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The active trapping of doxorubicin in liposomes by pH gradient: photon correlation spectroscopy and fluorimetric study

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Summary

Changes in liposome diameter in response to a pH gradient during uptake of doxorubicin were studied. After an initial increase liposomes underwent shrinkage which was dependent on lipid composition and temperature of incubation. Changes in fluorescence undergone by the drug during the active trapping process were also monitored. The observed self-quenching of doxorubicin was consistent with the kinetics of drug entrapement within the internal aqueous compartment of the vesicles except when incubation was carried out at 37°C in the presence of cholesterol.

Introduction

In liposome technology there are several methods for encapsulating active compounds, all with the same objective: to improve the encapsulation efficiency of drugs in vesicles.

In passive methods to enhance trapping efficiencies two strategies have been assayed. The first consists of methods which yield large unilamellar vesicles (LUVs) or multilamellar vesicles (MLVs). While LUVs entrap greater volumes of water than small unilamellar vesicles (SUVs), MLVs obtained under appropriate conditions show higher encapsulation efficiencies than those generated by simple dispersion in excess water (Mayer et al., 1986a). The second strategy is related to poorly soluble or insoluble compounds, which could be partly or completely encapsulated into the bilayer. As an example, methotrexate could be covalently coupled to phosphatidylethanolamine, which is in fact treated as a lipid occurring during vesicle formation (Noé et al., 1988). Therefore, in such cases, we would expect to achieve a higher concentration of drugs irrespective of the method chosen.

At the other extreme, with amphiphilic cationic drugs, it is possible to encapsulate them in response to pH or potential gradients between the interior and exterior of vesicles (Bally et al., 1985; Mayer et al., 1986b). Active methods resolve two main pharmaceutical difficulties both because they lead to more efficient encapsulations and since they allow the design of stable liposome formulations which are prepared as required.

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Doxorubicin (DXR), a well-known antineoplastic agent, has been extensively studied during the last decade, since (liposomally encapsulated) it has been shown to have beneficial effects including the enhancement of its therapeutic index and the concomitant decrease in drug cardiotoxicity (Gabizon et al., 1990). This is the reason for the increasing interest in new methods of encapsulation (Amselem et al., 1990). On the other hand, since the physical characteristics of a liposome formulation seem to exert an extensive effect on the pharmacological properties of liposomes (Mayer et al., 1990a), several studies have focused on DXR-lipid interaction (Murphree et al., 1982; Constantinides et al., 1988–89). Moreover, because DXR is an amphiphilic cation it has been successfully encapsulated by active methods in response to both pH and potential gradients (Mayer et al., 1986a; Bally et al., 1985).

The two main aims of this study were to determine how physicochemical conditions (temperature and lipid composition) affect the morphological properties of liposomes during the active trapping procedure and to investigate how DXR is internalized by liposomes, taking advantage of the intrinsic fluorescence of the drug. Furthermore, this is of interest as the method appears to form the basis of a new pharmaceutical preparation which allows encapsulation of DXR in empty liposomes just prior to use. We have also compared the efficiency of encapsulation of these liposomes with that of liposomes obtained by passive methods.

Materials and Methods

Materials

L-α-Dipalmitoylphosphatidylcholine (DPPC) and cholesterol (CHOL), both specified as 99% pure, were obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Doxorubicin-HCl was donated by Farmitalia-Carlo Erba (Barcelona, Spain). Deionized water was distilled from sodium permanganate in an all-glass apparatus and further purified by reverse osmosis through a Milli-Q system (Millipore, U.S.A.). All other common chemicals were of reagent grade or better and all organic solvents were redistilled. Gel for chromatography was obtained from Pharmacia (Uppsala, Sweden).

Methods

Preparation of multilamellar vesicles

The interior of a conical tube was coated with a thin lipid film by evaporation to dryness under a stream of nitrogen of a chloroform solution containing either 40 μmol of DPPC or an equimolar mixture of DPPC and CHOL. After lyophilization overnight, MLVs were prepared by hydration in an appropriate buffer and vortexing vigorously for several minutes.

Passive encapsulation

After redispersion in 145 mM NaCl, 10 mM Tris (pH 7.4) buffer (in the presence or absence of DXR), vesicles were extruded 10 times through 100 nm polycarbonate membranes (Nuclepore, U.S.A.) following a method described elesewhere (Hope et al., 1985). The DXR content of liposomes was determined after the separation of free DXR from liposome-entrapped DXR by gel filtration on Sephadex G-50.

