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Spectroscopic studies of amphotericin B solubilized in nanoscale bilayer membranes

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Abstract

Nanodisks (ND) are discrete nanometer scale phospholipid bilayers whose perimeter is circumscribed by amphipathic apolipoproteins. The membranous environment of ND serves as a matrix for solubilizing the polyene antibiotic amphotericin B (AMB). The spectral properties of AMB in ND are dependent upon AMB concentration. Whereas AMB-ND prepared at a concentration of 2.5 mg AMB per 10 mg phospholipid are consistent with AMB self association in the ND membrane environment, AMB-ND prepared at 0.25 or 0.025 mg AMB per 10 mg phospholipid give rise to spectra reminiscent of AMB in organic solvent. Incubation of ND prepared at a phospholipid/AMB ratio of 400:1 (w/w) at 37 °C for 1 h induced a shift in absorbance and near UV circular dichroism spectra consistent with antibiotic self-association. The kinetics of this spectral transition were investigated as a function of incubation temperature. While no change in A_{388} nm occurred in incubations at 20 °C, a time-dependent decrease in A_{388} nm was observed at 25, 30 and 37 °C. Inclusion of ergosterol in the ND membrane attenuated temperature-induced AMB spectral changes. In *Saccharomyces cerevisiae* growth inhibition assays, ND containing self associated AMB were somewhat less effective than ND possessing a greater proportion of monomeric AMB. On the other hand, inclusion of ergosterol or cholesterol in the ND particle did not alter the growth inhibition properties of AMB-ND. The miniature membrane environment of ND provides a novel milieu for solubilization and characterization of lipophilic biomolecules.

Keywords: Amphotericin B; Phospholipid; Apolipoprotein; Spectroscopy; Nanodisk

1. Introduction

The macrolide polyene antibiotic, amphotericin B (AMB) is a potent antifungal agent that functions by complexing with the fungal membrane sterol, ergosterol, forming pores that result in leakage of cellular contents [1]. Although its therapeutic effectiveness is hampered by dose-limiting toxicity in vivo, AMB has seen widespread use in the treatment of systemic fungal infections among the rapidly increasing population of immune compromised individuals. Over the past 10 years, it has been demonstrated that combining AMB with lipid significantly decreases its toxicity, thereby enhancing its therapeutic index. These AMB lipid formulations include a colloidal suspension of AMB and cholesteryl sulfate [2] and AMB/dimyristoylphosphatidylglycerol (DMPG)/dimyristoylphosphatidylcholine (DMPC) complexes [3]. A third liposomal formulation of AMB comprised of hydrogenated soy phosphatidylcholine, cholesterol and distearoylphosphatidylglycerol is also commercially available [4]. Recently, we described a novel lipid formulation of AMB [5]. When complexed with phospholipid and amphipathic apolipoprotein, AMB stably integrates into nanometer scale, disk-shaped reconstituted high-density lipoproteins termed nanodisks (ND). Studies suggest that AMB molecules interact with phospholipids in the ND bilayer at amounts up to 2.5 mg AMB/10 mg phospholipids [5]. Furthermore, AMB-ND are effective inhibitors of yeast and pathogenic fungal growth in vitro and display decreased toxicity toward erythrocytes and cultured hepatoma cells.

Abbreviations: AMB, amphotericin B; ND, nanodisk; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; PBS, phosphate buffered saline; DMSO, dimethylsulfoxide; PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism; apo, apolipoprotein

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Table 1
Effect of phospholipid and apoA-I on AMB solubility

Components [a]	Solubilization efficiency [b] (%)
AMB+PL+ApoA-I	98
AMB+PL	9
AMB+ApoA-I	1.5
AMB	0.5

[a] The indicated components were added to PBS and processed as described in Materials and methods for formation of AMB-ND. Individual samples contained 2.5 mg AMB alone or 2.5 mg AMB plus 10 mg phospholipid and/or 4 mg apoA-I. Following bath sonication, the samples were centrifuged at 13,000×*g* for 1 min and an aliquot of the supernatant transferred to DMSO for determination of AMB content.

[b] Solubilization efficiency (%)=the amount of AMB in solution after centrifugation/AMB added to the incubation ×100.

AMB can exist in alternate states, including monomeric, aggregated and super-aggregated states [6–8]. From studies with liposomal membranes, it is known that AMB self-association is concentration and temperature dependent [9,10]. Since ND are fully soluble particles whose AMB content is easily manipulated, this system offers a means to examine concentration- and temperature-dependent spectral properties of this antibiotic as well as the effect of AMB aggregation state on its biological activity. In the present study, we report studies of AMB self-association in the ND membrane environment together with the finding that temperature-induced aggregation of AMB in ND membranes decreases its in vitro yeast growth inhibition activity.

