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International Journal of Pharmaceutics 309 (2006) 234–240
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Encapsulation of amphotericin B in poly(ethylene glycol)-block-poly(ε-caprolactone-*co*-trimethylenecarbonate) polymeric micelles

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Received 25 August 2005; received in revised form 21 November 2005; accepted 26 November 2005
Available online 6 January 2006

Abstract

The aim of this work was to evaluate the potential of self-assembling poly(ethyleneglycol)$_{750}$-block-poly(ε-caprolactone-*co*-trimethylenecarbonate)$_{4500}$ 50/50 copolymers (PEG-p(CL-*co*-TMC)) to solubilize amphotericin B in polymeric micelles and to disaggregate the drug to the less toxic monomeric form. Amphotericin B was encapsulated in the micelles upon dilution of a mixture of the liquid polymer and the drug in water. Its solubility was increased by two orders of magnitude depending on polymer concentration. The aggregation state of amphotericin B was decreased by PEG-p(CL-*co*-TMC). The preparation method and the loading of the polymeric micelles influenced it. The antifungal activity of the drug was reduced by encapsulation in the polymeric micelles whereas the onset of amphotericin B-induced hemolysis was delayed. PEG-p(CL-*co*-TMC) micelles could be an easy method for amphotericin B encapsulation.

Keywords: Polymeric micelles; Amphotericin B; mmePEG750-block-poly(ε-caprolactone-*co*-trimethylenecarbonate)

1. Introduction

Amphotericin B is a drug of choice against systemic mycosis but it has several dose-limiting effects including nephrotoxicity. Due to its poor water-solubility, it was formulated with sodium deoxycholate (Fungizone®) for market introduction but nephrotoxicity is a frequent complication. Other formulations have also been commercialized including: a lipid complex, Abelcet®; a liposomal product, Ambisome®; a colloidal dispersion, Amphocil®, and an emulsion product in association with Intralipid®. The nephrotoxicity of all these formulations is reduced and they are better tolerated allowing the administration of higher doses. However, cost and other factors limit their widespread use (Hillery, 1997; Andrès et al., 2001; Hann and Prentice, 2001). Hence, other non-commercialized formulations of various types (micelles, nanospheres, conjugates) have been tested.

Due to its amphiphilic structure (Fig. 1A), amphotericin B tends to aggregate in aqueous solution. It exists as a combination of monomers, soluble and non-water soluble aggregates (Legrand et al., 1992). Amphotericin B associates with membrane sterol to form amphotericin B–sterol complexes that then associate to form transmembrane pores. The selectivity of amphotericin B for fungi is attributed to its higher affinity for ergosterol than cholesterol. Amphotericin B toxicity is thought to be mediated by the relative aggregation state of the drug as its non-selective toxicity can be attributed to the aggregated form of the drug (Bolard et al., 1991). Surfactants and some amphiphilic polymers have been demonstrated to reduce the toxicity of amphotericin B by decreasing its state and extent of aggregation. Previous studies have shown that micellar solubilization with surfactants such as Mirj 59 or copolymers such as various Pluronic®, poly(ethylene oxide)-block-poly(*N*-hexyl stearate L-aspartamide), poly(ethylene oxide)-block-poly(β-benzyl-L-aspartate) or monoglycerides decreased hemolysis

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0378-5173/$ – see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.ijpharm.2005.11.031

