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RESEARCH PAPER

Amphotericin B/Sterol Co-loaded PEG-Phospholipid Micelles: Effects of Sterols on Aggregation State and Hemolytic Activity of Amphotericin B

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ABSTRACT

Purpose To elucidate the effect of sterols on the aggregation of amphotericin B (AmB) in PEG-phospholipid micelles and its consequences on the hemolytic activity of AmB.

Methods AmB-incorporated PEG-phospholipid micelles co-loaded with ergosterol, cholesterol, or 7-dehydrocholesterol were prepared at 4:1:1 and 20:5:1 ratios of polymer-to-sterol-to-AmB. The aggregation state of AmB was elucidated by UV–vis spectroscopy. AmB/sterol co-loaded PEG-phospholipid micelles were incubated with red blood cells and the hemolytic activity of AmB assessed by measurement of free hemoglobin.

Results AmB in PEG-phospholipid micelles stayed mostly in a deaggregated state in the absence of sterol or with cholesterol, but aggregated in the presence of ergosterol or 7-dehydrocholesterol. The fraction of aggregated AmB in PEG-phospholipid micelles was lower at the 20:5:1 ratio. In an aggregated state or in the absence of sterol, AmB caused rapid and complete hemolysis. In contrast, deaggregated AmB co-loaded with cholesterol caused slower and incomplete hemolysis, especially at a 20:5:1 ratio.

Conclusions The aggregation state of AmB in PEG-phospholipid micelles was sterol dependant. AmB/cholesterol co-loaded PEG-phospholipid micelles caused low *in vitro* hemolysis due to deaggregation of AmB and micellar stability, presumably owing to cholesterol/phospholipid *versus* cholesterol/AmB interactions in the interior core region.

KEY WORDS amphotericin B · aggregation state · hemolysis · micelle · sterol

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INTRODUCTION

Over the past two decades, the frequency of sepsis has increased significantly in the United States, with systemic candidiasis accounting for the fourth most common infection in immunocompromised patients (1,2). Amphotericin B (AmB) is a common choice for the treatment of systemic fungal diseases despite causing severe toxic side effects (3). In a drug delivery approach, liposomal AmB has reduced the dose-limiting renal toxicity over AmB deoxycholate (AmB-D), its standard formulation, but still, mortality rates for systemic fungal diseases remain largely unchanged and high, ca. 40%, providing solid rationale for progress in the delivery of this potent, membrane-active antifungal agent.

In an alternative strategy based on the aggregation state hypothesis, monomeric (i.e. deaggregated) AmB is selective for ergosterol in fungal cell membranes, forming channels by the classic barrel stave motif (Fig. 1b). In contrast, soluble aggregated species of AmB are non-selective and form channels in both fungal and mammalian cell membranes, causing host toxicity. Due to its amphiphilic nature, AmB has a high propensity towards self-aggregation in water and for poor water solubility. 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (ammonium salt) (PEG-DSPE) micelles solubilized AmB in a largely monomeric state, evidenced by a loss of a peak in the UV–vis spectrum at 328 nm, characteristic of aggregated AmB (Fig. 1c). As a result, AmB caused low *in vitro* hemolysis relative to free drug (4). In contrast, serum proteins quickly disrupted the integrity of PEG-DSPE micelles, resulting in the rapid release of AmB, its reaggregation, and potential for host toxicity (5). More recently, co-incorporation of cholesterol along with AmB enhanced the structural stability of PEG-DSPE