2. Material and methods

2.1. Materials

AMB (USP grade) was obtained from Research Organics Inc. DMPC and DMPG were from Avanti Polar Lipids Inc. Recombinant apolipoprotein (apo) A-I was produced as previously described [11].

2.2. AMB-ND preparation

Phospholipid vesicle dispersions were prepared as described earlier [5]. To the dispersed lipid a given amount of AMB from a stock solution (30 mg/ml in dimethylsulfoxide; DMSO) was added in a subdued light environment. Subsequent addition of apoA-I in buffer leads to a time-dependent decrease in sample turbidity with full sample clarity achieved by bath sonication with the sample temperature maintained below 25 °C. Preparations were dialyzed overnight at 4 °C against PBS and filter sterilized before use.

2.3. Analytical procedures

Protein concentrations were determined by the bicinchoninic acid assay (Pierce Chemical Co.) with bovine serum albumin as standard. Nondenaturing PAGE was performed on 4–20% acrylamide slab gels using a Phast gel system (Pharmacia). Gels were stained with Gel Code Blue (Pierce Chemical Co.) and the relative mobility of ND complexes compared with standards of known size.

2.4. UV/Vis absorbance spectroscopy

Absorbance spectroscopy was performed on a Perkin Elmer Lambda 20 spectrometer with a thermostated cell holder maintained with a temperature controlled circulating water bath. The AMB content of samples was determined by transferring an aliquot to a solution of DMSO and measuring absorbance at 416 nm (AMB extinction coefficient at 416 nm=1.214×10^5 M^{-1} cm^{-1} in DMSO [5]. Scans of intact ND samples in aqueous media were obtained in PBS.

2.5. Light scattering and circular dichroism spectroscopy

Right angle light scattering measurements were obtained on a Perkin-Elmer LS50B luminescence spectrometer. Excitation and emission wavelengths were set at 600 nm (3.0 nm slit width). Near UV circular dichroism (CD) spectra were collected on an Applied Photophysics Pi-Star 180 spectrometer interfaced to an Acorn computer controlled by API software. Spectra were obtained at 20 °C. The instrument was calibrated with ammonium d-(+) camphorsulfonate at 290.5 and 192 nm.

2.6. Yeast growth inhibition assays

Cultures of the yeast, *Saccharomyces cerevisiae* were grown in yeast extract peptone glucose broth media (YEPD; Teknova, Hollister, CA). Eight μl of a saturated overnight culture was used to inoculate 2 ml YEPD media in the absence or presence of indicated amounts of a given AMB-ND preparation. Cultures were grown for 36 h at 18 °C with rotation and the extent of culture growth monitored by measuring sample turbidity at 600 nm. In the case of ergosterol and cholesterol containing ND, *S. cerevisiae* growth inhibition assays were conducted at 30 °C for 16 h.

3. Results

3.1. AMB solubilization in nanodisk particles

To assess the ability of apoA-I to solubilize a mixture of phospholipid and AMB, various components of the system

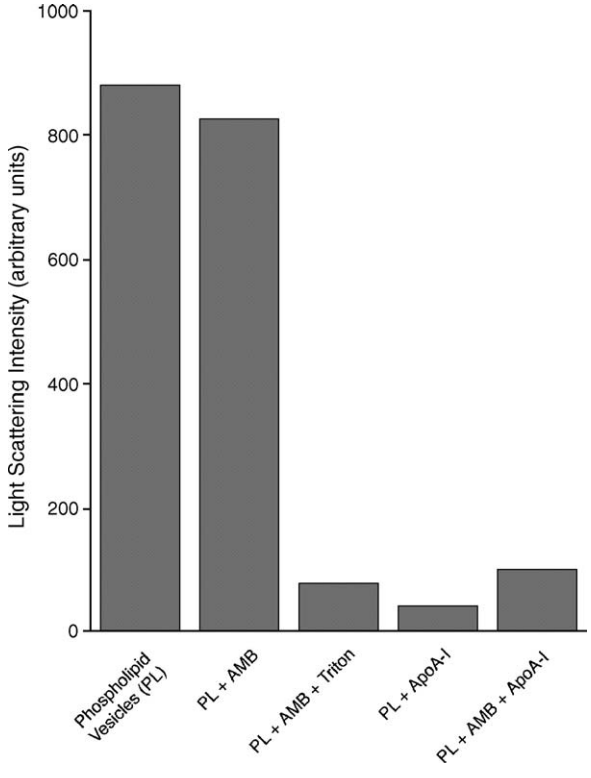

Fig. 1. Effect of apoA-I on the light scattering intensity of phospholipid and AMB. DMPC vesicles, in the absence or presence of AMB (PL:AMB, 4:1 w/w), were incubated alone or with apoA-I (4 mg protein per 10 mg DMPC) as described in Material and methods. An aliquot of each sample was subjected to right angle light scattering intensity analysis on a Perkin-Elmer LS50B luminescence spectrometer. Baseline light scattering was determined by inclusion of 1% Triton X-100.

