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Encapsulation of Mithramycin in Liposomes in Response to a Transmembrane Gradient of Calcium Ions

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Abstract. The recent discovery that mithramycin (MTR) in aqueous solution forms a high affinity $[Ca(MTR)_4]^{2-}$ complex led us to the idea that Ca^{2+}-loaded liposomes might be able to accumulate MTR in their aqueous internal compartment. We therefore investigated the uptake of MTR into large unilamellar vesicles (LUV) containing NaCl or $CaCl_2$. Our data show that MTR was efficiently accumulated within LUV made from dipalmitoylphosphatidylcholine and cholesterol, only when the liposomes contained Ca^{2+} and were resuspended in a Ca^{2+}-free medium. A drug encapsulation efficiency as high as 60% was achieved, at a drug to lipid molar ratio of 1/18. The circular dichroism and fluorescence excitation spectra of liposome-encapsulated MTR (LMTR) displayed strong similarities with those of the $[Ca(MTR)_4]^{2-}$ complex. LMTR was found to be stable, when submitted to conditions that destabilized the $[Ca(MTR)_4]^{2-}$ complex. Upon dilution and incubation for 24 h at 37 °C, MTR-containing liposomes did not release a significant amount of MTR. These properties were attributed to the formation of a high affinity complex between MTR and Ca^{2+} in the aqueous compartment of liposomes.

Key words: Mithramycin, calcium ion gradient, liposomes, encapsulation.

1. Introduction

Liposomes are self-assembling colloidal particles in which a lipid bilayer encapsulates a fraction of the surrounding aqueous medium. They have been used successfully as carriers for drugs and biological molecules *in vivo*. They have been shown to alter the pharmacokinetics and *in vivo* localization of encapsulated drugs and, in many cases, to reduce their side effects and/or enhance their efficacy [1].

A key step in the development of a liposomal drug formulation is the selection of an appropriate lipid composition and encapsulation procedure, mainly on the

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basis of the drug encapsulation efficiency, the retention of encapsulated drug, and the physical and chemical stabilities of liposomes. In conventional encapsulation protocols, drug encapsulation occurs passively during the formation of liposomes. Unfortunately, these often lead to low encapsulation efficiencies, especially in the case of water-soluble drugs. Furthermore, subsequent surface modifications [2] may lead to membrane disturbances and result in the partial release of liposome-encapsulated material. On the other hand, a recently introduced active loading method does not display these shortcomings [3–5]. By exploiting a pH gradient across the liposome membrane, it provides an extremely efficient drug encapsulation into preformed liposomes. This procedure, however, is restricted to small solutes with a pH-dependent change in hydrophobicity.

Mithramycin (MTR) is an anti-cancer agent used for the treatment of testicular cancer, but whose wider use is limited because of its undesired side effects [6, 7]. It is therefore a good candidate for liposome encapsulation.

We looked for an encapsulating method that could produce a high level of MTR incorporation within preformed liposomes. The recent discovery that MTR forms in aqueous solutions a high affinity $[Ca(MTR)_4]^{2-}$ complex [8] led us to the idea that Ca^{2+}-loaded liposomes might be able to accumulate MTR in their internal aqueous compartment. In this paper, we report the efficient and stable inclusion of MTR in liposomes submitted to a transmembrane gradient of Ca^{2+}.

2. Experimental

2.1. Drugs and chemicals

Mithramycin (MTR), L-α-dipalmitoylphosphatidylcholine (DPPC), cholesterol (CHOL), and calf thymus deoxyribonucleic acid (DNA) were purchased from Sigma Chemical Co. All other reagents were of the highest quality available, and deionized, double-distillated water was used throughout the experiments.

