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Drug interaction and location in liposomes: correlation with polar surface areas

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Abstract

An important step in liposome characterization is to determine the location of a drug within the liposome. This work thus investigated the interaction of dipalmitoylphosphatidylcholine liposomes with drugs of varied water solubility, polar surface area (PSA) and partition coefficient using high sensitivity differential scanning calorimetry. Lipophilic estradiol (ES) interacted strongest with the acyl chains of the lipid membrane, followed by the somewhat polar 5-fluorouracil (5-FU). Strongly hydrophilic mannitol (MAN) showed no evidence of interaction but water soluble polymers inulin (IN) and an antisense oligonucleotide (OLG), which have very high PSAs, interacted with the lipid head groups. Accordingly, the drugs could be classified as: hydrophilic ones situated in the aqueous core and which may interact with the head groups; those located at the water–bilayer interface with some degree of penetration into the lipid bilayer; those lipophilic drugs constrained within the bilayer.

Keywords: Liposomes; High sensitivity differential scanning calorimetry; Polar surface areas; Drug–liposome interaction.

1. Introduction

Determination of the location of drugs within liposomes and their interactions with the lipid bilayer is important in liposome characterization. Differential scanning calorimetry (DSC) has been used to investigate the interactions of molecules such as cholesterol, surfactants, penetration enhancers, drugs and proteins with lipid membranes (e.g. Papahadjopoulos et al., 1975; Ganesan et al., 1984; Rolland et al., 1991; Spink et al., 1991; McMullen et al., 1993, 1999; Lo and Rahman, 1995; Castile et al., 1998). DSC was also employed to investigate protein location in liposomes (Papahadjopoulos et al., 1975; Ganesan et al., 1984; Lo and Rahman, 1995; Zhao and Feng, 2004; Zhao et al., 2004). The technique also monitored drug release from

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vesicles and liposome–polymer interaction (Savva et al., 1999; Hashizaki et al., 2003). Accordingly, we used high sensitivity differential scanning calorimetry (HSDSC) to probe the interactions of drugs with dipalmitoylphosphatidylcholine (DPPC) liposomes in a further attempt to specify the position of drugs in liposomes.

The model drugs included estradiol (ES), 5-fluorouracil (5-FU), inulin (IN) and an antisense oligonucleotide (OLG). In addition, mannitol (MAN), a membrane non-interacting drug that is known to localise in the aqueous domain of liposomes, was used as a control. Drugs were thus selected to provide a range of aqueous solubilities and of partition coefficients. It was decided to select a single quantitative parameter reflecting the physicochemical characters of all drugs to be correlated with the recorded interactions. The polar surface area (PSA) was used for this purpose. PSA is the sum of surfaces of polar atoms in a molecule. The decision was based on the fact that this parameter is now replacing traditional parameters such as partition coefficient and solubility in studying the transport of drug molecules through biological membranes. It can be applied for small as well as large molecules (Palm et al., 1997; Tronde et al., 2003) and an easy method for calculation of PSA is now available (Ertl et al., 2000). PSAs were calculated as the sum of fragment based contributions. The contribution of the individual fragment to the PSA is summarized in Table 1. The calculated PSA value were 40.5, 65.7, 121.4, 2770.3 and 2704.3 $Å^2$ for ES, 5-FU, MAN, IN and OLG, respectively. Within the hydrophilic class, molecules with different levels of PSA and hydrogen bonding sites were thus chosen. It was hoped that some parameters could be derived that would predict the interaction of a drug with the lipid membrane and its location in liposomes.

DPPC was used as a sample phospholipid as it is one of the most widely used models for studying bilayer interactions. Its transition temperature (T_m) can be easily measured as it has a narrow main endothermic peak (Montenegro et al., 1996).

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC, 99% purity), estradiol (at least 98%), 5-fluorouracil (99%) and inulin were purchased from Sigma Chemical Company (UK). Mannitol was obtained from BDH Chemicals Ltd. (England). The antisense oligonucleotide was a gift from Roche Drug Company (England). Fig. 1 shows their chemical structures.

