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Liposomes as Carriers of Anticancer Medicines

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The aim of the work was the successful encapsulation of etoposide (ETO) and vinorelbine (VIN). Liposomes made from dipalmitylphosphatidylcholine (DPPC), liposomes containing both drugs (DPPC/ETO/VIN) and control liposomes DPPC/ETO and DPPC/VIN were prepared by the mREV method at 328 K. Liposome entrapped drugs were separated from free drugs by ultracentrifugation. The degree of encapsulation of drugs into liposome vesicles DPPC/ETO, DPPC/VIN and DPPC/ETO/VIN was estimated using UV spectrofluorescence after ultracentrifugation. The phase transition temperature was determined for all types of liposomes. The encapsulation efficiency, which is a measure of the percentage of the total compound entrapped within the liposome, is also determined.

Keywords Etoposide; liposomes; phase transition; vinorelbine

Introduction

Clinical application of liposomes as drug delivery systems concerns, among others, cancer treatment. However in vivo instability, short life-time in blood circulation and lack of transport specifity have impeded the clinical application of liposomes as anticancer drug delivery system. In the multidrug therapy used in cancer treatment the another difficulty appears: encapsulation of two drugs with different affinity to phospholipid membrane. Therapy using anticancer agents encapsulated in liposomes improves efficacy and reduces toxic side effects.

The aim of this study was to encapsulate etoposide and vinorelbine into liposomes in the process of modified reversed phases evaporation (mREV). The drugs demonstrate antineoplastic activity and are clinically approved. Vinorelbine is mainly used for treatment of metastatic breast cancer and non-small-cell lung cancer [1], etoposide is used in therapy of small cell lung carcinoma [2]. Vinorelbine is cycle-specyfic anticancer agent. Its antitumor activity relies on inhibition of cell proliferation in late G_2 and M phase. Vinorelbine

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interacts with tubuline and prevents creation of microtubules [3]. Etoposide forms a ternary complexes with topoisomerase II and DNA which results in DNA break leading to cell death [2]. The research was to verify VIN (Vinorelbine) and ETO (etoposide) competition in the encapsulation into liposomes in multidrug therapy. The results should allow setting concentration and the percentage of encapsulation (%E) of the medicines in liposomes.

Materials and Methods

1. Reagents

To receive liposomal membranes dipalmitylphosphatidylcholine 99% (DPPC) [Sigma-Aldrich Chemical Co, Germany]; buffer PBS of pH 7,4 prepared from K_2HPO_4 [Polish Chemical Reagents S.A. POCH Gliwice] and NaH_2PO_4 [Polish Chemical Reagents S.A. POCH Gliwice]; chloroform [Polish Chemical Reagents S.A. POCH Gliwice]; dichloromethan 99% HPLC [Sigma- Aldrich Chemical Co, Germany]; chloroform-d 99,8 Atom% D stab. Mit Ag [ARMAR AG Chemicals, Dottingen, Switzerland]; cholesterol (3β – hydroxy – 5 cholestene 5 – cholesten – 3β – ol) [Sigma-Aldrich Chemical Co, Germany] were used. VIN 2264 and ETO E1383 were purchased from Sigma.

2. Procedure of Preparation of Liposomes

Lipsomes have been obtained in the process of modified reversed phase evaporation method (mREV) which is based on permanent mixing of defined concentration of phospholipids dissolved in excess amount of organic liquid phase. 2.0 ml of buffer of pH 7.4 and 3.5 ml of organic phase (dichloromethane/chloroform), in which phospholipidis have been dissolved, were put in the preparatory thermostated at 55°C. Then the mixture was stirred extensively. After evaporation of organic solvents the obtained liposome emulsion was centrifuged at 10000 rpm. Liposomes were then separated from supernatant where un-embedded drugs remained.

In this way liposomes made of DPPC, DPPC/ETO, DPPC/VIN, DPPC/VIN/ETO have been prepared. Similarly liposomes containing different content of cholesterol were prepared. Molar ratio of DPPC to cholesterol were 6,5:1, and 2:3. The amount of drugs used separately for each preparation was $4{,}5 \times 10^{-4}$ mol and for composition of both drugs it was 9×10^{-4} mol.

