ELSEVIER

Biophysical Chemistry 130 (2007) 32 – 40

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

Phase separation is induced by phenothiazine derivatives in phospholipid/sphingomyelin/cholesterol mixtures containing low levels of cholesterol and sphingomyelin

Andrzej B. Hendrich *, Krystyna Michalak, Olga Wesołowska

Department of Biophysics, Wrocław Medical University, ul. Chałubińskiego 10, 50-368 Wrocław, Poland

Received 27 March 2007; received in revised form 2 July 2007; accepted 3 July 2007
Available online 10 July 2007

Abstract

Lipid rafts are membrane structures enriched in cholesterol, sphingomyelin and glycolipids. In majority raft-mimicking model systems high contents of cholesterol and sphingomyelin (approximately 30 mol%) are used. Existence of raft-like structures was, however, reported also in model and natural membranes containing low levels of cholesterol and sphingomyelin. In the present work differential scanning calorimetry and fluorescence spectroscopy with the use of Laurdan probe was employed to demonstrate the existence of phase separation in model systems containing DPPC with addition of 5 mol% or 10 mol% of both cholesterol and sphingomyelin. Additionally, the influence of three phenothiazine derivatives on phase separation in mixed DPPC/cholesterol/sphingomyelin bilayers was investigated. Chlorpromazine, thioridazine and trifluoperazine were able to induce phase separation in DPPC and DPPC/cholesterol/sphingomyelin bilayers in temperatures below lipid main phase transition. However, only trifluoperazine induced phase separation in temperatures close to or above main phase transition. Trifluoperazine also induced phase separation in bilayers composed of egg yolk PC or DOPC mixed with cholesterol and sphingomyelin. We concluded that presence of lipid domains can be observed in model membranes containing low levels of cholesterol and sphingomyelin. Among three phenothiazine derivatives studied, only trifluoperazine was able to induce a permanent phase separation in phosphatidylcholine/cholesterol/sphingomyelin systems.

Keywords: Lipid rafts; Phenothiazine derivatives; Laurdan generalized polarization; Phase separation

1. Introduction

During the last decade lipid rafts attracted attention of many laboratories investigating biological membranes because they offer an excellent possibility to explain mechanisms of many membrane-related processes like transport, sorting, signal transduction and others [1,2]. Rafts are observed in natural membranes but they can be also obtained in models like giant unilamellar vesicles [3], supported lipid monolayers or bilayers [4]. In case of lipid rafts heterogeneous distribution of their main components: sphingomyelin and cholesterol is caused by the differences in physical properties of those compounds and their surrounding. For this reason in majority of model systems mimicking raft behavior besides sphingomyelin and cholesterol, highly fluid (at the temperature of experiment) lipids are also used. Such a composition of model system provides favorable circumstances for phase separation. Another feature of most commonly explored raft model systems is that cholesterol and sphingomyelin are present in high concentrations (approx. 30% of total lipid content, see [5] for most recent study in this field). It has been shown, however, that phase separation can be observed in model membranes containing much smaller cholesterol and sphingomyelin amounts. Using fluorescence correlation spectroscopy Kahya et al. [6] observed phase separation in giant unilamellar vesicles containing even less than 10% of cholesterol in DOPC/SM/Chol mixture. This finding demonstrates that presence of lipid rafts (or more general phase separation) is possible even in situations in which cholesterol

Abbreviations: Chol; cholesterol; CPZ; chlorpromazine; DOPC; dioleoyl phosphatidylcholine; DPPC; dipalmitoyl phosphatidylcholine; EYPC; egg yolk phosphatidylcholine; GP; generalized polarization; SM; sphingomyelin; TDZ; thioridazine; TFP; trifluoperazine.

* Corresponding author. Tel.: +48 71 784 1416; fax: +48 71 784 0088.
E-mail address: hendrich@biofiz.am.wroc.pl (A.B. Hendrich).

0301-4622/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2007.07.001

content in the membrane is small. Low cholesterol level was observed in disk membranes of the rod cells of retina [7]. Such a composition of disks does not exclude, however, the presence of lipid rafts in these membranes [8,9]. On the other hand, it was also found that low levels of membrane cholesterol appear in certain stages of development of B cells [10].

Phase separation can be observed in systems containing at least two lipid components differing in physical properties but can also be induced in mono-component systems under certain experimental conditions. As we have shown some years ago phenothiazine derivative — trifluoperazine, can induce phase separation in phosphatidylcholine bilayers in temperatures below the main phase transition [11]. The fact that phenothiazines alter the properties of lipid bilayers may correlate with their ability to modulate the activity of multidrug-related transport proteins [12] and therefore they became the object of our studies. In present work we describe the studies on the influence of three phenothiazine derivatives (trifluoperazine, chlorpromazine and thioridazine) on phase separation in mixtures containing low levels of cholesterol and sphingomyelin. The fluorescent indicator Laurdan was employed, whose generalized polarization is an ideal tool to follow the formation of domains in lipid systems [13,14]. It was found that all studied drugs induced phase separation, however the appearance of this effect depended on the phase state of the bilayer.

2. Materials and methods

2.1. Chemicals

Drugs used in the present study: chlorpromazine (CPZ), trifluoperazine (TFP) and thioridazine (TDZ) were products of ICN Biomedicals (Costa Mesa, CA, USA). Lipids: 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), L-α-phosphatidylcholine from egg yolk (EYPC), sphingomyelin (SM) from egg yolk and cholesterol (Chol) were purchased from Sigma (St. Louis, MO, USA). Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) was from Molecular Probes (Eugene, OR, USA). All other chemicals used in experiments were of analytical grade.

2.2. Microcalorimetry

DPPC was mixed with chloroform solutions of cholesterol and/or sphingomyelin to obtain the desired molar ratios. Lipid mixture was then evaporated under stream of nitrogen and placed under vacuum for a minimum of 2 h to remove the traces of organic solvent. Next, each sample (1.5 mg of lipid) was hydrated with 15 μl of buffer (20 mM Tris–HCl 0.5 mM EDTA, 150 mM NaCl, pH 7.4) containing the appropriate amount of CPZ, TFP or TDZ. The samples were heated to the temperature *c.a.* 10 °C higher than the temperature of main phase transition of DPPC and vortexed. Optically homogenous lipid mixtures were transferred into aluminum pans and sealed.