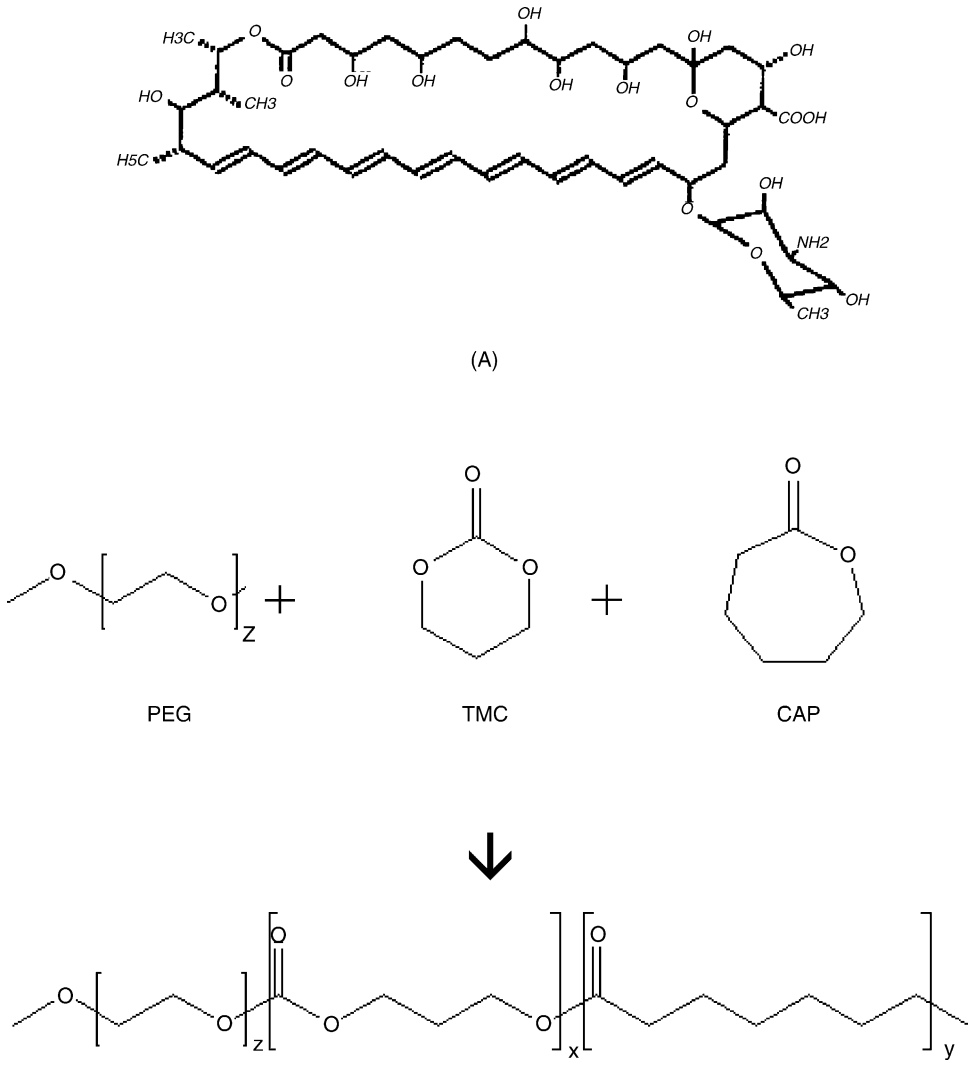

Fig. 1. (A) Amphotericin B structure. (B) Synthesis and structure of PEG-p(CL-*co*-TMC).

and/or nephrotoxicity while retaining the antifungal activity of amphotericin B (Forster et al., 1988; Kirsch et al., 1988; Tasset et al., 1990, 1991; Yu et al., 1998a,b; Lavasanifar et al., 2001, 2002; Adams and Kwon, 2003; Esposito et al., 2003; Vakil and Kwon, 2005).

Recently, novel diblock copolymers made up of ε-caprolactone (CL) and trimethylenecarbonate (TMC) and mmePEG$_{750}$ (Fig. 1B) (PEG-p(CL-*co*-TMC)) have been shown to form stable micelles spontaneously upon gentle mixing with water in the absence of solvent and to increase the solubility of poorly soluble-drugs without significant cytotoxicity (Ould-Ouali et al., 2004, 2005).

The aim of this work was to investigate if the self-assembling PEG-p(CL-*co*-TMC) 50/50 system can be used both to solubilize amphotericin B and to disaggregate the drug to reduce its non-selective toxicity (Ernst et al., 1981). Therefore, the solubility of amphotericin B in the PEG-p(CL-*co*-TMC) micellar solutions was determined. The aggregation state of amphotericin in the micelles was also studied. The effect of encapsulation on the efficacy and on the hemolysis induced by the drug was evaluated.

2. Methods

2.1. Polymer synthesis and characterization

The copolymer was synthesized by ring-opening polymerization as described previously (Ould-Ouali et al., 2004) (Fig. 1B). Briefly, the reaction was initiated by PEG monomethylether of 750 Da (mmePEG750) at a molar monomer/initiator ratio of 13.3/1. ε-Caprolactone and trimethylene carbonate were added at 1:1 molar ratio. The reaction was catalyzed by stannous octoate and was allowed to run for 24 h. The molecular weight of the diblock polymer was determined by gel permeation chromatography and the monomer ratio in the polymer by NMR. The critical micellar concentration (CMC) was determined by the pyrene fluorescence method (Ould-Ouali et al., 2004).