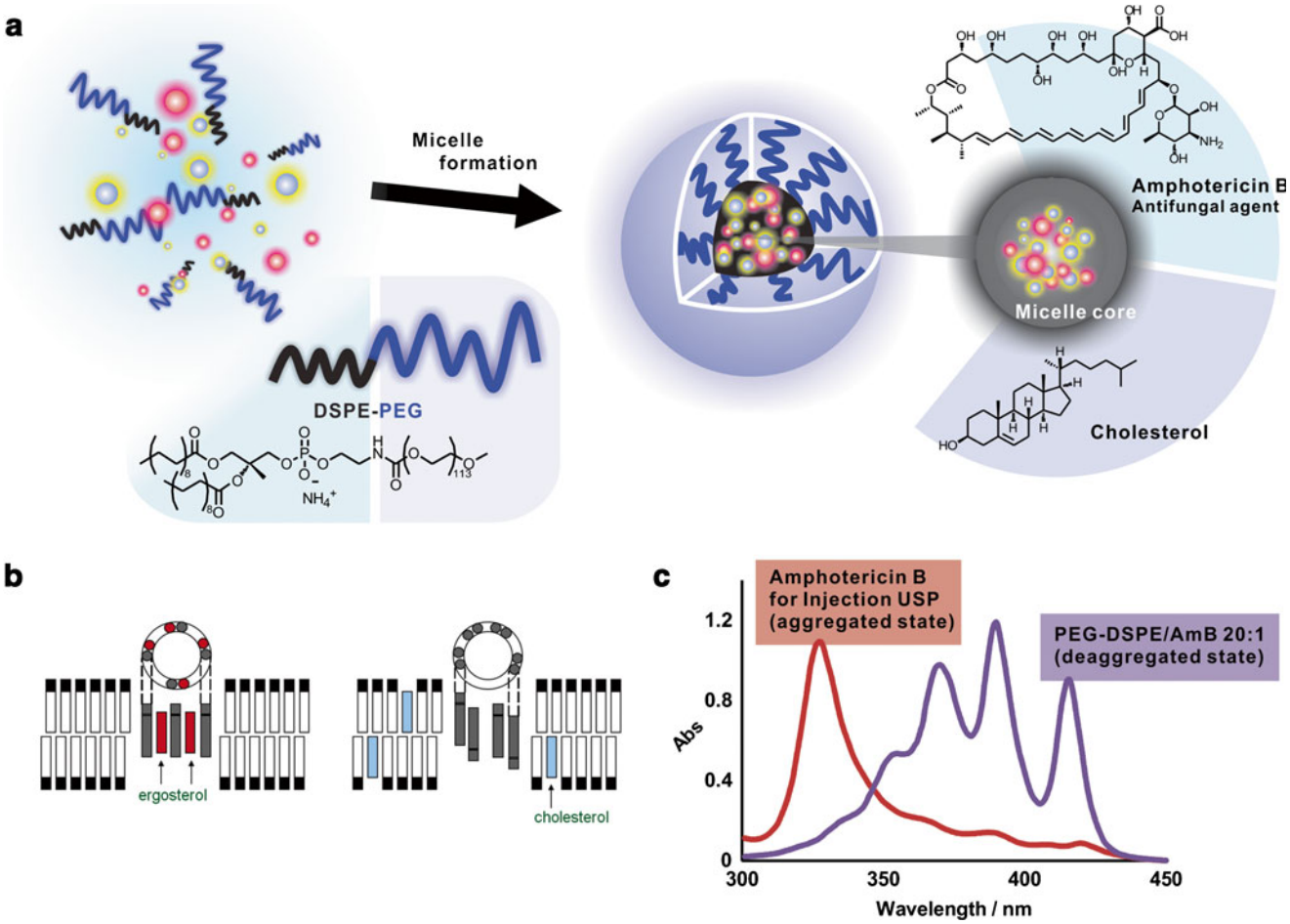


Fig. 1 PEG-DSPE, cholesterol, and AmB assembly into micelles (**a**), aggregation state hypothesis for AmB in ergosterol and cholesterol containing membranes showing pore formation of monomeric AmB in ergosterol containing mambranes and pore formation by aggregated AmB in cholesterol containing membranes (**b**), and absorption spectra of aggregated and deaggregated AmB (**c**).

micelles in the presence of serum albumin, owing to cholesterol/phospholipid interactions that increase core viscosity (6).

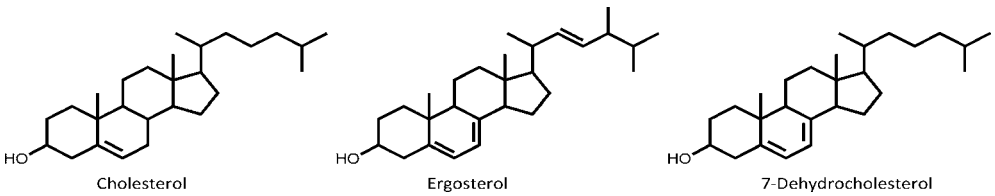
The goal of this work was to characterize the effects of various co-loaded sterols on the aggregation state and hemolysis of AmB incorporated in PEG-DSPE micelles: Ergosterol, cholesterol, and 7-dehydrocholesterol (7-DHC) (Fig. 2). Ergosterol and cholesterol were chosen because they represent the native membrane sterols in fungal cells and mammalian cells, respectively. AmB has been shown to preferentially interact with ergosterol over cholesterol in lipid environments, when AmB is in a deaggregated state, while losing interaction specificity at higher concentration, when AmB begins to self-associate (7,8). 7-DHC has been previously shown to bind AmB in a deaggregated state in a co-solvent system (9).

MATERIALS AND METHODS

Materials

AmB was a gift from X-GEN Pharmaceuticals Inc. (Big Flats, NY). Glacial acetic acid, phosphoric acid, HPLC-grade acetonitrile, methanol, and chloroform were purchased from Sigma-Aldrich Inc. (St. Louis, MO) and used as received.

Fig. 2 Chemical structure of cholesterol, ergosterol, and 7-dehydrocholesterol.



