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Paclitaxel interaction with phospholipid bilayers: high-sensitivity differential scanning calorimetric study

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Abstract

High-sensitivity differential scanning calorimetry (DSC) has been used to study the interaction of the low water-soluble anticancer agent Paclitaxel with multilamellar (MLVs) and unilamellar (ULVs) phospholipid vesicles. Three different lipid matrices have been investigated: pure di-palmitoyl-phosphatidyl-choline (DPPC), a mixture of DPPC with 3 mol% of the polymer-lipid poly(ethylene glycol:2000)-dipalmitoyl-phosphatidyl-ethanolamine (PEG:2000-DPPE) and a mixture of DPPC with 10 mol% of dipalmitoyl-phosphatidyl-glycerol (DPPG). Each lipid matrix has been investigated with a Paclitaxel concentration ranging from 0 to 5 mol%.

For MLVs dispersions, irrespective of the lipid matrix, the presence of Paclitaxel from 1 to 3 mol% causes the down shift of both pre- (T_p) and main-phase (T_m) transition temperatures and the broadening of the thermograms. The effects are, however, more pronounced on the pre-transition. The interaction of the drug with the lipid multilamellar vesicles is reduced at 5 mol% of Paclitaxel. In ULVs containing charged lipids, i.e., DPPC/PEG:2000-DPPE and DPPC/DPPG mixtures, the presence of Paclitaxel at concentrations $\geq$3 mol% affects significantly the main transition endothermic scans, with the appearance of side shoulders. The results suggest that the interaction of Paclitaxel is favoured with bilayer vesicles of low radius of curvature and with those containing lipids bearing a net negative charge on the polar heads.
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Keywords: Paclitaxel; Phospholipid bilayers; PEG-lipid; DSC

1. Introduction

Paclitaxel, also known as Taxol, is a novel drug showing particular anticancer activity [1,2]. It has been approved by US FDA for treatment of ovarian, breast, lung cancer and AIDS-related cancer Kaposi's sarcoma. It prevents the growth of cancer cells by binding and stabilizing the microtubules and inhibiting their disassembly into tubulins at the end of mitosis process, so cancer cells cannot divide further [3]. Paclitaxel is a complex natural diterpenoid molecule (Fig. 1a), isolated from the bark of the Pacific yew tree *Taxus brevifolia* [4]. In the exsiccated form it is a white to off-white crystalline powder mainly hydrophobic, having a poor solubility in water due to the presence of aromatic rings, but dissolves in organic solvents. It was also established that the molecule undergoes self-aggregation at concentration $\geq$3 mol% [5].

Due to its low solubility in water, for intravenous administration in cancer treatments, Paclitaxel was dissolved in 1:1 ethanol/polyethoxylated castor oil (Cremophor EL) [6] and then diluted 5–20 fold in saline solutions. The solvent caused allergic reactions [7] and toxic effects [8].

Recently, to eliminate Cremophor vehicle, formulations based on nano-structures dissolved in water have been developed to encapsulate the drug. They encompass uni- (ULVs) and multilamellar vesicles (MLVs) made of a variety of phospholipids [9–11], phospholipid micelles [12], emulsions [13] and nanospheres of biodegradable polymers [14]. To prolong the circulation lifetimes, some formulations contain appropriate amount of polymer-lipids, i.e. lipids derivatised on the polar heads with water-soluble polymer *N*-poly(ethylene

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glycol) (PEG) [15–18]. The polymeric coating acts as a steric barrier that stabilizes lipid bilayer against attack by elements of the immune system [19]. This new class of liposomal drug carriers is known as sterically stabilized liposomes (SSLs). Moreover, it has been shown that the inclusion of newly synthesized phosphatidyl polyglycerols in liposomes prolongs their circulation time in vivo via an electrostatic effect [20]. All the proposed formulations have been studied mainly by a pharmacological point of view. Only few studies have been made to gain further insight into the structural modifications and perturbation produced by Paclitaxel molecule on lipid matrices, mainly focusing on multilamellar dispersions.

