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Effect of Cholesterol on the Release of Amphotericin B from PEG-Phospholipid Micelles

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Abstract: Micelles formed from PEG-DSPE solubilize high levels of the poorly water-soluble antifungal amphotericin B (AmB). AmB release from PEG-DSPE micelles is slow in buffer but remarkably rapid in the presence of bovine serum albumin (BSA). Sequential changes in the absorbance spectrum of AmB in PEG-DSPE micelles point to a rapid dissociation of incorporated drug in the presence of BSA. In this context, we have studied micelles formed from PEG-DSPE which coincorporate cholesterol (PEG-DSPE|cholesterol). ^{1}H NMR measurements point to a lower mobility of lipid in PEG-DSPE|cholesterol micelles compared to PEG-DSPE micelles. The absorbance spectrum of AmB incorporated in PEG-DSPE|cholesterol micelles is distinct from that in PEG-DSPE micelles, which may point to differences in the drug−micelle interaction. AmB release from PEG-DSPE|cholesterol micelles is slow in buffer and in the presence of BSA. The absorption spectrum of AmB in PEG-DSPE|cholesterol micelles remained unchanged in BSA, further supporting stable incorporation and the slow release from the carrier.

Keywords: PEG-lipid; cholesterol; micelle; condensing effect; polyene macrolide; amphotericin B; drug delivery; serum albumin; in vitro release

Introduction

Amphotericin B (AmB) is a poorly soluble antifungal drug used to treat systemic fungal diseases, despite severe toxicities. AmB toxicity is thought to be mediated by the relative aggregation state of the drug.[1–4] A goal of our research is to explore the utility of polymeric micelles to solubilize AmB and to reduce its toxicity without an appreciable loss in antifungal activity. We have recently described our efforts to study PEG-DSPE micelles for the delivery of AmB.[5] PEG-DSPE micelles could incorporate and deaggregate AmB on the basis of absorption spectroscopy. AmB incorporated in PEG-DSPE micelles simultaneously (1) exerted less hemolytic activity than free AmB and (2) retained potent antifungal activity. This 2-fold characteristic suggested that AmB is released from PEG-DSPE micelles in a monomeric form, which is selectively active against fungal cells. However, we found that the *in vivo* toxicity of this formulation correlated poorly with *in vitro* assays and was comparable to the standard formulation, D-AmB (AmB solubilized by sodium deoxycholate), possibly due to rapid release of AmB in blood.

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In this work, we have investigated AmB release in buffer and in the presence of serum albumin using a dialysis setup. PEG-DSPE micelles release AmB slowly in buffer, presumably in a monomeric form. However, there is rapid dissociation of incorporated AmB in a physiologically relevant concentration of serum albumin. Clinical research has recently shown that administration of D-AmB as a continuous infusion over 24 h is better tolerated compared to an equal dose administered as a standard 2–4 h infusion.[6–10] It is becoming increasingly clear that slow delivery of AmB reduces toxic manifestations of this important antifungal drug. In this context, we have prepared novel PEG-DSPE micelles coincorporating cholesterol (PEG-DSPE|cholesterol) with the objective of designing a polymeric micelle carrier with slow AmB release in buffer and in serum albumin.

Materials

AmB was obtained as a gift from Alpharma (Copenhagen, Denmark) and was stored at −20 °C until use. 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-methoxy(polyethylene glycol) (M_n = 5800 g/mol) (PEG-DSPE) was obtained from Avanti Polar Lipids (Alabaster, AL). Cholesterol and deuterium oxide were obtained from Aldrich (Milwaukee, WI). All other reagents used were of analytical grade and were used without further purification.

Micelle Preparation and Incorporation of AmB

PEG-DSPE (6.0 mg/mL in chloroform), cholesterol (1 mg/mL in chloroform), and AmB (0.25 mg/mL in methanol) stock solutions were mixed in a round-bottom flask to obtain the desired AmB:PEG-DSPE:cholesterol ratio. The organic solvent mixture was evaporated under high vacuum to produce a thin film of coprecipitated drug and polymer. This film was dissolved in 10 mM HEPES, pH 7.0 and incubated at 50 °C for 10 min to allow for complete equilibration. The solution was filtered through a 0.45 μm polyethersulfone (PES) syringe filter. The concentration of AmB was quantified by diluting a 50 μL aliquot of AmB in 1.95 mL of DMF and by observing absorbance at 413.5 nm. This assay was tested for linearity in the 0.02–0.8 mg/mL range.

