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Comparison of the effect of 4-hydroxycoumarin and umbelliferone on the phase transition of dipalmitoylphosphatidylcholine (DPPC) bilayers

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Abstract:
The study compares the effect of the addition of two coumarins: 4-hydroxycoumarin (4-HC) and 7-hydroxycoumarin (umbelliferone; UMB) on dipalmitoylphosphatidylcholine (DPPC) membranes. The study was based on microcalorimetric and fluorescence measurements. The examinations have shown that 4-HC changes parameters of phase transition of DPPC membranes to a greater degree than UMB. It is associated with different location of each coumarin in the lipid membrane, which is caused by different orientation of polarity of coumarin molecules. 4-HC molecules that are amphiphilic "along" incorporate inside the membrane interacting with lipid carbohydrate chains. UMB molecules amphiphilic "across" the molecule are not incorporated inside the membrane and do not interact with acyl chains.

Key words:
4-hydroxycoumarin, umbelliferone, dipalmitoylphosphatidylcholine, DSC, drug-membrane interaction

Abbreviations: DPPC – dipalmitoylphosphatidylcholine i.e. 1,2-dihexadecanoyl-rac-glycerol-3-phosphocholine, DSC – differential scanning calorimetry, 4-HC – 4-hydroxycoumarin, UMB – umbelliferone

Introduction

Coumarins are a big group of compounds whose molecular structure is based on a common skeleton of benzo-α-pyrone. Its chemical structure is presented in Figure 1.

Almost all coumarins have ligands, mainly in C-7 position, but also in other positions. These are most often -OH, -OCH_3 groups and aliphatic lateral chains. 4-Hydroxycoumarin (4-HC) and umbelliferone (UMB), examined in this study, have hydroxyl group at C-4 and at C-7 position, respectively. The result is that 4-HC molecules are amphiphilic "along" the molecule, i.e. molecules have one of the shorter sides polar and the other apolar, and UMB molecules have am-

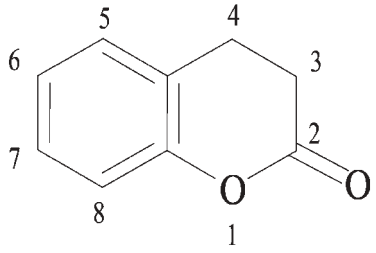

Fig. 1. Structure of benzo-α-pyrone molecule

phiphilic character „across" the molecule, namely longer sides of UMB molecule are amphiphilic. The aim of the study was to examine the effect of these differences on thermodynamic properties of lipid membranes modified by one of these compounds.

Coumarins are a group of compounds which are characterized by a wide variety of pharmacological activities. Among the most important properties of these active substances is their antithrombotic activity. This activity is the result of coumarin-induced inhibition of the hepatic synthesis of certain blood clotting factors, especially prothrombin, which, in turn, impairs the process of coagulation. Derivatives of 4-HC are usually anticoagulant drugs, but they also have antitumor effect [8]. UMB is one of the most common coumarins in nature and a drug presenting many effects. It is used as a spasmolytic drug. Recently, its antitumor activity has been accentuated [10, 20]. The study of Stefanova et al. [20] examined the antitumour effect of UMB against Sarcoma 180 in mice. The study revealed the inhibition of tumor growth and increased survival time of tumor-bearing animals. The antitumor effect of UMB was studied *in vitro* and *in vivo* [10]. Authors have reported a cytostatic and apoptotic activity of UMB on lung cancer cell lines. They observed inhibition of cell proliferation, identified the phase in which cell cycle arrest occurs, and evidenced the induction of apoptosis. Some authors [13–17] point to its antidiabetic effect. In the study by Ramesh et al. [13], it was found that treatment with UMB decreased plasma glucose and increased insulin, total proteins, and albumin, apart from the food intake or body weight of diabetic rats. In UMB-treated diabetic rats, plasma and tissue cholesterol, triglycerides, and free fatty acids returned to near normal levels. A protective effect of UMB on membranous fatty acid composition has also been observed [17]. UMB has an antioxidant effect [14]. Treatment with UMB brought lipid peroxidation markers, nonenzymic and enzymatic antioxidants back to near normal values. The toxicity of UMB has been discussed in a previous work [18]. A cytotoxic effect has been found on cultured rat hepatocytes. UMB has also antifungal properties [21].