In this paper we focus on three lipid matrices, which are interesting each one for a different reason: pure DPPC (Fig. 1b), for its temporal stability; a mixture of DPPC with 3 mol% of PEG:2000-DPPE (Fig. 1c), for the enhanced lifetimes in circulation; and a mixture of DPPC with 10 mol% of DPPG (Fig. 1d), for its high drug encapsulation efficiency (see Table 1 in [11]). The aim is to test the degree of perturbation of Paclitaxel on the three lipid matrices when organised in multilamellar vesicles and to compare the observed behaviour when organised in a unilamellar structure. To do this we use high-sensitivity differential scanning calorimetry (DSC). The results show a concentration dependent effect of Paclitaxel on the characteristics of both pre- and main transitions of the lipid vesicles whose extent depends on the lipid mesophase and lipid composition. Indeed, the interaction drug/lipid is augmented in aggregates with lower curvature radius (i.e., ULVs) and it is enhanced by the presence of charged lipids (i.e., PEG:2000-DPPE and DPPG) in the host lipid matrix.

2. Materials and methods

2.1. Materials

The synthetic lipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (DPPG), as well as Paclitaxel (purity >99%) were from Sigma (St. Louis, MO).

High purity (>99%) poly(ethylene glycol)-lipid (PEG-lipid), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-poly(ethylene glycol) with PEG of average molecular weight of 2000 Da (PEG:2000-DPPE), was from Avanti Polar Lipids (Birmingham, AL). The reagent-grade salts for the 10 mM phosphate buffer solution (PBS) at pH = 7.5 were

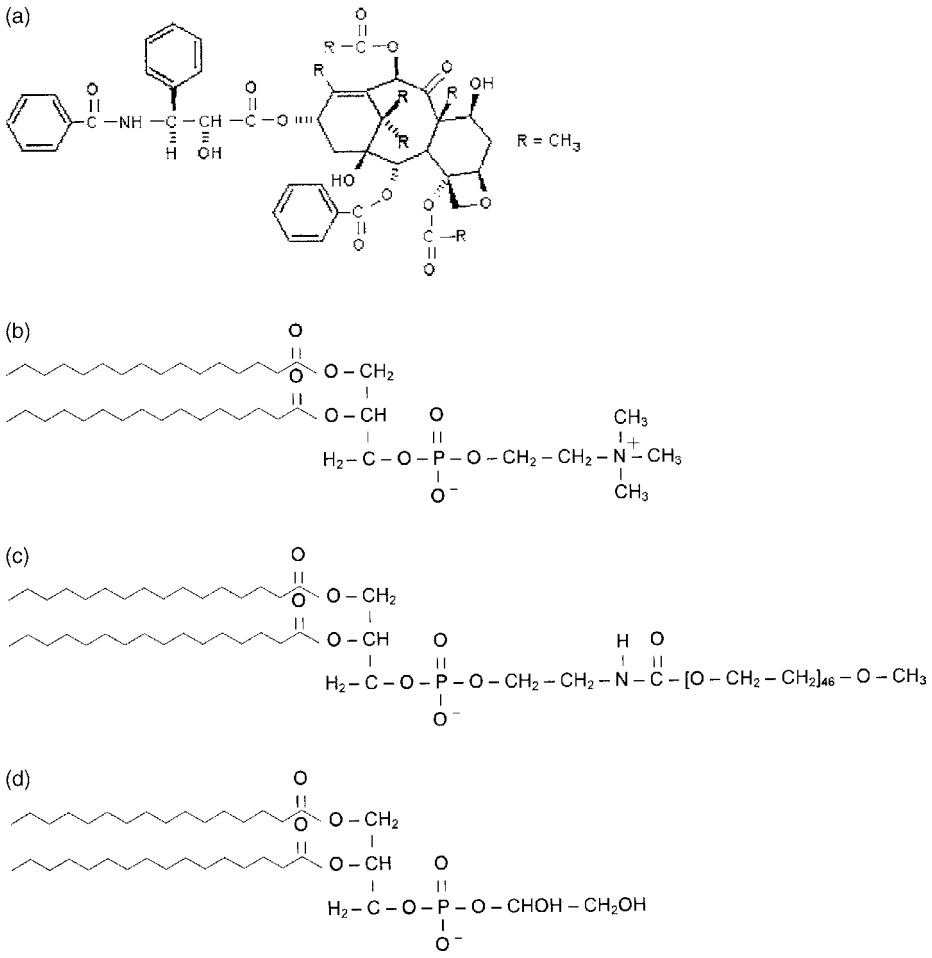

Fig. 1. Chemical structure of the molecules used: (a) Paclitaxel, (b) DPPC, (c) PEG:2000-DPPE and (d) DPPG.