Phosphate buffered saline 1 × (pH 7.4) (PBS) was purchased from Cellgro (Mediatech Inc., Manassas, VA). Ergosterol, cholesterol, and 7-DHC were purchased from Sigma-Aldrich Inc. (St. Louis, MO) and used as received. Sterols were stored at −20°C. PEG-DSPE (5801 g/mol) with a 5000 M_n PEG block was purchased from Avanti Polar Lipids Inc. (Alabaster, AL) and stored at −20°C. Bovine red blood cells were purchased from Innovative Research (Novi, MI), stored at 4°C, and used within 2 weeks of receipt.

Preparation of PEG-DSPE/sterol/AmB Micelles

Micelles were prepared using a solvent evaporation method similar to ones previously described (6,10,11). Briefly stated, PEG-DSPE was weighed in a 10 mL round bottom flask (RBF). AmB (0.6 mg/mL in methanol) and sterol (1.0 mg/mL in chloroform) were added to achieve 4:1:1 or 20:5:1 polymer to sterol to AmB molar ratios. One mL of chloroform was added to aid in the dissolution of the polymer. The organic solvents were removed under reduced pressure at 60°C to leave behind a thin film of polymer, sterol, and AmB. Any residual solvent was removed by purging the RBF with argon gas. The thin film was then warmed to 60°C, and PBS heated to 60°C was added to achieve a 10 mg/mL polymer concentration. The RBF was rotated at room temperature for 10 min. Finally, the sample was sonicated for 1 min, followed by filtration through a 0.22 μm nylon syringe filter (Whatman, Kent, UK). Micelles not containing sterols or AmB were prepared in the same way.

Dynamic Light Scattering Measurements of PEG-DSPE/sterol/AmB Micelles

The hydrodynamic diameters of micelles were determined by dynamic light scattering using a ZETASIZER Nano-ZS (Malvern Instruments Inc., Worcestershire, UK) equipped with a He-Ne laser (4 mW, 633 nm) light source and a 173° angle scattered light collection configuration. Micelle samples were diluted in PBS to approximately 0.5 mg/mL polymer concentration, and the samples were equilibrated for 2 min at 25°C before measurements were taken. The correlation function was curve fitted by the Cumulants analysis method to calculate Z-average size through the use of the Stokes-Einstein equation and the polydispersity index (PDI) from the slope of the correlation function. PDI is a measure of the relative variance of the Z-average size, assuming a Gaussian distribution. All measurements were performed in triplicate.

Quantification of AmB and Sterol Loading in PEG-DSPE Micelles

The HPLC system used for quantification was a Shimadzu prominence HPLC system (Shimadzu, JP) that consisted of a LC-20AT pump, a SIL-20 AC HT autosampler, a CTO-20 AC column oven, and a SPD-M20A diode array detector.

For AmB detection, PEG-DSPE micelles were diluted 10-fold in the mobile phase, and a sample of 10 μL was injected into an Eclipse XDB-C8 cartridge (4.6× 150 mm, 5 μm) (Agilent Technologies, Palo Alto, CA). The column temperature was maintained at 40°C throughout the run. The mobile phase consisted of a gradient of 5% (v/v) acetic acid in methanol and 5% (v/v) acetic acid in doubly distilled water (ddH_2O). The eluent composition was maintained at 5% organic phase for 2 min, then linearly increased to 95% organic phase over 6 min, and then held at 95% organic phase for 6 min. The flow rate was 1 mL/min. The assay was tested for linearity in the 0.1-100 μg/mL AmB range. AmB was detected at 406 nm and eluted at 9.2 min.

A second HPLC method, which was developed to quantify ergosterol, cholesterol, and 7-DHC, was adapted from a similar method that was published previously (12). The HPLC system used for quantification was a Shimadzu prominence HPLC system (Shimadzu, JP) that consisted of a LC-20AT pump, a SIL-20 AC HT autosampler, a CTO-20 AC column oven, and a SPD-M20A diode array detector. Micelles were diluted 5-fold in the mobile phase, and a sample of 10 μL was injected into a Zorbax 300SB-C18 cartridge (4.6×150 mm, 3.5 μm) (Agilent Technologies, Palo Alto, CA). The column temperature was maintained at 40°C throughout the run. The mobile phase was an isocratic mixture of 95% acetonitrile and 5% aqueous phase containing 0.1% phosphoric acid and 1% methanol. The run time was 12 min. The flow rate was 1 mL/min. The assay was tested for linearity in the range of 2.5-200 μg/mL for all three sterols. Ergosterol and 7-DHC were detected at 282 nm and eluted at 6.25 and 6.2 min, respectively. Cholesterol was detected at 202 nm and eluted at 7.7 min.

Aggregation State Determination of AmB in PEG-DSPE Micelles by UV–vis Spectroscopy

UV–vis spectra of AmB were obtained on a CARY 50 BIO (Varian, Palo Alto, CA). Samples were diluted to a 0.1 mg/mL AmB concentration in PBS and transferred into a quartz cuvette of 1 mm path length (Starna Cells Inc., Atascadero, CA) for measurement. UV–vis absorbance spectra were obtained from 300–450 nm. PeakFit v. 4.12 (Systat Software Inc., Chicago, IL) was used to deconvolve absorbance spectra of AmB using a Gaussian response function with a Fourier deconvolution/filtering algorithm. The graphical fitting of peaks to the second derivative was performed. $R^2 > 0.98$ could be achieved for all spectra.

