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A morphological study of an amphotericin B emulsion-based delivery system

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Abstract

The structure of an amphotericin B emulsion-based delivery system (AmB-E) was investigated using spectroscopic methods (electronic absorption and circular dichroism (CD)), photon correlation spectroscopy (PCS) and transmission electron microscopy (TEM) at various amphotericin B (AmB) concentrations, in comparison with AmB-deoxycholate micelles, Fungizone® (Fungi-SS) and free AmB suspension (AmB-SS). Our results show that AmB-E absorption and CD spectra, mean particle size determination by PCS and morphological examination are only weakly concentration-dependent, between 5.10^{-5} and 5.10^{-7} M, as compared with Fungizone® and free AmB suspension. The only difference observed was at high dilution (5.10^{-8} M), where AmB dissociation appeared in spectroscopic studies, together with emulsion droplet fusion observed in PCS. These data seem to indicate that AmB could be located in the droplets of the emulsion, preferentially in the phospholipid layer, because AmB is not soluble in the oil inner phase. All these results suggest that AmB-E is a system which has potential applications and merits further evaluation. Copyright © 1996 Elsevier Science B.V.

Keywords: Amphotericin B; Absorption spectra; Circular dichroism spectra; Spontaneous emulsification; Submicronic emulsions

Abbreviations: AmB, amphotericin B; AmB-E, amphotericin B emulsion-based delivery system; AmB-SS, stock solution of free amphotericin B; CD, circular dichroism; DMSO, dimethylsulfoxide; Fungi-SS, Fungizone® stock solution; PCS, photon correlation spectroscopy; TEM, transmission electron microscopy.

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1. Introduction

Opportunistic infections are a major problem in oncology, especially in patients suffering from leukemias and lymphomas (Bodey, 1977; Singer et al., 1977), as well as in individuals with congenital

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and acquired immunodeficiencies (Verhoeff et al., 1980).

Currently, amphotericin B remains one of the most effective and widely used agents for treating systemic mycoses caused by opportunistic fungi. The clinical use of this agent, however, is problematic. Several reports (Bodey, 1966; Winston et al., 1979) indicate that patients with deep mycoses do not respond to amphotericin B therapy. Moreover, postmortem examination of patients with apparent clinical resolution of infection after amphotericin B therapy often reveals the presence of *Candida* pseudohyphae at sites of diagnosed infection (Krick and Remington, 1976). The reasons for the poor therapeutic efficacy of amphotericin B in patients suffering from established infections remain unclear. In most cases, the clinical use of amphotericin B is limited by its dose-related nephrotoxicity, which occurs in more than 80% of patients (Perfect et al., 1991). Although nephrotoxicity is largely reversible, most patients who complete a full course of amphotericin B therapy have residual impairment of kidney function (Barnhart, 1985).

Amphotericin B is a lipophilic drug that binds to sterols and intercalates into lipid bilayers (De Kruijff and Demel, 1974). This suggests that amphotericin B would be particulary suitable for use with lipid-based delivery systems. Indeed, a large amount of research (Graybill et al., 1982; Lopez-Berestein et al., 1983, 1984; Mehta et al., 1984; Lopez-Berestein, 1987; Janoff et al., 1988; Gondal et al., 1989; Tyle and Frank, 1991) has shown that incorporating amphotericin B into liposomes significantly reduces the systemic toxicity of this agent without a concomitant loss of efficacy against experimental fungal infections. Recently, clinical trials with liposomal formulations of this agent have been carried out (Speller and Warnock, 1991).

Other groups have shown that formulating amphotericin B in a lipid-emulsion carrier does not alter its antifungal activity; however, there is a significant reduction in acute lethality and nephrotoxicity (Kirsh et al., 1988) and the interaction with red blood cells is reduced (Davis and Washington, 1988; Souza et al., 1993)

The characteristic optical properties of amphotericin B conferred by the presence of conjugated double bonds, make the study of environmental modifications by spectroscopic methods particularly appealing. In fact, Bolard et al. (1980) have shown that the electronic absorption and circular dichroism spectra of the amphotericin B are concentration-dependent.

Jullien et al. (1989, 1990) demonstrated that the toxicity of liposomal amphotericin B preparations towards cells is due to the levels of amphotericin B remaining free (unbound to the lipids) in these preparations. This binding is easily determined by electronic absorption and circular dichroism (CD) spectra.