Table 1
Pre-transition temperature and enthalpy values from endotherms in Fig. 2 for multilamellar vesicles of DPPC, DPPC/PEG:2000-DPPE and DPPC/DPPG in presence of Paclitaxel

Paclitaxel (mol%)	DPPC		DPPC + 3 mol% DPPE-PEG:2000		DPPC + 10 mol% DPPG	
	T_p (°C)	ΔH_p (kJ/mol)	T_p (°C)	ΔH_p (kJ/mol)	T_p (°C)	ΔH_p (kJ/mol)
0	33.7 ± 0.03	3.31 ± 0.20	32.0 ± 0.03	1.55 ± 0.05	32.4 ± 0.04	1.80 ± 0.09
1	30.7 ± 0.05	0.59 ± 0.03	29.7 ± 0.04	0.54 ± 0.02	30.3 ± 0.03	0.96 ± 0.06
3	30.2 ± 0.04	0.33 ± 0.02	30.3 ± 0.05	0.88 ± 0.04	29.9 ± 0.05	0.92 ± 0.04
5	31.5 ± 0.05	1.05 ± 0.06	30.7 ± 0.05	0.67 ± 0.04	30.4 ± 0.04	0.63 ± 0.03

from Merck (Darmstadt, Germany). Distilled water was used throughout.

2.2. Preparation of the lipid dispersions

Multilamellar dispersions (MLVs), heterogeneous in size, ranging from 0.1 to 5.0 μm, have been obtained first by dissolving previously prepared stock solutions of DPPC or of the mixtures DPPC/polymer-lipids and DPPC/DPPG together with Paclitaxel in chloroform/ethanol solutions. The solvent was successively evaporated with a stream of dry nitrogen and any residual trace was completely removed by pumping the samples overnight. The dried lipids were then dispersed at complete hydration in 10 mM phosphate buffer solution (PBS) at pH 7.5 to a final lipid concentration of 0.5 mg/ml (0.68 mM) by periodically vortexing and mixing at 50 °C. The resulting multilamellar vesicles were kept overnight at 4 °C before measuring.

Large unilamellar vesicles (ULVs) have been obtained passing 21 times the MLVs dispersions through a LiposoFast extruder (Avestin, Ottawa, Canada) equipped with 100 nm polycarbonate filters. The extrusion process creates a population of unilamellar vesicles distributed around an average diameter value of 80 nm [21].

2.3. DSC measurements

High-resolution heating scans were recorded using a VP-DSC microcalorimeter (MicroCal Inc., Northampton, MA). Heating rate was 4 °C/h. Data were analysed using MicroCal ORIGIN dedicated software. Samples were scanned almost three times to ensure reproducibility of the endotherms.

3. Results

3.1. DSC measurements in multilamellar dispersions

From DSC excess heat capacity curves, multilamellar dispersions of DPPC, in absence of Paclitaxel, show a main transition, from the ripple gel phase ($P_{\beta'}$) to the liquid crystalline one (L_α) centred at about T_m = 41.5 °C (ΔH_m = 33.45 kJ/mol) and a pre-transition from the gel phase ($L_{\beta'}$) to the ripple gel phase ($P_{\beta'}$) at 33.7 °C (ΔH_m = 3.31 kJ/mol). The characteristics of these transitions are in agreement with those reported in literature using the same experimental method and the same lipid mesophase ([22] and references therein). On increasing Paclitaxel concentration up to 3 mol% (Fig. 2a), a slight decrease of T_m to 41.2 °C (Fig. 3a) and of the enthalpy of the main transition to 30.14 kJ/mol (Fig. 3b) is observed; further addition of the drug up to 5 mol% leads to an increase of the main transition temperature (T_m = 41.3 °C), whereas the enthalpy continues to decrease to 24.78 kJ/mol. Moreover, from the plots of the main transition width at half-peak height, $\Delta T_{1/2}$, versus [Paclitaxel] in Fig. 3c, it comes out that the main endothermic peak of pure DPPC multilayers broadens slightly from 0 to 1 mol%, more abruptly up to 3 mol% and then sharpens at 5 mol% of Paclitaxel.

Pre-transition of pure MLVs of DPPC, with respect to the main one, results much more influenced by the presence of Paclitaxel. Indeed, up to 3 mol% of the drug T_p decreases from 33.7 to 30.2 °C and the pre-transition peak progressively broadens. At 5 mol% of Paclitaxel, the pre-transition endotherm behaves similarly to the main one, in that T_p increases at 31.5 °C (Table 1) and the pre-transition endothermic peak becomes more pronounced. In Table 1, it can be seen that the pre-transition enthalpy, ΔH_p, is notably reduced from 0 to 3 mol% and then it raises at 5 mol%.

