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Laurdan properties in glycosphingolipid-phospholipid mixtures: a comparative fluorescence and calorimetric study

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Abstract

Laurdan (6-dodecanoyl-2-dimethylamine-naphthalene) is a fluorescent membrane probe of recent characterization. It was shown that this probe discriminates between phase transitions, phase fluctuations and the coexistence of phase domains in phospholipid multilamellar aggregates. We measured the excitation and emission generalized polarization (GP_{ex} and GP_{em}) of Laurdan in aggregates of complex glycosphingolipids in their pure form and in mixtures with dipalmitoylphosphatidylcholine (DPPC). Our results show that Laurdan detects the broad main phase transition temperature of the neutral ceramide-tetrasaccharide Gg_4Cer (asialo-G_{M1}) and shows a value of GP_{ex} in between that of DPPC and that of ganglioside G_{M1}. In contrast, Laurdan was unable to detect the thermotropic phase transition of G_{M1}. The probe also appears to be unable to detect phase coexistence in both types of pure glycolipid aggregates. Deconvolution of the excess heat capacity vs. temperature curves of pure Gg_4Cer and DPPC/Gg_4Cer mixtures indicates that the thermograms are composed by different transition components. For these cases, Laurdan detects only the high cooperativity component of the transition of the mixture. The peculiar behaviour of Laurdan in aggregates containing complex glycosphingolipids may result from the inherent topological features of the interface that are conferred by the bulky and highly hydrated polar head group of these lipids.

Keywords: Ceramide tetrasaccharide; Ganglioside; Differential scanning calorimeter; Generalized polarization; Lipid domain; Lifetime distribution

1. Introduction

Laurdan (6-dodecanoyl-2-dimethylamine-naphthalene) is an amphiphilic fluorescence probe synthesized by Weber and Farris to study the effect of solvents with different polarity on the characteristics of its fluorescence emission properties [1]. In solvents of high polarity, Laurdan shows a considerable shift

Abbreviations: Laurdan, 6-dodecanoyl-2-dimethylamine-naphthalene; Gg_4Cer (asialo G_{M1}), Galβ1 → 3Gal-NAcβ1 → 4Galβ1 → 4Glcβ1 → Cer; G_{M1}, Galβ1 → 3Gal-NAcβ1 → 4Gal(3 ← 2αNeuAc)β1 → 4Glcβ1 → Cer; DPPC, dipalmitoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene

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of the emission spectra to higher wavelengths due to dipolar relaxation processes [2]. When the local environment of Laurdan is a phospholipid phase, the emission depends strongly on the physical state of the lipid aggregate. The dipolar relaxation in a given phase physically corresponds to the reorientation of the surrounding dipoles around the Laurdan excited-state dipole. At temperatures below the lipid phase transition (gel state) this process is slower than the Laurdan fluorescence lifetime and the probe emits with an emission spectra localized at high energy frequencies (blue region). At temperatures above the phase transition (liquid crystalline state) this process is faster and comparable to the Laurdan excited state lifetime and the emission spectrum shifts about 50 nm from 440 nm to 490 nm [2,3].

The fluorescence steady-state parameter Generalized Polarization (*GP*) relates quantitatively these spectral changes by taking into account the relative fluorescence intensities of the blue and red edge regions of the emission and excitation spectra, respectively [2,3]. For phospholipids, a characteristic *GP* value was found for the gel and liquid crystalline phases independent on the polar head group of the phospholipid [2]. The *GP* values obtained for phospholipids undergoing phase changes are related to the different motional freedom of water molecules around the fluorescent group inserted in the lipid membrane [2].