In this report we describe the spectroscopic characteristics of an amphotericin B emulsion-based delivery system as studied by electronic absorption and CD spectra, and the morphology of this emulsion as revealed by transmission electron microscopy. The results are compared with those of the commercial form of amphotericin B (Fungizone®).

2. Materials and methods

2.1. Materials

Amphotericin B was purchased from Sigma, St. Quentin Fallavier, France. Amphotericin B as the Fungizone® preparation (containing sodium deoxycholate and sodium phosphate) was supplied by the Pharmacie Centrale des Hôpitaux, Paris, France. The following chemicals were obtained from commercial sources and used without further purification: the oily phase, a mixture of saturated medium-chain fatty acids, (Mygliol 812) came from Huls, Puteaux, France; the lipophilic surfactant, egg lecithin (Lipoid E80) was from D3F, Paris, France; and the organic solvents (methanol and DMSO) were obtained from Prolabo, Paris, France.

2.2. Preparation of solutions

A stock solution of amphotericin B (AmB-SS) at 10^{-3} M was obtained by dissolving the drug in

dimethyl sulfoxide (1 mg in 100 μl) and then in methanol. This solution was used in the 2 h following its preparation.

A stock solution of Fungizone® (Fungi-SS) at 5.10^{-3} M was prepared by dissolving the powder in 5% glucose solution.

2.3. Preparation of injectable emulsions

Amphotericin B emulsions (AmB-E) were produced as described previously by Tabosa do Egito et al. (1994). Briefly, amphotericin B (50 mg) was dissolved in methanol (100 ml at 40°C), and Mygliol (5 g) and Lipoid E80 (1.2 g) were added. The oily alcoholic solution was slowly injected into the aqueous phase (200 ml) under moderate magnetic stirring. The aqueous phase immediately turned milky with bluish opalescence as the result of the formation of an emulsion. The mixture was evaporated down to 30–40 ml at 45–50°C in order to remove the methanol.

2.4. Electronic absorption and circular dichroism (CD) spectra of amphotericin B delivery systems

Absorption and CD spectra of the AmB-SS, Fungi-SS and AmB-E were obtained by dispersion of the stock solutions in phosphate-buffered saline (PBS), pH 7.4, at various amphotericin B concentrations. Amphotericin B emulsions were recorded against a blank consisting of amphotericin B-free emulsion, in the reference beam. The path-lengths of the cuvettes used were 0.1 cm, 1 cm and 10 cm for the concentrations of 5.10^{-5} M, 5.10^{-6} M and 5.10^{-7} M and 5.10^{-8} M, respectively. All spectra were recorded at 25°C.

Electronic absorption spectra were recorded with a Varian Cary 219 spectrophotometer, Orsay, France.

CD spectra were recorded with a Jobin-Yvon Mark V. dichrograph, Longjumeau, France, equipped with a thermostat.

2.5. Analytical ultracentrifugation experiments

Analytical ultracentrifugation experiments were carried out on a Hettich (Tuttingen, France) ultracentrifugation equipped with a type Microliter 15000 t/min ($12000 \times g$) rotor. The samples of AmB-E dispersed in PBS as a 5.10^{-6} M solution were ultracentrifuged for 10 min at $12000 \times g$. The spectral alterations were recorded.

2.6. Transmission electronic microscope (TEM) analysis

Morphological examination of various concentrations of Fungi-SS and AmB-E was carried out using a transmission electron microscope (TEM) JEOL 1010 following negative staining with uranyl acetate solution (1.5% w/v).

2.7. Mean particle size studies

The mean particle size of dispersed AmB-SS, Fungi-SS and AmB-E in PBS, was estimated by photon correlation spectroscopy using a Super Nanosizer N4MD, Coultronics, Andilly, France.