3. Physicochemical Properties of Drugs

Drugs solubility in different solvents was considered before preparation of liposomes. Etoposide is poorly soluble in water but can be successfully dissolve in organic solvents. In order to increase liposomal etoposide formulation the drug was added to water/organic emulsion in form of etoposide solution in $CH_2Cl_2/CHCl_3$ (1:1 v/v). Drug incorporation into liposome membrane was predicted. Vinorelbine is strongly lipophilic but due to its relatively good solubility in water (1000 mg per ml) [4] drug was added in water solution. It is said that vinorelbine is the most lipophilic representant of *Vinca* alkaloids which can cause an increased membrane permeability affecting more rapid release from liposomes and reduced encapsulation efficiency [3].

Results and Discussion

1. Encapsulation of Etoposide, Vinorelbine and the Mixture of Drugs to Lipsosmes

Liposomes formed from DPPC and DPPC with cholesterol in different molar ratios (6,5:1 and 2:3) were prepared by mREV method. Liposomes containing etoposide and vinorelbine encapsulated separately and together were formed. Liposomes without drugs were also prepared. UV-Vis spectroscopy was used to prove encapsulation of drugs in liposomes. UV-Vis absorption spectrum of DPPC liposomes containing mixture of etoposide and vinorelbine (Fig. 1a) was compared with spectrum registered for supernatant remained after centrifugation of preparation mixture (Fig. 1b). Absorbance 1.08 at 280 nm is observed for mixture of drugs incorporated in liposomes (DPPC/ETO/VIN) (Fig. 1a). In the spectrum of supernantant absorbance at 280 nm is lower by 0.48 (Fig. 1b). Therefore we can state that majority of drug introduced into preparation mixture was encapsulated into liposomes.

This is an evidence of the presence of drugs in DPPC liposomes, resulted by efficient drugs encapsulation. Similar comparison was made for liposomal formulations of etoposide and vinorelbine encapsulated separately.

2. Determination of Phase Transition Temperature (T_c) in Liposomes

In order to study the changes in drug release caused by temperature absorption UV-Vis spectra in the temperature range 298-320 K were recorded (Fig. 2). Temperature does not affect the UV spectra of free ETO and VIN (spectra not shown) but in the spectra of these drugs encapsulated in liposomes separately or together the dependence on temperature is observed. Significant variation of absorbance observed in small temperature range corresponds to phospholipids phase transition temperature (T_c). The temperature of phase transition is characteristic for different lipids. Variations of temperature cause structural changes in lipid bilayers. During heating in excess water, multiple phase transitions can be observed in bilayers formed of one type of phospholipids. There are four phases of lipids conformation in bilayer which are separated by three temperatures of phase transition. Between lamellar crystalline phase (L_c) and tilted gel phase ($L_{\beta'}$) is temperature of subtransition (T_I), pretransition temperature (T_{II}) separates tilted gel phase from ripple

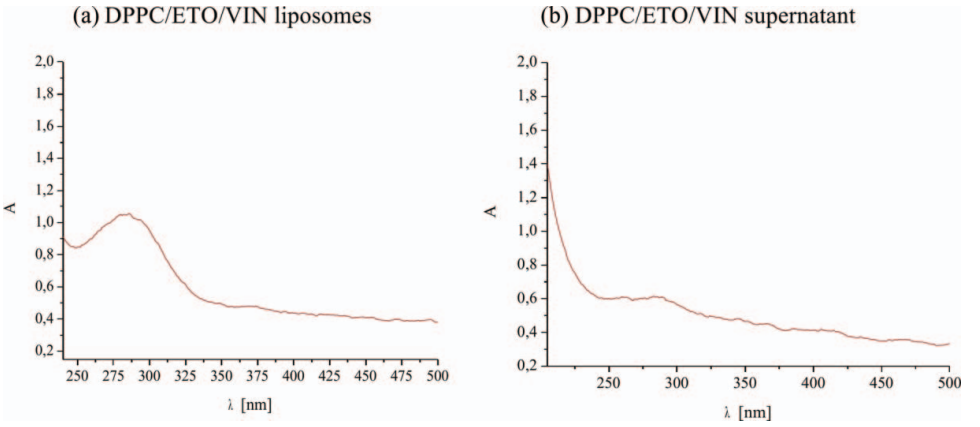

Figure 1. UV-Vis absorption spectrum of: a) DPPC/ETO/VIN liposomes, b) DPPC/ETO/VIN supernatant.

