Block Copolymer Micelles for the Encapsulation and Delivery of Amphotericin B

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Purpose. To assess the effect of fatty acid substitution of a micelle-forming poly(ethylene oxide)-*block*-poly(*N*-hexyl stearate-L-aspartamide) (PEO-*b*-PHSA) on the encapsulation, hemolytic properties and antifungal activity of amphotericin B (AmB).

Methods. PEO-*b*-PHSA with three levels of stearic acid substitution were synthesized and used to encapsulate AmB by a solvent evaporation method. Size exclusion chromatography and UV spectroscopy were used to confirm and measure levels of encapsulated AmB. The hemolytic activity of encapsulated AmB toward human red blood cells and its minimum inhibitory concentration against *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans* were obtained and compared to AmB alone.

Results. An increase in the level of stearic acid substitution on PEO-*b*-PHSA improved the encapsulation of AmB while reducing its hemolytic activity. PEO-*b*-PHSA micelles having 50% and 70% stearic acid substitution (mol fatty acid: mol reacted and unreacted hydroxyls) were completely non-hemolytic at 22 μg/ml. At 11% stearic acid substitution, AmB caused 50% hemolysis at 1 μg/ml. AmB in PEO-*b*-PHSA micelles was as effective as AmB alone against pathogenic fungi.

Conclusions. PEO-*b*-PHSA micelles with a high level of stearic acid side chain substitution can effectively solubilize AmB, reduce its hemolytic activity yet retain its potent antifungal effects.

KEY WORDS: block copolymer micelles; amphotericin B; systemic fungal diseases; drug delivery; hemolysis.

INTRODUCTION

Amphiphilic synthetic polymers self-assemble into micellar structures that can encapsulate water insoluble drugs in an aqueous environment (1,2). Among several structures designed and studied for this purpose, poly(ethylene oxide)-*block*-poly(L-aspartic acid)s (PEO-*b*-PLAA)s form micellar carriers with unique properties for drug delivery. Owing to a nanoscopic size and a PEO shell, PEO-*b*-PLAA micelles may resemble biologic carriers, namely serum lipoproteins, by having a long circulation in blood while carrying insoluble molecules in a hydrophobic core. In addition, free carboxyl groups on the core-forming poly(L-aspartic acid) block provide functional sites for the attachment of drugs (3,4) or drug-compatible moieties (5–7) in PEO-*b*-PLAA micelles. Finally, PEO-*b*-PLAA micelles may easily be sterilized by filtration, freeze-dried, reconstituted and administered safely (8).

To enhance the encapsulation of aliphatic drugs such as amphotericin B (AmB), we have synthesized micelle-forming PEO-*b*-PLAA block copolymers with fatty acid side chains on the PLAA block and assessed the effect of polymer structure on micellar properties (7,9). AmB has been encapsulated by poly(ethylene oxide)-*block*-poly(*N*-hexyl stearate-L-aspartamide) (PEO-*b*-PHSA) micelles and its hemolytic activity was reduced, particularly after encapsulation by a solvent evaporation method (10). We have now focused on the effect of polymer structure on the encapsulation, hemolytic and antifungal activity of AmB. We have pursued an optimum structure for PEO-*b*-PHSA micelles by changing the level of stearic acid conjugated as a side chain, obtained adequate encapsulation, and reduced hemolytic activity of AmB. The latter may be due to control over the rate of AmB release. AmB encapsulated in PEO-*b*-PHSA micelles having different levels of stearic acid substitution was found active against fungal cells. The attachment of a chemically compatible side chain at a varied degree of substitution onto a core-forming block may be used as a tailoring strategy to control encapsulation and release of water insoluble drugs from polymeric micelles.