Calorimetric measurements were performed using Rigaku calorimeter, which was partially rebuilt in our laboratory. Samples were scanned immediately after preparation with scanning speed of 1.25 °C/min. For each lipid mixture studied at least two samples were prepared, each sample was scanned at least four times.

2.3. Fluorescence spectroscopy

Phospholipids (DPPC, DOPC or EYPC) were mixed with appropriate amounts of cholesterol and/or sphingomyelin in chloroform. Organic solvent was then evaporated under stream of nitrogen and the samples were kept under vacuum for at least 2 h. Unilamellar liposomes were obtained by sonication of lipid suspensions in 20 mM Tris–HCl buffer, 0.5 mM EDTA, 150 mM NaCl (pH 7.4). Sonication was performed using UP 200s sonicator (Dr Hilscher, Berlin, Germany) operating at 50/50 pulse mode. Samples were sonicated on ice until the liposome suspension became opalescent. Sonicated samples were not checked for remaining multilamellar vesicles and integrity. Laurdan (1 mM) was dissolved in dimethyl sulfoxide, all drugs (10 mM) were dissolved in water. Liposomes (final phospholipid concentration 200 μM) were incubated with fluorescent probe (concentration 3 μM) in darkness for 30 min at room temperature. Next phenothiazine derivative was added (in amounts that gave final drug concentration in samples of 100 μM) and the incubation was continued for a further 15 min under the same conditions.

Laurdan emission spectra were collected using LS 50B spectrofluorimeter (Perkin-Elmer Ltd., Beaconsfield, UK). Emission and excitation slits were set to 5 nm. Temperature was controlled by a water-circulating bath and the actual temperature was measured directly in the cuvette using a platinum thermometer. The content of the cuvette was continuously mixed. In GP(λ_{ex}) experiments excitation wavelength for Laurdan was 320–400 nm and fluorescence emission spectra were recorded in the range of 410–540 nm, while in GP(T) experiments Laurdan was excited at 390 nm and emission spectra were collected in the wavelength range 410–540 nm. Data were collected and processed with FLDM Perkin-Elmer software. Laurdan generalized polarization was calculated according to the equation [15]:

$$GP = \frac{I_B - I_R}{I_B + I_R} \quad (1)$$

where I_B and I_R were the fluorescence emission intensities at the blue and red edges of the emission spectrum, respectively. The GP values were calculated using emission intensities at 440 nm (I_B) and 490 nm (I_R). Since studied phenothiazines showed only negligible fluorescence in the wavelengths range used in experiments (less than 1% of Laurdan intensity) and we have not recorded any significant influence of these compounds on Laurdan spectral properties, the recorded data were not corrected for the presence of phenothiazines.

2.4. Partition coefficient calculations

Octanol/water partition coefficients of phenothiazine derivatives studied in present work were calculated by computer

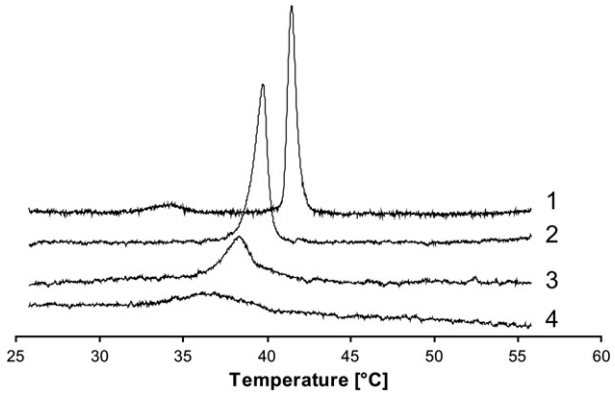

Fig. 1. Thermograms exemplifying the impact of composition on thermal behavior of studied lipid mixtures. 1 — pure DPPC, 2 — DPPC+5 mol% Chol+5 mol% SM, 3 — DPPC+5 mol% Chol+5 mol% SM+2.5 mol% TFP, 4 — DPPC+10 mol% Chol+10 mol% SM+2.5 mol% TFP.

software Titan (Wavefunction, Inc., Schrödinger Inc., USA). Calculations were made using AM1 semi-empirical molecular orbital method.

3. Results

Calorimetric investigations were performed to give a preliminary description of phase behavior of DPPC/SM/Chol mixtures and to characterize the impact of phenothiazine derivatives on these mixtures. Fig. 1 exemplifies the character of changes induced by addition of first cholesterol and sphingomyelin and then trifluoperazine to DPPC. As can be seen there, increasing amounts of Chol and SM in samples shifted the main transition temperature towards lower values and broadened the transition profile. The presence of the drug in DPPC/Chol/SM mixtures further broadened the transition profile. Due to the presence of cholesterol and sphingomyelin in studied samples the pretransition of DPPC was completely abolished and only the main phase transition peaks were present in the recorded thermograms. The results of these measurements are presented

