

A photophysical study of the polyene antibiotic filipin Self-aggregation and filipin–ergosterol interaction

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Abstract

Filipin, a macrolide polyene antibiotic, is known to interact selectively with ergosterol, a constituent of fungi membranes. In this work, the fluorescence resonance energy transfer (FRET) between a fluorescent analog of ergosterol, dehydroergosterol (DHE), and filipin was measured in small unilamellar vesicles of dipalmitoylphosphatidylcholine at 25°C. The time-resolved FRET results were rationalized in the framework of the mean concentration model, and were complemented with steady-state fluorescence intensity, anisotropy and absorption measurements. The results point to the formation of both DHE–filipin aggregates (evidence from static quenching of DHE fluorescence by filipin) and filipin–filipin aggregates (evidence from: (i) the FRET acceptor concentration distributions; (ii) spectral changes of filipin absorption in the vesicles, the excitonic interaction suggesting a stack arrangement; (iii) filipin fluorescence self-quenching), even in presence of DHE and low antibiotic mole fractions (<1 mol%). These results point out that apparently contradictory biochemical models for the action of filipin (some based on the presence of sterols, others not) can be equally valid. Moreover, since results (ii) and (iii) are also observed when a sterol is present, both models of action can actually coexist in membranes with a low sterol content. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Energy transfer; Ergosterol; Filipin; Fluorescence; Membrane; Polyene antibiotic

1. Introduction

Filipin is a macrolide antifungal agent, characterized by very low antibacterial activity. Its structure

includes a pentaene chromophore (Fig. 1) and it is intrinsically fluorescent. The biochemical action site of filipin is the cell membrane, causing leakage of cellular components [1]. The presence of sterol affects filipin partition into lipidic bilayers [2]. Recently [3], it was proposed that the effect of filipin on phospholipid bilayer membranes is mainly regulated by the concentrations of filipin in the aqueous solution and by the sterol concentration in the bilayer. More precisely, it was proposed that the filipin–phospholipid interaction is regulated by the filipin aggregation

Abbreviations: DHE, dehydroergosterol ($\Delta^{5,7,9(11),22}$ -ergostatrien-3 β -ol); DPPC, dipalmitoylphosphatidylcholine; FRET, fluorescence resonance energy transfer; SUV, small unilamellar vesicles

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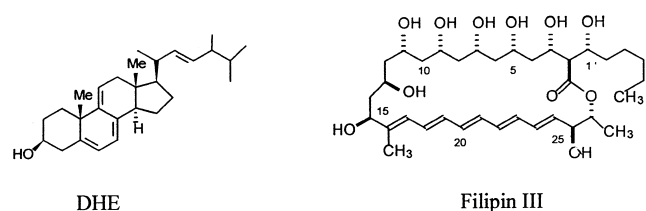


Fig. 1. Structures of DHE and filipin III.

state in the aqueous medium and the sterol domain formation in the membranes. The filipin organization inside the membranes is an open question. In this work, dehydroergosterol (DHE, see Fig. 1), a fluorescent analog of ergosterol, was used as a donor in fluorescence resonance energy transfer (FRET) experiments involving filipin. We apply the method of recovery of acceptor concentration distribution using Gaussian-type functions [4,5] to the analysis of time-resolved FRET data. As shown in these works, this method is particularly sensitive to aggregation. The information obtained is complemented by steady-state FRET and filipin absorption and fluorescence self-quenching studies.

2. Theory

The basic equation for FRET to an ensemble of acceptors [6] is given below:

$$i_{\text{DA}}(t) = A \cdot \exp(-t/\tau - C(t/\tau)^{d/6}) \quad (1)$$

where $i_{\text{DA}}(t)$ is the time-resolved donor fluorescence intensity in the presence of acceptor, A is a constant, τ is the donor fluorescence lifetime in the absence of acceptor, and d is the dimensionality of the system. For integer dimensionality, C is given by Eq. 2 [7],

$$C = \Gamma(1-d/6) \cdot n_{\text{dA}} \cdot V_d \cdot R_0^d \quad (2)$$

Γ being the complete gamma function, n_{dA} the number of acceptors per d -space volume unit (e.g., $n_{2\text{A}}$ is the number of acceptors per area unit), V_d is the d -dimensional unit sphere ($V_1 = 2$, $V_2 = \pi$, $V_3 = 4\pi/3$), and R_0 is the Förster radius. The surface of phospholipid vesicles, even small unilamellar vesicles (SUV) obtained by sonication, can be treated as planar ($d = 2$) from the standpoint of FRET, because the curvature effect is negligible when the sphere diameter D and the Förster radius R_0 obey the relationship $D/R_0 > 1.5$ [8]. Being $D \cong 25$ nm for SUV of dipalmitoylphosphatidylcholine (DPPC) [9], this is clearly

the case (see Section 4 for the R_0 calculation). However, FRET in lipid membranes usually requires two alterations in the equation given above. First, the donor decay, even in the absence of acceptor, is seldom monoexponential. In fact, for DHE, we recovered two lifetime components (see Section 4). The alterations in the FRET decay law that this fact introduces have been described [10]. Second, the donor and acceptor molecules may be located at different depths in the bilayer. We assume that the location of DHE in the membrane is the same as that of cholesterol, i.e., the hydroxyl group is in the immediate vicinity of the phospholipid ester carbonyl [11,12]. From fluorescence quenching studies, filipin is thought to be located near the center of the bilayer [3]. This gives an approximate distance between the DHE and filipin planes in DPPC gel phase bilayers $w = 12.4$ Å (for this calculation, we considered the depth of the chromophore of DHE as being the center of the conjugated system). The decay law for such a system is also described elsewhere [13]. In these conditions, it becomes

