**P-054** **Evaluation of amphotericin B "superaggregates" micelle systems.**

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## INTRODUCTION AND OBJECTIVES

The amphotericin B in a micellar system (AmB), Fungizon®, is a system largely used for the treatment of systemic fungal infections, mainly in immune-compromised patients. Its use is limited due to its high toxicity, which is the main cause of its side effects such as nephrotoxicity (Cheron 2003).

According to the literature, in aqueous solution Fungizon® has monomeric and aggregated forms of AmB, these latter being responsible for its side effects. There are several less toxic amphotericin B lipidic formulations, but at a high cost that hinders their use worldwide. It has been observed that the heating of AmB micelles generates "superaggregates" forms, which are produced by the condensation of monomeric and aggregate forms. This new state has been demonstrated to be less cytotoxic while keeping its activity (Gaboriou 1997).

The aim of this work was to investigate the changes in the size and in the UV-VIS spectra of the unheated (AmB-DOC) and heated (AmB-DOC-H) AmB micelle systems and to correlate them with their toxicity against Red Blood Cells (RBCs).

## MATERIALS AND METHODS

***Heat treatment***
The heated AmB (AmB-DOC-H) was prepared by treatment of AmB-DOC (at $5x10^{-3}$M) with moderate heat treatment at 70°C for 20 minutes.

***Size analysis***
The particle size analysis of AmB-DOC and AmB-DOC-H, purchased from Cristália SP-Brazil, at $5x10^{-3}$M (5,000mg.$L^{-1}$) was performed by Dynamic Light Scattering (DLS) in a Delsa™nano (Belckman Coulter).

***Spectra analysis***
Scanning spectra of both AmB-DOC and AmB-DOC-H at four study concentrations were taken by using a Varian-model Cary 1E – UV-VIS Spectrophotometer (Mulgrave). The optical path of the quartz cuvettes used was 0.1, 1, and 10 cm for the concentrations of $5 \times 10^{-5}$ M (50mg.$L^{-1}$); $5 \times 10^{-6}$ M (5mg.$L^{-1}$), and $5 \times 10^{-7}$ M (0.5mg.$L^{-1}$) and $5 \times 10^{-8}$ M (0.05mg.$L^{-1}$), respectively. These paths were chosen to obtain spectra with absorbance values less than 0.8. Their molar extinction coefficients (ε) were calculated using the Beer-Lambert equation. All spectra were recorded at 25 ± 0.1° C with a 300 - 450 nm range (Egito 2002).

***Toxicity assay***
Four mL of RBCs ($5x10^{7}$cells.$mL^{-1}$) were incubated for 1 hr at 37ºC with the control or with different concentrations (50, 5, 0.5 and 0.05 mg.$L^{-1}$) of both AmB-DOC and AmB-DOC-H. The RBCs were then centrifuged for 5 min at 1,100g and washed three times with normal saline. The pellet of RBC was lysed by adding 4 mL of distilled water. Stirring and centrifuging (1,100g for 10 min) were used in order to remove membranes. Potassium ($K^+$) content of the supernatant was determined using a Flame Photometer 7000 (910M Analyser) calibrated with $K^+$ reference at 5 mEq.$L^{-1}$. Hemoglobin was estimated from its absorption at 540nm recorded on a Spectrophotometer Varian-model Cary 1E – UV-VIS (Mulgrave) (Araújo 2005).

## RESULTS AND DISCUSSION

Concerning the size, due to the weak signal from the Delsa™nano equipment the sample of AmB-DOC was not measured. However, when the sample was submitted to moderate heating (AMB-DOC-H), the appearance of a new aggregate state size of 240 nm was observed. (Table 1).

**Table 1 : Dynamic Light Scattering from AmB-DOC and AmB-DOC-H at $5x10^{-3}$M**

| | Ave. Diameter | Polidispersity index |
|---|---|---|
| AmB-DOC | no detected | no detected |
| AmB-DOC-H | 240.9 nm | 0.532 |

Regarding the UV-Vis spectrophotometric analysis, similar to AmB-DOC, AmB-DOC-H presents a spectrum that were concentration dependent (Figure 1 and Figure 2). At low concentration, the spectra were similar to those obtained in organic solvents like methanol, showing maxima at 363, 385 and 408 nm and a shoulder around 347 nm. The band at 408 nm, traditionally assigned to monomeric AmB, is mainly responsible by the activity against fungal cells (Gaboriou 1997). As the concentration of AmB-DOC increase, a new band appeared at 327 nm at the expense of the one assigned to monomeric AmB. The increase of amplitude of the band at 327 nm is due to the presence of self-associated species (Gaboriou 1997). Concerning the AmB-DOC-H spectra, this band at 327 nm was slightly blue shifted (4nm) and became located at 323 nm. Several authors stated that this band is characteristic of the formation of superaggregates (Gabo-

riou 1997). The most important difference among the AmB-DOC and AmB-DOC-H spectra occurred at the concentration of 5 x $10^{-7}$ M, in which the band at 323 nm presented a high molar extinction coefficient (ε = 71,000) compared to the one assigned for AmB-DOC (ε = 48,666). It should be emphasize that the spectra at 5 x $10^{-8}$ M were given to illustrate the tendency of concentration dependence. In fact, they should not be considered on a quantitative basis because of the weakness of the signals at low wavelength region (maximum absorbance at 408 nm was 0.071 for AmB-DOC).