3. Results

3.1. Electronic absorption and circular dichroism (CD) spectra of amphotericin B delivery systems

3.1.1. Dilution of Amphotericin B stock solutions and Fungizone® in PBS

The CD and electronic absorption spectra of the AmB-SS (Fig. 1) and Fungi-SS (Fig. 2) are concentration-dependent. At low concentrations (5.10^{-8} M) the spectra were similar to those obtained with methanol or other polar organic solvents with only small red shifts (spectra not shown). These spectra were characteristic of the monomeric form of AmB in an aqueous environment. As the concentrations increased, the spectra were progressively modified and above 5.10^{-5} M a completely new spectrum was observed with a very intense bisignated curve, called a dichroic doublet, characteristic of AmB self-association. Its center corresponded to the absorption peak at 340 nm (AmB-SS) and at 330 nm (Fungi-SS). In the case of Fungi-SS, the band at 340 nm (electronic absorption) and the dichroic doublet (CD) centered at 340 nm were more intense than those observed with AmB-SS.

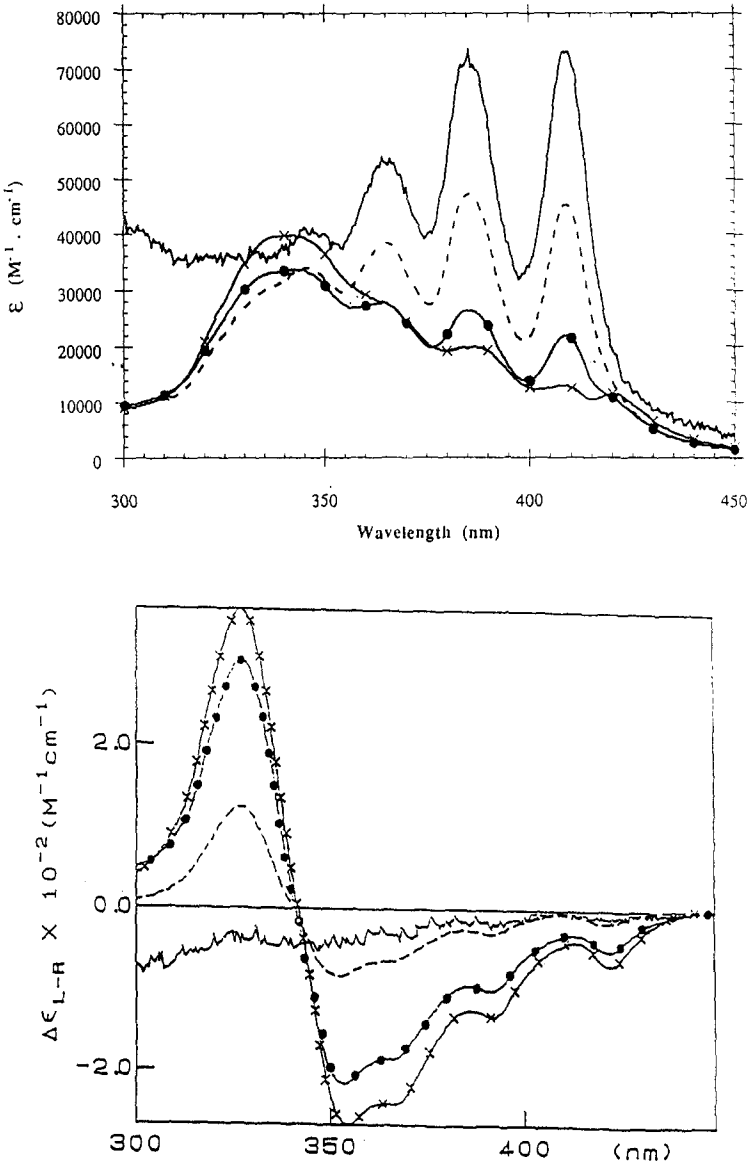

Fig. 1. Absorption and circular dichroism spectra of the AmB stock solution (AmB-SS) in PBS. The AmB concentrations were 5.10^{-5} M (– × –), 5.10^{-6} M (– • –), 5.10^{-7} M (- -) and 5.10^{-8} M (——).

3.1.2. Dispersion of amphotericin B emulsions in PBS

The electronic absorption and CD spectra (Fig. 3) were poorly concentration-dependent except at very low AmB concentration. At high AmB concentration (5.10^{-5} M), the electronic absorption and CD spectra showed that the position of the band and the center of the dichroic doublet, characteristic of self-associated AmB (327 nm), were shifted with respect to the aqueous suspension of AmB (340 nm) and to Fungi-SS (330 nm). Electronic absorption spectra revealed that the monomeric AmB band at 409 nm was shifted to 415 nm. Dilutions below 5.10^{-5} M led to both a