Substantial work from our laboratory has unravelled several biophysical properties of a series of chemically related glycosphingolipids ranging from simple cerebrosides to complex gangliosides. Gangliosides contain one or more sialic acids attached to a neutral oligosaccharide chain of varied complexity in their polar head group. The hydrophilic moiety of these lipids reaches a length similar to that of the hydrocarbon portion and, in turn, influences dramatically their surface, thermotropic and topological properties [4–9]. The number and type of carbohydrates in the polar head group of these glycosphingolipids have also a direct influence on the extent of the interfacial hydration or micropolarity [9–11] and on the polarity gradient taken from the polar head group to the hydrophobic core of the lipid aggregate [12]. Considering that Laurdan is particularly sensitive to interfacial water motion in phospholipid aggregates, we explored the behaviour of this solvatochromic probe in interfaces containing glycosphingolipids. In the present work we studied the behaviour of Laurdan in micelles of ganglioside G_{M1} in pure aggregates of the neutral tetrasaccharide Gg_4Cer (asialo G_{M1}) and mixed Gg_4Cer-DPPC systems. We compared the Laurdan behaviour with that obtained by differential scanning calorimetry and with the properties showed by a more hydrophobic probe, DPH, whose lifetime is also sensitive to the physical-state of the membrane.

2. Materials and methods

2.1. Materials

Laurdan was purchased from Molecular Probes (Eugene, OR), DPH was from Sigma (St. Louis, MO) and DPPC was from Avanti Polar Lipids (Alabaster, AL). G_{M1} and Gg_4Cer were obtained as described previously [4,13,14].

2.2. Methods

Stock solutions of Laurdan were made in absolute ethanol, G_{M1} and Gg_4Cer in chloroform/methanol/water (60:30:4.5, v/v) and DPPC in chloroform/methanol (2:1, v/v). Lipid-probe dispersions were made with a lipid/probe ratio of 400:1 (Laurdan final concentration was 0.5 μM). The solvent was evaporated under N_2 and the dried samples were left for at least 4 h under high vacuum. The dry lipid mixtures were hydrated in 20 mM Tris-HCl-50 mM NaCl (pH 7.4) for 2 h above the main lipid phase transition and vortexed. All samples were prepared and stored in dark and the spectra measured immediately after preparation. A similar procedure was used for DPH samples (lipid/probe ratio was 400:1, DPH final concentration was 1 μM).

Steady state excitation and emission spectra were measured with a SLM 4800C fluorometer (SLM/AMINCO®, Champaign, IL) equipped with a Xenon-arc lamp using 8 nm bandwidth. The excitation spectra were corrected with a quantum counter (Rhodamine B in ethylene glycol 3 g/ml) in the reference cell [15] and the emission spectra were corrected for instrument response with SLM software. We carried out a blank subtraction in all samples.

The generalized polarization (GP_{ex}) is given by:

$$GP = \frac{I_g - I_{lc}}{I_g + I_{lc}}$$

where I_g and I_{lc} are the fluorescence intensities at the maximum emission in the gel and in the liquid crystalline phase, respectively (excitation GP). For phospholipids both distinguishable fluorescence excited states were defined as non-relaxed (gel) and relaxed (liquid crystalline) state of Laurdan by Gratton's group [3] and a similar meaning was used in this paper. In addition a GP value can also be obtained using the fluorescence intensities at the maximum excitation in the gel phase and in the liquid crystalline phase (emission GP) [2,16,17]. To obtain GP_{ex} spectra of pure Gg_4Cer, G_{M1} and DPPC/Gg_4Cer mixtures at different temperatures, the intensities of the emission at wavelengths of 440 nm and of 500 nm were chosen and GP_{ex} values were calculated using excitation wavelengths from 320 nm to 420 nm. Laurdan in G_{M1} aggregates already shows a relaxed behaviour in the gel state (see Fig. 1), with practically similar values of its lifetime below or above its mean phase transition temperature (see Table 1), and to a minor extent in Gg_4Cer aggregates. In order to evaluate comparatively the Laurdan GP_{ex} values in phospholipid and glycosphingolipid aggregates we kept the above emission wavelengths for both types of lipids. To obtain the GP_{em} spectra at different temperatures the intensities of the excitation at 390 nm and 360 nm were chosen and GP_{em} values were calculated using emission wavelengths from 420 nm to 550 nm.

The temperature was controlled by a refrigerated thermocirculating bath and temperature was directly measured in the sample cuvette with a digital thermometer.