In Vitro Hemolysis

For hemolysis analysis, a method similar to the one previously described was adapted (13). Bovine red blood cells were washed three times with PBS. Cells were diluted in PBS to achieve a working suspension of 0.8 absorbance at 542 nm (1 mm path length). Cell suspensions were diluted 50:50 with AmB-containing formulations. The final AmB concentration was 50 μg/mL. Samples were incubated in a VWR Hybridization Oven set to 37°C (30 rpm rotation speed). At specific time points, samples were removed and set on ice for 5 min to halt hemolysis. Samples were then spun down for 10 min at 13200 rpm in an Eppendorf Centrifuge 5415 D (Brinkmann Instruments Inc., Westbury, NY) to settle intact cells and cell debris. The supernatant was collected and plated in 96 well plates in duplicate. Sample hemoglobin absorbances were measured at 542 nm. The percentage of hemolysis was calculated as: $100 \times \frac{Abs_{Sample} - Abs_0}{Abs_{100} - Abs_0}$, where Abs_{Sample}, Abs_0, and Abs_{100} refer to the absorbances of sample supernatants incubated with micelle solutions, PBS, or water, respectively. Hemolysis studies were performed in triplicate.

RESULTS AND DISCUSSION

Physical Properties of PEG-DSPE/Sterol/AmB Micelles

PEG-DSPE, AmB, and sterols assembled into fairly monodisperse micelles of less than 50 nm Z-average hydrodynamic diameter (Table I), which corresponded well with previous reports of drug-loaded PEG-DSPE micelles (6,14). PEG-DSPE micelles co-loaded with ergosterol or 7-DHC (4:1:1 ratio) were ca. 10 nm larger than cholesterol-loaded PEG-DSPE micelles. PEG-DSPE micelles loaded with only AmB were approximately 20 nm in diameter. The smaller size of the cholesterol and sterol-free micelles may have been due to differences in aggregation state of AmB and core packing. The 5-fold reduction in AmB loading from a 4:1:1 to a 20:5:1 ratio caused a reduction in micellar dimensions for all sterol-containing PEG-DSPE micelles. PDI was also reduced, except for ergosterol-loaded PEG-DSPE micelles which saw an increase in size distribution. All PEG-DSPE micelles successfully solubilized AmB, achieving around 0.5 mg/mL at a 4:1:1 ratio and ca. 0.2 mg/mL at a 20:5:1 ratio. PEG-DSPE micelles could also be successfully prepared above 1 mg/mL AmB concentration at a 4:1:1 ratio (data not shown).

All sterols co-loaded with AmB in PEG-DSPE micelles had a loading efficiency of approximately 90% at a 4:1:1 ratio (Table I). At a 20:5:1 ratio, the loading efficiency dropped to 37% for ergosterol and to 80% for 7-DHC, but stayed high for cholesterol, ca. 96%. At a ratio of 4:1:1, the final ratio of sterol to AmB for ergosterol, cholesterol, and 7-DHC was similar: 0.650, 0.687, and 0.636, respectively. At a 20:5:1 ratio, final ratios of sterol to AmB for ergosterol, cholesterol, and 7-DHC differed: 1.33, 3.41, and 2.79, respectively. A previous report describing sterol structure requirements for incorporation in lipid membranes showed a similar trend in incorporation efficiency of ergosterol, cholesterol, and 7-DHC (15). The higher incorporation efficiency of the ergosterol co-loaded with AmB in PEG-DSPE micelles at a 4:1:1 *versus* 20:5:1 ratio implies that solubilization of ergosterol by PEG-DSPE micelles was increased by interaction with AmB. The absence of AmB in the micelle core upon reduction of loading may be reducing the propensity of ergosterol to incorporate parallel to the lipid chains in the core, thereby increasing polydispersity of the micelles.

Monomeric AmB Content of PEG-DSPE Micelles

The level of monomeric species of AmB in PEG-DSPE micelles was calculated from the monomer UV–vis peak intensity at 415 nm compared to monomer peak intensity in

Table I Physical and Composition Properties of Sterol/AmB Co-loaded PEG-DSPE Micelles. % Sterol Loading and AmB Concentration were Determined by Reverse Phase HPLC. Results are Presented as the Mean±Standard Deviation ($n = 3$). $n = 1$ for Sterol-free Micelles Loaded at the 4:0:1 Ratio

