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The Effect of Polyethylene Oxide on the Aggregation State and Toxicity of Amphotericin B

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폴리에틸렌 옥사이드가 암포테리신-B의 응집 특성 및 독성에 미치는 영향

유 봉 규

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ABSTRACT–Amphotericin B (AmB) is a drug of choice for the treatment of systemic fungal diseases, but its use is considerably limited due to a high incidence of toxicity, particularly nephrotoxicity. It has been demonstrated that the toxicity of AmB is caused by self-aggregated species of the drug and that unaggregated (monomeric) drug is nontoxic but still expresses antifungal activity. Poly (ethylene oxide) (PEO) is a water-soluble polymer, which may impact the aggregation state of AmB. We have studied the aggregation state of AmB as a function of PEO molecular weight and concentration. At 3,000 and 8,000 g/mole, there was minimal or no change of critical aggregation concentration (CAC) of AmB regardless of the concentration of polymer. By contrast at 20,000 g/mole, the CAC of AmB strikingly increased to 24.3 and 37.5 μM at 5.0% and 10 % w/v of polymer, respectively. The critical overlap concentration (COC) of PEO 20,000 g/mole was 5.5 %. It appears that an interaction between monomeric AmB and polymer coil increases above the COC, competing with self-aggregation of the drug. Accordingly, the degree of aggregation of AmB stayed low and the toxicity became less. There was no such effect at 3,000 and 8,000 g/mole of PEO, owing perhaps to small dimensions in comparison to AmB. Based upon these findings, less toxic AmB formulation may be developed by a pharmaceutical technique such as solid dispersion system containing both AmB and PEO 20,000 g/mole.

Keywords–Amphotericin B; Poly (ethylene oxide), Critical aggregation concentration, Hemolysis, Critical overlap concentration

Amphotericin B (AmB) is a membrane active polyene macrolide antibiotic which is a drug of choice for the treatment of systemic fungal diseases, particularly in immuno-compromised patients including AIDS patients. [1,2] However, its use is considerably limited due to its high incidence of toxicity, particularly nephrotoxicity. Efforts to reduce its toxicity have been ongoing for more than a decade. Chemical modifications of the molecular structure of AmB and formulation with various carriers are two major strategies to increase the therapeutic index of this drug. [3-5]

Recent findings on AmB activity at a membrane level have provided a rationale for design of less toxic drug delivery systems of the drug. Monomeric AmB is non-toxic toward mammalian cells, but permeabilizes fungal cells, while the self-aggregated species of AmB are non-selective. [6-8] Thus, researchers are pursuing delivery of AmB in a monomeric or partially monomeric form. However, since AmB is amphiphilic molecule, it readily forms micelle-like self-aggregates at very low concentration such as 1 μM. Furthermore, it is known that about one thousand monomers stack into a self-aggregate with molecular weight of 10^6 g/mole at 10 μM. [9]

There have been reports about the association between small amphiphilic molecules and water-soluble polymers, e.g., PEO. [10] We have found that PEO impacts the aggregation state of AmB, leading to a monomeric or significantly less aggregated form. In contemplating the amphiphilic properties of AmB, it may be a result of an interaction of AmB and PEO. We have hypothesized that the supramolecular overlap of entangled polymer coils may more efficiently prevent the formation of self-aggregates, leading to a decrease of AmB toxicity. In this paper, we address the relevance of the molecular size and concentration of PEO.

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Experimental

Materials

AmB was generously donated from Dumex (Denmark). PEO 3000 g/mole and PEO 8000 g/mole were purchased from Sigma. PEO 20000 g/mole was purchased from Spectrum Chemical Manufacturing Corp. All other chemicals were analytical grade.