Heating DSC scans of multilamellar mixtures of DPPC containing 3 mol% of PEG:2000-DPPE and DPPC containing 10 mol% of DPPG (Fig. 2b and c, respectively) suggest that the width of the endothermic peaks changes in the order: DPPC ≪ DPPC/PEG:2000-DPPE < DPPC/DPPG. The

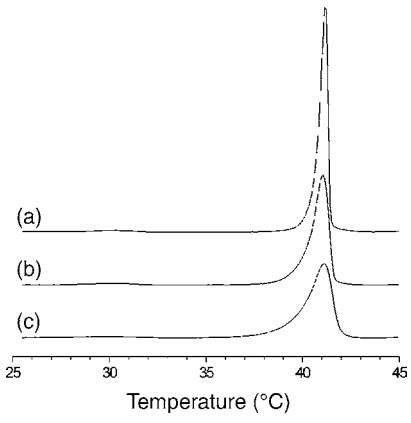

Fig. 2. DSC excess heat capacity curves for multilamellar dispersions of pure DPPC (a), mixture of DPPC with 3 mol% PEG:2000-DPPE (b), and mixture of DPPC with 10 mol% DPPG (c), in presence of Paclitaxel at 3 mol%.

addition to DPPC of 3 mol% of the polymer-lipid and of 10 mol% of DPPG leads to a slight decrease of the main transition temperature, in keeping with literature data. Indeed, by spin-label electron spin resonance spectroscopy (ESR) and spectrophotometry the down shift of both the pre- and the main transition temperatures as well as the loosening of the lipid packing density in DPPC/PEG:2000-DPPE at 3 mol% has been shown [23–25]. This was ascribed to the area expansion of the lipid membranes by the lateral pressure exerted among polymeric PEG chains in the brush regime. Moreover, Blume and co-workers [26] using DSC reported a slight variation of the main transition temperature in mixtures of phosphatidylcholine–phosphatidylglycerol.

The effect of the Paclitaxel content on the main transition temperature of the charged lipids containing two binary mixtures is rather similar to that observed for DPPC dispersions, with the T_m values in the order: T_m (DPPC) > T_m (DPPC/DPPG) ≫ T_m (DPPC/PEG:2000-DPPE) (Fig. 3a). Moreover, the endothermic peaks for the main transition of DPPC/PEG:2000-DPPE mixtures broaden up to 1 mol% and then sharpen progressively without reaching the starting value; for DPPC/DPPG instead, they slightly broaden up to 3 mol% and then sharpen (see $\Delta T_{1/2}$ plots in Fig. 3c).

Differences emerge from the comparison of ΔH_m values in Fig. 3b: unlike DPPC values, that decrease from 0 to 5 mol% of drug, for DPPC/PEG:2000-DPPE dispersions ΔH_m starts from a lower value (25.70 kJ/mol at 0 mol%) and increases till 28.00 kJ/mol. The ΔH_m behaviour in MLVs of DPPC/DPPG is instead multiphasic.

A comparison of the values of the temperatures and of the enthalpies of the pre-transition of the three lipid matrices investigated both in absence and in presence of Paclitaxel is given in Table 1. For any system considered, the trend of T_p versus [drug] is rather similar to that of T_m (see also Fig. 3a).

