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Relative aggregation state and hemolytic activity of amphotericin B encapsulated by poly(ethylene oxide)-*block*–poly(*N*-hexyl-L-aspartamide)-acyl conjugate micelles: effects of acyl chain length

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Abstract

We systematically altered the chemical structure of the core-forming poly(L-amino acid) block of an amphiphilic diblock copolymer series based on poly(ethylene oxide)-*block*–poly(*N*-hexyl-L-aspartamide), PEO-*b*–p(*N*-HA), acyl esters by varying the length of the attached acyl side chain. Drug-loaded micelles were prepared in good yield by a modified solvent evaporation procedure. In addition, the relative aggregation state and hemolytic activity of encapsulated amphotericin B (AmB) were analyzed by absorption spectroscopy. The length of the attached acyl side chain in PEO-*b*–p(*N*-HA) acyl ester micelles modulates the relative aggregation state of encapsulated AmB. Furthermore, acyl chain length appears to have a profound influence on the time-dependent hemolytic profile of encapsulated AmB toward bovine erythrocytes. For all acyl conjugate micelle–AmB formulations, the onset of hemolysis is delayed relative to free AmB. Particularly in the case of stearate ester micelles, the incomplete and gradual build-up of hemolysis might reflect the sustained release of drug over a period of 24 h. Based on the corresponding absorption spectrum, we speculate that encapsulated AmB may interact strongly with stearate side chains, resulting in sustained release. Via chemical manipulation of the core-forming region, it may be possible to fine-tune the release of encapsulated AmB from PEO-*b*–p(*N*-HA)-acyl ester micelles.

Keywords: Polymeric micelles; Poly(L-amino acid); Amphotericin B; Sustained release

1. Introduction

AB-type amphiphilic diblock copolymers contain a hydrophobic portion that is chemically attached to a hydrophilic block, thereby preventing phase separation of the blocks. The functional properties of micelles based on amphiphilic diblock copolymers render them quite useful for encapsulation and delivery of hydrophobic agents. Acyl esters of poly(ethylene oxide)-*block*–poly(6-hydroxyhexyl-L-aspartamide), PEO-*b*–p(6-HHA), form micelle-like supramolecular structures in aqueous medium [1]. The shell, or corona, is comprised of the hydrophilic, highly hydrated PEO block while the hydrophobic core block is derived from poly(L-aspartate), p(L-Asp). In this series, the L-Asp residues are conju-

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gated with 6-amino-1-hexanol, which can then be acylated with carboxylic acids or acid anhydrides under standard reaction conditions to yield a system of esters containing saturated aliphatic side chains of various lengths (Fig. 1a). The length of the attached acyl chain can be adjusted in order to impact the properties of the hydrophobic core region [1]. Thus, it is possible to utilize chemical means to 'tailor' the micellar core and, thereby, enhance the properties of the micelle system with respect to encapsulation and delivery of physically encapsulated compounds with poor aqueous solubility. Previous work demonstrated the utility of PEO-*block*–poly(*N*-hexylstearate-L-aspartamide) micelles as carrier systems for amphotericin B (AmB) [2,3]. Chemical tailoring of the core-forming block of PEO-*block*–poly(*N*-hexyl-L-aspartamide), PEO-*b*–p(*N*-HA), acyl esters may provide a viable means for improving the therapeutic effectiveness of AmB.

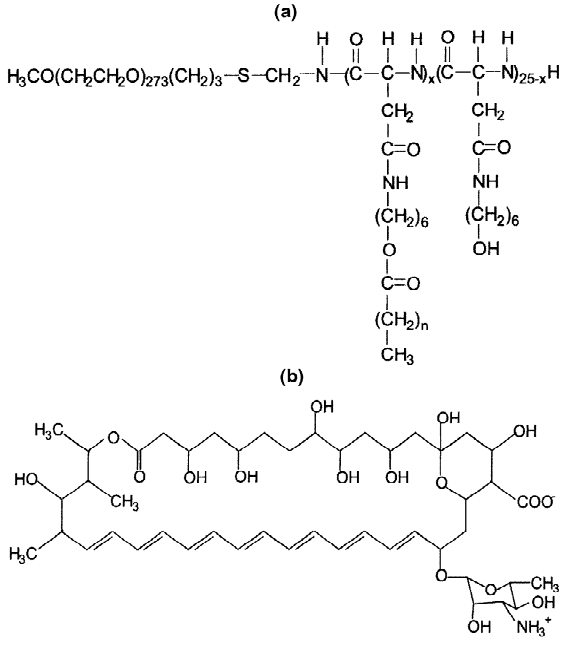

Fig. 1. (a) Chemical structure of PEO-*b*–p(*N*-HA)-acyl conjugates. The final block copolymer products are named as poly(ethyleneoxide)-*block*–poly(*N*-hexyl-L-aspartamide-Z-acid) where Z designates the length of the esterified chain, i.e. acetic, hexanoic, lauric, or stearic, and *x* corresponds to the average number of acyl groups attached per 25 L-Asp residues as determined by ^{1}H NMR. For simplicity, the copolymers are abbreviated as PEO-*b*–p(HAZA). (b) Chemical structure of amphotericin B.