MATERIALS AND METHODS

Synthesis of PEO-*b*-PHSA

PEO-*b*-PHSA was synthesized from PEO-*b*-poly(β-benzyl-L-aspartate) (PEO-*b*-PBLA) (PEO M_n = 12,000 g mol^{-1}; M_w/M_n = 1.05; number of BLA units = 24) as described previously (7,9). Briefly, PEO-*b*-PBLA was reacted with 6-aminohexanol at 25°C in the presence of 2-hydroxypyridine, as a catalyst. Free hydroxyl groups on PEO-*b*-poly(hydroxyhexyl-L-aspartamide) (PEO-*b*-PHHA), were reacted with stearic acid in dry dichloromethane in the presence of dicyclohexylcarbodiimide and dimethylaminopyridine. The reaction time was kept between 2 to 72 h to achieve varied substitution levels of stearic acid on the PHHA block (mol stearic acid: mol hydroxyl). The level of stearic acid substitution on PEO-*b*-PHSA was estimated by ^{1}H-NMR in chloroform-*d* (AM-300 MHz).

AmB Encapsulation in PEO-*b*-PHSA Micelles by a Solvent Evaporation Method

AmB (470 μg or 2.0 mg) and PEO-*b*-PHSA (20 mg) were dissolved in methanol (5 or 10 ml depending on AmB level) in a round bottom flask. Methanol was evaporated under vacuum at 40°C. Distilled water was added to the PEO-*b*-PHSA/drug film, and the solution was incubated at 40°C for 10 min and vortexed for 30 s afterwards. The micellar solution was filtered (0.22 μm) and freeze-dried. An aliquot of the micellar solution in water was diluted with an equal volume of *N, N*-dimethylformamide (DMF) and the drug content mea-

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ABBREVIATIONS: PEO-*b*-PLAA, Poly (ethylene oxide)-*b*-poly(L-aspartic acid); PEO-*b*-PHSA, PEO-*b*-poly(*N*-hexyl stearate-L-aspartamide); PEO-*b*-PBLA PEO-*b*-poly(β-benzyl-L-aspartate), PEO-*b*-PHHA, PEO-*b*-poly(hydroxyhexyl-L-aspartamide); AmB, Amphotericin B; DMSO, *N*,*N*-dimethylsulfoxide; DMF, *N*,*N*-dimethylformamide; RBC, Red blood cell; PBS, Phosphate buffer saline; MIC, Minimum inhibitory concentration.

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sured from the UV/VIS absorbance of AmB at 412 nm (Pharmacia Biotech Ultraspec 3000).

Size Exclusion Chromatography (SEC)

AmB was dissolved in 0.10 M phosphate buffer, pH = 7.4, with the aid of DMSO, providing levels at 1.0 to 100 μg/ml. The amount of DMSO in the final samples was less than 1% (v/v). Freeze-dried PEO-*b*-PHSA micelles with encapsulated AmB were dissolved in 0.10 M phosphate buffer, pH = 7.4, at 0.50 mg/ml for the polymer and 6 to 10 μg/ml for AmB. The samples at 125 μl were injected into a SEC column (Hydrogel 2000, Waters) after having been equilibrated with 0.10 M phosphate buffer, pH = 7.4, at a flow rate of 0.80 ml/min (Waters B15 LC system). PEO-*b*-PHSA and AmB were detected by UV/VIS at 210 and 410 nm, respectively.

Aggregation State of AmB – UV/VIS Spectroscopy

Freeze-dried samples of AmB in PEO-*b*-PHSA micelles with 11% and 70% of stearic acid substitution were dissolved in phosphate buffered saline (PBS), pH = 7.4, at 4 μg/ml of AmB. DMSO was used to solubilize AmB in PBS, pH = 7.4, at a similar drug level. The level of DMSO in the final sample was <1% (v/v). The UV/VIS spectra of AmB in different samples were recorded from 300 nm to 450 nm (Milton Roy 3000).

Hemolytic Activity of AmB toward Human Red Blood Cells

Human blood was collected and centrifuged (2000 rpm). Supernatant and buffy coat were removed. Red blood cells (RBCs) were washed and diluted with PBS, pH = 7.4. The proper dilution factor for RBCs was estimated from the absorbance of hemoglobin at 576 nm in the supernatant in samples lysed by 20 μg/ml of AmB. A properly diluted sample of RBCs gave an absorbance of 0.4–0.5. AmB solutions were incubated with diluted RBCs at 37°C for 30 min and placed in ice to stop hemolysis. The unlysed RBCs were removed by centrifugation (14,000 rpm for 20 s), and the supernatant was analyzed for hemoglobin by UV/VIS spectroscopy at 576 nm. The percent of hemolysed RBCs was determined using the following equation:

$$\% \text{ Hemolysis} = 100(\text{Abs} - \text{Abs}_o)/ (\text{Abs}_{100} - \text{Abs}_o). \quad (1)$$

Abs, Abs_o and Abs_{100} are the absorbance for the sample, control with no AmB and control in the presence of 20 μg/ml AmB, respectively.