Empty micelles were prepared by using an identical procedure without drug. Samples for ^{1}H NMR were prepared by dissolving the coprecipitated film of PEG-DSPE and cholesterol in deuterium oxide allowing for equilibration at 50 °C for 10 min.

Micelle Characterization

Dynamic Light Scattering. Micelle diameters were determined by using the NICOMP ZLS380 particle sizer (Particle Sizing Systems, Santa Barbara, CA). Data was acquired to have at least 100K counts in Channel 1. The light scattering data was interpreted by using NICOMP analysis, which permits deconvolution into multimodal distributions. Particle sizes were expressed as volume-weighted diameters.

^{1}H NMR Spectroscopy. ^{1}H NMR spectra were acquired at 25 °C on a 400 MHz Varian spectrometer equipped with Varian qn4549 probe (Varian, Palo Alto, CA) using the standard two-pulse sequence.[11] Peak line-widths ($\Delta\nu_{1/2}$) were determined using the Varian software (version 6.1 revision C, Palo Alto, CA).

In Vitro Release Study of Micelle Encapsulated AmB Using Equilibrium Dialysis. Drug release was assessed using a setup similar to that described in the literature.[12,13] AmB stock solutions (micelle encapsulated or dissolved in DMSO) were diluted to 30 μg/mL in HEPES buffer or in 4% BSA. These solutions were put in dialysis cassettes (MWCO 7000 g/mol, Pierce) and were placed in excess buffer (5 mM HEPES, pH 7.0). The dialysis buffer was degassed overnight, and 20 μg/mL of propyl gallate was added to prevent drug degradation.[14] AmB concentrations in the dialysis cassettes were determined at fixed time points using a reversed-phase HPLC method. Protein from samples containing serum albumin was first precipitated by addition of 400 μL of cold methanol and centrifugation at 13 200 rpm (16.1×10^3 *g*) for 10 min.

AmB release was fit to a first-order process of the form $A_t/A_0 = a \cdot e^{-k \cdot t}$ by nonlinear regression (SigmaPlot v.9.0). A_t corresponds to AmB concentration in the dialysis cassette at time t, and A_0 is the initial concentration. The half-life $t_{1/2}$ for AmB release was calculated using $t_{1/2} = 0.693/k$.

HPLC Assay for Estimation of AmB Content. For determination of AmB content, 15 μL samples were injected into a 4.6 mm × 150 mm Eclipse XDB-C8 reversed-phase column (Agilent Technologies) and AmB absorbance de-

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tected at 412 nm using the diode array detector. The mobile phase consisted of a linear gradient of methanol−(5% v/v acetic acid). The column was maintained at 25 °C, and the flow rate of the mobile phase was 2 mL/min. The assay was tested for linearity in the 0.1−100 μg/mL range.

Electronic Absorption Kinetics. Stock solutions containing AmB (micelle encapsulated or dissolved in DMSO) were added to 4% BSA, buffered to pH 7.0 with 10 mM HEPES. The final concentration of AmB was 10 μg/mL. For AmB dissolved in DMSO, the final DMSO content was less than 0.2%. Absorbance spectra were recorded from 300 to 450 nm at room temperature by using the Cary 50 spectrophotometer (Varian, Palo Alto, CA). Sequential spectra were acquired at 15 s intervals for the first 5 min and at 1 min intervals for an additional 40 min. A scan for baseline absorbance was taken before addition of AmB and was subtracted from each spectrum. The change in absorbance at 412 nm, $A_\infty - A_t$ was fit to a first-order process of the form $A_\infty - A_t = a \cdot e^{-k \cdot t}$ using nonlinear regression (SigmaPlot v.9.0).

Results and Discussion

Micelle Preparation and Characterization. PEG-DSPE micelles with a narrow size distribution were obtained by dissolving the film of coprecipitated AmB and PEG-DSPE at 25 °C. Samples prepared by dissolving coprecipitated AmB, PEG-DSPE, and cholesterol at 25 °C resulted in turbid solutions with variable mean particle sizes. PEG-DSPE|cholesterol micelles with a narrow particle distribution could be obtained when the thin film was dissolved at 50 °C, allowing 10 min for equilibration, presumably due to greater mobility of lipid chains at this elevated temperature.