2.2. Liposome preparation and MTR encapsulation

Large unilamellar vesicles (LUV) were prepared according to the reverse-phase evaporation method [9], from DPPC and CHOL (1 : 0.75 molar ratio) using, as the aqueous phase, a Ca^{2+}-containing buffer (100 mmol/L $CaCl_2$, 20 mmol/L Hepes, pH 7.5) or a Ca^{2+}-free buffer (150 mmol/L NaCl, 20 mmol/L Hepes, pH 7.5). LUV were then extruded twice, successively through three Nucleopore polycarbonate filters of decreasing pore size (0.8 μm, 0.4 μm and 0.2 μm) in order to obtain a liposome population of relatively homogeneous size distribution [10, 11]. External calcium ions were eliminated by running the liposome suspension through a Sephadex G-50 column equilibrated with Buffer A. Buffer A was prepared as 130 mmol/L NaCl, 50 mmol/L Hepes at pH 7.5, and was finally treated with chelex 100 (Bio-Rad Co.), a chelating ion exchange resin that displays a strong affinity for calcium ions. In a final step, the liposome suspension was treated twice with chelex

100 resin, in order to ensure a low extraliposomal Ca^{2+} concentration. At this stage, the DPPC concentration was determined in the liposome suspension according to Ref. [12].

Encapsulation of MTR in liposomes was performed by adding MTR (from a frozen 2 mmol/L MTR stock solution prepared with Buffer A) to the liposome suspension previously diluted in Buffer A at the appropriate lipid concentration, in the absence or in the presence of 10 mmol/L $CaCl_2$. The final MTR concentration was 0.5 mmol/L. The MTR-liposome mixture was then incubated for 5 min at 60 °C (unless otherwise stated). Liposome-encapsulated MTR (LMTR) was separated from unencapsulated drug by ultracentrifugation at 100 000$\times g$ for 1 hour at 25 °C.

2.3. Characterization of liposome-encapsulated MTR

After ultracentrifugation, LMTR was quantified by measuring MTR visible absorption on a Cary 219 spectrophotometer, either of the supernatant (using ϵ_{400} = 10 000 L $mol^{-1}cm^{-1}$) or of the liposome pellet previously dissolved in methanol (using ϵ_{410} = 10800 L $mol^{-1}cm^{-1}$). Both methods gave comparable results within experimental error (±5%). Two parameters were further determined: the percentage of entrapped MTR and the trapped MTR to DPPC molar ratio.

In the following experiments, the behaviour of LMTR was compared to that of MTR in the form of the $[Ca(MTR)_4]^{2-}$ complex [8]. For this purpose, a solution of $[Ca(MTR)_4]^{2-}$ was prepared by diluting MTR stock solution in Buffer A containing 10 mmol/L $CaCl_2$ (final MTR concentration of 0.5 mmol/L), and submitting the resulting solution to the same incubation step used for the preparation of LMTR (5 min at 60 °C).

The physicochemical state of LMTR (prepared at a MTR : DPPC molar ratio of 1/27) was assessed by fluorescence spectroscopy, using a Perkin Elmer spectrofluorometer model LS 50B (excitation and emission slits of 10 nm) and by circular dichroism spectroscopy, using a Jobin Yvon Mark V dichrograph. The liposome pellet was diluted to a final MTR concentration of 2 μmol/L in the buffer: 130 mmol/L NaCl, 20 mmol/L Hepes, 20 mmol/L citric acid, pH 7.5. Fluorescence excitation (emission at 540 nm) and circular dichroism (CD) spectra were recorded. The CD spectrum of LMTR was subtracted from that of a suspension of MTR-free liposomes (at the same final lipid concentration). Results are expressed in terms of $\Delta\epsilon = \epsilon L - \epsilon R$ (molar CD coefficient). The values of $\Delta\epsilon$ are expressed in terms of [MTR], the molar concentration of MTR.

2.4. Stability of the MTR-Ca^{2+}-liposome complex

LMTR (prepared at a MTR to DPPC molar ratio of 1/27) was submitted to three potentially destabilizing conditions. Firstly, LMTR (and $[Ca(MTR)_4]^{2-}$ complex as a control) was diluted in Buffer B (130 mmol/L NaCl, 20 mmol/L Hepes, 20 mmol/L citric acid, 1 mmol/L $CaCl_2$, pH 7.5) to a final MTR concentration of 2 μmol/L.