Active encapsulation by pH gradient (ΔpH)

Multilamellar vesicles were generated by redispersion in 0.3 M citric acid (pH 3.7) buffer. After extrusion 10 times through 100 nm filters, the exterior pH of empty vesicles was titrated with 1.0 M NaOH until a ΔpH of 4.1 was reached (inside acidic). DXR in citrate buffer (pH 7.8) was then added to liposomes to yield a final concentration of 0.3 mM. Samples were heated at 60°C for 100 min to determine morphological changes during loading and unentrapped DXR was separated by molecular exclusion chromatography on Sephadex G-50.

In all cases, the temperature during agitation and extrusion was kept above the transition temperature (T_m) of the lipid.

Fluorescence

To follow the kinetics of drug entrapment in liposomes, DXR was added to a liposome solu-

tion to obtain a lipid/DXR ratio of 2.88:1 (mol/mol). The solution was placed in a quartz cuvette in the jacketed cuvette holder of the fluorometer, which was mantained at the desired temperature (37 or 60°C). Fluorescence measurements were performed using a Kontron spectrofluorometer. Doxorubicin was excited at 470 nm with a slit width of 0.5 mm, and fluorescence was monitored at 552 nm at constant temperature. In all cases, measurements were corrected for scattering changes of the samples.

In both active and passive encapsulation, Triton X-100 was added (1% v/v) to an aliquot of the effluent, after chromatographic separation to disrupt the vesicles. The DXR released was then assayed spectrofluorimetrically. Determination of total phospholipid was performed according to a colorimetric method (Stewart, 1980), with a minor modification: centrifugation for 10 min at 2000 rpm was introduced to improve phase separation before spectrophotometric determination of the organic extract. Encapsulation efficiency was presented as mol of DXR entrapped per mol of total lipid recovered (Hernàndez et al., 1987).

Photon correlation spectroscopy

For particle-size analysis in solution, photon correlation spectroscopy (PCS), also known as quasielastic light scattering (QELS), was used to calculate normalized autocorrelation functions, which typically show exponential decay (McConnell, 1981). According to the theory of dynamic light scattering, the time constant of this decay is directly related to the translational diffusion coefficient. Then, if we assume that particles (liposomes) are spherical, the liposome diameter can be obtained from the Stokes-Einstein relationship. Light scattering measurements were performed in an Autosizer IIc photon correlation spectrometer (Malvern Instruments, U.K.) using a helium-neon laser as a source of incident light ($\lambda = 632.8$ nm), operating at 5 mW. As in fluorimetry, DXR was added to a liposome solution to yield a total lipid/DXR ratio of 2.88:1 (mol/mol) and the cuvette was placed inside the temperature-regulated cell-scattering enclosure. The first measurement was performed at 25°C and the mean diameter of the liposomes was taken as a reference value. Immediately after the first measurement (instrumental delay time of 2.59 min) the solutions were heated to 37 or 60°C. The time interval required to reach the desired temperature had previously been monitored with a thermistor in direct contact with the full cuvette. After approx. 60 min incubation, samples were cooled to the initial temperature and their final size was determined. Other experimental conditions were: viscosity, 0.899×10^{-3} Pa s and refractive index, 1.330. The data were collected at a scattering angle of 90° and the autocorrelation function was determined via a Malvern 7032-N, 72-channel multibit correlator. Assuming a log-normal size distribution of particles, the method of cumulant analysis (Koppel, 1972) was available, while for broad unimodal distributions of the model, independent analysis which does not assume any particular form of the distribution was used (Ostrowski et al., 1981).

Osmometry

In order to determine the osmolarity of solutions 10 μl of sample was transferred to a Fiske Osmometer.

Results and Discussion

In response to transmebrane pH gradients (inside acidic), DXR moves to the vesicle interior. However, DXR uptake appears to depend on a more complex process, which involves an equilibrium between entrapped and insoluble forms of the drug (Mayer et al., 1990a). Irrespective of the mechanism involved, it is assumed that the drug remains mainly in the aqueous spaces in the protonated form, since it has been demonstrated that trapping efficiencies can approach 100% (Mayer et al., 1990a,b). In concordance with this, the encapsulation efficiencies for neutral DXR liposomes obtained by passive and active methods are listed in Table 1. Results of passive DXR encapsulation methods have previously been published (Martí et al., 1992). Indeed, as can be observed, for both lipid compositions active encapsulation became 5-fold greater than that obtained using passive methods. Moreover, it is