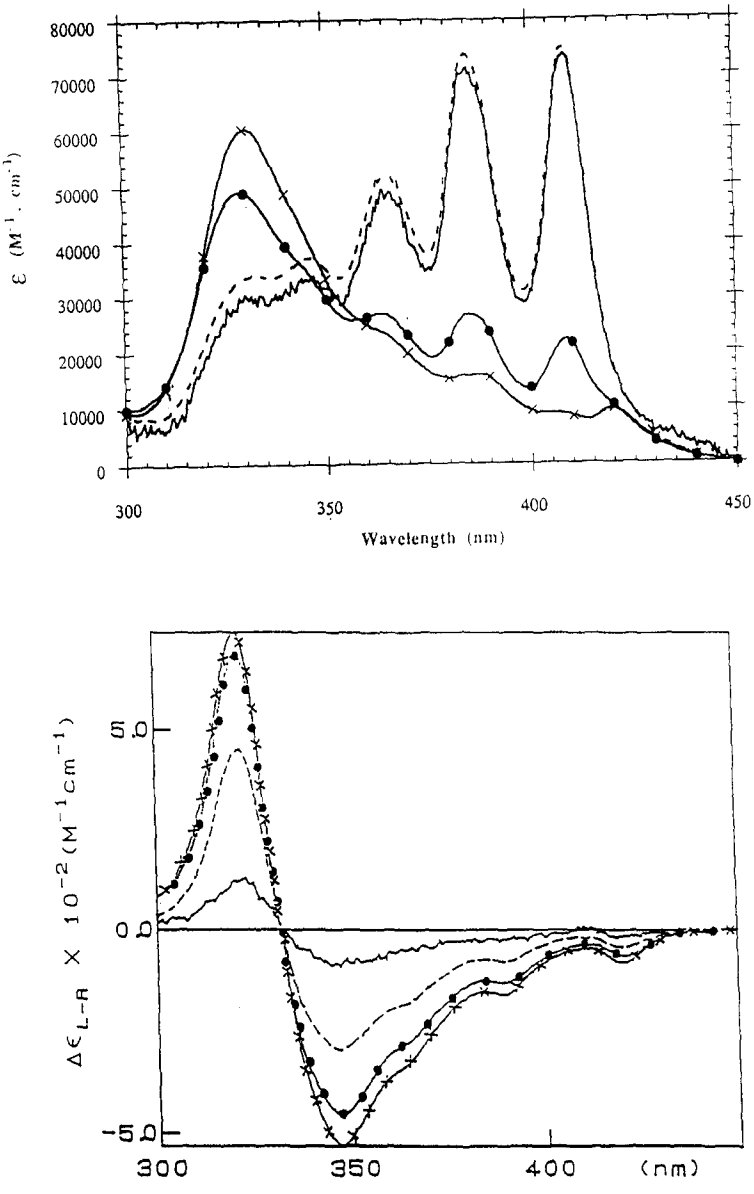

Fig. 2. Absorption and circular dichroism spectra of the Fungizone® stock solution (Fungi-SS) in PBS. The AmB concentrations were 5.10^{-5} M (– × –), 5.10^{-6} M (– • –), 5.10^{-7} M (- -) and 5.10^{-8} M (——).

slight increase of the amplitude of the band at 409 nm and a decrease of the intensity of the band at 327 nm (electronic absorption) and to a slight reduction in the amplitude of the dichroic doublet in CD. Although this reduction of AmB self-association was more pronounced at very high AmB dilution (5.10^{-8} M), the AmB aggregation band and doublet were still observed, while these had disappeared in AmB in aqueous suspension at 5.10^{-7} M.

No changes in the CD or electronic absorption spectra were observed after analytical ultracentrifugation of the AmB emulsions (Fig. 4).