were incubated under conditions identical to those used to prepare AMB-ND. Following this, the samples were centrifuged at 13,000×*g* for 1 min and the amount of AMB retained in the supernatant determined. In incubations containing AMB, phospholipid and apoA-I, 98% of the original AMB was recovered in the supernatant fraction (Table 1). By contrast, in incubations containing AMB and phospholipid, AMB and apoA-I or AMB alone, far less AMB was recovered in the supernatant fraction. In light scattering studies phospholipid vesicles alone or a phospholipid vesicle/AMB mixture displayed significant sample light scattering (Fig. 1). Addition of Triton X-100 (1%; v/v) induced a large reduction in light scattering intensity consistent with solubilization of the components in detergent micelles. ApoA-I induced a comparable decrease in the light scattering intensity of the phospholipid vesicles, consistent with formation of ND. ApoA-I induced a similar decrease in light scattering intensity of a phospholipid vesicle/AMB mixture, indicating solubilization the reaction components.

3.2. Effect of AMB concentration on antibiotic self-association in ND

Absorbance spectroscopy was performed to investigate the organization of AMB in the soluble ND particles. It is known that monomeric AMB (dissolved in DMSO) displays a characteristic set of absorbance maxima [5]. In aqueous media or membrane environments, characteristic changes in AMB absorbance spectra have been ascribed to self-association of the antibiotic [9,10,12,13]. Consistent with this, AMB-ND formulated at a phospholipid:AMB ratio of 4:1 (w/w) gives rise to a spectrum consistent with AMB self association [5]. It is likely that, compared to an organic solvent environment, sequestration of AMB in the ND membrane environment has the effect of concentrating the antibiotic. To evaluate the effect of AMB concentration on the extent of antibiotic self-association within the ND membrane environment, AMB-ND were prepared with different amounts of AMB. The phospholipid:AMB weight ratio was increased from 4:1 to 40:1 or 400:1 by decreasing the AMB content of the ND. Compared to ND prepared with 2.5 mg AMB per 10 mg phospholipid, absorbance spectra of ND containing less AMB revealed differences consistent with the presence of AMB monomers (Fig. 2). The spectrum of ND containing 0.25 mg AMB per 10 mg phospholipid displayed an increase in absorbance peaks characteristic of monomeric AMB. At a 400:1 phospholipid:AMB weight ratio (0.025 mg AMB per 10 mg phospholipid), an even greater proportion of AMB was present as monomer, as judged by the enhancement in absorbance peaks at 422 and 388 nm at the expense of the absorbance peak at 340 nm. The higher absorbance in the short wavelength region of spectrum c arises from tryptophan residues in apoA-I. ND harboring different amounts of AMB had similar particle diameters (8.5–9.0 nm), as judged by non-denaturing gradient PAGE (Fig. 2, inset) with minor particle size heterogeneity noted for ND prepared with different phospholipid:AMB ratios.