AmB is a potent, membrane-active polyene macrolide antibiotic widely used in the treatment of systemic fungal infection. The unique molecular structure (Fig. 1b) of AmB imparts broad-spectrum antifungal activity by enabling drug molecules to concentrate in cell membranes. Although AmB is an effective fungicide, there are several major clinical limitations to its use. Due to the amphiphilic nature of AmB, the drug tends to aggregate in aqueous solution, resulting in poor water solubility. In addition, AmB shows low selective toxicity for fungal versus mammalian cells. Generally, the selectivity of AmB has been attributed to differences in membrane sterol content. The affinity of AmB for different sterols varies depending on the chemical nature of the sterol [4,5]. Consequently, ergosterol-containing membranes are more sensitive to AmB than cholesterol-containing membranes [6,7]. In fact, the concentration of AmB required to obtain the same permeability is much higher for cholesterol-containing phospholipid vesicles than for those containing ergosterol [8]. The binding affinity constant of AmB for ergosterol is much higher than that of AmB for cholesterol in egg phosphatidylcholine vesicles [9]. In addition, theoretical studies have revealed that AmB-ergosterol complexes are more stable than AmB-cholesterol complexes [10]. Hence, fungal cells are more susceptible to the toxic effects of AmB to due the dominant presence of ergosterol, rather than cholesterol, in the cell membrane.

The mechanism of action of AmB on both cell types is similar. AmB associates with the membrane sterol component to form AmB–sterol complexes. These complexes then associate with one another to form transmembrane pores through which leakage of monovalent ions and small molecules occurs, ultimately leading to cell death. Although the mechanism of action is not completely understood, it is widely accepted that the toxicity of AmB is in some way mediated by the relative aggregation state of the drug [11–13]. The same relationship exists for derivatives of AmB in which modifications have been made to the polar head region while the polyene structure is conserved [14]. Nevertheless, the self-association and aggregation behavior of AmB is quite complicated and depends on concentration as

well as local environment. Legrand et al. demonstrated that AmB can exist as a combination of species (monomers and/or soluble or insoluble aggregates) in aqueous solution and that water-soluble, self-associated AmB induces leakage in human erythrocytes [13]. Essentially, the toxic effects of AmB can be attributed to the aggregated form of the drug [11].

Certain carrier vehicles seem to affect the aggregation state and activity of AmB [3,15–17]. Barwicz et al. demonstrated the ability of surfactants to reduce the toxicity of AmB via decreasing the aggregation state of the drug [12]. As the equilibrium between aggregated and monomeric forms of AmB seems to be crucial with respect to nonselective toxicity, a carrier capable of minimizing the aggregation state of AmB is desirable. Due to stealth exteriors formed by PEO brushes, low critical micelle concentrations (CMCs), hydrophobic core regions, and nanoscopic dimensions, polymeric micelles might be effective drug-delivery systems for poorly water-soluble compounds such as AmB. It is generally accepted that AmB associates with the sterol components of cell membranes, resulting in the formation of hydrophilic pores. However, AmB interacts to some extent with aliphatic chains [18–20]. Due to the presence of aliphatic ester core-forming regions, micelles based on PEO-*b*–p(*N*-HA)-acyl esters might be appropriate delivery vehicles for AmB. In the present work, AmB-loaded polymeric micelles of PEO-*b*–p(*N*-HA)-acyl conjugates were prepared in order to determine the effects of acyl chain length on the relative aggregation state of encapsulated AmB. In addition, the hemolytic activity of encapsulated AmB was determined as a function of acyl core structure and time.

2. Materials and methods

2.1. Preparation of methoxypoly(ethylene oxide)-block–poly(6-hydroxyhexyl-L-aspartamide)-acyl conjugates, PEO-b–p(HAZA)

For simplicity, the hydrophilic blocks will be referred to as PEO although the blocks are actually composed of methoxypoly(ethylene oxide), mPEO. The preparation of methoxypoly(ethylene oxide)-*block*–poly(6-hydroxylhexyl-L-aspartamide), PEO-*b*–p(6-HHA), and methoxypoly(ethylene oxide)-*block*–poly(*N*-hexyl-L-aspartamide)-Z-acid conjugates, PEO-*b*–p(HAZA), was described previously [1]. The esterification products are referred to as PEO-*b*–p(HAZA) esters where Z denotes acetic, hexanoic, lauric, or stearic, thereby designating the length of the attached acyl side chain. Thus, the esterified products in this polymer series can be abbreviated as PEO-*b*–p(HAZA) conjugates. However, the intermediate aminolysis product (no esterification) is referred to PEO-*b*–p(6-HHA). In subsequent discussions, the esterification products as a group will be referred to as PEO-*b*–p(HAZA) or PEO-*b*–p(*N*-HA)-acyl esters as mentioned in the Introduction.

