

# Influence of some physical properties of 5-fluorouracil on encapsulation efficiency in liposomes

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**Abstract** The aim of this study is to encapsulate two drugs: 5-fluorouracil (5-FU) with the hydrophobic properties and 1- $\beta$ -D-arabinofuranosylcytosine (Ara-C) with the amphiphilic properties into liposomes prepared by the modified reverse-phase evaporation method (mREV) from L- $\alpha$ -phosphatidylcholine dipalmitoyl (DPPC). We studied the thermotropic phase behavior of liposome entrapped 5-FU and Ara-C. It is known 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 competition of these two drugs entrapped in liposomes was analyzed by the use of two spectroscopies:  $^1\text{H}$  NMR and UV on the basis of the analysis of the signals of each drug in the liposome—drug system. The percent of encapsulation in DPPC/Ara-C/5-FU liposome obtained by the use of UV spectroscopy amounted 93.84 and 96.05% for 5-FU and Ara-C, respectively. Phase transition temperature  $T_c$  of liposomes containing Ara-C did not significantly change while for the liposomes containing 5-FU it increased in comparison with  $T_c$  of the reference liposomes formed from DPPC.

**Keywords** Liposome · Multidrug therapy · mREV · NMR · UV

## Introduction

Liposomes, vesicles enclosing an aqueous solution with membranes of phospholipids, have been extensively studied as microparticulate carriers for the efficient delivery of therapeutic agents since the first report by Bangham et al. [1]. The superiority of liposomes as drug carriers is now widely recognized and great advances in the liposome field have resulted in the development of some approved liposomal products [2]. However, it is still not easy to prepare acceptable aseptic liposomal drugs with desirable properties, high encapsulation efficiency, and long-term stability without drug leakage and loss [3]. When liposomes are used as drug carriers, their size is of major importance, since this influences the behavior of liposomes in biological systems [4, 5].

Many efforts have been made to improve the properties of liposomal preparations, especially the encapsulation efficiency, vesicle size, and stability, which are still major problems standing in the way of new advances in this field. Up to now, a number of different methods has been successfully developed for the preparation of liposomes. These methods can be classified for convenience into three categories based on the dispersion technology: (1) mechanical dispersion methods, for example, hand shaking, or vortexing, sonication, and the use of a French press; (2) detergent-solubilizing dispersion methods including solubilizing lecithin with sodium cholate or octylglucoside; (3) solvent dispersion methods, such as ethanol injection, ether infusion, and reverse-phase evaporation (REV). Liposomes prepared by the REV method are known to have a higher encapsulation efficiency for water-soluble agents than those prepared by other methods [6].

In this report, we have presented the small liposomes prepared by the modified REV method [7] with the

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desirable properties of the relatively high encapsulation efficiency of antitumor drugs Ara-C and 5-FU and a mean diameter less than 100 nm [5]. The drugs are used in the anticancer multidrug therapy F-MACHOP (fluorouracil, methotrexate, Ara-C, cyclophosphamide, doxorubicin, vincristine, and prednisone).

For liposomes used in the analytical and bioanalytical applications, the main characteristics include the encapsulation efficiency, the ratio of phospholipids to encapsulant concentration, and 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, they often undergo multiple thermotropic phase transitions [8]. These transitions maybe detected by a wide variety of physical techniques such as DSC, dilatometry, X-ray diffraction, neutron diffraction, dynamic light scattering, and NMR [9–11].

## Experimental

### Chemicals

L- $\alpha$ -phosphatidylcholine dipalmitoyl (1,2-dihexadecanoyl-*sn*-glycerol-3-phosphocholine) 99% (DPPC), 2,4-dihydroxy-5-fluoropyrimidine (5-FU), 1- $\beta$ -D-arabinofuranosylcytosine (Ara-C) were purchased from Sigma-Aldrich Chemical Co., chloroform, dichloromethane, hydrochloric acid, and phosphate buffered saline (buffer PBS:  $K_2HPO_4$ ,  $NaH_2PO_4$ ) from POCH, Gliwice, Poland.  $D_2O$  99%, chloroform- $d$  99%, stab. with Ag, dimethyl sulfoxide (DMSO), and 4,4-dimethyl-4-silapentane sodium sulfonate (DSS) were purchased from ARMAR Chemicals, Switzerland.