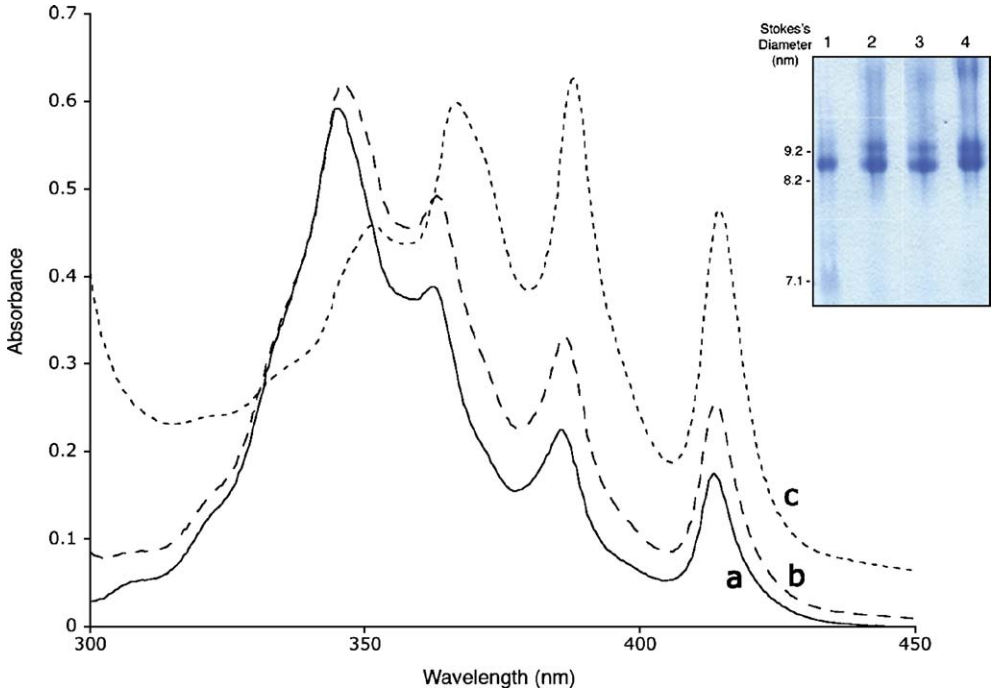

Fig. 2. AMB-ND UV/Vis absorbance spectral properties. ND prepared at different AMB: phospholipid ratios were scanned from 300 to 450 nm. In each case the amount of ND scanned was adjusted to include 8 μg AMB. (Curve a) AMB-ND (4:1 phospholipid/AMB; w/w); (Curve b) AMB-ND (40:1 phospholipid/AMB; w/w); Curve c) AMB-ND (400:1 phospholipid/AMB; w/w). Inset: native PAGE analysis of AMB-ND. ND were prepared at different AMB: phospholipid ratios. (Lane 1) ND lacking AMB; (Lane 2) AMB-ND (4:1 phospholipid/AMB; w/w); (Lane 3) AMB-ND (40:1 phospholipid/AMB; w/w); (Lane 4) AMB-ND (400:1 phospholipid/AMB; w/w).

3.3. Temperature-induced AMB self-association

Using ND wherein a large proportion of AMB is present as monomer, aspects of antibiotic self-association were investigated. All studies described below were conducted using AMB-ND prepared at a phospholipid/AMB ratio of 400:1 (w/w). Incubation of AMB-ND at 37 °C for 1 h induced a shift in the absorbance spectrum indicative of antibiotic self-association within the ND membrane (Fig. 3). To verify that AMB had interacted with ND particles and not residual phospholipid vesicles that may have contaminated the preparation, ND lacking AMB were isolated by Sepharose 6B gel permeation chromatography. Subsequent addition of AMB resulted in a spectral properties that were indistinguishable from those presented in Fig. 3. Others have shown that, in addition to characteristic changes in UV/Vis absorbance spectra, AMB self-association induces significant near UV CD spectral changes, characterized by the appearance of a large dichroic shift between 325 and 350 nm [12,13]. Near UV CD spectra of AMB-ND at 20 °C gives rise to a spectrum characteristic of monomeric AMB (Fig. 4). Upon incubation of AMB-ND at 37 °C for 1 h, a significant spectral change is observed, including the appearance of a large dichroic shift between 325 and 350 nm. Thus, evidence from CD spectroscopy and absorbance spectroscopy indicate AMB present in the ND membrane environment as monomers can be induced to self-associate as a function of increasing temperature.

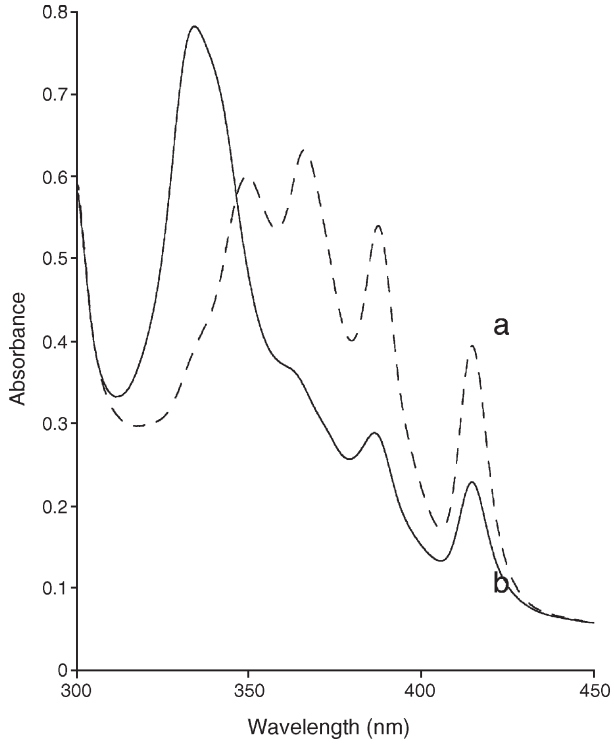

Fig. 3. Effect of temperature on AMB-ND spectral properties. AMB-ND were prepared at a 400:1 phospholipid/AMB (w/w) ratio. The resulting AMB ND were incubated at 4 °C and 37 °C for 1 h. Following this the samples were adjusted to 20 °C and absorbance spectra were obtained from 300 to 450 nm. (Curve a) AMB-ND incubated for 1 h at 4 °C; (Curve b) AMB-ND incubated at 37 °C for 1 h. The AMB concentration of the sample was 0.01 mg/ml.