2.2. Particle size

The size of the particles was determined by photon correlation spectroscopy using a Malvern autosizer 4700 at 25 °C. The measurements were carried out at a scattering angle of 90°.

2.3. Solubility of amphotericin B in the micelles

The solubility of amphotericin B (Sigma–Aldrich, cell culture tested) in 5, 10 and 20% (w/v) PEG-p(CL-*co*-TMC) in water was determined. A stock solution of amphotericin B (10 mg/ml) in DMSO was evaporated under vacuum, at 60–65 °C. The liquid polymer without any solvent was added to the vial and mixed overnight protected from light. Ultrapure water was then added drop-wise to form the micellar solution. The excess drug was removed by filtration through 0.45 μm PVDF filters. The amount of solubilized amphotericin B was quantified with a UV–vis spectrometer HP8453 from Hewlett Packard at λ = 388 nm after dilution of the solutions with the corresponding polymeric solution such that the absorbance was between 0.2 and 0.8. The blank was a solution containing the same polymer concentration than the analyzed sample. The calibration curve was also prepared with the micelles to get the monomeric form of the drug.

2.4. Aggregation state of amphotericin B

As it is widely reported that the toxicity and the activity of amphotericin B depend on its aggregation state and that the spectroscopic properties correlate with the aggregation state of amphotericin B molecules (Tancrède et al., 1990; Legrand et al., 1992; Gaboriau et al., 1997; Aramwit et al., 2000; Esposito et al., 2003), UV spectroscopic studies were carried out.

Samples containing 10 μg/ml of amphotericin B and 1 or 10% (w/v) of polymer were prepared. The influence of the preparation method was analyzed.

- *Method A*. Micellar solutions containing amphotericin B were prepared by mixing 1 mg of amphotericin B with the polymer for 24 h. Ultrapure water was then added to form 1 or 10% (w/v) micellar solution.
- *Method B*. The drug was first dissolved in DMSO (10 mg/ml). After the evaporation of the DMSO, the liquid polymer was added and mixed overnight, at room temperature. The micellar solution was then formed by addition of ultrapure water.
- *Method C*. A solution of DMSO containing 10 mg/ml of amphotericin B was added to a vial and the solvent evaporated. A 1 or 10% (w/v) micellar solution was added and mixed for 24 h.

Solutions of Fungizone® diluted in water and amphotericin B dissolved in DMSO were also prepared.

The spectrum of amphotericin B in the different samples was taken by UV spectroscopy with a UV–vis spectrometer HP8453 from Hewlett Packard. The blank was the corresponding micellar solution without amphotericin B.

The ratio of the absorbance of the first peak (I) to last peak (IV) was used to monitor the aggregation state of amphotericin B (Adams and Kwon, 2003).

2.5. Hemolytic activity

In order to determine the effect of the micellar encapsulation of amphotericin B on erythrocyte lysis induced by the drug, the hemolysis induced by different concentrations (0, 3, 6, 12, 18, 24 μg/ml) of a water-soluble formulation of the drug and of the drug encapsulated in 1 and 10% (w/v) solutions of PEG-p(CL-*co*-TMC) was compared. The toxicity of the excipients alone (sodium deoxycholate (Sigma)) and PEG-p(CL-*co*-TMC) was also determined.

For the water-soluble formulation, 50 mg Fungizone® was solubilized in 10 ml water for injection (Mini-Plasco®). Different concentrations were obtained by dilution of this solution with isotonic phosphate buffer (pH 7.41). Amphotericin B encapsulated in a 1 and 10% micellar solution of PEG-p(CL-*co*-TMC) was prepared by the method B (described under Section 2.4).

Blood from three rats were obtained by intracardiac puncture and collected in three citrated tubes. The blood was then centrifuged and the plasma eliminated. Complete hemolysis was induced using a hypoosmotic aqueous solution containing 24 μg/ml Fungizone®. Red blood cells were diluted with isotonic phosphate buffer in order to obtain an absorbance between 0.8 and 1 upon 100% hemolysis. 2.5 ml of this erythrocyte solution were incubated for 30 min at 37 °C under agitation with 2.5 ml of the different solutions. After centrifugation, the absorbance was measured at 576 nm (Tasset et al., 1990).

The hemolysis % was determined by the following formula (Lavasanifar et al., 2002):

$$\text{hemolysis}\,\% = 100\,(\text{abs} - \text{abs}_0)/(\text{abs}_{100} - \text{abs}_0)$$

where abs = absorbance of the sample, abs_{100} = absorbance at 100% hemolysis, abs_0 = absorbance at 0% hemolysis.