The mean diameter for empty PEG-DSPE micelles as determined using DLS was ca. 16 nm, consistent with previous reports.[15,16] The diameter of PEG-DSPE|cholesterol micelles increased in a composition-dependent manner and reached ca. 60 nm for a 1:1 molar ratio of PEG-DSPE:cholesterol (Figure 1). At a higher level of cholesterol, i.e., PEG-DSPE:cholesterol ratio of 1:2.5, DLS reported two populations−one that corresponded to PEG-DSPE|cholesterol micelles (ca. 60 nm) and another to aggregates with sizes greater than 150 nm. TEM images indicated a narrow, unimodal distribution of particles (data not shown). Increased end-to-end thickness of cholesterol containing lipid vesicles has been attributed to restricted trans-gauche isomerization in the diacyl lipid caused by hydrophobic interaction with

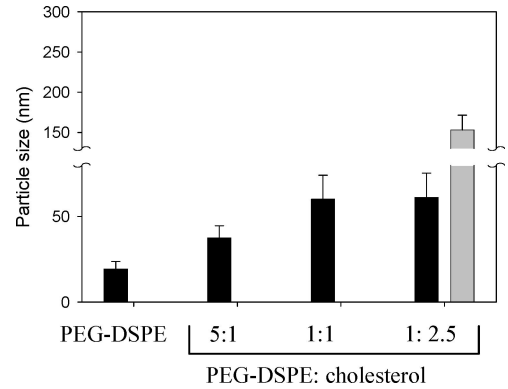

Figure 1. Effect of PEG-DSPE/cholesterol molar ratio on the volume-weighted diameters of PEG-DSPE|cholesterol micelles.

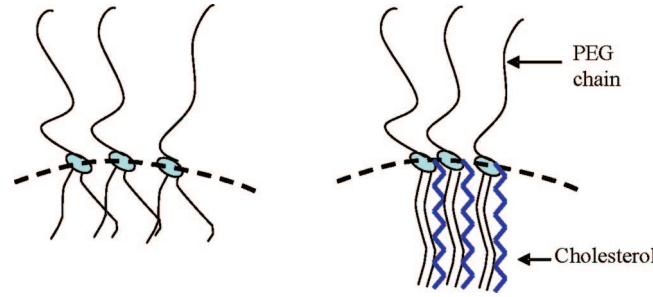

Figure 2. Structuring effect of cholesterol in PEG-DSPE|cholesterol micelles.

cholesterol.[17–19] In the context of PEG-DSPE|cholesterol micelles, we propose a similar interaction between cholesterol and PEG-DSPE resulting in a significant, composition dependent increase in the micelle diameter (Figure 2).

Restricted mobility in the core of polymeric micelles results in broadened line-widths of the core-forming moiety in a ^{1}H NMR spectrum.[11,20] Figure 3a shows the ^{1}H NMR spectrum of PEG-DSPE micelles in D_2O. The widths-at-half-height ($\Delta\nu_{1/2}$) for 1.26 and 0.8 ppm peaks were 8.9 and 13.7 Hz, respectively. High mobility in the core of PEG-DSPE micelles was inferred from the relatively sharp peaks corresponding to protons from the core-forming diacyl chains at 1.26 and 0.8 ppm. The high degree of mobility in PEG-lipid micelles compared to bilayers of similar composition has been attributed to the bulky poly(ethylene glycol)

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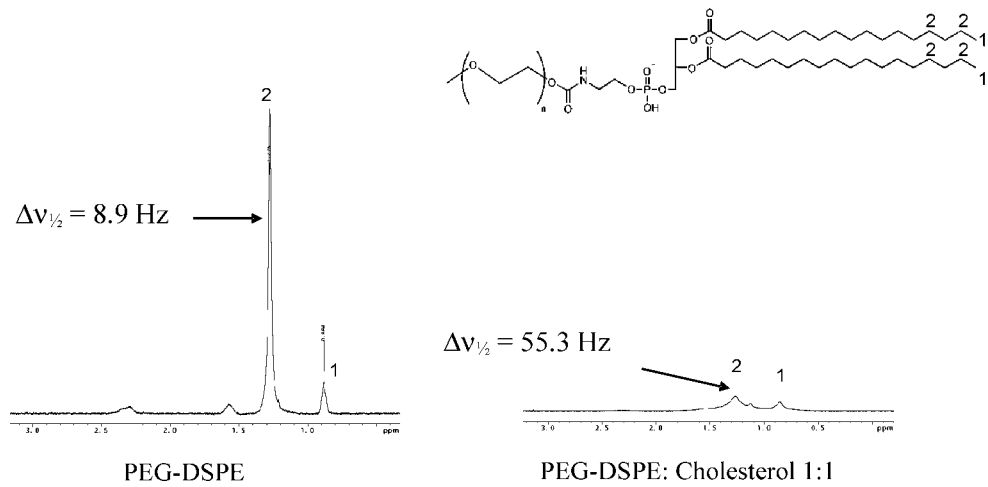