UMB was subjected to spectroscopic tests in earlier studies [2, 9]. In the study by Biswas et al. [2], it was stated, among other things, that maximum absorption of UMB in a phosphate buffer medium was 324 nm at pH up to 7 and 367 nm at pH equal 8 or more. This absorption of ultraviolet light suggests the usefulness of UMB derivatives as ultraviolet filters.

Measurements of UMB absorption and fluorescence in different polar and non-polar solvents made it possible for researchers to determine excited state dipole moments of the UMB molecule [9].

Lipid bilayers in the form of liposomes are a simple model of a cellular membrane. Therefore, much attention has been paid to the properties of bilayers. This paper describes studies of liposomes obtained on the basis of dipalmitoylphosphatidylcholine (DPPC). Bilamellar phospholipids with chains of saturated carbohydrates, including DPPC, are characterized by phase transition. The main phase transition $P_{\beta'}$–L_{α} of lecithin layers, called gel-liquid crystalline transition, consists, among others, in an increase in *trans-gauche* isomerization of acyl chains making up the hydrophobic part of lipid layers. In the case of multilamellar DPPC liposomes, phase transition occurs at 41.5°C. The addition of a modifier usually changes this temperature. Besides the main phase transition, there also exists pretransition $L_{\beta'}$–$P_{\beta'}$ consisting in the modification of bilayer arrangement.

Differential scanning calorimetry (DSC), the method applied in this study, is widely used for monitoring of the phase transition of lipid bilayers [1, 11]. Lipid membranes are also examined with the use of fluorescence measurements [12].

Materials and Methods

1,2-Dihexadecanoyl-rac-glycerol-3-phosphocholine (DPPC), used for the preparation of liposomes, was manufactured by Sigma (St. Louis, MO, USA). 4-HC, UMB and Tricine buffer were also purchased from this company. Spectrally pure methanol and chloroform delivered by POCh S.A. (Gliwice, Poland) were used as lipid and coumarin solvents.

Multilamellar liposomes with and without coumarin were obtained according to the following procedure. The lipid was dissolved in 1:1 chloroform-methanol solutions possibly with an additive of either 4-HC or UMB. Afterwards a thin lipid film was obtained by evaporating the solvent in the atmosphere of dry nitrogen. Residues of solvents were removed in a vacuum.

Liposomes were obtained by adding 10 mM Tricine buffer (pH = 7.6) to the film and shaking for 1 h at a temperature above that of the phase transition.

Liposome solutions at 1.5 mM/l concentration with the addition of one of the coumarins at 1–30 mol% ratio to DPPC were analyzed and compared with liposomes without coumarins.

Calorimetric measurements were performed using a differential scanning calorimeter (UNIPAN 605M, Poland). Lipid suspensions (120 μl) were transferred into a steel measurement pan, and the same volume of buffer was in the reference pan. The scan rate employed was 1°C/min in the temperature range 30–55°C. The phase transition temperature was calculated as the temperature at the minimum value of calorimetric signal within the endotherm, and the enthalpy from the area of the peak relevant to the phase transition. Microcalorimetric measurements were repeated 5–7 times.

Fluorescence intensity in DPPC vesicles with the concentration 0.3 mM/l was measured using spectrofluorometer Shimadzu RF-5000 in temperature range 20–50°C. The measurements were performed at established wave lengths of excitation and emission, 374 nm and 452 nm, respectively, for 4-HC, and 382 nm and 459 nm for UMB. These wavelengths correspond to the maximum of absorption and maximum of fluorescence emission, respectively.