Briefly, methoxypoly(ethylene oxide)-*block*–poly(β-benzyl-L-aspartate), PEO-*b*–PBLA, 12:25 (Nanocarrier, Kashiwashi Chiba, Japan), containing a PEO block with a molecular weight of 12 000 g/mol and an average of 25 L-Asp repeat units was reacted with 6-amino-1-hexanol in freshly distilled dimethylformamide (DMF) in the presence of 2-hydroxypyridine to prepare PEO-*b*–p(6-HHA). The loss of the aromatic protons attributed to the phenyl group was verified by ^{1}H NMR (spectra not shown). In addition, the methylene peaks centered at 1.25 and 1.40 ppm were integrated relative to the PEO polyether methylene resonances at 3.6 ppm in order to determine the percent substitution of the aminolysis product (Table 1). The percent yield was approximately 94%. This product was esterified at ambient temperature with excess stearic, lauric, or hexanoic acid in dried CH_2Cl_2 in the presence of dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP). Acetylation of PEO-*b*–p(6-HHA) was accomplished with excess acetic anhydride in the presence of DMAP. A general chemical structure for the final block copolymer products is given in Fig. 1a. Relative acyl attachment was quantified from the ^{1}H NMR spectra (spectra not shown) of the conjugates by comparing the relative intensity of the PEO peak at 3.6 ppm to the terminal methyl peak of the acyl chains at 0.9 ppm for the fatty acid conjugates or 2.0 ppm for the acetate methyl (Varian, 500 MHz, Palo Alto, CA, USA). On average, the acyl conjugates were prepared in 85

Table 1
PEO-*b*–p(L-Asp) derivatives used for the encapsulation of AmB: abbreviations and structures (n and x refer to Fig. 1a)

Polymer	Acyl chain length (no. C)	n $(CH_2)^a$	x^a	Substitution level (%)
PEO-*b*–p(6-HHA)	0	N/a	24.8	99.3±0.1
PEO-*b*–p(HAAA)	2	0	23.7	94.6±0.6
PEO-*b*–p(HAHA)	6	4	21.9	87.4±0.5
PEO-*b*–p(HALA)	12	10	21.4	85.6±0.5
PEO-*b*–p(HASA)	18	16	22.7	90.7±0.9

[a] Refers to Fig. 1a.

(±7)% yield. The average degree of substitution for each esterified product is given in Table 1.

2.2. Drug loading

PEO-*b*–p(6-HHA), 80 mg, or 5 mg of PEO-*b*–p(HAZA) conjugate was dissolved in 2.0 ml of methanol (MeOH) containing 0.3125 mg/ml of AmB (Chem-Impex, Wood Dale, IL, USA). Deionized water (dH_2O) was added dropwise to the stirring solution at a rate of 0.075–0.090 ml/min (1 drop/10–12 s) to obtain a 50:50 MeOH–dH_2O mixture. An additional 1.0 ml of dH_2O was added directly to the stirring solution. All samples were sonicated as necessary to obtain clear solutions. Trehalose dihydrate (0.75 g) was then dissolved in the 40:60 MeOH–dH_2O polymer mixtures. In the case of PEO-*b*–p(6-HHA), 0.6 g of trehalose dihydrate was added instead. The volume was reduced to approximately 1.5 ml via rotary evaporation. The aqueous solution was collected and diluted to a final volume of 5.0 ml with dH_2O for all copolymers except PEO-*b*–p(6-HHA), which was diluted to 4.0 ml. An additional 0.25 g of trehalose dihydrate was added to the acyl ester solutions and dissolved with the aid of slight stirring. In the case of PEO-*b*–p(6-HHA), 0.2 g of trehalose dihydrate was added and dissolved. The aqueous polymer solutions were sterile-filtered. Aliquots (0.5 ml) were placed in 10-ml freeze-drying flasks, dipped in liquid N_2 until frozen, and then freeze-dried (Freeze Dry System/Freezone 4.6, Labconco Corporation, Kansas City, MO, USA). The freeze-dried samples were stored at 5 °C until use. 'Blank' micelles containing only block copolymer were also prepared as described above except that the polymers were initially dissolved in MeOH containing no AmB.

2.3. Determination of aggregation state and AmB loading

The freeze-dried formulations were reconstituted in 1.0 ml dH_2O. In order to quantify AmB content, the reconstituted solutions were diluted two-fold with DMF, then diluted appropriately into the linear range. AmB concentration was quantified via absorbance of the monomeric species at 412 nm (Amersham Pharmacia Biotech Ultraspec 4000, Piscataway, NJ, USA). Spectra were acquired from 320.0 to 450.0 nm at a rate of 405 nm/min and a scan step of 0.1 nm. In order to assess the relative aggregation state of AmB, formulations were reconstituted in dH_2O and diluted as necessary. A spectrum of 3 μg/ml AmB in PBS (0.0375% DMSO) was also acquired using a 1.0-mm cell (data not shown). All spectra were acquired as described above. In spectra in which the final absorbance band was centered around 409 nm, the ratio of the first to last peak in the absorbance spectrum served as an indicator of relative aggregation state. Spectra of 5 μg/ml AmB in DMF and 10 μg/ml AmB in dH_2O containing 1% DMF were also acquired.

2.4. Determination of micelle size by size-exclusion chromatography

All measurements were taken using an Agilent 1100 series HPLC system equipped with a refractive index detector and Agilent GPC analysis software (Agilent Technologies, Palo Alto, CA, USA). Freeze-dried PEO-*b*–p(HAZA) samples, with or without AmB, were reconstituted to 0.5 mg/ml polymer with phosphate-buffered saline, pH 7.2 (PBS), whereas PEO-*b*–p(6-HHA) formulations were reconstituted to 10 mg/ml. Formulations were injected (100 μl) in

duplicate onto a Shodex SB-806M HQ OHpak size exclusion column equipped with a Shodex OHpak SB-G guard column (Showa Denko, New York, NY, USA). The column was equilibrated with PBS mobile phase and calibrated with dextran standards, $M_w = 1–7 \cdot 10^6$ g/mol (JM Science, Grand Island, NY, USA). The flow-rate and column compartment temperature were set to 0.8 ml/min and 37 °C, respectively.

