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Thermotropic Phase Behavior of Liposome Entrapped 5-FU and LCV

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The aim of this work was to encapsulate two drugs: 5-Fluorouracil (5-FU) with the hydrophobic properties and 5-Formyl-5,6,7,8-tetrahydropteroyl-L-glutamic acid calcium salt (leucovorin) with the hydrophilic properties into liposomes prepared by the modified reverse-phase evaporation method (mREV) from L-α-phosphatidylcholine dipalmitoyl (DPPC). The drugs are used in the anticancer multidrug therapy FLv (5-fluorouracil, leucovorin) and ELF (etoposide, leucovorin, 5-fluorouracil). We studied the competition for their encapsulation in liposomes by the use of two spectroscopies: ^1H NMR and UV on the basis of the analysis of the signals of each drug in the liposome – drug system. Liposomes are highly versatile structures for research, therapeutic and analytical applications. We concluded that the liposomes obtained by the mREV method may transport more than one drug simultaneously.

Keywords Liposome; mREV; multidrug therapy; NMR; UV

Introduction

Liposomes are highly versatile structures for research, therapeutic and analytical applications. They are composed of a lipid bilayer with the hydrophobic chains of the lipids forming the bilayer and the polar headgroups of the lipids oriented towards the extravesicular solution and inner cavity. Their structure is similar to that of cells, and thus can be used as more easily characterized vessel for studying interactions between membrane lipids and biomolecules such as DNA [1,2] and proteins [3], permeability of ions [4] and drugs [5,6].

When liposomes are used as drug carriers, their size is of major importance, since this influences the behavior of liposomes in biological systems [7]. In order to extravasate into the disease site, the liposomes should be smaller than 120 nm. This requires very efficient loading, otherwise, either therapeutic levels of drug cannot be reached or

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a very large amount of lipids is needed to obtain these levels. Furthermore, inefficient loading will lead to a great loss of the active agent during loading and complicate the preparation procedure by making it difficult to remove the free agent [8].

As liposomes are usually made of phospholipids which are susceptible to light, heat and oxygen, it is usually not acceptable to sterilize the products for parenteral use by autoclaving. Furthermore, when liposomes exist in aqueous solutions, they may be subject to a series of adverse effects such as aggregation, fusion, phospholipid hydrolysis, and drug leakage, which lead to a short-shelf life [9].

For liposomes used in analytical and bioanalytical applications, the main characteristics include the encapsulation efficiency, the ratio of phospholipids to encapsulant concentration, the temperature of phase transition (T_c).

When lipid bilayers prepared from pure one-component phospholipids are subjected to heating under the ambient pressure condition, these lipid bilayers often undergo multiple thermotropic phase transitions. These transitions may be detected by a wide variety of physical techniques such as DSC, dilatometry, X-ray diffraction, neutron diffraction, dynamic light scattering, NMR [10].

Materials and Methodology

Chemicals

L-α-phosphatidylcholine dipalmitoyl (1,2-dihexadecanoyl-*sn*-glycerol-3-phosphocholine) 99% (DPPC), 2,4-dihydroxy-5-fluoropyrimidine (5-FU), leucovorin 5-Formyl-5,6,7,8-tetrahydropteroyl-L-glutamic acid calcium salt (LCV) were purchased from Sigma-Aldrich Chemical Co., chloroform, dichloromethane, hydrochloric acid, buffer PBS: K_2HPO_4, NaH_2PO_4 from POCH, Gliwice, Poland. D_2O 99%, chloroform-d 99%, stab. with Ag, were purchased from ARMAR Chemicals, Switzerland.

Liposome and Liposome/Drug Preparation

We obtained small liposomes (DPPC; DPPC/5-FU; DPPC/LCV; DPPC/LCV/5-FU) by the modified reverse-phase evaporation method (mREV) [11,12]. PBS buffer pH 7.4 was applied.

For NMR and UV study $5 * 10^{-3}$ M 5-FU in DMSO and $5 * 10^{-3}$ M LCV in D_20 instead of the solutions of these drugs in the buffer were added to the preparation mixture. The preparation was carried out at 317 K. Liposome entrapped 5-FU and LCV were separated from free 5-FU and free LCV by dialysis through Servapor dialysis tubing against several changes of buffer at 277 K.