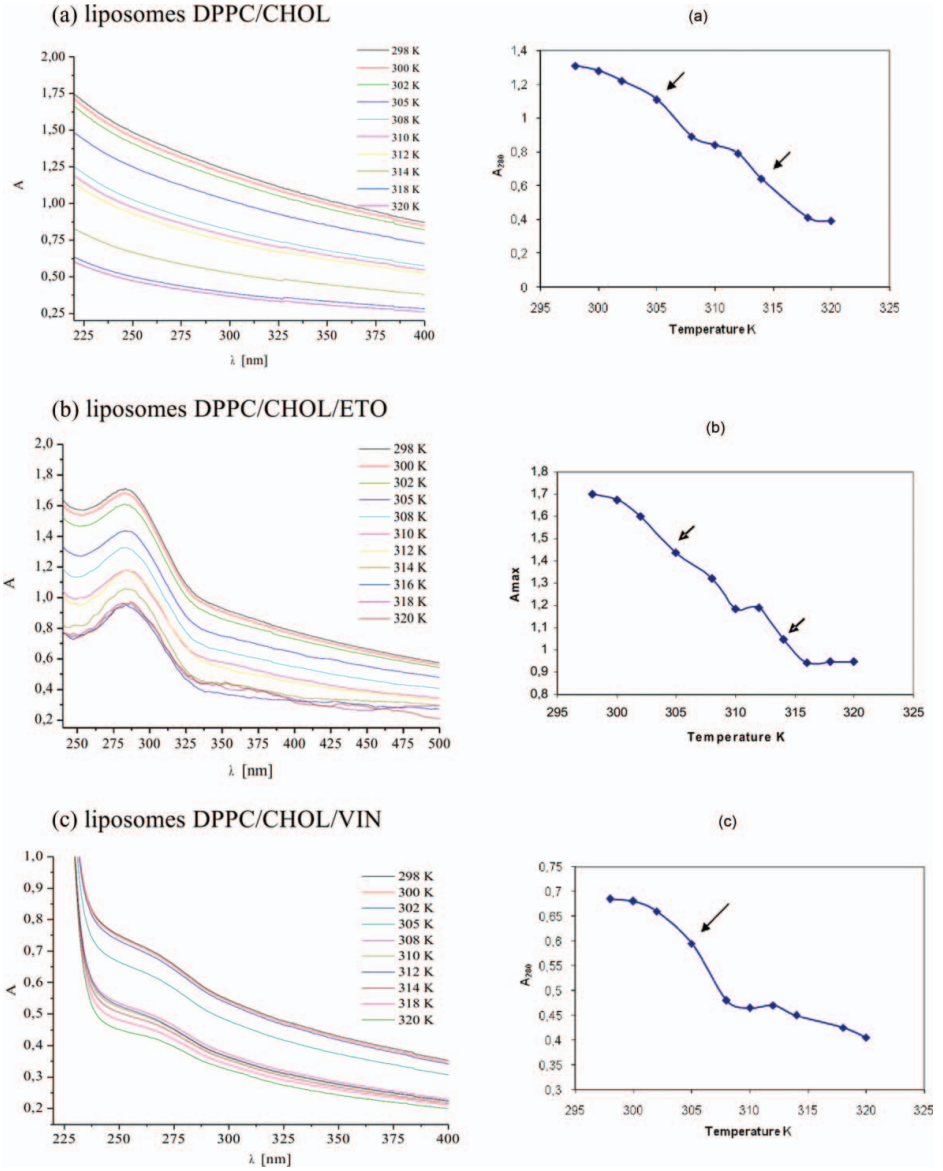

Figure 2. The influence of temperature rise on absorbance of encapsulated drugs: a) un-embedded liposomes DPPC/CHOL, b) DPPC/CHOL/ETO liposomes entrapped-etoposide, c) DPPC/CHOL/VIN liposomes entrapped-vinorelbine, d) DPPC/CHOL/ETO/VIN liposomes loaded with both drugs; molar ratio of DPPC : CHOL is 2:3.

