 4:1:1, respectively.

UV−vis absorption and fluorescence measurements were carried out as described in ref 10. Namely, second rank order parameters, $\langle P_2 \rangle$, were obtained from electronic absorption in aligned multibilayers. Fourth rank order parameters, $\langle P_4 \rangle$, were obtained from fluorescence emission in aligned multibilayers, provided that $\langle P_2 \rangle$ is known. Excitation and emission wavelengths were, respectively, 337 and 480 nm.

Details regarding this methodology can be found elsewhere.[10] Buffer: Tris 50 mM pH 7.4, 150 mM NaCl. All measurements were carried out at room temperature.

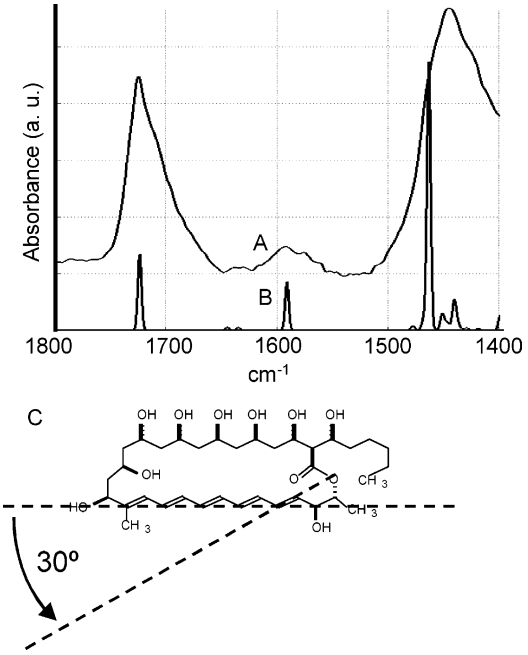

Figure 2. Theoretical (B) and experimental infrared spectra of [filipin] = 10 mg/mL in a Germanium plate (A). Filipin molecules (C) have C=C double bonds forming a 30° angle relative to the pentaene axis, which was considered to convert $\langle P_2 \rangle'$ in $\langle P_2 \rangle$ (see ATR-FTIR studies section).

ATR-FTIR Studies. A germanium plate was used for the ATR-FTIR measurements and its cleaning and sample films preparation, obtained by evaporation of the solvent, were done as described in ref 11. Buffer: HEPES 2mM, pH 7.4. All measurements were carried out at room temperature.

The final molar ratios of lipid to filipin and lipid/cholesterol/filipin were 5:1 and 4:1:1, respectively, as in UV−vis Linear dichroism studies.

ATR-FTIR spectra were recorded on a Bruker Equinox IR spectrophotometer equipped with a liquid nitrogen cooled detector and a polarizer mount assembly under computer control. Backgrounds of the internal reflection element were collected for each polarization and subtracted to the samples spectra afterward. A total of 256 scans were averaged for each spectrum to improve signal/noise ratio with a 4 cm^{-1} resolution. The spectrometer was continuously purged.

A more detailed description of the data analysis methodologies can be found elsewhere.[11] Second rank order parameters are calculated from IR absorption of selected bands with orthogonal incident beam polarizations. Simulated filipin molecular structure (see description in this section) shows that C=C bonds orientation relative to the pentaene axis varies between 28° and 31°. Second rank order parameters relative to C=C stretching, $\langle P_2 \rangle'$, was converted to the pentaene axis (the electronic absorption molecular axis), $\langle P_2 \rangle$, using

$$\langle P_2 \rangle = \frac{3\cos^2\theta' - 1}{2}\langle P_2 \rangle' \qquad (1)$$

where $\langle P_2 \rangle$ and $\langle P_2 \rangle'$ refer to axis with relative orientation θ' between them. θ' was considered 30° (Figure 2).

Anisotropy Decay Studies. Anisotropy decay measurements were carried out with a time-correlated single-photon counting system. For excitation of the systems with filipin at 324 nm, a frequency doubled, cavity pumped dye laser of 4-dicyannomethylene-2-methyl-6-(*p*-dimethylaminostyryl)-4H-pyran (DCM), synchronously pumped by a mode-locked Ar^+ laser (514.5 nm, Coherent Innova 400−10) was used.