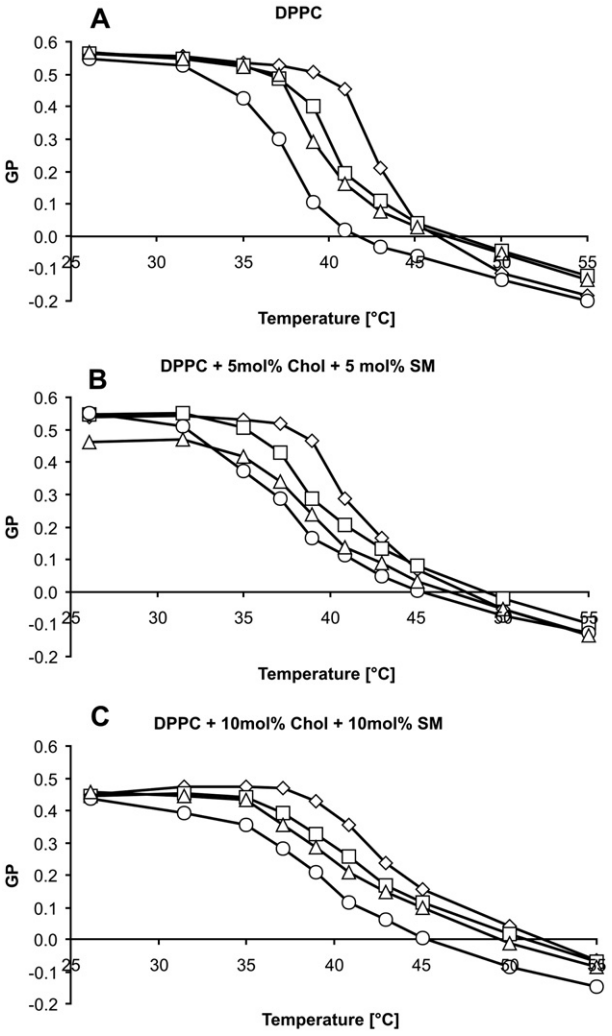

Fig. 2. The effect of phenothiazines on the dependence of Laurdan generalized polarization on temperature in DPPC liposomes containing different amounts of cholesterol and sphingomyelin. (A) — pure DPPC, (B) — DPPC+5 mol% Chol+5 mol% SM, (C) — DPPC+10 mol% Chol+10 mol% SM. In each panel: ◇ — pure DPPC or DPPC/Chol/SM mixture, □ — lipids+chlorpromazine, △ — lipids+thioridazine, ○ — lipids+trifluoperazine. Concentration of phenothiazine derivatives in samples was 100 μM.

Table 1
The influence of trifluoperazine, thioridazine and chlorpromazine on the parameters of main phase transition of DPPC mixed with cholesterol and sphingomyelin

Phenothiazine derivative [2.5 mol%]	Cholesterol [mol%]	Sphingomyelin [mol%]	T_m [°C]	ΔH [kJ/mol]	$\Delta T_{1/2}$ [°C]
None	0	0	41.5	36.5	0.6
	5	5	40.0	18.1	1.1
	10	10	39.6	14.1	1.6
Chlorpromazine	5	5	38.6	18.4	4.0
	10	10	n.d.	n.d.	n.d.
Trifluoperazine	5	5	38.2	16.5	3.0
	10	10	n.d.	n.d.	n.d.
Thioridazine	5	5	40.2	13.6	1.4
	10	10	n.d.	n.d.	n.d.

T_m — transition temperature, ΔH — transition enthalpy, $\Delta T_{1/2}$ — half-height width of the transition peak.
n.d. — not determined due to the excessive broadening of transition peaks.

in Table 1. When the concentration of cholesterol and sphingomyelin in studied mixtures was increased we observed the gradual decrease of DPPC's main phase transition temperature and enthalpy. Simultaneously the transition peaks were broadened, as illustrated by the increase of the half-height width of transition peaks. When phenothiazine derivatives were added to the studied lipid mixtures further alteration of phase transition parameters was recorded. In mixtures containing 5 mol% of Chol and SM trifluoperazine and chlorpromazine induced additional phase transition temperature decrease followed by significant broadening of transition peaks. Transition enthalpy was decreased by TFP but not by CPZ. Presence of thioridazine has not changed the transition temperature, caused only slight increase of the transition half-height width but substantially decreased the transition enthalpy. The thermograms, recorded for lipid mixtures as well as for samples with addition

of phenothiazine derivatives, were composed of single peaks, no humps on the shoulders or double peaks were observed. For phenothiazine-lipid mixtures containing 10 mol% of both Chol and SM the transition peaks become so broad that determination of the transition parameters was not possible (see thermogram 4 in Fig. 1).

To broaden the description of the DPPC/Chol/SM mixtures phase behavior we measured the dependence of Laurdan generalized polarization on temperature in liposomes containing different amounts of cholesterol and sphingomyelin. The influence of CPZ, TFP and TDZ on GP(T) dependencies obtained for liposomes composed of pure DPPC, 5 mol% and 10 mol% of both cholesterol and sphingomyelin are plotted in Fig. 2A, B and C, respectively. In all cases a drop of GP values was observed in a certain range of temperatures. The narrowest temperature range of this drop was recorded in liposomes composed of pure DPPC, while the range of GP values' decrease was much bigger for liposomes containing increasing amounts of cholesterol and sphingomyelin. Also addition of phenothiazine derivatives increased this range and induced its shift towards lower temperatures. Among the three studied phenothiazines TFP exerted the biggest influence on this increase of GP drop range and shift of this drop in all examined lipid combinations.

In further experiments we measured the dependence of Laurdan generalized polarization on excitation wavelength in the liposomes containing 5 mol% or 10 mol% of cholesterol and sphingomyelin. These experiments were performed at room temperature, i.e. the temperature in which DPPC (as well as sphingomyelin) was in the gel phase. As presented in Fig. 3A for pure DPPC and for mixtures DPPC/Chol and DPPC/SM containing 5 mol% of cholesterol or sphingomyelin, respectively, GP was almost constant for all excitation wavelengths, with values lying in the range between 0.5 and 0.6. Only for mixtures of DPPC with 5 mol% of both Chol and SM we observed a gradual GP increase from 0.32 to 0.46, when the excitation wavelength was elevated from 310 nm to 390 nm. Simultaneously for all mixtures containing 10 mol% of cholesterol and/or sphingomyelin we recorded the ascending GP(λ_{ex}) dependence — see Fig. 3B. It is worth to notice that in Fig. 3B for each of the excitation wavelengths GP values for DPPC/Chol mixtures were higher (in the range between 0.4 and 0.5) than those for DPPC/SM or DPPC/Chol/SM.

