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Cellular association of liposomal formulations with in THP-1 cells : influence of lipid composition

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INTRODUCTION AND OBJECTIVES

Paromomycin (PRM) is an aminoglycoside indicated for treatment of infectious diseases namely leishmaniasis and tuberculosis. The main drawbacks associated to the use of this antibiotic are the reduced half-life in blood circulation, the rapid renal excretion and consequently insufficient intracellular concentration of the drug.

One strategy to change the biodistribution profile of antibiotics may be achieved through their association to liposomes. These systems may be envisaged as a natural system for targeting intracellular infections as after i.v. administration they are taken up by mononuclear phagocytic system (MPS).

Our strategy, in the present work was to encapsulate PRM in liposomes, to study the parameters affecting the encapsulation efficiency such as lipid charge and pH medium and select PRM formulations with higher loadings. In addition, Liposome-cell interactions are also an important issue. With this purpose, cellular association studies were carried out by using THP-1 cells, a human monocytic leukemia cell line. Rhodamine labeled liposomes at different lipid concentrations were incubated with differentiated THP-1 cells and the influence of the lipid composition on liposome binding and internalization into the cells was evaluated either by flow cytometry or by fluorescence microscopy.

MATERIALS AND METHODS

PRM was obtained from Fluka, Biochemika, pure phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA). All the other chemicals and reagents used were of analytical grade.

Liposome preparation: PRM liposomes were prepared by the dehydration-rehydration method (Cruz 1993). In brief, the lipid mixtures (18 µmol/ml) were dried under a N_2 stream and the lipid film was hydrated with a PRM solution. The liposome suspension was lyophilized overnight and the lyophilized powder was rehydrated in two steps with a 150 mM NaCl in 10mM Hepes buffer pH 6.0 or 8.0. The so formed liposomes were diluted in the same buffer, extruded through polycarbonate membranes of different porosities and the non-encapsulated PRM was separated by ultracentrifugation.

***Liposome characterization*:** PRM in liposomal form was determined by a spectrophotometric assay after disruption of the lipid membranes with 20% Triton X-100 (Mimoso 1997). Liposome mean diameters were determined by dynamic light scattering (Zetasizer Nano-S, Malvern Instruments, UK). Zeta potential was calculated by using Smoluchowsk`s equation (ZetaSizer 2000, Malvern, UK).

***Cellular association studies*:** To assess liposome-cell interactions, liposomes were labeled by incorporating phosphatidylethanolamine –N-lissamine rhodamine B sulfonyl (rhodamine PE) at a concentration of 0.2 mol% of total lipid (Pires, 1999) to the lipid mixtures. Differentiated THP-1 cells at a concentration of $4x10^6$ cells/ml were incubated with liposomes, prepared with selected lipid mixtures, in order to obtain a final lipid concentration of 0.4 and 1.6 mM. For binding and uptake cell association studies, cells were incubated at 4 and 37ºC, respectively, for 2 h. After incubation period cells were washed 3 times with PBS and fixed with paraformaldehyde at final concentration of 2%. Mean fluorescence intensity of the cells was analysed by flow cytometer (FACSCalibur, BD FACSDiva Software, BD Biosciences, USA). Cellular uptake of liposomes was also assessed by fluorescence microscopy (Axioskop 40, Zeiss, Germany).

RESULTS AND DISCUSSION

PRM was encapsulated in liposomes composed of negative, neutral and positively charged lipid mixtures and the effect of the lipid composition on the encapsulation parameters was evaluated. In Table 1 the obtained results are shown. The size of all liposomal formulations presented a mean diameter of 0.10 to 0.14 µm and a polydispersity index below 0.15 demonstrating a very high homogeneity. The theoretical initial PRM to lipid ratio for all formulations was 40 µg per µmol of lipid. According to the obtained results negatively charged formulations presented the highest encapsulation parameters with Encapsulation Efficiency (E.E.) higher than 80%. The results were independent on the phase transition temperature of the phospholipids, ranging from 23ºC for dimiristoyl phosphatidylcholine and dimiristoyl phosphatidylglycerol (DMPC : DMPG) to 42ºC for dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidylglycerol (DPPC :DPPG) lipid mixtures.