2.2. Preparation of lipid vesicles

Liposomes containing DPPC (5 mg/ml) were prepared by mechanical shaking. The phospholipid was dissolved in chloroform:methanol (2:1). For ES, ethanolic drug solution was added to the lipid solution to produce a drug concentration of 0.1% (w/v) in the final liposome suspension. The organic solvent was evaporated under a stream of nitrogen at 50 °C (above the lipid transition temperature). The deposited lipid film was hydrated with distilled water (for the control or OE liposomes) or drug solution (for 5-FU, IN, OLG or MAN liposomes) for 1 h by intermittent vortexing and heating at 50 °C. 5-FU was used as a saturated aqueous solution (1.22%, w/v) and IN, OLG and MAN were 0.5, 1 and 10% (w/v) aqueous solutions. Liposome dispersions were swollen at room

Table 1
Atomic contribution to PSA ($Å^2$) (according to Ertl et al., 2000)

Atom type[a]	PSA contribution	Atom type[a]	PSA contribution
[NH_2]—*	26.02	[*n*](:*)(:*):*	4.41
[*n*H](:*):*	15.79	[*n*](—*)(:*):*	4.93
[O](—*)—*	9.23	[O]=*	17.07
[OH]—*	20.23	[O—]—*	23.06
[P](—*) (—*)(—*)=*	9.81		

[a] (*) Stands for any non-hydrogen atom, (—) for a single bond, (=) for a double bond, (:) for an aromatic bond; atomic symbol in lowercase means that the atom is part of aromatic system.

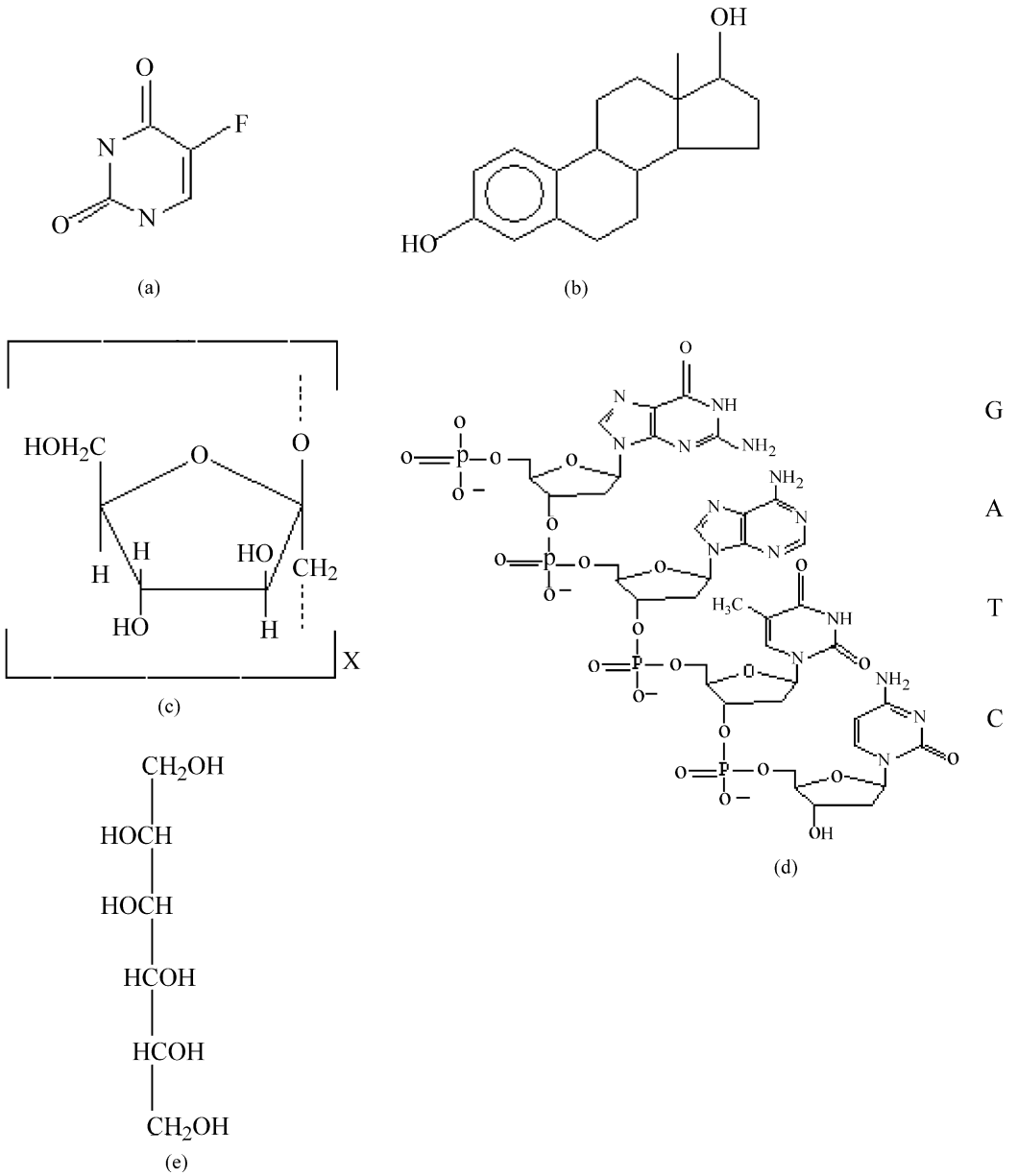

Fig. 1. Chemical structures of the drugs used (a) 5-fluorouracil, (b) estradiol, (c) inulin where $X \cong 35$, (d) the building units of antisense oligonucleotides where a molecule is 20 mer and (e) mannitol.