Determination of CAC

AmB (10 mg) was dissolved into 1.0 ml of dimethyl sulfoxide (DMSO) and was followed by dilution with PEO solutions in distilled water, giving an AmB level of 6.0 μM. DMSO is better solvent for the drug than dimethyl formamide (DMF). This was followed by a two-fold dilution until an AmB level of 0.10 μM was obtained. After incubation at 37 °C for 5 min, the molar absorptivity at 409 nm (ε) was measured by UV/VIS spectroscopy (Ultrospec 4000, Pharmacia). The ε was plotted as a function of the reciprocal of AmB level. The intercept of the two linear plots was obtained and the corresponding level of AmB was defined as the CAC. [11] For AmB alone, it was dissolved into DMSO and diluted with distilled water without PEO. The level of DMSO was always maintained less than 1.0 %v/v to avoid the effect of the organic solvent on the molar absorptivity. For 5.0% and 10% PEO 20000, CAC was measured by light scattering method using fluorescence spectrophotometer at 450 nm (F-3010, Hitachi). Level of AmB where the intensity of the scattered light emerges was assigned as the CAC. The detailed procedure of light scattering method is described elsewhere. [12] All tests were repeated at least three times.

Degree of aggregation

AmB was first dissolved into DMSO and then diluted with distilled water or PEO solution so that the level of DMSO is less than 1.0%v/v. Further, it was subjected to a two-fold dilution. After incubation at 37 °C for 5 min, the absorbances of the first peak (I) and the fourth peak (IV) were measured by absorption spectroscopy at around 348 nm and 409 nm, respectively. The ratio of the two absorbances (I/IV) was plotted as a function of AmB level and used as a measure of the degree of aggregation. [13]

Hemolysis

The hemolysis of erythrocytes was performed as previously described. [14] Briefly, erythrocytes were diluted into isotonic PBS, pH 7.0 with varied levels of PEO, giving an absorbance of 0.40 at 576 nm in the presence of 20 μg/ml of AmB as a lysis inducer. Aliquots (5.0 ml) of the diluted erythrocytes containing varied levels of AmB were incubated at 37 °C for 30 min with gentle shaking. Unlysed erythrocytes were removed by centrifugation. The supernatant was collected and analysed for hemoglobin by absorption spectroscopy at 576 nm. The percent of lysed RBC was determined by the following equation: % hemolysis=100(Abs-Abs_0)/(Abs_{100}-Abs_0), where Abs, Abs_0, and Abs_{100} are the absorbances of the sample, control with no AmB and control in the presence of 20 μg/ml AmB, respectively. For Abs_{100}, erythrocytes were diluted into