For positively charged formulations, containing stearylamine (SA), the influence of the buffer was very important. Changing the pH from 6.0 to 8.0 resulted in an in-

crease on the final PRM to lipid ratio and E.E. from 5 to 11 µg/ µmol of lipid and 9 to 58%, respectively.

Table 1 : Influence of the lipid composition on encapsulation parameters of PRM in liposomes.

Lipid Composition (molar ratio)	**$(PRM/Lip)_f$** (µg/µmol)	**E.E. (%)**	**ζ Pot. (mV)**
DMPC	3±1	16±5	-3±1
DMPC :Chol (8 : 2)	7±1	17±3	-2±1
DMPC :DMPG (7 : 3)	19±1	82±5	-24±10
DPPC :DPPG (7 : 3)	13±1	86±5	-35±5
DMPC :SA* (9.75 : 0.25)	5±1	9±2	+2±1
DMPC :SA** (9.75 : 0.25)	11±1	58±2	+9±2

E.E. (%) – $(PRM/Lip)_f$ / $(PRM/Lip)_i$ x 100
*- pH buffer 6.0 / ** - pH buffer 8.0

Cellular association studies

In order to obtain insights into the ability of liposomes to internalize into THP-1 cells, three different liposomal formulations containing the fluorescent label rhodamine-PE were tested: DMPC:DMPG (LIP A); DPPC:DPPG (LIP B) and DMPC:SA (LIP C). Cells were incubated with liposomes at 4ºC and 37ºC and the difference between the fluorescent intensity in percentage was used as a measure for cellular internalization as at 4ºC no endocytosis is expected to occur (Kamps, 2010).

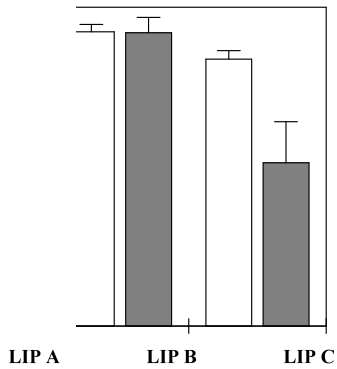

Figure 1. Influence of different liposomal formulations on cellular internalization : DMPC :DMPG (LIP A), DPPC :DPPG (LIP B) and DMPC :SA (LIP C). Two different lipid concentrations were tested 1.6 mM (white columns) and 0.4mM (grey columns)

For all liposomal formulations under study two lipid concentrations were used 1.6 and 0.4 mM

The obtained results are shown in Figure 1 and according to data all the formulations presented a higher association rate at 37ºC than at 4ºC. Particularly for DPPC: DPPG liposomes (LIP B) the cellular internalization showed the highest levels in percentage, more than 90% independent on the lipid concentration.

For the positively charged formulation, DMPC:SA (LIP C) the percentage of the cellular internalization was lipid concentration dependent: ranging from 50 to 84% for lipid concentrations of 0.4 and 1.6 mM, respectively.

The obtained results by flow cytometry are in accordance with acquired images by fluorescence microscopy. In Figure 2 are shown images of THP-1 cells after incubation at 37ºC with DPPC: DPPG liposomes where a high internalization of labeled liposomes is observed.

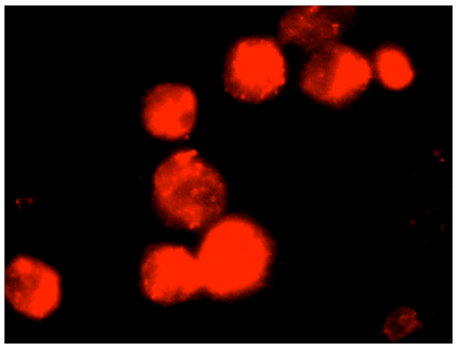

Figure 2. Association of fluorescent labeled liposomes prepared with DPPC:DPPG to THP-1 cells

CONCLUSIONS

PRM was efficiently encapsulated in liposomes particularly when negatively charged phospholipids were used.
All the formulations under study presented a higher cellular association rate at 37ºC than at 4ºC and the percentage of internalized labeled liposomes was higher for DPPC: DPPG liposomes. This liposomal formulation appears a very good candidate formulation for efficient treatment of intracellular infections.

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