TABLE 1

Encapsulation efficiency of DXR in DPPC and DPPC:CHOL (1:1, mol/mol) liposomes and standard deviation obtained by passive and active method

Lipid composition	Encapsulation efficiency (μmol DXR/μmol lipid)	
	Passive method	Active method
DPPC	0.0094 ± 0.005	0.0476 ± 0.019
DPPC-CHOL	0.0098 ± 0.002	0.0454 ± 0.013

Data are the mean values of three different experiments. Both methods were carried out at room temperature with an initial lipid-drug ratio of 2.88.

shown that encapsulation is insensitive to the presence of CHOL, as was expected for these neutral lipids (Mayer et al., 1989). This is not surprising at least when samples were incubated at high temperature (60°C), because the permeability properties of these membranes could be independent of vesicle lipid composition. However, as similar results are obtained at 37°C, factors other than a simple increase in permeation may be involved in the mechanism of internalization. Indirect confirmation of this behaviour was found in the study of the interaction between DXR and monolayers of similar lipid composition to liposomes (Hernàndez et al., 1991).

The above results and calculated trapping efficiency are in agreement with previous reports (Mayer et al., 1986b). Nevertheless, although the system is technically feasible, we have no information about the effect of the drug on vesicle morphology while loading takes place, nor whether it could perturb lipid packing at the end of the process. These points require further study because size is a variable which plays an important role in the pharmacological properties of vesicles (Mayer et al., 1989) and furthermore can affect DXR capture (Amselem et al., 1990).

Uptake of DXR into liposomes depends on temperature, being more efficient at higher temperatures (Mayer et al., 1990b). Because this method has been proposed for clinical use, it is held that after loading, samples should be cooled before administration. Consequently, because of the thermal dependence of the physical state of bilayers, several changes in liposome size could be expected throughout the overall process of loading.

In the present work, liposomes were incubated after drug addition at 37 or 60°C (below and above the transition temperature of DPPC) and

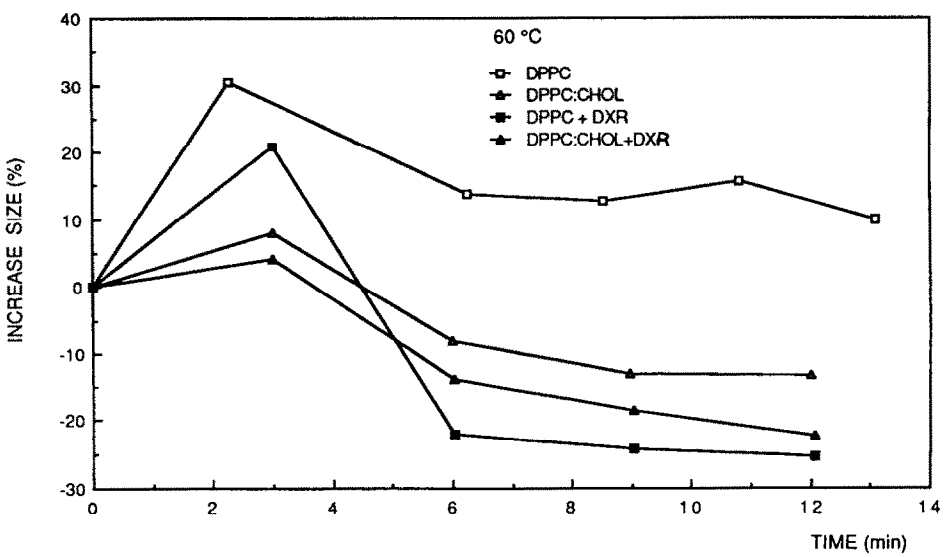

Fig. 1. Kinetics of mean diameter variation expressed as a percentage of the initial size at 60°C for DPPC and DPPC-CHOL liposomes in the absence and presence of DXR.

the temperature was maintained for 60 min. Samples were then cooled to room temperature. Since uptake at both temperatures takes place in the first few minutes (Mayer et al., 1990b) no further incubation was needed. However, it is demonstrated that longer incubations do not affect encapsulation efficiencies or final liposome size.