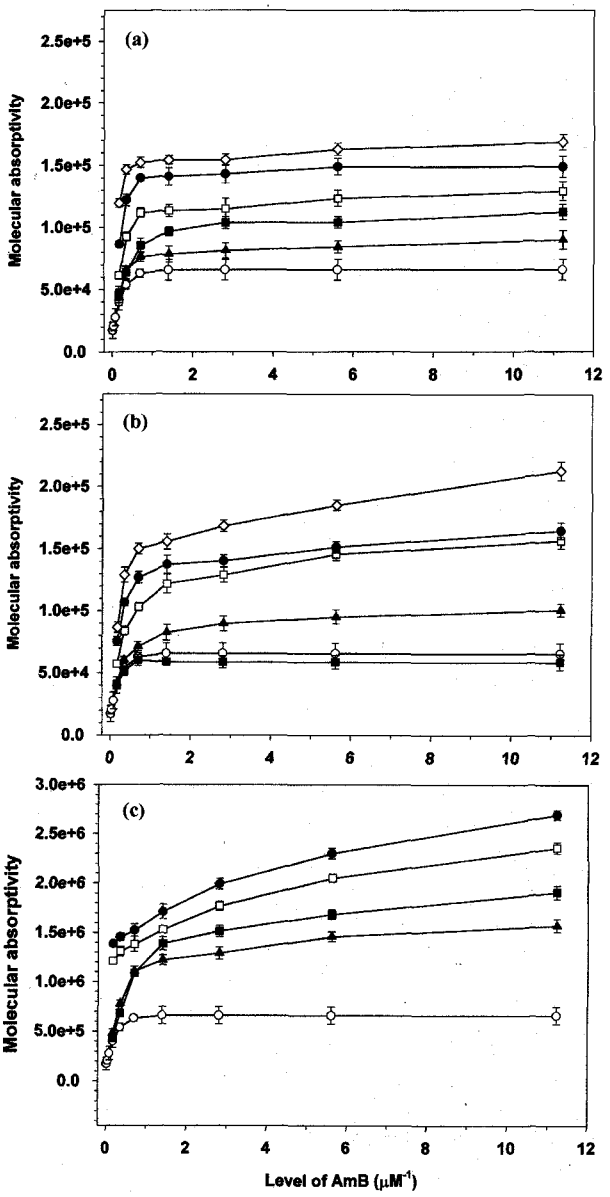

Figure 1–Molecular absorptivity of AmB as a function of reciprocal concentration in (a) PEO 3000, (b) PEO 8000, and (c) PEO 20000 at 20°C. ◆; 25%, ◇; 15%, ●; 10%, □; 5%, ■; 0.5%, ▲ ; 0.1%, O; without PEO.

PBS, pH 7.0 without PEO.

Results

The molar absorptivity at 409 nm (ε) of AmB is plotted as a function of the reciprocal of AmB level (Fig ure 1). Within the concentration range tested (0.1-6.0 μM), ε does not follow Beer-Lambert law and shows an abrupt decrease at some critical concentration. In our previous paper,[14] we assigned this point as the critical aggregation concentration which corresponds to the change of molecular configuration caused by self-aggregation. As dilution times increase in distilled water, ε is leveled off at 67,400 $cm^{-1}mole^{-1}$. This is approximately a half of ε of completely monomeric AmB in DMF (145,800 $cm^{-1}mole^{-1}$). This suggests that AmB may exist in a dimeric form in very dilute aqueous solution.

In the presence of PEO 3000, ε of AmB increases with concentration of PEO. At 10% PEO 3000 and PEO 8000 solution, the molar absorptivities in dilute condition are 148,000 and 165,000 cm^{-1} $mole^{-1}$, respectively, which are close to that of the monomeric AmB in DMF. In the case of PEO 20000 solution, ε of AmB in dilute condition is about 150,000 $cm^{-1}mole^{-1}$ even at the lowest concentration of PEO (0.1%). The increase of ε suggests that the molecular conformation of AmB changes into monomeric form as it does in organic solvents. The increase of ε is proportional to the polymer concentration except for slight reversal at 0.5% PEO 8000. For the three different PEOs tested, the higher molecular weight PEO shows the higher ε at the same concentrations of AmB. Taking into consideration of interaction between PEO and amphiphiles, we suspect this reflects the interaction of PEO and monomeric AmB.

CAC of AmB in PEO solution is summarized in Table I. In PEO 3000 and PEO 8000, there is minimal or no change. In 5.0% and 10% PEO 20000 solution, however, CAC is beyond the highest concentration tested, i.e., 6.0 μM. Due to the limit of UV/VIS absorbance scale, CAC higher than 6.0 μM was not measurable by absorption spectroscopy. Therefore, we additionally used light scattering method for the measurement of CAC beyond 6.0 μM. They were 24.3 μM and 37.5 μM for 5.0% and 10% PEO 20000 solution, respectively.

