

Interaction of Doxorubicin and Dipalmitoylphosphatidylcholine Liposomes

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Abstract The interaction between doxorubicin (DOX), an anthracycline antibiotic frequently used in chemotherapy, and zwitterionic dipalmitoylphosphatidylcholine (DPPC) was investigated using Fourier transform infrared (FTIR) spectroscopy, differential scanning calorimetry (DSC), and rheological measurements. FTIR results showed that DOX shifted the wavenumber of the PO_2^- band for pure DPPC to a higher wavenumber. This may have been because of the strong interactions between the NH_3^+ group in DOX and the phosphate (PO_2^-) group in the polar head of DPPC. The main transition temperature of DPPC liposomes was slightly shifted to a lower temperature for DPPC liposome-encapsulated DOX. This suggested that DOX had a significant effect on the acyl chains in the DPPC bilayers, and that its presence decreased the transition cooperativity of lipid acyl chains. There was also the appearance of an additional transition peak at nearly 136°C for the DPPC/DOX sample. These interactions between DOX and DPPC phospholipid would cause a decrease in the DPPC liposomes plastic

viscosity and increase membrane fluidity. A better understanding of the interactions between DOX and lipid bilayers could help in the design and development of improved liposomal drug delivery systems.

Keywords Liposomes · Doxorubicin · Interaction · DPPC · Viscosity · FTIR · DSC

Introduction

Anthracycline-based antibiotics such as doxorubicin (DOX), pirarubicin and daunorubicin have found widespread application in cancer chemotherapy. DOX is an anthracycline commonly used to treat several solid tumors, acute leukemia and malignant lymphoma [1, 2]. DOX can inhibit the replication process of cells by crossing the tumor cellular membrane and intercalating into the base pairs of DNA [3]. However, severe clinical side effects, such as cardiotoxicity and myelosuppression, are of major concern. The prevailing liposomal drug delivery system can provide a solution to this problem [2, 4].

The side effects of DOX can be greatly reduced when the drug is encapsulated into liposomes [2]. It is interesting to note that the interaction between DOX and lipids can inhibit the formation of a DOX and cardiolipin complex, which might play a role in cardiotoxicity [5]. Therefore, elucidation of the interactions between anthracycline and phospholipids can have a profound role to play in the understanding of the mechanisms responsible for the enhanced safety profile of anthracycline liposomes. Furthermore, such interactions determine the partitioning, allocation, orientation and conformation of exogenous substances in the bilayers, thus, playing an important role in the liposomal drug delivery system [6–8].

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Until now, only limited data have been available with regard to the interaction of anthracycline with phospholipids from the perspective of the thermotropic phase behavior of phospholipids [9, 10]. There have been no systematic investigations in relation to anthracycline and phospholipids using differential scanning calorimetry (DSC) and Fourier transform infrared (FTIR) spectroscopy. These two techniques are highly effective in probing the interaction between drugs and phospholipids [11, 12].

FTIR can be used to monitor subtle changes in the structure and function of the lipid assemblies, by analyzing the frequency or the bandwidth changes of the different vibration modes representing the acyl chains, the interfacial region and the head group region of lipid molecules. Various kinds of information can be derived from these bands. The measuring of some spectral parameters such as band frequency, width and intensity change provides information regarding the possible structural interactions and conformational rearrangements taking place [13, 14].

DSC is generally used to measure a number of characteristic properties of a sample. It is possible to observe fusion, crystallization and even oxidation and other chemical reactions. Furthermore, the glass transition temperature (T_g), crystallization temperature (T_c) and melting point (T_m) of a sample can be determined. The fluidity of lipid bilayers depends on the lipid or combination of lipids used and their fluid gel transition temperature [15]. T_m represents the peak temperature of the endotherm for the lipid gel-to-fluid phase transition recorded during the heating scan.

The pre and main transitions are often observed in PC lipids. The pre-transition, which corresponds to the conversion of a lamellar gel phase to a rippled gel phase, is mainly related to the polar region of phospholipids, whereas the main transition reflects the change from a gel phase to a liquid crystal phase. It has also been suggested that the main transition process is closely related to the acyl chains of phospholipids bilayers, which can probe the interaction between the acyl chains of phospholipids and exogenous substances.