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The emission wavelength was 478 nm, and a Hamamatsu R-2809 MCP photomultplier was used for the detection.

Samples were obtained by the extrusion method[13] with filipin and lipid (or lipid + cholesterol) concentrations of 0.1 mM and 3 mM, respectively. Data treatment was made as described in ref 14 but accounting for the fraction of filipin in the aqueous phase. When the fluorophores partition between lipidic (L) and aqueous (w) phases, the average ensemble anisotropy (r_{total}) is an intensity-weighted average of the contribution from the fluorophores in each environment

$$r_{total}(t) = r_w(t)f_w(t) + r_L(t)f_L(t) \quad (2)$$

where $r_w(t)$ and $r_L(t)$ are the anisotropy decays in aqueous and lipid phase, respectively. $f_i(t)$ stands for the fractional time-dependent intensities for filipin in each environment (aqueous, w, or lipid, L) and is determined by the decay functions. Considering that filipin in lipidic/aqueous systems has multiexponential fluorescence decays

$$f_w(t) = \frac{\sum X_w \times \alpha_{w,i} \exp(-t/\tau_{w,i})}{\sum \alpha_{total,j} \exp(-t/\tau_{total,j})} \quad (3)$$

(the molar fraction of filipin in aqueous phase, X_w, can be calculated from the published partition coefficients;[9,15] parameters α and τ are the normalized preexponential and lifetime parameters obtained from the fit of multiexponential functions to the fluorescence decay of filipin in buffer or lipidic vesicles suspension − subscripts w and total, respectively). Thus the meaningful fluorescence anisotropy decay, which is selectively related to the filipin inserted in the lipid phase is

$$r_L(t) = \frac{r_{total}(t) - f_w(t) \times r_w(t)}{1 - f_w(t)} \quad (4)$$

Although it was not possible to calculate the fourth rank order parameter from complex decays, which could not be fitted by the Mitchell and Litman[14] equations, the second rank order parameter was obtained from the fluorescence anisotropy, r, in the limit of infinite time, r_∞[16]

$$r_\infty = r_0\langle P_2\rangle_r\langle P_2^*\rangle_r \approx \langle P_2\rangle_r^2 \quad (5)$$

where r_0 is r at time 0 and equals 0.4 if the absorption and emission transition moments are parallel, which is a reasonable approximation for filipin.[17] The subscript r refers to the director axis in macroscopically isotropic samples, which may eventually differ from the one in aligned samples (not the case in this work − see results section). The superscript * refers to the excited-state orientational distribution. We assume $\langle P_2\rangle = \langle P_2^*\rangle$.

All studies were made at pH 7.4 in TRIS 50 mM NaCl 150 mM buffer solutions. Fluorescence anisotropy studies in suspended lipidic vesicles of DPPC or POPC (with or without cholesterol) were carried out at 50 °C or room temperature, respectively.

Computational Details and Theoretical Results. To investigate the vibrational spectrum of the filipin molecule, a full geometry optimization based on Density Functional Theory (DFT)[18] was carried out. In this approach, the total energy of the system is represented by

$$E = V_{NN} + H^{CORE} + V_{ee} + E_{xc}[\rho] \quad (6)$$

where V_{NN} is the nuclear−nuclear interaction, H^{CORE} is a monoelectronic contribution to the total energy, including electron kinetic and electron−nuclear interaction energies, and V_{ee} is the Coulombic interaction between the electrons. $E_{xc}[\rho]$ is the exchange-correlation functional of the electronic density ρ. For this last contribution we have adopted the Becke's 3-parameter hybrid exchange-correlation functional[19] with the Lee−Yang−Parr (LYP),[20] a combination usually represented by Becke3LYP. The DFT calculations have been carried out with the 6-31G(d) basis set.[21] This theoretical level is denoted by Becke3LYP/6-31G(d).

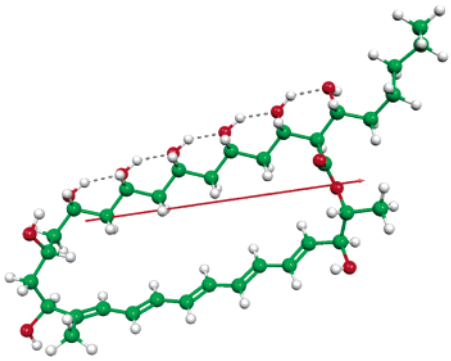

Figure 3. Orientation of the dipole moment (μ = 12.6 D) of filipin.