temperature for 2 h, stored at 4 °C overnight to ensure complete hydration, degassed by bath sonication for 3 min and analysed by HSDSC. These liposomes have an average size of 1038 nm as determined by photon-correlation spectroscopy (El Maghraby et al., 1999).

2.3. High sensitivity differential scanning calorimetry

A Micro Calorimetry System Differential Scanning Calorimeter (MCS DSC), MicroCal Inc., Northampton, MA, USA, was controlled by MicroCal observer

data acquisition software linked to the MicroCal Origin data analysis software. The MCS unit comprises two identical coin shaped cells enclosed in an adiabatic jacket for reference and for sample. Degassed sample and reference (water) were loaded into the corresponding cell and were heated from 10 to 60 °C at 1 °C/min.

A baseline, run before each determination with water loaded in sample and reference cells, was subtracted from individual results on data analysis.

2.4. Data analysis

The excess heat capacity function was normalised for phospholipid concentration. The HSDSC trace is a plot of the excess heat capacity as a function of temperature. According to Lo and Rahman (1995), the following parameters were calculated:

(i) The transition midpoint, T_m, is the temperature at which the transition is half completed (the peak point).
(ii) The transition enthalpy, ΔH, is the actual heat required for the entire transition, normalised per mol. This is calculated from the area under the transition peak.
(iii) The temperature width at half peak height, $\Delta T_{1/2}$, is the sharpness of the phase transition. This parameter is very sensitive to the presence of any additives. It will be taken as a measure for the co-operativity of the transition. $\Delta T_{1/2}$ is inversely proportional to the co-operativity. Co-operative units measure the number of phospholipids undergoing simultaneous transition.

Statistical analysis used the Student's *t*-test.

3. Results and discussion

Table 2 presents the parameters calculated from the HSDSC traces of DPPC liposomes containing different drugs and Fig. 2 shows examples of these traces.

The HSDSC traces of pure DPPC vesicles (control; Fig. 2a and Table 2) showed a typical profile with a pre-transition peak at 35.58 °C and the main transition at 41.43 °C in agreement with published data (e.g. Mabrey-Gaud, 1981; Rolland et al., 1991; Lo and Rahman, 1995; El Maghraby et al., 2004; Zhao et al., 2004). No post-transition peaks were detected by this technique compared with those recorded by FT-Raman spectroscopy (Lawson et al., 1998). The pre-transition can be due to rotation of the phospholipid head groups or to conformational changes in the phospholipid bilayer structure. The highly ordered gel state (L_β) with the hydrocarbon chains in an all-trans configuration

Table 2
The phase transition parameters of dipalmitoylphosphatidylcholine liposomes containing different drugs as assessed by high sensitivity differential scanning calorimetry

Drug	T_m (°C)	Enthalpy (kcal/mol)	$\Delta T_{1/2}$ (°C)
Pre-transition			
Control	35.58 (0.35, 5)	0.7880 (0.24, 5)	1.977 (0.27, 5)
Estradiol	32.04 (0.20, 3)*	0.87333 (0.20, 3)	2.327 (0.16, 3)
5-Fluorouracil	33.83 (0.066, 3)*	0.7833 (0.10, 3)	1.933 (0.37, 3)
Mannitol	35.19 (0.054, 3)	0.7516 (0.16, 3)	1.930 (0.14, 3)
Inulin	35.26 (0.076, 3)	1.279 (0.044, 3)*	1.402 (0.11, 3)*
Antisense oligonucleotide	35.87 (0.0058, 3)	1.645 (0.033, 3)*	1.676 (0.14, 3)*
Main transition			
Control	41.43 (0.028, 5)	8.424 (1.1, 5)	0.7242 (0.0030, 5)
Estradiol	40.44 (0.064, 3)*	9.911 (0.38, 3)	0.8086 (0.080, 3)
5-Fluorouracil	40.90 (0.066, 3)*	9.631 (0.55, 3)	0.7330 (0.0018, 3)
Mannitol	41.40 (0.0024, 3)	9.292 (0.31, 3)	0.7247 (0.0015, 3)
Inulin	41.40 (0.0068, 3)	9.342 (0.42, 3)	0.7260 (0.0012, 3)
Antisense oligonucleotide	41.40 (0.0035, 3)	9.631 (0.36, 3)	0.7250 (0.0058, 3)

$\Delta T_{1/2}$ is the temperature width at half peak height. Values between brackets are S.D. and number of replicates, respectively. Values are presented as four significant figures to allow presentation of S.D.
* Significantly different from the control.