NMR Analysis

All spectra were obtained on 9.4 Tesla Bruker Avance UltraShield using a 5-mm broad band inverse probe. NMR spectra were recorded at the temperature range 298–320 K. Temperature of the studied samples was controlled by air and monitored by Bruker thermal control system. The samples were heated at a rate of about 1 K/min up and were left at this temperature for approximately 20 min to attain the equilibrium condition which was monitored by the invariability of the free induction decay (FID) signal. The temperature was maintained at ±0.1 K. For water suppression, the presaturation method was used. Spectra processing were performed with TOPSPIN 2.1 Bruker software. Apparatus error was ±0.0001 ppm.

UV Analysis

UV spectra of all prepared liposomes DPPC, DPPC/5-FU, DPPC/LCV, DPPC/LCV/5-FU and drugs 5-FU and LCV were obtained on spectrophotometer LAMBDA BIO 40 PERKIN ELMER using the quartz cells 1.0 cm × 0.5 cm × 4.0 cm. The investigations were carried out in temperature range 298–320 K. Apparatus error was ±1nm and ±0.00001 for the wavelength λ and for the absorbance A, respectively. The spectra were recorded in the range from 200 nm to 400 nm.

To determine the concentration of the drugs incorporated into liposome vesicles the three steps dialysis in PBS buffer were done. Each dialysis went on 60 min. at 277 K in dialysis sack Servapor with pore diameter 2.5 nm.

Results and Discussion

The objective of this work was to incorporate the drugs with hydrophilic (LCV) and hydrophobic (5-FU) properties into liposome vesicles, prepared by the modified reverse-phase evaporation method (mREV). We tried to estimate the competition between these drugs for encapsulation in liposome vesicles, efficiency of drug encapsulation and to estimate the temperature phase transition of phospholipids. Two spectroscopic methods: proton nuclear magnetic resonance (^{1}H NMR) and UV were used. Both spectroscopic methods allowed us to investigate the formation of liposome/drug systems by the analysis of the signals assigned to each drug. Temperature of phase transition was determined by using of NMR spectroscopy.

Nuclear Magnetic Resonance Study

In this study, NMR technique is described that can be used to estimate the phase transition temperature (T_c) of phospholipid. Figure 1 shows the high-resolution ^{1}H NMR spectra of liposome obtained from: (A) DPPC; (B) DPPC/5/FU; (C) DPPC/LCV; (D) DPPC/LCV/5-FU.

The estimation of the temperature T_c value uses the sequence of spectra for each thermally equilibrated system as shown in Figure 1.

Figure 2 shows that increase of temperature leads to a slight increase of area of the signal of protons of methyl groups of ammonium of phospholipids $-N^+(CH_3)_3$.

Derivative of NMR peak area was determined from the signals (Fig. 1). The peak maximum (Fig. 2) shows the phase transition temperature (T_c).

T_c obtained with this method for: DPPC; DPPC/5-FU; DPPC/LCV; DPPC/LCV/5-FU is 314.95 K, 315.42 K, 314.98 K, 315.24 K respectively.

The determination of the temperature T_c with the use of NMR is not as convenient as with DSC but allows for determination of the main phase transition of phospholipid. This method made possible the study of the effect of the encapsulation of drug to liposome on the phase transition temperature.

UV Study

The vesicle size and number of bilayers influence drug encapsulation in the liposomes and has been shown to be an important factor in the efficient delivery of an antitumor agent to a tumor. Our previous study allowed us to obtain liposomes of diameter <100 nm and of regular spherical structure [13]. In this study the effect of liposome encapsulation is discussed.