We assessed the degree of aggregation by absorption spect - roscopy measuring the ratio of absorbances of the first peak (I) to the fourth peak (IV) at 348 nm and 409 nm, respectively. The higher I/IV ratio represents the higher degree of aggregation of AmB molecules and vice versa. A reference value of I/IV ratio

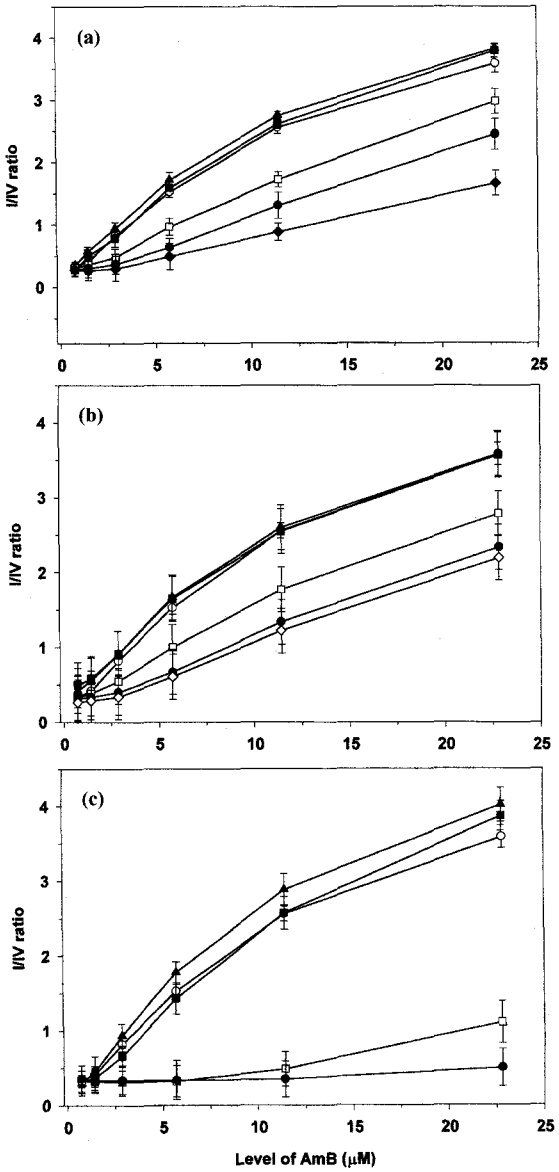

Figure 2–I/IV ratio as a function of level of AmB in (a) PEO 3000, (b) PEO 8000, and (c) PEO 20000 at 20°C. ◆; 25%, ◇; 15%, ● ; 10%, □; 5%, ■ ; 0.5%, ▲ ; 0.1%, O; without PEO.

Table I–*Critical Aggregation Concentration (CAC, μM) of AmB in PEO Solution*

PEO (g/mole)	polymer-free (distilled water)	0.1%	0.5%	5%	10%	15%	25%
3000	1.36	1.44	1.16	1.47	1.47	-	2.60
8000	1.36	1.09	1.57	1.09	1.31	2.26	-
20000	1.36	1.05	1.27	24.3[a]	37.5[a]	-	-

[a] measured by light scattering method using 450 nm.

Table II–*Hydrodynamic Properties of Polyethylene Oxide (PEO) in Distilled Water at 20°C*

PEO molecular weight (g/mole)	specific volume (υ, ml/g)	intrinsic viscosity (η, ml/g)	critical overlap concentration (COC, %)	hydrodynamic radius[a] (r_h, nm)
3000	0.70	9.7	17.9	0.7
8000	0.75	16.8	11.2	1.1
20000	0.70	31.7	5.5	1.7

[a] Length of AmB molecule is 1.6 nm.[17]

for monomeric AmB is 0.25 in DMF. At concentrations of PEO greater than 0.5 %, I/IV ratio of AmB is lower than in the polymer-free solution, while it is same or slightly higher at 0.1 % and 0.5% (Figure 2). In the case of PEO 3000 and PEO 8000 solution, the I/IV ratios remain still high (1.7 and 2.2 at 22.8 μM) even above their critical overlap concentrations (COC) (refer to Table II and Discussion). At 5%-10% PEO 20000 solution, however, I/IV ratio is significantly lowered (0.5-1.0), indicating that AmB exists as a monomeric or significantly less aggregated state.

