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New lipid formulation of amphotericin B: spectral and microscopic analysis

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Abstract

UV–visible and dichroic spectrum analysis and electron microscopy have been used to characterize a new amphotericin B (AmB) lipid formulation prepared by a solvent displacement process. The composition was dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) in molar ratio DMPC/DMPG/AmB 7:3:5, a similar composition to that of Abelcet®. Although the latter has a "ribbon-like" structure, our process gave a thin disc-like structure. Analysis of circular dichroism (CD) and UV–visible spectra of formulations containing different percentages of AmB revealed that a minimum of AmB self-association was observed with 7:3:5 molar ratio. Varying the lipid ratio (DMPC/DMPG) while maintaining the fixed ratio of AmB yielded similar results when DMPC was in excess (DMPC/DMPG from 10:0 to 6:4). However, when the ratio was between 5:5 to 3:7, AmB self-aggregation increased. For compositions rich in DMPG (2:8 and 0:10), inversion of the CD spectrum was observed. The influence of the lipid composition on the morphology of the complex was also evident in electron microscopy. DMPC/DMPG/AmB (10:0:5) gave large unfracturable lamellae. The presence of DMPG shortened the lamellae, which often appeared as disc-like structures. AmB content, the presence of DMPG and the preparation process all contribute to generating these original structures with particular CD spectra.

Keywords: Amphotericin B; Circular dichroism spectrum; UV–visible spectrum; Electron microscopy; Interdigitated structure; DMPC; DMPG

1. Introduction

Amphotericin B (AmB) is considered to be the antifungal agent of choice for the treatment of disseminated infections in immunocompromised patients (cancer, transplants or AIDS), despite its toxicity.

In fact, both the activity and the toxicity of AmB are related to its structure (Fig. 1) and its tendency to associate with itself and with lipids. Structurally, this antibiotic possesses a macrolide ring, containing an internal ester or lactone, a heptane chromophore, and a hydroxyl hydrophilic region. The head of the molecule consists of a carboxyl group and an amino sugar, the latter being attached to the macrolide ring through a glycosidic linkage.

The therapeutic potential of AmB and other polyene antibiotics is determined by their affinity for cholesterol and ergosterol, the principal sterols in eucaryote cell membranes. The selectivity for these sterols depends on the aggregation state of AmB [1]. Monomeric AmB associates with the sterols in fungal cell membranes, whereas self-associated AmB can also form pores in cholesterol-containing membranes, leading to toxicity towards host cells.

Strategies aimed at improving the therapeutic index of AmB have attempted to minimise the amount of self-associated AmB by complexing the antibiotic within lipid-based formulations (liposomes, complexes, emulsions) from which it is released progressively in the

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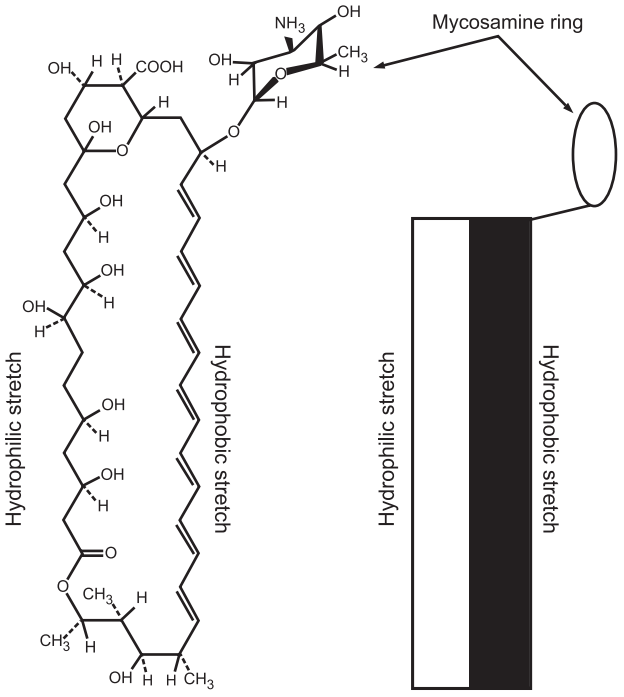

Fig. 1. Structure of AmB.

monomeric form. Many clinical trials have shown a reduction of AmB toxicity with this type of formulation, and three of them are now available commercially. In the last decades, many studies have addressed the interactions between AmB and lipids, especially those involved in the toxicity of AmB and in pore formation (sterols). Different techniques have been employed, the most frequently used being UV–visible absorbance, circular dichroism (CD), electron microscopy, NMR, X-ray diffraction and differential scanning calorimetry (DSC). These studies of the interactions between AmB and lipids were particularly useful during the development of the commercial formulations of AmB: AmBisome® [2], Abelcet® [3] and Amphotec® [4]. One of these formulations, Abelcet®, consists of AmB associated with two phospholipids: dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG), in a ratio of 7:3, organized in a "ribbon-like" structure [3,5]. However, the large size of this lipid assembly, about 3 μm, favours its rapid clearance from blood and accumulation in organs rich in phagocytic cells, leading to a reduction in its antibiotic activity [6,7].

In the present work, a solvent displacement process had been used to prepare a colloidal dispersion of AmB and phospholipids with the same composition as Abelcet®, but with a smaller, controlled, particle size. To characterize this formulation, among the techniques mentioned above, we chose to use spectroscopic measurements: electronic absorption and CD [8]. The particular structure of AmB molecule means that such techniques are especially appropriate to study its aggregation state under different conditions. Of the two, CD spectroscopy is more suitable for detecting the aggregated form of AmB.

We have correlated the results of spectroscopic analysis with zeta potential measurements and electron microscopy in order to characterize this original formulation and to explain its mechanism of formation.

