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International Journal of Pharmaceutics 338 (2007) 258–266
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

DSC and EPR investigations on effects of cholesterol component on molecular interactions between paclitaxel and phospholipid within lipid bilayer membrane

Lingyun Zhao [a], Si-Shen Feng [a,b,*], Nikolai Kocherginsky [a], Iouri Kostetski [b]

[a] *Department of Chemical & Biomolecular Engineering, Faculty of Engineering, National University of Singapore, Block E5, 02-11, 4 Engineering Drive 4, Singapore 117576, Singapore*
[b] *Division of Bioengineering, Faculty of Engineering, National University of Singapore, Block E5, 02-11, 4 Engineering Drive 4, Singapore 117576, Singapore*

Received 5 June 2006; received in revised form 18 January 2007; accepted 27 January 2007
Available online 2 February 2007

Abstract

Differential scanning calorimetry (DSC) and electron paramagnetic resonance spectroscopy (EPR) were applied to investigate effects of cholesterol component on molecular interactions between paclitaxel, which is one of the best antineoplastic agents found from nature, and dipalmitoylphosphatidylcholine (DPPC) within lipid bilayer vesicles (liposomes), which could also be used as a model cell membrane. DSC analysis showed that incorporation of paclitaxel into the DPPC bilayer causes a reduction in the cooperativity of bilayer phase transition, leading to a looser and more flexible bilayer structure. Including cholesterol component in the DPPC/paclitaxel mixed bilayer can facilitate the molecular interaction between paclitaxel and lipid and make the tertiary system more stable. EPR analysis demonstrated that both of paclitaxel and cholesterol have fluidization effect on the DPPC bilayer membranes although cholesterol has more significant effect than paclitaxel does. The reduction kinetics of nitroxides by ascorbic acid showed that paclitaxel can inhibit the reaction by blocking the diffusion of either the ascorbic acid or nitroxide molecules since the reaction is tested to be a first order one. Cholesterol can remarkably increase the reduction reaction speed. This research may provide useful information for optimizing liposomal formulation of the drug as well as for understanding the pharmacology of paclitaxel.

Keywords: Anticancer drugs; Differential scanning calorimetry (DSC); Electron paramagnetic resonance spectroscopy (EPR); Liposomes; Nanomedicine; Taxol®

1. Introduction

Paclitaxel (structure shown in Fig. 1) is one of the best antineoplastic drugs found from nature in the past decades, with excellent therapeutic effects for a wide spectrum of cancers including breast cancer, ovary cancer, small/non-small cell lung cancer, and AIDS-related Kaposi's sarcoma (Eric, 1994). In order to eliminate the serious side effects associated with its current clinical adjuvant called Diluent 12 (Cremophor EL and ethanol; 1:1,v/v) liposomal formulation of paclitaxel has been intensively investigated as an alternative formulation as well as for controlled and targeted delivery (Nussbaum et al., 2004; Streith et al., 2004; Fetterly and Straubinger, 2003; Kunstfeld et al., 2003; Schmitt-Sody et al., 2003; Dosio et al., 1997; Greenwald et al., 1996; Dordunoo et al., 1995; Rodrigues et al., 1995; Onyuksel et al., 1994; Bartoli et al., 1990; Tarr et al., 1987). Molecular interaction between the drug and the lipid bilayer membrane has decisive influence on the liposomal formulation process and the drug release from the liposomes, which determine partitioning, allocation, orientation, and conformation of the drug in the bilayer membrane and thus plays an important role in transport, distribution, accumulation, and eventually, efficacy of the drug (Joachim et al., 1992, 1994; Raimund et al., 2002).

Abbreviations: DSC, differential scanning calorimetry; EPR, electron paramagnetic resonance; DPPC, dipalmitoylphosphatidylcholine; CD, circular dichroism; GS, gramicidin S; PC, phosphaticylcholine; 5-DSA, 5-doxyl stearic acid; 16-DSA, 16-doxyl stearic acid

* Corresponding author at: Division of Bioengineering, Faculty of Engineering, National University of Singapore, Block E5, 02-11, 4 Engineering Drive 4, Singapore 117576, Singapore. Tel.: +65 6516 3835; fax: +65 6779 1936.
E-mail address: chefss@nus.edu.sg (S.-S. Feng).

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doi:10.1016/j.ijpharm.2007.01.045

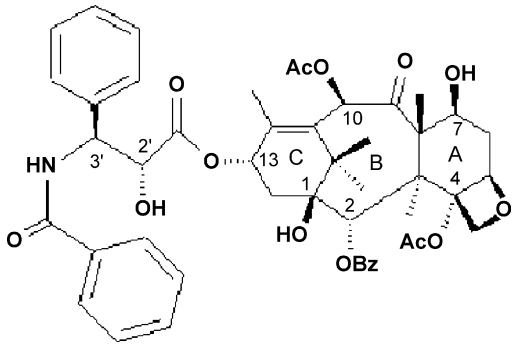

Fig. 1. Molecular structure of paclitaxel.