Figure 3 shows the hemolytic activity of AmB at varied concentrations of PEO. In the case of PEO 3000 and PEO 8000 solution, AmB lyses erythrocytes although their COCs were reached (Table II). At 25% PEO 3000, hemolysis started at around 7 μM and reached up to 50% at 10 μM. PEO 8000 did not prevent hemolytic activity of AmB even above its COC, either. However, in contrast, PEO 20000 completely inhibited the hemolytic activity of AmB up to 80 μM once COC (5.5%) is reached. In 5.0% PEO 20000 (below COC), lysis started at 8 μM and leveled off at 50%.

To understand the driving force of interaction of AmB and PEO, we measured the change of CAC in PEO 20000 solution (5.0% and 10%) at different temperatures. If the driving force of this interaction is hydrogen bond, CAC will decrease as temperature increases, while it will increase if hydrophobic binding is the case. CAC of AmB in 10% PEO 20000 solution at 40°C is 59.4 μM, which is about 1.5 times greater than that at 20°C, i.e., 37.5 μM (Table III). This result indicates that driving force in this interaction is hydrophobic binding. In the self-aggregation process, driving force is known to be hydrogen bond.

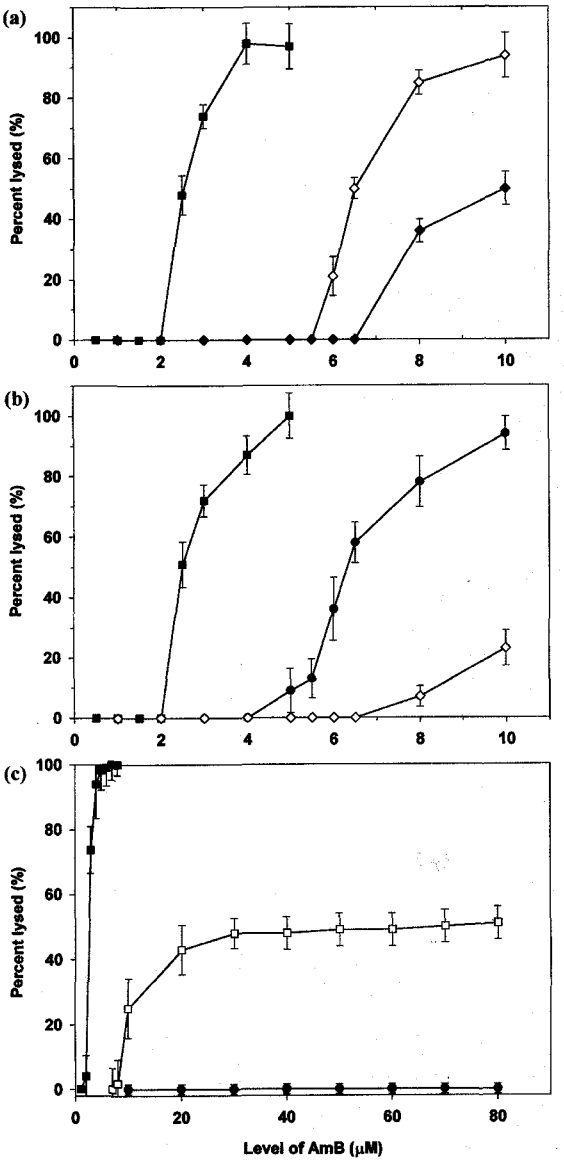

Figure 3–Hemolytic activity of AmB at varied concentrations of (a) PEO 3000, (b) PEO 8000, and (c) PEO 20000. Erythrocytes were incubated at 37°C for 30 min with gentle stirring. ◆; 25%, ◇; 15%, ●; 10%, □; 5%, ■ ; 0.5%.