The release of drug from semi-solid carriers is influenced by rheological behavior. The effect of certain parameters such as storage time and temperature on the quality of liposomes as pharmaceutical products can be investigated by means of rheological measurements. Rheological analysis can be employed as a sensitive tool in predicting the physical and pharmaceutical properties of liposomal-encapsulated DOX.

The objectives of this study were as follows: (1) to observe changes in the acyl chain conformations and characteristic PO_2^- bands in the polar heads of DPPC in the presence and absence of DOX using FTIR spectroscopy and (2) to investigate the thermotropic phase behavior of DPPC in the presence of DOX using the DSC technique. (3) The

interaction between DOX and DPPC lipids was also investigated using rheological parameters measurement.

Materials and Methods

Materials

L- α -dipalmitoyl phosphatidylcholine (DPPC) in powder form and of purity 99% was purchased from Lipoid KG, Ludwigshafen, Germany. Adriblastina that consists of DOX hydrochloride was obtained from Pharmacia Italia, SPA, Italy. Triton X-100 was purchased from Sigma, St. Louis, MO, USA. Trizma buffer with a molecular weight of 121.1 was purchased from Sigma Chemicals, Steinheim, Germany. Ethanol used in the study was of analytical grade and was obtained from Merck, Heliopolis, Cairo, Egypt. All other reagents and solvents were of analytical grade and were used without further purification.

Liposomal Preparation

We prepared multilamellar vesicles (MLV) of DPPC liposomes according to the reverse phase evaporation method [16]. The lipids were first dissolved and mixed in ethanol to ensure a homogeneous mixture of lipids. The organic solvent was removed by rotary evaporation to yield a thin lipid film on the sides of a round bottom flask. Hydration of the dry lipid film was accomplished by simply adding trisma-buffer to the container of dry lipid and agitating at a temperature greater than the phase transition temperature of the lipid. Multilamellar vesicles (MLV) were formed with a final lipid concentration of 2 mg/ml. For the DPPC/DOX liposomal sample, hydration of the dry lipid thin film was achieved with 5 ml of trisma-buffer (pH 7.4) containing DOX (drug to lipid molar ratio = 0.28:1) [17]. The flask was mechanically shaken for 15 min at 45°C. The suspension was then centrifuged at 11,000 rpm three times for 15 min each to remove non-encapsulated drug. The non-encapsulated drug concentration was calculated from a calibration curve (a relation between drug concentrations and the absorbance at 485 nm). The encapsulation efficiency was calculated from the following formulation [18]:

$$\text{Encapsulation efficiency} = \frac{[\text{Total drug}] - [\text{Non-encapsulated drug}]}{[\text{Total drug}]} \times 100$$

Encapsulation efficiency was 72%.

FTIR Spectroscopy

FTIR spectra of lyophilized samples of DPPC liposomes and DPPC liposomes encapsulated DOX deposited in KBr

disks were recorded on a NICOLET 6700 FTIR spectrometer (Thermo Scientific, Cambridge, England). Scanning was carried out at room temperature, in the range 400–4,000 cm^{-1} at a speed of 2 mm/s and a resolution of 4 cm^{-1} . A final lipid concentration of 2 mg/ml was used.

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was carried out using a DSC-50 (Shimadzu, Kyoto, Japan) to investigate the thermal behavior of lyophilized DPPC in pure samples of liposomes and MLV DPPC liposome-encapsulated DOX. The thermograms covered the 25–200°C temperature range at a 5°C/min heating rate. A final lipid concentration of 2 mg/ml was used.

Rheological Measurements

DPPC liposomes alone and DPPC liposomes encapsulating DOX were analyzed using a Physica type MCR 301 (Anton Paar, Graz, Austria), where the sample was introduced between two plates at a distance of 0.5 mm. The flow curves were plotted for shear stress (N/cm^2) and shear rate (s^{-1}) at room temperature for each sample. Plastic viscosity and yield stress were calculated from the linear-fitted flow curves [19, 20]. The rheological properties of liposomal formulations can be described by the power law model [19] as follows:

$$\tau = k\gamma^n, \quad (1)$$

where τ is the shear stress, k is the consistency index, γ is the shear rate and n is the flow behavior index. To obtain the flow behavior index (n) and consistency index (k) values, a final lipid concentration of 2 mg/ml was used.

