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JOURNAL OF DRUG DELIVERY SCIENCE AND TECHNOLOGY (2010)
20
DOI: 10.1016/S1773-2247(10)50028-5

Amphotericin B loaded SLN prepared with the coacervation technique

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ABSTRACT: Stearic acid solid lipid nanoparticles (SLN) were prepared according to the coacervation technique, consisting in fatty acid precipitation of alkaline salt from a solution. SLN can encapsulate Amphotericin B with high efficiency in its monomeric form. SLN markedly increase in vitro drug toxicity towards Saccharomyces cerevisiae (taken as model yeast), expressed as potassium leakage. Yeast susceptibility towards Amphotericin B is increased when the drug is loaded in nanoparticles compared to the free drug, even if inhibition is limited by drug encapsulation within lipid matrix.

Solid lipid nanoparticles (SLN) are disperse systems suitable for drug delivery owing to their biocompatibility [1]. Several methods are described in literature to produce SLN, such as cold and hot homogenization [2], microemulsion dilution [3], microemulsion cooling [4], solvent evaporation [5], solvent injection [6], o/w [7, 8] and w/o/w [9] emulsions solvent dilution. Each of the mentioned techniques presents certain disadvantages, such as high operative temperatures, the use of toxic solvents, the need for sophisticated apparatus. Recently [10], a new, solvent-free SLN production technique was developed, and it was defined as "coacervation": briefly, when the pH of a micellar solution of fatty acid alkaline salt is lowered by acidification, fatty acid precipitates owing to proton exchange between the acid solution and the soap.

As this technique is based on the acidic precipitation of soap micellar solutions, it has great perspectives for the encapsulation of Amphotericin B (AmB) as a model drug, which is well known to form mixed micelles with surfactants: in fact the most widespread vehicle for AmB delivery is a sodium deoxycholate micellar solution (Fungizone).

AmB is a macrocyclic polyene considered as the drug of choice in the treatment of invasive fungal infections, in particular pathogen yeasts. In order to kill various species of fungi, AmB must first bind to ergosterol, the main sterol in fungal cell membranes; as AmB binds, it displaces the sterol from its normal phospolipid interactions to form sterol/AmB complex. Aggregation of sterol/AmB complexes eventually forms transmembrane pores, leading to enhanced membrane permeability and ultimately to cell lysis and death [11]. The selectiv- ity of AmB for fungi is attributed to its higher affinity for ergosterol than cholesterol in cellular membranes. Nonetheless, AmB can also damage mammalian membranes by a similar mechanism, causing serious side effects to the host. Therefore, the use of AmB is limited by the frequent complication of cumulative nephrotoxicity.

Because of its low water solubility, AmB should be administered in lipid formulations: in recent decades a number of studies have been performed to develop lipid preparations of AmB, aimed both at increas- ing its efficacy towards pathogen fungi and reducing its renal toxicity. Three lipid formulations of the drug are currently present in the market: AmB colloidal dispersion (Amphocil), AmB lipid complex (Abelcet) and liposomal AmB (Ambisome). The main limitation in their use is their high cost [12-14]. Moreover, drug resistance phenomena can be involved in AmB therapy, reducing its efficacy against pathogen fungi [15]. For all these reasons, other non-commercialized formulations of various types (micelles, nanospheres, conjugates) have been tested [16, 17].

The aim of this work was to develop a lipid colloidal system as a vehicle for AmB, which could enhance its efficacy against fungi *in vitro*. In this study, *Saccharomyces cerevisiae* was identified as model yeast which is sensitive to AmB and can be readily manipulated.

Due to its amphiphilic structure, AmB aggregates in aqueous solu- tion, where it exists as a combination of monomers and self-aggregates [18]. Different AmB aggregation states can be easily detected, because monomer and aggregates lead to different absorption and fluorescence spectra [19]. AmB toxicity seems to be restricted to self-associated AmB (dimers, oligomers) [19, 20]. Surfactants and some amphiphilic polymers have been demonstrated to reduce nephrotoxicity, while retaining the antifungal activity of AmB, by decreasing its state and extent of aggregation [16, 21]. In this study AmB aggregation state within nanoparticles was investigated through fluorescence spectroscopy.