A two-way ANOVA was used to compare the formulations.

2.6. In vitro antifungal activity

In order to study the influence of the encapsulation of amphotericin B in PEG750-p(CL-*co*-TMC) on the antifungal activity of the drug, the minimal inhibitory concentration (MIC), defined as the lowest drug concentration inhibiting clearly visible growth of the fungi, with slight turbidity being ignored (Lennette et al., 1985), was determined by a dilution method in Sabouraud liquid medium (Tasset et al., 1990). A suspension of *Candida albicans* (5×10^6/ml) was incubated with different concentrations of amphotericin B for 30 h at 31 °C.

A stock solution of Fungizone®, was prepared by dilution in ultrapure water. The polymeric formulations were prepared by adding ultrapure water to polymer PEG-p(CL-*co*-TMC) containing Amphotericin B (method B described under Section 2.4). Both solutions were sterilized through a 0.22 μm filter. Two by two dilutions in a Sabouraud liquid medium were then performed in a 19–0.037 μg/ml concentration range. *C. albicans*-containing tubes without antifungal were used as a control.

3. Results

3.1. Polymer characterization

The PEG-p(CL-*co*-TMC) diblock copolymer initiated with mmePEG_{750} had a M_W of 5250 with a polydispersity index of

Table 1
Amphotericin B solubility and in vitro antifungal efficacy at different PEG-p(CL-*co*-TMC) concentrations

	Solubility (μg/ml)	MIC (μg/ml)[a]
Water	1[b]	
Fungizone®	>1000	0.15–0.15
PEG-p(CL-*co*-TMC)		
1%[c]	ND	0.30–0.59
5%	85	ND
10%	104	0.59–1.19
20%	122	ND

[a] Minimal inhibitory concentration on *C. albicans*.
[b] Lavasanifar et al. (2002).
[c] The polymer was dissolved in water.

1.8. The monomer ratio in the final polymer was 49.1% CL, 50.7% TMC (mol%). Its CMC was 2×10^{-5} g/ml. The polymer was liquid at room temperature (Ould-Ouali et al., 2004).

3.2. Particle size

The size of PEG-p(CL-*co*-TMC) micelles that formed spontaneously upon dilution in water was 22 nm (Ould-Ouali et al., 2004). Their size increased to 41 nm at a concentration of 10% PEG-p(CL-*co*-TMC) after amphotericin B encapsulation at saturation.

3.3. Solubility of amphotericin B in the micelles

Results of the solubility study are reported in Table 1. PEG-p(CL-*co*-TMC) increased the solubility of amphotericin B by two orders of magnitude. The higher the polymer concentration, the higher the solubility.

Below the solubility, amphotericin B was encapsulated with a yield of 100% by a simple procedure: mixing the liquid polymer containing amphotericin B with water.

3.4. Aggregation state of amphotericin

The UV spectrum of amphotericin B depends on its aggregation state. In aqueous solution (Fungizone®), amphotericin B is aggregated. The absorption spectrum presents a major broad band at 328 nm. In organic solvents such as DMSO, amphotericin B is present as a monomer and the absorption spectrum is composed of four peaks with maxima at 350, 368, 388 and 412 nm (Fig. 2a). The aggregated form is thought to be more toxic than the monomeric form (Legrand et al., 1992; Aramwit et al., 2000). Hence, the aggregation state of amphotericin B was assessed by UV spectroscopy.

The spectra of amphotericin B encapsulated in PEG-p(CL-*co*-TMC) micelles by the method B at different concentrations presented all four bands typical of the monomeric form of the drug. The ratio of the first to the fourth peak was taken as a measure of the relative aggregation state of amphotericin B: this ratio is low (<0.25) for unaggregated monomeric form and as high as 2 for aggregated species. Fig. 2b demonstrates that the aggregation remains low for amphotericin B encapsulated in