Paclitaxel–biomembrane interactions can be investigated by using the lipid monolayer at the air–water interface and the bilayer vesicles (liposomes) as model cell membrane. Various characterization techniques, such as Langmuir film balance technique, atomic force microscopy (AFM), circular dichroism (CD), DSC, fluorescence polarization and X-ray diffraction, have been employed in the literature. Paclitaxel was found to partition into the lipid membrane, perturbing the hydrocarbon chain conformation and inducing a broadening of the lipid phase transition. Incorporation of paclitaxel into the lipid bilayer also affects other physical properties of the bilayer such as the lipid order parameter (fluidising effect) (Balasubramanian and Straubinger, 1994; Wenk et al., 1996; Bernsdorff et al., 1999; Ali et al., 2000; Belsito et al., 2005). Recently, we have carried out serial research to investigate molecular interactions between paclitaxel and the cell membrane by adopting both lipid monolayer and liposomes as model membrane. It was demonstrated that the molecular structure of phospholipids, such as lipid chain length, chain unsaturation and head group type have a profound effect on the paclitaxel–biomembrane interactions (Feng et al., 2002; Zhao et al., 2004; Zhao and Feng, 2004; Zhao and Feng, 2005; Zhao and Feng, 2006). Nevertheless, effects of the cholesterol component in the lipid bilayer membrane on the molecular interaction between paclitaxel and lipid membrane have rarely been addressed in the literature. As a major component of the cell plasma membrane, cholesterol has unique cellular functions, such as stabilizing membrane fluidity and filling spaces between neighbouring phospholipids (Yeagle, 1995). It also has been found that cholesterol may have different effects on the interactions between biomolecules and biomembranes, depending on the nature of the biomolecules (Gallois et al., 1998; Ghannam et al., 1999; Prenner et al., 2001). In brief, there is a large body of evidence showing that cholesterol may affect a number of processes of drug–membrane interactions and the interactions between guest molecules and cholesterol-contained lipid membranes are much more complex than those found from the neat lipid membranes (Soderlund et al., 1999).

In this paper, effects of the cholesterol component on molecular interactions between paclitaxel and phospholipid within lipid bilayer membrane were investigated by DSC and EPR techniques. DSC is a thermal analytical technique, which is used most often in studies of lipid thermotropic phase behaviour of liposomes and biological membranes, from which the effects of cholesterol component on the molecular interactions between the drug paclitaxel and the phospholipids within the bilayers membrane can be quantitatively investigated. Of the various spectroscopic techniques, spin-label electron paramagnetic resonance (EPR) has proved to be one of the most useful tools applicable in this field. Its dynamic sensitivity is optimally matched to the timescale of the rotational motions of the lipids in biological membranes (Lange et al., 1985; Moser et al., 1989). From EPR analysis it is possible to quantify both the stoichiometry and the selectivity of the interactions of different spin-labelled lipids with the protein and to study the dynamics of the protein-associated lipids (Lange et al., 1985; Moser et al., 1989; Marsh and Horvath, 1998). It also can provide decisive information on the localization the drug molecules in the liposomal membrane and reflect the changes in the membrane mobility (Budai et al., 2003, 2004). This spectroscopic technique allows us to detect changes in the spin tropic movement of an unpaired electron. Biomolecules such as PC or cholesterol with no unpaired electrons can still be studied by EPR when they are surrounded or chemically bonded to a stable free radical. This radical or spin label produces a sharp and simple EPR spectrum that yields information about the molecular environment of the label (Ahlin et al., 2000). In the present study, 5-doxyl stearic acid (5-DSA) and 16-doxyl stearic acid (16-DSA) were chosen as the free radicals, which are oriented like the lipids in the bilayer. The radical in the 5- or 16-position of the alkyl chain can thus determine local motional profiles in the two main regions of the lipid bilayer near the polar head group (5-DSA) or at the end of the hydrophobic chain.

In this research, we reported for the first time in the literature how DSC and EPR were applied to investigate the effects of cholesterol component on molecular interactions of paclitaxel with the lipid membrane. The results can provide comprehensive insights and guidance on the liposomal formulation of paclitaxel for chemotherapy of cancer and other diseases such as cardiovascular restenosis and AIDS.

2. Materials and methods

2.1. Materials

DPPC (1,2-dipalmitoyl-sn-glycerol-3-phosphocholine) was purchased from Avanti Polar Lipid, Inc. (Alabaster, Al, USA). Paclitaxel was obtained from Yunnan Hande Bio-Tech Co. Ltd. (Kunming, PR China). Cholesterol was purchased from Sigma (St. Louis, MO, USA). 5-DSA and 16-DSA were obtained from Aldrich Chem. Co. (Milw, WI, USA). Ascorbic acid was manufactured by NACALAI TESQUE (KYOTO, Japan).

2.2. Liposome preparation

Liposomes were prepared by the traditional thin-film method. Briefly, the lipid and paclitaxel stock solutions were prepared in chloroform and appropriate amounts of each were pipetted into a 25 ml flask. After vortexing to ensure thorough mixing, the solvent was allowed to evaporate by applying a nitrogen stream. This resulted in the formation of a thin lipid film on the inside

wall of the flask. The film was stored overnight in a vacuum desiccator to ensure complete evaporation of chloroform. One millilitre of Milli-Q water was added into the flask, which was then vigorously vortexed for 1 min. The flask was placed in a bath-type sonicator at temperature above the main phase transition of the lipid. The solution was sonicated for 5–10 min. The flask was then placed in a shaking water bath at 37 °C for 1 h to ensure complete mixing of the contents. For EPR analysis, liposomes were formed to achieve a final lipid concentration of 5×10^{-3} M with pH value around 6.5.