2.5. Assessment of hemolytic activity of AmB–polymer formulations

EDTA-anticoagulated bovine blood was diluted in PBS, pH 7.2. The erythrocytes were separated by centrifugation for 10 min at 3000 rpm. The supernatant was removed and discarded. The packed cell volume (PCV) was washed three more times with PBS. The PCV was diluted appropriately in PBS to obtain suspensions of $8 \cdot 10^7$ and $1.48 \cdot 10^8$ cells/ml. The AmB–polymer formulations and polymer blanks were equilibrated to ambient temperature and reconstituted with 1.0 ml of PBS just prior to use. 1.85 ml of the 8×10^7 cells/ml suspension was incubated at 37 °C in a shaking water bath (75 rpm) with 150 μl of AmB–polymer formulation, polymer blank containing no AmB, or PBS buffer (control) for 24 h. A solution containing 8 mg/ml of AmB in dimethylsulfoxide (DMSO) was prepared and diluted appropriately with buffer to yield 6 μg/ml AmB in PBS containing 0.075% DMSO. In addition, 1.0 ml of the $1.48 \cdot 10^8$ cells/ml suspension was incubated with 1.0 ml of 6 μg/ml AmB in PBS (0.075% DMSO) or PBS at 37 °C in the shaking water bath (75 rpm) for 24 h. In all cases, the final AmB and erythrocyte concentrations in the incubated samples were approximately 3 μg/ml and $7.4 \cdot 10^7$ cells/ml, respectively. Samples containing AmB–polymer formulations, AmB-free polymer blanks, and buffer controls were withdrawn in triplicate at 1, 9, 16 and 24 h and centrifuged at 3000 rpm for 10 min. The supernatant was collected, and hemoglobin content was determined by absorbance at 542 nm. The value for total cell lysis was obtained by hypotonic hemolysis. Percent hemolysis is reported by $100(Abs_s - Abs_b)/(Abs_l - Abs_b)$ where Abs_s is the absorbance of the sample, Abs_b is the average absorbance of the buffer, and Abs_l is the average absorbance of the lysed samples. All values are reported as mean±standard deviation.

3. Results and discussion

With seven conjugated double bonds, AmB is a very strong chromophore and is easily detected by UV–Vis spectroscopy. The typical absorption spectrum of AmB contains four primary peaks (Fig. 2). Although the positions and intensities of the absorption bands vary with respect to local environment, the ratio of the first (346 nm) to the fourth (409 nm) peaks, I/IV, can be taken as a measure of relative aggregation state of AmB. This ratio can be quite low (<0.25) for unaggregated, or monomeric, AmB and as high as 2.0 for highly aggregated species [12]. Under the experimental conditions, the levels of encapsulated drug and the encapsulation efficiency were similar for all of the polymer–AmB formulations (Table 2). The absorption spectrum of AmB encapsulated by PEO-*b*–p(*N*-HA)-acyl conjugates is given in Fig. 3. The effects of acyl chain length are quite apparent. The absorption spectrum of AmB encapsulated by PEO-*b*–p(6-HHA) contains four absorption bands centered at 346, 362, 385 and 409 nm with a I/IV ratio of 1.2. Although this value indicates a low level of AmB aggregation compared to some of the micelle formulations, the polymer concentration in the PEO-*b*–p(6-HHA) formulation is much greater than for the acyl conjugates. Previously, we determined that the CMC of PEO-*b*–p(6-HHA) micelles is substantially higher (approximately 0.5 mg/ml) than those of the acyl conjugates, which had CMCs on the order of 20 μg/ml [1]. Therefore, PEO-*b*–p(6-HHA) was reconstituted to 10 mg/ml in order to ensure that the polymer concentration was well above the CMC. Consequently, the drug-to-polymer ratio for this formulation is quite low (Table 2) compared to those of the PEO-*b*–p(HAZA) formulations. Because the CMC is related the thermodynamic equilibrium that exists between the micelles and unimers, or thermodynamic stability, we hypothesized that PEO-*b*–p(6-HHA) might not be stable enough to withstand dilution. Therefore, even with low levels of drug aggregation, this formulation might not be a suitable delivery system for parenteral administration of AmB. In fact, the