Results

Figure 2 and 3 present the calorimetric scans of vesicles of "pure" DPPC and with addition of 4-HC (Fig. 2), or UMB (Fig. 3). The minimum of these scans corresponds to the $P_{\beta'}$–L_{α} phase transition temperature of DPPC in lipid bilayers. The phase transition in this case is related to a cooperative increase in the *trans-gauche* isomerization of acyl chains of lipid molecules. This results in a lipid membrane fluidization, so the temperature region of the process occurrence is often referred to as "melting".

The presented scans show that the addition of 4-HC causes a distinct broadening of the transition peak and gradual decrease in the phase transition temperature from about 41.5°C to below 38°C with the increase in coumarin concentration from 0 mol% to 30 mol% relative to DPPC. Additive change in transition temperature and peak broadening for UMB is considerably smaller. The addition of UMB decreases the phase transition temperature of DPPC membranes not more than by 1°C but yet concentrations of 1–2 mol% UMB increase slightly the phase transition temperature. These dependencies of phase transition temperature *vs.* coumarin concentrations are shown in Figure 4. Similar results were obtained when the effect of the described coumarins on DPPC membrane phase transition was examined by means of the ultrasound method [23].

Figure 5 shows the dependence of phase transition enthalpy on coumarin concentrations. The enthalpy was determined from the area between endotherm (Fig. 2 and 3) and a baseline which was constructed

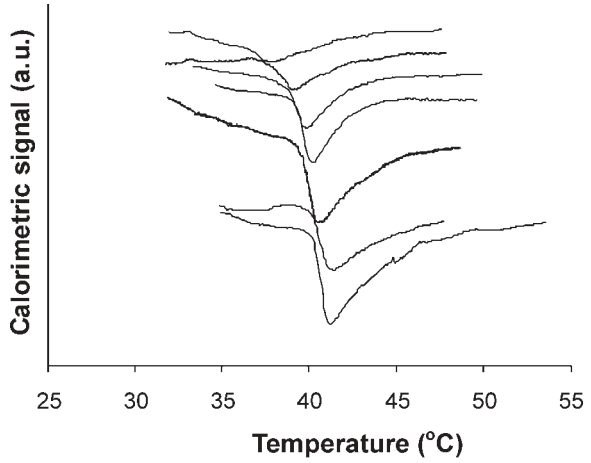

Fig. 2. Calorimetric scans of DPPC liposomes containing different molar percentage of 4-hydroxycoumarin. From the bottom: 0 mol%, 1%, 2%, 5%, 10%, 20% and 30 mol% relative to DPPC

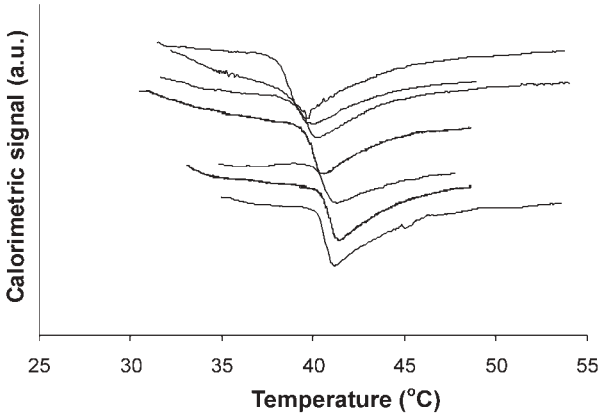

Fig. 3. Calorimetric scans of DPPC liposomes containing different molar percentage of umbelliferone. From the bottom: 0 mol%, 1%, 2%, 5%, 10%, 20% and 30 mol% relative to DPPC

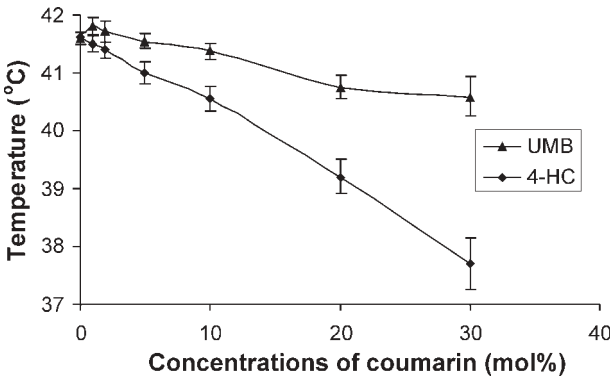

Fig. 4. The phase transition temperature of DPPC bilayers versus concentration of coumarins umbelliferone (UMB) and 4-hydroxycoumarin (4-HC)