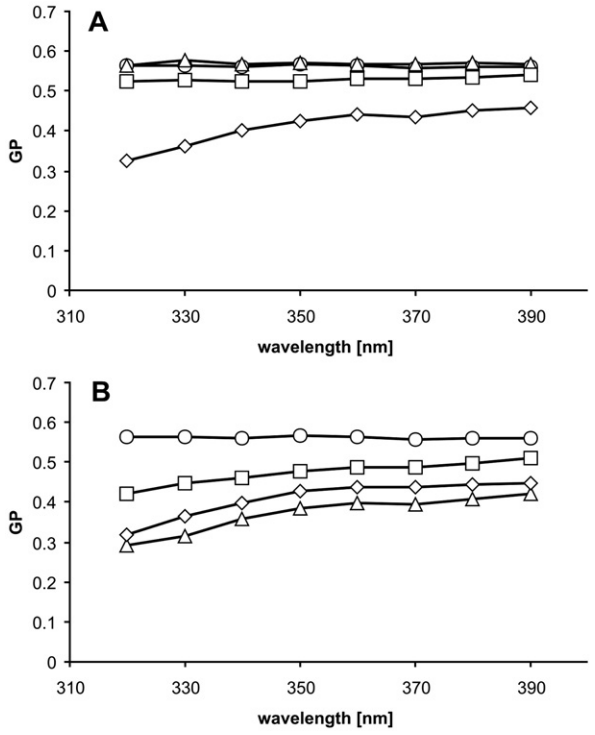

Fig. 3. The dependence of Laurdan generalized polarization on the excitation wavelength – GP(λ_{ex}) – in DPPC liposomes containing 5 mol% (A) and 10 mol% (B) of cholesterol and/or sphingomyelin. ○ — pure DPPC, □ — DPPC/cholesterol, △ — DPPC/sphingomyelin, ○ — DPPC/cholesterol/sphingomyelin. Measurements were performed at room temperature.

In the next step we used Laurdan generalized polarization to characterize the influence of three phenothiazine derivatives: chlorpromazine, thioridazine and trifluoperazine on the phase behavior of DPPC/Chol/SM mixtures. Since from the calorimetric experiments we already knew that presence of phenothiazine derivatives alters the main phase transition temperature of studied systems the experiments were performed at three temperatures at which the mixtures were well below (26.1 °C), close to (40.9 °C) and well above (55 °C) the main phase transition temperature, respectively. According to our calorimetric data phenothiazines affect main phase transition of lipids. Spectroscopic experiments were performed also in temperature close to the main phase transition because we wanted to check if phenothiazines induce (or not) any additional phase separation effects when bilayer lipids are changing their packing mode. The results of these measurements are summarized in Fig. 4. As follows from that figure for the temperature at which mixtures were in the gel phase (26.1 °C, left column), we found that addition of phenothiazines altered the character of GP(λ_{ex}) dependence. Generally the increase of GP values following the elevation of excitation wavelength was recorded. For all DPPC/Chol/SM mixtures, however, the slope of this dependence was much steeper for lower wavelengths, while less significant GP values increase was observed for wavelengths closer to 390 nm. The extent of the total GP change depended also strongly on the type of phenothiazine derivative used. For all studied DPPC/Chol/SM mixtures the weakest impact on GP(λ_{ex}) dependence was exerted by CPZ, TDZ affected this dependence more strongly, and the strongest influence was recorded when TFP was added to studied lipid mixtures. The extent of GP alteration by phenothiazine derivatives was also dependent on the composition of the mixtures. For the increasing amounts of cholesterol and sphingomyelin in mixtures we observed a downward shift of GP(λ_{ex}) plots.

When the experiments were performed at temperature close to the main phase transition (40.9 °C), the character of the recorded GP(λ_{ex}) dependencies has changed. Chlorpromazine or thioridazine added to DPPC liposomes caused the decrease of GPs as the excitation wavelength was increased. This effect was weaker for mixtures containing 5 mol% of cholesterol and sphingomyelin, because only a minute decrease of GP values