2. Materials and methods

2.1. Materials

Amphotericin B powder and Fungizone® were purchased from Squibb (Neuilly, France). Abelcet® (3 μm diameter), AmB lipid complex preparation based on DMPC:DMPG (7:3, mol/mol) was provided by the Liposome Company Ltd. (London, UK). Amphotec was obtained from Liposome Technology Inc. (Menlo Park, CA, USA). DMPC and DMPG were purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA). Solvents and other reagents were obtained from Carlo Erba reagenti (Val de Reuil, France).

2.2. Preparation of lipid complex of AmB (LC-AmB)

A colloidal dispersion was prepared using the solvent displacement process described by Stainmesse et al. [9]. AmB (3.5 mg in 10 ml of methanol, 0.35 mg/ml) was added to organic solvent containing different ratios of DMPC/DMPG (5 ml of methanol) at 40 °C. This organic phase (15 ml) was then added to a water phase (MilliQ® water, 15 ml, Waters Millipore, France) under magnetic stirring at room temperature (around 20 °C). The volume was reduced by low pressure solvent evaporation until a volume of 5 ml. The AmB/phospholipids ratios varied between 5% and 50% (w/w; at ratios higher than 50% the formulation precipitated) and DMPC/DMPG ratios between 10:0 and 0:10, mol/mol. As a control, AmB was also precipitated alone, without lipid, by the same process.

2.3. Size measurement

The mean particle diameter was measured by photon correlation spectroscopy (PCS) with a Nanosizer N4 (Coultronics, Margency, France).

2.4. Zeta potential

The zeta potential of the formulations was determined by Doppler velocimetry and PCS on a Zetasizer 4 (Malvern Instruments, UK). The different formulations were analysed without further dilution. AmB precipitated alone at a final concentration of 5×10^{-4} M or less did not yield sufficient light-scattering properties to make a valid measurement. The pH of all formulations was similar to the pH of MilliQ® water, pH = 6.25, at room temperature.

2.5. CD and UV–visible spectroscopy

Absorbance measurements were made by using a Cary 1E UV–visible spectrometer (Varian, France). The drug concentrations in dispersions were calculated from absorbance at 405 nm after appropriate dilution in methanol ($\varepsilon_{methanol}$ 150 000 M^{-1} cm^{-1}). The CD spectra were recorded with a Jobin–Yvon Mark V dichrograph, and expressed as $\Delta\varepsilon$ (M^{-1} cm^{-1}), is the differential molar absorption dichroic coefficient.

These spectroscopic measurements were made at room temperature (around 20 °C) after dilution in water to final AmB concentrations of 50, 10, 5 and 1×10^{-6} M (pathlengths of quartz cuvettes: 0.2, 1, 2, 5 and 10 cm) to evaluate the aggregation state of AmB in the different formulations.

2.6. Electron microscopy

Freeze Fracture: a drop of the suspension containing 30% glycerol as a cryoprotectant was deposited on a thin copper planchett and rapidly frozen in liquid propane. Fracturing and shadowing using Pt-C were performed in a Balzers BAF 310 freeze-etch unit. The replicas were examined with a Philips 410 electron microscope.

Air Drying: the sample was deposited on a fresh cleaved mica plate, dried at room temperature and shadowed in the Balzers Units. The shadowing using Pt-C was performed in a Balzers BAF 310 freeze-etch unit. The replicas were examined with a Philips 410 electron microscope.

3. Results

3.1. Size and polydispersity

The size and polydispersity of AmB lipid preparations depended on the AmB/phospholipids ratio when the phospholipid composition was DMPC/DMPG 7:3 (molar ratio). Optimal size and minimal polydispersity were obtained with AmB at 35% of total weight of phospholipids (Table 1A).

Modification of the proportions of the two phospholipids (DMPC/DMPG) modified the size and the polydispersity, the optimal ratio giving small size and polydispersity being the ratio 7:3. This optimal ratio is the same as that in Abelcet® (Table 1B).

The optimal formulation (DMPC/DMPG/AmB 7:3:5 referred to as LC-AmB) was stable for 6 months after preparation when stored at + 4 °C, with no change in size. Other formulations increased in size a few days after the preparation with or without precipitation.

3.2. Zeta potential

The zeta potential of AmB of LC-AmB: − 43.8 mV, Table 1A, was intermediate between that of AmB precipitated alone (around − 27 mV, Table 1C) and the lipids alone (− 55 mV, Table 1A). Increasing the proportion of AmB up to 30% (w/w) reduced the absolute value of the zeta potential (Table 1A).

3.3. Spectroscopic analysis of AmB alone

3.3.1. UV–visible absorbance of AmB alone

When AmB from a stock solution in DMSO was diluted in water at a sub-micellar concentration ($< 10^{-7}$ μg/ml, 10^{-7} M) the absorbance spectrum showed three bands at 410, 385, 365 nm and a smaller band at 344 nm characteristic of the monomeric form [8]. As the AmB concentration was increased up to 10^{-5} M, the small band at 344 nm increased in intensity and the other three bands underwent red shifts from 410 to 420 nm, from 385 to 390 nm and from 365 to 368 nm, together with a reduction in intensity, indicating the appearance of the self-associated form [8].

AmB was also prepared by the solvent displacement process at concentrations corresponding to 10%, 35% and 50% (w/w) in the complex but without phospholipids. The spectra of these preparations, recorded at a final concentration of 5×10^{-5} M in water, were different from those of AmB solubilized in methanol at a final concentration of 5×10^{-5} M in water. For the preparations corresponding to the two highest ratios, four absorption bands were seen, the main one at 344 nm and three others at 365, 385 and 420 nm in decreasing order of intensity (Fig. 2A). A similar spectrum was seen for AmB prepared at the lowest concentration, but with a blue shift of the principal band to 326 nm.