I. MATERIALS AND METHODS

1. Materials

Lactic acid was from A.C.E.F. (Fiorenzuola d'Arda, Italy), 80 % hydrolyzed PVA 9000-10000 MW (PVA 9000), was kindly donated by Kuraray Europe GmbH; sodium stearate (SS) and AmB were from Fluka (Buchs, Switzerland). D(+)glucose monohydrate for microbiol- ogy, granulated agar and peptone digested from soymeal papain were from Merck (Darmstadt, Germany). De-ionized water was obtained by a MilliQ system (Millipore, Bedford, MO, United States). All other chemicals were analytical grade and used without any further purification.

2. SLN preparation

Stearic acid (SA) SLN were prepared as described in a previous work [10]. Briefly, SS was dispersed in a PVA 9000 aqueous solution and the mixture was heated under stirring (300 rpm) just above the Krafft point of SS (48 °C) in order to obtain a clear soap micellar solution. AmB was then added and stirring was maintained until AmB was completely solubilized. A proper lactic acid solution (coacervating solution) was then added drop-wise until pH 4.0 was reached and the obtained suspension was cooled to 15 °C in a water bath under stirring at 300 rpm.

3. SLN characterization

Particle size and polydispersity of SLN dispersions were determined by the laser light scattering technique (LLS) (90 Plus, Brookhaven, United States). Measurements were obtained at a 90° angle on the appropriate water-diluted samples.

Thermal analysis was performed with a DSC 7 (Perkin-Elmer, United States). Lipid bulk material and SLN suspensions were placed in conventional aluminum pans and heated from 30 to 90 °C at 2°C min^{-1} scan speed.

4. AmB encapsulation efficiency

AmB encapsulation efficiency (EE %) in SLN was calculated as the ratio between AmB amount in SLN and that in the starting micellar solution. SLN suspension was centrifuged at 57,000 g for 15 min (Allegra 64 centrifuge, Beckman Coulter, United States), the precipitate was dried under vacuum overnight and then treated as follows: a) a weighted amount was dissolved in 1 mL CH_3OH:DMSO 1:1 v/v opportunely diluted with methanol and used to record its absorption spectrum in 350-500 nm range (DU 730 Spectrophotometer, Beckman Coulter, United States); b)

an equal amount was washed with 0.01 N NaOH to remove surface-adsorbed drug and then treated as in a).

5. AmB stability

The main chemical modifications of AmB upon heating and pH shifting that can occur in SLN production process would be the oxidation of the polyene backbone or the cleavage of the lactone ring. The former would lead to serious alterations of the characteristic spectrum and the latter would be expected to modify the retention time in HPLC. Therefore, EE % was also determined by direct phase HPLC equipped with a LC9 pump, a SPD10AV UV-visible lamp and a C-R5A integrator (Shimadzu, Kyoto, Japan). Experimental conditions were as follows: column Hypersil5Silica 25 × 4.6 mm; mobile phase CH_3OH; flow 1 mL/min; λ 405 nm.

6. AmB aggregation state

From now on all experiments onAmB-loaded SLN were performed on 2 % SA 4 % PVA SLN.

AmB aggregation state was determined by fluorescence analysis [19], directly on SLN suspension: excitation wavelengths were 408 and 350 nm and emission wavelengths were 560 and 471 nm for the monomer and the dimer, respectively; fluorescence spectra were recorded by RF-551 Fluorimeter (Shimadzu, Kyoto, Japan) and monomer to dimer ratio was calculated by the ratio of emissions at 560 and 471 nm: monomer/dimer = [UF = 408 λ_{em} = 560]/[UF λ_{ecc} = 350 λ_{em} = 471]

7. Yeast strains and growth conditions

Saccharomyces cerevisiae (strain MUT 955) obtained from Mycotheca Universitatis Taurinensis (MUT) was used. Culture was kept in agar Saboraud. Cells were cultured in Saboraud medium for 24 h at 28 °C in a SBS30 shaker bath (Stuart Scientific, NJ, United States). Growth was monitored by cell counting through a Coulter Multisizer II (Beckman Coulter, United States).

8. Effect of AmB on retention of K^+ by yeasts

Twenty-four hours yeast culture was properly diluted in Sabo- raud medium to 2 × 10^7 CFU/mL [21]: cell counting was performed with Coulter Multisizer II. AmB-loaded SLN and Fungizone used as reference formulation n were added to 30 mL yeast culture, which was incubated for 1 h at

37 ± 0.1 °C. Cells were then harvested by centrifugation at 1,500 g for 10 min, re-suspended in 1 mL water and lysed in an autoclave (30 min at 120 °C) to extract intracellular K^+, which was then analyzed through Flame Photometer 410 (Prolabo, Paris, France).