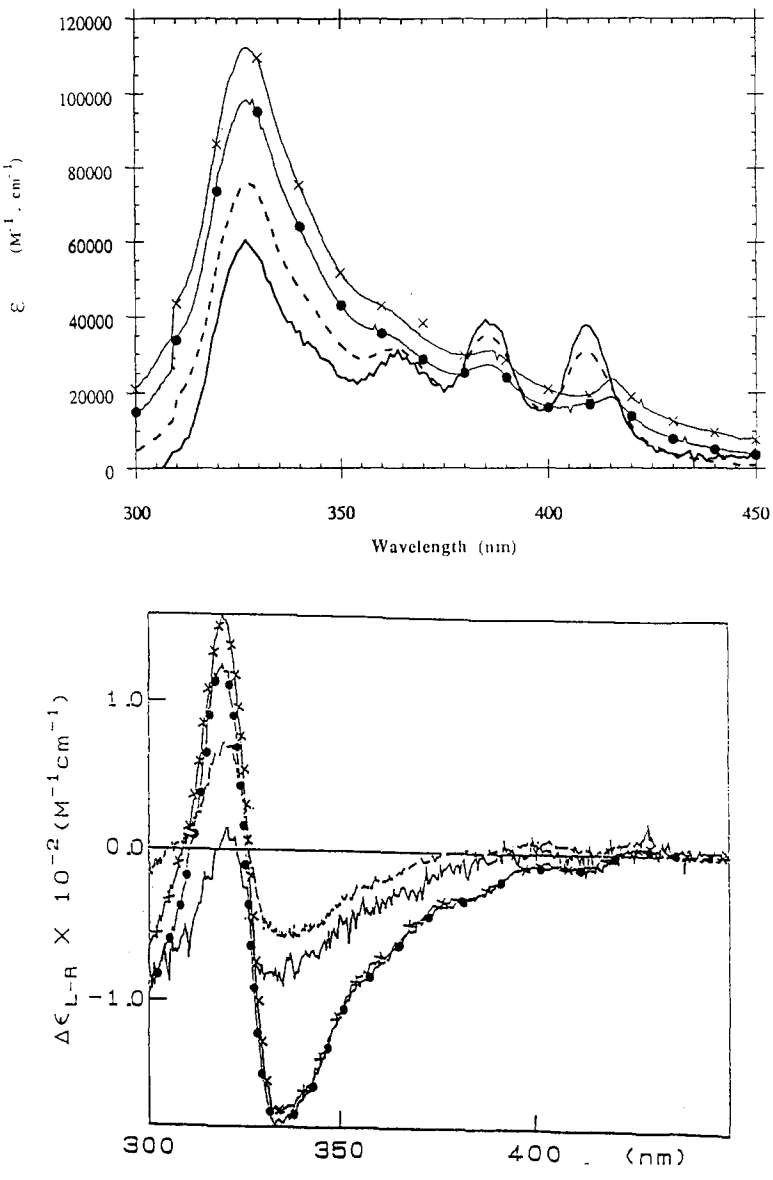

Fig. 3. Absorption and circular dichroism spectra of the amphotericin B emulsion (AmB-E) in PBS. The AmB concentrations were 5.10^{-5} M (– × –), 5.10^{-6} M (– • –), 5.10^{-7} M (- -) and 5.10^{-8} M (——).

3.2. Mean particle size studies

No significative changes in the AmB emulsion particle size (60 ± 49 nm to 182 ± 119 nm) were observed between 5.10^{-5} and 5.10^{-7} M. At 5.10^{-8} M the size increased to about 2300 nm (Table 1).

The particle size of AmB-SS was concentration-dependent. For low concentrations (5.10^{-8} M) the size was 91 nm, however at high concentrations it was higher than the upper limit of the equipment (> 10 000 nm).

At high concentration Fungizone® aggregates were also very large (> 10 000 nm). Dilutions to