The pH was then lowered to 5 by the addition of an aliquot of 2 mol/L HCl. This procedure is known to induce the transformation of the $[Ca(MTR)_4]^{2-}$ complex into non-fluorescent aggregates of protonated MTR and free Ca^{2+}[8,13]. The extent of dissociation of the $[Ca(MTR)_4]^{2-}$ complex was then assessed by recording the fluorescence quenching (excitation wavelength = 440 nm, emission wavelength = 540 nm) induced by acidification. Secondly, LMTR was diluted in Buffer B containing $MgCl_2$ (8 mmol/L) and DNA (base pair concentration = 30 μmol/L) to a final MTR concentration of 2 μmol/L. These experimental conditions are known to induce the dissociation of the $[Ca(MTR)_4]^{2-}$ complex, in favor of a ternary MTR–Mg–DNA complex which exhibits differential spectral characteristics [13]. This transformation was then assessed by recording the fluorescence excitation spectrum of the diluted preparation and comparing it to that of the preparation diluted in Buffer B. Thirdly, LMTR was diluted in Buffer B to a final MTR concentration of 2 μmol/L, and incubated for 24 h at 37 °C. Under these conditions, a dilution-induced drug release was expected to occur, because of the increased concentration gradient of MTR across the liposome membrane. The extent of released MTR was then evaluated by lowering the pH of the diluted preparation to 5 and recording the drop in fluorescence intensity. Considering that the intraliposomal pH is only slightly modified when the external pH is strongly altered [14], the extent of fluorescence quenching is directly related to the concentration of external MTR.

2.5. Calculation of the capture volume of liposomes

The calculation of the expected capture volume of the resulting liposomes, based on the liposome diameter following extrusion and on the total surface area of the lipid, can be made assuming that the liposomes are unilamellar [9] and form a uniform population of spherical vesicles [10, 11].

The internal volume of a liposome (V_{L}) can be expressed as a function of its radius (R):

$$V_{\mathrm{L}} = (4/3)\pi R^3 \tag{1}$$

The surface area of a liposome (S_{L}) can be expressed as:

$$S_{\mathrm{L}} = 4\pi R^2 = 0.5 N n_{\mathrm{DPPC}}(A_{\mathrm{DPPC}} + 0.75 A_{\mathrm{CHOL}}) \tag{2}$$

where N is Avogadro's number (6.022×10^{23}), n_{DPPC} is the number of moles of DPPC per liposome, and A_{DPPC} and A_{CHOL} represent the area per polar head group for DPPC and CHOL, respectively.

From Equations 1 and 2, we can determine the capture volume of liposomes per mole of DPPC (V):

$$V = V_{\mathrm{L}}/n_{\mathrm{DPPC}} = RN(A_{\mathrm{DPPC}} + 0.75 A_{\mathrm{CHOL}})/6 \tag{3}$$

Values for R, A_{DPPC} and A_{CHOL} were obtained from the literature: R = 130 nm [10, 11], A_{DPPC} = 0.41 nm^2 [15], A_{CHOL} = 0.28 nm^2 [10]. Using these values, we used Equation (3) to calculate the capture volume of liposomes per mol of DPPC:

$$V = 8.1\mathrm{L/mol}$$

It is noteworthy that the calculated value is in good agreement with values determined experimentally for liposomes prepared by the same method as that used in this work [11].

3. Results and Discussion

3.1. Encapsulation efficiency of MTR in $CaCl_2$- and NaCl-containing liposomes

Table I displays the efficiencies of MTR encapsulation within $CaCl_2$- and NaCl-containing liposomes, respectively, for a DPPC concentration of 8 mmol/L and a MTR : DPPC molar ratio of 1/16. $CaCl_2$- and NaCl-containing liposomes incorporated respectively 61% and 7% of total MTR, when calcium ions were removed from the extraliposomal medium. On the other hand, when calcium ions were added to the external medium at a final concentration of 10 mmol/L, the percentages of entrapped MTR were lower than 10% for both liposome preparations. The present data demonstrate that, in order to efficiently encapsulate MTR in liposomes, at least two conditions must be fulfilled: calcium ions must be present in the internal compartment of liposomes and calcium ions must be removed from the external medium. The fact that MTR accumulated to a much higher extent in $CaCl_2$-containing liposomes than in NaCl- containing ones may be attributed to the formation of the high affinity $[Ca(MTR)_4]^{2-}$ complex [8] in the internal compartment of the $CaCl_2$- containing liposomes. The formation of this complex, in the external medium, may prevent MTR from crossing the membrane and, in the internal compartment of liposomes, may permit MTR accumulation by maintaining a high concentration gradient of free MTR across the liposome membrane. A similar mechanism of drug accumulation was previously described for anthracycline antibiotics. These drugs were shown to strongly accumulate into DNA-containing liposomes because of their high affinity binding to DNA [16].