Figure 3. ^{1}H NMR spectra for (a) PEG-DSPE micelles and (b) PEG-DSPE|cholesterol micelles (PEG-DSPE: cholesterol = 1:1) at 25 °C.

Table 1. Solubilization of AmB by PEG-DSPE and PEG-DSPE|cholesterol Micelles

molar ratio AmB/PEG-DSPE/cholesterol	fraction of initial AmB encapsulated	AmB loading (% w/w)	[AmB] (mg/mL)	diameter (nm)
2:1:0	0.78	24.2	0.39 ± 0.02	21.8 ± 6.1
0.5:1:0	0.78	7.21	0.38 ± 0.12	19.3 ± 4.4
0.5:1:0.125	0.82	7.53	0.41 ± 0.09	39.3 ± 4.7
0.5:1:0.25	0.88	7.93	0.44 ± 0.12	64.6 ± 9.2
0.5:1:1	0.84	7.27	0.42 ± 0.02	64.0 ± 9.6

headgroup.[21–23] PEG-DSPE|cholesterol micelles had broad peaks at 1.26 ppm ($\Delta\nu_{1/2} \sim 55.3$ Hz) and 0.88 ppm ($\Delta\nu_{1/2} \sim 31.9$ Hz), pointing to hindered motion of the core-forming diacyl chains in these micelles (Figure 3b).

AmB Encapsulation and Analysis of Absorbance Spectra of Encapsulated Drug. Table 1 summarizes AmB incorporation into PEG-DSPE and PEG-DSPE|cholesterol micelles. PEG-DSPE micelles could be loaded with high levels of AmB using the solvent evaporation method, consistent with our previous report.[5] From DLS measurements, the size of PEG-DSPE micelles increased from 16 to 20 nm on incorporation of AmB. Clinically relevant levels of AmB could be solubilized by dissolution of the thin film of coprecipitated AmB, cholesterol, and PEG-DSPE. The size of PEG-DSPE|cholesterol micelles was dependent on the level of cholesterol incorporation and reached ca. 60 nm at a molar ratio of AmB:PEG-DSPE:cholesterol = 0.5:1:1.

The absorbance spectrum for AmB (10 μg/mL) incorporated in PEG-DSPE micelles in 10 mM HEPES buffer was sensitive to the initial AmB:PEG-DSPE ratio. At low levels of AmB incorporation (AmB/PEG-DSPE = 0.5:1), bands II, III, and IV at 368, 388, and 417 nm were prominent (Figure 4). At higher levels of AmB incorporation (AmB: PEG-DSPE = 2:1), band I at 328 nm was prominent relative to higher wavelengths. The ratio of intensity of band I to band IV (i.e., I/IV ratio) has been taken to be indicative of the degree of AmB self-association.[24] The absorbance spectrum of AmB in PEG-DSPE|cholesterol micelles (AmB: PEG-DSPE:cholesterol = 0.5:1:1) displayed prominent bands II, III, and IV. The low peak I/IV ratio in the absorbance spectrum of AmB was characteristic of diminished self-aggregation.

In addition, the absorption spectrum of AmB in PEG-DSPE|cholesterol micelles displayed sharper absorption bands compared with AmB in PEG-DSPE micelles. The

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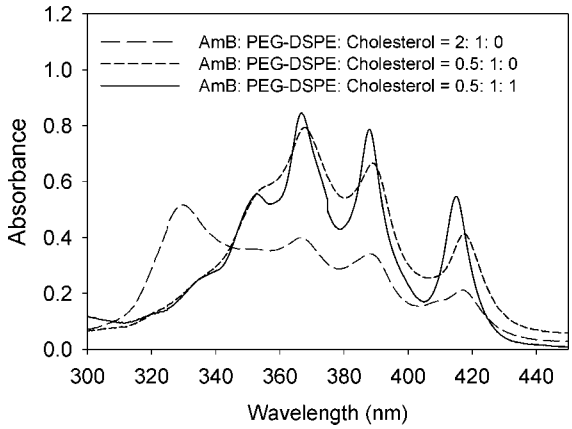

Figure 4. Absorption spectra of AmB solubilized by PEG-DSPE and PEG-DSPE|cholesterol micelles.