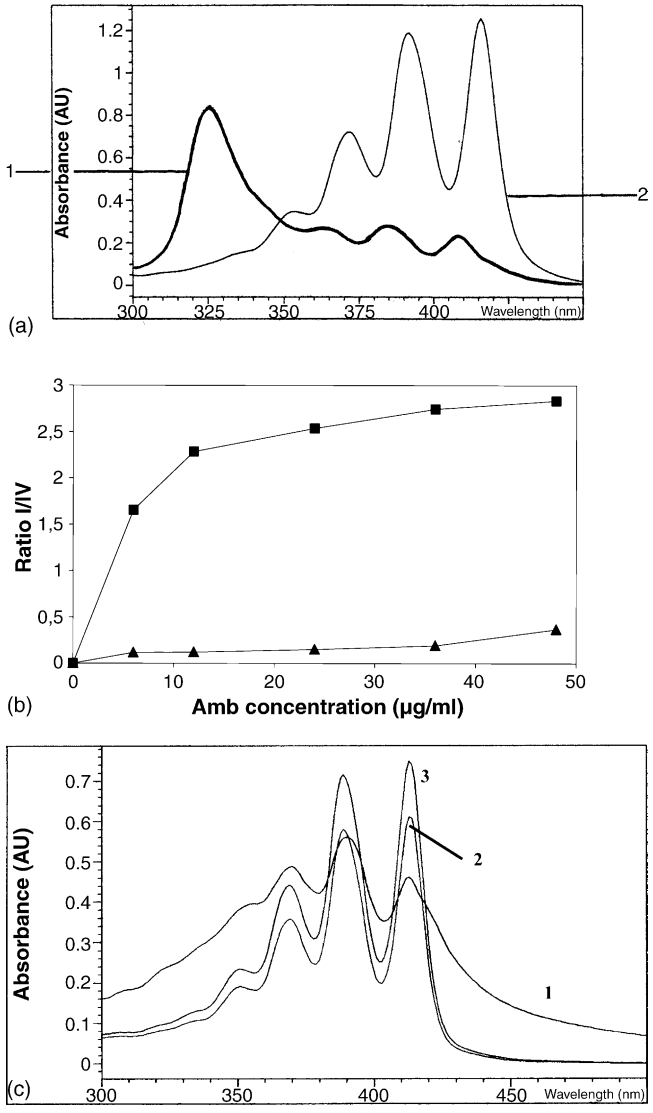

Fig. 2. (a) UV absorption spectra of amphotericin B at a concentration of 10 μg/ml in (1) aqueous solution (Fungizone®) and (2) DMSO. (b) Ratio of peak I to peak IV in the UV absorption spectra of amphotericin B as indicator of relative aggregation state as a function of amphotericin B concentration for Fungizone® (■) and a 10% polymeric micelles solution PEG-p(CL-*co*-TMC) (▲) where amphotericin B, first dissolved in DMSO before its evaporation, was added to the polymer and then water (method B). (c) UV absorption spectra of 10 μg/ml amphotericin B encapsulated in 10% PEG-p(CL-*co*-TMC) by (1) method A (amphotericin B + polymer + water); (2) method B and (3) method C (amphotericin B, first dissolved in DMSO before its evaporation + polymeric micelles).

PEG-p(CL-*co*-TMC) whereas amphotericin B in Fungizone® is highly aggregated.

To determine if the method of encapsulation of the drug in the micelles had an influence on the aggregation state of amphotericin B, three different methods were selected (as described under Section 2.4). The spectra of amphotericin encapsulated in 10% (w/v) PEG-p(CL-*co*-TMC) solutions by these three methods are reported in Fig. 2c and are similar to those obtained in 1% (w/v) PEG-p(CL-*co*-TMC) solutions. The method of encapsulation of the drug in the PEG-p(CL-*co*-TMC) did influence the aggregation of the amphotericin B: the monomeric form

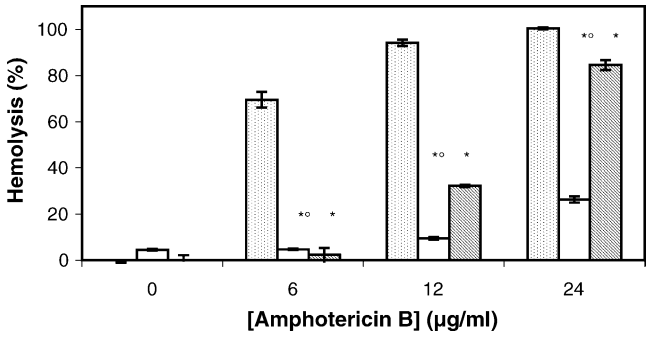

Fig. 3. Hemolysis as a function of amphotericin B concentration in Fungizone® (dotted bars), 10% PEG-p(CL-*co*-TMC) (empty bars) and 1% PEG-p(CL-*co*-TMC) (dark bars) ($n = 3$). $^{*}p < 0.05$ vs. Fungizone®; $^{\#}p < 0.05$ vs. 1% PEG-p(CL-*co*-TMC).

of the drug was present in the micelles when DMSO was first removed before polymer or micelles were added. The spectra obtained when the drug was solubilized directly by mixing with the polymer seem to correspond to a mixture of the aggregated and non-aggregated forms.