Table 1A
Evolution of size, polydispersity and zeta potential with the ratio of AmB (w/w) of LC-AmB with ratio DMPC/DMPG 7:3, as described in Materials and methods

AnmB/Lipid ratio (w/w)	0%	2.5%	5%	10%	20%	30%	35%	40%	45%	50%
Size (nm)	>1000	>1000	>1000	>1000	690 ± 129: 79% 184 ± 57: 21%	559 ± 161: 66% 157 ± 46: 34%	303 ± 53	368 ± 115	248 ± 158: 87% >1000: 13%	275 ± 78: 93% >1000: 7%
Polydispersity	0.68	0.512	0.45	1.2	0.512	0.56	0.16	0.278	0.35	0.45
Zeta potential (mV)	− 55.1	− 67.1	− 60.8	− 55.0	− 46.3	− 46.3	− 43.8	− 42.8	− 43.2	− 45.9

Table 1B
Evolution of size and polydispersity as a function of the molar ratio of lipids (DMPC/DMPG) of LC-AmB as described in Materials and methods with the total lipids/AmB molar ratio fixed to 10:5

DMPC/DMPG	10:0	9:1	8:2	7:3	6:4	5:5	4:6	3:7	2:8	1:9	0:10
Size (nm)		>1000: 87%			311 ± 128: 91%					284 ± 75: 79%	
	>1000	209 ± 42: 13%	>3000	303 ± 53	>1000: 9%	381 ± 207	436 ± 273	339 ± 131	418 ± 131	93 ± 21: 21%	396 ± 101
Polydispersity	1.5	0.51	2.1	0.16	0.35	0.49	0.58	0.27	0.35	0.48	0.56

3.3.2. CD of AmB alone

The monomeric form of AmB yielded a CD spectrum with three weak positive bands at 410, 385 and 365 nm. On the other hand, the CD spectrum of the aggregated form of AmB displayed a very intense dichroic doublet centered at 344 nm as well as negative bands at 420, 390 and 368 nm [8].

The CD spectrum of AmB prepared according to the solvent displacement process starting with various quantities of AmB (10, 35, and 50%) showed a modification of the center of the dichroic doublet (327, 335, 337, respectively) and of its intensity ($\Delta\varepsilon$ (M^{-1} cm^{-1}): 4500, 2500, 2800, respectively) for a diluted to give a final AmB concentration of 5×10^{-5} M. The negative minimum of the CD doublet band was at 350 nm for 35%, and 50% but was at 336 nm for 10% w/w (Fig. 2B).

3.4. Effect of varying AmB–lipid ratio

3.4.1. UV–visible absorbance

Spectra were recorded for the LC-AmB formulations prepared at a constant lipid concentration (ratio: DMPC/DMPG 7:3) with different percentages of AmB, diluted to give an final AmB concentration of 5×10^{-5} M. For the ratio 10%, the bands at 333 and 344 nm had similar intensity and were more intense than the other bands (365, 385 and 415 nm; Fig. 3). However, for AmB ratios between 20% and 50% (w/w), the intensity of the whole spectrum decreased with increasing proportions of AmB, and a minimum intensity was obtained with the 35% ratio, as shown in Fig. 3.

3.4.2. CD

When the AmB–lipid complexes (LC-AmB) were examined by CD (10% to 50% AmB, Fig. 4), the dichroic doublet characterising the aggregation state of AmB was still observed, but the intensity and the center of the dichroic band varied according to the percentage of AmB. The dichroism spectra of the complexes with AmB ratios up to and including 20% were similar to the spectrum of AmB alone (Fig. 4): a doublet centered on 340 nm and negative bands at 368, 389 and 419 nm. For ratios above 20%, the intensity was lower and the negative bands were progressively replaced by three positive bands (420, 392, 375 nm) which had a similar intensity whatever the ratio. The

Table 1C
Evolution of size, polydispersity and zeta potential with the ratio of AmB without lipids prepared by the process like LC-AmB as described in Materials and methods

AmB (w/w) without lipids	10%	35%	50%
Size (nm)		1900 ± 355: 60%	2750 ± 250: 65%
	>1000	275 ± 40: 40%	350 ± 100: 35%
Polydispersity	0.35	0.62	1.2
Zeta potential (mV)	− 29.6	− 28.7	− 24.3

