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Interaction of Doxorubicin with phospholipid monolayer and liposomes

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Abstract

The effect of Doxorubicin which is (an anthracycline antibiotic with a broad spectrum of antitumor activity) on the monolayer and bilayer in the form of large Multilamellar Vesicles (MLV's) of Dipalmitoyl phosphatidylcholine (DPPC) were studied by means of monolayer techniques (surface pressure, penetration kinetics, and association constant) and light scattering technique. The monolayer technique showed that addition of DXR to a lipid film composed of (DPPC/CHOL/PEG-PE) at a molar ratio of (100:0:0) produced a less condensed Monolayer. In the (π–A) curves, DXR induced shift towards larger area/molecule, where the area/molecule was shifted from 61 to 89 A^2, and 116 A^2 in the presence of 20 and 40 nM DXR, respectively. The three curves collapsed at a pressure $\pi = 45$ mN/m. In penetration kinetics experiment ($\Delta\pi$–t), the change in pressure with time was 8 and 14 mN/m for a DXR concentration of 20 and 40 nM, respectively, and the increase in surface pressure presented a plateau over a period of 30 min. The measured association constant (K) was found to be 5×10^5/M. In the light scattering experiment, there was a shift of the transition temperature (T_m) of (MLV's) of the same composition of the monolayer towards a smaller value from 40.5° to 34.5°C. Incorporation of CHOL and PEG-PE as DPPC/CHOL/PEG-PE at a molar ratio of (100:20:0), (100:0:4) and (100:20:4) greatly counteracted the effect of DXR and made the lipid membrane more condense and rigid. Moreover, the penetration of DXR into the membrane was greatly reduced. There was a very small shift for the (π–A) and ($\Delta\pi$–t) curves, and the association constant of the drug for these different lipid compositions was greatly reduced down to 2.5×10^5/M and the transition temperature (T_m) was increased up to (42.5°C) in the presence of 40 nM DXR. Our results suggest that DXR has a great effect on the phospholipid membrane, and that addition of CHOL or PEG-PE to the phospholipid membrane causes stabilization for the membrane, and reduces the interaction with Doxorubicin. © 1998 Elsevier Science B.V.

Keywords: Doxorubicin; Monolayer; Liposomes; Drug–lipid interaction; Cholesterol

1. Introduction

Doxorubicin hydrochloride (DXR) is a cancer chemotherapeutic agent with an anthracycline structure, which consists of an aglycon, adriamycinone, combined with an amino sugar, daunosamine [1]. Doxorubicin interacts widely with some membrane models such as lipid monolayers, lipid bilayers and

Abbreviations: DXR, Doxorubicin; MLV's, Multilamellar Vesicles; DPPC, Dipalmitoylphosphatidylcholine; CHOL, Cholesterol; PEG-PE, Distearoylphosphatidylethanolamine derivatized at the amino position with polyethylene glycol; K, association constant; T_m, transition temperature

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liposomes [2,3]. The affinity of (DXR) to lipid membranes which bear no net charge is moderate. This interaction is believed to be dominated by forces other than ionic ones, since the affinity is only slightly dependent on the concentration of metal ions in the solution [4]. In any case, an electrostatic interaction is observed between the ammonium group of DXR and the phosphate group of the neutral phospholipids [5]. In contrast, DXR interacts strongly with positively or negatively charged liposomes. However, binding of Doxorubicin to charged vesicles is not strictly dependent on electrostatic interactions because the binding characteristics of a DXR derivatives, daunorubicin, which present a comparable basicity, were totally different from those of DXR [6]. Moreover, studies on model membranes suggest that adriamycin exhibits a specific affinity to membrane lipid domains [7,8], and modifies the lipid thermotropic properties [2,9]. Many studies suggested that phospholipid bilayers become more fluid upon interaction with DXR and that the latter binds with the same affinity to liposomes of various compositions [10]. In this study, we determined the influence of DXR on membrane model with different compositions, namely (DPPC) pure and mixed with different stabilizing lipids such as CHOL and PEG-PE, as well as on liposomes of the same composition as a curved membrane model (bilayer). This is an aspect that can contribute to a better understanding of the drug membrane interactions.