$$i_{\text{DA}}(t) = \rho_1(t) + \rho_2(t) \quad (3)$$

where

$$\rho_1(t) = \exp(-t/\tau_1) \cdot \exp\left\{-\frac{2c}{\Gamma(2/3) \cdot b} \int_0^1 [1 - \exp(-tb^3 \alpha^6)] \alpha^{-3} d\alpha\right\} \quad (4)$$

$$\rho_2(t) = q \cdot \exp(-t/\tau_2) \cdot \exp\left\{-\frac{2c}{\Gamma(2/3) \cdot b} \int_0^1 [1 - \exp(-tb^3 \alpha^6)] \alpha^{-3} d\alpha\right\} \quad (5)$$

In these equations, τ_1 and τ_2 are the two lifetime components, $q = A_2/A_1$ is the ratio of pre-exponential factors A_i associated to each component, $c = C/\tau^{1/3}$ for each component, and $b = (R_0/w)^2 \tau^{-1/3}$.

The formalism described above is valid for random distribution of donors and acceptors. However, it is common that in membrane studies (especially in the gel phase) at least one of these species is distributed non-randomly [5,10]. One way to treat this deviation

from ideality is to consider a continuous distribution of acceptor concentrations [4,14]. In a microheterogeneous system, it may be no longer true that all donor molecules should have a uniform concentration c of acceptors in their vicinity. For example, if acceptor aggregation takes place, some donors, located closer to a cluster of acceptors, may be surrounded by a large concentration of acceptors in their surroundings ($c > \text{average concentration}$). Conversely, other donor molecules may be isolated ($c = 0$) in the sense that they have no acceptors in the FRET distance range ($\sim 2R_0$). In fact, it is possible that there is non-zero probability of a donor molecule to sense an arbitrary concentration c of acceptors, which results in a continuous distribution function, and leads to the following decay law:

$$i_{\text{DA}}(t) = \int_{c_{\min}}^{c_{\max}} f(c) \cdot (\rho_1(t, c) + \rho_2(t, c)) \cdot dc \quad (6)$$

where $f(c)$ is the acceptor concentration distribution function, and ρ_1 and ρ_2 , given in Eqs. 4 and 5, respectively, are now two-variable (depending also on acceptor local concentration) functions. c_{\min} and c_{\max} represent the lower and upper limit values for acceptor concentration, respectively. Of course, it should be $c_{\min} = 0$ and $c_{\max} = +\infty$. However, for numerical integration purposes, c_{\max} has to be kept at a finite (but sufficiently large so that $F(c_{\max})$ is negligible) value. $f(c)$, which, for the uniform distribution model of Eqs. 3–5, is a discrete function, becomes a continuous function when non-randomness of probe distribution occurs. In a recent work [4] we tested the ability of Gaussian (Eq. 7, below, with $r=0$) and sum-of-two-Gaussians (Eq. 7, below, with $r \neq 0$) functions to successfully recover simulated distributions.

$f(c) =$

$$A(\exp(-(c-c_1)^2/(2\sigma_1^2)) + r\exp(-(c-c_2)^2/(2\sigma_2^2))) \quad (7)$$

In the cited work, the two-Gaussian function was shown to be particularly versatile, being able to differentiate wide from narrow and unimodal from bimodal distributions. We were able to extract relevant information regarding the distribution of common spectroscopic bilayer probes in DPPC large unilamellar vesicles. In this work, we apply the same contin-

uous distribution of acceptor concentration formalism to the DHE-filipin pair. The system (DPPC SUV) is chosen so that DHE is known to be randomly dispersed (unlike for DPPC large unilamellar vesicles, where dimer formation is reported [15]) and the low donor concentration (≤ 1 mol%) prevents biasing of the DHE-filipin FRET results due to energy migration among the DHE molecules (which could become significant for higher concentrations [15]). Hence, we know that eventual recovery of wide or bimodal distributions (which could, in principle, be due to either donor aggregation, acceptor aggregation, or both) should point to filipin aggregation. In that case, the actual pattern of the distribution (i.e., the number of peaks, their location, their relative weight) and its dependence on the acceptor concentration can give insight on the nature of the aggregation process.

We would like to stress that for spectroscopic measurements based on filipin absorption and emission properties, the results analysis is rendered difficult, because of the partition of filipin between water and the lipid bilayer, and due to fact that filipin emits fluorescence in both aqueous and bilayer phases with similar quantum yields. FRET between DHE and filipin has the advantage of being sensitive only to filipin concentration in the bilayer (as FRET to filipin molecules in the water phase has essentially zero efficiency), thus allowing the direct study of filipin aggregation without need to correct the data for the fraction in water.