Minimal Inhibitory Concentration (MIC) of AmB

AmB in PEO-*b*-PHSA micelles was dissolved in isotonic sodium chloride solution; giving an AmB level of 200 μg/ml. AmB was dissolved in DMSO and diluted further with sodium chloride solution to give the same concentration. The level of DMSO in the final solution was <1% v/v. Samples of PEO-*b*-PHSA micelles in sodium chloride solution were also used as a control. Solutions of 20 μl from these samples were diluted with the broth medium (RPMI 1640) (80 μl) in the first microwell. The next 11 microwells had two-fold diluted solutions. To each microwell, 100 μl of the inoculum containing 5×10^3 cfu/ml of fungal organisms (*Candida albicans*, *Aspergillus fumigatus* or *Cryptococcus neoformans*) in broth medium was added, giving a total volume of 200 μl per well. Microwell containers were incubated at 35°C for 24 h. Organism and medium controls were performed simultaneously to check the growth of organisms and sterility of broth medium, respectively. The MIC was defined as the minimum concentration of AmB that shows a full inhibition of the fungus growth in the well, examined by an inverted microscope (×40). All tests were repeated three times.

RESULTS AND DISCUSSION

PEO-*b*-PHSA was synthesized at three levels of stearic acid substitution (Fig. 1). The level of fatty acid substitution was determined by ^{1}H NMR, comparing the characteristic peak intensity of PEO (-CH_2-CH_2O-, δ = 3.65 ppm) to the peak intensity of the methyl group of stearic acid (-CH_3, δ = 0.8 ppm) (data not shown). The levels of stearic acid substitution on PEO-*b*-PHSA were 11, 50 and 70%. In an earlier study, the level of fatty acid substitution had a dramatic effect on the thermodynamic stability of micelles composed of fatty acid conjugates of PEO-*b*-PHHA (9). We hypothesized that this structural parameter would influence the encapsulation of AmB, the drug's toxicity as judged by hemolysis and its antifungal activity.

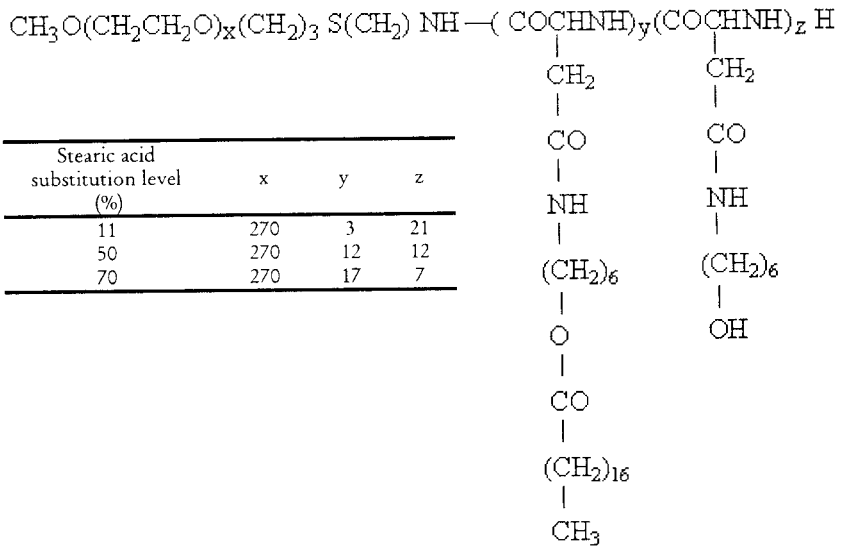
SEC confirmed the encapsulation of AmB by PEO-*b*-PHSA micelles. AmB eluted with PEO-*b*-PHSA micelles at 50 and 70% stearic acid substitution at 10.68 and 10.62 min (on average), respectively, with no evidence of unencapsulated drug (data not shown). AmB alone injected into the SEC column at 1.0 μg/ml eluted at 17.36 min, but at 10 and 100 μg/ml, it eluted at 17.28 and 16.48 min, respectively, owing to the self-aggregation of the drug. We were unable to directly confirm the encapsulation of AmB by PEO-*b*-PHSA micelles at 11% by SEC, owing to its dissociation and elution as unimers.