2. Materials and methods

2.1. Materials

L-α-Dipalmitoyl phosphatidylcholine (DPPC) specified 99% pure, and Cholesterol (CHOL) type 99 + % pure were purchased from Sigma (St. Louis, MO, USA). Distearoylphosphatidylethanolamine derivatized at the amino position with 2000 molecular weight segment of poly(-ethylene glycol), PEG-PE, was obtained from Calbiochem (La Jolla, CA). Organic solvents (chloroform and ethanol) were of analytical grade and obtained from Merck and were without surface active impurities and used without further purification. Tris buffer, molecular weight (121.14) was purchased from BDH limited poole (England). Water was triple distilled and then ultrapurified by a Millipore system (Mill-Q system).

2.2. Monolayer experiments

Films of DPPC/CHOL/PEG-PE (100:0:0), (100:20:0), (100:0:4) and (100:20:4) mole ratio dissolved in chloroform/ethanol (5:1, v/v) were spread over the subphase of a Teflon Trough filled with 10 mM Tris and 145 mM NaCl buffer (pH 7.4) solution. Compression isotherms were measured using an electromicrobalance, (Sartorius A-120-S) based on the Wilhelmy method and coupled to a Chart recorder to give a continuous reading of the force on the dipping plate. The dimensions of the Teflon Trough were $28.5 \times 16.2 \times 2.5$ cm. Compression started at least 30 min after spreading, and the compression rate was of 5 A^2/molecule/min. For each experiment, DXR was injected into the aqueous subphase beneath the lipid film and the subphase solution was stirred well by a magnetic stirrer. The final concentration of the bulk was (20 or 40 nM). Penetration kinetics were performed by spreading the necessary amount of DPPC to obtain monolayer of an initial pressure of 5 or 10 mN/m, and the area of the Teflon Trough was 245 cm^2. Different volumes of DXR solution were injected into the subphase with a Hamiltonian Syringe to attain DXR concentrations of 20 and 40 nM. Association constant (K) of the drug–phospholipid measurements were carried out consisting of measuring the change in film surface pressure with increasing drug concentration in the subphase at constant molecular area. The lipid were spread at an initial surface pressure of 10 mN/m, subsequently, the drug was added stepwise into the subphase, and the surface pressure was measured at each step. The association constant was obtained from the resulting curve as the concentration at which half maximum film expansion occurs (correction was made to overcome the non zero slope of the curves). All data reported here are the average of three measurements, and the temperature was kept at 25°C. The surface pressure was measured to an accuracy of 0.1 mN/m.

2.3. Liposomes preparation

Multilamellar vesicles were prepared by the thin film method [11]. The lipids were mixed in chloro-

form and the solvent was removed under reduced pressure. Multilamellar vesicles were formed by vigorous shaking of the lipid film in an aqueous solution of 10 mM Tris buffer and NaCl (145 mM) (pH 7.4). Liposomes of different lipid composition were prepared by the same manner by adding cholesterol, and PEG-PE purified before use [12] to DPPC at the desired molar ratios.

3. Results

3.1. Measurements of compression isotherms

Measurements of the surface pressure–area (π–A) of DPPC films are shown in Fig. 1. The Doxorubicin is added to the monolayer in the region of the liquid expanded state (surface pressure 5:10 mN/m) at which the monolayer is sensitive to any interaction [13–15]. Fig. 1A shows the effect of DXR on a monolayer composed of DPPC. The DXR was injected in the subphase (Tris buffer, at pH = 7.4) at a concentration of 20 and 40 nM. From Fig. 1, it is clear that the adsorption of DXR to the lipid film produced a less condensed monolayer, which is a consequence of the drug–lipid interaction. This finding was more evident at a surface pressure of 10 mN/m. At this pressure, which corresponds to the liquid expanded phase (LE) transition of pure DPPC monolayers, the area per molecule was nearly 61 A^2, while in the presence of 20 and 40 nM DXR, it rose to 89 and 114 A^2, respectively. However, the three curves tended to collapse at $\pi = 45$ mN/m. This results are in good agreement with those described else where [13–16]. Fig. 1B shows the effect of DXR on a mixed monolayer film composed of