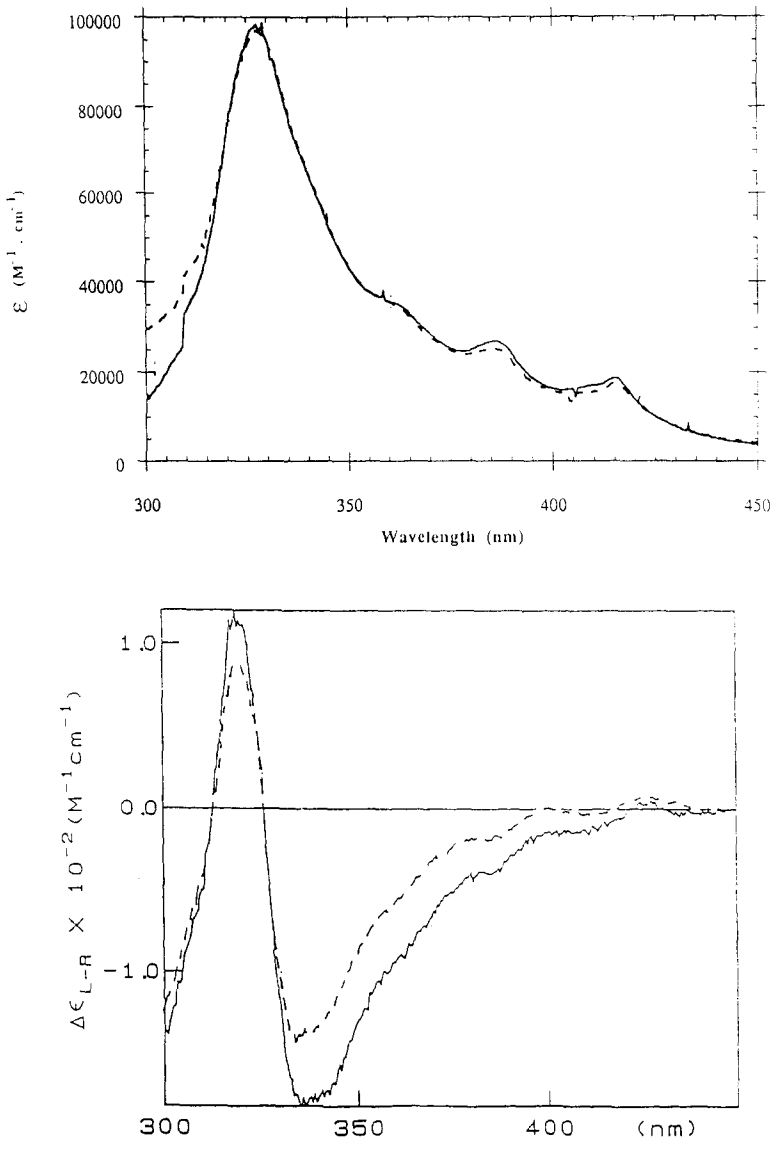

Fig. 4. Absorption and circular dichroism spectra of the amphotericin B emulsion (5.10^{-6} M) before (——) and after (- -) analytical ultracentrifugation experiments.

lower concentrations decreased their size which, however, remained at about 2 μm.

3.3. Transmission electronic microscope (TEM) analysis

The emulsions with (Fig. 5) or without (Fig. 6) AmB had very different aspects. Indeed, the free AmB emulsions were less electron-dense than AmB-loaded emulsions. Nevertheless, no changes in particle size were observed.

4.Discussion

The aim of new amphotericin B formulations is

to reduce the toxicity of this potentially useful anti-fungal drug. Many studies have suggested that this toxicity is related to the physico-chemical state of the drug, and particularly to self-associated forms which are able to complex cholesterol in mammalian cell membranes. Therefore, it seemed important to determine the state of the amphotericin B molecules in the emulsions. Electronic absorption and circular dichroism spectra are powerful tools for this sort of analysis.

Our studies of an aqueous solution of amphotericin B (AmB-SS) confirm the observations of Mazerski et al. (1990), that the spectra are concentration-dependent. The monomeric form, characterized by a band at 409 nm, is most prominent in dilute aqueous preparations. At higher concentrations, self-associated forms are present, as evidenced by the very intense doublet in the CD spectra (Ernst et al., 1981; Mazerski et al., 1982; Strauss and Kral, 1982).

When the proprietary form of AmB, Fungizone®, was diluted to 5.10^{-6} M in PBS, the band characteristic of self-associated forms was shifted to a slightly lower wavelength. We interpret this as indicating that these forms are complexed with deoxycholate. However, this shift disappeared on further dilution. Since the deoxycholate concentration in Fungizone® diluted to 5.10^{-7} M AmB is very low and probably below its critical micellar concentration (CMC), it is possible that detergent molecules dissociate from the AmB complex and dissolve in the water. Thus, at high dilutions, Fungizone® behaves like an aqueous solution of AmB (Lamy-Freund et al., 1991).

Table 1
Mean particle sizes of AmB-SS, Fungi-SS and AmB-E at different AmB concentrations

AmB concentration (M)	Particle size (nm)		
	AmB-SS[a]	Fungi-SS[b]	AmB-E[c]
5.10^{-8}	91±25	2397±560	2323±1767
5.10^{-7}	687±223	2787±707	182±119
5.10^{-6}	2527±623	2230±703	139±84
5.10^{-5}	>10000	>10000	60±49

[a]Amphotericin B stock solution.
[b]Fungizone® stock solution.
[c]Amphotericin B emulsion.