Sterol	Initial Loading Ratio	Z-Average Particle Diameter (nm)	PDI	% Sterol Incorporated	AmB (mg/mL)	Final Sterol:AmB Molar Ratio
Erg	4:1:1	48.6 ± 12.6	0.22 ± 0.03	91.5 ± 2.3	0.56 ± 0.01	0.650 ± 0.001
	20:5:1	41.7 ± 11.0	0.40 ± 0.07	37.3 ± 3.8	0.23 ± 0.02	1.33 ± 0.24
Chol	4:1:1	36.6 ± 0.2	0.21 ± 0.01	88.0 ± 2.5	0.51 ± 0.02	0.687 ± 0.005
	20:5:1	26.7 ± 0.6	0.12 ± 0.05	96.3 ± 3.9	0.22 ± 0.01	3.41 ± 0.03
7-DHC	4:1:1	44.0 ± 2.5	0.22 ± 0.01	84.8 ± 4.7	0.53 ± 0.01	0.636 ± 0.051
	20:5:1	27.4 ± 0.8	0.13 ± 0.04	79.9 ± 1.3	0.23 ± 0.00	2.79 ± 0.07
No Sterol	4:0:1	20.8 ± 2.1	0.11 ± 0.09	–	0.48	–
	20:0:1	22.6 ± 0.8	0.07 ± 0.03	–	0.22 ± 0.00	–

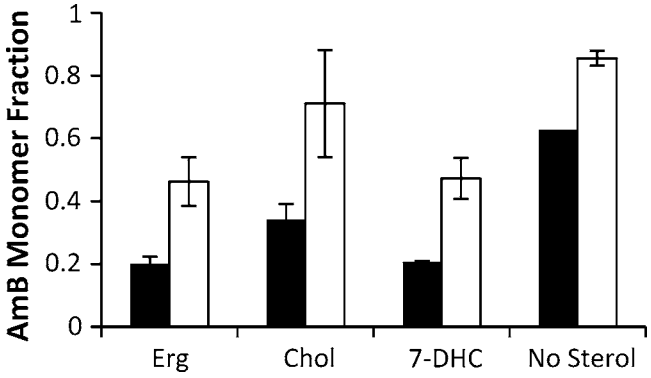


Fig. 3 Fraction monomeric AmB in PEG-DSPE micelles co-loaded with ergosterol (Erg), cholesterol (Chol), or 7-dehydrocholesterol (7-DHC) at a polymer-to-sterol-to-AmB ratio of 4:1:1 (black bars) and 20:5:1 (white bars). Sterol-free micelles are loaded at a polymer-to-AmB ratio of 4:1 (black bar) and 20:1 (white bar). Error bars represent standard deviation ($n = 3$).

DMSO:H_2O (95:5 v/v). In 95% (v/v) DMSO, AmB is a completely monomeric state (data not shown). Figure 3 provides the fraction of monomeric AmB in PEG-DSPE micelles. Sterol-free PEG-DSPE micelles contained the highest fraction of monomeric AmB, ca. 90%. At a 4:1:1 ratio, ergosterol and 7-DHC co-loaded PEG-DSPE micelles had the lowest monomer content, indicating that AmB interacts with ergosterol and 7-DHC and forms aggregated species. Forces between the sterol skeleton and the alkyl chain and the rigid polyene backbone of AmB are thought to play a major role in the attraction between sterol molecules and AmB. The additional double bonds at C7 and C22 in ergosterol and at C7 in 7-DHC further rigidifies these sterols, allowing for favored Van der Waals forces and π-π interactions with the polyene backbone of AmB relative to cholesterol and thus forming AmB aggregate structures within PEG-DSPE micelles (9,16). At a 20:5:1 ratio, the fraction of monomeric AmB in the sterol co-loaded PEG-DSPE micelles doubled and increased by about 20% in the sterol-free PEG-DSPE micelles.

Characterization of Aggregated Species of AmB in PEG-DSPE Micelles

Figure 4 depicts UV–vis spectra for AmB co-loaded with ergosterol, cholesterol, or 7-DHC in PEG-DSPE micelles at a 4:1:1 ratio. The aggregation of AmB results in a hypsochromic shift in its UV–vis spectrum: a loss of the monomer absorbance at ca. 415 nm and increased absorbance below 350 nm. Shifts to 340–350 nm have been attributed to non-covalent dimers of AmB, while shifts to approximately 330–340 nm and 320–330 nm have been attributed to soluble aggregates of AmB, (17,18), referred to here as aggregate 1 and aggregate 2, respectively. While the number of AmB molecules in aggregates 1 and 2 are unknown, it has been postulated that aggregate 2 is the larger species, and it is the major aggregate in AmB-D, based on its UV–vis spectrum. In this study, UV–vis absorbance spectra of AmB loaded in PEG-DSPE micelles were