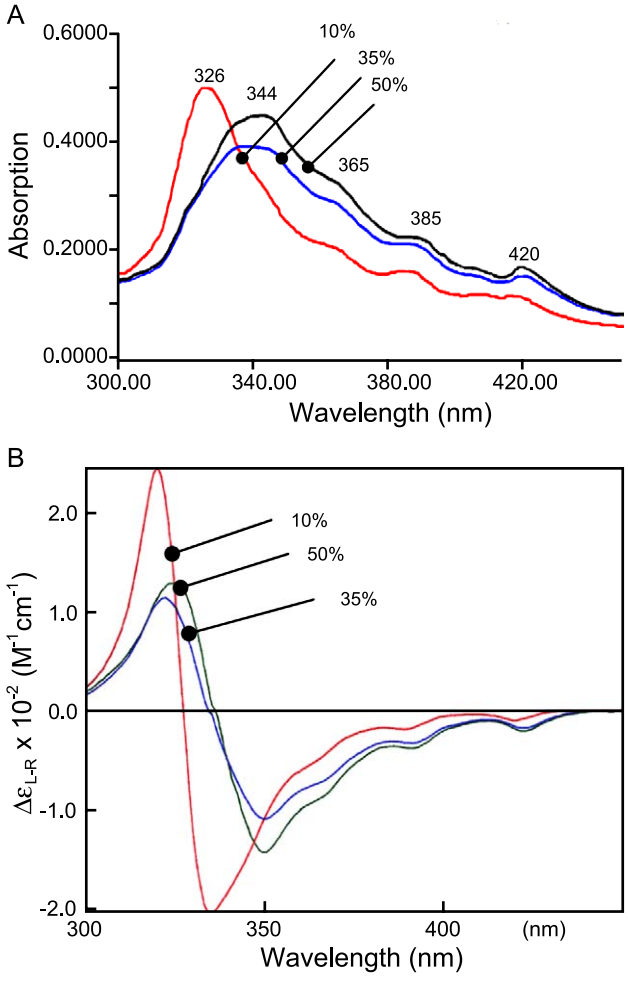

Fig. 2. (A) Evolution of electronic absorbance spectra of AmB obtained by displacement solvent procedure (at different initial concentration: 1.0, 3.5 and 5.0 mg) as described in Materials and methods. (B) Evolution of CD spectra ($\Delta\varepsilon_{L-R} \times 10^{-3}$ (M^{-1} cm^{-1})) of AmB obtained by displacement solvent procedure at different initial concentration as described in Materials and methods.

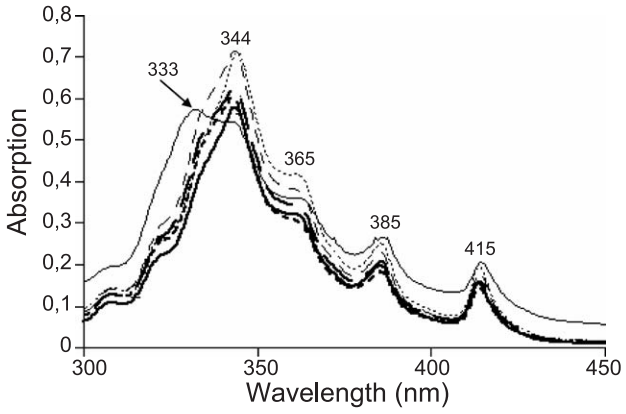

Fig. 3. Evolution of electronic absorbance spectra with different percentages of AmB in AmB/lipid preparations (w/w) with a molar ratio of DMPC/DMPG 7:3, obtained as described in Materials and methods. (10%: ——; 20%: . . . . .; 30%: – – –; 35%: ——; 40%: – – –; 50%: ·····).

minimum doublet intensity was reached with the 35% ratio. These positive bands are characteristic of interactions of AmB with other molecules (in this case, lipids).

3.4.3. Influence of the dilution on CD and UV–visible spectra

The LC-AmB 7:3:5 formulation was diluted 10 and 1000 times to give AmB concentrations of 5×10^{-6} and 5×10^{-8} M. There was no modification of the spectra, indicating that AmB remained associated with the lipid. Incubation for 1 h at 37 °C did not affect its spectrum (data not shown).

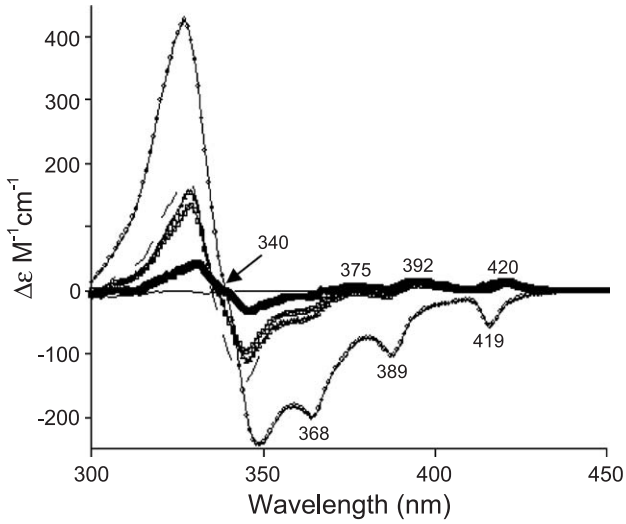

Fig. 4. Evolution of CD spectra ($\Delta\varepsilon_{L-R} \times 10^{-2}$ (M^{-1} cm^{-1})) with different percentages (w/w) of AmB in AmB/lipid preparations with molar ratios DMPC/DMPG 7:3, as described in Materials and methods. (20%: –○–○–○–○–; 30%: –□–□–□–; 35%: ——; 40%: –Δ– Δ– Δ–; 50%: - - - -).

3.5. Effect of DMPC/DMPG ratio with a fixed AmB ratio (total lipids/AmB 10:5)

3.5.1. UV–visible absorbance

The UV–visible spectra observed for DMPC/DMPG ratios between 10:0 and 6:4 were very similar to that observed with the ratio 7:3:5 (Fig. 5). For the lipid ratios between 5:5 and 0:10 (Fig. 5), the intensity of the band at 333 nm increased with increasing DMPG up to the ratio 2:8 (Fig. 5).

3.5.2. CD

Changing the proportions of the two phospholipids (DMPC/DMPG) with the same ratio of AmB: 35% w/w or 5 molar ratio) between 10:0 and 6:4 yielded similar results to the 7:3:5 formulation (Fig. 6A): the doublet was centered around 335 nm with similar intensity, except for the 6:4:5 formulation in which it was slightly lower, and three similar positive bands (420, 395, 376 nm). The ratios 5:5:5 and 4:6:5 showed spectra similar to that of AmB alone: the dichroic doublet centered around 345 nm and three negative bands at 368, 389, 419 nm (Fig. 6B). For the lipid ratios 3:7:5 to 0:10:5, the large positive band of the dichroic doublet became negative and the positive bands (420, 389, 368, 352 nm) were very intense (50 times higher than the ratio 7:3:5).