The results obtained on dilution of the emulsion were in complete contrast. Firstly, except at very low AmB concentration (5.10^{-8} M), neither the electronic absorption spectra nor the CD spectra varied greatly as a function of the AmB concentration. The band due to self-associated AmB (at 327 nm in this preparation) was maintained at high dilutions, while the proportion of the monomeric form remained low. This suggests that AmB is strongly associated with one component of the emulsion. The fact that analytical centrifugation did not change the spectra confirms that this is a stable association. Theoretically, AmB could be located either in the oily core of the emulsion droplets or at the interface. In experiments not reported here we found that AmB was not soluble in the oil used (Mygliol 812); therefore we assume that AmB is located in the phospholipid layer, as already suggested by Washington et al. (1988).

Particle size analysis confirmed the stability of the amphotericin B emulsion. Droplet aggregation was only observed at high AmB dilutions. This makes the emulsion very suitable for parenteral administration. In contrast, both the solution of amphotericin B and the commercial preparation (Fungizone®) produced very large particle sizes (>10 000 nm) at 5.10^{-5} M AmB. The instability of Fungizone® after dilution could be one factor determining its toxicity (Lamy-Freund et al., 1991).

Liposomal and other lipid-based forms of amphotericin B have been extensively studied, and some are already in clinical use (Graybill et al., 1982; Lopez-Berestein et al., 1983, 1984; Mehta et al., 1984; Janoff et al., 1988). This sort of formulation is able to reduce the toxicity of AmB, compared to Fungizone®, while conserving its therapeutic activity. According to Jullien et al. (1990) these forms act as a reservoir of monomeric amphotericin B, which binds to the ergosterol of fungal cells but not to the cholesterol of mammalian cells. The physico-chemical data obtained in the present study suggest that a simi-