3. Materials and methods

DHE and filipin were purchased from Sigma Chemical Co. (St. Louis, MO) and DPPC was obtained from Avanti Polar Lipids (Birmingham, AL). These materials were used as received. Filipin is a mixture of four macrolides [1] with minor differences in their structures, having a pentaene chromophore as a common feature. The fraction known as filipin III (the one depicted in Fig. 1) is the major component. Tris-HCl from BDH (London, UK) was used to prepare the buffer (Tris-HCl 50 mM (pH 7.4), NaCl 100 mM, EDTA 0.2 mM). NaCl (p.a.) and all organic solvents were from Merck (Darmstadt, Germany). Deionized water was used throughout.

DPPC/DHE SUV were prepared as described elsewhere [15]. The final DPPC concentration was approximately 1 mM. Filipin was added to the lipid dispersions from a stock solution in Tris–HCl buffer, the samples being then left in the dark for 2 h [16]. The concentrations of the chloroform solutions of DPPC and DHE were determined by phosphorus analysis [17] and absorption ($\epsilon(\lambda_{\max} = 327.8 \text{ nm}) = 11\,200 \text{ M}^{-1} \text{ cm}^{-1}$ [18]), respectively. The concentration of the filipin stock solution was analyzed by absorption ($\epsilon(\lambda_{\max} = 337 \text{ nm}) = 47\,000 \text{ M}^{-1} \text{ cm}^{-1}$ [2]). The DHE to lipid ratio was kept below 1:100, so that energy migration among DHE molecules is negligible [15]. Several samples with different filipin content were prepared, the filipin to lipid ratio being kept between 0 and 0.03 in the FRET experiment and between 0 and 0.06 in the filipin absorption and fluorescence intensity measurements.

Fluorescence decay measurements were carried out with a time-correlated single-photon counting system. The instrument has been described [10]. Emission (centered at 370 nm, bandwidth 30 nm) was detected at the magic angle relative to the vertically polarized excitation beam ($\lambda_{\text{exc}} = 300 \text{ nm}$). The number of counts on the peak channel was 7000, the number of channels per curve used for analysis was 400, and the timescale was 4.6 ps/channel. Data anal-

ysis was carried out using non-linear, least squares iterative convolution software based on the Marquardt algorithm [19]. The fluorescence decays of the different samples were all analyzed simultaneously using global analysis [5,10]. The goodness of the fit was judged from the global (χ^2_{G}) and individual (χ^2) chi-square values, and weighted residuals and autocorrelation plots.

Fluorescence steady-state measurements were carried out with a SPEX F112 A Fluorolog spectrofluorometer, with a double emission monochromator, in a right-angle geometry. Excitation light ($\lambda = 300 \text{ nm}$ for DHE excitation in the FRET experiment, $\lambda = 338 \text{ nm}$ for the filipin self-quenching experiment) was vertically polarized and fluorescence was detected in a magic angle arrangement. Correction of spectra was performed using a Rhodamine B quantum counter solution and a standard lamp, respectively [20]. Quartz cuvettes ($5 \times 5 \text{ mm}$) were used, and in these experimental conditions, no correction for artifacts was needed [21]. All measurements were carried out at room temperature (the vesicles being thus in the gel phase, in order to maximize the partition of filipin to the vesicles [2]). To minimize photobleaching of DHE, narrow slits in the excitation monochromator were used. In these conditions, fluorescence was largely independent of time, even when

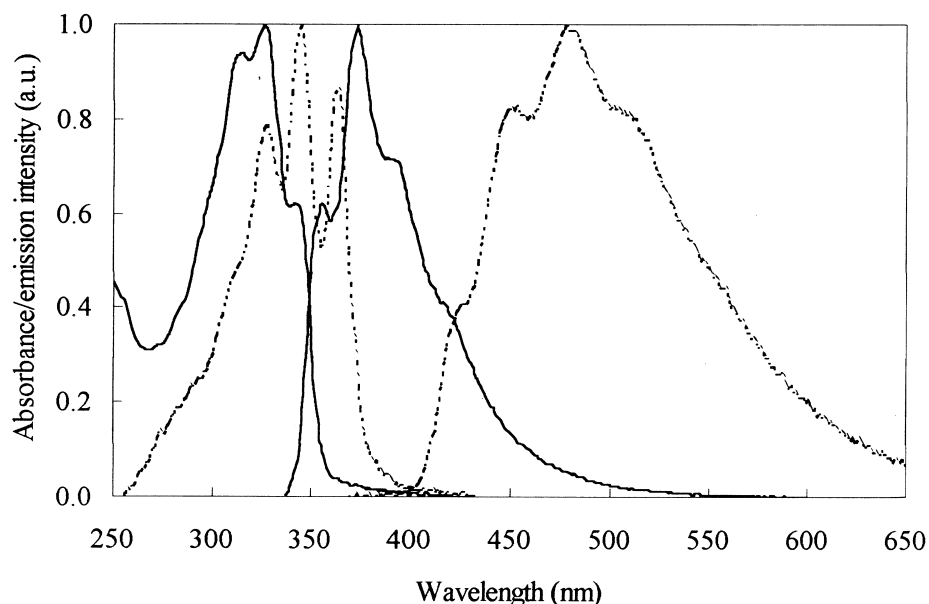


Fig. 2. Normalized absorption and corrected emission spectra of DHE (—) and filipin (---) in DPPC SUV. For the emission spectra, $\lambda_{\text{exc}} = 310 \text{ nm}$ (DHE) and $\lambda_{\text{exc}} = 344 \text{ nm}$ (filipin).