Consequently, the hemolytic activity and antifungal activity of PEG-p(CL-*co*-TMC) micelles loaded with amphotericin B were tested only for polymeric micelles prepared by the method B.

3.5. Hemolytic activity

In order to determine the effect of the encapsulation of amphotericin B on the erythrocyte lysis induced by the drug, hemolysis induced by different concentrations of amphotericin B solubilized in deoxycholate (Fungizone®) or in 1 and 10% PEG-p(CL-*co*-TMC) were compared.

Sodium deoxycholate and PEG-p(CL-*co*-TMC) 1% induced no hemolysis. At a concentration of 10%, PEG-p(CL-*co*-TMC) induced a very low hemolysis (5%) (data not shown). Consequently, the hemolytic activity observed in the presence of amphotericin B can be attributed to the action of the drug.

As shown in Fig. 3, Fungizone® resulted in hemolysis at low concentration. The onset of amphotericin B-induced hemolysis was delayed by PEG-p(CL-*co*-TMC) relative to Fungizone®. The higher the PEG-p(CL-*co*-TMC) concentration, the lower the amphotericin B-induced hemolysis. At 12 μg/ml of amphotericin B, the percentage of hemolysis was 94, 32 and 9 for, respectively, Fungizone®, 1% and 10% PEG-p(CL-*co*-TMC) formulations.

3.6. In vitro antifungal activity

In order to assess in vitro the antifungal activity of amphotericin B after encapsulation in the micelles, the MIC was determined in the presence of *C. albicans*, a fungi sensitive to amphotericin B.

Amphotericin B was slightly less effective in vitro when encapsulated in the polymer (Table 1). The MIC of the commercialized formulation Fungizone®, 0.15 μg/ml, increased to 0.30–1.19 μg/ml with the PEG-p(CL-*co*-TMC) formulation, depending on polymer concentration. The polymer had no effect on *C. albicans* growth.

4. Discussion

Diblock copolymers made of caprolactone and trimethylenecarbonate and mmePEG self-assemble to form spontaneously stable micelles by gentle mixing with water. They have been identified as interesting drug delivery systems for oral delivery of poorly water-soluble drugs (Ould-Ouali et al., 2004, 2005). In order to evaluate their potential for drug delivery, amphotericin B was formulated in polymeric micelles made of PEG-p(CL-*co*-TMC) (M_W 5250). The solubility and aggregation state of amphotericin B in these formulations as well as the effect of encapsulation of amphotericin B on the antifungal activity of the drug and on the hemolysis induced by the drug were evaluated.

Compared to other polymers forming polymeric micelles, the major advantage of PEG-p(CL-*co*-TMC) is that neither organic solvent nor a dialysis or evaporation step is required for the encapsulation of the drug. As the micelles are formed by the addition of water to the mixture of the liquid polymer and drug, the yield of amphotericin B was 100%.

The encapsulation of amphotericin B in self-assembling polymeric micelles of PEG-p(CL-*co*-TMC) increased its solubility significantly from less than 1 μg/ml (Lavasanifar et al., 2001) up to 122 μg/ml in the presence of 20% polymer. Other micellar polymeric systems made of poly(ethylene oxide)-block-poly(β-benzyl-L-aspartate) presented similar solubilities (57–141 μg/ml) (Yu et al., 1998b). Amphotericin B solubility reached more than 250 μg/ml in freeze-dried poly(ethylene oxide)-block-poly(*N*-hexyl stearate-L-aspartamide) micelles with a yield of up to 77% (Lavasanifar et al., 2002).

The toxicity and the activity of amphotericin B depend on its aggregation state: the aggregation of amphotericin B seems to decrease its antifungal activity and increase its toxicity (Legrand et al., 1992; Yu et al., 1998a; Aramwit et al., 2000). In addition, several authors have seen a correlation between the spectroscopic properties and the aggregation state of amphotericin B molecules (Tabosa do Egito et al., 1996; Gaboriau et al., 1997; Ernst et al., 1981). Therefore the effect of PEG-p(CL-*co*-TMC) on the aggregation state of amphotericin B was evaluated. The spectral features of amphotericin B encapsulated in PEG-p(CL-*co*-TMC) indicate that the polymer disaggregates the drug. The method of encapsulation of the drug in the PEG-p(CL-*co*-TMC) influenced the aggregation of the amphotericin B: the monomeric form of the drug was present in the micelles when the drug was first solubilized in DMSO which was removed before adding the polymer or micelles. Lavasanifar et al. (2001) have also reported that the preparation method of the poly(ethylene oxide)-block-poly(*N*-hexyl stearate L-aspartamide) micelles influenced amphotericin B solubility and toxicity.