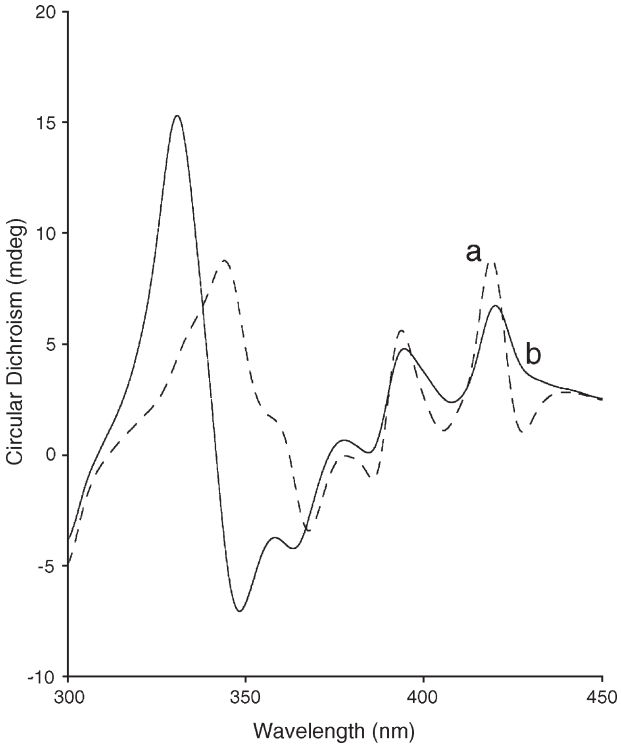

Fig. 4. Effect of temperature on near UV CD spectra of AMB in ND. AMB-ND prepared at a starting phospholipid/AMB ratio of 400:1 (w/w) in PBS were scanned from 300 to 450 nm. (Curve a) AMB-ND incubated for 1 h at 4 °C; (Curve b) AMB-ND incubated at 37 °C for 1 h. Spectra were obtained at 20 °C. The AMB concentration of the sample was 0.01 mg/ml.

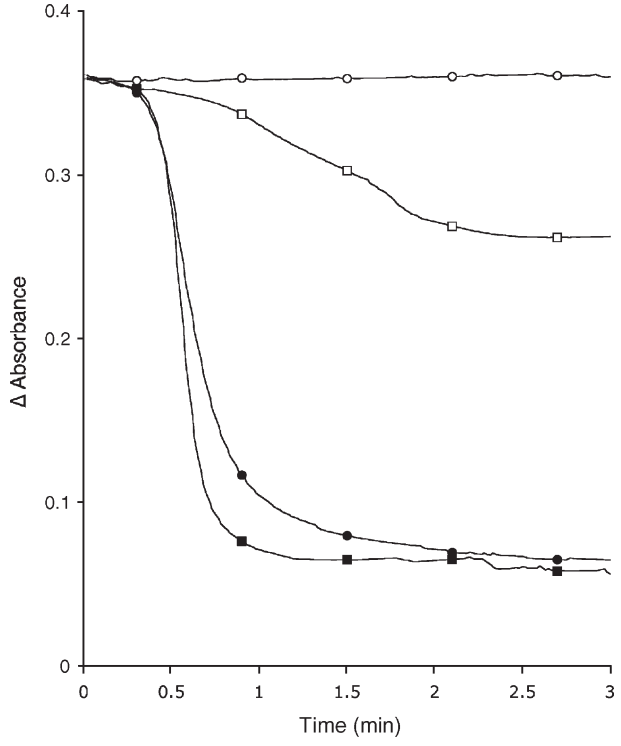

Fig. 5. The effect of temperature on spectral properties of AMB-ND. AMB-ND (0.01 μg/ml AMB) samples were transferred from 4 °C to the indicated temperature in the thermostated cell holder of a Perkin-Elmer Lambda 20 UV/Vis spectrophotometer. Sample absorbance at 388 nm was monitored as a function of time at 20 °C (○), 25 °C (□), 30 °C (●) and 37 °C (■).