For EPR studies, liposome samples were labelled with 5-DSA or 16-DSA. The spin-label solution was added to the lipids before taken to dryness with label concentration of 3×10^{-4} M.

2.3. DSC measurement

DSC measurements were performed by using Perkin Elmer Pyris 6 DSC (USA) with empty hermetically sealed aluminium pans as reference. The lipid concentration was made 20 mg/ml and 10 μl of liposome suspension was carefully placed and sealed in the aluminium hermetic pans. The scan rate was set at 2 °C/min.

2.4. EPR analysis

The EPR measurements were performed using a Bruker (Bruker BioSpin Gmbh, Rheinstetten/Karlsruhe, Germany) Elexsys Series E500 CW-EPR X-band (9–10 GHz) spectrometer. The measurements were carried out with a standard quartz EPR Flat cell of 21 cm length and 1.5 mm inner diameter. The spin-labelled liposomes were drawn in the Flat cell for analysis. All measurements were carried out at 23 °C.

2.5. Kinetics of reduction of nitroxides by ascorbic acid

Ascorbic acid solution was added into the liposome suspensions. The mixture was drawn with a syringe into a gas-permeable teflon (polytetrafluoroethylene) capillary (PTFE, 0.81 mm i.d., 0.86 mm o.d.; Zeus Industrial Products, NJ) and the ends of capillary (length ca. 3 cm) were closed by crimping. The capillary was inserted into a standard EPR quartz tube with holes in the bottom of the tube. Oxygen content was bubbled out by regulating nitrogen flux.

3. Results and discussion

3.1. DSC study of DPPC/paclitaxel liposomes

Fig. 2 shows the DSC thermographs of paclitaxel/DPPC liposomes of various mole fractions of the drug. Two thermal transitions were observed for the DPPC liposomes in the absence of paclitaxel: a sharp acyl chain melting transition at 42.3 °C and small broad pretransition at 35.4 °C. Presence of small amount of paclitaxel can eliminate the pretranstion of DPPC liposomes. The pretransition arises from the transformation from an $L_\beta{}'$ bilayer structure to the $P_\beta{}'$ conformation. Because the pretransition is highly sensitive to the presence of other molecules in the polar region of the phospholipids, the loss of the pretransition cannot be ascribed to any specific molecular changes. It can be observed from Fig. 2 that at lower paclitaxel concentration, the main transition temperature is shifted slightly to a lower temperature and the sharp main transition of the DPPC bilayer is broadened. This can be demonstrated from the transition width at half-peak height ($\Delta T_{1/2}$), which increases from 0.3 °C for the pure DPPC vesicles to 0.5 °C for the DPPC bilayer vesicles of 1 mol% paclitaxel and 0.6 °C for those of 10% paclitaxel. $T_{1/2}$ is a measure of destabilization of the phospholipid assemblies, indicating a decrease in size of the cooperative unit. Our results showed that incorporation of paclitaxel into DPPC bilayers caused a reduction in the cooperativity of the transition, leading to a looser and more flexible bilayer. The main transition marks the transition of the bilayer from a highly hindered all-trans hydrocarbon chain conformation to a state in which some acyl chains exist in the gauche (kinked) conformation, resulting in greater phospholipid rotational freedom.

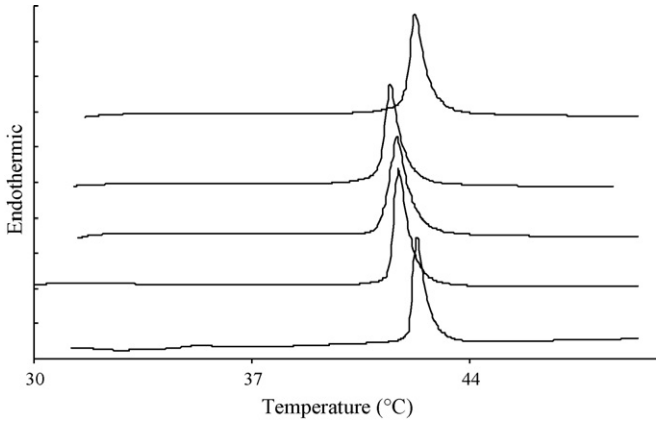

Fig. 2. DSC thermographs of DPPC/paclitaxel liposomes of (from bottom to top) 0%, 1%, 2%, 5%, 10% paclitaxel.

The observed paclitaxel induced changes in the main phase transition provide further information on the hydrophobic location of the drug in the bilayer matrix. Such an effect would be expected if paclitaxel were localized in the outer hydrophobic cooperative zone of the bilayer, i.e., in the region of the C1–C8 carbon atoms of the acyl chain. Such a location for paclitaxel in the bilayer could be anticipated from the structure of the drug. The C13 side chain of the paclitaxel is relatively hydrophobic because of the two aromatic rings, while the main taxane ring bears substituents that have comparatively greater propensity for polar interactions.