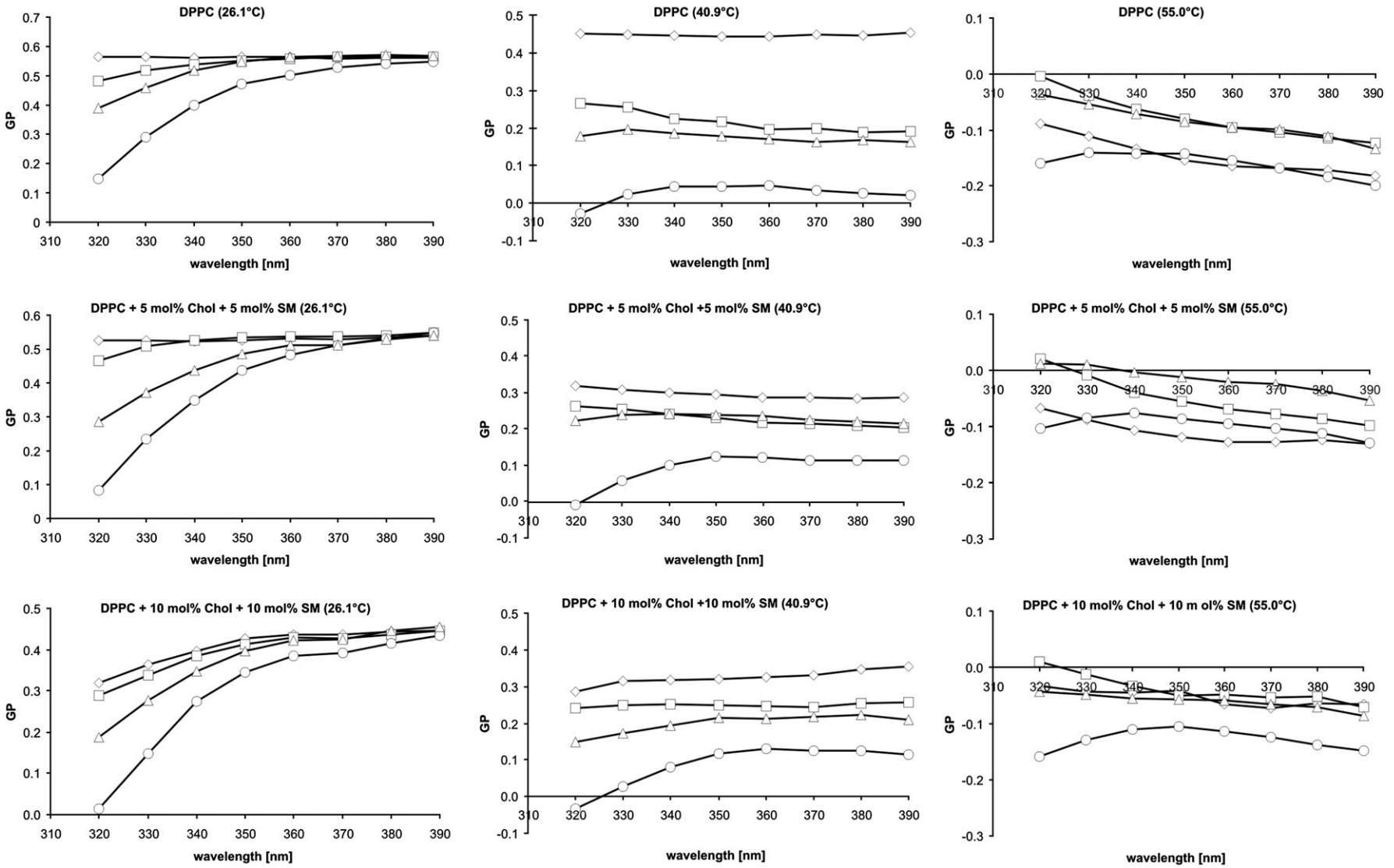

Fig. 4. The influence of phenothiazine drugs on the GP(λ_{ex}) dependence in DPPC liposomes (upper row) containing 5 mol% (middle row) and 10 mol% (lower row) of sphingomyelin and cholesterol. The experiments were performed in temperatures below (26.1 °C, left column), close to (40.9 °C, middle column) and above (55 °C, right column) main transition temperature of DPPC. In each panel: ◊ — pure DPPC or DPPC/Chol/SM mixture, □ — lipids+chlorpromazine, △ — lipids+thioridazine, ○ — lipids+trifluoperazine. Concentration of phenothiazine derivatives in samples was 100 μM.

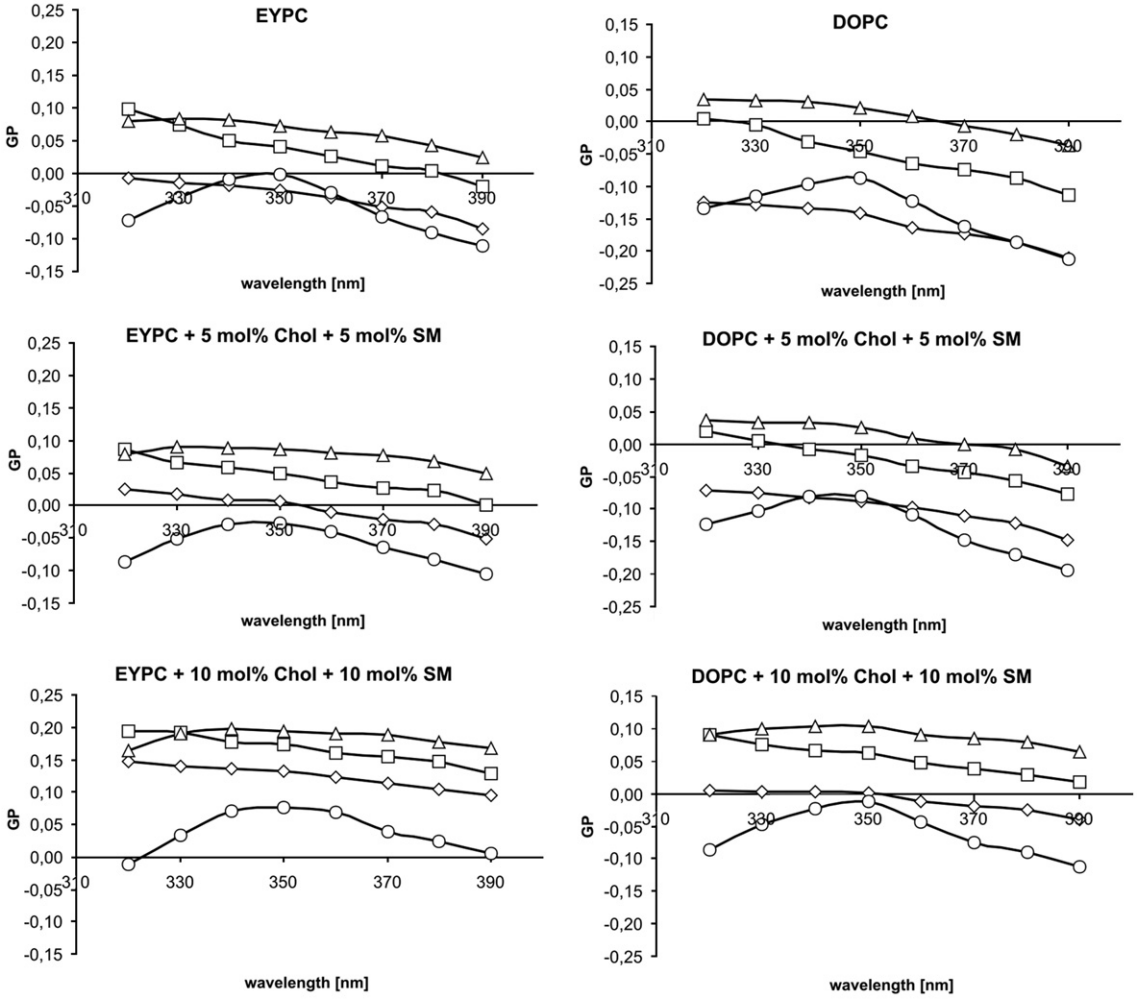

Fig. 5. The effect of phenothiazine drugs: CPZ (□), TDZ (△) and TFP (○) on the Laurdan GP(λ_{ex}) dependence in EYPC (left column) and DOPC (right column) liposomes (upper row) containing 5 mol% (middle row) and 10 mol% (lower row) of sphingomyelin and cholesterol. In each panel ◊ represents control samples (liposomes without addition of drugs). Experiments were performed at room temperature. Concentration of phenothiazine derivatives in samples was 100 μM.

was found. For mixtures containing 10 mol% of Chol and SM we observed a slight increase of GP as the excitation wavelength was elevated. Different effects were recorded when trifluoperazine was added to the studied mixtures. In pure DPPC liposomes as well as in those formed from DPPC/Chol/SM mixtures TFP induced an increase of GP for the wavelength increasing in the range 320–350 nm, while for bigger excitation wavelengths a very small decrease of GP was recorded.