Under these conditions, morphological changes during loading (size and polydispersity) were continually monitored to study the effect of DXR on preformed liposomes. As can be seen in Fig. 1, liposomes undergo an increase in *z*-average mean diameter within the initial period of incubation at 60°C, immediately after DXR solution or buffer is added. Similar but less pronounced effects were observed for DPPC and DPPC-CHOL liposomes incubated at 37°C (data not shown). The initial swelling of these liposomes was dependent on the lipid composition and also on the presence of drug. Results show that the smallest increases were undergone by DPPC-CHOL liposomes in the presence of DXR while the highest values were attained by empty DPPC liposomes. Independently of lipid composition, the presence of DXR led to smaller variations in size than those undergone by empty liposomes (Fig. 1). These results are consistent with the well-known condensing effect of CHOL on DPPC bilayers, and may be related with the interdigitazion of the neutral form of the drug into the bilayer, which can have an additional condensing effect on the lipid bilayer (Dupou-Cézanne et al., 1989). On the other hand, size changes could in part be attributed to a slight degree of liposome aggregation or fusion in the presence of DXR (Murphree et al., 1982; Constantinides et al., 1988–89).

It is known that the loading process is completed in less than 3 min at 60°C and in approx. 10 min at 37°C (Mayer et al., 1990a). These results correlate very well with the initial swelling of liposomes (Fig. 1) and can explain in elementary terms of bilayer permeability why at 60°C (fluid state) the rate of the loading process is faster than at 37°C. Furthermore, it was found that an initial increase in diameter precedes a shrinkage of liposomes, which ceases after 6–10 min of incubation (Fig. 1). Note that within this interval encapsulation has finished and correlates with the minimum values of diameter observed. Table 2 shows absolute values of the mean diameter and standard deviation for DPPC and DPPC-CHOL liposomes during the main steps of the process studied at 37 and 60°C. Four experiments were performed separately but Table 2 only shows the results for one. There, we can see the moderate shrinkage undergone by the vesicles after 60 min incubation (cf. steps II and III in Table 2) and sizes attained when samples were cooled back and allowed to equilibrate at room temperature (IV in Table 2).

It is well known that liposome size is sensitive to changes in extravesicular tonicity and, since incubation solutions were slightly hypertonic (683 and 728 mOsmol/kg), no major size modifica-

TABLE 2

Variation of mean diameter and standard deviation of DPPC and DPPC-CHOL (1:1) liposomes at 37 and 60°C in the absence and presence of DXR at steps I–IV (I, before heating at room temperature; II, in the first 3 min, heating; III, at 60 min, heating; and IV, after cooling to room temperature)

	Step			
	I	II	III	IV
DPPC 37°C	120.3 ± 26.9	121.5 ± 20.3	129.9 ± 23.4	143.8 ± 23.5
DPPC + DXR 37°C	139.3 ± 20.8	133.7 ± 22.0	169.5 ± 30.5	173.8 ± 37.2
DPPC 60°C	125.3 ± 27.5	121.4 ± 19.9	144.5 ± 28.6	161.0 ± 29.4
DPPC + DXR 60°C	116.1 ± 18.8	120.7 ± 21.0	160.7 ± 19.3	239.5 ± 69.2
DPPC-CHOL 37°C	127.8 ± 21.2	131.4 ± 23.3	167.5 ± 35.2	199.1 ± 59.7
DPPC-CHOL + DXR 37°C	137.8 ± 28.6	143.2 ± 23.5	181.1 ± 34.4	207.2 ± 72.6
DPPC-CHOL 60°C	127.1 ± 23.4	131.9 ± 21.6	147.5 ± 24.5	150.3 ± 30.0
DPPC-CHOL + DXR 60°C	140.0 ± 25.6	136.2 ± 1.5	138.5 ± 24.1	202.4 ± 72.0