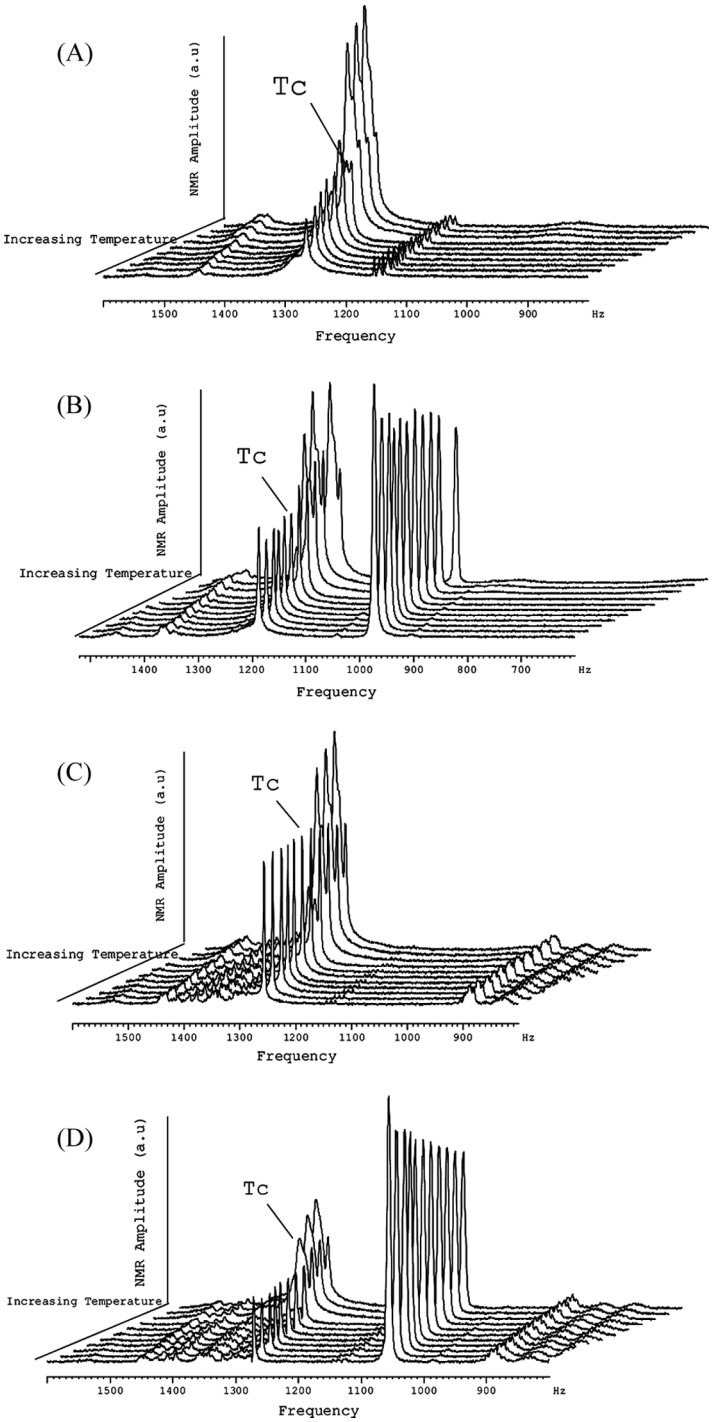

Figure 1. ^{1}NMR spectra presenting the thermal transitions in liposomes obtained from (A) DPPC; (B) DPPC/5-FU; (C) DPPC/LCV; and (D) DPPC/LCV/5-FU. Variation of the NMR signal of $-N^{+}(CH_3)_3$ with increasing temperatures.