Results and Discussion

FTIR Studies

Figure 1 shows the full FTIR spectrum of the DPPC liposomal sample. The spectrum of the DPPC liposomes displayed the main characteristic bands, especially those that were because of the following: The symmetric and antisymmetric stretching vibrations of the CH_2 in the acyl chain (2,850 and 2,920 cm^{-1} , respectively), the OH stretching and bending vibrations (3,470 and 1,640 cm^{-1} , respectively), the carbonyl stretching vibration $\text{C}=\text{O}$ (1,734 cm^{-1}), the CH_2 bending vibration CH_2 (1,470 cm^{-1}) and the symmetric and antisymmetric PO_2^- stretching vibrations (1,090 and 1,220 cm^{-1} , respectively). These findings are in good agreement with the data reported in the literature [21–25].

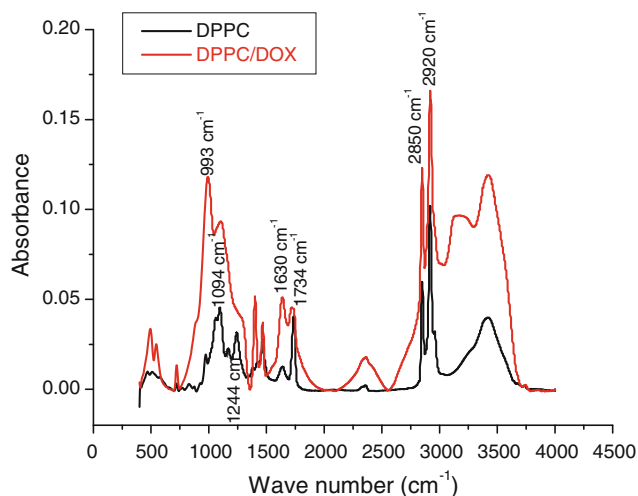


Fig. 1 The full FTIR spectra of DPPC and DPPC/DOX liposomal samples

For DOX/DPPC FTIR spectrum, there was no significant change in the frequency of the CH_2 stretching bands observed (Fig. 1), implying that DOX does not change the number of gauche conformers. In other words, it did not have any significant effect on the order of the membrane. Investigation of the $\text{C}=\text{O}$ band revealed that there was no hydrogen bond between DOX and the $\text{C}=\text{O}$ group in the phospholipid.

The interaction between DOX and the head group of DPPC liposomes was monitored by means of the PO_2^- symmetric and antisymmetric stretching bands, which were located at 1,094 and 1,244 cm^{-1} , respectively. Figures 1 and 2 show the PO_2^- symmetric stretching band for DPPC liposomes in the absence and presence of DOX. As can be seen from the figures, the wavenumber was shifted to higher values (1,107 cm^{-1}) after the encapsulation of DOX into DPPC liposomes. This implied the presence of hydrogen bonding between the liposome head group and DOX [26–28]. Also, the $\text{N}^+(\text{CH}_3)_3$ asymmetric stretching band at 975 cm^{-1} was shifted to a higher wave number of 993 cm^{-1} in the DPPC/DOX sample. The PO_2^- antisymmetric double-bond stretching band (at 1,244 cm^{-1}) disappeared in the DPPC/DOX liposomal sample. These results implied that DOX was located in the interfacial region of the membrane.

The strong interactions (electrostatic interaction and hydrogen bonding interaction) between the phospholipid PO_2^- group and the DOX NH_3^+ group should not be ignored, as they might play a vital role in the interactions of DOX and phospholipids. The existence of a $\text{N}(\text{CH}_3)_3^+$ group in the polar head of DPPC might prevent the DOX NH_3^+ group from getting close to the PO_2^- group, because of the electrostatic repulsive force between the DPPC $\text{N}(\text{CH}_3)_3^+$ group and the DOX NH_3^+ group; thus, weakening the