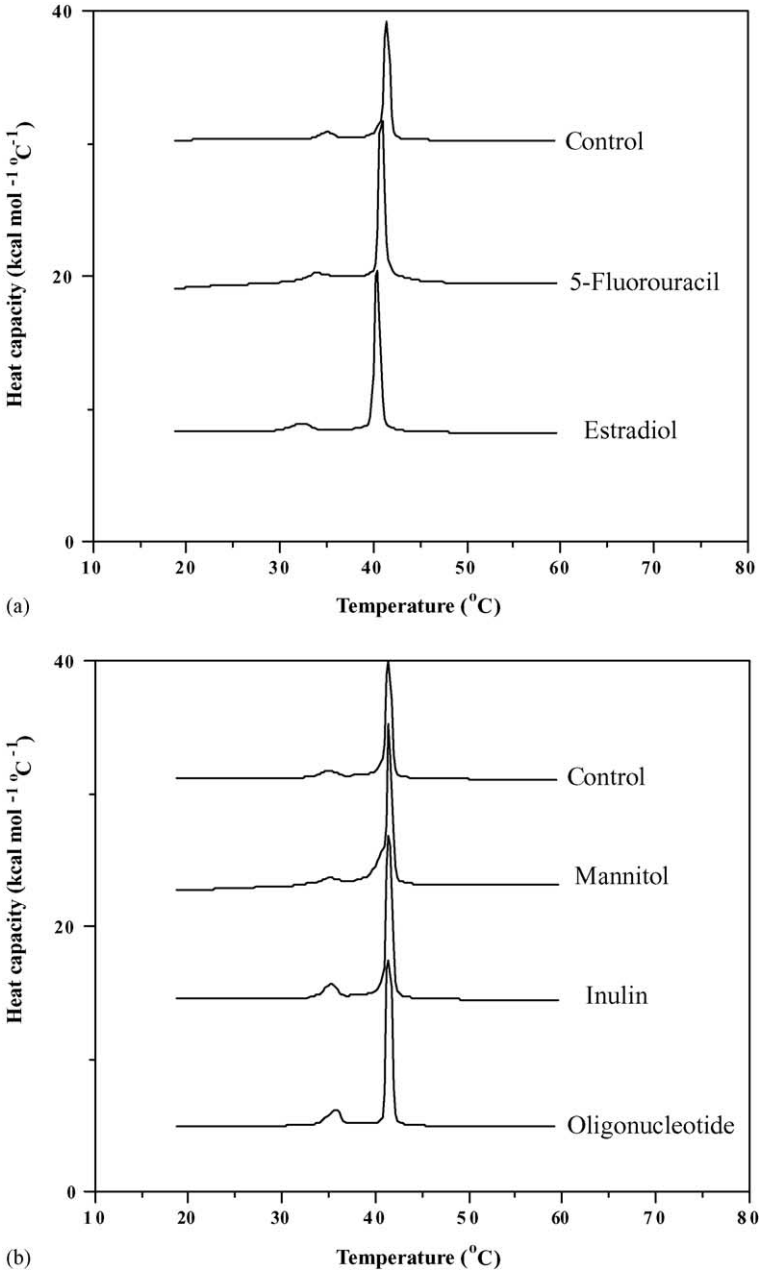

Fig. 2. Examples of high sensitivity differential scanning calorimetry traces of dipalmitoylphosphatidylcholine liposomes containing different drugs. Control is DPPC empty liposomes dispersed in distilled water.

(tilted one-dimensional arrangement) changes to a two-dimensional arrangements with periodic undulations (rippled gel phase, P_β). This means that any compound that interacts with the head groups will affect the pre-transition. The main transition arises from chain melting; thus above T_m, trans/gauche rotational isomerisation along the chains laterally expands and decreases the thickness of the bilayer and the system reverts

to one-dimensional arrangements. Thus, the lipids become more fluid (liquid crystalline phase, L_α) (Janiak et al., 1976; Mabrey-Gaud, 1981; Rolland et al., 1991).