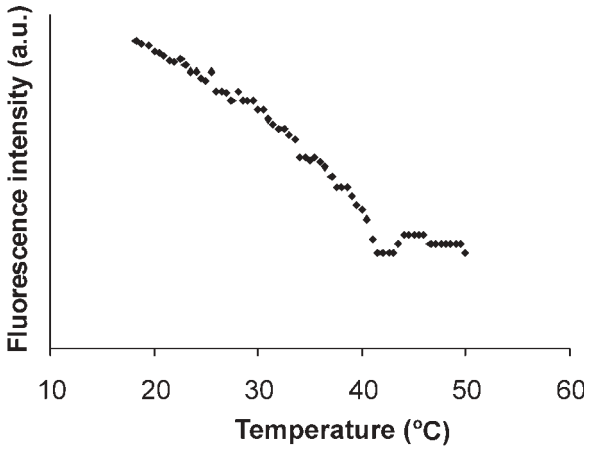

Fig. 6. Fluorescence intensity of 4-hydroxycoumarin in DPPC membranes versus temperature at the coumarin concentration of 5 mol% relative to DPPC

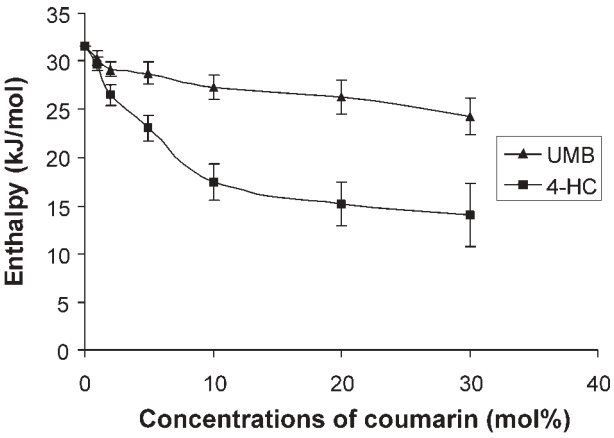

Fig. 5. Plots of the transition enthalpy of DPPC as a function of coumarin concentration

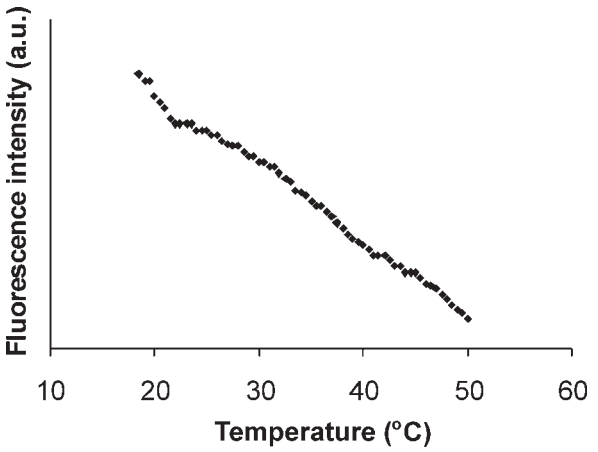

Fig. 7. Fluorescence intensity of umbelliferone in DPPC membranes versus temperature at the coumarin concentration of 5 mol% relative to DPPC

by extrapolating it to the scan beyond the endotherm. It can be seen that the addition of each coumarin decreases phase transition enthalpy. Here one can also observe a greater effect of 4-HC than the effect of UMB addition. The enthalpy of DPPC phase transition for 4-HC changes from 31.5 kJ/mol for "pure" DPPC to about 14 kJ/mol for DPPC with the addition of 30 mol% coumarin. UMB supplementation changes this value to about 24 kJ/mol at 30 mol% concentration.