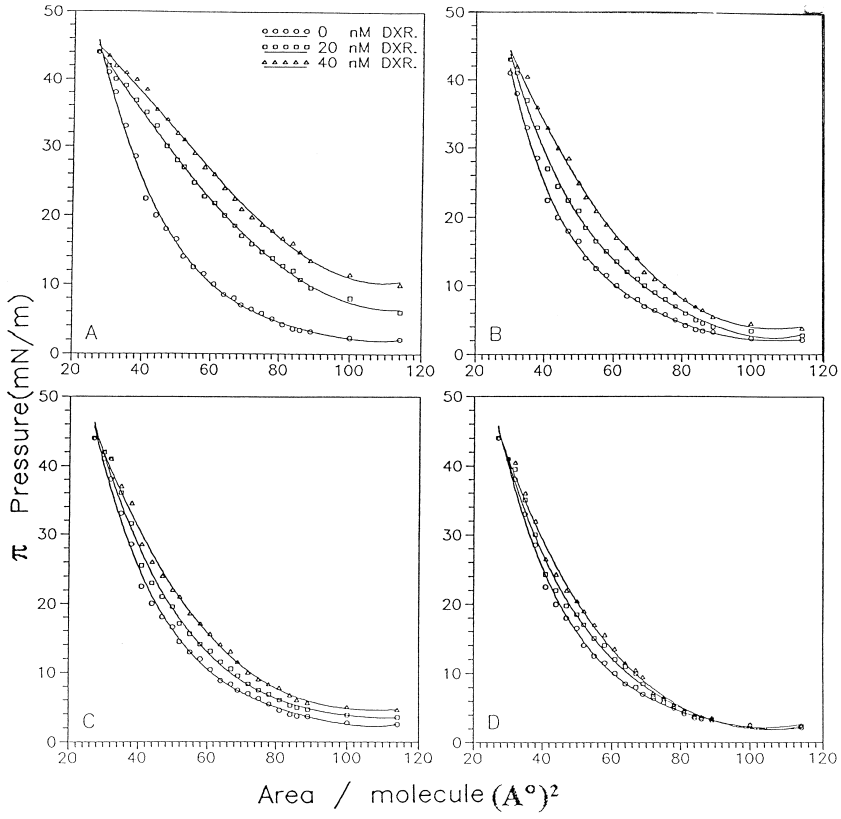

Fig. 1. Surface pressure–molecular area isotherms for adsorbed films of Doxorubicin (0, 20, 40 nM) at the DPPC/CHOL/PEG-PE–water (145 mM NaCl, 10 mM Tris–HCl, pH 7.4) interface. The molar ratios for the lipids were: (A) 100:0:0, (B) 100:20:0, (C) 100:0:4, and (D) 100:20:4, respectively.

DPPC/CHOL/PEG-PE at (100:20:0) molar ratio. It is clear from Fig. 1 that at the same DXR concentrations, there is a small shift to larger areas per molecule, where the areas per molecule were shifted to 69 and 77 A^2/molecule in the presence of 20 and 40 nM DXR, respectively. Fig. 1C shows the effect of DXR (at the same concentrations) on mixed monolayer composed of DPPC/CHOL/PEG-PE at (100:0:4) molar ratio. It is clear from Fig. 1 that there is a small shift to a larger area per molecule, where the area per molecule were shifted to 68 and 73 A^2/molecule in the presence of 20 and 40 nM DXR, respectively. Fig. 1D shows the effect of DXR on a mixed monolayer film composed of DPPC/CHOL/PEG-PE at a molar ratio of (100:20:4). It is clear from Fig. 1 that the shift in the area per molecule due to the effect of DXR is very small compared to the previous curves, where the area per molecule were shifted to 65 and 67 A^2/molecule in the presence of 20 and 40 nM DXR, respectively. Moreover, the curves tended to collapse especially at the liquid expanded phase, as clearly seen in Fig. 1.

3.2. Penetration kinetics

Initially, we determined the adsorption kinetics of DXR to the air–water interface in the absence of the lipid monolayer. DXR concentrations ranging from 10 to 100 nM were injected into the subphase. Above 90 nM, the increase in the surface pressure presented a plateau (steady state), but the increase never exceed 0.96 mN/m. This plateau was reached in 15–20 min, which is in good agreement with the results described elsewhere [15].