It is noticed in Fig. 2 that increasing the amount of paclitaxel beyond 5% does not cause any further disturbance to the DPPC vesicles, as the thermograms are more similar to those of the liposomes in the absence of paclitaxel. This result is in good agreement with previous DSC data on the effect of paclitaxel in liposomes of phosphatidylcholine as concerns the effects of the drug concentration on the main phase transition temperature (Balasubramanian and Straubinger, 1994; Bernsdorff et al., 1999; Belsito et al., 2005). This may imply that there exists a maximum solubility of paclitaxel in the DPPC bilayer. Paclitaxel above this concentration (5%) may sequester at the

water–lipid interface in the liposomes, or form micelles or emulsions with phospholipids, which are not detectable under the DSC conditions. Such structures have been reported for paclitaxel associated with phospholipids (Kan et al., 1999; Burt et al., 1999). Another possible explanation is that due to the bulky structure of the paclitaxel molecule, it may form a screen that limits a deeper penetration of other paclitaxel molecules.

3.2. DSC study of cholesterol effect on the DPPC/paclitaxel liposomes

The effect of the cholesterol component on the transition parameters of the lipid/paclitaxel mixed bilayer was investigated. The phase transition of the cholesterol/DPPC is modified to show a progressive broadening of the transition peak. The results in Fig. 3 show that when cholesterol is incorporated into the lipid bilayer, it exerts strong perturbing effects on the DPPC/paclitaxel mixed bilayer systems. The phase transition of the tertiary systems is modified to show a progressive broadening of the transition peaks. It is also significant to note that cholesterol causes a decrease in ΔH of the main transition endotherm of the DPPC/paclitaxel/cholesterol tertiary system. The effect on the enthalpy of the transition depends on the localization of the molecule in the phospholipid bilayers. When changes are observed in transition enthalpy, it is normally attributed to the location of molecules within the hydrophobic interior of the phospholipid array, and not superficially in the vicinity of the polar groups or at the interfacial region of the phospholipids.

3.3. Stability of the DPPC/paclitaxel bilayer vesicles and effect of cholesterol component

DPPC vesicles (free of cholesterol or with 10 mol% cholesterol) with 2% paclitaxel were further investigated over a period of several days to investigate the long-term stability of the vesicles. The vesicles were kept in the round bottom flask and put in the shaker. The temperature was kept constant at 37 °C and the shaking speed was set to be 120 rpm.

Fig. 4 shows that the phase transition shifts as the temperature gradually increases, which indicates that paclitaxel is released from the DPPC vesicles with time goes on. This may suggest that DPPC/paclitaxel vesicles are not stable over long period of time. Bernsdorff et al. studied the time dependent DSC thermographs of DPPC/paclitaxel vesicles of various molar ratios (Bernsdorff et al., 1999). Their results showed that the pretransition of DPPC liposomes could be observed 7 days after the preparation of the samples, which suggested that the DPPC/paclitaxel liposomes were not stable in such a long period. Our findings are in good agreement with their results. However, we did not observe the re-appearance of the pretransition, probably due to the shaking of the samples. Control DPPC vesicles were also maintained under the same conditions but no significant change was found for their thermographs.

When 10% cholesterol was incorporated into DPPC liposomes, both the thermograph shape and phase transition temperature of the vesicles did not change noticeably as compared with the cholesterol free liposomes. It may thus suggest

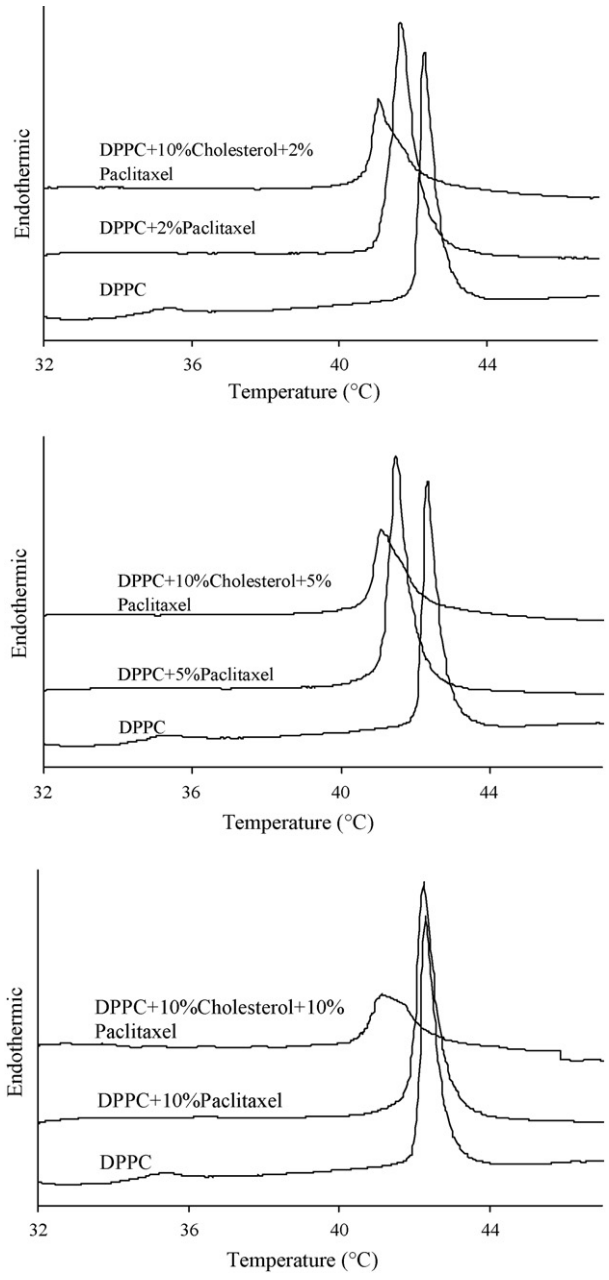

Fig. 3. DSC thermographs of DPPC/10% cholesterol/paclitaxel liposomes.

that cholesterol makes the vesicles more stable. When cholesterol is present in the mixed DPPC/paclitaxel bilayer, it would fill the space between the DPPC and paclitaxel molecules due to its small size and amphiphilic nature. In this case, cholesterol can be functioned as "paste" or "dynamic glue" in a sandwich, making the tertiary system more condensed and therefore enhancing the intermolecular forces between DPPC and paclitaxel.