9. Antifungal susceptibility test

The susceptibility of yeasts towards AmB was tested according to a method that was slightly modified from the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) guidelines [22]. Briefly, 24 h yeast culture was diluted in Saboraud medium to 2 × 103 CFU/ mL. AmB-loaded PVA SLN, Fungizone and a free AmB aqueous suspension (used as controls) were added to culture media to 0.5-20 μg/mL final concentration. According to the CLSI-recommended endpoint for AmB, the MIC (minimum inhibitory concentration) is the drug concentration that shows complete growth inhibition: cell proliferation after 24 and 48 h should be measured by optical density at 540 nm. In this case, since optical density of the medium can be influenced by SLN suspension turbidity, yeast cell proliferation was measured by cell counting, using Coulter Multisizer II. AmB inhibitory concentration that gave 90 % growth reduction compared to control (IC90) was used as the MIC endpoint [23].

II. RESULTS AND DISCUSSION

1. SLN characterization

Several SA SLN suspensions with different lipid concentrations were obtained, containing 0.05 % w/w AmB, according to *Table I*. Two percent SA SLN was stabilized with 2 and 4 % PVA, while 5 % SA SLN was stabilized with 5 % PVA. It was not possible to obtain stable AmB loaded 1 % SA SLN, as they formed aggregates.

	Blank				0.05 % AmB-loaded			
% SA- % PVA	1-1	2-2	2-4	5-5	1-1	2-2	2-4	5-5
SS	107 mg*	215 mg**	215 mg**	430 mg***	107 mg*	215 mg**	215 mg**	430 mg***
PVA 9000	100 mg	200 mg	400 mg	500 mg	100 mg	200 mg	400 mg	500 mg
1M lactic acid	0.5 mL				0.5 mL			
2M lactic acid		0.5 mL	0.5 mL			0.5 mL	0.5 mL	
5M lactic acid				0.5 mL				0.5 mL
AmB					5 mg	5 mg	5 mg	5 mg
Water	to 10 mL	to 10 mL	to 10 mL	to 10 mL	to 10 mL	to 10 mL	to 10 mL	to 10 mL
Mean size (nm)	285 ± 11	373 ± 18	421 ± 48	419 ± 50	-	355 ± 17	407 ± 45	422 ± 51
Polydispersity	0.008	0.079	0.131	0.222	-	0.211	0.147	0.307

*Corresponding to 100 mg SA. **Corresponding to 200 mg SA. ***Corresponding to 400 mg SA.

Table 1 Composition and mean sizes of blank and 0.05 % AmB-loaded SLN.

LLS mean diameters of AmB loaded SLN were quite similar to those of blank SLN, indicating that mean sizes were not affected by drug encapsulation, while in some cases a certain increase in polydispersity was observed (*Table I*).

Blank and AmB-loaded SLN DSC patterns are shown in *Figure 1*. It should be noted that in SLN SA is in B-form (m.p. 53 °C), even in the presence of drug. The precipitation of SA in B form is typical of coacervation process, as previously described [10] and it is not influenced by drug encapsulation within the lipid matrix.