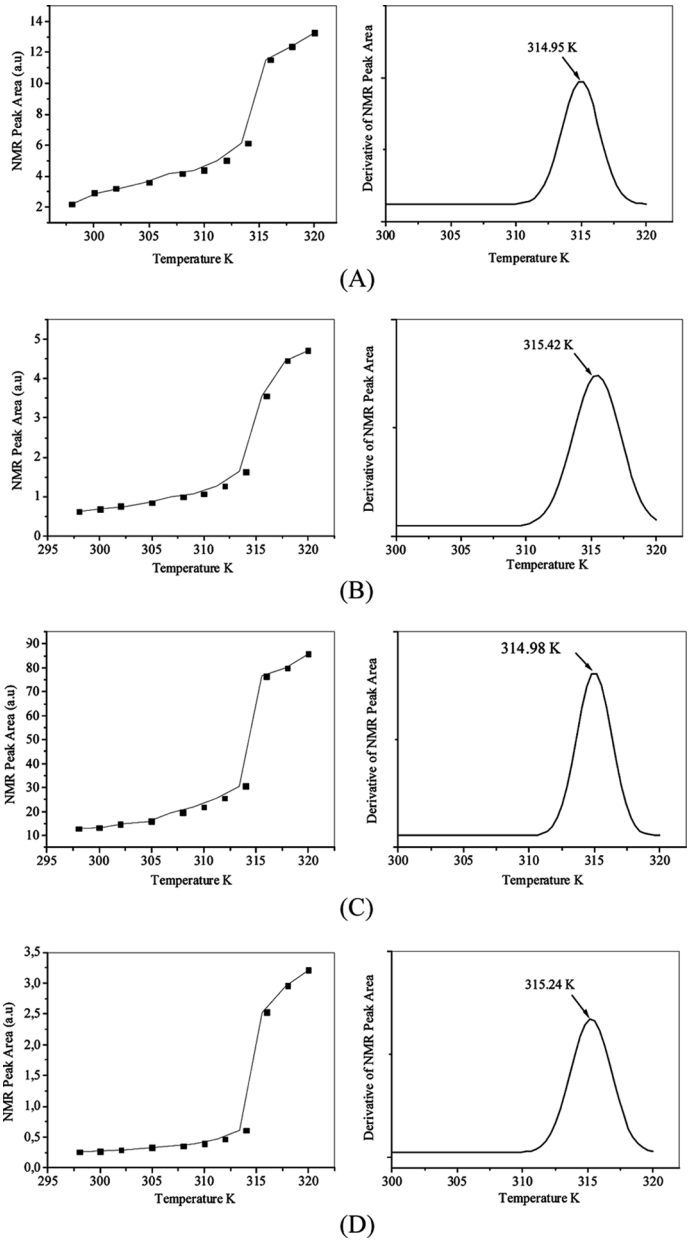

Figure 2. Variation of the area of the peak assigned to $-N^+(CH_3)_3$ protons of liposomes obtained from (A) DPPC; (B) DPPC/5-FU; (C) DPPC/LCV; (D) DPPC/LCV/5-FU. Determination of T_c.

The degree of the incorporation of drug into liposome vesicles DPPC/LCV DPPC/5-FU, DPPC/LCV/5-FU defined as a concentration of LCV and 5-FU in phospholipide carriers, is estimated using UV spectroscopy after dialysis.

We compared UV spectra of LCV and 5-FU incorporated together into the liposome vesicles with that of each of those drugs incorporated separately in the

temperature range 298–320 K. Temperature has no effect on the UV spectra of LCV and 5-FU. However temperature affects the spectra of LCV and 5-FU incorporated into liposome vesicles.

The encapsulation efficiency, i.e., the percentage of the total compound entrapped within the liposome, is an important parameter in liposomal characterization. It amounts 3.75×10^{-4} M and 4.37×10^{-4} M for 5-FU and LCV, respectively. In the liposome vesicles DPPC/5-FU, % of encapsulation 5-FU amounts 99.17%. In the liposomes vesicles DPPC/LCV, % of encapsulation LCV amounts 91%. DPPC/LCV/5-FU liposome containing both drugs encapsulates 89.00% of 5-FU and 60.96% of LCV.

One can conclude that there is only a slight influence of LCV on the 5-FU encapsulation in liposome vesicle while 5-fluorouracil affects significantly the LCV content in the liposome vesicles.

Conclusions

Liposomes are vesicle-like structures basically constituted of phospholipids organised as concentrical bilayers containing an aqueous compartment in their interior. Due to their amphipatic characteristics, they can incorporate substances in the aqueous compartment, the lipidic bilayer, or even distributed in both compartments. Considering this particularity, they have been recognised for their great potential as drug delivery systems [14].

Liposomes prepared by the modified reverse-phase evaporation method (mREV) have proven to be excellent drug carriers, demonstrating an ability to decrease the toxicity of many cytotoxic drugs while retaining or augmenting their therapeutic efficacy.

We conclude that the stability of liposomes depends not only on the method of chemical gradient loading, the use of membrane stabilizer such as sterols but also on the phase transition temperature (T_c) of phospholipids, which undergoes an alteration after encapsulation of drugs to liposomes. The encapsulation of LCV to DPPC liposomes does not affect the T_c value in comparison with the reference liposomes formed from DPPC. Phase transition temperature T_c increases however for the liposomes containing 5-FU. The analyse of competition between 5-FU and LCV shows that 5-FU incorporates in higher degree to liposome than LCV and affect the T_c value of phospholipid forming liposomal membrane.

One can conclude that the used spectroscopic techniques are useful for an estimation the temperature phase transition (T_c) of phospholipid and the % of encapsulation of drugs in liposomes. The use of NMR and UV spectroscopy in the *in vitro* investigations of competitive drugs incorporation and their transport into liposome vesicles can be the basis for the analysis of changes which take place in the *in vivo* conditions.

Acknowledgments

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