The DMPC/DMPG proportions were also varied for other AmB ratios (10%, 40% and 50%), yielding similar results to those for 35%.

3.6. Electron microscopy

AmB prepared by the solvent displacement method without lipids appeared to be aggregated like a bunch of grapes after air drying and shadowing (not shown). Freeze-

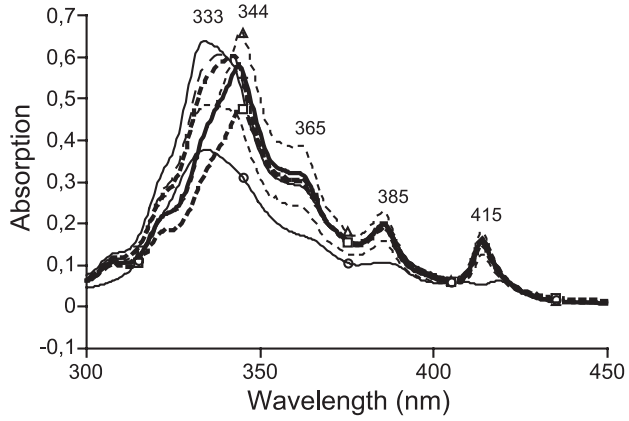

Fig. 5. Evolution of electronic absorbance spectra with different molar ratios of DMPC/DMPG in the AmB/lipid preparations with 5 mol of AmB for 10 mol lipid, obtained as described in Materials and methods. (10:0, —○—; 8:2, - -□- - -; 7:3, ——; 6:4, ·····Δ·····; 5:5, ·····; 3:7, – – –; 2:8, ——; 0:10, – – – –).

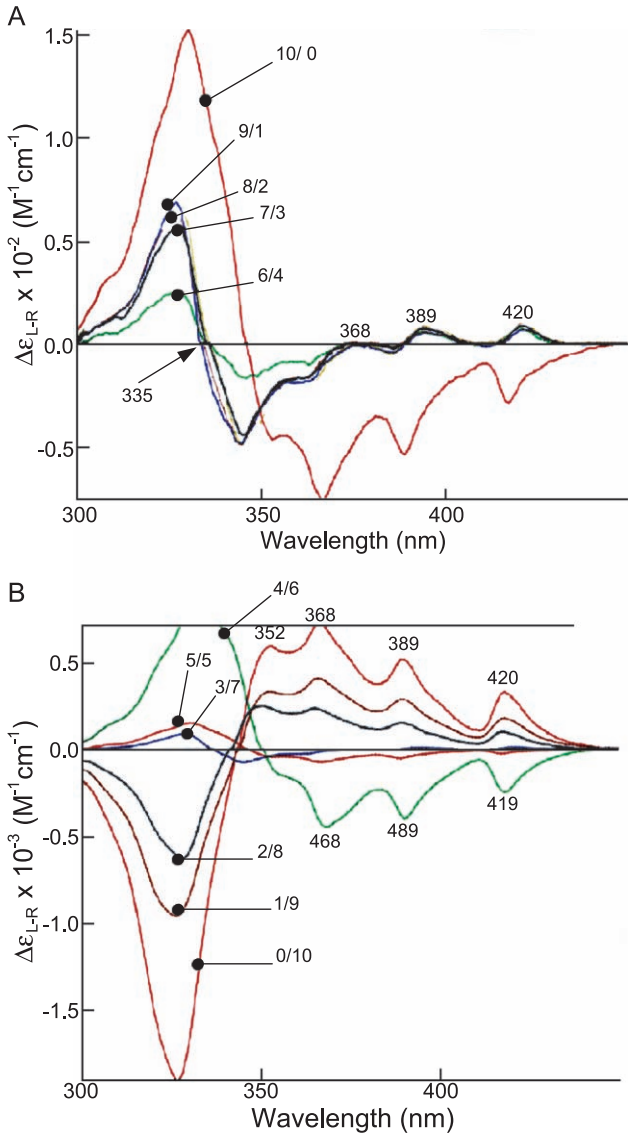

Fig. 6. (A) Evolution of CD ($\Delta\varepsilon_{L-R} \times 10^{-2}$ (M^{-1} cm^{-1})) spectra with different molar ratios of DMPC/DMPG for 5 mol of AmB for 10 mol lipid, obtained as described in Materials and methods. (B) Evolution of CD ($\Delta\varepsilon_{L-R} \times 10^{-3}$ (M^{-1} cm^{-1})) spectra with different molar ratios of DMPC/DMPG for 5 mol of AmB for 10 mol of lipids, as described in Materials and methods.

fracture electron microscopy of the same preparation yielded a string-of-pearls structure of several microns (Fig. 7A). The addition of the lipid to the formulation in the ratio 7:3:5 resulted in non-fracturable disc structures as shown by freeze-fracture (Fig. 7B) and shadowing after drying (Fig. 7C). The thickness of the disc was evaluated from the angle of shadowing and the length of the shadow to be about 29 Å.

Modification of the ratio of the two phospholipids yielded different structures in freeze-fracture electron microscopy. Without DMPG (molar ratio DMPC/DMPG/AmB 10:0:5) large unfracturable lamellae were observed (Fig. 7D). The presence of DMPG favoured the formation of shorter lamellar structures, both stacked and fused in places (Fig. 7E) or a predominantly disc morphology (Fig. 7B, C, F).