3.4. EPR study on DPPC/paclitaxel liposomes

The EPR parameters obtained at room temperature for the DPPC liposomes of 5-DSA and 16-DSA are presented in Fig. 5. It is well documented that the EPR spectra of 5-DSA incorporated into the lipidic membranes show an anisotropic motion,

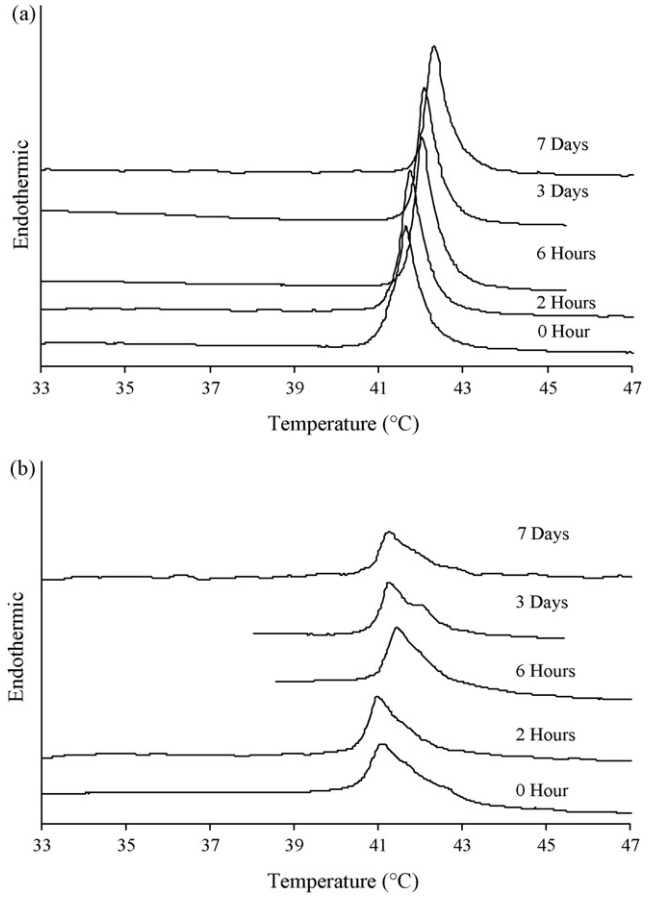

Fig. 4. DSC thermographs of DPPC vesicles with 2% paclitaxel as a function of time: (a) free of cholesterol; (b) with 10% cholesterol.

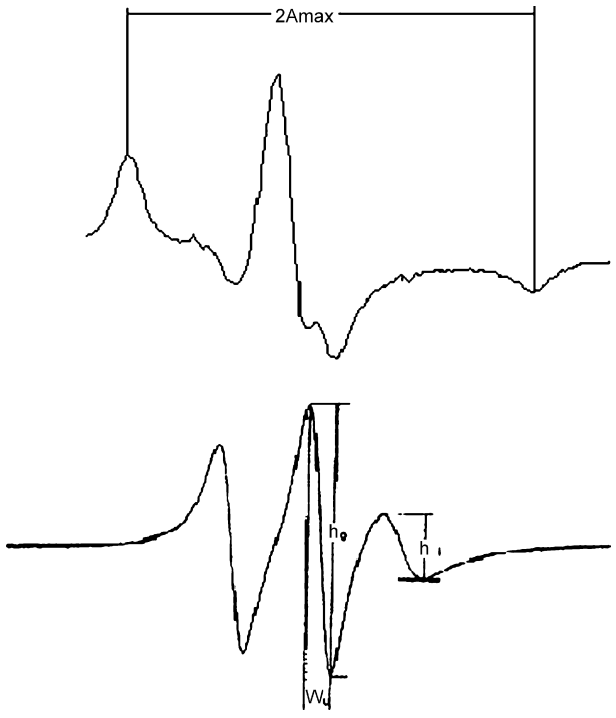

Fig. 5. Typical EPR spectra of 5-DSA and 16-DSA labelled DPPC liposomes.

Table 1
EPR parameters of 5-DSA and 16-DSA labelled DPPC/paclitaxel liposomes

	$2A_{max}$ (G)	τ (s)
DPPC	62.27	4.24×10^{-9}
2% paclitaxel	61.52	4.26×10^{-9}
20% paclitaxel	61.26	4.24×10^{-9}

and the fluidity of the membrane can be estimated from the outermost separation between the spectral extremes, i.e., the maximum hyperfine splitting ($2A_{max}$). The value of $2A_{max}$ reflects the rotational freedom of DPPC close to the polar head groups in the bilayer. This value increases with the decrease in fluidity.