The starting point for the DFT geometry optimization of filipin was based on the AM1 semiempirical method.[22,23] Harmonic frequency calculations were then carried out and the optimized geometry has been characterized as a local minimum on the potential energy surface, i.e., all frequencies are real. Harmonic DFT frequencies were not scaled although some authors[24,25] indicate the DFT frequencies should be scaled to correct for anharmonicity effects. The calculations (AM1 and DFT) were carried out with the Gaussian-98 program.[26]

The geometry of filipin is characterized by a chain of OH...O hydrogen bonds (Figure 3). OH...O distances are quite similar and equal to 1.803 Å. This value is shorter than the OH...O distance in the water dimer (~1.93 Å) and it is related to nonadditive polarization effects induced by the chain of OH...O hydrogen bonds. Polarization effects and the structure of the OH...O chain are at the origin of the strong dipole moment of filipin (μ = 12.6 D). The orientation of μ is represented in Figure 3.

The three most intense vibrational modes of filipin are associated with internal modes of the OH...O chain. They are the OH stretching (ν_{str}(O−H) = 3549 cm^{-1}), and two OH bending modes (ν_{bend}(O−H)) at 1524 and 582 cm^{-1}, respectively. C=C stretching modes in filipin are characterized by harmonic frequencies in the 1600−1700 cm^{-1} range. However, these modes show a relatively low intensity (less than 5%).

Results

(1) UV−Vis Linear Dichroism. The molar fraction of filipin incorporated in aligned multibilayers of DPPC is >0.99,

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Table 1. Second and Fourth Rank Order Parameters ($\langle P_2 \rangle$ and $\langle P_4 \rangle$, respectively) Values Obtained for Filipin Inserted in Selected Lipid Membranes from Different Optical Spectroscopic Techniques[a]

system	phase description	$\langle P_2 \rangle$	$\langle P_4 \rangle$	techniques involved
DPPC	gel crystalline	0.328	0.169	ATR-FTIR/UV−vis LD
		0.346	0.215	UV−vis LD
DPPC + 33%Chol	liquid ordered	0.732	0.888	ATR-FTIR/UV−vis LD
		0.660	0.713	anisotropy decay/ UV−vis LD
DPPC 50 °C	liquid crystalline	0.427	0.543	anisotropy decay
POPC	liquid crystalline	0.625	0.547	anisotropy decay

[a] When $\langle P_2 \rangle$ and $\langle P_4 \rangle$ were obtained from different techniques, the last column indicates both techniques separated by "/" (the technique involved in $\langle P_2 \rangle$ determination is always mentioned first).

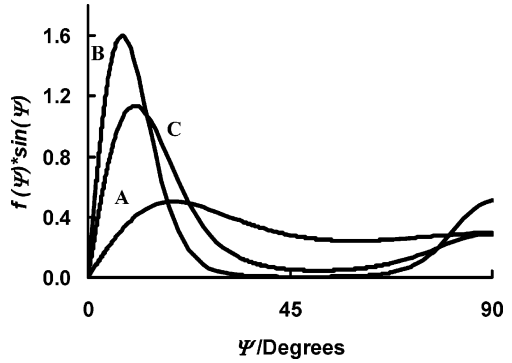

Figure 4. Probability density functions for filipin in DPPC multilayers at room temperature (gel crystalline phase; function obtained from ATR-FTIR) (A), DPPC + 33% Cholesterol (function obtained from anisotropy decay) (B) and POPC (liquid crystalline phase; function obtained from anisotropy decay) (C). DPPC 50 °C distribution is similar to C. A and B are similar to the functions obtained from the alternative methods mentioned in Table 1. Ψ is the angle between the polyene chain axis and the director axis (bilayer's surface normal axis).