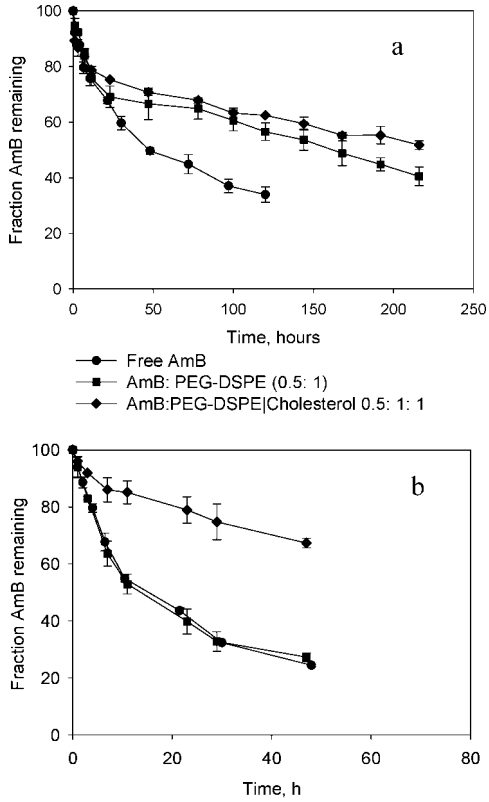

Figure 5. AmB release profiles at 25 °C in (a) 5 mM HEPES and (b) 4% BSA.

width-at-half-height for band IV of the AmB absorbance was estimated by fitting to a Gaussian curve of the form

$$A = A_0 + A_{\max} \exp\left(\frac{-(\lambda - \lambda_{\max})^2}{2w^2}\right) \qquad (1)$$

where A_0 is the baseline absorbance value and $A_{\max}$ is the peak absorbance value, where $\lambda_{\max}$ is the wavelength corresponding to band IV maximum, and where w is the width-at-half-height in nm. The $\lambda_{\max}$ for AmB in PEG-DSPE micelles was 417.2 ± 0.04 nm compared to 415.1 ± 0.03 nm for AmB in PEG-DSPE|cholesterol micelles. The bandwidth w for AmB in PEG-DSPE micelles was 6.34 ± 0.04 nm compared with 4.66 ± 0.04 nm for AmB in PEG-DSPE|cholesterol micelles.

These distinct absorption spectra may point to differences in the association of AmB with the micelle core. However, the role of cholesterol in this interaction is poorly understood. AmB is a rigid molecule due to the conjugated polyene system. Greater interaction of the rigid AmB molecule with ordered lipids, such as in PEG-DSPE|cholesterol micelles, has been proposed by Bolard et al.[2,25] Alternatively, AmB may interact with cholesterol in PEG-DSPE|cholesterol micelles. The absorbance spectrum of AmB in cholesterol containing propanol-water mixtures, in which AmB is presumably associated with cholesterol, displayed narrow bands similar to that of AmB in PEG-DSPE|cholesterol micelles.[26,27] Regardless of the origin of these spectral differences, cholesterol seems to influence the microenvironment of the encapsulated AmB.

In Vitro Release Study of Micelle Encapsulated AmB Using Equilibrium Dialysis. For the *in vitro* release study, free or micelle-encapsulated AmB diluted to 30 μg/mL in HEPES or 4% BSA was dialyzed against excess HEPES buffer. AmB concentration in the dialysis cassettes was assayed at periodic intervals using a HPLC method. The $t_{½}$ for free AmB release from the dialysis cassette was 69 h in HEPES and 18.6 h in 4% BSA. This apparent discrepancy in the AmB release profile can be explained considering that AmB is highly self-aggregated above its CAC, which is ca. 1 μM in HEPES compared to 10 μM in 4% BSA. Above the CAC, monomeric AmB coexists with aggregates with varying association numbers according to an open association model.[3] The slow release of AmB in HEPES may be explained on the basis that a greater fraction of the AmB would exist in a self-aggregated form in HEPES than in 4% BSA and only monomeric AmB or small aggregates would be free to diffuse out of the dialysis membrane.