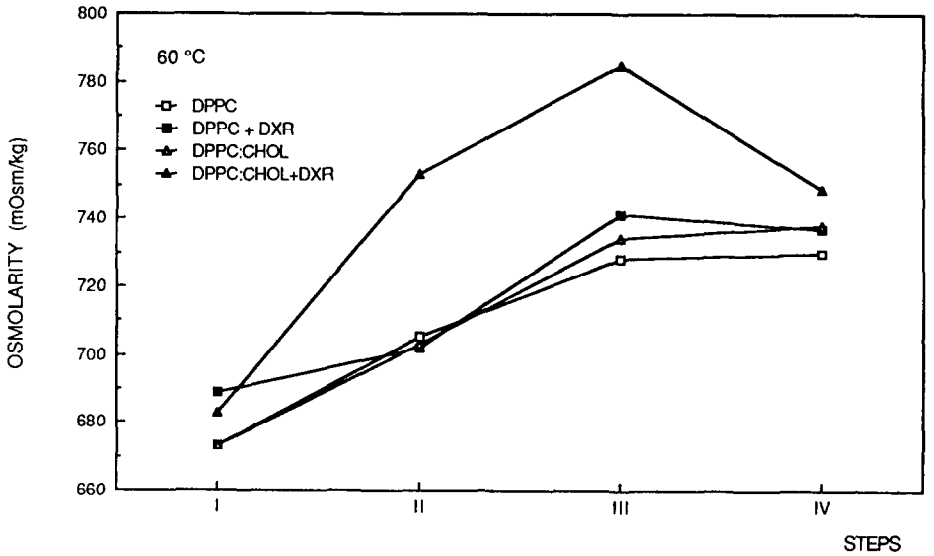

Fig. 2. Values of osmolarity at 60°C for DPPC and DPPC-CHOL liposomes in the absence and presence of DXR in the four steps explained in Table 2.

tions or slight shrinkage of liposomes should be expected. Thus, Fig. 2 reveals that osmotic pressures undergo a perceptible increase throughout 60 min incubation. It is interesting to observe that at the same step, the liposome sizes monitored show only a very small decrease at the end of incubation (III in Table 2). It is difficult to correlate these two results but if we deduce that mean diameter stabilizes between 4 and 6 min of incubation at both temperatures, then further increases in osmolarities (Fig. 2) could be attributed to variations due to modifications in tonicity. This would be due to partial leakage of DXR under stress temperature conditions. In

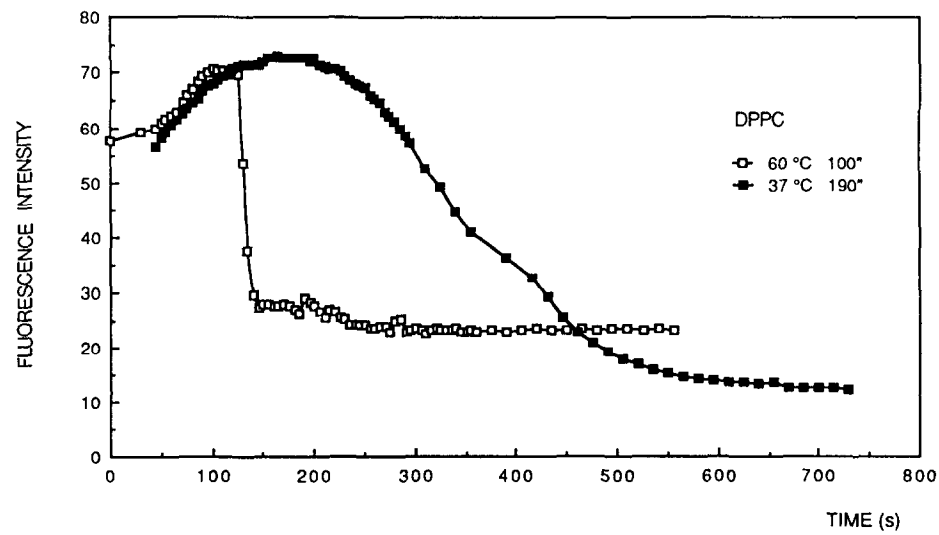

Fig. 3. Time dependence of the fluorescence intensity of DXR trapped in DPPC vesicles at 37 and 60°C. The time (in s) at which the maximum values of fluorescence intensity are reached is indicated.

agreement with this, we found that, after samples were cooled to room temperature, liposomes reached similar values of tonicity (range between 730 and 749 mOsmol/kg) (IV in Fig. 2), which matches with the widespread variations in size observed (IV in Table 2). It is not ruled out that the recooling process could lead to greater absolute sizes (Table 2) due to the presence of some interdigitated DXR which has been demonstrated to exert an ordering effect at the bilayer level. This finding is consistent with the observed expansion in monolayers of similar composition (Hernàndez et al., 1991) but the data on the two membrane models would not be directly correlated (Rand and Parsegian, 1984). However, all these results reinforce the earlier conclusion that during the loading process local variations in tonicity, lipid-drug interactions and lipid packing modifications are all involved in changes in liposome morphology.