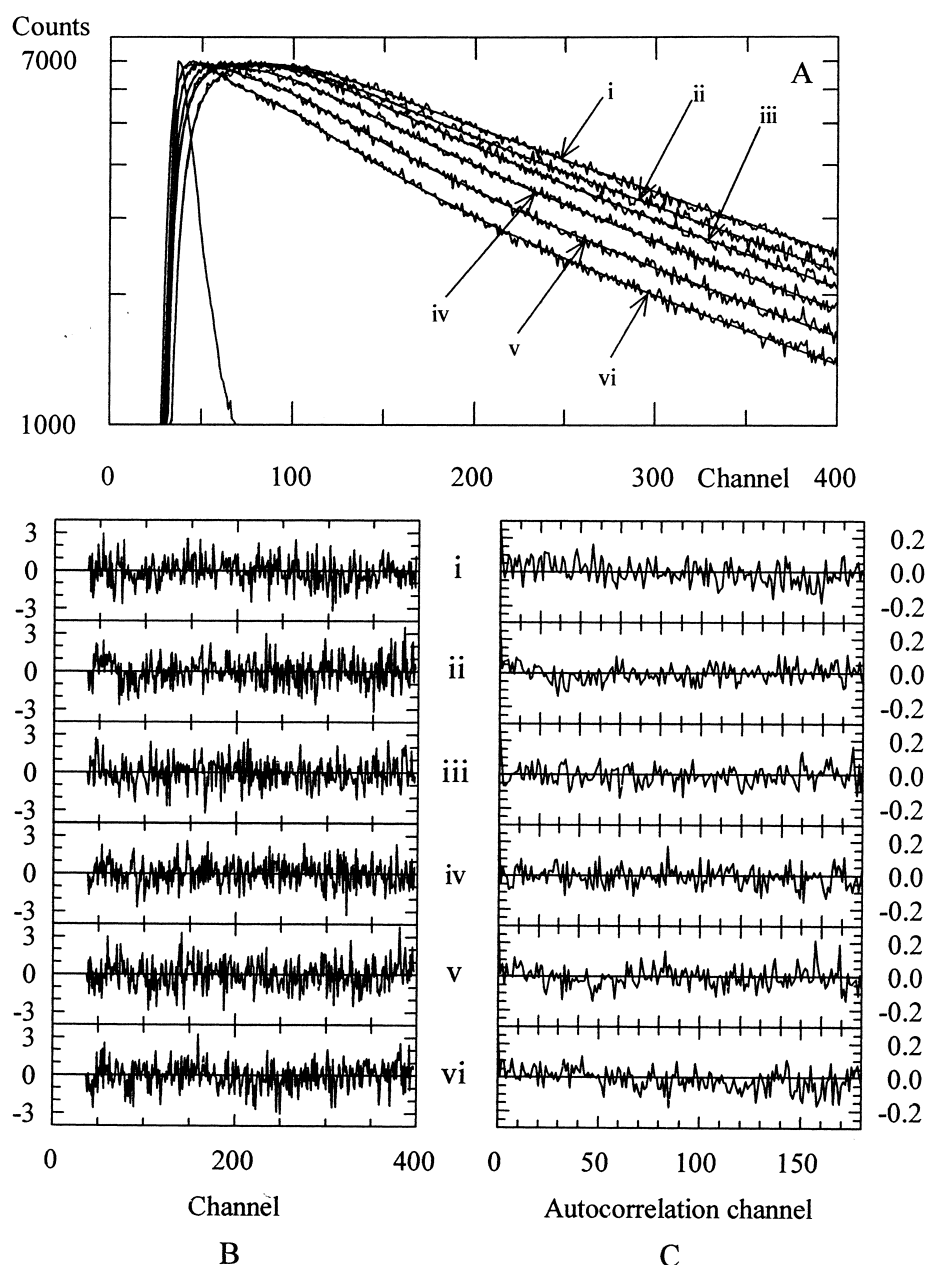


Fig. 3. (A) DHE (0.6 mol%) fluorescence decays in the presence of varying amounts of filipin (filipin:DPPC ratios: i, 0.000; ii, 0.003; iii, 0.007; iv, 0.013; v, 0.022; vi, 0.032) in DPPC SUV at 25°C. The smooth lines are fits to Eqs. 6 and 7. The instrumental response function is also shown. One channel = 4.6 ps. (B) Residual distributions. (C) Residual autocorrelation functions.

excitation was made at the absorption maximum. The bandwidths were 4.5 nm for both excitation and emission monochromators in the FRET experiment and 1.8 nm for excitation and 2.25 nm for emission in the filipin fluorescence intensity measurements. Blank subtraction was carried out. Absorption spectra were recorded on a Jasco V-560 spectrophotometer and corrected for light scattering

artifacts (not too important in small vesicles) according to Castanho et al. [22].