These results allow us to conclude that a different association between the surfactant and the AmB molecules occurred when the system is heated, and such changes remained over the whole range of concentrations

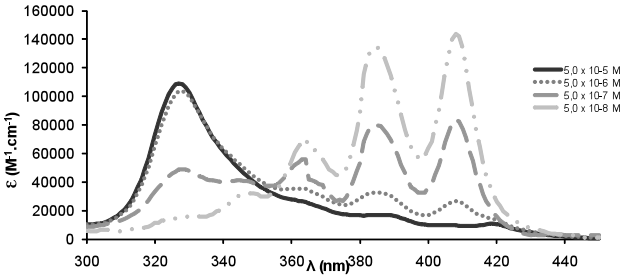


**Figure 1 : concentration-induced changes in the AmB-DOC spectra at 25ºC**

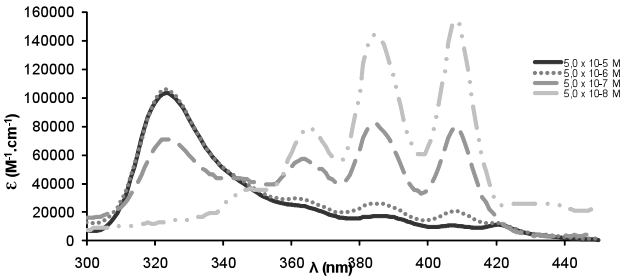


**Figure 2 : concentration-induced changes in the AmB-DOC-H spectra at 25ºC**

The toxicity of both AmB-DOC and AmB-DOC-H against RBCs were quite interesting. Concerning $K^+$ leakage, both profiles were quite similar (Figure 3).

On the other hand, concerning hemoglobin leakage both AmB-DOC and AmB-DOC-H presented no significant release below 0.5mg.$L^{-1}$ (Figure 4). In fact, AmB-DOC-H showed to be no toxic for over the whole range of concentration tested (from 0.05 to 50 mg.$L^{-1}$. $p<0.001$). Inversely, AmB-DOC became quite toxic at 5mg.$L^{-1}$, (100 ± 0.36%) when the full bulk of RBCs was lysed and the total of entrapped hemoglobin was leaked to the external media.

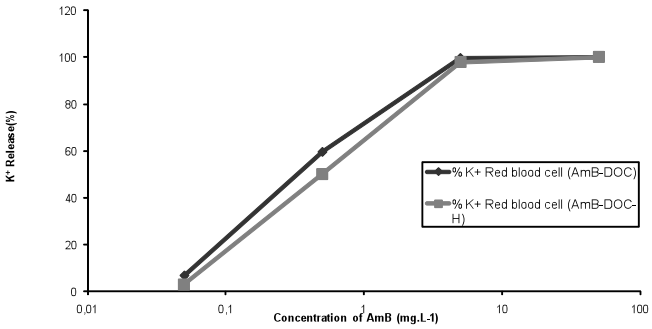


**Figure 3 : release of $K^+$ from RBCs induced by AmB-DOC and AmB-DOC-H.**

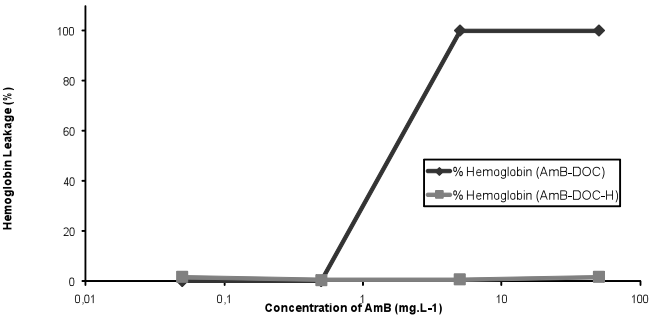


**Figure 4 : release of hemoglobin from RBCs induced by AmB-DOC and AmB-DOC-H.**

## CONCLUSIONS

All the results together suggest that the AmB-DOC-H was able to reduce the toxicity of its unheated form (AmB-DOC) probably by changing the AmB aggregation state. Also, it demonstrates that a simple way to rebuilt micelles systems and generates new entities at a nanoscale domain is possible by simple heating. Therefore, we can speculate that this strategy open a way to create nanocarriers by changing the manufacturing parameters and process of micelles production.

Indeed, the AmB-DOC-H demonstrated to be much less toxic than AmB-DOC highlighting the importance of this new procedure as a simple, inexpensive and safe alternative for the future treatment of systemic fungal infections.

## REFERENCES


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