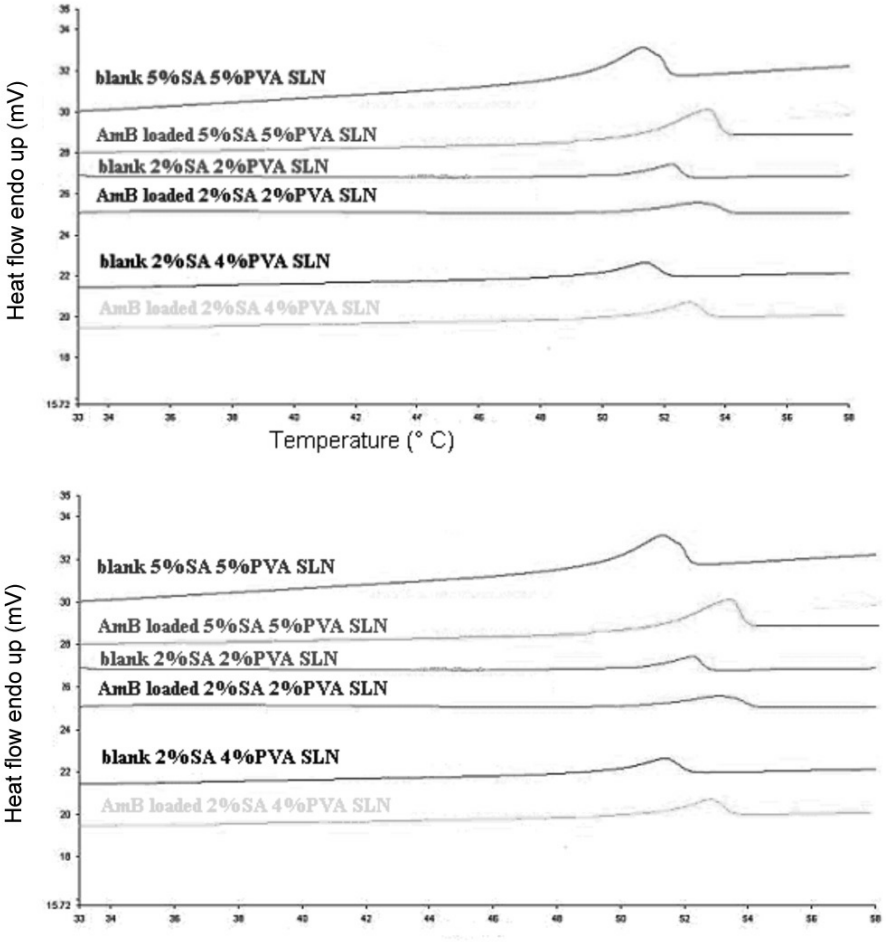

Figure 1 SLN DSC patterns.

AmB encapsulation efficiency (EE %), determined according to method a) and b), is reported in *Figure 2*. EE % was nearly 100 % of the total dose in all centrifuged SLN and lowered to nearly 70 % after washing with 0.01N NaOH: the washing procedure enabled the removal of AmB adsorbed onto SLN surface and the following determination of the amount of the drug actually present in the lipid core. HPLC analysis confirmed the chemical integrity of AmB as a single peak at 9.0 min was observed.

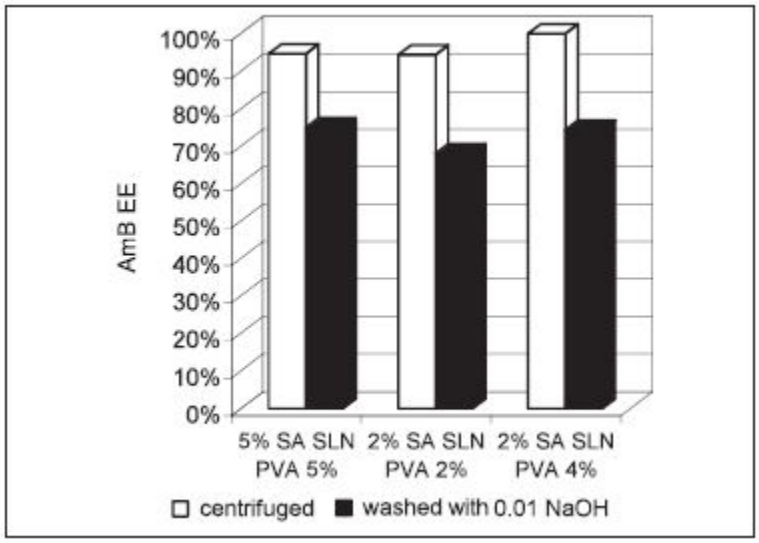

Figure 2 AmB encapsulation efficiency (EE %) in SA-SLN.

2. AmB aggregation state

AmB is characterized by two different absorption spectra [16], corresponding to different aggregation states: the monomeric form, occurring in diluted organic solutions shows three characteristic peaks at 412, 388, 368 nm [17], while the dimeric form, occurring in aqueous solution (as in the case of Fungizone micellar solution) shows a broad band at 325 nm (*Figure 3*). Unfortunately, the spectrophotometric method cannot be used for the determination of aggregation state in SLN, because of the opacity of the lipid suspension. A fluorimetric determination can be used, since emission and excitation spectra of monomer and dimer are also different [19].