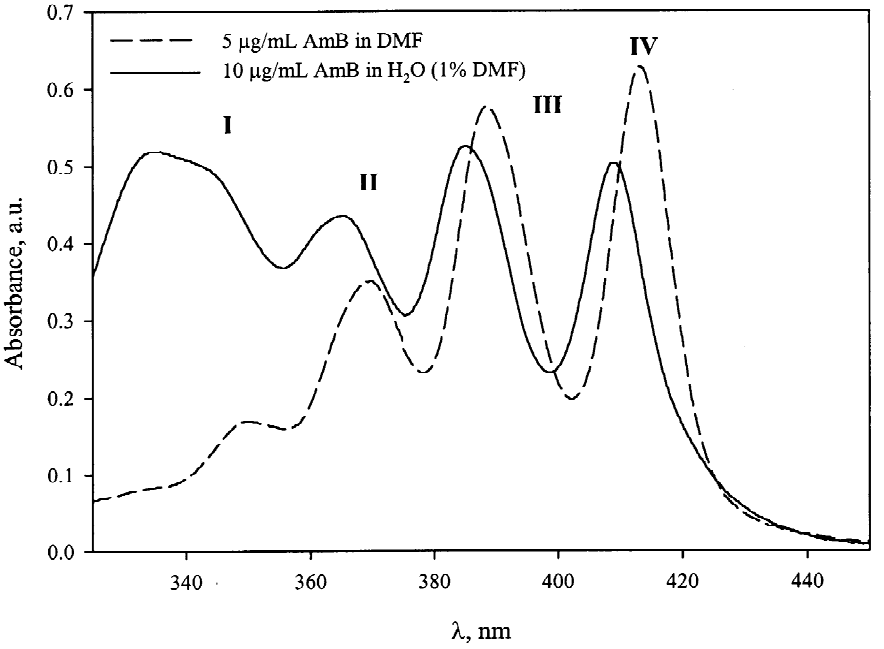

Fig. 2. Solvent/environmental effects on the typical four-peak AmB absorption spectrum.

formulation did not maintain integrity during SEC analysis. All of the acyl conjugates, with and without AmB, formed micelles on the order of 10^6 g/mol (data not shown), which was consistent with previous results for PEO-*b*–p(HASA) ester micelles [2]. However, PEO-*b*–p(6-HHA) micelles did not remain intact on the column and could not be detected. As expected, the relatively unstable PEO-*b*–p(6-HHA) micelles did not withstand dilution conditions.

The absorption spectrum of 3 μg/ml AmB in PBS (0.0375% DMSO) was characteristic of aggregated AmB (data not shown), while the absorption spectrum of AmB encapsulated in PEO-*b*–p(*N*-HA)-acyl conjugate micelles varies considerably (Fig. 3). In PEO-*b*–p(HAAA), PEO-*b*–p(HAHA), and PEO-*b*–p(HALA) micelles, encapsulated AmB appears to be quite aggregated. In contrast, the spectrum observed for the PEO-*b*–p(HASA) formulation is rather distinctive compared to those of the other acyl conjugate formulations. The AmB/PEO-*b*–p(HASA) spectrum shares three bands with the AmB/PEO-*b*–p(HALA) spectrum in which the absorption bands are red-shifted compared to the shorter acyl chain formulations. The λ_{max} of the AmB/PEO-*b*–p(HALA) and AmB/PEO-*b*–p(HASA) formulations are 347 and 368 nm, respectively. For the shorter chain lengths, λ_{max} is of the order of 331–338 nm. The peak at the longest wavelength in the absorption

Table 2
Results of AmB encapsulation

Polymer	[Polymer] (mg/ml)	[AmB] (μM[a])	AmB–polymer (mol:mol)	Encapsulation efficiency
PEO-*b*–p(6-HHA)	10	44.9±0.8	0.08±0.01	0.66±0.01
PEO-*b*–p(HAAA)	0.5	53.2±2.1	1.94±0.08	0.79±0.03
PEO-*b*–p(HAHA)	0.5	36.9±0.8	1.43±0.03	0.55±0.01
PEO-*b*–p(HALA)	0.5	53.2±0.8	2.25±0.03	0.70±0.03
PEO-*b*–p(HASA)	0.5	42.7±0.3	1.96±0.01	0.63±0.01

[a] Designates the level of AmB upon reconstitution with 1 ml of dH_2O.

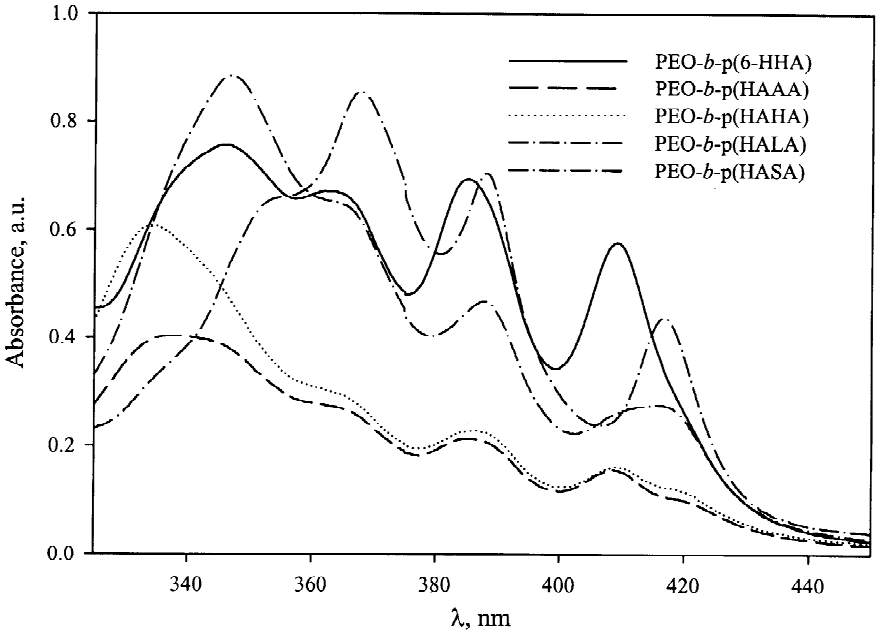

Fig. 3. Absorption spectrum of amphotericin B encapsulated in PEO-*b*–p(6-HHA) and PEO-*b*–p(HAZA) ester micelles as a function of acyl composition. Block copolymer concentrations are as follows: 1.25 mg/ml for PEO-*b*–p(6-HHA), 0.0625 mg/ml for PEO-*b*–p(HAAA); 0.125 mg/ml for PEO-*b*–p(HAHA), PEO-*b*–p(HALA) and PEO-*b*–p(HASA).