4. Discussion

The amphotericin B molecule (Fig. 1) has a particular cyclic structure with seven conjugated double bonds which exhibit intense peaks of absorption between 250 and 450 nm. This means that electronic absorption and CD spectroscopy are ideal tools to study the organisation of this antibiotic. Such studies are useful both for attempting to understand the interactions of AmB with fungal and mammalian cell membranes and, as in this study, for characterising lipid formulations of AmB designed to reduce its toxicity. In consequence, the protocols used in the literature are variable. Most studies have used low AmB/lipid ratios (<0.02), only those involving the commercial formulations referred to above have used higher ratios similar to those in this study. In some cases, such as the studies of Chen and Bittman [10] and Jullien et al. [11], AmB was added to preformed lipid membranes. However, in the case of pharmaceutical formulations, the antibiotic is in contact with the lipids and organic solvents during the preparation process.

The molecular state of AmB, and in particular its self-aggregation in aqueous medium, depends on a number of factors: its final concentration, pH, temperature and ionic strength of the aqueous phase, the concentration and nature of the original organic solution. Even in organic solvent where AmB is in its monomeric form, the absorption and CD spectra of AmB vary according to the medium, because of influences on the $\pi^* \leftarrow \pi$ transition. In our study, AmB dissolved in a large volume of methanol either alone or with lipids is added to an equal volume of water to form an aqueous dispersion. This study shows a difference in the aggregation state between AmB prepared by the solvent displacement process and AmB dispersed in water from a concentrated stock solution in methanol, with evidence of AmB self-association (absorption band at 344 nm and a dichroic doublet) after solvent displacement. Similar results concerning the influence of the original solvent medium on the state of AmB in aqueous dispersion were obtained by Espuelas et al. [12]. The blue shift of the center dichroic doublet from 337 to 327 nm can be interpreted as a super-aggregated form of AmB, as observed by Gaboriau et al. [13] for heat-treated AmB.

When AmB was formulated with lipids by the solvent displacement process, the absorption and CD spectra indicated an influence of the lipid on the aggregation state of the AmB, which depended on the AmB/lipid ratio. Kawabata et al. [14] observed that the amount of non-self-associated AmB associated with lipids was inversely proportional to the AmB/lipid ratio when the antibiotic was incorporated into small unilamellar vesicles composed of DMPC/DMPG in a 7:3 molar ratio. However, the maximum AmB/lipid

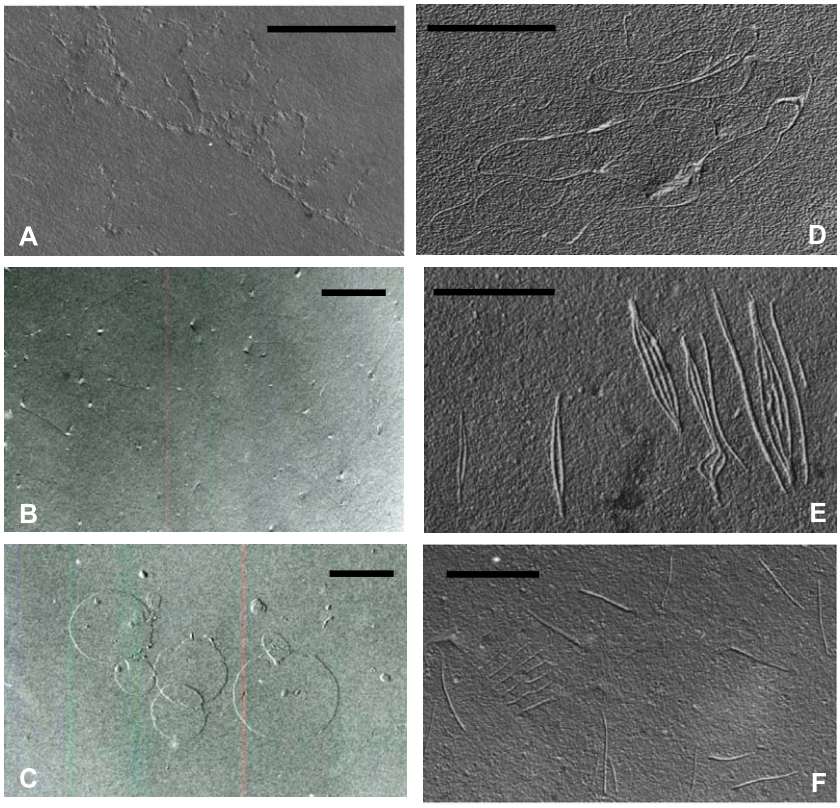

Fig. 7. Electron microscopy of AmB alone (A): prepared by solvent displacement evaporation, (freeze fracture, bar = 500 nm); AmB – lipid formulation with the ratio DMPC/DMPG/AmB 7:3:5: (B) (freeze fracture, bar = 200 nm) and (C) (air drying and shadowing bar = 200 nm); 10:0:5: (D) (freeze fracture, bar = 500 nm); 5:5:5: (E) (freeze fracture, bar = 500 nm); 1:9:5: (F) (freeze fracture, bar = 500 nm). In some cases, for better visualisation, the samples were etched before shadowing (D, E, F).

ratio in this study was 0.1. In our study, AmB prepared with lipids at 10% or less gave spectra similar to those of AmB prepared alone and the band corresponding to monomeric AmB associated with the lipids at 415 nm did not change, suggesting a saturation of the interaction between the monomer and lipids. This is correlated with the reduced intensity of the dichroic doublet and the appearance of positive bands in the higher range of the CD spectrum of AmB at contents above 20% (Fig. 4).