DPH and Laurdan fluorescence lifetimes were measured in a multifrequency phase shift and modulation fluorometer (model GREG 200 ISS, Champaign, IL) equipped with a Xenon Arc Lamp and He/Cd Liconix laser as light source and the emission was observed through a KV399 cutoff filter. A solution of 1,4-bis-2-(4-methyl-5-phenyloxazolyl) bencene (dimethyl-POPOP) in ethanol was used as a reference ($\tau = 1.45$ ns) [15]. Data were collected until the standard deviation from each measurement of phase and modulation were at most 0.2° and 0.004, respectively. Data acquisition and analysis were performed using software from ISS Instruments.

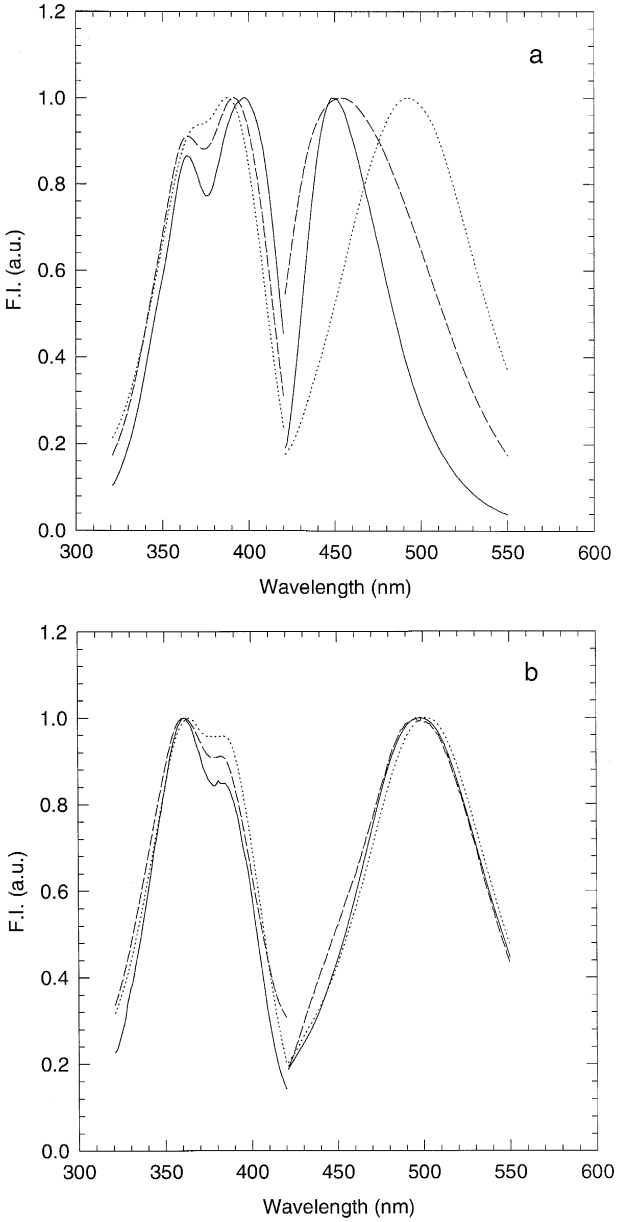

Fig. 1. Laurdan fluorescence excitation and emission spectra in DPPC (————), Gg_4Cer (---) and G_{M1} (· · ·) at below (a) or above (b) their respective main phase transition temperature. The temperatures below and above the main transition temperature were 10 and 60°C for DPPC, 10 and 65°C for Gg_4Cer and 2 and 50°C for G_{M1}. Buffer was 20 mM Tris-HCl-50 mM NaCl (pH 7.4). Laurdan concentration was 0.5 μM, lipid/probe ratio was 400:1.

Table 1
Laurdan fluorescence lifetimes

Sample	τ (ns) (below phase transition)	τ (ns) (above phase transition)
DPPC	7.51	3.32
DPPC-Gg_4Cer 6%	7.80	3.47
DPPC-Gg_4Cer 36.4%	7.50	3.22
DPPC-Gg_4Cer 69.7%	6.17	3.50
Gg_4Cer	4.30	3.40
G_{M1}	3.93	3.56

Calorimetric measurements were made using a Microcral MC-2D differential scanning calorimeter (Northampton, MA) and samples were prepared as described by Maggio et al. [18].