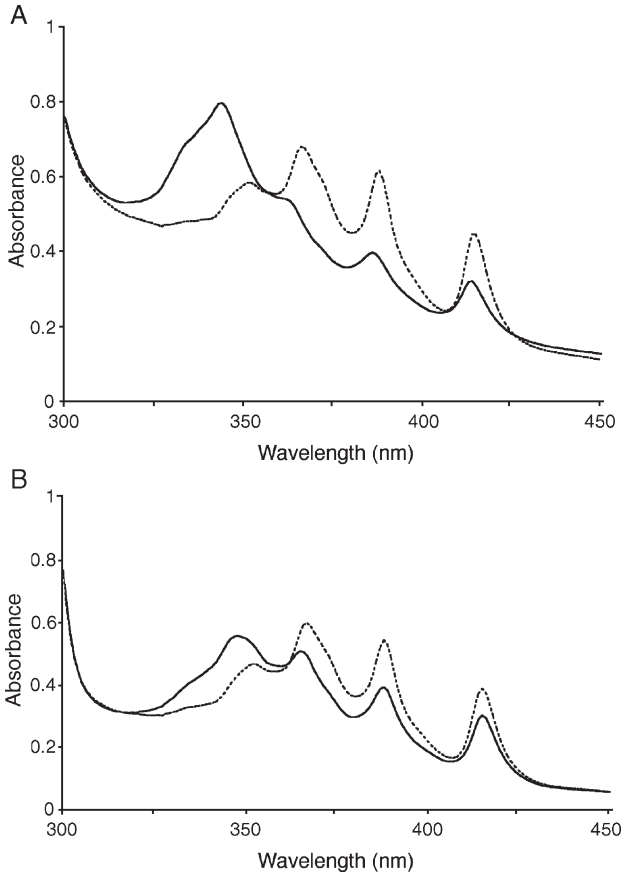

Fig. 6. Effect of sterol content on temperature-induced AMB-ND spectral properties. AMB-ND (400:1 phospholipid:AMB (w/w) were prepared with cholesterol or ergosterol (2% by weight versus phospholipid). UV/Vis absorbance scans were collected from 300 to 450 nm following incubation at 4 °C or 37 °C for 1 h. Scans were obtained at 20 °C. (Panel A) Cholesterol containing AMB-ND following incubation at 4 °C (dashed line) and 37 °C (solid line). (Panel B) Ergosterol containing AMB-ND following incubation at 4 °C (dashed line) and 37 °C (solid line).

One of the characteristic effects of AMB self-association on the absorbance spectrum of AMB-ND in buffer is a decrease in absorbance peak intensity at 388 nm with a corresponding increase in the absorbance maximum at ~340 nm. To examine the kinetics of spectral changes ascribed to AMB within the ND membrane environment, AMB-ND absorbance at 388 nm was monitored as a function of time at specified temperatures. Fig. 5 presents plots of absorbance at 388 nm versus time in incubations of AMB-ND at 20, 25, 30 and 37 °C. Whereas little or no change in A_{388} nm occurred upon incubation at 20 °C, a time-dependent decrease in A_{388} nm was observed at 25, 30 and 37 °C. Although the change in absorbance was more rapid at 37 °C than at 30 °C, both samples reached the same end point absorbance value. In further studies, the reversibility of the spectral transition was examined (data not shown). Whereas spectral changes induced by incubation of AMB-ND at 25 °C were reversible upon decreasing the temperature to 18 °C, the spectral change induced upon incubation at 37 °C was unaffected by decreasing temperature. It is noteworthy that the gel to liquid crystalline phase transition temperature of DMPC is 24 °C and that of DMPG is 23 °C. Thus, it is conceivable that the thermotropic phase behavior of the phospholipid component of ND is related to the temperature specific alterations in AMB spectral changes that correlate with antibiotic self-association state.

3.4. Influence of cholesterol and ergosterol on temperature-dependent AMB spectral changes

AMB-ND prepared with 2% cholesterol or 2% ergosterol, were indistinguishable from AMB-ND lacking these sterols in terms of particle size. Compared to spectra of AMB-ND lacking sterol (see Fig. 3), with the exception of background light scattering, introduction of cholesterol had little effect on the starting or final AMB spectra. On the other hand, ergosterol attenuated the temperature induced spectral shift (37 °C), resulting in a final spectrum that displays a less pronounced decrease in absorbance intensity at 422 nm and 388 nm together with an attenuated temperature-induced increase in the absorbance peak intensity at ~340 nm (Fig. 6). In other studies, the effect of including ND lacking AMB in a 1:1 ratio with AMB-ND on the kinetics of temperature-induced AMB self-association was examined. The presence of ND lacking AMB had no effect on the rate of AMB absorbance changes characteristic of self association induced upon incubation of ND at 37 °C.