The importance of the concentration of AmB for its interaction with phospholipids had already been demonstrated by Chen and Bittman [10] and Jullien et al. [11], but for different lipid compositions and lower AmB/lipid ratios. In our study, the ratios between 10% and 50% did not allow all the AmB to be "dissolved" in the lipid in the monomeric form. However, the interaction between the lipid and the aggregated AmB was very stable since the absorption and CD spectra of the preparation containing 35% AmB with lipid were not affected by dilution down to 5×10^{-8} M AmB. This proportion of AmB could be considered optimal as a pharmaceutical formulation because it is generally accepted that the origin of toxicity towards mammalian cell membranes is free, self-associated AmB [1]. On the other hand, the CD spectrum of Abelcet® was different from that of our formulation, with a more intense dichroic doublet, similar to that obtained with the composition 5:5:5 prepared by the solvent displacement method. However, Perkins et al. [15] have shown a reduction of the intensity of dichroic doublet with the increase of AmB weight ratio with ABLC™ (Abelcet®, which was correlated with a reduction of the AmB toxicity of this formulation [5].

In order to determine the role of the lipid composition in the interactions with AmB, we varied the proportion of the two phospholipids while keeping the total AmB/lipid ratio at 35% AmB. Both spectral analysis and electron microscopy indicate that the type of objects obtained was strongly influenced by the lipid composition. As far as the aggregation state of AmB is concerned, similar results were obtained for the DMPC/DMPG ratios between 10:0 and 6:4. However, when the proportion of DMPG, a phospholipid with a net negative charge on the polar head group at physiological pH, was increased further, dramatic changes were observed in the CD spectra. At ratios between 5:5 and 3:7, there was a red shift of the center of the positive part of the dichroic doublet and a large increase in its intensity (maximal at the ratio 4:6, peak height out of the scale), while the negative part of the doublet resembled that obtained with AmB alone. This could be interpreted as a

decrease in the interaction between AmB and lipids leading to an absence of incorporation of the antibiotic molecule in the complex. The instability and precipitation observed for these preparations corroborate this hypothesis.

DMPG/DMPC ratios of 3:7 and 0:10 gave a completely new CD spectrum which was inverted, with the negative part at lower wavelengths and the positive part at higher ones. An inverted spectrum for AmB was observed by Milhaud and Michels [16], but under very different conditions: AmB was associated with multilamellar vesicles of dilauroylphosphatidylcholine at a very low AmB/lipid ratio of 0.005. The spectrum obtained was very different from the one presented here. Another study by the same group concerning the interaction of the polyene antibiotic filipin III with multilamellar vesicles of DMPC at an antibiotic/lipid ratio of 0.25 may be more relevant [17]. Filipin differs from AmB in that it is a smaller molecule, with five rather than seven conjugated double bonds and in that it is uncharged, lacking the amino sugar and the carboxyl group. The spectrum obtained for filipin/DMPC had a negative band at 308 nm and three positive bands at 324, 340 and 362 nm.

The strong association of filipin with DMPC was confirmed by DSC measurement of the phospholipid phase transition, the enthalpy of which was reduced in parallel with increasing antibiotic content [17]. We have observed (unpublished results) similar decreases induced by AmB in DMPC/DMPG mixtures at all lipid ratios, as did Janoff et al. [3]. However, CD spectrum inversion only occurred with high proportions of DMPG. This could indicate an electrostatic interaction between the negatively charged phospholipid and the amino group of AmB. However, spectrum inversion was not dependent on the AmB/lipid ratio: similar results were observed with AmB contents of 10%, 40% and 50% (results not shown). Another explanation could be that DMPG is in the gel state in water under the temperature and pH conditions of this study. This could reduce the mobility of AmB within the formulation and block it in a particular configuration.

Electron microscopy also demonstrated the critical role of the lipid composition. Although the spectra were not influenced by the proportion of DMPG up to 40% of total lipid, the morphology of complexes formed from AmB and DMPC alone was completely different from that of ternary AmB/DMPC/DMPG complexes. Molecular modelling studies show that AmB can form stable aggregates with DMPC by electrostatic interactions between the amino group and the phosphate of the lipid head-group and that this could contribute to pore formation in cholesterol-containing membranes [18]. Similar structures have already been seen by electron microscopy with this composition [3,19]. The addition of DMPG to the system allows a disc-like structure to be formed when DMPC remains in excess. Amphotericin B is essential for this structure, which is not seen with the lipids alone (results not shown). In particular, the ''optimal'' formulation (7:3:5) yields a disc structure. An interdigitated structure was observed by Perkins et al. [15] for the same composition, but in the form of ''ribbons''. These authors only observed this structure for the 7:3 ratio, but we cannot exclude the possibility that it could also be formed for ratios 9:1, 8:2 and 6:4 in our study because these compositions were not examined by electron microscopy. The gel state of DMPG might induce the disc structure; however, at equimolar proportions of DMPC and DMPG, a very particular structure with discs fused at their extremities was observed, while the 1:9 ratio again showed individual discs.

Another AmB formulation which has a small disc-like structure is Amphotec®, AmB and cholesteryl sulfate in a 1:1 molar ratio [20]. However, this shows a bilayer disc by freeze fracture electron microscopy and not an interdigitated structure. The CD and UV–visible spectra for Amphotec® were similar to that of AmB–cholesterol described in the literature (UV–visible spectrum, Fig. 8A: one large band at 332 and three smaller at 365, 386 and 406; CD spectra, Fig. 8B: 100 times less intense than that of LC-AmB, a dichroic doublet center at 342 and three bands at 381, 400 and 426). These differences indicate that the interactions between AmB and the lipids were very different. In spite of the difference in structure between Abelcet® [15] and LC-AmB, spectrum analysis of the bands shows a similar interaction (interdigitated) and organisation. However, the different intensity of the bands reflects the different process of preparation.