### Liposome and liposome/drug preparation

We obtained small liposomes (DPPC; DPPC/5-FU; DPPC/Ara-C; DPPC/Ara-C/5-FU) by the modified reverse-phase evaporation method (mREV) [12, 13]. PBS buffer pH 7.4 was applied. For  $^1H$  NMR and UV study  $5 \times 10^{-3}$  M 5-FU in DMSO and  $5 \times 10^{-3}$  M Ara-C in  $D_2O$  were added to the preparation mixture. The preparation was carried out at 317 K. Liposome entrapped 5-FU and Ara-C were separated from free 5-FU and free Ara-C 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 (400.130 MHz for  $^1H$ ) using a 5-mm broad band inverse probe.  $^1H$  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  $\pm 0.1$  K. For water suppression, the presaturation method was used. 32 transients were accumulated with a  $^1H$  pulse length of 10.70  $\mu s$  pulse and 5 s relaxation delay, 2.044 s acquisition time, 32768 data points, and 0.30-Hz line broadening.  $^1H$  chemical shift values were referred to DSS as external reference.

Spectra processing was performed with TOPSPIN 2.1 Bruker software. Apparatus error was  $\pm 0.01$  ppm.

### UV analysis

UV spectra of all prepared liposomes DPPC, DPPC/5-FU, DPPC/Ara-C, and DPPC/Ara-C/5-FU and drugs 5-FU and Ara-C were recorded on spectrophotometer LAMBDA BIO 40 PERKIN ELMER using the quartz cells  $1.0 \times 0.5 \times 4.0$  cm. The investigations were done within the temperature range 298–320 K. Apparatus error was  $\pm 1$  nm and  $\pm 0.004$  for the wavelength  $\lambda$  and absorbance A, respectively. The spectra were recorded in the range from 200 to 400 nm. The maximum of absorption for Ara-C and 5-FU was determined from the obtained spectra at  $\lambda = 271$  nm and  $\lambda = 268$  nm, respectively.

To determine the concentration of the drugs incorporated into liposome vesicles the three step dialysis in phosphate buffered saline (PBS) were done. Each dialysis went on for 60 min at 277 K in dialysis sack Servapor with pore diameter 2.5 nm. The percent of the drug encapsulation was determined on the basis of the calibration curves.

## Results and discussions

The objective of this study was to incorporate the drugs with amphiphilic (Ara-C) 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 of phase transition of phospholipids.

The study performed with 5-FU and Ara-C allowed us to make a comparative analysis with the data obtained previously for 5-FU and 5-formyl-5,6,7,8-tetrahydropteroyl-L-glutamic acid calcium salt (leucovorin; LCV) [14].

Two spectroscopic methods: proton nuclear magnetic resonance ( $^1H$  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 using  $^1\text{H}$  NMR spectroscopy.

#### Nuclear magnetic resonance study

In this study,  $^1\text{H}$  NMR technique is described that can be used to estimate the phase transition temperature ( $T_c$ ) of phospholipid. Figure 1 shows the high-resolution  $^1\text{H}$  NMR spectra of liposome obtained from: (a) DPPC; (b) DPPC/5-FU; (c) DPPC/Ara-C; (d) DPPC/Ara-C/5-FU.

To estimate the temperature  $T_c$  value the sequence of spectra for each thermally equilibrated system was used as shown in Fig. 1.