Discussion

Hydrodynamic radius and critical overlap concentration (COC) of PEO were calculated based upon the following equations. Briefly, we used two equations: $[\eta]=\nu(V_h/V_d)\upsilon$ and $[\eta]=2.0+0.016M^{0.76}$, where $[\eta]$, ν, V_h, V_d, υ, and M are intrinsic viscosity, shape factor (2.5 when assumed spheric), hydrodynamic volume, dry volume, and specific volume, and molecular weight of PEO, respectively.[15,16] From two equations above, V_h/V_d was calculated and used for the calculation of COC. COC was defined as the percent dry volume versus the

Table III–*Critical Aggregation Concentration[a] (CAC, μM) of AmB in PEO 20000 Solution as a Function of Temperature*

	20°C	30°C	40°C
5% PEO 20000	24.3	34.7	42.5
10% PEO 20000	37.5	49.1	59.4
distilled water	1.3	1.6	1.4

[a] measured by light scattering method using 450 nm. Excitation and emission band widths were 3 nm and 10 nm, respectively.

hydrodynamic volume, i.e., COC (%)=100 $\times(V_d/V_h)$. When the polymer concentration is increased, a critical concentration (COC) is reached and the individual coils overlap, form many contacts, and become entangled. Hydrodynamic radius was estimated by the following equations: $r_h \approx {}^2/_3 r_G$, $r^2_G = {}^1/_6 h^2$, $h^2 = nA^2$.[15] In these equations, r_h, r_G, h, n, and A are hydrodynamic radius, gyrometric radius, end-to-end distance, number of repeating units, and length of single unit, respectively.

The hydrodynamic properties of PEO thus obtained are summarized in Table II. The hydrodynamic radii of PEO 3000, PEO 8000, and PEO 20000 in distilled water at 20 °C are 0.7, 1.1, and 1.7 nm, respectively. The COCs are 17.9%, 11.2%, and 5.5% for 3000, 8000, and 20000 g/mole of PEOs, respectively. The end-to-end distance of conjugated double bonds of AmB molecule is found to be 1.6 nm long by X-ray crystallography.[17] The finding that AmB became significantly less aggregated only in 5.0% and 10% PEO 20000 solutions suggests that both hydrodynamic radius and concentration of PEO play an important role in the aggregation behavior of AmB molecules (Figure 2). In PEO 3000 and 8000 solution whose hydrodynamic radii are smaller than the dimension of drug molecule, the degree of aggregation in terms of I/IV remains still high although their COCs are reached. In PEO 20000, however, AmB became strikingly less aggregated above its COC, leading to a speculation that hydrodynamic radius of PEO needs to be greater than the dimension of drug molecule.

Based upon earlier researchers finding, the increased molar absorptivity of AmB in PEO solution is considered to be due to monomeric form of the drug.[18, 19] Since PEO does not have any absorbance at UV/VIS absorbing region of the drug, i.e., 348-409 nm, the increase of ε at 409 nm represents the preferential existence of the drug as monomeric form in the presence of PEO. The ε increased in every PEO solution (Fig. 1). In PEO 20000 solution, particularly, ε is 135,000 $cm^{-1}mole^{-1}$ even at extremely low level of PEO such as 0.004 % (data not shown). From these results, it is evident that PEO impacts the aggregation state of the drug. In light of exceedingly high costs of lipid formulations of AmB, it is worthwhile to pursue less toxic formulation of the drug simply by using PEO 20000.

In conclusion, hydrodynamic radius and COC of PEO play an important role in the interaction between AmB and PEO, leading to a monomeric or significantly less aggregated form of the drug. When r_h of the polymer is smaller than the drug molecule, AmB always forms self-aggregates regardless of the concentration of PEO. However, when r_h is larger than the drug molecule, AmB prefers monomeric interaction with PEO above COC of the polymer. It is notable that AmB stays at significantly less aggregated form and has no hemolytic activity up to 80 μM in 10% PEO 20000 solution.

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