estimated from a partition coefficient of $K_p = (3.4 \pm 0.8) \times 10^3$ for filipin in DPPC small unilamellar vesicles[9] and considering equal volumes of aqueous and lipidic phases.[27] The small fraction of filipin molecules in aqueous phase prevents biasing of $\langle P_2 \rangle$ results. Moreover, the small quantum yield of filipin in aqueous phase makes its contribution to emission UV−vis linear dichroism ($\langle P_4 \rangle$) even less important. The second and fourth rank order parameters of filipin in aligned DPPC multibilayers are presented in Table 1. The corresponding orientational probability density function is presented in Figure 4. This function needs no correction because the molecular polyene axis is almost parallel to the electronic transition moment.[17] $\langle P_2 \rangle$ of NBD-cholesterol ($\lambda = 425$ nm) in multibilayers of DPPC is 0.5, regardless of the presence of filipin and cholesterol content.

(2) Fluorescence Anisotropy Decays. The application of UV−vis linear dichroism to DPPC membranes containing cholesterol did not suffice to obtain $\langle P_2 \rangle$ and $\langle P_4 \rangle$ due to the significant turbidity of the samples, which largely bias electronic absorption data. Fluorescence anisotropy decay analysis was used to overcome this problem. Second and fourth rank order parameters can be directly obtained from the fitting of anisotropy decay data in suspended vesicles (LUVs) with the equations presented by Mitchell and Litman[14] with adaptations to account for the significant contribution of filipin molecules presented in the aqueous phase at the early stages of the decay (eq 4). The results are in Table 1. Nevertheless, one should be cautious when interpreting fluorescence anisotropy dichroism data because the reference orientation axis (director axis) is not always perpendicular to the lipidic bilayer plane.[28] In this work, the director axis in suspended vesicles and macroscopically aligned samples are coincident because the orientational parameters obtained in both systems (from fluorescence anisotropy decays and linear dichroism methodologies, respectively) results are in close agreement (Table 1).

Order parameters were also calculated for filipin inserted in POPC membranes (Table 1), which are in liquid crystal state. Incorporation in LUV's in this case is 63% molar ($K_p = (7.7 \pm 2.2) \times 10^2$). However, both POPC and DPPC membranes containing cholesterol led to complex filipin fluorescence anisotropy decays, which could not be adequately fitted by the modified Mitchell and Litman equations (Figure 5). In these cases $\langle P_4 \rangle$ cannot be known but $\langle P_2 \rangle$ can be calculated from the anisotropy limit at $t = \infty$ (eq 5).

(3) ATR-FTIR Linear Dichroism. Theoretical background studies of the filipin vibrational dynamics and simulated IR spectrum (Figure 2) enabled band assignment. The C=C stretching band is located at 1600 cm^{-1} in agreement with ref 29 and its intensity depends on the exciting beam polarization (Figure 6). The corresponding second rank order parameters are presented in Table 1 for several multibilayers lipidic systems. The $\langle P_2 \rangle$ values obtained by ATR-FTIR spectroscopy are in close agreement to the ones obtained with UV−vis Linear dichroism in DPPC multilayers with and without cholesterol.

Discussion

(1) Role of the Lipid Phase. Filipin has the unusual property of having a larger partition coefficient to the gel crystalline phase than to liquid crystal phase (K_p(gel crystalline phase) = $(3.4 \pm 0.8) \times 10^3$ and K_p(liquid crystal phase) = $(7.7 \pm 2.2) \times 10^2$ for filipin in DPPC small unilamellar vesicles),[9] so studying its orientation in the gel crystalline phase is of major importance. Discussion in the literature has been mainly dedicated to the cholesterol presence, but lipidic phase may also play a role. Recently, a similar effect was proposed for the polyene antibiotic Amphotericin B.[4]

Second and fourth rank order parameters ($\langle P_2 \rangle$ and $\langle P_4 \rangle$, respectively) of filipin inserted in the gel crystalline phase bilayers (DPPC) obtained by different techniques (see Table 1) lead to similar density probability functions. These functions are broad and there is no clear preferred orientation. In gel crystalline phase lipids without cholesterol, filipin is in an essentially random organization in the lipidic matrix (Figure 4). At variance, filipin in liquid crystalline bilayers (both DPPC at 50 °C and POPC at room temperature, Table 1) lies preferentially almost perpendicular to the membranes plane (Figure 4). The broad distributions in the gel crystalline phase systems become significantly narrower in the fluid systems.