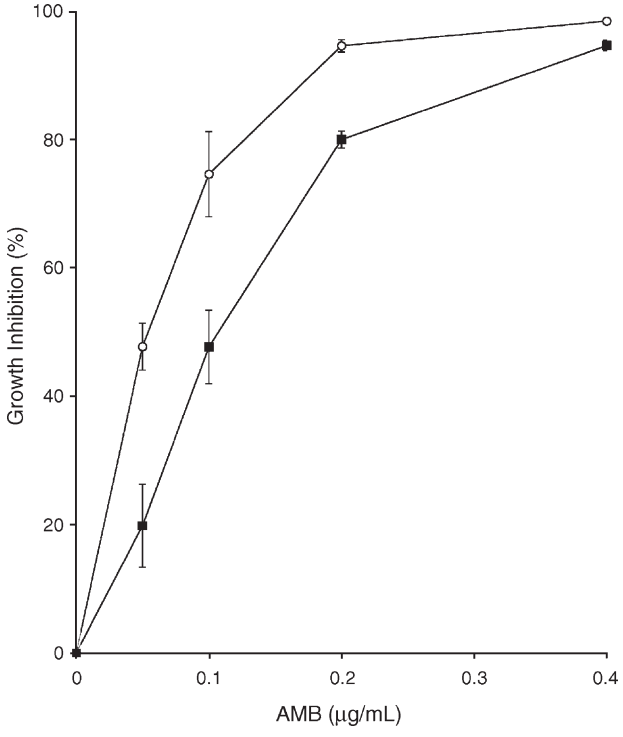

Fig. 7. Effect of AMB-ND on yeast growth. Eight μl of a saturated overnight culture of *S. cerevisiae* grown in YEPD media was used to inoculate 2 ml YEPD media in the presence of indicated amounts of specified AMB-ND samples. Cultures were incubated at 18 °C for 36 h at which time the extent of culture growth was determined spectrophotometrically. AMB-ND pre-incubated at 4 °C (○); AMB-ND pre-incubated for 1 h at 37 °C (■). Values reported are the mean±S.D. (n=3).

3.5. Effect of AMB self-association on S. cerevisiae growth inhibition activity

Having established that AMB self-association in the ND membrane environment proceeds in seconds at 37 °C and, once achieved, is irreversible we used this property to assess the effect of AMB self-association state on its biological activity. AMB-ND preparations harboring self-associated or largely monomeric AMB were employed in *S. cerevisiae* growth inhibition assays. A saturated yeast culture, grown at 20 °C, was used to inoculate YEPD media to which increasing amounts of specified AMB-ND preparations were added. The yeast were cultured at 18 °C (to prevent temperature-induced aggregation of AMB in ND harboring largely monomeric AMB) and, after 36 h, yeast growth was measured by absorbance spectroscopy (Fig. 7). The data show that ND containing self-associated AMB is a less effective growth inhibitor than ND possessing a greater proportion of monomeric AMB.

3.6. Effect of ergosterol or cholesterol on AMB-ND growth inhibition

AMB concentration-dependent *S. cerevisiae* growth inhibition assays were conducted with AMB-ND lacking sterol, cholesterol bearing AMB-ND and ergosterol bearing AMB-ND (Fig. 8). The data revealed no significant differences in the growth inhibition properties of these ND as a function of cholesterol or ergosterol content.

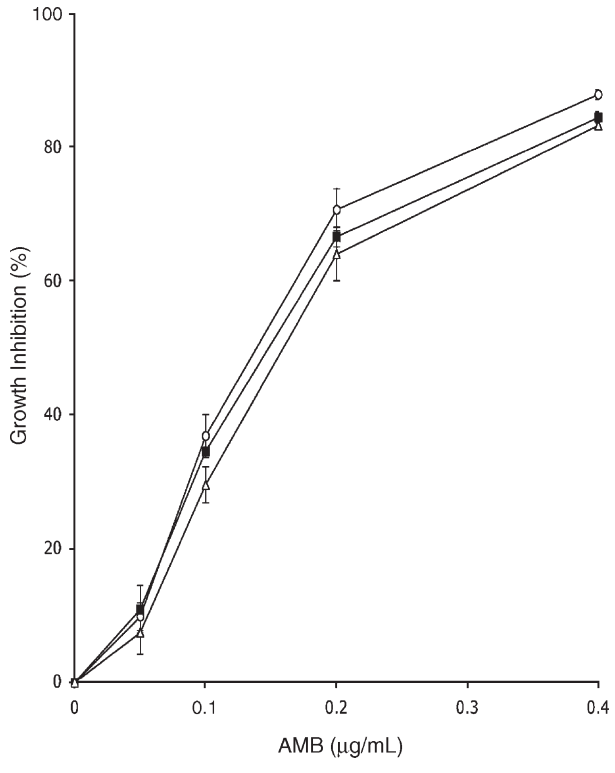

Fig. 8. Effect of cholesterol and ergosterol on AMB-ND yeast growth inhibition. Cultures of *S. cerevisiae* were grown in YEPD media in the presence of indicated amounts of specified AMB-ND preparations. Cultures were grown for 16 h at 30 °C and the extent of yeast growth determined spectrophotometrically. AMB-ND without sterol (○); AMB-ND containing 2% cholesterol (■) and AMB-ND containing 2% ergosterol (△). Values reported are the mean±S.D. (n=3).