Incorporation of ES the most lipophilic of the tested drugs ($\log P = 2.3$, PSA = 40.5 Å^2), into DPPC liposomes, reduced the T_m of the pre-transition endotherm and broadened its peak. It also significantly reduced the T_m, broadened the peak (reduced the co-operativity as indicated by increased $\Delta T_{1/2}$) but did not affect the enthalpy of the main transition endotherm, compared with the empty liposome control (Fig. 2a and Table 2). This negative effect on the enthalpy of the main transition is not expected for such a compound, which interacts with the acyl chains of the phospholipids.

5-FU, a more hydrophilic molecule relative to ES ($\log P = -0.824$, PSA = 65.7 Å^2) showed a similar qualitative behaviour to that of ES. However, the reduction in T_m was less than that obtained with ES, although 5-FU was used at a higher molar concentration (Fig. 2a and Table 2). This demonstrated the dependence of the interaction on the partition coefficient of the drug, with stronger interaction for lipophilic drugs with smaller PSAs.

Mannitol ($\log P = -2.47$, PSA = 121.4 Å^2) liposomes showed a thermotropic phase behaviour similar to that of the negative control (Fig. 2b and Table 2). Similar behaviour was reported for glucose-containing liposomes (Ganesan et al., 1984).

With inulin (water soluble polysaccharide with many possible sites for hydrogen bonding, PSA = 2770.3 Å^2) and the antisense oligonucleotide (charged large molecular weight very water soluble drug, $\log P = -3.5$, PSA = 2704.3 Å^2), there were no significant effects on the main transition endotherm in terms of T_m, enthalpy or $\Delta T_{1/2}$ indicating no interactions with the acyl chains of the bilayer itself. However, for the pre-transition, the enthalpy significantly increased and $\Delta T_{1/2}$ decreased. The former effect was greater with the charged drug (Fig. 2b and Table 2). This may indicate interaction at the interface site (with the polar head groups).

Thus, drug lipophilicity leads to interaction with the acyl chains of the phospholipid, but hydrophilicity implies no interaction or interaction with the head groups of the phospholipids, depending on the functional groups that contribute to the PSA in the polar compound. Here, the very hydrophilic mannitol showed no HSDSC interaction with liposomes, but inulin and the oligonucleotide revealed some interaction with the head groups as indicated by the changes in the pre-transition endotherm. This interaction can be attributed to the very high PSA values of IN and OLG relative to that of the other hydrophilic drug, MAN. This can be explained further by the presence of more hydrogen bonding sites in IN and OLG compared to MAN. It is thus possible to infer that increasing the PSA will reduce the interaction of drugs with the acyl chain of the bilayer, with high PSA values producing interactions with the head groups.

Proteins were classified, on the basis of their effects on the thermotropic behaviour of lipids, into three categories (Papahadjopoulos et al., 1975). Category 1 are water soluble proteins that can be adsorbed on the bilayer surface exerting electrostatic interactions with a strong effect on charged phospholipids. Category 2 consists of proteins that are adsorbed on the surface but also partly embed into the bilayer due to combined electrostatic and hydrophobic interactions. Category 3 defines proteins, which penetrate into the bilayer due to hydrophobic interactions. Lo and Rahman (1995) monitored protein location in liposomes with the aim of testing the validity of this classification. They found that the classification needed some modification in the way that hydrophilic proteins do not bind exclusively on the surface by electrostatic interactions. We have also found a hydrophilic drug (mannitol), which showed negligible interaction.

Accordingly, drugs can similarly be classified into three categories. The first one comprises those hydrophilic drugs that localise in the aqueous compartment of liposomes. They may exert some degree of interaction with the head group depending on their PSA, with high PSA indicating interactions with the head groups (e.g. IN and OLG). The second group consists of those partially hydrophilic, partially lipophilic drugs that arrange at the bilayer–water interface with some penetration into the lipid domain (e.g. 5-FU). The third category includes those lipophilic drugs that mainly locate into the lipid membrane, exerting hydrophobic interactions (e.g. ES).

4. Conclusion

Drug–liposome interaction depends not only on the partition coefficient of the drug but also on its func-

tional groups, such as hydrogen bonding sites and its polar surface area.

Polar surface area of drugs can correlate with their interaction with liposomes and hence with biological membranes. Increasing the PSA reduces the magnitude of interaction with the lipid bilayer and raises the tendency for surface interaction with the head groups.

With regard to liposome interaction, drugs can be classified into three categories; the strongly hydrophilic ones, which are localized in the aqueous domains of liposomes and can exert some interaction with the head groups if they have a very high PSA, the less hydrophilic, more balanced molecules that adsorb at the water–lipid bilayer interface with some degree of penetration into the bilayer, and the strongly lipophilic drugs, which locate in the bilayer itself.

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