A small contribution at angles $60° \leq \Psi \leq 90°$ is obtained in POPC and DPPC + Cholesterol (33%). As discussed in previous works[5,30] one cannot rule out the hypothesis that this contribution

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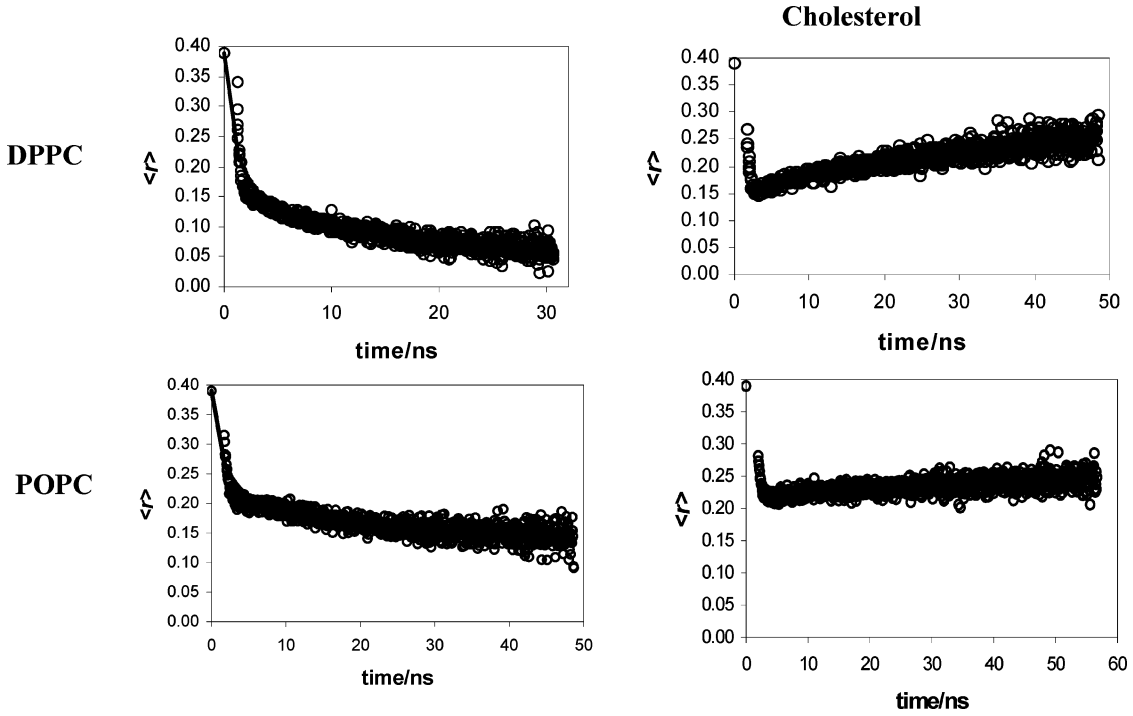

Figure 5. Fluorescence anisotropy decays (open circles) for filipin inserted in LUV's of POPC or DPPC, with and without cholesterol ([filipin] = 0.1 mM; [lipid] or [lipid + cholesterol] = 3 mM). DPPC - 50 °C; POPC - room temperature. Anisotropy decays in POPC and DPPC LUVs without cholesterol were fitted (solid line) as described in the Experimental Section and detailed in Supporting Information document in the internet. Cholesterol-containing samples data showed complex decays and could not be fitted.

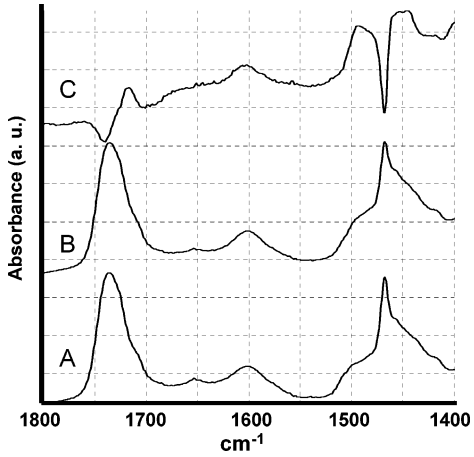

Figure 6. C═C stretching band located at ≈ 1600 cm^{-1} for filipin in DPPC/Cholesterol (33%) multilayers at pH 7.4 in 2mM HEPES buffer and its intensity dependence on the excitation beam polarization. (A − Parallel Polarization; B − Perpendicular Polarization and C − Dichroic ratio; the yy scales were differentially shifted for the sake of clarity).

is spurious. The typical random errors may turn unimodal functions into apparent bimodal ones.[31] However the relative area of the 90° contribution is small and therefore disregarded in our analysis.