However, since a minimum of 2.59 min were required to perform two consecutive PCS measurements, no information was available within this interval when samples were heated from 25 to 37 or 60°C. Therefore, we have exploited the intrinsic fluorescence of the drug to reveal information during shorter time intervals concerning the active trapping of DXR by liposomes. Figs 3 and 4 demonstrate the variations in fluorescence intensity with time at 37 and 60°C for DPPC and DPPC-CHOL liposomes, respectively. As can be seen, the fluorescence intensity for DPPC at 37 and 60°C and DPPC-CHOL at 60°C increases, reaches a maximum and then declines to lower intensities than the initial values. Kinetic curves also show that the process is faster at 60°C than at 37°C, the most rapid fall being observed on a straight line for DPPC liposomes at the highest temperature. Conversely, upon mixing of DXR and DPPC-CHOL vesicles at 37°C, the increase in fluorescence is not followed by a decay, and the intensity approaches a limiting value. The reason for this different behaviour should be related with the lipid composition itself and the transition temperature of the phospholipid. Thus, as Fig. 3 shows, for DPPC liposomes active encapsulation is faster above T_m than below T_m. On the other hand, while at 60°C DPPC-CHOL liposomes (Fig. 4) show similar behaviour, a different mechanism appears to be involved at 37°C. In this case, the lack of decay of fluorescence intensity could be explained as a consequence of a different kind of liposome-DXR binding. However, because encapsulation efficiencies were insensi-

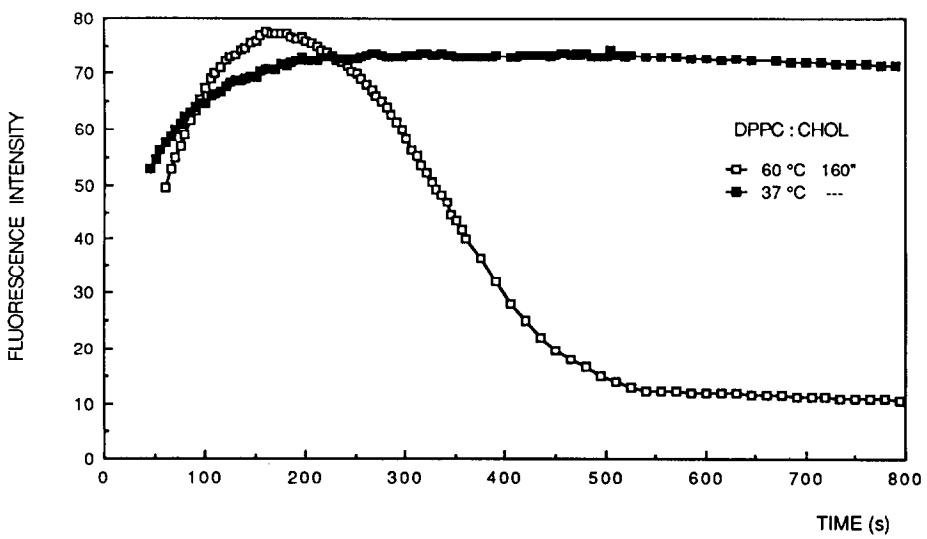

Fig. 4. Time dependence of the fluorescence intensity of DXR trapped in DPPC-CHOL vesicles at 37 and 60°C. The time (in s) at which the maximum values of fluorescence intensity are reached is indicated.

tive to lipid composition (Table 1), DXR may remain associated at the bilayer level when liposomes are loaded at 37°C.

The increase in fluorescence is consistent with earlier studies (Karczmar and Tritton, 1979), which showed that liposome-bound DXR has a higher fluorescence intensity than free DXR. On the other hand, this increase can be interpreted as a consequence of the release of fluorescence when the free drug was diluted with liposome solution and buffer (1:300, v/v) to obtain a correct fluorescence reading. It should be borne in mind that, at working concentrations (above 1×10^{-5} M), the free drug shows an inner filter effect by self-association (Bosanquet, 1986). Therefore, the increase observed in fluorescence should be explained by either one or a combination of both mechanisms. On the other hand, a further decrease observed in fluorescence intensity can be interpreted in terms of self-quenching of the drug by self-association as a consequence of concentration inside the vesicles, as can be demonstrated by fluorescence release after liposome solubilization. However, it cannot be excluded that other mechanisms of self-quenching by energy transfer may play a role in the resulting fluorescence decrease (Goren et al., 1990).

In summary, while from a pharmaceutical viewpoint, the active trapping procedure is a powerful method to encapsulate amphiphilic drugs, the biophysical aspects of drug loading remain unclear. These need to be resolved before the mechanism of encapsulation itself can be understood and, moreover, because the interaction between the drug and the phospholipids at the biophase level is essential for anthracyclin antitumor activity. Our results thus indicate the importance of the presence of sterol, temperature and physicochemical characteristics of the drug in the process of loading and drug-lipid association.

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