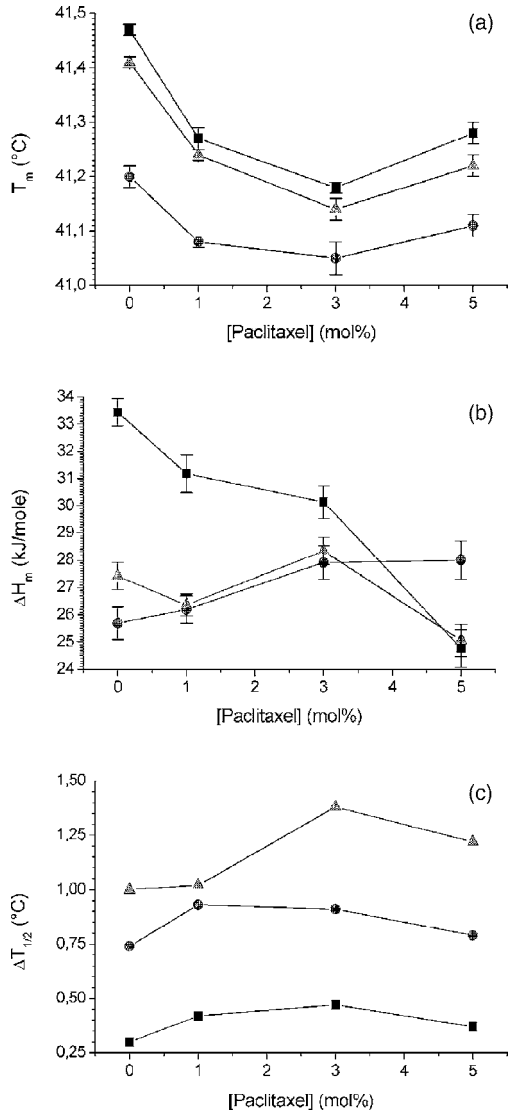

Fig. 3. Plots vs. Paclitaxel concentration of main-phase transition temperature, T_m, (a), main transition enthalpy, ΔH_m, (b), and $\Delta T_{1/2}$ (c) for multilamellar dispersions of pure DPPC (■), mixture of DPPC with 3 mol% PEG:2000-DPPE (●), and mixture of DPPC with 10 mol% DPPG (▲). The errors of $\Delta T_{1/2}$ values are smaller than the symbols.

3.2. DSC measurements in unilamellar dispersions

The thermograms of extruded unilamellar vesicles relative to the corresponding scans of MLV dispersions show broader and more asymmetric endothermic peaks for the main transition, the absence of the pre-transition (Fig. 4) and a down shift of the main transition temperature, as expected [22]. This could be due to the polydispersity of unilamellar lipid systems and to the reduced co-operativity of the vesicles due to the increased curvature and the smaller size of the ULVs limiting the maximum size of cooperative units [27].

For pure DPPC unilamellar dispersions T_m versus [Paclitaxel] show a trend similar to that observed in multilamellar ones. T_m goes from about 41.3 °C at 0 mol% to 41.1 and 40.6 °C at 1 and 3 mol% of Paclitaxel, respectively. The introduction of 5 mol% of the drug slightly moves the main transition peak to higher temperature.

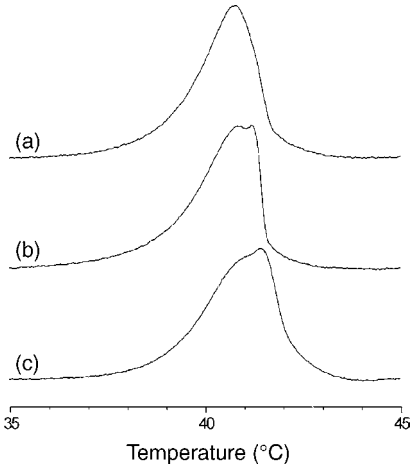

Fig. 4. DSC excess heat capacity curves for unilamellar dispersions of pure DPPC (a), mixture of DPPC with 3 mol% PEG:2000-DPPE (b), and mixture of DPPC with 10 mol% DPPG (c), in presence of Paclitaxel at 5 mol%.

In the absence of Paclitaxel, the DSC profile of ULVs of DPPC/PEG:2000-DPPE is broad and asymmetric with the main transition temperature slightly downward shifted to $T_m = 40.9\,^{\circ}C$ due to the presence of polymer-lipid. Paclitaxel at 1 mol% promotes a further decrease of T_m to 40.5 °C. Composite main peaks are, instead, evident in the DSC scans as the drug concentration is increased to 3 mol% and even more at 5 mol% (Fig. 4).

In contrast with the other mixtures, for DPPC/DPPG unilamellar dispersions, the main transition temperatures increase from 40.9 to 41.5 °C on going from 0 to 1 mol% of Paclitaxel. At 3 mol%; a sharpening of the transition and a down shift of the main peak temperature are present, whereas at 5 mol% of Paclitaxel; the thermogram becomes broad again with the appearance of two peaks (Fig. 4c).