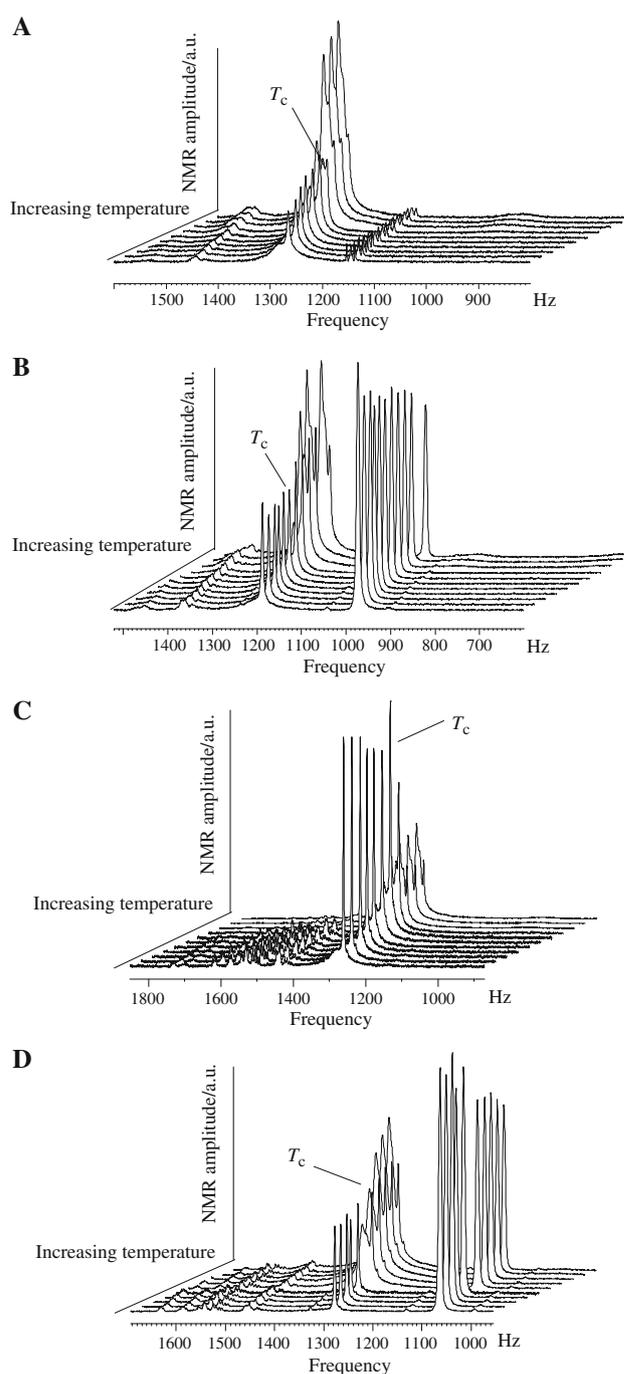
Figure 2 shows that increase of temperature leads to a slight increase of area of the signal of protons of methyl groups of ammonium of phospholipids  $-\text{N}^+(\text{CH}_3)_3$ .

Derivative of  $^1\text{H}$  NMR peak area was determined from the signals (Fig. 1). The peak maximum (Fig. 2) showed the phase transition temperature ( $T_c$ ).

$T_c$  obtained with this method for: DPPC; DPPC/5-FU; DPPC/Ara-C; DPPC/Ara-C/5-FU is 315.0, 315.4, 315.1, and 312.7 K, respectively.

The fraction of drug encapsulated liposomes depends on the physico-chemical properties of the drugs used in multidrug therapy [14].