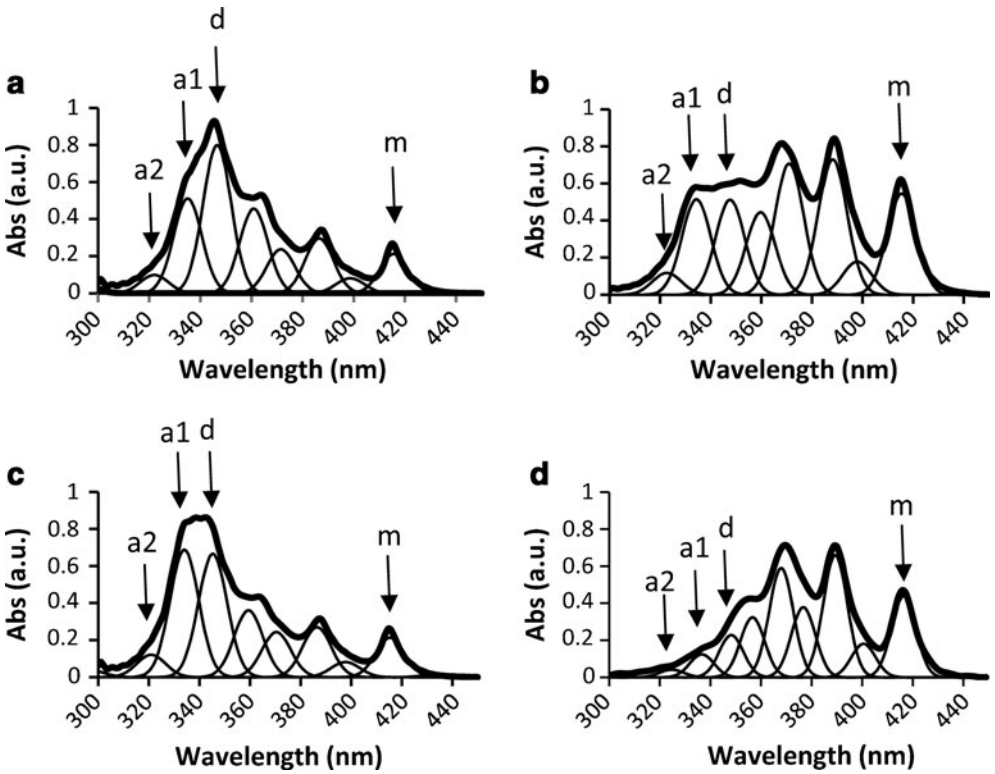


Fig. 4 Example absorption spectra of AmB in PEG-DSPE micelles co-loaded with ergosterol (**a**), cholesterol (**b**), or 7-dehydrocholesterol (**c**), and sterol-free PEG-DSPE micelles (**d**) in PBS. Loading ratio is 4:1:1 (polymer-to-sterol-to-AmB) for sterol co-loaded micelles and 4:1 (polymer-to-AmB) for sterol-free micelles. Spectra include deconvoluted peaks. Labeled arrows correspond to the peaks of monomeric (m), dimeric (d), aggregate 1 (a1), and aggregate 2 (a2) forms of AmB.