In Fig. 2A–D, the increase in surface pressure of lipid monolayers produced by solutions of DXR at

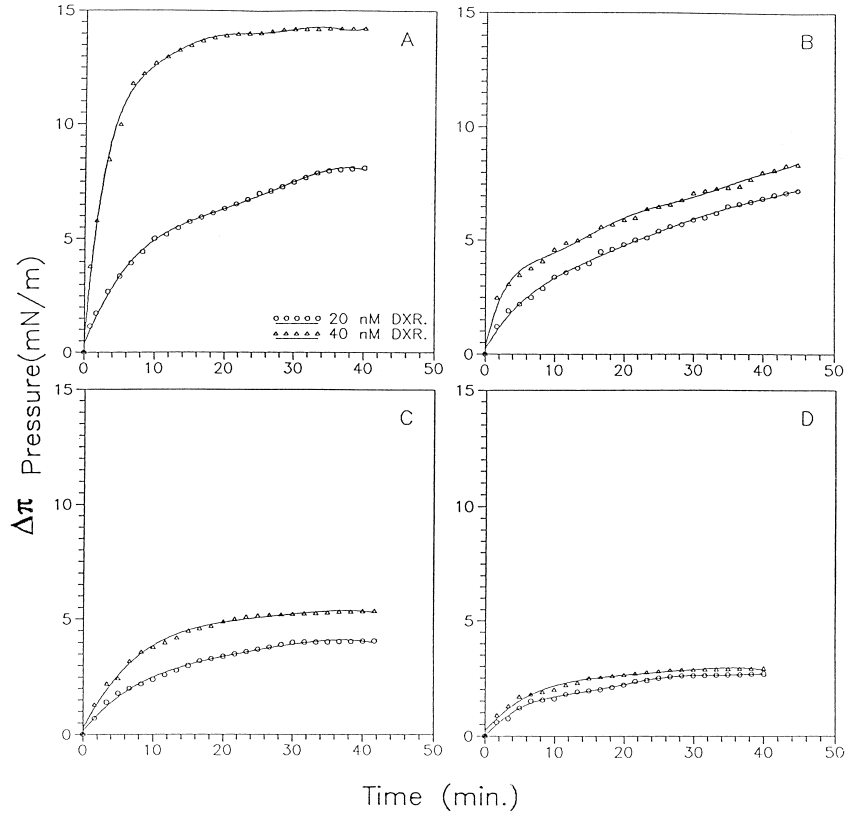

Fig. 2. Variation in surface pressure ($\Delta\pi$) with time for the interaction of Doxorubicin at two concentrations (20, 40 nM) with monolayers composed of DPPC/CHOL/PEG-PE at molar ratios of: (A) 100:0:0, (B) 100:20:0, (C) 100:0:4, and (D) 100:20:4. Subphase buffer: 145 mM NaCl, 10 mM Tris–HCl (pH 7.4). The initial pressure of the film was 5 mN/m.

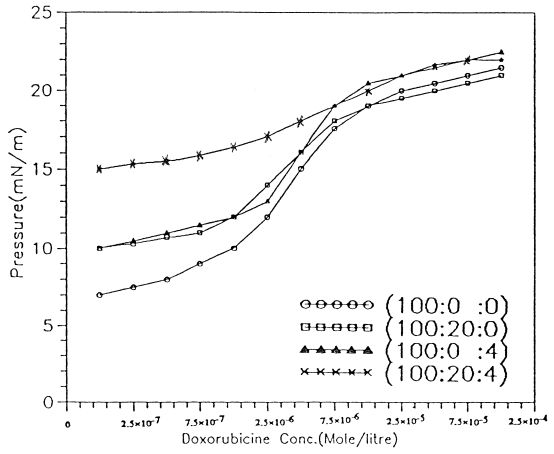

Fig. 3. Changes in the surface pressure of films composed of DPPC/CHOL/PEG-PE (at the indicated molar ratios) with increasing Doxorubicin concentration in the subphase at constant molecular area. The initial surface pressure of the film was 5 mN/m.

20 and 40 nM concentration are drawn as a function of time. Previously, it has been checked that monolayers of DPPC/CHOL/PEG-PE with different molar ratios do not exhibit any change in its surface pressure over a period of 30 min. Fig. 2A refers to DPPC monolayer at initial surface pressure (π_i) of 5 mN/m [15]. The increase in the surface pressure ($\Delta\pi$) was 8 and 14 mN/m for DXR concentration of 20 and 40 nM, respectively, and this increase in surface pressure presented a plateau over a period of 30 min. Fig. 2B,C,D refers to monolayers composed of DPPC/CHOL/PEG-PE at (100:20:0), (100:0:4) and (100:20:4) molar ratios, respectively. The increase in surface pressure was (7, 8.2), (4.2, 5.4) and (2.5, 3) mN/m at DXR concentrations of (20, 40) nM for the previous lipid monolayer compositions, respectively. It is clear from Fig. 2 that the curves became closer to each other and the change in surface pressure was reduced to a greater extent after the addition of CHOL and PEG-PE to the DPPC monolayer.