Critical radii of energy transfer were calculated using a rewritten Förster's formula [23]:

$$R_0 = 0.2108 \cdot \left[\kappa^2 \cdot \Phi_F \cdot n^{-4} \cdot \int_0^\infty I(\lambda) \cdot \epsilon(\lambda) \cdot \lambda^4 \cdot d\lambda \right]^{1/6} \quad (8)$$

where R_0 is expressed in Å units, κ^2 is the orientation factor, Φ_F is the quantum yield of the donor probe, n is the refractive index of the medium, $I(\lambda)$ is the normalized emission spectrum of donor and $\varepsilon(\lambda)$ is the molar absorption coefficient of acceptor in $\text{M}^{-1}\text{cm}^{-1}$ units. Wavelength is expressed in nm units. For the orientation factor, the value $\kappa^2 = 2/3$ (corresponding to a dynamic isotropic regime of transfer) was assumed, and $n = 1.44$ was considered [13].

4. Results and discussion

4.1. Time-resolved FRET

Fig. 2 shows the normalized absorption and emission spectra of DHE and filipin in SUV of DPPC. For the calculation of the Förster radius, the DHE fluorescence quantum yield value in DPPC vesicles of $\Phi_F = 0.13$ at 25°C [15] was used, and the filipin absorption coefficient in DPPC SUV was estimated as $\varepsilon(344\text{ nm}) = 49 \times 10^3\text{ M}^{-1}\text{cm}^{-1}$. On substitution in Eq. 1, one obtains $R_0 = 26.4\text{ Å}$.

In Fig. 3 is depicted the time-resolved fluorescence of DHE (0.6 mol%) in presence of variable filipin concentration, the parameters being shown in Table 1. Even in the absence of acceptor, the decay is not monoexponential. There are two recovered lifetime components, $\tau_1 = 1.43\text{ ns}$ (77%) and $\tau_2 = 0.29\text{ ns}$ (23%). This decay complexity is common for fluorescent probes in interaction with microheterogeneous

systems [10], and specifically for DHE, biexponential decays in gel phase vesicles have been reported by Smutzer et al. [18]. Our value for τ_1 coincides with the main component reported by this author for DHE in DMPC multilamellar vesicles. Although the origin of the faster component is not clear, this does not hamper the FRET study, as shown in Section 2 [10]. From the R_0 , d and τ values, $b = 4.07\text{ ns}^{-1/3}$ was calculated for use in Eqs. 4 and 5.

In Fig. 4 are shown the recovered acceptor concentration distributions using three different analysis formalisms: discrete, single Gaussian, and sum-of-two-Gaussians functions. The discrete distribution model should return c values proportional to the amount of filipin in the sample (Eq. 2). However, this is only verified for the two most dilute samples. For higher filipin concentrations, the variation of c versus filipin concentration has negative curvature (not shown). Additionally, the decay statistics (Table 1) worsen. This means that filipin is aggregating and the model described by Eqs. 1 and 2 is no longer valid. The next step is to use a Gaussian acceptor distribution analysis. Although better χ^2 values are generally obtained, for concentrations as low as Filipin:DPPC = 0.013 anomalous distributions, which are in fact tails of broad Gaussians centered at negative values, are recovered. This kind of recovered distribution usually hides a bimodal function [4]. Moreover, the statistics for the most concentrated samples are still unsatisfactory.

This led us to analyze the data with the sum-of-two-Gaussians formalism. For the most dilute sam-

Table 1
Comparison of the recovered linking parameters and χ^2 values recovered using the three described analysis methods

Analysis method	Discrete concentration	Single Gaussian	Sum of two Gaussians
Linking parameters	$\tau_1 = 0.21\text{ ns}$ $\tau_2 = 1.45\text{ ns}$ $q = 2.70$	$\tau_1 = 0.21\text{ ns}$ $\tau_2 = 1.44\text{ ns}$ $q = 2.87$	$\tau_1 = 0.21\text{ ns}$ $\tau_2 = 1.44\text{ ns}$ $q = 3.18$
Individual χ^2 values			
Filipin:DHE = 0.000	1.62	1.45	1.05
Filipin:DHE = 0.003	1.43	1.32	1.19
Filipin:DHE = 0.007	1.35	1.24	1.08
Filipin:DHE = 0.013	1.00	0.99	1.00
Filipin:DHE = 0.022	1.95	1.72	1.22
Filipin:DHE = 0.032	2.66	2.04	1.07
χ_G^2	1.65	1.44	1.09