3. Results

3.1. Fluorescence properties of Laurdan in different aggregates constituted by DPPC, Gg_4Cer and G_{M1}

The Laurdan emission and excitation spectra were measured in pure Gg_4Cer and G_{M1} ganglioside aggregates and compared to those obtained with pure DPPC. The excitation was set at 360 nm for the emission spectra and the emission wavelength was fixed at 440 nm for the excitation spectra.

Fig. 1 shows the Laurdan excitation and emission spectra in DPPC, Gg_4Cer and G_{M1} aggregates below (Fig. 1a) and above (Fig. 1b) their respective main phase transition temperature. Laurdan emission spectra show a progressive red shift from DPPC to G_{M1} aggregates below the phase transition (Fig. 1a). Above the phase transition the position of Laurdan emission maximum is the same, independent of the lipid tested. In ganglioside micelles, the gel → liquid phase transition induces a relatively small red shift of the emission spectra by 8–9 nm whereas for Gg_4Cer and DPPC vesicles the red shifts are 43 and 53 nm, respectively (compare Fig. 1a and b). At temperatures below the phase transition the intensity of the red band of the excitation spectra, centred at 390–400 nm, is higher in DPPC than in glycosphingolipid aggregates compared to the blue band centred at 360–370 nm. A slight blue shift was observed in the excitation spectra of Laurdan in glycolipid aggregates