Since maximum hyperfine splitting is inversely related to the fluidity, a logical decrease in $2A_{max}$ is observed for all the samples when the paclitaxel is incorporated into the DPPC liposomes. This suggests that addition of paclitaxel can make the liposome more fluidized. This observation is in good agreement with our DSC results.

The EPR spectrum of 16-DSA incorporated into the lipidic bilayer reflects an isotropic motion of the phospholipid acyl chain. In this case, the rotational correlation time (τ) is the parameter that can be used to measure the motion of the phospholipid acyl chains near the hydrophobic end. This empirical parameter can be calculated by the equation from Coderch et al. (2000):

$$\tau = (6.5 \times 10^{-10}) \times W_0 \left[\left(\frac{h_0}{h_{-1}} \right)^{0.5} - 1 \right] \quad (1)$$

The relevant parameters derived from EPR spectra of 16-DSA labelled liposomes can be found in Table 1. As in the case of $2A_{max}$, τ increases with the decrease in fluidity. No noticeable change can be observed for the τ parameter when paclitaxel is added to the DPPC liposomes. This implies the molecular dynamics around C16 in the acyl chain of DPPC is rarely perturbed by paclitaxel.

It should be pointed out that there may be a problem with the determination of the correlation time by using EPR spectra. According to the linewidth-theory, Eq. (1) is valid if and only if the lineshape can be taken as Lorentzian. To our experiences, although the ratio of the amplitudes (h_0/h_{-1}) can be used to describe differences in the fluidity between control and treated samples, absolute values of the correlation times might become incorrect if the conditions for the evaluation were not met.

Our EPR results agree well with those obtained from the DSC study in the previous section. The minor change of the EPR parameters caused by paclitaxel suggests that the molecular interaction between paclitaxel and phospholipid is insignificant or non-specific. Paclitaxel causes an increase in the local membrane fluidity and accordingly, a decrease in the viscosity of the bilayer membrane. Compared with the 16-DSA labelled liposomes, greater change of the EPR parameters can be observed for the 5-DSA labelled liposomes. This observation strongly suggests that the binding site of paclitaxel to the phospholipid

vesicles is in the outer hydrophobic cooperative zone of the bilayer, probably in the region of the C1–C4 carbon atoms of the acyl chain.

3.5. EPR study on DPPC/cholesterol liposomes

Changes in membrane fluidity caused by cholesterol near the hydrophobic end of the acyl chains have been reported to be somewhat different from those observed near the polar groups (Nagumo et al., 1991). A number of studies have indicated that cholesterol greatly interferes with the most hydrophobic part of the bilayer. In the present study, the two doxyl nitroxide 5-DSA and 16-DSA with the radical at the beginning and at the end of the hydrophobic changing were chosen to monitor the changes in fluidity of the DPPC/cholesterol liposomes of various molar ratios.

Table 2 shows the EPR parameters of the DPPC/cholesterol liposomes at various ratios at 23 °C, which are labelled with 5-DSA and 16-DSA. As greater change of the EPR parameters has been noticed for the DPPC/cholesterol liposomes than that for the DPPC/paclitaxel liposomes, indicating cholesterol has greater effect on the lipid membrane than paclitaxel does.

The effect of the cholesterol component on the lipid membranes fluidity is temperature dependent: the fluidity of the membrane increases with cholesterol incorporation below the phase transition temperature of the lipid bilayer while the contrary effect can be observed at temperature higher than the phase transition temperature. For the DPPC/cholesterol bilayer, our results show that the $2A_{max}$ and τ parameter decrease with cholesterol molar ration since the EPR analysis was operated at room temperature (23 °C), which is lower than the phase transition temperature of the DPPC bilayer (about 42 °C). The $2A_{max}$ value can also be used to judge if the prepared liposomes are single-layered unilaminar vesicles (SUVs) or multilayered vesicles (MLVs). From the literature, liposomes prepared by the thin-film method could be a mixture of MLVs and SUVs. The $2A_{max}$ values shown in Table 2 imply that the liposomes we obtained could be a mixture with more MLVs and less SUVs. The possible reason may be the ineffective sonication.

The evaluation of bilayer fluidity is based on the measurements with two probes at different acyl chains regions. Our results show that the effect of cholesterol on the bilayer fluidity is similar, though not the same, in the different acyl chain sections. It is demonstrated that cholesterol has greater effect on the hydrophobic areas since when 20% cholesterol was added to the liposomes, the τ parameter decreased by 40%, while the $2A_{max}$ decreased by 2.1%. This finding confirms our DSC observations that the location of cholesterol is within the hydrophobic interior of the phospholipid but not in the locality of the polar groups or at the surface region of DPPC bilayer. Compared with paclitaxel, cholesterol can penetrate more deeply into the hydrophobic core of the liposomes. Nagumo et al. also found by EPR more significant effect of cholesterol near the hydrophobic end, which resulted in fluidization below T_m and condensation above T_m (Nagumo et al., 1991).