4. Discussion

Our DSC results on MLVs suggest that Paclitaxel added from 0 to 3 mol% to pure zwitterionic DPPC dispersions or to mixed dispersions containing charged lipids interacts, although with different extent, with the bilayers favouring the fluid state (i.e., lowering T_m) and decreasing the cooperativity of the main transition (i.e., broadening the transition). These results are in good agreement with previous DSC data on the effect of Taxol in liposomes of phosphatidylcholine and of phosphatidylcholine mixed with anionic lipids [10,27,28] as concerns the effects of the drug on the main transition temperature. These studies, furthermore, proof the total disappearance of the pre-transition endothermic peak upon addition of 1 mol% of drug. In contrast, in our thermograms the pre-transition is still evident, even in mixed lipid systems of DPPC/PEG:2000-DPPE and of DPPC/DPPG.

At 5 mol%, instead, the drug perturbs less the bilayers as the thermograms are more similar to those of the MLVs in the absence of Paclitaxel. The behaviour at high loading could be due to the self-aggregation of the drug. The possibility of concentration dependent self-aggregation for the drug in aqueous solution was suggested by Balasubramanian et al. [5], and was recently confirmed by Krishnadas et al. [12]. It is therefore plausible that the bilayers are less perturbed by the drug in the aggregated state at high concentration.

On the whole, for each multilamellar dispersion, we observe a more remarkable effect of Paclitaxel on the pre-transition, rather than on the main transition. This suggests that the preferential site of action of the drug is the surface of the phospholipid vesicles. This mechanism of interaction was also proposed by Balasubramanian et al. [28]. Paclitaxel, due to its bulkiness, is not able to force the lipid molecules away for inserting in the bilayer interior. It has only a limited access in the hydrophobic region of the bilayer, incorporating some taxane rings (Fig. 1a) at the level of first carbons of lipid acyl chains.

As for MLVs of DPPC, also for ULVs we report a down-shift of the temperature of the main peaks up to 3 mol% of Paclitaxel, and the successive slight raising up at 5 mol%. However, for unilamellar dispersions the extent of T_m downward shift is bigger being $\Delta T_m \cong 0.6\,^{\circ}C$ and $\cong 0.3\,^{\circ}C$ for uni- and multilamellar DPPC dispersions, respectively. Such evidences suggest that the increased bilayer curvature allows a bigger interaction and an augmented incorporation of drug molecules in the upper hydrophobic bilayer zone. All are in keeping with literature data reporting that micellar aggregates improve drug incorporation efficiency [12]. This could be due to the lower cohesion and enhanced flexibility in the hydrophobic region of such lipid vesicles.

Another interesting feature emerging in the endotherms of unilamellar vesicles containing charged lipids is the occurrence of side peaks at high Paclitaxel concentrations. This could be due to an inhomogeneous mixing of the drug with the lipids as a consequence of the enhanced capability of incorporating Paclitaxel in the dispersions containing charged lipids. The two components in the thermograms evident in ULVs of DPPC/PEG:2000-DPPE and of DPPC/DPPG could therefore be ascribed to the formation of domains of charged lipids of PEG:2000-DPPE and of DPPG interacting with Paclitaxel, and regions of DPPC with few Paclitaxel molecules.

In conclusion, our study point out that the lipid mesophase and the composition of the lipid matrix play an important role in Paclitaxel/lipid association. The interaction is augmented in lipid vesicles with smaller radius of curvature and it is enhanced in presence of lipids bearing a net charge on the polar head. The study gives also support to the development and optimisation of mixed lipid formulations of proper size and composition suitable for Paclitaxel delivery and in vivo administration.

To increase Paclitaxel amount vehicolated by liposomes, another way to consider could be the synthesis of novel hydrophobic [29,30] or hydrophilic [31] prodrugs, i.e. Paclitaxel derivatives, capable of improving bilayer anchoring or entrapment, respectively.

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References

[1] R.M. Straubinger, Biopharmaceutics of Paclitaxel (Taxol): formulation, activity, and pharmacokinetics, in: M. Suffness (Ed.), Taxol: Science and Applications, CRC Press Inc., Boca Raton FL, 1995 (Chapter 9).
[2] M.E. Wall, M.C. Wani, J. Ethnopharmacol. 51 (1996) 239–253.
[3] G.I. Georg, T.C. Boge, Z.S. Cheruvallath, J.S. Clowers, G.C.B. Harriman, M. Hepperie, H. Park, The medicinal chemistry of Taxol, in: M. Suffness (Ed.), Taxol: Science and Applications, CRC Press Inc., Boca Raton FL, 1995 (Chapter 13).