(2) Role of Cholesterol. Second and fourth rank order parameters of filipin inserted in DPPC + 33% molar cholesterol (liquid-ordered phase) obtained by different techniques (see Table 1) show no significant differences. Both probability density functions are narrow and bimodal. It is clear from Figure 4 that the sterol contributes not only to a reorientation of filipin (average orientation angle) but also to its order degree (distribution broadness). It can be argued that this is a consequence of the reorientation not only of filipin molecules, but also of the cholesterol molecules. To clarify this point, we performed UV−vis Linear Dichroism experiments of NBD-cholesterol inserted in DPPC multilayer systems with or without filipin and cholesterol. $\langle P_2 \rangle$ is constant (∼0.5) in all cases, meaning that cholesterol it-self is not reoriented by filipin, maintaining its perpendicular orientation relative to the bilayer surface.[7] Our results (Figure 4) are in agreement with the proposed model by Elias et al.[6] in orientational terms. Nevertheless, previous results[15] show that filipin location is not the one predicted by Elias et al. The De Kruijff's model[3] is better in this regard, although it does not consider the correct orientation of filipin. Moreover, subtraction of cholesterol from the palisade (Figure 1B) does not seem reasonable.[32] A new model is proposed, which considers the lipidic phase and cholesterol presence as key modulators of filipin action (Figure 7). Our results are also in agreement with previous published results[33] that show the extent of filipin incorporation in lipidic bilayers is also dependent on the membrane phase and sterol presence.

Pore-like structures are not formed by filipin,[1,3] at variance with other polyene antibiotics such has Nystatin and Amphotericin B, probably due to the lack of charged hydrophilic bulk groups, which would enable anchoring at the membrane interface. It was recently proposed that membrane phase plays a role in the biological action of Amphotericin B.[4] Combined with our findings, this information suggests that membrane fluidity changes are ubiquitous modulators of the polyene antibiotic action.

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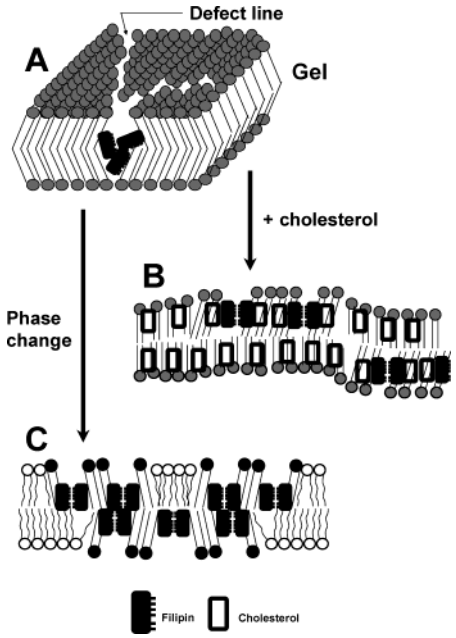

Figure 7. Filipin model of action**.** Gel crystalline phase membranes (A) are characterized by the presence of line defects,[37] which tend to host filipin randomly oriented at the core. When the lipidic phase changes to a liquid crystal (C), filipin is under the influence of the orientational potentials imposed by the acyl chains.[39] The antibiotic lays perpendicular to the membrane surface and remains at the center of the bilayer.[34] Milhaud et al.[8] suggested that filipin would form ordered and rigid domains (darker circles) No extensive alteration of the membrane morphology is observed when cholesterol is not present.[40] However, cholesterol interacts with filipin[15,41] forming extensive protrusions in the membrane surface.[40] Panel B combines this information with orientational data. The simultaneous effects of filipin presence closer to the interface and fluidity heterogeneity[41] generates surface tension in the bilayers and concomitant appearance of protrusions, as seen in microscopy experiments (ref 40 and references therein). In agreement with our reasoning, membrane protrusions are most likely to occur in the interface between membrane regions having different fluidities.[42]