Table 2
EPR parameters of 5-DSA and 16-DSA labelled DPPC/cholesterol liposomes

	$2A_{max}$ (G)	τ (s)
DPPC	62.27	4.24×10^{-9}
20% cholesterol	60.99	2.55×10^{-9}
50% cholesterol	57.69	1.26×10^{-9}

3.6. Reduction kinetics of nitroxides by ascorbic acid

As it is observed that EPR spectrum of DPPC liposomes is not changed remarkably with the incorporation of paclitaxel, another set of experiment was carried out to study the reduction kinetics of nitroxides by ascorbic acid. Since it has been demonstrated that paclitaxel may be localized in the polar region of the DPPC liposomes, 5-DSA was chosen as the probe for this analysis. Reduction of nitroxides by ascorbic acid is of particular interest because of its potential role in biological systems and its widespread use in model systems (Kocherginsky and Swartz, 1995; Kristl et al., 2003). Such investigation has been carried out extensively, especially for its pH dependence (Perkins et al., 1980). The only product of stoichiometric reduction by ascorbate in the absence of oxygen is the dydroxylamines (Kocherginsky et al., 1981):

$$2 > NO^- + \text{asorbate} \rightarrow 2 > NOH + \text{dehydroascorbate}$$

Reduction by ascorbate has been suggested as a method of quantitative determination of nitroxides, but in the presence of oxygen, the apparent rate of reduction may decrease due to oxidation of ascorbate and/or reoxidation of the hydroxylamine. Before measuring the rate of interaction of nitroxides with ascorbate, nitrogen has to be bubbled vigorously through the solutions of both reagents to remove dissolved oxygen. Experiment was carried out by measuring the peak height of the EPR spectrum changing with time. The concentration of nitroxides is decreased because nitroxides are reduced by ascorbic acid and this will lead the EPR spectrum intensity to become weaker with time goes on.

Fig. 6 shows the reduction kinetics of nitroxides by ascorbic acid inside the DPPC liposomes with and without paclitaxel. κ is defined as the slope of the curves and can be regarded as the rate constant of the reduction:

$$\kappa = \frac{\mathrm{d}\ \ln\left(\frac{I_t}{I_0}\right)}{\mathrm{d}t} \quad (2)$$

where I_t is the peak height of the EPR spectrum at given time during the reaction and I_0 is the peak height of the EPR spectrum at the beginning of the reaction. It is clearly demonstrated in Fig. 8 that both of the reaction speed and the extent are reduced noticeably by the presence of paclitaxel in the DPPC liposomes. The reaction barely takes place when 5% paclitaxel is incorporated. The reaction takes place simultaneously when the 5-DSA labelled vesicles are put in the ascorbic acid solution since there is no lag time shown in Fig. 8. The ascorbic acid molecule will penetrate the headgroup layer of the DPPC liposomes because

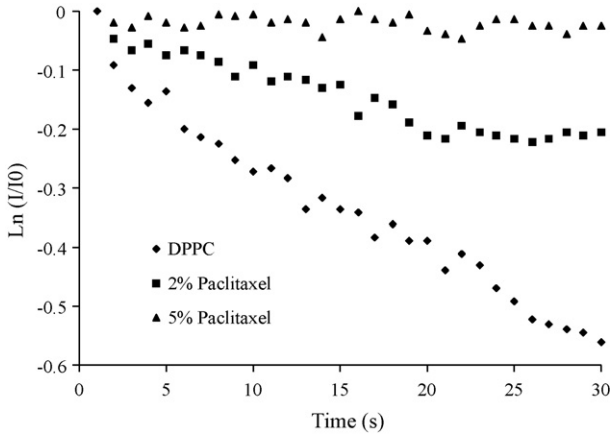

Fig. 6. Semilog plot of the ascorbic acid induced decrease of the peak heights of EPR spectrum of 5-DSA in a suspension of liposomes with ascorbic acid. Ascorbic acid concentration: 3×10^{-4} mol/l.

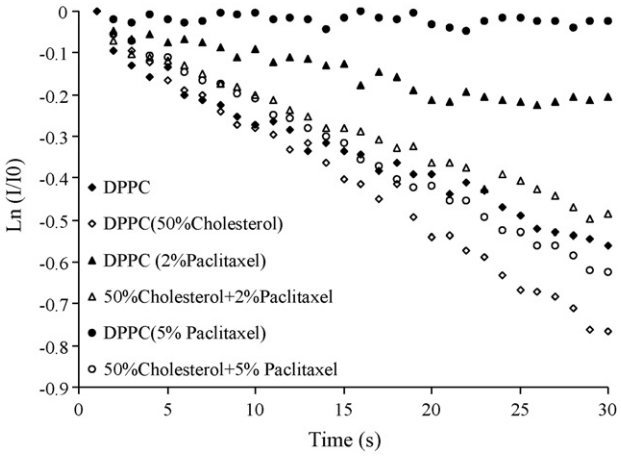

Fig. 8. Semilog plot of cholesterol effect on the ascorbic acid induced decrease of the peak heights of EPR spectrum of 5-DSA in a suspension of liposomes with ascorbic acid. Ascorbic acid concentration: 3×10^{-4} mol/l.

the nitroxides are located in the fifth carbon of the acyl chain length, or the nitroxides will be released from the acyl chain to the surface. The retard effect of paclitaxel on the reaction suggests that paclitaxel may block the penetration of ascorbic acid into the lipid bilayer or the release of the nitroxides. This observation implies that paclitaxel is localized superficially at the interfacial region of DPPC, or form a screen to inhibit the penetration or the release of ascorbic acid and nitroxide molecules. This finding supports our DSC and EPR parameter analysis obtained earlier.