AmB incorporated in PEG-DSPE micelles was released slowly in HEPES ($t_{½}$ of 184 h), presumably in a monomeric form. We were surprised to find that release of AmB in PEG-DSPE micelles in the presence of 4% BSA was greatly enhanced ($t_{½}$ of 18.4 h) and was comparable to that for free drug. This finding pointed to the destabilizing effect of BSA on AmB incorporation in PEG-DSPE micelles. In HEPES buffer, the release of AmB incorporated in PEG-DSPE|cholesterol micelles was slower ($t_{½}$ of 259 h) compared with the release of AmB incorporated in PEG-DSPE micelles or

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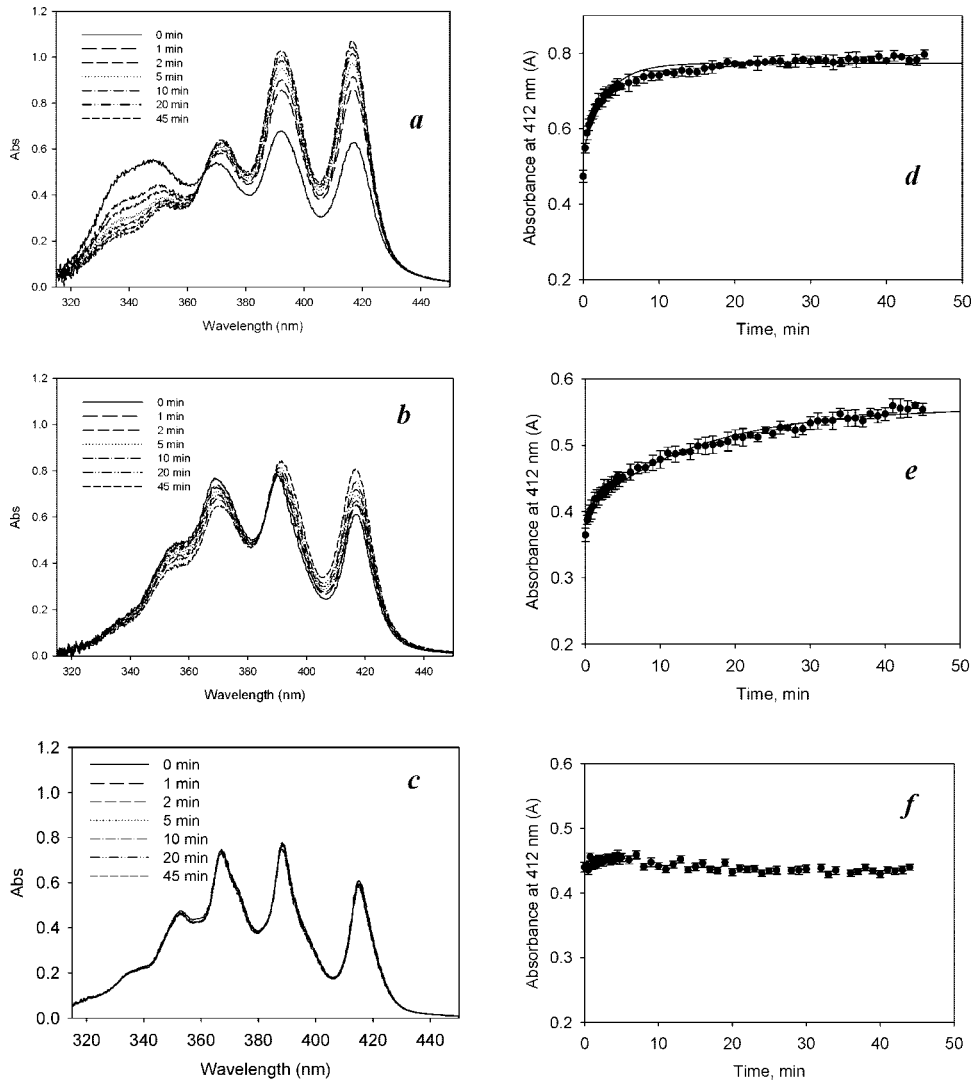

Figure 6. Absorbance spectra of micelle encapsulated AmB in 4% BSA:[a] (a) free AmB; (b) AmB in PEG-DSPE micelles (AmB:PEG-DSPE = 0.5:1); (c) AmB in PEG-DSPE|cholesterol micelles (AmB:PEG-DSPE:cholesterol = 0.5: 1: 1); (d−f) absorbance at 412 nm for as a function of incubation time corresponding to parts a−c. [a]AmB concentration was 10 μg/mL.