The level of AmB encapsulated in PEO-*b*-PHSA micelles at 11% stearic acid substitution was 0.22 mol drug: mol PEO-*b*-PHSA, and the level increased to 0.35 and 0.36 at 50 and 70% of stearic acid substitution, respectively (Table 1). The yield of encapsulated AmB for PEO-*b*-PHSA micelles was 51, 73 and 77%, respectively. In each case, freeze-dried product could be reconstituted with an aqueous vehicle at 200 μg/ml of AmB, the initial level of drug used for the MIC experiments, indicating encapsulation of AmB at all three levels of stearic acid substitution. An increase in the initial amount of AmB used for encapsulation lead to a higher content of AmB encapsulated in PEO-*b*-PHSA micelles at 50% stearic acid substitution (0.89 mol drug: mol PEO-*b*-PHSA).

Hemolysis of AmB encapsulated in PEO-*b*-PHSA micelles at 11% stearic acid substitution was similar to AmB itself, causing 50% hemolysis at 1 μg/ml and 100% hemolysis at about 3 μg/ml (Figs. 2 and 3). In contrast, AmB encapsulated in PEO-*b*-PHSA micelles at 50 and 70% stearic acid substitution was completely non-toxic (no lysis) at levels reaching 22 μg/ml.

The effect on hemolysis, however, was dependent on the content of AmB in the PEO-*b*-PHSA micelles (Fig. 3). PEO-*b*-PHSA micelles (50% stearic acid substitution) at 0.36 mol drug: mol polymer were completely non-hemolytic at 22 μg/ml of AmB. On the other hand, PEO-*b*-PHSA micelles at 0.89 mol drug: mol polymer caused 80% hemolysis at a similar level of drug. Notably, PEO-*b*-PHSA by itself exerted no

PEO-*b*-PHSA



Stearic acid substitution level (%)	x	y	z
11	270	3	21
50	270	12	12
70	270	17	7

AmB

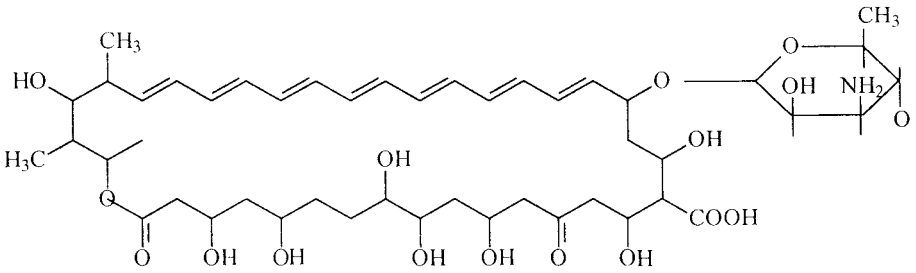


Fig. 1. Chemical structure of PEO-*b*-PHSA and AmB.

Table I. The Effect of Fatty Acid Substitution on the Encapsulation of AmB by PEO-b-PHSA Micelles by a Solvent Evaporation Method

Stearic acid substitution level (%)	PEO-b-PHSA (mg)	Initial level of AmB (μg)	Am*B*[a] (μg)	AmB: PEO-b-PHSA[a] (mol:mol)	Yield[a] (%)
11	20	470	240	0.22	51
50	20	470	340	0.35	73
70	20	470	360	0.36	77
50	20	2000	942	0.89	53

[a] Data is the average of two replicates.