We increased the ascorbic acid concentration from 3×10^{-4} to 10^{-3} M. The kinetic results are shown in Fig. 7. The rate constant κ was increased from 0.0011 to 0.0058. It can be seen that the kinetics is almost proportional to the concentration, which suggests a first order reaction.

As discussed before, paclitaxel can increase the fluidity of the lipid membranes. It should be reasonable to anticipate that the incorporation of paclitaxel into the DPPC liposomes can increase the reaction speed since the membrane becomes more flexible. One possible reason for the contradiction observation is that the fluidization effect of paclitaxel is not the dominant factor of the transport process for either nitroxide or ascorbic acid molecules since only minor change of EPR parameters was noticed due to the insignificant interaction between paclitaxel and DPPC molecules. Whereas the blockage function effect of paclitaxel may play the most important role since this is the first order reaction, the diffusion of the reactant is the control process.

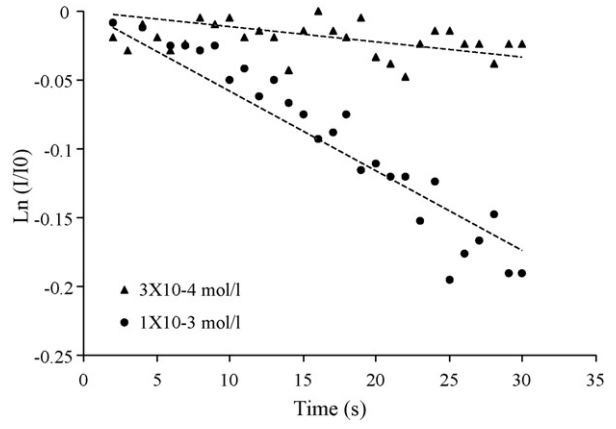

Fig. 7. Semilog plot of the ascorbic acid induced decrease of the peak heights of EPR spectrum of 5-DSA in a suspension of liposomes with ascorbic acid. Paclitaxel concentration: 5%.

Fig. 8 shows the effects of cholesterol on the reduction kinetics of DPPC/cholesterol bilayers with various molar ratios of paclitaxel. The reaction rate was increased remarkably with 50% cholesterol incorporated. The possible explanation is that cholesterol has a great effect on the lipid bilayer by increasing its fluidity, permeability and the phase transition temperature, which can facilitate the diffusion of the reactants. A comparison between the curves in Fig. 8 shows that the retard effect of paclitaxel is not as significant. One possible reason for this is that the effect of cholesterol on the membrane is much greater than that of paclitaxel. Therefore, the paclitaxel effect is not so significant with 50% cholesterol incorporation. The other implication is that paclitaxel may penetrate into the inner core of the DPPC acyl chain with the presence 50% cholesterol.

4. Conclusions

DSC and EPR techniques have been applied to study the molecular interactions between paclitaxel and phospholipid within lipid bilayer membranes with emphasis on the effects of the cholesterol component. Our DSC results show that incorporation of paclitaxel into the DPPC bilayer caused a reduction in the cooperativity of the phase transition, leading to a looser and more flexible bilayer. Time dependent DSC thermographs of DPPC/paclitaxel vesicles without or with 10% cholestrol suggest that paclitaxel-containing liposomes may not be stable over long time range of days. Cholesterol can facilitate the molecular interaction between paclitaxel and lipid bilayer and make the tertiary system more stable. EPR parameter analysis demonstrates that both of paclitaxel and cholesterol have fluidization effect on the lipid membranes although cholesterol has a greater effect

than paclitaxel does. For DPPC/paclitaxel liposomes, greater change of the EPR parameters was found for 5-DSA labelled liposomes. This observation strongly suggests that the binding site of paclitaxel to the phospholipid vesicles is in the outer hydrophobic cooperative zone of the bilayer, probably in the region of the C1–C4 carbon atoms of the acyl chain or just attaching to the headgroup of the liposomes. Opposite trend was found for the DPPC/cholesterol liposomes. The EPR findings confirm the results obtained from the DSC analysis that the location of cholesterol is within the hydrophobic interior of the phospholipid but not in the locality of the polar groups or at the surface region of DPPC. Compared with paclitaxel, cholesterol can penetrate more deeply into the hydrophobic core of the liposomes. The reduction kinetics of nitroxides by ascorbic acid shows that paclitaxel can inhibit the reaction by blocking the diffusion of either ascorbic acid or nitroxide molecules. When 50% cholesterol incorporated into the DPPC liposomes, the reaction speed is increased remarkably and the possible explanation is that cholesterol increases the fluidity and permeability of the lipid bilayers below the phase transition temperature and this effect can facilitate the diffusion of the reactants. This research may provide useful information for optimizing liposomal formulation as well as for understanding the pharmacology of paclitaxel and other anticancer drugs.

Acknowledgements

The authors would like to express their sincere thankfulness to the Reviewers for their thoughtful comments, which significantly contribute to the final version of this paper. This research was supported by NUS Grant R-379-000-014-112, National University of Singapore and Singapore Cancer Syndicate (SCS) Grant UU0028 (S.S. Feng, PI). LYZ is grateful of the National University of Singapore for the financial support for her PhD study.

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