spectrum of the AmB–polymer formulations is also of particular interest. For the AmB/PEO-*b*–p(HALA) and AmB/PEO-*b*–p(HASA) samples, the last absorption band lies at 416 and 417 nm, respectively, compared to 409 nm for the shorter acyl chain length formulations. The spectrum of the AmB/PEO-*b*–p(HASA) formulation is quite similar to that observed when AmB is complexed with ergosterol, while the AmB/PEO-*b*–p(HALA) spectrum is similar to that observed when AmB is complexed with cholesterol [4,21]. Due to striking similarities between our spectra and those in the literature, we propose that the interactions between AmB and PEO-*b*–p(HALA) are weaker than those between AmB and PEO-*b*–p(HASA). It is possible that increasing acyl chain length results in stronger drug–polymer interactions, whereas AmB self-association predominates at lower acyl chain lengths. Furthermore, increased interaction between the polymer side chains and AmB might result in a more gradual or sustained release profile for encapsulated AmB.

Hemolysis was not observed in the 'blank' polymer formulations containing no AmB. However, very slight hemolysis (~4%) was observed in the presence of PEO-*b*–p(HASA) after 16 h (data not shown). Consequently, the hemolytic activity observed in the presence of AmB–polymer formulations can be attributed to the action of AmB. Incubation with free AmB results in immediate hemolysis, while encapsulated AmB exerts hemolysis more gradually (Fig. 4). It is possible that the relatively low toxicity of the acyl conjugate formulations observed toward bovine erythrocytes is the result of slow diffusion of AmB from the PEO-*b*–p(HAZA) ester micelles. Free AmB caused 40% hemolysis after 1 h and 80% hemolysis at 16 h. A similar profile was observed for the less stable PEO-*b*–p(6-HHA) formulation which caused 15% hemolysis at 1 h. Furthermore, hemolysis in the presence of AmB/PEO-*b*–p(6-HHA) builds over time in a manner similar to free AmB with 70% hemolysis at 16 h. It is quite likely that AmB is released rapidly from PEO-*b*–p(6-HHA) micelles due to the relatively low stability indicated by the high CMC value. This explanation is in line with our SEC findings.

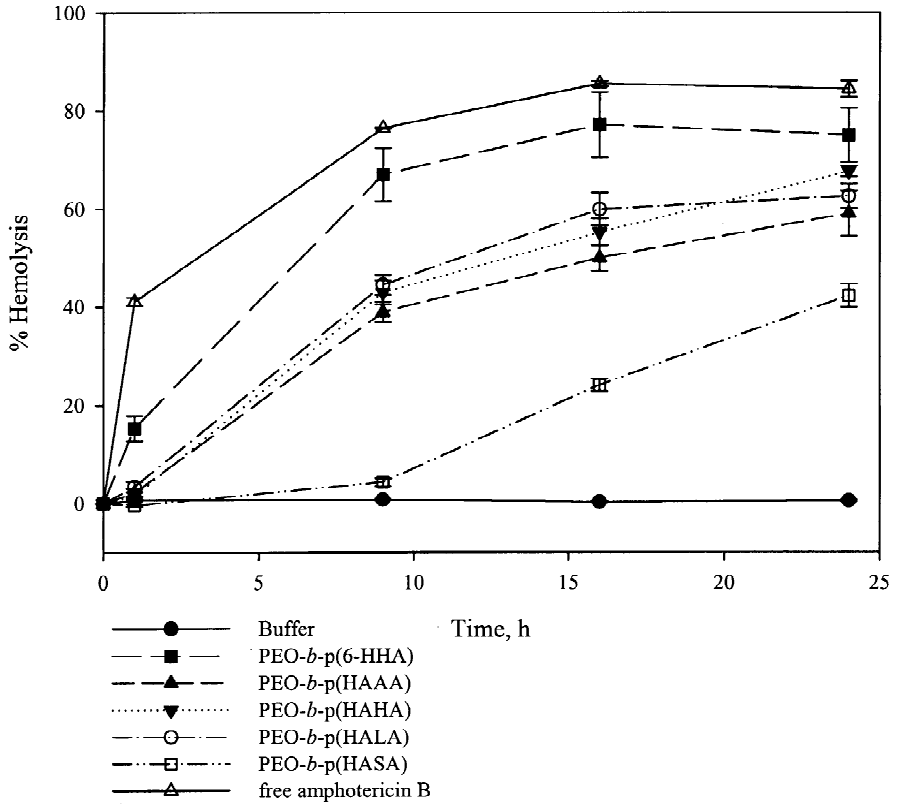

Fig. 4. Time-dependent hemolytic profile of AmB (~3 μg/ml) encapsulated in PEO-*b*–p(6-HHA) and PEO-*b*–p(HAZA) ester micelle formulations.