The polymer exerted a protective effect on the amphotericin B in vitro hemolytic activity. The delay in hemolysis onset increased with the increase in polymer concentration. This reduction in amphotericin B toxicity could be explained by

the presence of monomeric amphotericin B and/or the lower free non-micellar amphotericin B concentration. The toxicity of the 1% polymer formulation was comparable to that of a cyclodextrin formulation (Chakraborty and Naik, 2003) and of poly(ethylene oxide)-block-poly(β-benzyl-L-aspartate) micellar system (Yu et al., 1998a). The toxicity induced by the 10% PEG-p(CL-*co*-TMC) formulation was better than these systems. Depending on the fatty acid substitution level, the amphotericin B induced hemolysis was higher or lower when amphotericin B was encapsulated in PEG-b-poly(*N*-hexyl stearate-L-aspartamide). No hemolysis at 20 μg/ml of the drug was observed at a substitution of 50% (Lavasanifar et al., 2002). The PEG-p(CL-*co*-TMC) 10% formulation has however a higher hemolytic activity than lipid formulations (Esposito et al., 2003).

The studied formulations decreased the in vitro antifungal activity of the drug: the MIC increased from 0.15 to 1.19 μg/ml in the presence of 10% polymer. Data on the efficacy of amphotericin B in surfactant or polymeric micelles have been reported and are highly variable. Lower, equivalent or higher activities have been published (Tasset et al., 1991; Yu et al., 1998b; Lavasanifar et al., 2002).

The interaction of amphotericin B with PEG-p(CL-*co*-TMC) was not studied extensively. However, due to its amphiphilic properties, it is very likely that amphotericin B is incorporated in the core of the micelles. The changes in its spectral features and its solubility confirm this hypothesis. Increasing the CL ratio in the hydrophobic moiety of the polymer could enhance the solubility and the drug/polymer ratio (Latere Dwan'Isa et al., 2005). However, as for most of the polymeric micelles developed up to now, the low solubility of amphotericin B (<1 mg/ml) prevents their use in vivo. Further studies are still required to improve the polymer design to enhance the drug solubility while maintaining the thermodynamic and kinetic stability of polymeric micelles and the easiness of drug encapsulation.

5. Conclusion

PEG-p(CL-*co*-TMC) micelles for amphotericin B delivery have been investigated as a mean to: (i) inhibit its self-aggregation and (ii) enhance its solubility. They were formulated by simple addition of the polymer and the drug to water. They solubilized amphotericin B in a monomeric form. Consequently, they drastically reduced the hemolysis induced by amphotericin B as compared to Fungizone®. The encapsulation decreased the efficacy of the drug as measured by the MIC.

Acknowledgements

Vlaams Instituut voor de aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen is gratefully acknowledged for the financial support of this research project. The authors also wish to thank the team of J. Rosenblatt at Johnson & Johnson Center of Biomaterials and Advanced Technology, Somerville, NJ, US, for the synthesis of the polymers and Prof. M.Delmée (Université catholique de Louvain) for the measurement of MIC.

References

Adams, M., Kwon, G., 2003. 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. J. Contr. Release 87, 23–32.

Andrès, E., Tiphine, M., Letscher-Bru, V., Herbrecht, R., 2001. Nouvelles formes lipidiques de l'amphotéricine B. Revue de la littèrature. Rev. Med. Int. 22, 141–150.

Aramwit, P., Yu, B.G., Lavasanifar, A., Samuel, J., Kwon, G., 2000. The effect of serum albumin on the aggregation state and toxicity of amphotericin B. J. Pharm. Sci. 89, 1589–1593.

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

Chakraborty, K., Naik, S., 2003. Therapeutic and hemolytic evaluation of in-situ liposomal preparation containing amphotericin-B complexed with different chemically modified β-cyclodextrins. J. Pharm. Pharm. Sci. 6, 231–237.

Ernst, C., Grange, J., Rinnert, H., Dupont, G., Lematre, J., 1981. Structure of amphotericin B aggregates as revealed by UV and CD spectroscopies. Biopolymers 20, 1575–1588.