The influence of the incubation temperature of the MTR-liposome mixture on the uptake of MTR in liposomes was also investigated. In order to illustrate the temperature-dependence of the rate of MTR incorporation, we determined at different temperatures the necessary incubation time to incorporate 20% of MTR ($t_{20\%}$). The values obtained for $t_{20\%}$ were 1 min at 60 °C, 12 min at 50 °C, 110 min at 40 °C, 20 h at 30 °C, and more than 72 h at 20 °C. Such effect of temperature on the rate of MTR incorporation most probably arises from the high temperature dependence of the membrane permeability to MTR. It is noteworthy that the experimental conditions chosen for encapsulating MTR in liposomes (an

Table I. Entrapment percentages of MTR in $CaCl_2$-containing and NaCl-containing liposomes, in the presence (+Ca) and absence (−Ca) of external $CaCl_2$ (10 mmol/L). Conditions of encapsulation: MTR concentration = 0.5 mmol/L, DPPC concentration = 8 mmol/L, incubation of the MTR-liposome mixture for 5 min at 60 °C.

Liposome preparation	% Entrapped MTR
$CaCl_2$-containing liposomes (−Ca)	61%
$CaCl_2$-containing liposomes (+Ca)	5%
NaCl-containing liposomes (−Ca)	7%
NaCl-containing liposomes (+Ca)	7%

incubation for 5 min at 60 °C) were sufficient to reach the steady-state of MTR incorporation.

As an attempt to further improve the encapsulation efficiency of MTR in $CaCl_2$-containing liposomes, we investigated the influence of the phopholipid concentration upon the drug encapsulation efficiency. The results of this study are presented in Figure 1. The percentage of entrapped MTR increased from 27 to 60%, when the DPPC concentration was raised from 1.2 to 5 mmol/L. However, for higher DPPC concentrations (5–11 mmol/L), its value remained in the range of 55 to 61%. Figure 1 also shows that the trapped MTR to DPPC molar ratio decreased from 1/9 to 1/37, when the DPPC concentration was increased from 1.2 to 11 mmol/L. It was also possible to estimate the trapped MTR to Ca^{2+} molar ratio ($R_{\mathrm{MTR/Ca}}$), from the trapped MTR to DPPC molar ratio ($R_{\mathrm{MTR/DPPC}}$), the intraliposomal Ca^{2+} concentration (C_{i} = 0.1 mol/L), and the capture volume of the liposomes (V = 8.1 L/mol of DPPC, calculated as described in Experimental):

$$R_{\mathrm{MTR/Ca}} = R_{\mathrm{MTR/DPPC}}/VC_{\mathrm{i}} \tag{4}$$

Calculation of $R_{\mathrm{MTR/Ca}}$ from Equation 4 showed that the trapped MTR : Ca^{2+} molar ratio decreased from 0.137 to 0.033, when the DPPC concentration was increased from 1.2 to 11 mmol/L. It is noteworthy that the values obtained for $R_{\mathrm{MTR/Ca}}$, being less than 4, are consistent with our hypothesis of the formation of a $[Ca(MTR)_4]^{2-}$ complex.

These data also indicate that the ability of $CaCl_2$-containing liposomes to accumulate MTR is reduced at high phospholipid concentrations. A possible explanation of this phenomenon may be that the concentration of calcium ions remaining in the extraliposomal medium increased with phospholipid concentration. These residual calcium ions may have come from membrane-adsorbed Ca^{2+} that were not removed during the treatment with chelex 100 resin (see Experimental Section), or from encapsulated Ca^{2+} that were released from liposomes during the incubation step at 60 °C.