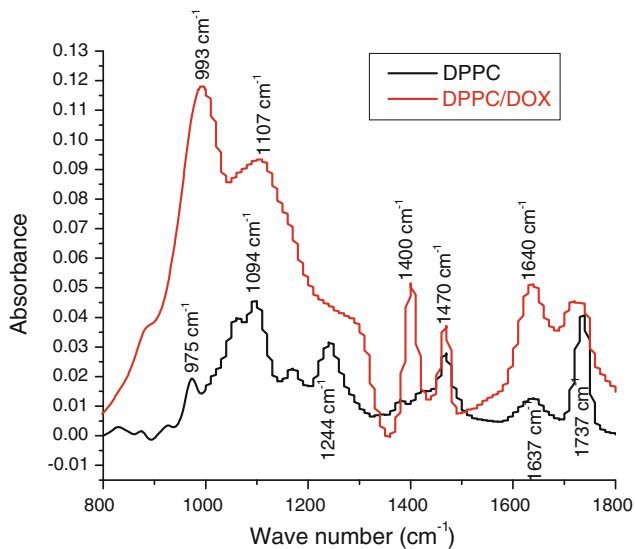


Fig. 2 The magnified region (1,800–800 cm^{-1}) of the FTIR spectra of DPPC and DPPC/DOX liposomal samples

interactions between phospholipid PO_2^- and DOX NH_3^+ groups. This could explain why the wave number of the PO_2^- band of DPPC was shifted to a higher value, from 1,094 to 1,107 cm^{-1} [29].

The Phase Behavior of DPPC/DOX

Figure 3 shows the thermograms of lyophilized DPPC liposomes and DPPC/DOX liposomes. The pre-transition temperature was around 60°C for pure DPPC liposomes. The T_m of DPPC was 106°C on dehydration, in accordance with previous studies [30, 31].

The pre-transition temperature peak for DPPC/DOX liposomes disappeared, which revealed that the amino sugar component of DOX interacted with the polar head

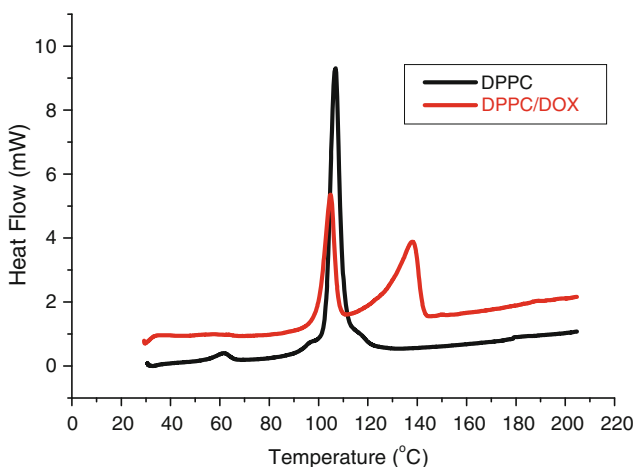


Fig. 3 DSC thermograms illustrating the effect of DOX on the gel/liquid-crystalline phase transition of DPPC

group of the phospholipids [32]. The disappearance of the pre-transition is a sensitive criterion for the incorporation of substances into lipid bilayers [33]. This fact is interpreted as a consequence of the drug insertion into the bilayer of the membrane disturbing the hydrophobic interactions between the lipid molecules [34]. The main transition temperature of DPPC liposomes (106°C) was slightly shifted to a lower temperature (104°C) for DPPC liposome encapsulated DOX, which suggested that DOX had a significant effect on the acyl chains of DPPC bilayers, and that its presence decreased the cooperative transition of the lipid acyl chains. The lowered temperature of the main DPPC transition process indicated that the incorporation of DOX is more favorable to the formation of acyl chains in a disordered and loose state. The insertion of the drug between the polar heads of DPPC could favor the development of a liquid crystalline phase less ordered than the gel phase and slightly decreases the gel-to-liquid crystal phase transition temperature as observed by DSC [34].

The above observation is in accordance with previous finding [35] regarding the fact that interaction of the encapsulated drug with the lipid components of liposomes may alter the physicochemical properties of liposomes. There was the appearance of an additional transition peak at nearly 136°C for the DPPC/DOX sample. It may be assumed that the micro-heterogeneous distribution of DOX interacting with DPPC led to the formation of lateral domains.