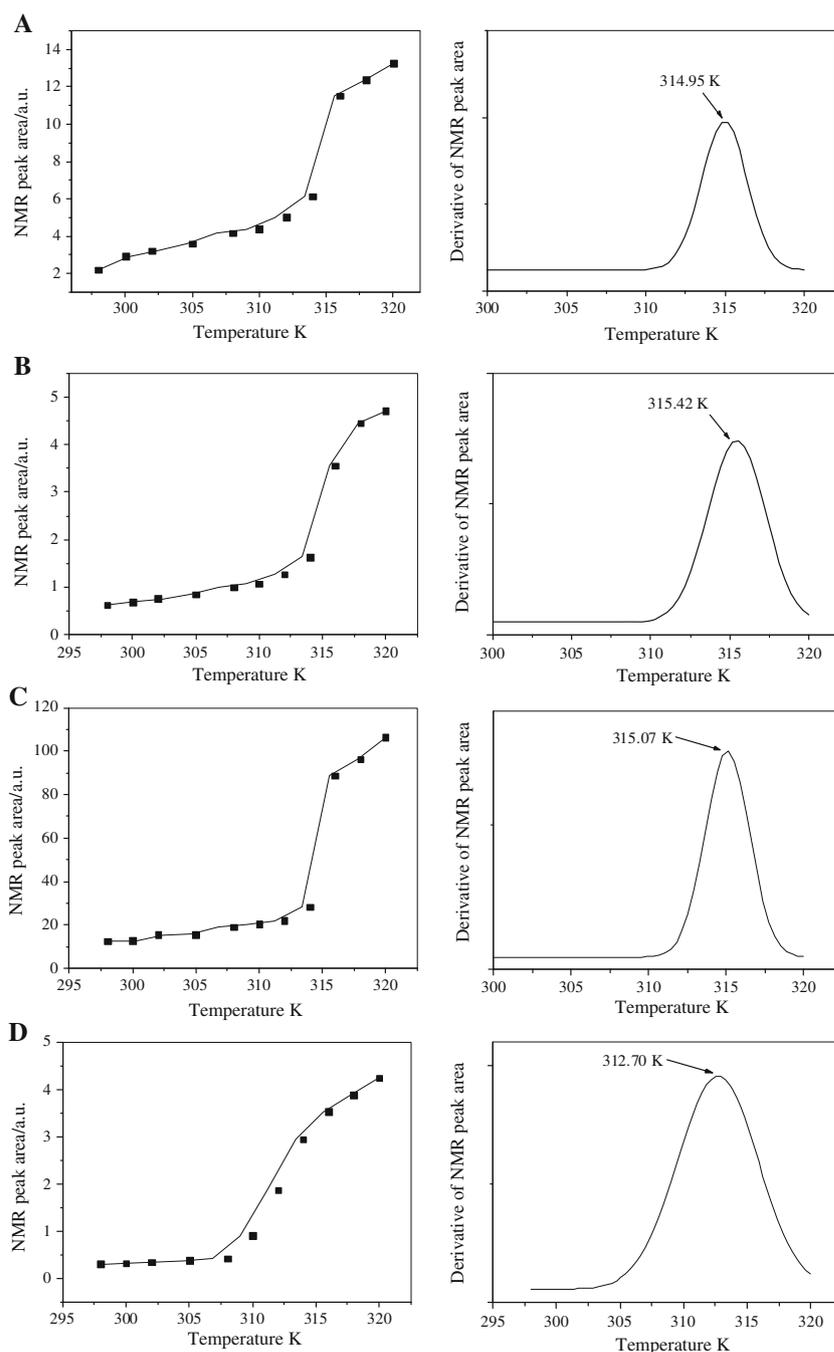
The most significant competition in encapsulation to liposome has been previously observed for hydrophilic–hydrophobic (LCV–5-FU) drugs encapsulated together [14]. In that case, the difference in encapsulation amounted to 10.17%, i.e. it is higher than 5.33% determined for hydrophobic–amphiphilic (5-FU–Ara-C) pair of drugs. The content of encapsulated drugs affected the phase transition temperature ( $T_c$ ) of phospholipids. When only hydrophobic drug was encapsulated then  $T_c$  amounted to 315.4 K. An addition of hydrophilic LCV did not affect markedly  $T_c$  and it was 315.2 K. Probably, the competition between 5-FU and LCV was significant and LCV had only slight effect on  $T_c$ . LCV due to its physico-chemical properties can be encapsulated only within aqueous region of liposome and it was not incorporated into phospholipids membrane. In this study, there was a difference when both 5-FU and Ara-C were together introduced to liposome. Between these drugs there was only 5.33% of difference in encapsulation to liposome. However, the difference in phase transition temperature  $T_c$  was more significant.  $T_c$  of phospholipids vesicle containing only hydrophobic (5-FU) drug amounted to 315.4 K. An addition of amphiphilic Ara-C caused a decrease of temperature up to 312.7 K. This means that there is a division of encapsulation site of both drugs (5-FU and Ara-C) between the phospholipide molecules. A



**Fig. 1**  $^1\text{H}$  NMR spectra investigating the thermal transitions in liposomes obtained from (A) DPPC; (B) DPPC/5-FU; (C) DPPC/Ara-C; (D) DPPC/Ara-C/5-FU. Variation of the NMR signal of  $-\text{N}^+(\text{CH}_3)_3$  with increasing temperatures.

simultaneous location of 5-FU and Ara-C in the membrane affected substantially the distance between phospholipids (i.e. packing) and their mobility. The above parameters decide about the increase or decrease of temperature  $T_c$  of phospholipids phase transition [14].