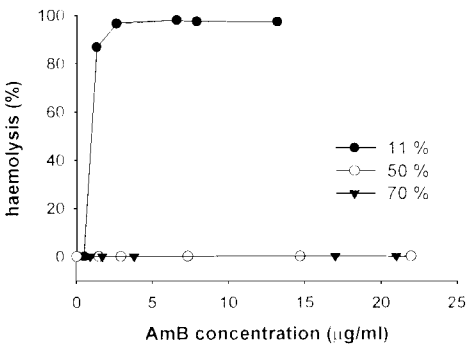


Fig. 2. The effect of fatty acid substitution level in PEO-*b*-PHSA micelles on the hemolytic activity of AmB encapsulated by a solvent evaporation method (n = 3).

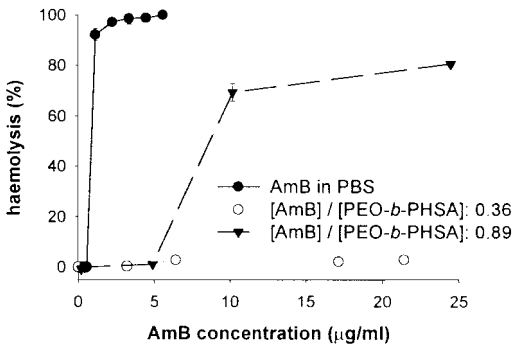


Fig. 3. The effect of drug loading on the hemolytic activity of AmB encapsulated in PEO-*b*-PHSA micelles (50% of stearic acid substitution) by a solvent evaporation method (n = 3).

Table II. The Effect of Fatty Acid Substitution of the Core-Forming Block on the *in Vitro* Antifungal Activity of AmB Encapsulated by PEO-*b*-PHSA Micelles through Solvent Evaporation in Comparison to AmB Alone

AmB in:	MIC ± SD (μg/ml) n = 3		
	C. albicans	*C. neoformans*	*A. fumigatus*
PBS	0.30 ± 0.00	0.30 ± 0.00	0.45 ± 0.00
PEO-b-PHSA 11%	0.35 ± 0.09	0.18 ± 0.04	0.6 ± 0.00
PEO-b-PHSA 50%	0.27 ± 0.04	0.18 ± 0.05	0.6 ± 0.00
PEO-b-PHSA 70%	0.33 ± 0.11	0.23 ± 0.07	0.35 ± 0.09

hemolytic effects at all three levels of stearic acid substitution (data not shown).

While there was a decrease of hemolysis induced by AmB, the antifungal activity of encapsulated AmB was comparable to AmB itself or better based on measurements of MICs against the growth of three pathogenic fungi. Fungi growth was examined with an inverted microscope (×40). AmB in an isotonic solution inhibited the growth of *C. albicans, C. neoformans* and *A. fumigatus* at 0.30, 0.30 and 0.45 μg/ml, respectively (Table 2). AmB encapsulated in PEO-*b*-PHSA micelles was as effective as free AmB in most of the cases. At 11 and 50% of stearic acid substitution, encapsulated AmB was even more effective than AmB itself, inhibiting the growth of *C. neoformans* at a level of 0.18 μg/ml (Unpaired *t* test, $P < 0.01$). PEO-*b*-PHSA micelles without AmB were unable to inhibit the fungal growth at 5-mg/ml levels or below (data not shown).

The reduction of hemolysis but with a maintenance or even improved antifungal activity in terms of MIC of AmB encapsulated in PEO-*b*-PHSA micelles may reflect the sustained release of the drug and/or the release of the drug in a monomeric (non-aggregated) state. Monomeric AmB is nontoxic toward mammalian cells, but causes leakage of fungal cells, presumably due to its selective interaction with ergosterol. Aggregated AmB, on the other hand, is nonselective, i.e., forms pores in both mammalian and fungal cell membranes (11).