Conclusions

The orientation of filipin in gel crystalline phase membranes is determined by the presence/absence of cholesterol;

Cholesterol maintains its orientation regardless of filipin presence in the system;

Cholesterol modulates the antibiotic orientation, narrowing its distribution and reducing the mean angle;

The two models proposed so far[3,6] for the action of filipin at the molecular level are not satisfactory. While De Kruijff's model proposes the correct filipin location (bilayer core)[15] but incorrect orientation, Elias' model proposes the correct orientation but incorrect location. Neither model accounts for filipin interaction with sterol free membranes, nor consider that the peculiar feature of filipin preference for more rigid membranes may play a role. Milhaud[34] proposed a topologically structured action of filipin in sterol-free membranes were filipin-rich (rigid) and filipin-depleted (fluid) areas would coexist. The borders of these areas are putatively responsible for permeation. However, no information on the molecular organization of filipin was presented. Again, no preference of filipin for rigid membrane areas[33] was accounted for. Recently, Waheed[35] and Simons[36] speculated that filipin could have its action attributed to lipid rafts destruction in biological membranes. Those authors overlooked filipin preference for rigid membranes but this may be a key experimental result supporting their ideas. Although a filipin orientation perpendicular to the membrane surface is compatible with a pore-forming model similar to the one adopted for other polyene antibiotics such as Amphotericin B and Nystatin,[5] permeability, and other data are not.[3] It is reasonable to expect that filipin action interferes with lipidic rafts due to the mixed effect of cholesterol presence and increased bilayer rigidity.[33] Nevertheless, this interference probability does not account for all the biological action of filipin because permeability changes are detected in artificial membranes where rafts do not exist. Moreover, this work shows that filipin forms highly organized structures in single-lipid bilayers, which suggest a specific function.

Figure 7 depicts our filipin model of action proposal. Gel crystalline phase membranes (Figure 7A) are characterized by the presence of line defects,[37] which tend to host foreign molecules, segregated by the ordered and rigid lipidic acyl chains palissades. Filipin is no exception; although a high partition coefficient into gel crystalline phase membranes was measured,[15] DSC techniques show that filipin is not miscible with gel crystalline phase bilayers.[34] Without the influence of the orientational forces imposed by the lipidic acyl chains, filipin is randomly oriented. Neverthless, the bilayer core center is the preferred location.[15,38] When the lipidic phase changes to a liquid crystal (Figure 7C), line defects disappear and the membrane is fluid. Filipin is not segregated and is thus under the influence of the orientational potentials imposed by the acyl chains.[39] Filipin is now oriented perpendicular to the membrane surface (Figure 4) at the center of the bilayer.[34] Milhaud et al.[8] suggested that filipin would form ordered and rigid domains (lipids with darker circles in panel C of Figure 7) in which borders leakage could occur. Our results, showing a highly ordered filipin orientational distribution, nearly perpendicular to the membrane surface, support this hypothesis. No extensive alteration of the membrane morphology is observed when cholesterol is not present.[40]

Cholesterol interacts with filipin[15,41] forming extensive protrusions in the membrane surface.[40] Panel B of Figure 7 combines this information with orientational data (Figure 4). Filipin is shifted by cholesterol toward the bilayer surface[15,32] and pure lipid phase transitions are partially recovered.[41] Filipin-rich areas of the membrane are therefore more rigid than the rest, which is liquid-ordered. The simultaneous effects of filipin presence closer to the interface and fluidity heterogeneity generates surface tension in the bilayers and concomitant appearance of protrusions, as seen in microscopy experiments (ref 40 and references therein). In agreement with our reasoning, membrane protrusions are most likely to occur in the interface between membrane regions having different fluidities.[42]

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Supporting Information Available: Further information on sample preparation and Linear Dichroism theory pertinent for data analysis. Artifact correction is addressed. An outline of fluorescence anisotropy decay data analysis to calculate the rank order parameters is also presented. This material is available free of charge via the Internet at http://pubs.acs.org.

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