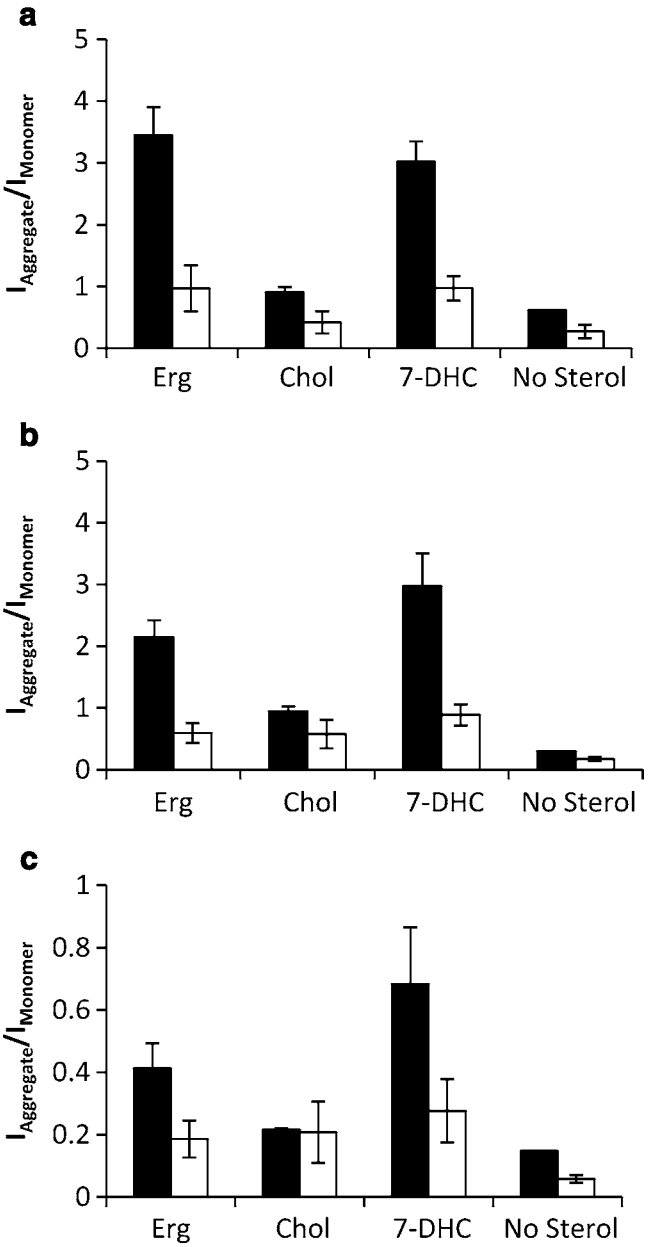


Fig. 5 Peak intensity of AmB dimer (**a**), aggregate 1 (**b**), and aggregate 2 (**c**) relative to monomer intensity for ergosterol, cholesterol, and 7-dehydrocholesterol loaded at a polymer-to-sterol-to-AmB ratio of 4:1:1 (black bars) and 20:5:1 (white bars), and sterol free PEG-DSPE micelles loaded at a polymer-to-AmB ratio of 4:1 (black bars) and 20:1 (white bars). Error bars represent standard deviation ($n = 3$).

deconvoluted to quantify differences in the relative aggregation states of AmB based on co-loaded sterols. Deconvolution was performed under the assumption of Gaussian peak distribution, with the peaks being of equal width.

Figure 5 depicts the AmB UV–vis absorbance peak intensities of dimer, aggregate 1, and aggregate 2 relative to the monomer peak intensity of AmB co-loaded with ergosterol, cholesterol, or 7-DHC in PEG-DSPE micelles at a 4:1:1 ratio. PEG-DSPE micelles co-loaded with ergosterol or 7-DHC have the greatest population of AmB dimers relative to monomer, while cholesterol and sterol-free PEG-DSPE micelles had a low content of AmB dimers. The ratio of aggregate 1 to monomer for AmB was high and similar for the ergosterol and 7-DHC co-loaded in PEG-DSPE micelles and low for cholesterol and sterol-free micelles. Similarly, the ratio of aggregate 2 to monomer for AmB was relatively high for the ergosterol and 7-DHC co-loaded in PEG-DSPE micelles and low for cholesterol and sterol-free micelles. Intermolecular interaction between ergosterol or 7-DHC and AmB in the cores of PEG-DSPE micelles appeared to favor the aggregation of AmB. These findings contradict previous reports of deaggregation of AmB in the presence of 7-DHC (9). In contrast, AmB co-loaded with cholesterol in PEG-DSPE micelles existed primarily in a monomeric state akin to AmB in PEG-DSPE micelles without co-loaded sterols. Thus, AmB in PEG-DSPE micelles co-loaded with cholesterol probably favored interaction with phospolipid (DSPE) over cholesterol, whereas AmB in PEG-DSPE micelles co-loaded with ergosterol or 7-DHC favored interaction with sterol, resulting in self-aggregation with some variation in aggregated species between sterols (Figs. 4 and 5). These results agree with previous reports of AmB incorporated in lysophosphatidylcholine micelles where AmB was shown to preferentially aggregate in micelles containing ergosterol but not with cholesterol (19).

At a 20:5:1 ratio, AmB co-loaded with ergosterol, cholesterol, or 7-DHC in PEG-DSPE micelles had a reduced degree of self-aggregation, also reflected in the increased fraction of monomer content (Figs. 3 and 5). PEG-DSPE micelles co-loaded with ergosterol or 7-DHC had a 3-fold reduction in dimer and aggregate 1 content and a 2-fold reduction in aggregate 2 content. The change for PEG-DSPE micelles co-loaded with cholesterol was less pronounced, and was absent for aggregate 2 *versus* the 4:1:1 ratio.

In Vitro Hemolysis

Hemolysis experiments are commonly used to assess the *in vitro* toxicity of AmB. The incubation of free AmB at 3 μg/mL and bovine red blood cells resulted in 50% hemolysis within 1 hour (data not shown), noting that the onset of hemolysis corresponds to the critical aggregation concentration of AmB, ca. 1 μg/mL (20). Empty PEG-DSPE micelles had no hemolytic activity (data not shown). Figure 6 depicts the hemolytic activity of AmB co-loaded with ergosterol, cholesterol, or 7-DHC in PEG-DSPE micelles at 4:1:1 and 20:5:1 ratios as a function of time. Up to 25 μg/mL, differences in hemolysis were minimal (data not shown) which is why a quite high level of 50 μg/mL of AmB was used in the hemolysis experiment. At a 4:1:1 ratio, hemolysis was rapid, complete, and in the order of sterol free,