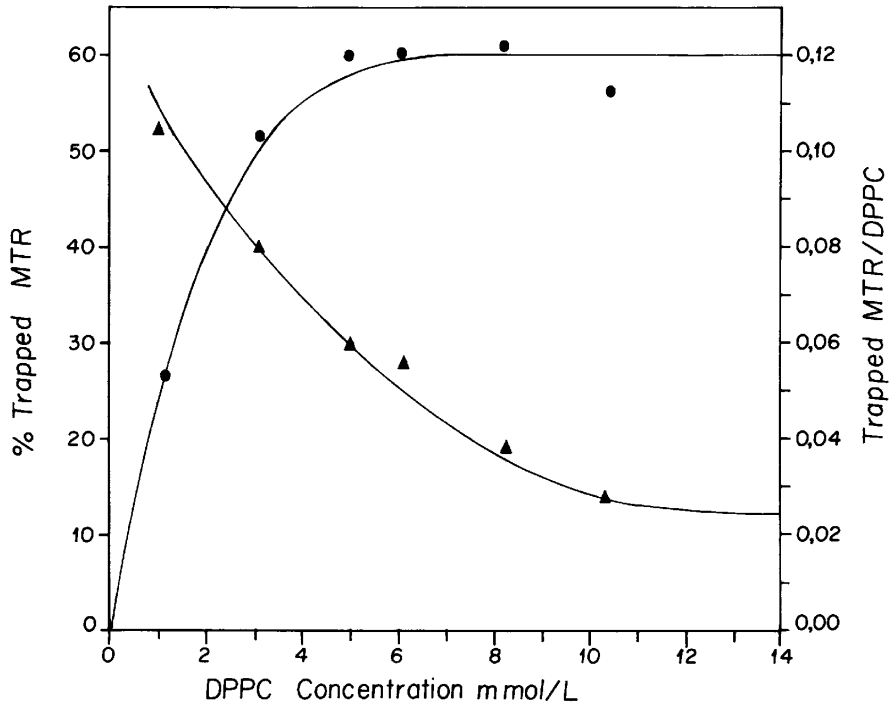

Figure 1. Influence of phospholipid concentration on the percentage of MTR entrapped within $CaCl_2$-containing liposomes (●, left), and on the trapped MTR to DPPC molar ratio (▲, right). Conditions of encapsulation: MTR concentration = 0.5 mmol/L, incubation of the MTR-liposome mixture for 5 min at 60 °C.

3.2. CHARACTERIZATION OF LIPOSOME-ENCAPSULATED MTR

The data presented above have demonstrated the selective inclusion of MTR in $CaCl_2$-containing liposomes. This second set of experiments was aimed to further characterize LMTR by fluorescence and CD spectroscopies. The CD and fluorescence excitation spectra of LMTR solution (Figures 2 and 3, respectively) were compared to those of a $[Ca(MTR)_4]^{2-}$ solution. The fluorescence excitation spectrum of LMTR was found to be very similar in shape to that of the $[Ca(MTR)_4]^{2-}$ complex, although its intensity was about 1.4-fold higher. More strikingly, the CD spectrum of LMTR displayed a strong couplet-type signal centered at 280 nm, that was previously shown to be characteristic of the $[Ca(MTR)_4]^{2-}$ complex [8]. The strong spectral similarities between LMTR and MTR in the form of the $[Ca(MTR)_4]^{2-}$ complex suggest that LMTR exists in the form of a $[Ca(MTR)_4]^{2-}$ complex. The few spectral discrepancies may be attributed to the fact that MTR was much more concentrated inside the liposome aqueous compartment than in the $[Ca(MTR)_4]^{2-}$ solution used for comparison.