With increasing acyl chain length, in vitro hemolysis proceeds at a much more gradual rate (Fig. 4). Perhaps the steady build up of hemolysis reflects the sustained release of AmB from PEO-*b*–p(*N*-HA)-acyl conjugate micelles. The hemolytic profiles of the PEO-*b*–p(HAAA), PEO-*b*–p(HAHA), and PEO-*b*–p(HALA) micelle formulations are quite similar, with relatively little hemolysis after 1 h, approximately 40% at 9 h, and around 60% at 24 h. PEO-*b*–p(HASA) micelles have the most dramatic effect on AmB-induced hemolysis with only slight hemolysis (~4%) at 9 h, approximately 20% at 16 h, and about 40% after 24 h. At the 24 h mark, the hemolytic activity of the PEO-*b*–p(HASA)–AmB formulation is approximately half that of free drug. We speculate that interaction between encapsulated AmB and the stearate side chains indicated by the corresponding absorption spectrum might result in slow, or sustained, drug release. In contrast, AmB encapsulated by PEO-*b*–p(HAAA), PEO-*b*–p(HAHA), and PEO-*b*–p(HALA) micelles interact to a lesser extent with the block copolymers, enabling drug-induced hemolysis to proceed more readily.

Several factors may be responsible for the observed time-dependent hemolytic activity of the AmB–polymer formulations. Perhaps the most obvious contribution is from micelle stability. Less stable micelles might rapidly dissociate upon dilution, resulting in a relatively high concentration of AmB in solution. Indeed, this behavior is likely responsible for the similarities observed in the time-dependent hemolytic profile of AmB encapsulated by PEO-*b*–p(6-HHA) and that of free drug. Another important factor could be time-dependent release. In other words, the presence of a lag phase can be explained in the context of an AmB threshold level necessary for pore formation in the target cells [17]. A carrier vehicle may delay the onset of pore formation by

limiting the diffusion of free AmB into the aqueous environment. If released AmB interacts to a greater extent with ergosterol-containing fungal cell membranes, a threshold concentration necessary for mammalian cell toxicity may not be reached. As AmB binds preferentially to ergosterol, slower release of AmB may help favor fungal cell selectivity. It seems plausible that the PEO-*b*–p(*N*-HA)-acyl conjugate micelle series might be capable of reducing the nonspecific toxicity of encapsulated AmB through such a mechanism. Furthermore, a carrier exhibiting an increased affinity for the drug would be capable of reducing the equilibrium concentration of AmB in the aqueous phase. In this way, encapsulation by PEO-*b*–p(HASA) might lower the nonselective toxicity of AmB to a greater extent than the other polymers in the series. However, the precise mechanism by which AmB interacts with stearate or other acyl substituent side chains in PEO-*b*–p(HAZA) micelles has not been fully evaluated in the present work and remains to be determined.

In the current work, it is unlikely that the effects of diffusion of AmB from the PEO-*b*–p(HAZA) micelles can be entirely separated from the relative aggregation state of encapsulated AmB. Specifically, whether AmB is released in a relatively monomeric or highly aggregated form could have a dramatic effect on toxicity to mammalian cells. If AmB is encapsulated in a highly aggregated state and released in a similar state, the formulation should be toxic to both cell types with minimal specificity. In the present study, formulations containing highly aggregated AmB did indeed exhibit increased hemolysis over the seemingly less-aggregated PEO-*b*–p(HASA) formulation. Therefore, it is likely that the requirement of a threshold level for AmB toxicity will apply more closely to polymeric micelle drug-delivery systems in which AmB is encapsulated in a relatively nonaggregated state. For example, the onset of hemolysis in the PEO-*b*–p(HASA) formulation is delayed more that observed with the other acyl conjugate formulations. Eventually, however, hemolytic activity is apparent. This observation supports the necessity of a threshold level of AmB in solution for effective pore formation. Then again, aggregation state is not the only consideration. Even though AmB was encapsulated in a fairly nonaggregated state by PEO-*b*–p(6-HHA) micelles, hemolysis occurred rapidly and in a similar manner to free drug. This observation indicates the need for relatively stable micellar structures in order for the aggregation state of encapsulated drug to serve as a predictor of hemolytic activity. Micelle stability, or low CMCs, is critically important for any polymeric micelle formulation subjected to dilution in the in vivo environment. In summary, it might be possible to reduce the toxicity of AmB to mammalian cells via sustained release of relatively nonaggregated AmB from stable micelle-like structures.

4. Conclusions

The interaction of AmB with itself, membrane sterols, and carriers is quite complex, yet the aggregation state of the drug has proven to be a good indicator of toxicity and hemolytic activity. Consequently, the ability to modulate the equilibrium between the different aggregates is of primary concern for AmB formulation development. The length of the esterified acyl side chain in PEO-*b*–p(*N*-HA)-acyl conjugate micelles has a profound influence on the aggregation state of encapsulated AmB. In turn, the relative aggregation state affects the hemolytic activity of AmB toward bovine erythrocytes. In particular, PEO-*b*–p(HASA) micelles have a pronounced effect on the time-dependent hemolytic profile of AmB. Through manipulation of the core properties, it might be possible to design drug-delivery systems with properties tailored to the application at hand. Specifically, it might be feasible to fine-tune the release of AmB from PEO-*b*–p(HAZA) micelles by adjusting the structure of the core-forming p(L-Asp) block. Stearate esterification might impart the stability necessary for a long-circulating vehicle and possibly lead to a decrease in toxicity in vivo through alteration of the equilibrium distribution of AmB in the aqueous phase.