gel phase ($P_{\beta'}$). The main phase transition temperature (T_c) causes transformation of lipid bilayer from ripple gel phase to liquid-crystalline phase (L_α) [5]. In majority of liposomal bilayers two temperatures of phase transition are observed. The first one is pretransition undergoing in temperature of 305 K and the second is the main phase transition at 314 K [6]. It is said that lipid bilayers are most permeable in Tc due to increased disordered and mobility of its components.

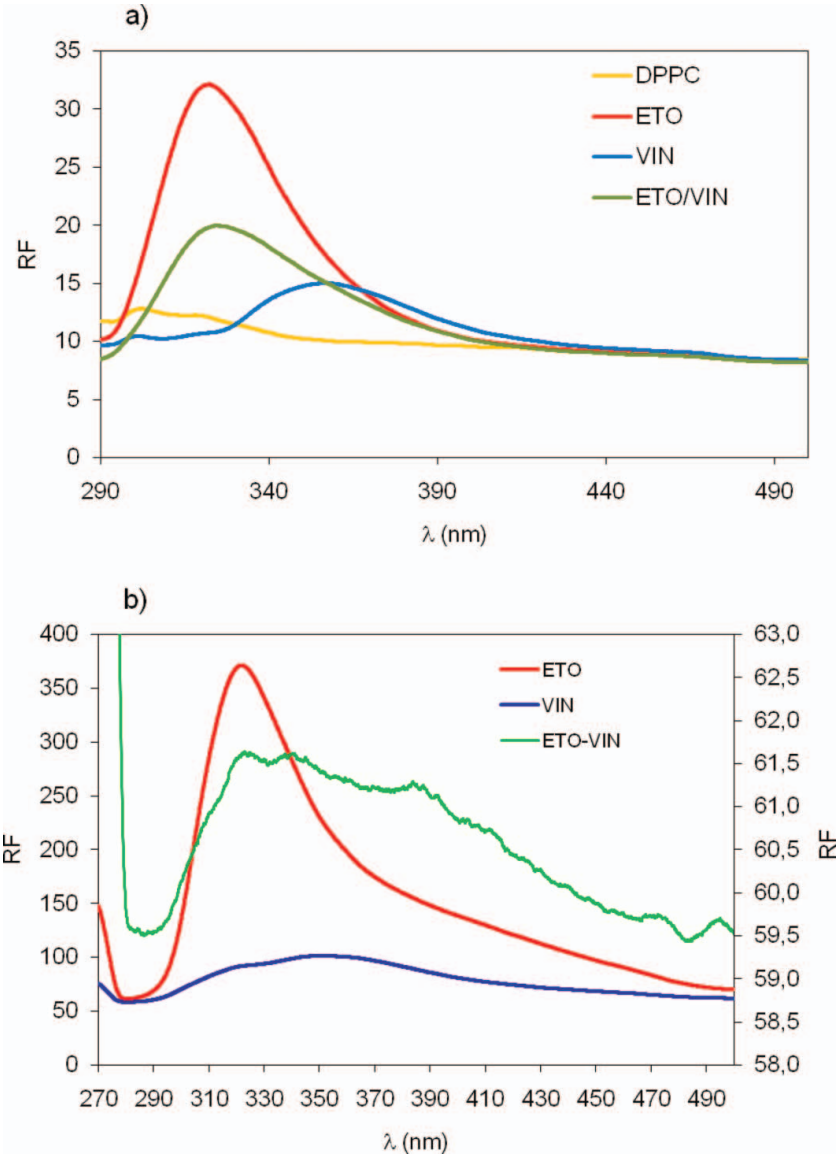

Figure 3. Fluorescence emission spectra of a) drugs remained in supernatant, b)standard drugs: ETO $c = 2\times10^{-3}$ M; VIN $c = 2\times10^{-3}$ M; ETO/VIN $c = 2{,}5\times10^{-3}$ M; $\lambda_{ex} = 270$ nm.