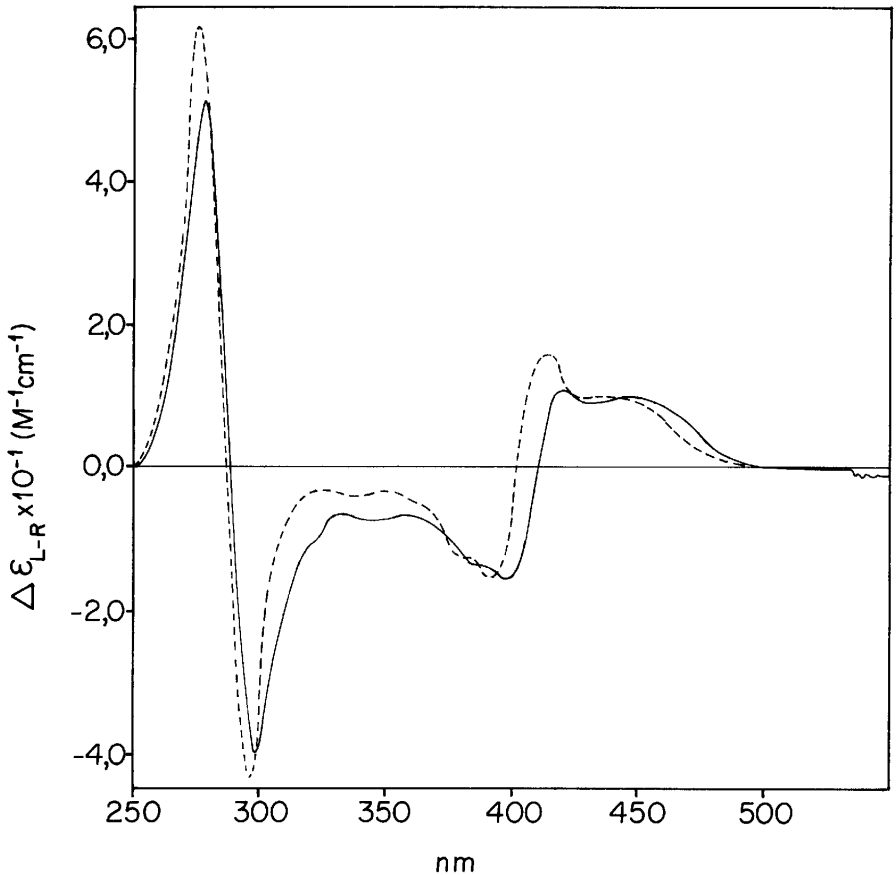

Figure 2. CD spectra of LMTR (solid line) and $[Ca(MTR)_4]^{2-}$ complex (dotted line). MTR concentration = 2 μmol/L.

3.3. STABILITY OF MTR ENCAPSULATION IN $CaCl_2$-CONTAINING LIPOSOMES

LMTR was submitted to two different conditions, that were previously shown to destabilize the $[Ca(MTR)_4]^{2-}$ complex.

First, LMTR and MTR in the form of the $[Ca(MTR)_4]^{2-}$ complex (as a control) were diluted to a final concentration of 2 μmol/L in a pH 7.5 buffer and the pH was then lowered to 5. Figure 4 displays the effect of the pH drop on the fluorescence intensity of the resulting solutions. The strong fluorescence quenching (68% of original fluorescence) in the case of the $[Ca(MTR)_4]^{2-}$ complex can be attributed to its dissociation in favor of non-fluorescent aggregates of protonated MTR [8, 13]. In the case of LMTR, a fluorescence quenching of only 5% was recorded. These data indicate that LMTR, in contrast to the free $[Ca(MTR)_4]^{2-}$ complex in solution, underwent only minor physicochemical changes when the pH was varied from 7.5 to 5.