The spectra obtained with AmB alone prepared by the process of nanoprecipitation were characterised by the formation of a super-aggregate form in the formulation equivalent to 10% of AmB (1 mg) [13] while the AmB aggregation state with the concentration corresponding to 35% and 50% was similar to AmB in methanol diluted in water. The dried and shadowed electron microscopy of AmB alone prepared by the solvent displacement process shows structures resembling a bunch of grapes while freeze-fracture electron microscopy of the same preparation yielded a string-of-pearls structure of several microns (Fig. 7A). This is different from the structure observed for crystallised AmB, which is in the form of a bundle of needles [21]. Grant et al. [19] also observed a different the structure for AmB prepared like Abelcet® without lipids (film dried down, hydrated with warming and dialysed where appropriate: a suspension of amorphous material with no similarity to lipid bilayers). The cut surfaces of the resulting amorphous masses (fracture faces) were generally smooth and featureless, etching to expose their outer surfaces revealed the microspherular appearance, with grains 6.25–25 nm in size. This is probably the result of the preparation process which allows AmB to assume a double helical structure in aqueous dispersion as described by Millié et al. [22], in which the hydrophobic parts are hidden and the polar head groups are hydrated. This result could show the importance of hydrophobic interactions in the formation of AmB aggregates [23].

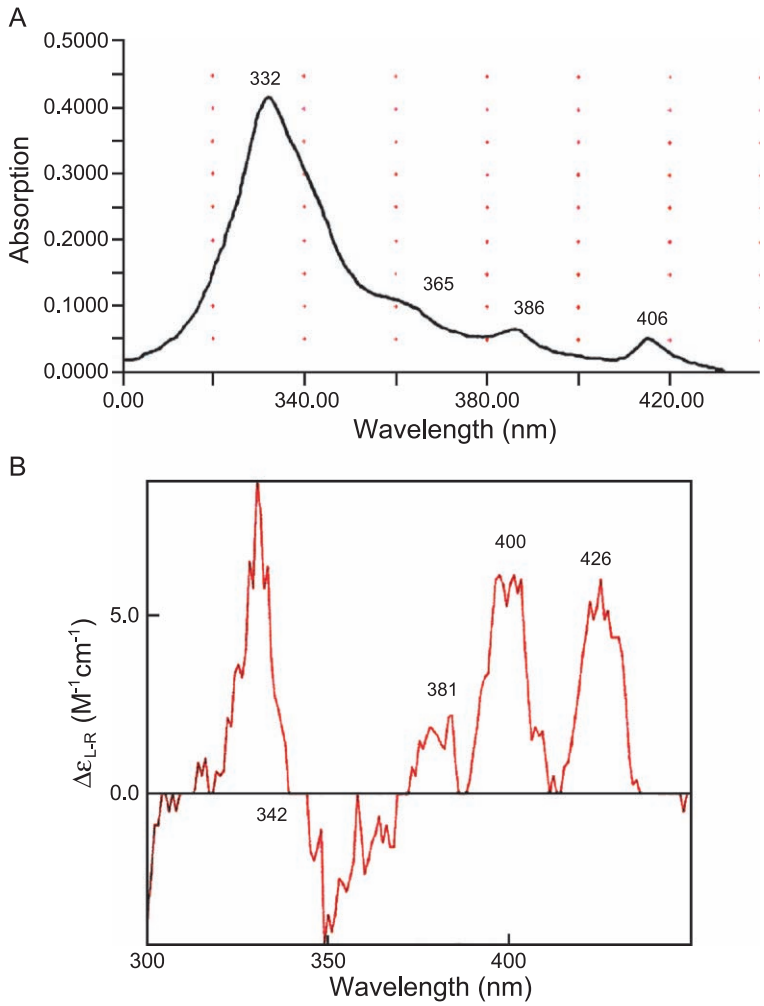

Fig. 8. (A) Electronic absorbance spectra of Amphotec diluted in water to give a final AmB concentration of 5×10^{-5} M obtained as described in Materials and methods. (B) CD ($\Delta\varepsilon_{L-R}$ (M^{-1} cm^{-1})) spectra of Amphotec diluted in water to give a final AmB concentration of 5×10^{-5} M obtained as described in Materials and methods.

Surprisingly, this AmB aggregate has a negative zeta potential (− 27 mV) at pH 6.25. Since the pK_a values of the amino group and the carboxyl group are 10 and 5.7, respectively [24,25], a net positive charge would be expected at this pH. However, a negative zeta potential for AmB aggregates has already been noted by Witzke and Bittman [26]. The explanation is that between pH 4 and 9 intermolecular electrostatic interactions within the aggregates mask some positive charge, leaving the surface of the aggregate negative [27,28]. Espuelas et al. [12] observed a negative zeta potential for AmB aggregates prepared by a process similar to that used in this study for external pH values of 5–10. Below pH 4, intermolecular interactions decrease and positively charged species can be detected.

As expected, the objects formed from DMPC and DMPG in the 7:3 molar ratio had a negative zeta potential (− 55 mV) because of the net negative charge on the DMPG head group. The interactions between the lipids would be essentially hydrophobic and van der Waals forces, rather than electrostatic interactions. Addition of AmB showed an increase in the absolute value of the zeta potential with increasing AmB ratio up to 35% AmB. Thereafter, the zeta potential value was stable, up to 50% AmB. However, it is difficult to conclude about the nature of the interaction between AmB and lipids using only zeta potential data, because the values could be an average of several different species.

These results clearly show that our solvent displacement process leads to the formation of AmB–lipid structures which are different are from the "ribbon-like" ones described by Janoff et al. [3,5] for the same composition. Our observations are consistent with a model of the antibiotic intercalated between the phospholipids in an interdigitated structure for the molar ratio DMPC/DMPG/AmB 7:3:5. There was a strong interaction between AmB and lipids (same analysis spectra after dilution suggest no dissociation). The reduction of free self-associated AmB and the small size of the complexes could explain why this new formulation has been found to be less toxic than Abelcet® and to have a different biological activity [29,30].

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