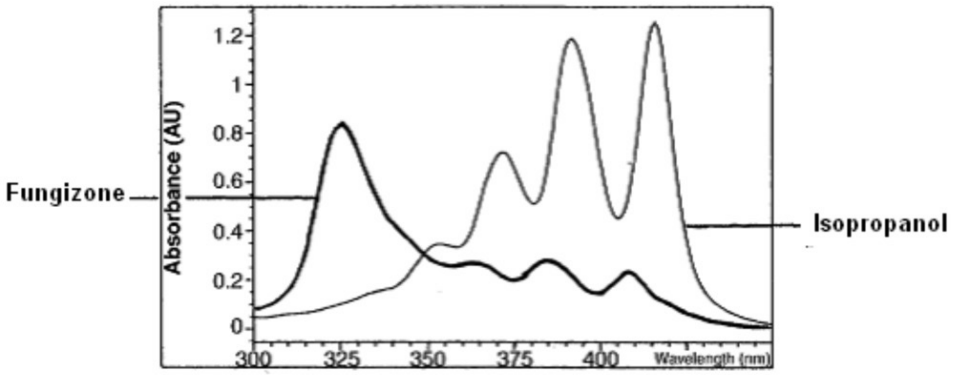

Figure 3 AmB UV-Vis spectra.

In *Figure 4* fluorescence emission spectra of AmB-loaded SLN, of AmB solution in isopropanol and Fungizone determined setting excitation wavelength at 408 nm are reported. Under these conditions, the monomeric form present in isopropanol solution shows three peaks in the 500-650 nm

range [19], while the dimeric form present in Fungizone shows a broad peak at 471 nm. It can be noticed that AmB-loaded SLN show an emission spectrum rather similar to that of AmB isopropanolic solution, suggesting that the drug is in its monomeric state within SLN. On the contrary the suspension obtained by spiking AmB to blank SLN shows a typical dimeric spectrum.

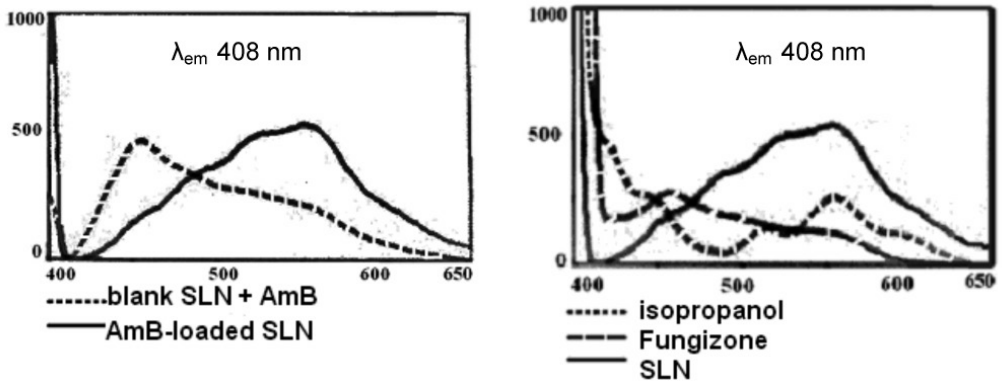

Figure 4 AmB fluorescence emission spectra.

The monomer to dimer ratio can be expressed as the ratio between the emission recorded at two different wavelengths, using different excitation wavelengths, as reported in the experimental section. In *Figure 5* calibration curve of versus isopropanol/water ratio is presented: as long as isopropanol increases, monomer/dimer ratio increases. The calculated ratios for Fungizone and AmB-loaded SLN are respectively 0.04 and 0.07, clearly indicating the prevalence of monomeric form in SLN compared to Fungizone.