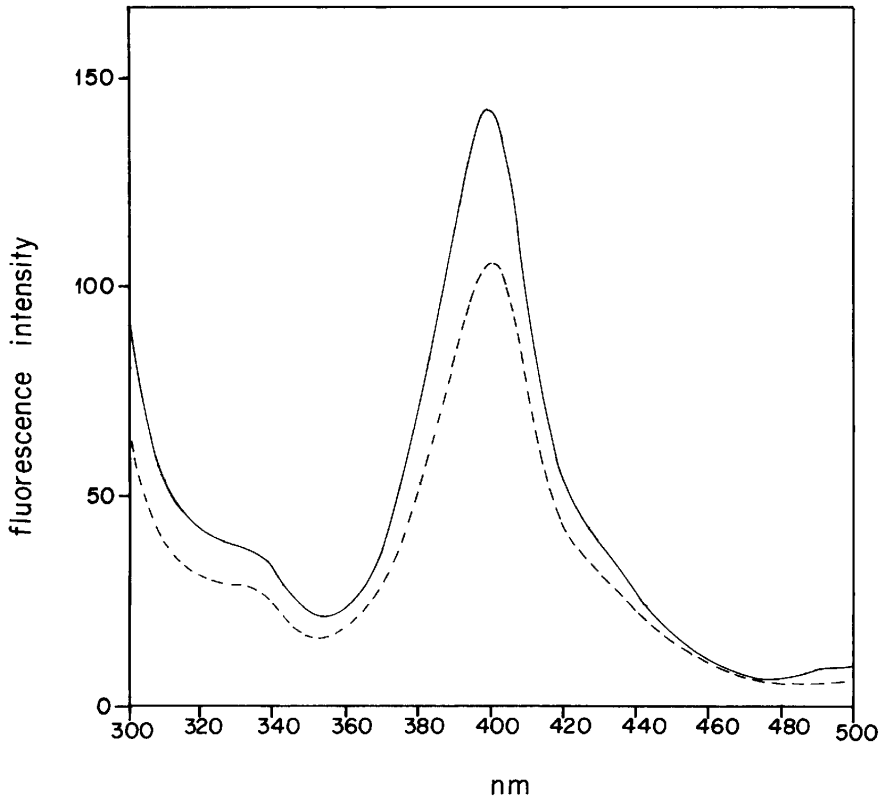

Figure 3. Fluorescence excitation spectra (emission wavelength = 540 nm) of LMTR (solid line) and the $[Ca(MTR)_4]^{2-}$ complex (dotted line). MTR concentration = 2 μmol/L.

In a second experiment, LMTR, as well as MTR in the form of the $[Ca(MTR)_4]^{2-}$ complex, were diluted in a pH 7.5 buffer containing Mg^{2+} and DNA to a final MTR concentration of 2 μmol/L. These experimental conditions are known to induce the complete dissociation of the $[Ca(MTR)_4]^{2-}$ complex in favor of a ternary MTR–Mg–DNA complex which exhibits differential spectral characteristics [13,17]. Figure 5 displays the fluorescence excitation spectra of MTR in the absence and in the presence of Mg^{2+} and DNA. As expected, the passage of the $[Ca(MTR)_4]^{2-}$ complex from a Mg^{2+}/DNA- free buffer to a buffer containing Mg^{2+} and DNA was accompanied by a strong red shift of the maximum fluorescence intensity from 400 nm to 440 nm. On the other hand, in the case of LMTR, only a slight quenching of the fluorescence signal was observed, with no apparent shift of the spectrum (data not shown). These data indicate that LMTR, in contrast to the free $[Ca(MTR)_4]^{2-}$ complex in solution, did not dissociate to interact with DNA.

The observation that LMTR and $[Ca(MTR)_4]^{2-}$ exhibited markedly different physicochemical behaviours with respect to their response to acidification and the

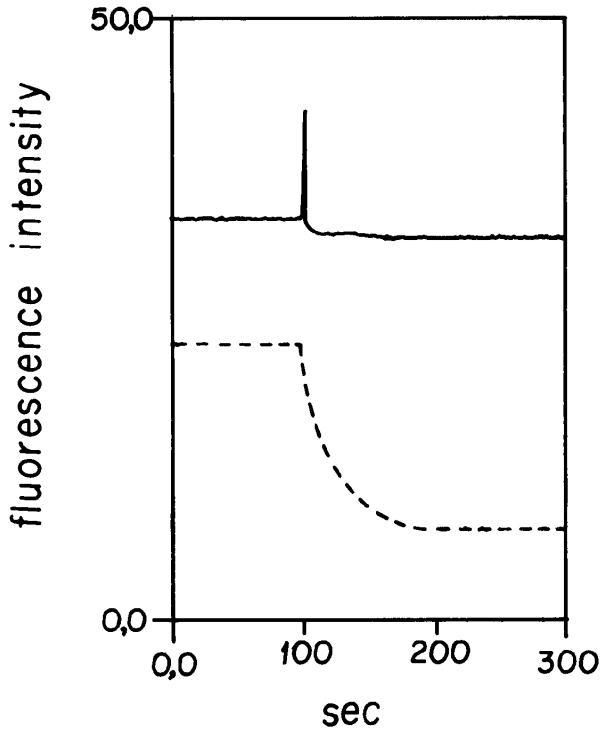

Figure 4. Effect of a pH drop from 7.5 to 5 on the fluorescence intensity (excitation at 440 nm, emission at 540 nm) of LMTR (solid line), and MTR initially in the form of a $[Ca(MTR)_4]^{2-}$ complex (dotted line). MTR concentration = 2 μmol/L.