Absorption spectra of DPPC liposomes show maximum at 220 nm [7]. To determine temperature of phase transition of DPPC/ETO/VIN liposomes the temperature dependences of absorbance were analyzed at 280 nm and λmax for un-embedded liposomes, VIN-liposomes and ETO-liposomes, ETO/VIN liposomes, respectively (Fig. 2).

Temperature of phase transition (T_c) is possible to determine as an inflection on the temperature dependence (arrows in the Fig. 2). Two phase transitions temperatures at 305 K and 314 K were determined for un-embedded DPPC/CHOL liposomes (Fig. 2a) and those containing etoposide (Fig. 2b). For liposomes containing vinorelbine only one

Table 1. Efficiency of encapsulation (E%) of drugs into liposomes differing with the composition of membrane

	Efficiency of drug encapsulation (E%)		
Liposomes	Etoposide	Vinorelbine	Etoside/ Vinorelbine
DPPC	82,34	56,7	19,45
DPPC/CHOL (6,5:1 mol)	74,63	49,01	24,75
DPPC/CHOL (2:3 mol)	78,98	77,28	85,78

phase transition at 306 K is determined (Fig. 2c). For liposomes containing two drugs (DPPC/CHOL/ETO/VIN) two phase transitions seem to be possible: at 305K and between 312 and 316 K (Fig. 2d). Worth noting is a distinct inflection for temperature dependence of vinorelbine embedded liposomes (Fig. 2c). The release of etoposide from liposomes undergoes in two stages. It seems to be less rapid than release of vinorelbine which gets out of liposomes mainly at phase transition temperature 305 K.

3. Efficiency of Encapsulation

Estimation of encapsulation efficiency was based on emission fluorescence spectroscopy. The comparison of emission fluorescence spectra registered for standard solutions of etoposide, vinorelbine, mixture of two drugs (Fig. 3b), with those of supernatants remained after ultracentrifugation of liposomes (Fig. 3a) allowed to determine drug loading into nanolipid carriers. Encapsulation efficiency was determined for preparations of liposomes with different composition of bilayers (Table 1), i.e. for lipsomes with membrane composed of DPPC, DPPC and cholesterol at molar ratio 6,5:1 and DPPC and cholesterol at molar ratio 2:3.

To calculate the concentration of drugs remained in supernatant the formula (1) was used:

$$c_s = \frac{RF_s}{RF_{st}} c_{st} \tag{1}$$

Where: c_s - concentration of sample, c_{st} - concentration of standard, RF_s - fluorescent intensity of sample at 270 nm, RF_{st} - fluorescent intensity of standard at 270 nm.

The amount of drug encapsulated in liposomes was determined by subtraction of amount of drug detected in supernatant from initial quantity of drug(s) used for preparation of each type of liposomes.

It appears that the most efficient encapsulation was obtained for liposomes with greatest percentage of cholesterol used for preparation.

Conclusions

We proposed mREV method to create liposomes able to transport anticancer drugs that are used in multidrug therapy of cancer. Etoposide and vinorelbine can be encapsulated in all types of prepared lipsomes, but the best efficiency was observed for liposomes

containing cholesterol added during mREV procedure. Liposomes prepared from DPPC and cholesterol of molar ratio 2:3 appeared to be the most efficient carriers of studied drugs.

It is shown that DPPC/VIN liposomes undergoes one phase transition at 305K while for DPPC/ETO lipsomes and DPPC/ETO/VIN two phase transitions at 305 K and 314 K were observed. Vinorelbine can pass faster than etoposide through liposomal bilayer. Fluorescent spectroscopy allowed to state that vinorelbine is significantly less efficient to encapsulate than etoposide. The percentage of encapsulated etoposide was the largest in all three types of prepared liposomes. Cholesterol increases most substantially efficiency of encapsulation of drug mixture from 19,45% for DPPC lipsomes to 85,78% for DPPC/CHOL liposomes (2:3 molar ratio).

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