DOX can be incorporated into the lipid bilayers, which might be closely related to its unique structure, namely, an anthraquinone group “chromophore,” a hydrophilic aminosugar that can be easily protonated and positively charged, and a hydrophobic tetrahydropyranyl group. Thus, DOX is an amphiphilic molecule and is soluble in water as well as in polar organic solvents. Lipid bilayers have hydrophobic acyl chains as well as the polar head group. There is also electrostatic interaction and hydrophobic interaction between DOX and phospholipids; the chromophore and tetrahydropyranyl parts connect with the acyl chains, whereas the aminosugar component interacts with the polar head group of the phospholipids [32]. Therefore, it can be inserted into the lipid bilayers, although the DOX molecule is relatively large.

Rheological Measurements

The rheological properties of liposomes were measured to enable evaluation of the interaction between DOX and DPPC liposomal membranes. Figure 4 shows the flow curves for DPPC and DPPC/DOX liposomal samples. Equation 1 was used to fit the experimental data from the different samples, and the rheological parameters are shown in Table 1.

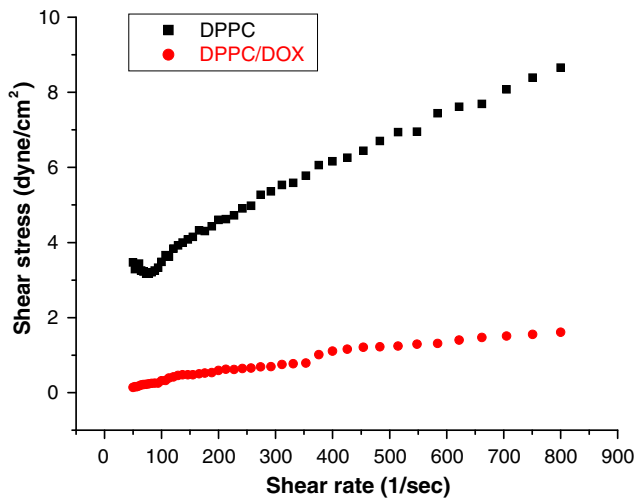


Fig. 4 The relationship between DPPC and DPPC/DOX liposome shear rate and shear stress at room temperature

Table 1 Rheological parameters for DPPC and DPPC/DOX liposomes

Sample	DPPC liposomes	DPPC/DOX liposomes
Plastic viscosity (cP)	0.76	0.67
Yield stress (dyne/cm ²)	0.28	0.12
Consistency index (cP)	1.22 ± 0.090	0.85 ± 0.095
Flow index	0.90 ± 0.010	0.80 ± 0.018
R ² -value	0.992	0.985

The yield stress (the minimum stress needed to cause a Bingham plastic to flow) decreases from 0.28 dyne/cm² for DPPC liposomes to 0.12 dyne/cm² for DPPC/DOX sample. The k and n values ranged from 1.22 to 0.85 and from 0.9 to 0.8, respectively. Liposome suspension exhibited a pseudoplastic behavior because the values of the flow behavior index (n), a measure of departure from Newtonian flow, were > 1 .

The consistency index (k), an indication of the viscous nature of liposomes [19, 20], can be used to describe the variation in plastic viscosity (a measure of the internal resistance to fluid flow of a Bingham plastic, expressed as the tangential shear stress in excess of the yield stress divided by the resulting rate of shear) after encapsulation of DOX into DPPC liposomes.

The plastic viscosity and consistency index of DPPC liposomes is decreased after DOX encapsulation into DPPC liposomes (from 0.76 to 0.67 cP), which indicates that the membrane fluidity is increased. Based on these results of liposomal rheological properties, it can be concluded that interaction between DOX and DPPC phospholipid would cause a reduction of the DPPC liposomes plastic viscosity and an increase the membrane fluidity.

Conclusions

The interaction of DOX with DPPC was investigated using non-invasive techniques, namely, FTIR, DSC and viscosity measurements. Based on these results, we propose that DOX can be easily incorporated into the DPPC bilayers, thus affecting its IR spectra, thermo tropic phase behavior and membrane fluidity. These findings facilitate a better understanding of the interactions between DOX and lipid bilayers and could help in the design and development of improved liposomal drug delivery systems.

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