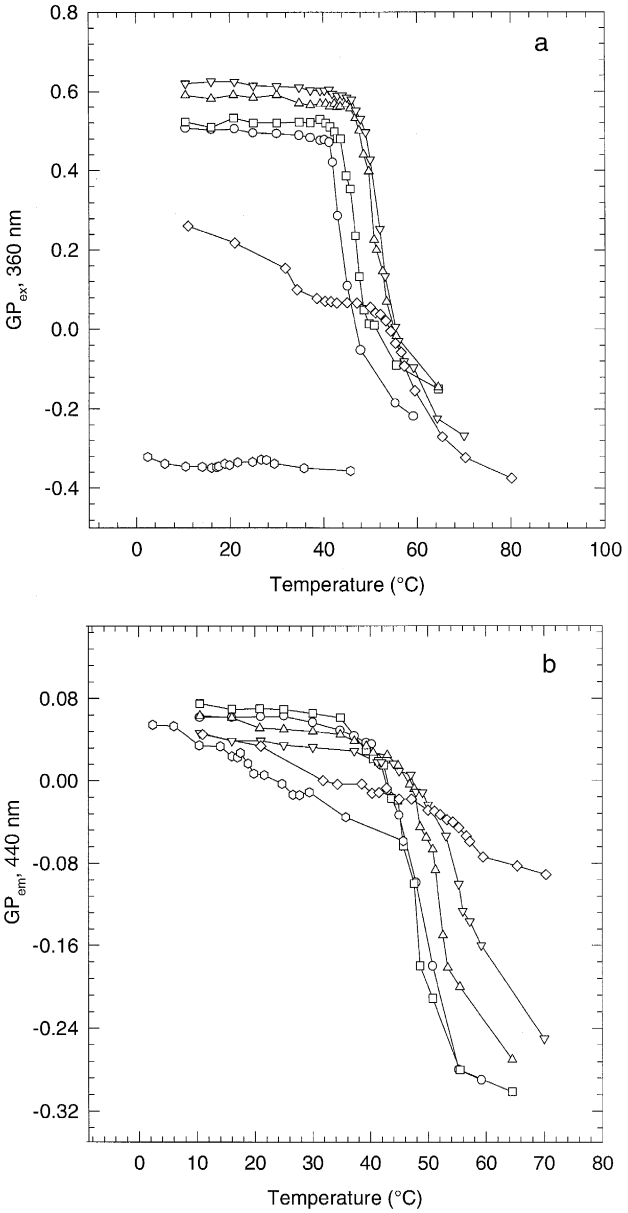

Fig. 2. GP_{ex} (a) and GP_{em} (b) values as a function of temperature for Laurdan in aggregates of: DPPC (○); DPPC/Gg_4Cer mixtures at 6 (□), 36.4 (△), 69.7 (▽) mol% of Gg_4Cer; Gg_4Cer (◇) and G_{M1} (⬡). The GP_{ex} values were calculated using an excitation wavelength of 360 nm. The GP_{em} values were calculated using an emission wavelength of 440 nm. The main phase transition temperatures were calculated from the dGP/dT and the values obtained were: pure DPPC = 41°C; DPPC/Gg_4Cer mixtures at a mol% of Gg_4Cer of: 6% = 42°C, 36.4% = 46°C and 69.7% = 52°C; pure Gg_4Cer = 56°C. Buffer was 20 mM Tris-HCl-50 mM NaCl (pH 7.4). Laurdan concentration was 0.5 μM, lipid/probe ratio was 400:1.

compared to that obtained in DPPC vesicles below the phase transition. As mentioned above and compared to DPPC, the intensity of red excitation band of Laurdan in the glycolipid aggregates decreases and the emission spectrum is red shifted below the phase transition, these changes are more marked for G_{M1} than Gg_4Cer (Fig. 1a). In addition, the halfwidth of the Laurdan emission spectra in the glycosphingolipid aggregates below the main phase transition temperature is higher than the value found for Laurdan in the gel state of DPPC aggregates (Fig. 1a). The higher interfacial polarity found for the glycosphingolipid interfaces, compared to phospholipids [10–12] together with the dipolar relaxation process may be responsible for the position of the Laurdan emission maximum in glycolipids below the phase transition (see below). As expected from a higher hydration, the values of Laurdan GP_{ex} in the gel phase of micelles of pure G_{M1} are lower than those observed with phospholipids and the values fall in the range of those obtained in the liquid phase of DPPC (see Fig. 2a). The previous calorimetric data for micelles of G_{M1} indicated a low cooperativity and broad phase transition with a T_m centred at 19°C [18] that is consistent with a highly hydrated and curved interface [8,19,20]. Even when the *GP* of Laurdan in G_{M1} shows a slight decrease with temperature the probe was insensitive for detecting the G_{M1} phase transition; this contrasts with the abrupt changes found at about 41°C for DPPC (Fig. 2, see also Ref. [16]).

The asialoganglioside Gg_4Cer, shows a broad transition with an average T_m centred at 54°C [18] that can be deconvoluted into four transition components (see below, Table 2 and Fig. 4, bottom right). The excitation *GP* values of Laurdan in Gg_4Cer at temperatures below the phase transition show an intermediate value between that exhibited by DPPC and ganglioside G_{M1} (Fig. 2a). The changes in the *GP* with the temperature are sensitive to the Gg_4Cer phase transition and are in keeping with the calorimetric data (compare Fig. 2 and Fig. 4, bottom right).

For pure Gg_4Cer, G_{M1}, DPPC and in mixtures of Gg_4Cer/DPPC we also measured the fluorescence lifetime τ of Laurdan inserted in these aggregates

Table 2
Calorimetric parameters of different DPPC/Gg_4Cer mixtures

Mol% of Gg_4Cer	Peak #	T_m	Cp_{max}	$T^{1/2}$	H_{cal}	Peak area (%)	Coop Unit	r^2
0	1	41.26	59.35	0.10	8.57	100	295	1.000
0.6	1	41.52	6.16	0.29	2.69	40.4	251	0.998
	2	41.68	6.63	0.46	3.96	59.6	83	
3	1	41.45	7.51	0.30	3.40	44.0	234	0.999
	2	41.56	11.54	0.31	4.32	56.0	121	
6	1	41.74	3.75	0.39	1.59	24.8	192	0.999
	2	42.12	4.75	0.75	4.80	75.2	41	
19.7	1	42.19	1.13	0.49	0.83	16.5	124	0.999
	2	43.0	0.43	2.54	1.17	23.1	62	
	3	45.0	0.65	4.43	3.06	60.5	14	
36.4	1	44.64	1.81	0.46	0.89	21.6	159	0.997
	2	44.85	0.52	4.02	2.38	57.5	73	
	3	49.19	0.26	3.11	0.86	20.9	18	
69.7	1	43.6	0.07	5.06	0.39	13.1	97	0.999
	2	48.89	0.24	5.81	1.46	48.2	58	
	3	52.43	0.18	6.18	1.18	38.8	25	
91.6	1	43.35	0.04	4.49	0.19	5.7	121	0.999
	2	49.14	0.17	6.78	1.21	36.5	56	
	3	54.55	0.19	4.28	0.85	25.6	34	
	4	57.51	0.18	5.79	1.17	32.2	24	
100	1	47.94	0.17	6.40	1.21	27.7	69	0.994
	2	51.74	0.17	3.67	0.67	15.3	32	
	3	55.39	0.23	3.42	1.03	23.6	24	
	4	58.42	0.20	6.58	1.45	33.3	19	