In most of the measurements performed at temperature above the main phase transition (55 °C) we found a descending relation between GP and excitation wavelength. The exception were samples with addition of TFP for which we observed biphasic GP(λ_{ex}) dependence: an increase of GP for λ_{ex} lying in the range 320–350 nm followed by the decrease for higher λ_{ex} values. Its worth to notice that at this temperature all of the recorded GP values were significantly smaller than those obtained at lower temperatures.

Finally, we determined the influence of phenothiazine derivatives on the Laurdan GP(λ_{ex}) dependencies in liposomes composed of EYPC and DOPC mixtures with cholesterol and sphingomyelin. These experiments were performed at room temperature because both phospholipids are in liquid-crystalline phase at this temperature. As it can be seen in Fig. 5, the results obtained in this part of work were qualitatively very similar to those obtained for DPPC/Chol/SM mixtures at 55 °C. When CPZ and TDZ were added to liposomes formed either from pure phospholipids or from lipid/Chol/SM mixtures the descending relation between GP and excitation wavelength was recorded. Similar to DPPC systems in case of TFP addition we observed biphasic GP(λ_{ex}) dependence, however the slope of the descending phase in EYPC and DOPC mixtures was greater than in DPPC ones. For mixtures of both phospholipids containing 10 mol% Chol/SM the recorded values of GP were slightly bigger than for 5 mol% mixtures.

4. Discussion

According to the thorough description of the influence of cholesterol on phase properties of DPPC presented by McMullen and McElhaney [16], in mixtures containing more than 5 mol% of this sterol the pretransition is completely abolished. Pretransition was absent also in the thermograms of

DPPC/Chol/SM mixtures studied in the present work. As it follows from the data presented in Table 1 the main phase transition of DPPC was affected by cholesterol and sphingomyelin in a similar way as it was described for the influence of cholesterol alone. The temperature and enthalpy of transition were decreased and transition peaks were broadened by addition of Chol and SM to the studied systems. This character of phase transition parameters alteration induced by presence of increasing amounts of cholesterol and sphingomyelin suggests that both regions (hydrophobic core and polar head-groups) of lipid bilayer were perturbed [17]. However, unlike the results presented by McMullen and McElhaney [16], we have not recorded thermograms containing two overlapping components: sharp and broad. The existence of only one component in our calorimetric results presumably was a caused by the fact that in our samples apart from cholesterol also sphingomyelin was present. In our experiments we used scanning rate higher than used in [16], what also can be a reason for appearance of only one component in thermograms. As a matter of fact also other authors, using low scanning rates have not recorded two components for cholesterol/DPPC mixtures [18]. According to the phase diagram proposed in [16] we may assume that DPPC/Chol/SM systems in temperatures below the phase transition were in liquid ordered gel phase and in liquid ordered liquid-crystalline phase in higher temperatures.

Our theoretical calculations have shown that octanol/water partition coefficients of CPZ (Log P=3.617), TFP (Log P=3.735) and TDZ (Log P=4.032) were very similar. Also the experimentally determined by Takegami et al. [19] as well as by Binford and Palm [20] water/lipid bilayer partition coefficients of CPZ, TFP and TDZ show only small differences. In our opinion possible differences of phenothiazine partition between water and lipid phases are not big enough to explain the differences of their effects observed in present work. The influence of chlorpromazine and trifluoperazine on the calorimetrically observed phase properties of DPPC/Chol/SM mixtures was different than that of thioridazine. CPZ and TFP present in mixtures containing 5 mol% Chol/SM induced only moderate decrease of transition enthalpy, while transition temperature was decreased more significantly. Simultaneously we observed pronounced broadening of transition peaks. On the other hand TDZ changed transition temperature and peak width only slightly but it decreased transition enthalpy much greater than CPZ and TFP. This results allow us to conclude that all three phenothiazine derivatives interact with DPPC/Chol/SM bilayers but CPZ and TFP are presumably located closer to the bilayer surface than TDZ.

The character of DPPC main phase transition changes induced either by alteration of liposome membrane composition or by the presence of phenothiazine derivatives revealed by microcalorimetry was confirmed by GP(T) measurements. GP drop present in all curves in Fig. 2 corresponded to the phase transition. Thus increased range of temperatures in which this drop was observed in the presence of cholesterol and sphingomyelin correlated with transition peak broadening observed calorimetrically. Simultaneously, the shift of GP drop range towards lower temperatures (observed in each panel of Fig. 2) represented the decrease of main phase transition temperature induced by phenothiazine derivatives. It is easy to notice that to obtain a similar magnitude of phase transition broadening and temperature shift in DSC and spectrofluorimetric measurements, in the latter case we had to use somehow higher drug/lipid molar ratios. This difference did not appear to be surprising because of the different water phase (buffer) content in both studied systems. It seemed obvious that in liposome suspensions which are more diluted than multilamellar DSC samples bigger drug/lipid molar ratio is necessary so obtain the same amounts of drug incorporated into bilayer.