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



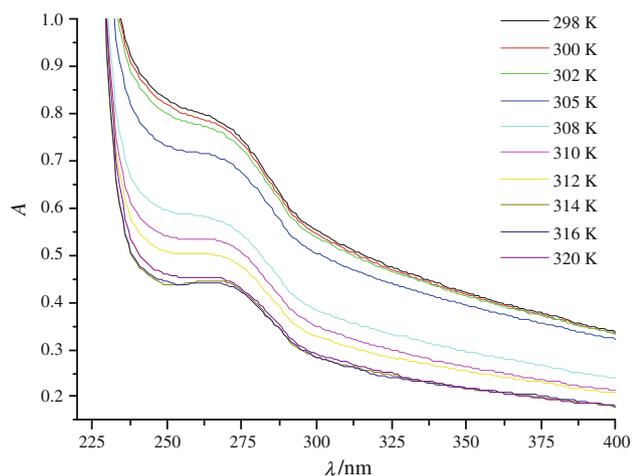
### UV study

In this study, the effect of liposome encapsulation was discussed. The degree of the incorporation of drug into liposome vesicles DPPC/Ara-C DPPC/5-FU, DPPC/Ara-C/5-FU, defined as a concentration of Ara-C and 5-FU in phospholipid carriers, was estimated using UV spectroscopy after dialysis.

The percent of encapsulation was determined on the basis of the calibration curves.

We compared UV spectra of Ara-C and 5-FU incorporated together into the liposome vesicles with the spectra of each drug incorporated separately in the temperature range 298–320 K. Temperature had no effect on the UV spectra of Ara-C and 5-FU. However, temperature affected the spectra of Ara-C and 5-FU incorporated together into liposome vesicles (Fig. 3).

The encapsulation efficiency, which is a measure of the percentage of the total compound entrapped within the liposome, is an important parameter in liposomal



**Fig. 3** Effect of temperature UV spectrum of liposomes obtained from DPPC/Ara-C/5-FU. (Color figure online)

characterization. It amounted to  $3.75 \times 10^{-4}$  M for 5-FU and Ara-C. In the liposome vesicles DPPC/5-FU, the percentage of encapsulation of 5-FU amounted to 99.17%. In the liposomes vesicles DPPC/Ara-C, the percentage of encapsulation of Ara-C amounts to 99.89%. DPPC/Ara-C/5-FU liposome containing both drugs, encapsulated 93.84% of 5-FU and 96.05% of Ara-C.

One can conclude that there is only a slight influence of 5-FU on the Ara-C encapsulation in liposome vesicle. The content of Ara-C decreased by 3.84% in liposomes DPPC/Ara-C/5-FU in comparison with liposomes formed of DPPC/Ara-C. Slightly higher changes were observed for the influence of Ara-C on 5-FU. Presence of Ara-C decreased the content of 5-FU by 5.33% in liposomes formed of DPPC/Ara-C/5-FU in comparison with those formed of DPPC/5-FU.

## Conclusions

The encapsulation of Ara-C to DPPC liposomes did not significantly affect the  $T_c$  value in comparison with the reference liposomes formed from DPPC. However, phase transition temperature  $T_c$  increased for the liposomes containing 5-FU. The analysis of competition between 5-FU and Ara-C showed that Ara-C incorporated to liposome in a higher degree than 5-FU and affected the  $T_c$  value of phospholipid forming liposomal membrane. Moreover, the presence of 5-FU in combination with Ara-C–5-FU caused a decrease of encapsulation of Ara-C and a decrease of temperature of phase transition  $T_c$ .

One can conclude that the used spectroscopic techniques are suitable for an estimation of the phase transition temperature ( $T_c$ ) of phospholipid and the percentage 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.

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