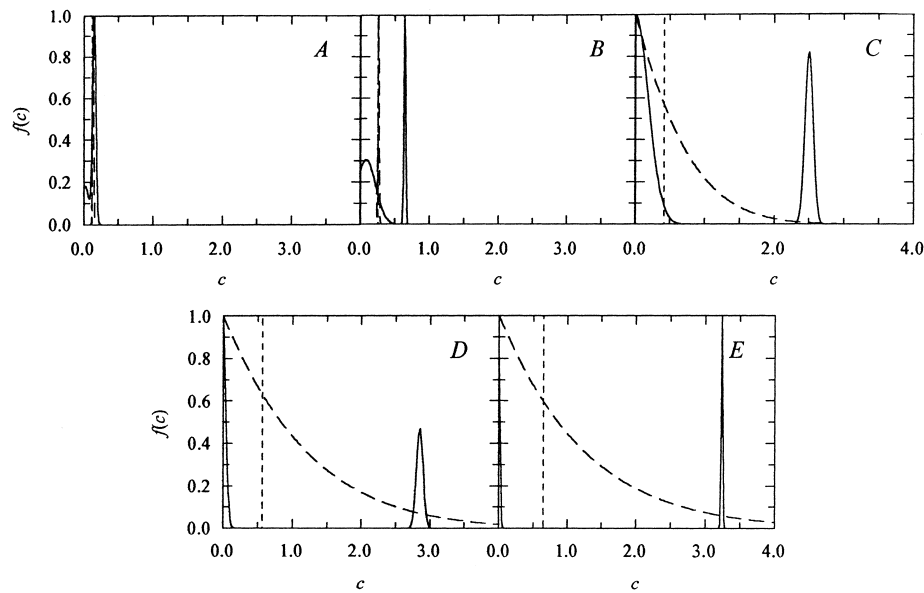


Fig. 4. Acceptor concentration distribution functions $f(c)$ recovered for the discrete (---, vertical lines), Gaussian (---) and sum-of-two-Gaussians (—) models. Filipin to DPPC ratios: A, 0.003; B, 0.007; C, 0.013; D, 0.022; E, 0.032.

ple, the results still resemble those obtained with the other analysis procedures, though a small fraction of donors already appears to be isolated, which indicates that some degree of aggregation is occurring. Major differences become apparent for filipin:DPPC=0.007. The fraction of isolated donors (first peak) increases, and the abscissa of the second peak increases non-linearly. This substantial increase in c_2 means that some donors sense more acceptors in their surroundings than expected and can be explained assuming aggregation of filipin: the c_2 peak is caused by donors which lie close to aggregates, while the c_1 peak is caused by donors which are left in an acceptor-rarefied environment due to this aggregation. For the remaining samples, the c_2 peak continues to shift to larger abscissas, possibly indicating increase of the aggregation number of filipin in the clusters (a donor close to an aggregate now 'sees' a higher filipin concentration than for more dilute samples).

Quantification of the clustering in terms of an aggregation number and/or association constant is not possible, because of the numerical limitations of the distribution recovery method. The problem is ill-conditioned, and parameter correlation may occur [4,14]. This means that peak widths and heights may be biased. In any case, the location of the peaks and the overall shape of the distributions are not ex-

pected to be considerably affected [4], and these undoubtedly point to aggregation of donor and/or acceptor. DHE–DHE aggregation can be ruled out, as we chose the system and DHE concentration in order to avoid this effect (see Section 2). Additionally, acceptable fits with similar c values are obtained with all formalisms for the DHE:filipin=0.003 sample, and the recovered two-Gaussian distribution is almost unimodal, apart from a minor fraction of isolated donors. The large deviations from unimodality occur upon increasing filipin concentration while keeping the DHE concentration at a constant value. This shows that the observed effect in the two-Gaussian distributions is due to acceptor (rather than donor) aggregation. For another FRET pair which showed donor aggregation, NBD-PE/Rh-PE in gel phase DPPC large unilamellar vesicles [4], a clearly bimodal distribution was recovered even for very low concentrations of acceptor, which is not the case in this work.

Note that even for the most dilute sample, the recovered concentrations are lower than the amount of filipin added to the sample. This is due to the partition of filipin between the aqueous medium and the vesicles. For that sample, and using Eq. 2, we should have $c=0.155$, assuming complete incorporation of filipin in the vesicles. The value $c=0.123$ was recovered from the discrete concentration fit,

which indicates that the fraction of incorporated filipin is ca. $0.123/0.155 = 79\%$. This (together with the concentration of DPPC and its molar volume) allows us to estimate $K_p = 7.1 \times 10^3$ for the partition constant. This value is of the same order of magnitude of that calculated by Castanho and Prieto [2] from anisotropy measurements, $(3.4 \pm 0.8) \times 10^3$. The discrepancy is probably due to (i) our value coming from only one sample measurement, instead of a fit to various measurements, which would improve its accuracy, and (ii) the c value which would produce $K_p = 3.4 \times 10^3$ is 0.110, only 10% less than our value. This difference could be due to random errors in the lipid or filipin concentration, or the c parameter recovery process.

One unresolved question is whether the filipin aggregate formation involves DHE molecules. Sterols are known to interact with polyene antibiotics such as filipin in model membranes. In fact, early works suggested that sterol presence is an essential factor for the interaction of filipin with membranes [24]. Although it is now established that filipin partitions into bilayers even in the absence of sterol [2,25], the filipin–sterol interaction should nevertheless be strong. The time-resolved FRET experiment is not able to differentiate between aggregation involving sterol and aggregation independent of sterol: a sterol molecule involved in a mixed DHE–filipin aggregate would be quenched almost instantly upon excitation, and its fluorescence would not be detected; on the other hand, the degree of quenching caused by DHE–filipin aggregates and pure filipin aggregates on a non-aggregated DHE molecule would be undistinguishable (provided that the filipin distribution function is the same in the two cases). Therefore, we need additional experiments to help answer this question.

4.2. Steady-state FRET

We carried out DHE steady-state fluorescence intensity measurements in the presence of filipin to determine whether if static quenching of DHE fluorescence occurs, thus revealing the formation of sterol–filipin complexes.