3.3. Association constant (K)

Fig. 3 shows the variation in the film surface pressure with increasing the DXR concentration in the subphase at constant molecular area (245 cm^2). The initial surface pressure of the film was 7, 10, 10 and 15 mN/m for monolayer composed of DPPC/CHOL/PEG-PE at (100:0:0), (100:20:0), (100:0:4) and (100:20:4) molar ratios, respectively. Addition of DXR up to a concentration of 10^{-4} M led to a progressive increase in surface pressure up to 20.8, 21.3, 21.8 and 22.3 mN/m for the different lipid compositions, respectively. An apparent drug-to-lipid binding constant (K) can be obtained from this curve as the concentration at which half-maximal film expansion occurs [17]. The K values obtained were found to be 5.10 ± 0.2, 4.71 ± 0.17, 4.41 ± 0.17 and $2.51 \pm 0.1 \times 10^5$/M for the different lipid compositions, respectively.

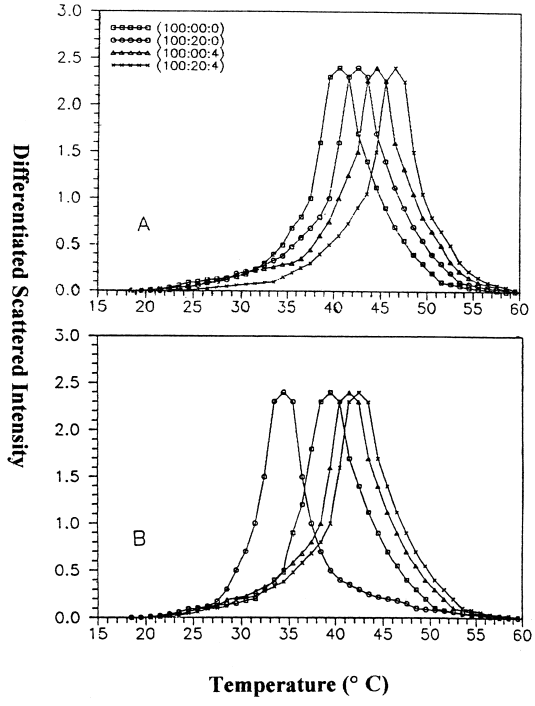

Fig. 4. Effect of Doxorubicin at a concentration of 40 nM on the scattered light intensity (graph A) of liposomes composed of DPPC/CHOL/PEG-PE at the indicated molar ratios as a function of temperature. The heating rate was 0.5°C/min. Graph (B) is the differentiated form of graph (A).

3.4. Phase transition temperature

The phase transition temperature of Liposomes (MLV's) composed of the same lipid composition used for monolayer experiments was determined through measurements of the scattered light intensity vs. temperature. Fig. 4A shows the differentiated scattered light intensity as a function of temperature for liposomes composed of DPPC/CHOL/PEG-PE at (100:0:0), (100:20:0), (100:0:4) and (100:20:4) molar ratio. Fig. 4B shows the effect of Doxorubicin (40 nM) on the differentiated scattered light intensity for the same liposome composition. It is clear that the characteristic main transition temperature (T_m) occurred at about 40.5°C for pure DPPC and that DXR at a concentration of 40 nM lowered the transition temperature to 34.5°C. However, Doxorubicin at the same concentration lowered the transition temperature from 42 to 40°C, from 45 to 42°C, and from 47 to 43°C in case of (100:20:0), (100:0:4) and (100:20:4), respectively.

4. Discussion

Doxorubicin have recently been shown to have a strong interaction with phospholipid membranes. These membrane interactions can result in changes in lipid organization, and are believed to play an important role either in its antitumoral effect or on its stability inside liposome vesicles [15]. For a complete understanding of the way in which this drug affect membrane structure, information is required at the molecular level on the interaction of this drug with different membrane models. The present study of monolayer experiments, and the phase transition for the prepared samples can all cooperate to give a clear understanding of the characteristics of the monolayer and liposome vesicles of the same composition, as well as the mode of action of the drug with phospholipid membranes.