Insight to the aggregation state of AmB can be obtained by UV/VIS spectroscopy. AmB easily forms aggregates (its critical aggregation concentration is 1.0 μg/ml). The UV/VIS spectrum of aggregated AmB is primarily characterized by a broad peak at approximately 330–340 nm and three other peaks at 364, 385, 409 nm (Fig. 4A). The spectral features of AmB encapsulated in PEO-*b*-PHSA micelles at 11% are quite similar to AmB alone (Fig. 4B), indicating weak interaction with the micelle core, which has little effect on the aggregation state of the drug. Thus, encapsulated AmB in PEO-*b*-PHSA micelles at 11% of stearic acid substitution might be quickly released and/or released in an aggregated state. In contrast, there was a bathochromic shift to 415 nm (approximately 6 nm) for AmB encapsulated in PEO-*b*-PHSA micelles at 70% stearic acid substitution (Fig. 4C). AmB exhibits a similar spectrum after its binding to serum lipoproteins, which have cores rich in triglycerides, or after its binding to sterols (12,13). In the latter case, AmB at a low degree of aggregation, probably a dimer, preferentially binds with ergosterol, the sterol of fungal cell membranes, whereas highly aggregated AmB has little tendency to bind ergosterol.

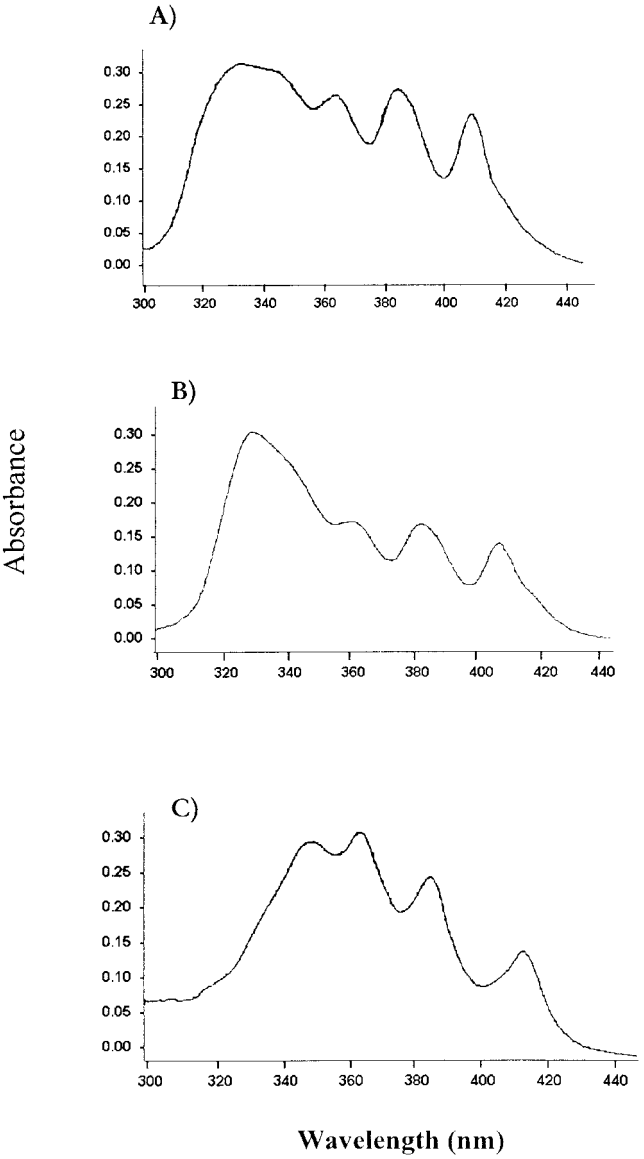


Fig. 4. Absorption spectra of AmB (4 μg/ml) in A) PBS, pH = 7.4, B) PEO-*b*-PHSA at 11% stearic acid substitution and C) PEO-*b*-PHSA at 70% stearic acid substitution.

Thus, spectral features of AmB encapsulated in PEO-*b*-PHSA micelles at 70% may reflect drug at a low degree of aggregation bound tightly in the micelle core. AmB encapsulated stably in PEO-*b*-PHSA micelles at 50 or 70% might be released gradually and/or in a monomeric state, increasing the *in vitro* efficacy of this important antifungal drug. This hypothesis is under further study.

CONCLUSIONS

The degree of fatty acid substitution of a micelle-forming PEO-*b*-PHSA can be altered to enhance the encapsulation of AmB and reduce the drug's membrane perturbing effects in terms of hemolysis, while retaining its *in vitro* antifungal activity. PEO-*b*-PHSA micelles at 50% and 70% stearic acid substitution effectively encapsulate AmB and increase its efficacy *in vitro.*

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