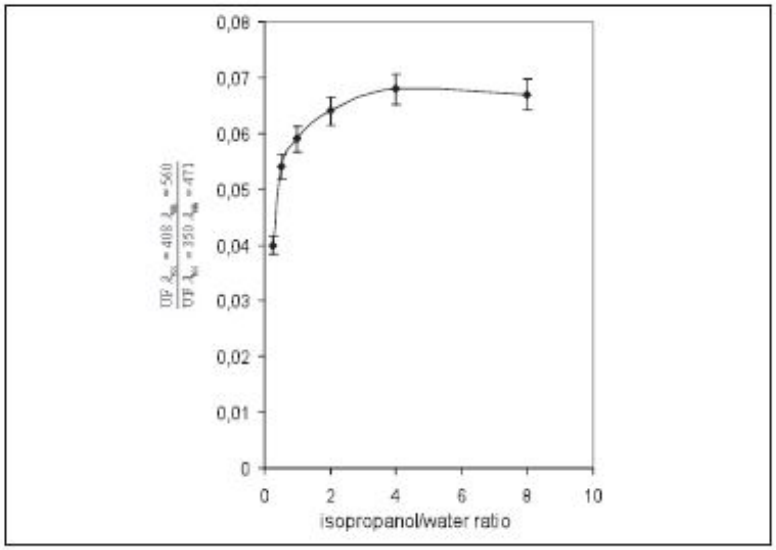

Figure 5 Calibration curve of monomer/dimer = [UF λ_{ecc} = 408 λ_{em} =560]/[UF λ_{ecc} = 350 λ_{em} = 471] vs isopropanol/water ratio.

As the monomeric form of AmB is well known to exert selective toxicity towards fungal cells, SLN can be considered as interesting vehicles for AmB administration [18].

3. Effect of AmB on retention of K^+ by yeasts.

The aggregation of sterol/AmB complexes leading to the formation of transmembrane pores and consequent enhanced membrane permeability is the main mechanism of AmB toxicity towards fungal cells [11]; potassium leakage is the most significant marker of this phenomenon. *Figure 6* displays the residual potassium amount within yeast cells after incubation with AmB-loaded SLN or Fungizone. It can be noted that the toxicity of AmB towards fungal cells increased dramatically when it was loaded in SLN, since a 10-fold higher dose of Fungizone is needed to obtain the same effect as AmB-loaded SLN. Blank SLN, on the contrary, caused no potassium leakage from yeast cells (data not shown).

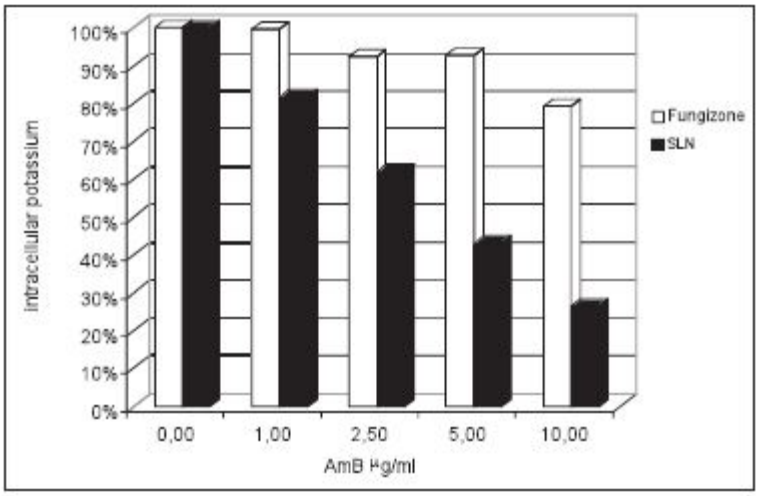

Figure 6 Retention of K^+ by yeasts incubated with AmB-loaded SLN and Fungizone.

4. Antifungal susceptibility test

The susceptibility test was performed with Fungizone,AmB-loaded SLN and an AmB water suspension (obtained by 5 min ultrasound treatment): cell growth was measured after 24 and 48 h and the results are reported in *Figure 7*. After 24 h MIC was lower than 0.5 μg/mL both for SLN and Fungizone, while AmB suspension did not inhibit cell growth in the whole range of concentrations studied. After 48 h SLN became no more effective in cell growth inhibition, and it was possible to identify MIC only in 20 μg/mL according to IC90 described in the experimental section.

Potassium leakage from yeast cells was effective after only 1 h incubation, indicating a quick toxic effect, whereas the antifungal susceptibility test showed comparable MIC between Fungizone and SLN only after 24 h. The lack of inhibition of yeast growth after 48 h, also confirmed by the high increase in MIC, agrees with literature data [24, 25], which show that entrapment of AmB within a nanoparticulate system reduces drug availability in culture medium, compared to Fungizone, where 100 % of the dose is immediately available.