[4] M.C. Wani, H.L. Taylor, M.E. Wall, P. Coggon, A.T. McPhail, J. Am. Chem. Soc. 93 (1971) 2325–2327.
[5] S.V. Balasubramanian, J.L. Alderfer, R.M. Straubinger, J. Pharm. Sci. 83 (1994) 1470–1476.
[6] D.S. Chervinsky, M.L. Brecher, M.J. Hoelcle, Anticancer Res. 13 (1993) 93–96.
[7] R.B. Weiss, R.C. Donehower, P.H. Wiernik, T. Ohnuma, R.J. Gralla, D.L. Trump, J.R. Baker Jr., D.A. Van Echo, D.D. Von Hoff, J. Clin. Oncol. 8 (1990) 1263–1268.
[8] R.T. Dorr, Ann. Pharmacother. 28 (1994) S11–S14.
[9] A. Sharma, R.M. Straubinger, Pharm. Res. 11 (1994) 889–896.
[10] C. Bernsdorff, R. Reszka, R. Winter, J. Biomed. Mater. Res. 46 (1999) 141–149.
[11] P. Crosasso, M. Ceruti, P. Brusa, S. Arpicco, F. Dosio, L. Cattel, J. Controlled Release 63 (2000) 19–30.
[12] A. Krishnadas, I. Rubinstein, H. Onyuksel, Pharm. Res. 20 (2003) 297–302.
[13] B.D. Tarr, T.G. Sambandan, S.H. Yalkowsky, Pharm. Res. 4 (1987) 162–165.
[14] S.S. Feng, G.F. Huang, L. Mu, Ann. Acad. Med. Singapore 29 (2000) 633–639.
[15] G. Blume, G. Cevc, Biochim. Biophys. Acta 1029 (1990) 91–97.
[16] D.D. Lasic, Liposomes: From Physics to Applications, Elsevier, Amsterdam, London, New York, 1993.
[17] D.D. Lasic, F. Martin, Stealth Liposomes, CRC Press, Boca Raton, London, Tokyo, 1995.
[18] D. Marsh, R. Bartucci, L. Sportelli, Biochim. Biophys. Acta Rev. Biomembr. 1615 (2003) 33–59.
[19] V.P. Torchilin, V.P. Omelyanenko, M.I. Papisov, A.A. Bogdanov Jr., V.S. Trubetskoy, J.N. Herron, C.A. Gentry, Biochim. Biophys. Acta 1195 (1994) 11–20.
[20] K. Maruyama, S. Okuizumi, O. Ishida, H. Yamauchi, H. Kikuchi, M. Iwatsuru, Int. J. Pharm. 111 (1994) 103–107.
[21] R.C. MacDonald, R.I. MacDonald, B.P. Menco, K. Takeshita, N.K. Subbarao, L.R. Hu, Biochim. Biophys. Acta 1061 (1991) 297–303.
[22] J.R. Silvius, in: P.C. Jost, O.H. Griffith (Eds.), Lipid-Protein Interactions, vol. 2, John Wiley & Sons, New York, 1982, pp. 239–281.
[23] S. Belsito, R. Bartucci, L. Sportelli, Biophys. Chem. 75 (1998) 33–43.
[24] S. Belsito, R. Bartucci, G. Montesano, D. Marsh, L. Sportelli, Biophys. J. 78 (2000) 1420–1430.
[25] G. Montesano, R. Bartucci, S. Belsito, D. Marsh, L. Sportelli, Biophys. J. 80 (2001) 1372–1383.
[26] P. Garidel, C. Johann, L. Mennicke, A. Blume, Eur. Biophys. J. 26 (1997) 447–459.
[27] D. Marsh, A. Watts, P.F. Knowles, Biochim. Biophys. Acta 465 (1977) 500–514.
[28] S.V. Balasubramanian, R.M. Straubinger, Biochemistry 33 (1994) 8941–8947.
[29] S. Ali, S. Minchey, A. Janoff, E. Mayhew, Biophys. J. 78 (2000) 246–256.
[30] B.B. Lundberg, V. Risovic, M. Ramaswamy, K.M. Wasan, J. Controlled Release 86 (2003) 93–100.
[31] M. Ceruti, P. Crosasso, P. Brusa, S. Arpicco, F. Dosio, L. Cattel, J. Controlled Release 63 (2000) 141–153.