Specific changes of Laurdan spectral properties are induced by the different rate of dipolar relaxation resulting from an alteration of access of water molecules to the fluorophore surrounding [13,15]. This property makes Laurdan an ideal tool to study the appearance of phase separation in lipid systems. The course of the dependence of GP on the excitation wavelength allows to find if the studied system is in gel, fluid phase or if different phases co-exist [13,21]. In brief, in gel phase GP does not depend on the excitation wavelength, in fluid phase descending GP(λ_{ex}) dependence is observed while in the bilayers with co-existing phases ascending GP(λ_{ex}) dependence should be observed. Recently the new parameter–generalized polarization slope (GPS) was also proposed for quantitative description of phase properties of lipid systems in which GP(λ_{ex}) dependence is linear [22]. Few years ago we used Laurdan generalized polarization to monitor the influence of TFP on the phase behavior of synthetic phosphatidylcholines: DPPC and DMPC [11]. In this work we observed that when bilayers were in the gel state the addition of trifluoperazine induced phase separation. Since domains easily appear in lipid/Chol/SM mixtures [3,23,24], in present work we also applied Laurdan GP to find if phase separation occurs in mixtures containing low concentrations of cholesterol and sphingomyelin. For the liposomes composed of DPPC with the addition of 5 mol% of Chol and/or SM we found that ascending and almost linear GP (λ_{ex}) dependence exists exclusively when both: cholesterol and sphingomyelin were added to the phospholipids. Simultaneously the GP values for this mixtures were generally smaller then for other 5 mol% mixtures. These results suggest that presence of 5 mol% cholesterol and sphingomyelin in DPPC bilayers, at room temperature, induced appearance of domains and increased average access of water molecules to the Laurdan fluorophore. For other 5 mol% mixtures GP was independent on excitation wavelength, what meant that at room temperature no phase separation occurred in these bilayers. Average GP values of these mixtures were in the range between 0.5 and 0.6, the values which are typical for the gel state of lipid bilayer [13].

Ascending GP(λ_{ex}) dependencies were recorded for all mixtures containing DPPC and 10 mol% of Chol and/or SM. Presence of domains in DPPC [16] or DOPC [6] bilayers containing 10 mol% of cholesterol was recorded also by other research groups. As it was shown by Dietrich et al. [3] the eventual appearance and size of rafts depended on temperature. In DOPC/Chol/SM mixtures they demonstrated that during heating from 22.8 °C to 30.5 °C rafts first decreased their size and than disappeared. To elucidate the role of temperature in the

phase behavior of DPPC/Chol/SM mixtures, with or without addition of phenothiazines, we performed GP(λ_{ex}) measurements at three temperatures. According to our calorimetric data we have chosen 26.1 °C (well below main phase transition of any of used mixtures), 40.9 °C (close to transition) and 55 °C (well above transition). With the exception of the bilayers formed from pure DPPC, for all other mixtures at 26.1 °C we observed ascending GP(λ_{ex}) dependencies. For samples containing also addition of phenothiazines these dependencies were not linear. This non-linearity depended on the amounts of cholesterol/sphingomyelin present in mixtures as well as on the type of phenothiazine derivative added. The strongest effect was recorded in 10 mol% mixtures containing 100 μM of trifluoperazine. Ascending and non-linear GP(λ_{ex}) dependencies were observed by Parasassi et al. [25] in DLPC-DPPC mixtures containing 10–30 mol% of cholesterol examined in temperatures in which both lipids were in liquid-crystalline state. The character of the non-linearity observed by Parasassi et al. [25] was similar to that recorded in present work, but these authors have not commented on the possible reasons of this observation.

For the temperatures close and above the main phase transition the non-linear but partly ascending GP(λ_{ex}) dependency was observed exclusively for mixtures with the addition of TFP. In other mixtures no indication of phase separation was found, moreover at 55 °C the descending GP(λ_{ex}) dependencies suggested that studied systems were in the fluid (liquid-disordered) phase. This conclusion was supported by the fact that for all mixtures, when increasing the temperature, we recorded also the subsequent decrease of average GP values — an effect informing that studied systems, including the mixtures containing TFP, become more fluid [13].

The most intriguing was the comparison of the GP(λ_{ex}) dependencies for TFP-containing 10 mol% DPPC/Chol/SM mixtures obtained at different temperatures. When the studied systems were in gel (or gel-like ordered) phase the ascending GP(λ_{ex}) dependency was recorded, while at much higher temperature (55 °C, liquid-crystalline phase) a biphasic (ascending–descending) GP(λ_{ex}) dependency was observed. It seems possible that these effects could be caused by overlapping of two separate mechanisms. First of them should be the TFP-induced phase separation which produces ascending GP(λ_{ex}) dependency, which is observed in all studied temperatures in DPPC/Chol/SM mixtures. The second one should be related to the temperature-induced increase of membrane disorder, producing descending GP(λ_{ex}) dependency in temperatures above the main phase transition. Such an explanation seems to be valid also for observations made in mixtures composed of EYPC or POPC and cholesterol/sphingomyelin (Fig. 5). Since biphasic GP(λ_{ex}) dependency was observed exclusively when TFP was added to these mixtures we may conclude that only this drug is able to induce a permanent phase separation in those systems.

In this work we found that phase separation can be induced by phenothiazine derivatives (CPZ, TDZ, TFP) in lipid mixtures containing low concentrations of cholesterol and sphingomyelin. While CPZ and TDZ exerted such effect exclusively when lipid bilayer was in the ordered gel phase, TFP-induced phase separation also in bilayers in liquid-disordered phase. This finding, in our opinion, suggests that different lipid domains could be observed under certain circumstances also in membranes which are not rich in cholesterol and sphingomyelin. Furthermore presence of phenothiazines could influence the structure of lipid rafts already present in cell membrane. This in turn can alter the activity of raft proteins and should be at least partially responsible for different biological effects exerted by phenothiazine derivatives — starting from the blocking of dopamine receptors up to modulation of multidrug resistance-related transport proteins.

Acknowledgements

The authors are grateful to Aneta Kalińska, Barbara Rajmund, Katarzyna Sidorska, Justyna Piegza and Piotr Walenców for technical assistance on the project. This work has been financially supported by the funds of Wrocław Medical University.