4. Discussion

AMB is a poorly soluble, amphoteric molecule that elicits its biological effects by membrane pore formation via sterol–AMB interactions [14]. Monolayer balance studies have revealed that the self-association state of AMB affects its interaction with cholesterol or ergosterol when present in a dioleoylphosphatidylcholine monolayer [15]. In the present studies, ND particles were employed as a discrete bilayer membrane environment capable of solubilizing AMB, thereby permitting study of antibiotic aggregation and/or sterol interactions. ND are distinct from liposomes in that they do not possess an internal aqueous space and the edges of the disc-shaped bilayer are stabilized by amphipathic apolipoproteins. Furthermore, ND are fully soluble particles that display low light scattering intensity. In keeping with results obtained with liposomes [9], at high phospholipid/AMB ratios, UV/Vis absorbance spectra of ND are characteristic of the presence of monomeric AMB [12,13].

From detailed studies of reconstituted high density lipoproteins (e.g., ND) lacking AMB, it can be concluded that ND are comprised of 2 molecules of apoA-I and approximately 160–200 phospholipid molecules, organized as a disk-shaped bilayer whose perimeter is circumscribed by apolipoprotein molecules [16,17]. The finding that AMB associates with these particles extends previous studies showing that native lipoproteins or reconstituted lipoproteins can serve as drug transport vehicles. For example, Kader and Pater [18] found that native lipoproteins can serve as carriers for anticancer drugs while Lacko et al. [19] and Lou et al. [20] successfully incorporated anti-tumor agents into spherical reconstituted HDL. While AMB is the first drug molecule to be incorporated into discoidal ND particles, it is noteworthy that others [21] have shown that the membrane interacting fluorescent probe, laurdan, can also be incorporated into ND particles. Given the structural properties of AMB, which include prominent polar and non-polar aspects, it is interesting to speculate about the interaction of AMB with ND. At low AMB concentrations, it is conceivable that monomeric AMB molecules orient perpendicular to phospholipid fatty acyl chains of the bilayer, inserting its hydrophobic conjugated heptaene structural region between polar head groups of the phospholipids, maintaining exposure of its hydoxyl-rich segment and mycosamine ring element to the aqueous milieu. AMB associated with a bilayer in this manner may be better able to interact with other AMB molecules located either in the same membrane or in a separate membrane. Although Figs. 2c and 3a suggest there may be some aggregated AMB present, transfer of AMB among ND particles would seem to be a prerequisite for AMB self association observed upon incubation of the sample at 37 °C. Since inclusion of ND lacking AMB had no effect on AMB self-association kinetics, it is evident that AMB exchange or transfer within or among ND is not a random process but rather, is likely controlled by an intrinsic affinity of this polyene for other AMB molecules.

The biological activity of AMB and its specificity for fungal membranes is thought to be due to a preferred interaction between AMB and ergosterol versus the cholesterol component of host membranes [1]. The present results provide evidence in favor of this concept. By monitoring the effect of temperature on AMB spectral changes correlated with self-association, it was observed that inclusion of cholesterol in the ND membrane had little or no effect on temperature-induced AMB spectral changes, compared to AMB-ND lacking sterol. By contrast, AMB-ND prepared with ergosterol display resistance to temperature-induced spectral changes. In this case, it is conceivable that interactions between AMB and ergosterol in a given ND inhibit inter-ND transfer of AMB, thereby affecting the rate and extent of temperature-dependent AMB self-association.

In an effort to extend the present results to the biological activity of AMB, growth inhibition studies were conducted with *S. cerevisiae*. From previous studies, it is known that AMB-ND are highly effective inhibitors of fungal growth [5]. To ascertain whether the extent of AMB aggregation within the ND membrane environment alters its growth inhibition properties, AMB-ND were incubated for 1 h at 37 °C to induce irreversible AMB spectral changes correlated with antibiotic aggregation. It was observed that ND containing aggregated AMB are less effective inhibitors of *S. cerevisiae* growth compared to ND possessing largely monomeric AMB. These results may be due to a decreased ability of aggregated versus monomeric AMB to transfer into the yeast membrane environment. Alternatively, it may be that self-associated AMB in ND is less able to recognize and interact with ergosterol in the yeast membrane. Although further studies are necessary to determine the precise orientation, alignment and organization of AMB in ND membrane environments, it is evident that these fully soluble, nanometer scale, uniform sized, miniature membranes are well suited to such analyses.

It is noteworthy that apolipoprotein stabilized phospholipid disk complexes (i.e., ND) have been well characterized in terms of their role as precursors of plasma high density lipoproteins [22] and have been employed as discrete miniature membranes for solubilization of membrane bound proteins [23]. In the present studies, a poorly soluble polyene antibiotic has been integrated into the bilayer environment of ND membranes, illustrating the potential utility of these particles for investigation of the spectroscopic properties of lipophilic chromophores and the effect of membrane lipid content and composition on spectral parameters. Further studies will reveal the extent to which ND can be used to characterize other membrane embedded solutes and, in a manner complementary to liposomal membranes, serve as vehicles for the solubilization, transport and delivery of hydrophobic drugs.

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