presence of DNA may seem, at first sight, to contradict our previous hypothesis that LMTR exists in the form of a $[Ca(MTR)_4]^{2-}$ complex. However, there is no contradiction, if we consider that LMTR exists in the form of a $[Ca(MTR)_4]^{2-}$ complex in the aqueous compartment of liposomes, where it is protected from variations of the external pH [14] and interaction with non-permeant species like DNA and Mg^{2+} present in the extraliposomal medium.

As a last experiment, we investigated the dilution-induced drug release, which is expected to occur following the intravenous administration of the liposome preparation. Therefore, LMTR was diluted in a pH 7.5 buffer to a final MTR concentration of 2 μmol/L, and incubated for 24 h at 37 °C. The extent of MTR release from liposomes was then evaluated by lowering to 5 the pH of the diluted preparation and recording the drop in fluorescence intensity (as described previously). A slight quenching occurred when the pH was lowered from 7.5 to 5. The quenching level was similar to that observed with the freshly prepared LMTR (see Figure 4). These data indicate that the amount of drug released during the 24 h incubation period at 37 °C was not significant.

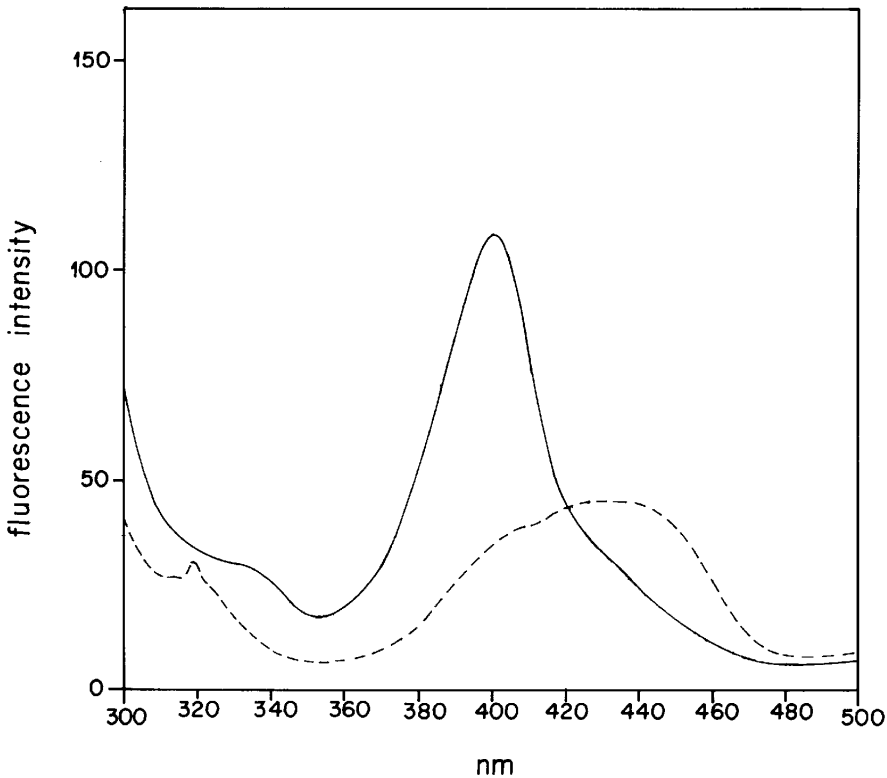

Figure 5. Fluorescence excitation spectra (emission wavelength = 540 nm) of MTR, initially in the form of a $[Ca(MTR)_4]^{2-}$ complex, in the absence (solid line) and presence (dotted line) of Mg^{2+} and DNA. MTR concentration = 2 μmol/L.

4. Conclusions

In this work we have developed a novel procedure for the efficient inclusion in preformed liposomes of a drug presenting strong metal-chelating properties. The method produced a very stable liposome–Ca^{2+}–MTR complex and should be applicable to other drug-metal systems.

Acknowledgements

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