Table 2. Fit Parameters for AmB Release from PEG-DSPE Micelles at 25 °C

	5 mM HEPES			4% BSA		
	$100k$ (h^{-1})	$t_{1/2}$ (h)	R^2	$100k$ (h^{-1})	$t_{1/2}$ (h)	R^2
free AmB	1.01 ± 0.10	68.5	0.94	3.72 ± 0.04	18.6	0.96
AmB:PEG-DSPE 0.5:1	0.38 ± 0.04	183.9	0.90	3.75 ± 0.52	18.4	0.94
AmB:PEG-DSPE:cholesterol 0.5:1:1	0.27 ± 0.03	259.2	0.89	8.08 ± 0.08	85.8	0.94

free drug. The release of AmB from PEG-DSPE|cholesterol micelles was also significantly slower in 4% BSA ($t_{½}$ of 85.4 h) compared to release of AmB in PEG-DSPE micelles or free drug.

Electronic Absorption Kinetics in Serum Albumin. The electronic absorption spectrum of AmB is uniquely sensitive to the environment of the molecule. The absorbance at peak IV increases when AmB dissociates from either vesicles or micelles and associates with serum albumin. Thus, increases in the absorbance intensity at 412 nm have been used both to evaluate drug dissociation from PEG-DSPE micelles and to quantify apparent rate constants. A similar method has been used by Witzke et al. to estimate the association of AmB in egg-PC vesicles.[28] Parts a−c of Figure 6 show representative AmB

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Table 3. Fit Parameters for Absorbance Change for PEG-DSPE Incorporated AmB at 412 nm upon Dilution in 4% BSA (Figure 6d−f)

	$100k$ (min^{-1})	$t_{1/2}$ (min)	R^2	A_∞
free AmB	3.25 ± 0.23	2.1	0.94	0.77
AmB/PEG-DSPE 0.5:1	6.63 ± 0.53	10.46	0.98	0.56
AmB/PEG-DSPE/cholesterol 0.5:1:1				0.42

absorbance spectra over time. Parts d−f of Figure 6 show the corresponding changes in the absorbance at 412 nm. Kinetic rate parameters that were obtained by fitting the absorbance values in parts d−f of Figure 6 to a first-order process are shown in Table 3.

The addition of free AmB to 4% BSA resulted in an initial broadband at ca. 350 nm, characteristic of self-aggregated AmB (Figure 6a). The intensity of this band diminished over time, with increasing intensity at higher wavelengths. This finding is consistent with drug deaggregation in 4% BSA.[29] The half-life for this association was estimated as 2.1 min, assuming first-order kinetics. Peak IV absorbance of AmB encapsulated in PEG-DSPE micelles increased over 45 min upon dilution in 4% BSA and absorbance maxima for peaks III and IV underwent minimal shifts (∼2 nm) (Figure 6b). The dissociation half-life, assuming first-order kinetics, was estimated as 10.5 min. This result was consistent with rapid AmB release from PEG-DSPE micelles in the presence of 4% BSA observed in dialysis studies. In contrast, there were minimal changes in the absorbance spectrum of AmB in PEG-DSPE|cholesterol micelles in serum albumin (Figure 6c). This finding indicates that AmB that was incorporated in PEG-DSPE|cholesterol micelles was not free to interact with serum albumin. The slow release of AmB from these micelles occurs presumably through a simple diffusion process.

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Conclusions

PEG-DSPE|cholesterol micelles prepared using a simple solvent evaporation method could solubilize high levels of deaggregated AmB. The coincorporation of cholesterol reduces core mobility of PEG-DSPE micelles, consistent with the structuring effect proposed in the literature. Differences in the absorption between AmB encapsulated in PEG-DSPE or PEG-DSPE|cholesterol micelles point to differences in the association of AmB with these micelles. Absorption kinetics and dialysis experiments indicate that while AmB in PEG-DSPE is free to interact with serum albumin, the drug is stably incorporated in PEG-DSPE|cholesterol micelles. Our results are consistent with greater interaction of the polyene antibiotic with structured lipids and might result in sustained release of AmB *in vivo*.

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