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References

[1] M.L. Adams, G.S. Kwon, The effects of acyl chain length on the micelle properties of poly(ethylene oxide)-*block*–poly(*N*-hexyl-L-aspartamide)-acyl conjugates, J. Biomater. Sci., Polym. Ed. 19 (9) (2002) 991–1006.

[2] A. Lavasanifar, J. Samuel, G.S. Kwon, Micelles self-assembled from poly(ethyleneoxide)-*block*–poly(*N*-hexyl stearate L-aspartamide) by a solvent evaporation method: effect on the solubilization and hemolytic activity of amphotericin B, J. Controlled Release 77 (2001) 155–160.

[3] A. Lavasanifar, J. Samuel, S. Satari, G.S. Kwon, Block copolymer micelles for the encapsulation and delivery of amphotericin B, Pharm. Res. 19 (4) (2002) 418–422.

[4] C. Charbonneau, I. Fournier, S. Dufresne, J. Barwicz, P. Tancrede, The interactions of amphotericin B with various sterol in relation to its possible use in anticancer therapy, Biophys. Chem. 91 (2001) 125–133.

[5] R. Seoane, J. Minones, O. Conde, M. Casas, E. Iribarnegaray, Molecular organisation of amphotericin B at the air–water interface in the presence of sterols: a monolayer study, Biochim. Biophys. Acta 1375 (1998) 73–83.

[6] W.C. Chen, R. Bittman, Kinetics of amphotericin B with vesicles, Biochemistry 16 (19) (1977) 4145–4149.

[7] A. Vertut-Croquin, J. Bolard, M. Chabbert, C. Gary-Bobo, Differences in the interaction of the polyene antibiotic amphotericin B with cholesterol- or ergosterol-containing phospholipid vesicles. A circular dichroism and permeability study, Biochemistry 22 (1983) 2939–2944.

[8] C. De Kruijff, W.J. Gerritsen, A. Oerlemans, R.A. Demel, L.L.M. Van Deenen, Polyene antibiotic–sterol interaction in membranes of *Acholeplasma Laidlawii* cells and lecithin liposomes I. Specificity of the membrane permeability changes induced by the polyene antibiotics, Biochim. Biophys. Acta 339 (1974) 30–43.

[9] J.D. Readio, R. Bittman, Equilibrium binding of amphotericin B and its methyl ester and borate complex to sterols, Biochim. Biophys. Acta 685 (1982) 57–70.

[10] J. Langlet, J. Berges, J. Caillet, J.-P. Demaret, Theoretical study of the complexation of amphotericin B with sterols, Biochim. Biophys. Acta 1191 (1994) 79–93.

[11] J. Bolard, P. Legrand, F. Heitz, B. Cybulska, One-sided action of amphotericin B on cholesterol-containing membranes is determined by its self-association in the medium, Biochemistry 30 (1991) 5707–5715.

[12] J. Barwicz, S. Christian, I. Gruda, Effects of the aggregation state of amphotericin B on its toxicity to mice, Antimicrob. Ag. Chemother. 36 (10) (1992) 2310–2315.

[13] P. Legrand, E.A. Romero, E. Cohen, J. Bolard, Effects of aggregation and solvent on the toxicity of amphotericin B to human erythrocytes, Antimicrob. Ag. Chemother. 36 (11) (1992) 2518–2522.

[14] J. Szlinder-Richert, J. Mazerski, B. Cybulska, J. Grzybowska, E. Borowski, MFAME, *N*-methyl-*N*-D-fructosyl amphotericin B methyl ester, a new amphotericin B derivative of low toxicity: relationship between self-association and effects on red blood cells, Biochim. Biophys. Acta 1528 (2001) 15–24.

[15] M.S. Espuelas, P. Legrand, M. Cheron, G. Barratt, F. Puisieux, J.-Ph. Devissaguet, J.M. Irache, Interaction of amphotericin B with polymeric colloids: a spectroscopic study, Col. Surf. B: Biointerfaces 11 (1998) 141–151.

[16] J. Brajtburg, J. Bolard, Carrier effects on biological activity of amphotericin B, Clin. Microbiol. Rev. 9 (4) (1996) 512–531.

[17] D. Forster, C. Washington, S.S. Davis, Toxicity of solubilized and colloidal amphotericin B formulations to human erythrocytes, J. Pharm. Pharmacol. 40 (1988) 325–328.

[18] I. Fournier, J. Barwicz, P. Tancrede, The structuring effects of amphotericin B on pure and ergosterol- or cholesterol-containing dipalmitoylphosphatidylcholine bilayers: a differential scanning calorimetry study, Biochim. Biophys. Acta 1373 (1998) 76–86.

[19] A.R. Balakrishnan, K.R.K. Easwaran, Lipid-amphotericin B complex structure in solution: a possible first step in the aggregation process in cell membranes, Biochemistry 32 (15) (1993) 4139–4144.

[20] E.J. Dufourc, I.C.P. Smith, H.C. Jarrell, Interaction of amphotericin B with membrane lipids as viewed by ^{2}H-NMR, Biochim. Biophys. Acta 778 (1984) 435–442.

[21] I. Gruda, N. Dussault, Effect of the aggregation state of amphotericin B on its interaction with ergosterol, Biochem. Cell Biol. 66 (1988) 177–183.