Fig. 5 compares the relative steady-state fluorescence intensity of DHE in the presence of filipin with the values obtained by integration of the time-

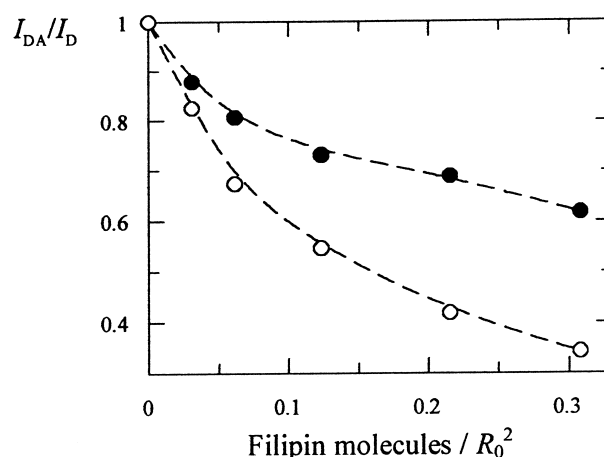


Fig. 5. Comparison of the fraction of unquenched fluorescence I_{DA}/I_D obtained from integration of the decays (●) and steady-state measurements (○). The lines are mere guides to the eye.

resolved curves. If the two quenching curves were identical, the existence of static quenching could not be concluded. However, if the steady-state quenching efficiency were greater than that resulting from integration of the time-resolved curves, this would probably mean that ground-state sterol–filipin complexes would be formed. As we mentioned above, these would not be detected in the time-resolved experiment, but the resulting fluorescence intensity decrease would be observed in the steady-state experiment. This is in fact observed, showing that at least part of the filipin aggregates involve DHE molecules.

4.3. Absorption spectra of filipin

UV absorption spectroscopy of polyene antibiotics has been useful in the past to probe aggregation of these molecules in presence of cholesterol [16]. The presence of excitonic bands, although not necessary to aggregation (there may be aggregation without the fulfillment of the strict geometric requirements for the excitonic interaction), is evidence for aggregation of the studied chromophore. For this experiment, we had to use ergosterol (ergosterol:DPPC ratio = 1:100) rather than DHE because of the overlap of DHE and filipin absorption. DHE and ergosterol are analog molecules (they have the same backbone, but ergosterol lacks the 9(11) double bond and so absorbs at lower wavelengths), which have been shown to behave identically in many different studies

(for a review, see [26]). We therefore assume that filipin absorption spectra are identical in the presence of either DHE or ergosterol.

As Fig. 6A shows, there are alterations in the filipin absorption spectrum upon increasing filipin concentration, namely a relative enhancement of the $\lambda \approx 325$ nm peak and a relative decrease in the $\lambda \approx 360$ nm peak intensity. This is probably due to the same type of filipin–filipin aggregate that is detected in the time-resolved FRET measurements. An enhancement in the high-energy region of the absorption spectrum points to formation of exciton aggregates in a stack geometry [27].

We investigated if the sterol presence was essential to the spectral changes. At variance with a previous report [16], in which correction of absorption spectra for light scattering was not fully developed, we found that even in the absence of sterol, identical alterations in the absorption spectra are observed (results not shown). Therefore it seems that filipin self-aggregates in DPPC membranes at low concentrations, without sterol presence being required.

Because of the incomplete incorporation of filipin into the vesicles, it could be argued that the alterations in filipin absorption were due to the aqueous fraction rather than the membrane-incorporated fraction. In order to clarify this, we measured the absorption of filipin in buffer, for the same concentration range as in the vesicle preparations. As Fig. 6B shows, apart from an anomalous spectrum observed for $[\text{Filipin}] = 2.1 \times 10^{-6}$ M (characteristic of

the pre-micellar filipin aggregates [16]), the variations in the shape of the absorption spectrum are much less pronounced than those in the buffer/vesicles system. The alterations observed (enhancement of both the small and large wavelength ranges of the spectrum relative to the maximum at $\lambda = 338$ nm) are probably due to a combination of the following possible effects. (i) Deviations from linearity between absorption at $\lambda = 338$ nm and $[\text{Filipin}]$, which become significant for $[\text{Filipin}] > 10^{-5}$ M (see Fig. 6C), may result from non-validity of the Lambert–Beer law at very high optical densities. Of course, at both the small and large wavelength ranges, where absorption is less intense, this loss of linearity would be less significant, thus resulting in a relative enhancement. (ii) Due to formation of filipin aggregates in aqueous buffer for $[\text{Filipin}] > 10^{-6}$ M [16], scattering of light probably biases the spectra, introducing a turbidity tail outside the absorption region ($\lambda > 400$ nm) and increasing the measured intensity at low wavelengths. (iii) Additionally, there may exist an actual excitonic interaction for filipin in buffer at high concentrations ($> 10^{-5}$ M). The conclusion of this study is that, in such an event, the alterations in absorption due to this eventual excitonic interaction would not justify the considerably more significant alterations upon increasing concentration in the buffer/vesicles system. Moreover, due to the partition between the aqueous and vesicle media, using the mentioned $K_p = (3.4 \pm 0.8) \times 10^3$ value [2], we expect the aqueous filipin concentration for the most concentrated sample