The interaction of Doxorubicin, with DPPC monolayer have been dominated largely by both hydrophobic interactions and electrostatic interactions, which appear to be involved in the interaction of anthracyclines with DPPC monolayers and bilayers [12,15]. It is clear, as shown in Fig. 1AFig. 2A, that due to this interaction, DXR produced a less condensed monolayer, thus, at concentrations 20, and 40 nM DXR, the compression isotherms were shifted to a larger area per molecule. Fig. 1AFig. 2A also indicate that the greater the concentration of the drug in the subphase, the greater the interaction obtained [15]. Doxorubicin, due to its structure, can penetrate into the hydrophobic core of the lipid layer [1]. This insertion into the hydrocarbon region explains the increased apparent area occupied in the presence of DXR which is clear from Fig. 1.

Doxorubicin, due to its localization in the acyl chains of the DPPC molecules, can decrease the enthalpy of the transition between the gel and the liquid crystalline phase, and subsequently, increases membrane fluidity [15]. The main transition temperature (T_m) was decreased as shown in Fig. 4A and B, where the transition temperature was shifted from 40.5°C for pure DPPC to 34.5°C for DPPC + DXR (40 nM).

The membrane affinity for DXR was calculated by measuring the association constant (K) of the drug which was found to be $5 \pm 0.2 \times 10^5$/M for pure DPPC as calculated from Fig. 3. Addition of cholesterol to the pure DPPC membrane increased the rigidity of the membrane, and for this reason, it was logical to suppose that the uptake of the drug molecules from the water phase was considerably slow. Fig. 3 shows also that the membrane affinity of the DXR to lipid membrane containing cholesterol is lowered and the value calculated of the association constant (K) was $4.7 \pm 0.17 \times 10^5$/M, which is lower than that of pure DPPC alone.

The stiffness of the DPPC:CHOL membrane was evident from Fig. 1B, where the curves became closer. Fig. 2B shows that the difference between the effect of the two DXR concentrations is small and the monolayer required larger time to uptake DXR.

Doxorubicin showed a dramatic effect on the phase transition temperature of multilamellar vesicles liposomes. However, this dramatic effect was reduced in the presence of cholesterol in the bilayer membrane. Fig. 4 shows that the transition temperature (T_m) for DPPC liposomes in presence of 40 nM DXR is 34.5°C, while that for DPPC + CHOL liposomes is 39°C at the same DXR concentration, this may be due to conformational changes aroused by cholesterol in the bilayer membrane [18].

The addition of polyethylene glycol (PEG-PE) by 4% mole ratio to the pure DPPC may decrease the motion of the choline methyl group, predominantly through coulombic and hydrophobic interaction forces, respectively [19]. The organization of PEG-PE in the lipid bilayer as a model membrane system was assumed to have part of the polymer incorporated into the hydrophobic portion of bilayer and the other part in the polar head group region (Choline head) [20]. Such organization can highly lower the DXR penetration to the lipid membrane. This was clear from Fig. 2CFig. 3C. The decrease of the drug lipid affinity was also indicated by the decrease of the association constant (K) which was $4.4 \pm 0.17 \times 10^5/\mathrm{M}$.

PEG-PE also affected the gel-to-liquid crystalline phase transition of vesicles membrane, where the incorporation of PEG-PE to large DPPC vesicles increased the phase transition temperature (T_m) from 39°C to 42°C for pure DPPC. The effect of DXR (40 nM) on the transition temperature of DPPC + PEG-PE liposome was shown in Fig. 4C,D where the transition temperature (T_m) was raised from 34.5°C to 41.5°C. This increase in the transition temperature may be attributed to the suppression of the molecular motion of lipids through the interaction with polymers, resulting in a reduced effect of DXR on lipid membranes.

Finally, the effect of DXR on mixed lipid system composed of DPPC plus CHOL (20%) and PEG-PE (4%) can be shown in Fig. 1DFig. 2DFig. 3DFig. 4D. Figs. 1–4 show that the addition of cholesterol and PEG-PE to the phospholipid system greatly stabilizes the membrane and reduces its interaction with DXR. This is clear from the measured association constant for DXR with such complex membrane model, which was found to be lower than the association constant in case of DPPC with CHOL alone or DPPC with PEG-PE alone ($2.5 \pm 0.1 \times 10^5/\mathrm{M}$), and from the phase transition temperature for such a complex system, which was found to be 42.5°C (in presence of 40 nM DXR) compared with 34.5°C in case of DPPC, 39°C in presence of 20% CHOL and 41.5°C in presence of 4% PEG-PE.

5. Conclusion

To sum up, we may conclude that in order to obtain a stable membrane composition used as a drug delivery system for Doxorubicin, we may include 20% CHOL and 4% PEG-PE in our liposome composition [21].

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