It should be noted, however, that the free drug suspension is not effective in inhibiting cell growth, even after 24 h. This probably depends on the low solubility of the drug which limits drug availability in culture medium independently from the administered dose. Refer- ring to SLN, yeast cell growth is dose-dependent and this can suggest that probably the inhibiting effect is limited by the high EE % within nanoparticles, which probably reduces drug availability in culture medium proportionally to the administered dose.

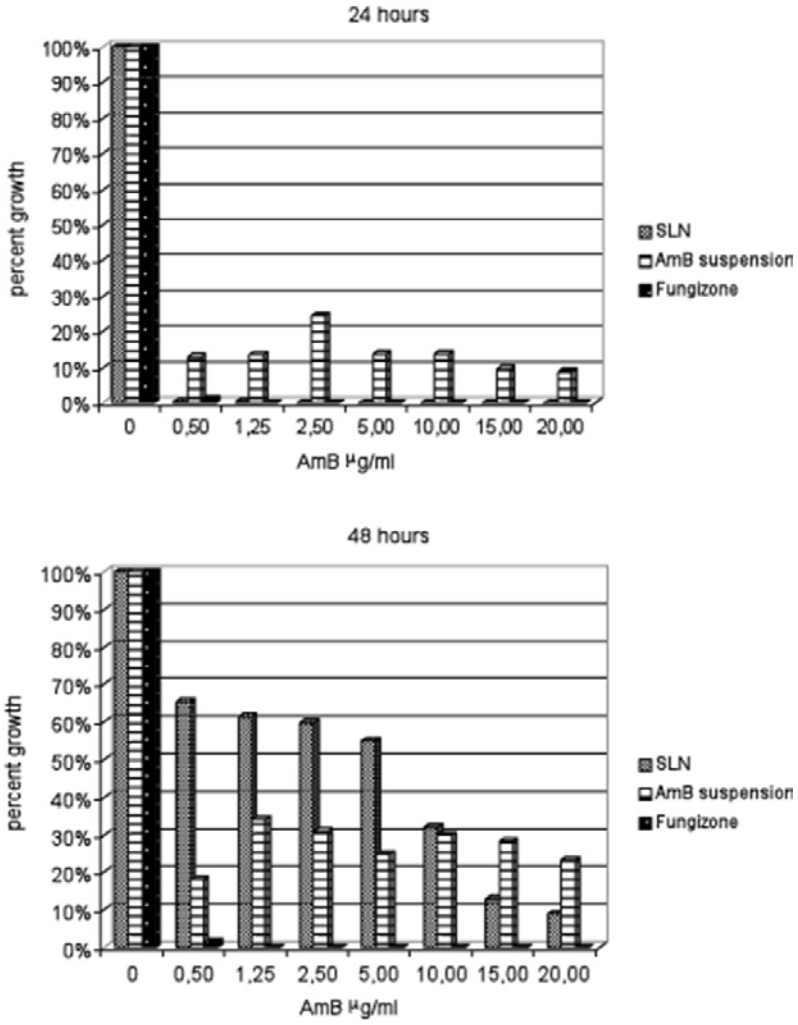

Figure 7 Antifungal susceptibility test of *Saccharomyces cerevisiae* incubated with AmB-loaded SLN, Fungizone and free AmB, **a)** after 24 h, **b)** after 48 h.

Although the precise mechanism of toxicity of AmB loaded SLN towards yeast cells needs further studies, we can assert that SLN are vehicles which can enhance *in vitro* activity of AmB against fungi compared to free drug, as previously reported in literature for polymeric nanoparticles carrying other anti-fungal drugs [26].

*

AmB (mainly in monomeric form) can be easily encapsulated within SLN prepared through the coacervation technique, increasing the toxicity towards yeast cells *in vitro*, expressed as potassium leakage from cells, compared to Fungizone. Although yeast susceptibility to AmB-loaded SLN is

probably limited by the high drug EE % within lipid matrix, SLN increase the inhibiting effect of AmB compared to that of free drug. Further *in vivo* studies are necessary to confirm *in vitro* data and to evaluate whether AmB-loaded SLN may improve the current treatment of pathogen fungi infections.

ACKNOWLEDGEMENT

We thank Kuraray Europe GmbH for support in the development of experimental work.

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