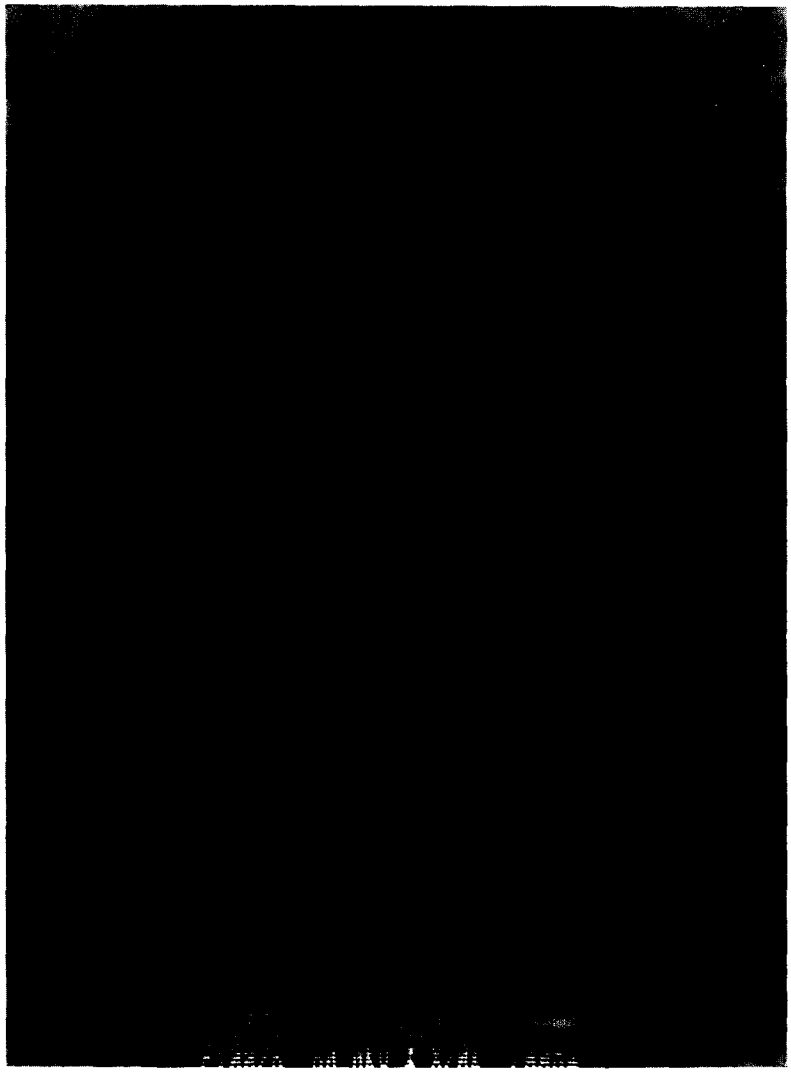

Fig. 5. Transmission electronic microscopy of the AmB emulsion (AmB-E) at $2.5 \cdot 10^{-6}$ M AmB concentration. Bar = 200 nm.

lar situation applies to the emulsion. Amphotericin B is tightly complexed within the droplets, and when it is released it is always in the monomeric form, below the critical concentration for self-association and hence toxicity. Preliminary studies in a mouse model have shown that this form is indeed less toxic than Fungizone® while retaining antifungal activity (Tabosa do Egito et al., 1996). Furthermore, the emulsion form may have some practical advantages over those previously described in that it does not include expensive semi-synthetic lipids and can be prepared by a simple spontaneous emulsification process which is amenable to scale-up. We therefore believe that further evaluation of this new amphotericin B delivery system is merited.

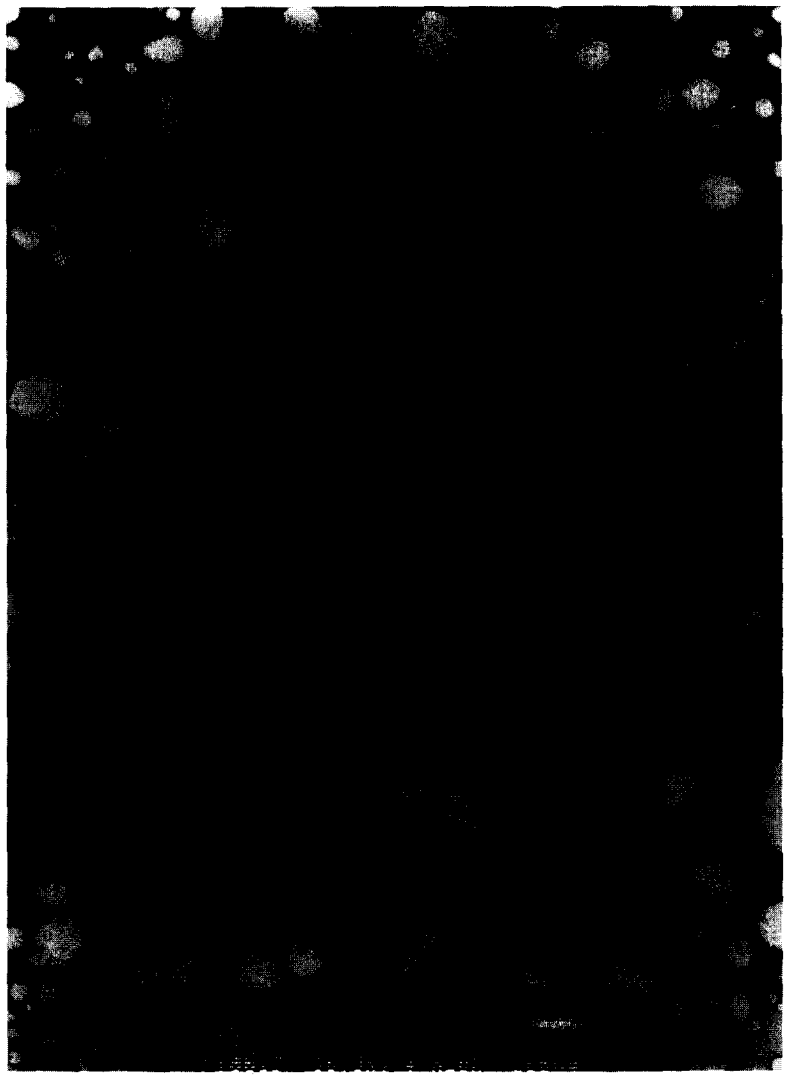

Fig. 6. Transmission electronic microscopy of the emulsion without AmB. Bar = 200 nm.

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