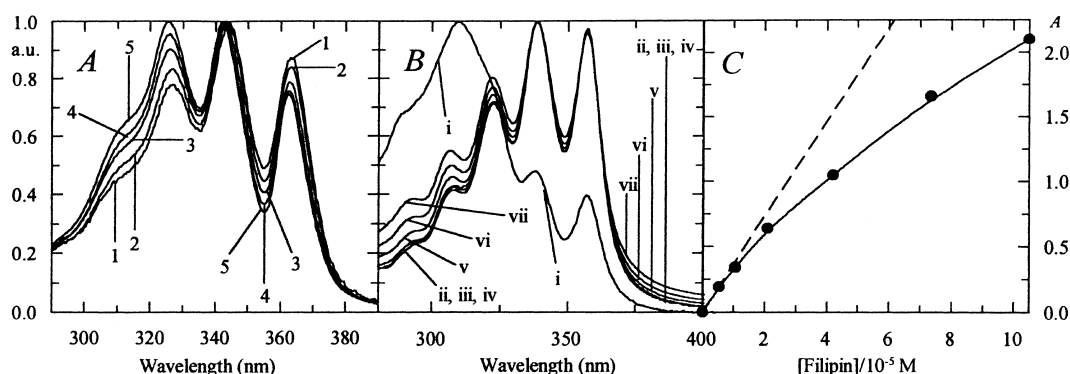


Fig. 6. (A) Normalized absorption of filipin in DPPC SUV, in the presence of 1 mol% ergosterol (in these plots, absorption due to filipin in the aqueous water is already subtracted, using the spectra in panel B and $K_p = 3.4 \times 10^3$). Filipin:DPPC molar ratios are: 1, 0.006; 2, 0.012; 3, 0.025; 4, 0.044; 5, 0.062. (B) Normalized absorption of filipin in buffer. $[\text{Filipin}]/10^{-5}$ M: i, 0.21; ii, 0.53; iii, 1.1; iv, 2.1; v, 4.2; vi, 7.4; vii, 10.5. (C) Absorbance ($\lambda = 338$ nm) of the samples referred to in B (except for the most dilute). The broken line is a mere guide to the eye, and the solid straight line's slope is equal to that of the absorbance curve for $[\text{Filipin}] = 0$.

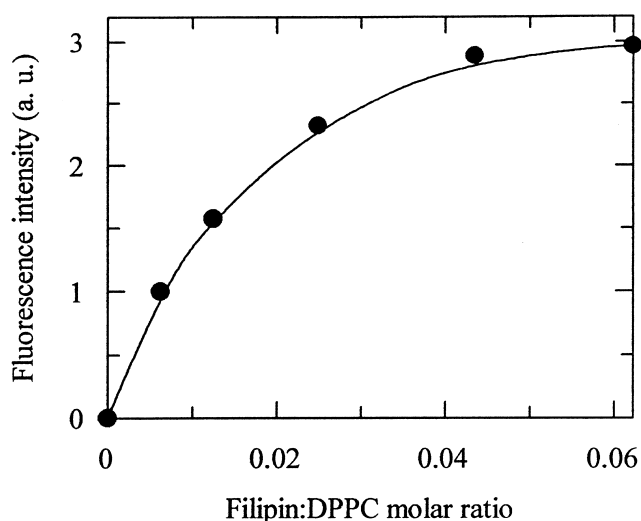


Fig. 7. Steady-state intensity fluorescence (corrected for inner filter effects) versus filipin concentration in DPPC SUV, in the presence of ergosterol (1 mol%). The curve is a mere guide to the eye.

to be $\sim 1.4 \times 10^{-5}$ M. For this concentration value, the changes in the absorption spectrum in buffer are still relatively small. We can therefore safely conclude that the alterations in absorption in the buffer/vesicles system are due to aggregation in the vesicles.

4.4. Filipin steady-state fluorescence intensity

Fig. 7 shows the filipin fluorescence steady-state intensity in DPPC SUV in presence of ergosterol (ergosterol:DPPC ratio = 1:100). This plot should be linear in the absence of filipin quenching, apart from the correction for the loss of linearity at very high absorbance values (in order for the plot to be linear, fluorescence intensities should be multiplied by $\ln 10 \cdot A_{\text{tot}} / (1 - 10^{-A_{\text{tot}}})$, where A_{tot} is the total optical density). Even taking into account this effect, a negative curvature remains, which, once again, also appears in the absence of ergosterol. This indicates that the mentioned filipin–filipin aggregates probably are non-fluorescent or less fluorescent than the monomeric form and/or absorb less than the monomer at the excitation wavelength.

The above results suggest that there are at least two types of aggregates to be considered: filipin–filipin aggregates (evidence: the negative curvature of the c plot and the spectral changes in filipin absorp-

tion, and probably the filipin self-quenching) and DHE–filipin aggregates (evidence: static quenching of DHE fluorescence by filipin). Further quantification of aggregation is not possible, because we do not know the stoichiometry (or even if there is a definite stoichiometry, rather than clusters with a broad distribution of sizes) of the filipin–filipin and DHE–filipin aggregates. In any case, we can safely conclude that for concentrations as low as 1 mol%, filipin aggregation in DPPC gel phase SUV is significant.

This work deals with interaction of filipin with low sterol content biomembrane model systems. The use of these peculiar experimental conditions, together with complementary photophysical techniques, enables one to distinguish between pure filipin and filipin–sterol interactions in a situation where both are possible. In fact, we concluded that both processes (filipin clustering and filipin–sterol association) coexist, meaning that the biochemical mode of action of filipin probably involves a ‘multiple-path strategy’, where several different phenomena may occur simultaneously, depending on the local filipin and sterol concentrations.

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