References

[1] E. Ikonen, Roles of lipid rafts in membrane transport, Curr. Opin. Cell Biol. 13 (2001) 470–477.
[2] D.A. Brown, E. London, Functions of lipid rafts in biological membranes, Annu. Rev. Cell Dev. Biol. 14 (1998) 111–136.
[3] C. Dietrich, L.A. Bagatolli, Z.N. Volovyk, N.L. Thompson, M. Levi, K. Jacobson, E. Gratton, Lipid rafts reconstituted in model membranes, Biophys. J. 80 (2001) 1417–1428.
[4] J.C. Lawrence, D.E. Saslowsky, J.M. Edwardson, R.M. Henderson, Real-time analysis of the effects of cholesterol on lipid raft behavior using atomic force microscopy, Biophys. J. 84 (2003) 1827–1832.
[5] C. Nicolini, J. Kraineva, M. Khurana, N. Periasamy, S.S. Funari, R. Winter, Temperature and pressure effects on structural and conformational properties of POPC/SM/cholesterol model raft mixtures — a FT-IR, SAXS, DSC, PPC and Laurdan fluorescence spectroscopy study, Biochim. Biophys. Acta 1758 (2006) 248–258.
[6] N. Kahya, D. Scherfeld, K. Bacia, B. Poolman, P. Schwille, Probing lipid mobility of raft-exhibiting model membranes by fluorescence correlation spectroscopy, J. Biol. Chem. 278 (2003) 28109–28115.
[7] A.D. Albert, K. Boesze-Battaglia, The role of cholesterol in rod outer segment membranes, Prog. Lipid Res. 44 (2005) 99–124.
[8] K. Boesze-Battaglia, R. Schimmel, Cell membrane lipid composition and distribution: implications for cell function and lessons learned from photoreceptors and platelets, J. Exp. Biol. 200 (1997) 2927–2936.
[9] R.E. Martin, M.H. Elliott, R.S. Brush, R.E. Anderson, Detailed characterization of the lipid composition of detergent-resistant membranes from photoreceptor rod outer segment membranes, Invest. Ophthalmol. Vis. Sci. 46 (2005) 1147–1154.
[10] F.G. Karnell, R. Brezski, L.B. King, M.A. Silverman, J.G. Monroe, Membrane cholesterol content accounts for developmental differences in surface B cell receptor compartmentalization and signaling, J. Biol. Chem. 280 (2005) 25621–25628.
[11] A.B. Hendrich, O. Wesołowska, K. Michalak, Trifluoperazine induces domain formation in zwitterionic phosphatidylcholine but not in charged phosphatidylglycerol bilayers, Biochim. Biophys. Acta 1510 (2001) 414–425.
[12] A.B. Hendrich, K. Michalak, Lipids as a target for drugs modulating multidrug resistance of cancer cells, Curr. Drug Target 4 (2003) 23–30.
[13] T. Parasassi, G. De Stasio, G. Ravagnan, R.M. Rusch, E. Gratton, Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of Laurdan fluorescence, Biophys. J. 60 (1991) 179–189.
[14] T. Parasassi, E. Gratton, W.M. Yu, P. Wilson, M. Levi, Two-photon fluorescence microscopy of laurdan generalized polarization domains in model and natural membranes, Biophys. J. 72 (1997) 2413–2429.

[15] T. Parasassi, G. De Stasio, A. dUbaldo, E. Gratton, Phase fluctuation in phospholipid membranes revealed by Laurdan fluorescence, Biophys. J. 57 (1990) 1179–1186.
[16] T.P.W. McMullen, R.N. McElhaney, New aspects of the interaction of cholesterol with dipalmitoylphosphatidylcholine bilayers as revealed by high-sensitivity differential scanning calorimetry, Biochim. Biophys. Acta 1234 (1995) 90–98.
[17] M.K. Jain, N.M. Wu, Effects of small molecules on the dipalmitoyl lecithin liposomal bilayer. III. Phase transitions of lipid bilayers, J. Membrane Biol. 34 (1977) 157–201.
[18] M.R. Vist, J.H. Davis, Phase equilibria of cholesterol/dipalmitoylphosphatidylcholine mixtures: 2H nuclear magnetic resonance and differential scanning calorimetry, Biochemistry 29 (1990) 451–464.
[19] S. Takegami, K. Kitamura, T. Kitade, K. Hasegawa, A. Nishihira, Effects of particle size and cholesterol content on the partition coefficients of chlorpromazine and triflupromazine between phosphatidylcholine–cholesterol bilayers of unilamellar vesicles and water studied by second-derivative spectrophotometry, J. Colloid Interface Sci. 220 (1999) 81–87.
[20] J.S. Binford, W.H. Palm, Absorption of surfactants by membranes: erythrocytes versus synthetic vesicles, Biophys. J. 66 (1994) 2024–2028.
[21] L. Bagatolli, T. Parasassi, G.D. Fidelio, E. Gratton, A model for the interaction of 6-lauroyl-2-(*N*,*N*-dimethylamino) naphthalene with lipid environments: implications for spectral properties, Photochem. Photobiol. 70 (1999) 557–564.
[22] J. Velazquez, M.S. Fernandez, GPS, the slope of laurdan generalized polarization spectra, in the study of phospholipid lateral organization and *Escherichia coli* lipid phases, Arch. Biochem. Biophys. 455 (2006) 163–174.
[23] S.L. Veatch, S.L. Keller, Organization in lipid membranes containing cholesterol, Phys. Rev. Lett. 89 (2002) 268101.
[24] R.F.M. de Almeida, A. Fedorov, M. Prieto, Sphingomyelin/phosphatidylcholine/cholesterol phase diagram: boundaries and composition of lipid rafts, Biophys. J. 85 (2003) 2406–2416.
[25] T. Parasassi, M. Di Stefano, M. Loiero, G. Ravagnan, E. Gratton, Influence of cholesterol on phospholipid bilayers phase domains as detected by Laurdan fluorescence, Biophys. J. 66 (1994) 120–132.