Esposito, E., Bortolotti, F., Menegatti, E., Cortesi, R., 2003. Amphiphilic association systems for amphotericin B delivery. Int. J. Pharm. 260, 249–260.

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

Gaboriau, F., Cheron, M., Leroy, M., Bolard, J., 1997. Physico-chemical properties of the heat-induced 'superaggregates' of amphotericin B. Biophys. Chem. 66, 1–12.

Hann, I., Prentice, H., 2001. Lipid-based amphotericin B: a review of the last 10 years of use. Int. J. Antimicrob. Agents 17, 161–169.

Hillery, A., 1997. Supramolecular lipidic drug delivery systems: from laboratory to clinic. A review of the recently introduced commercial liposomal and lipid-based formulations of amphotericin B. Adv. Drug Deliv. Rev. 24, 345–363.

Kirsch, R., Goldstein, R., Tarloff, J., Parris, D., Hook, J., Hanna, N., Bugelski, P., Poste, G., 1988. An emulsion formulation of amphotericin B improves the therapeutic index when treating systemic murine candidiasis. J. Infect. Dis. 158, 1065–1070.

Latere Dwan'Isa, J., Dinguizli, M., Préat, V., Arien, A., Brewster, M., 2005. Qualitative prediction of solubilization of highly hydrophobic drugs in block copolymer micelles. J. Contr. Release 101, 366–368.

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

Lavasanifar, A., Samuel, J., Kwon, G.S., 2002. The effect of fatty acid substitution on the in vitro release of amphotericin B from micelles composed of poly(ethylene oxide)-block-poly(*N*-hexyl stearate-L-aspartamide). J. Contr. Release 79, 165–172.

Legrand, P., Romero, E., Devissaguet, J.-P., Eleazar Cohen, B., Bolard, J., 1992. Effects of aggregation and solvent on toxicity of amphotericin B to human erythrocytes. Antimicrob. Agents Chemother. 36, 2518–2522.

Lennette, E., Balows, A., Hausler, W., Shadomy, H., 1985. Manual of Clinical Microbiology, American Society for Microbiology, Washington, DC.

Ould-Ouali, L., Arien, A., Rosenblatt, J., Nathan, A., Twaddle, P., Matalenas, T., Borgia, M., Arnold, S., Leroy, D., Dinguizli, M., Rouxhet, L., Brewster, M., Préat, V., 2004. Biodegradable self-assembling PEG-copolymers as vehicle for poorly water-soluble drugs. Pharm. Res. 21, 1581–1590.

Ould-Ouali, L., Noppe, M., Langlois, X., Willems, B., Te Riele, P., Timmerman, P., Brewster, M.E., Arien, A., Préat, V., 2005. Self-assembling PEG-p(CL-*co*-TMC) copolymers for oral delivery of poorly soluble drugs: a case study with risperidone. J. Contr. Release 102, 657–668.

Tabosa do Egito, E., Fessi, H., Appel, M., Barratt, G., Legrand, P., Bolard, J., Devissaguet, J.-P., 1996. A morphological study of an amphotericin B emulsion-based delivery system. Int. J. Pharm. 145, 17–27.

Tancrède, P., Barwicz, J., Jutras, S., Gruda, I., 1990. The effect of surfactants on the aggregation state of amphotericin B. Biochim. Biophys. Acta 1030, 289–295.

Tasset, C., Goethals, F., Préat, V., Roland, M., 1990. Effect of polyethyleneglycol (24) cholesterol on the solubility, toxicity and activity of amphotericin B. Int. J. Pharm. 58, 41–48.

Tasset, C., Préat, V., Roland, M., 1991. The influence of Myrj 59 on the solubility, toxicity and activity of amphotericin B. J. Pharm. Pharmacol. 43, 297–302.

Vakil, R., Kwon, G.S., 2005. PEG-phospholipid micelles for the delivery of amphotericin B. J. Contr. Release 101, 386–388.

Yu, B., Okano, T., Kataoka, K., Sardari, S., Kwon, G., 1998a. In vitro dissociation of antifungal efficacy and toxicity for amphotericin B-loaded poly(ethylene oxide)-block-poly(β-benzyl-L-aspartate) micelles. J. Contr. Release 56, 285–291.

Yu, B., Okano, T., Kataoka, K., Kwon, G., 1998b. Polymeric micelles for drug delivery: solubilization and haemolytic activity of amphotericin B. J. Contr. Release 53, 131–136.