Figures 6 and 7 show a dependence of fluorescence intensity on the temperature of 4-HC and UMB. Similar dependences were obtained for coumarins in the whole range of concentrations. For 4-HC, one can observe the phase transition of DPPC membranes. For UMB, these transitions are not expressed so distinctly.

Discussion

Such different effects of both coumarins can be explained in the following way.

Broadening of transition peaks on DSC scans, and lowering of transition temperature, which we can observe with 4-HC, are induced by addition of a modifier that incorporates in C_1–C_8 hydrocarbon region of lipid bilayer [6, 22]. In 4-HC molecules, shorter sides are amphiphilic, namely one of the shorter sides of the molecule is polar and the other apolar, due to the fact that the hydroxyl group is located next to the fourth carbon atom (see Fig. 1). Owing to this, it can incorporate into the lipid membrane hitching with its polar sides at the lipid polar heads, while the apolar part will be located between acyl chains disturbing their interactions and, consequently, decreasing the temperature and enthalpy of membrane phase transition. At high concentrations of coumarin the phase transition would be canceled. Such location of 4-HC is similar to positioning of phenothiazine derivatives in dimyristoylphosphatidylcholine [22] and sterols in phosphocholines [19]. Temperature dependence of fluorescence confirms such organization of 4-HC in lipid bilayers. The fact that in this dependence one can clearly see pretransition and the main phase transition confirms that hydrophobic and hydrophilic interactions can occur between 4-HC and lipid membranes. This type of interactions is observed with many amphiphilic drugs [7].

The UMB molecule has one of the longer sides polar and the other apolar, which is the result of a hydroxyl group location next to the seventh carbon atom. Thus, it can be said to have the amhiphilic character "across" the molecule. It is hard for such a molecule to incorporate itself into the lipid membrane and if it does so, this occurs very close to polar heads almost without interaction with lipid acyl chains and thus only slightly affecting phase transition parameters. This occurs at concentrations of UMB below 10 mol%. The interpretation of the obtained results at concentrations above 10 mol%, is not unequivocal. It is difficult to express unequivocally the organization of UMB in lipid bilayers. At these concentrations, one can observe slight, but noticeable lowering of phase transition parameters and broadening of transition peak on DSC scans, which may mean partial location of UMB in the acyl chain region. It is interesting that the similar problem was described by Hereć at al. [5] who studied the organization of amphotericin B in lipid bilayers. They found that amphotericin B at low concentration is in monomeric form and locates predominantly in the polar region of the lipid membrane. At concentrations above 1 mol% there is an incorporation of amphotericin B within acyl chain region of the lipid membrane with the process of amphotericin B aggregation. Amphotericin B molecules, like UMB, have an amphiphilic character "across" the molecule. Moreover, UMB molecules and amphotericin B significantly differ and it is not possible to compare, for instance the molecular aggregation mechanism of these two compounds, but it is possible that at higher concentrations of UMB, in partially aggregated state, its location is within acyl chain region of lipid bilayer. It seems that at pH level applied in this study, UMB may occur in monomeric and in dimeric form. This is confirmed by the measurements of absorption in the study by Biswas et al. [2]. The pH effect on absorption spectra observed in this study may be explained by aggregation of UMB molecules to the dimeric form. Analogical situation was observed by Gagoś et al. [3] in the studies of the pH influence on molecular organization of 2-(2,4-dihydroxyphenylo)-5,6-dichlorobenzothiazole. So, the influence of UMB on temperature and enthalpy of phase transition, at higher concentrations may be caused by aggregation of UMB molecules and the incorporation of dimers in the lipid membrane. Gagoś et al. [4] also proved the dimerization of 2-(2,4-dihydroxyphenylo)-5,6-dichlorobenzothiazole to be dependent on incorporation of the drug into DPPC membrane.

However, the temperature dependences of fluorescence do not confirm the location of UMB in the hydrophobic membrane core. The phase transition is not visible distinctly at these dependences. So, it seems that a probable aggregation of UMB and location in acyl chain region takes place only to a negligible degree.

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