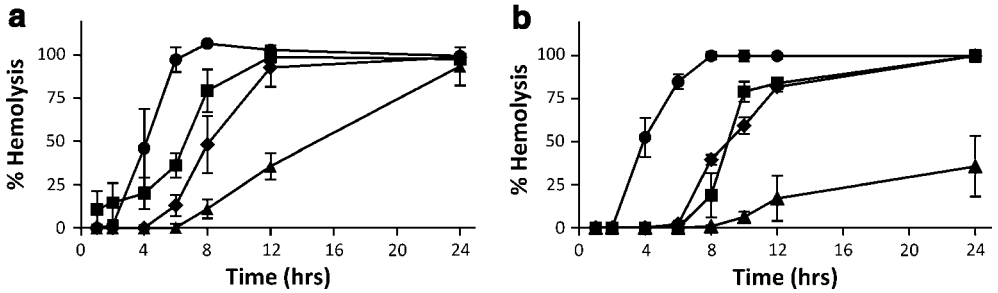


Fig. 6 Percent hemolysis for AmB loaded PEG-DSPE micelles (50 μg/mL) co-loaded with ergosterol (squares), cholesterol (triangles), or 7-DHC (diamonds) loaded at a polymer-to-sterol-to-AmB ratio of 4:1:1 (**a**) and 20:5:1 (**b**), and sterol-free micelles (circles) loaded at a polymer-to-AmB ratio of 4:1 (**a**) and 20:1 (**b**). Results are presented as the mean±standard deviation of three separate experiments.

ergosterol, and 7-DHC. In contrast, the hemolytic activity of AmB co-loaded with cholesterol was more gradual over 24 h. These results for the AmB co-loaded with ergosterol or 7-DHC in PEG-DSPE micelles corresponded well with the aggregation state hypothesis: aggregated species of AmB co-loaded ergosterol or 7-DHC in PEG-DSPE micelles are released and cause membrane damage and leakage of hemoglobin. On the other hand, the results for AmB co-loaded with cholesterol or sterol-free PEG-DSPE micelles were contrary, given that AmB exists in predominately a monomeric state yet causes hemolysis. At 50 μg/mL of AmB, we speculate that AmB is released from PEG-DSPE micelles in a monomeric state, but at this high level can self-aggregate after release, leading to hemolysis. It is noted that up to 25 μg/mL (above its critical aggregation concentration), AmB in sterol-free PEG-DSPE micelles did not cause significant hemolysis (data not shown). The difference between AmB co-loaded with cholesterol or sterol-free PEG-DSPE micelles in hemolysis is likely due to a slower rate of AmB release with co-loaded cholesterol in PEG-DSPE micelles (5). Cholesterol increased the core viscosity of PEG-DSPE micelles by its interaction with phospholipid (DSPE), resulting in enhanced micellar stability of PEG-DSPE micelles and slower rate of release for AmB relative to sterol-free PEG-DSPE micelles in the presence of serum albumin.

At a 20:5:1 ratio, the onset and degree of hemolysis for AmB co-loaded with ergosterol, cholesterol, or 7-DHC in PEG-DSPE micelles was reduced (Fig. 6b). At a 20:5:1 ratio, the fraction of monomeric AmB increased, resulting in reduced hemolysis in accord with the aggregation state hypothesis of AmB. The differences in hemolysis between the sterols, even though they all have a reduction in the aggregation of AmB, was likely due to differences in monomer content and enhanced micellar stability of PEG-DSPE micelles co-loaded with cholesterol over PEG-DSPE micelles co-loaded with ergosterol or 7-DHC. Cholesterol has a higher affinity for phospholipids over ergosterol and is present at a higher level in PEG-DSPE micelles over ergosterol and 7-DHC, resulting in micellar stability for ergosterol and 7-DHC co-loaded PEG-DSPE micelles that is intermediate between cholesterol or sterol-free PEG-DSPE micelles (Table I) (15). These results at a 20:5:1 ratio clearly showed that PEG-DSPE micelles co-loaded with AmB and cholesterol have low hemolysis even at 50 μg/mL of AmB, implying the dual requirement of sustained release of monomeric AmB for antifungal therapy.

CONCLUSIONS

PEG-DSPE micelles have a high capacity for AmB and co-loaded sterols, assembling into fairly mono-disperse micelles at high water solubility. AmB interacts with ergosterol and 7-DHC in PEG-DSPE micelles, favoring the aggregation of AmB into dimers and other higher-ordered aggregates and not the monomeric state. In contrast, AmB co-loaded with cholesterol in PEG-DSPE micelles favors a monomeric state. In this case, AmB and cholesterol prefer interaction with phospholipids (DSPE), resulting in deaggregation of AmB and enhanced integrity of PEG-DSPE micelles over sterol-free PEG-DSPE micelles. Accordingly, AmB co-loaded with cholesterol PEG-DSPE micelles causes low hemolysis even at 50 μg/mL. It is noted that the minimal inhibitory concentration of AmB against *Candida albi*cans is <1.0 μg/mL, i.e. beneath the critical aggregation concentration of AmB, and serum levels of AmB in patients is ca., 1.0 μg/mL. Thus, AmB PEG-DSPE micelles co-loaded with cholesterol satisfy requirements of safety and solubility for AmB and reduced its toxicity at a membrane level for the treatment of life-threatening systemic fungal diseases.

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