(Table 1). For pure DPPC Laurdan shows a value for τ of 7.51 ns in the gel phase and this changes to 3.32 ns in the liquid stated (Table 1, see Ref. [17]). In pure Gg_4Cer and G_{M1} aggregates the lifetime was between 3–4 ns at temperatures below their main phase transi-

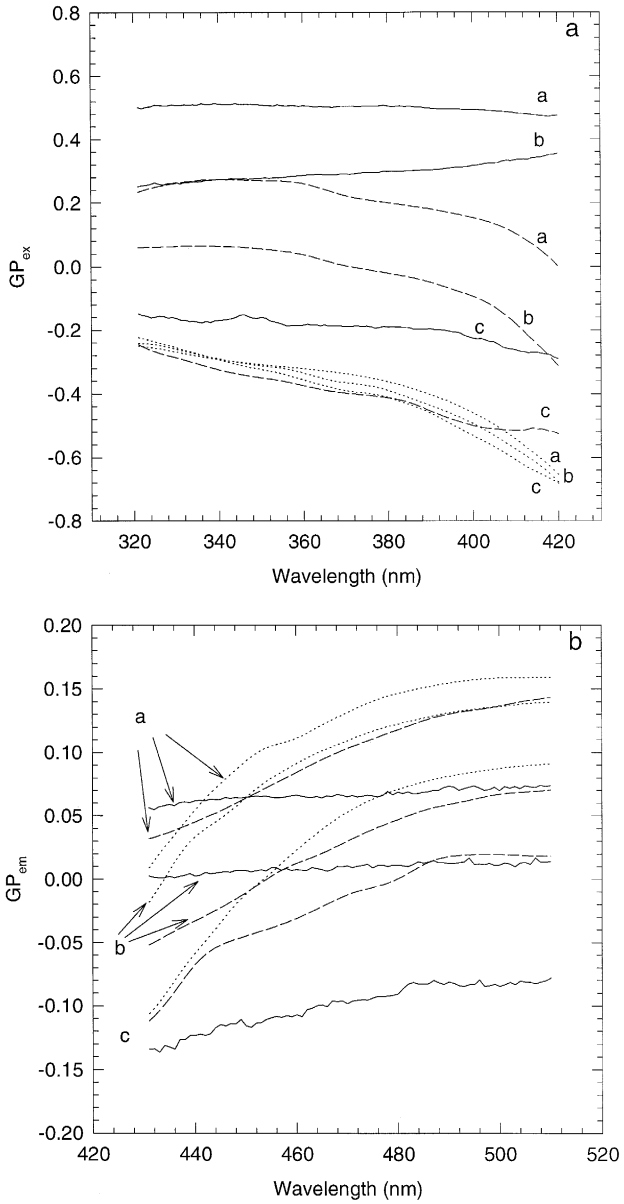

Fig. 3. Excitation (a) and Emission (b) Generalized Polarization values for Laurdan in aggregates of DPPC (————), Gg_4Cer (---) and G_{M1} (· · ·) at below of their respective main phase transition temperature (a), at their main phase transition temperature (b) and at above of their main phase transition temperature (c). Buffer was 20 mM Tris-HCl-50 mM NaCl (pH 7.4). Laurdan concentration was 0.5 μM, lipid/probe ratio was 400:1.

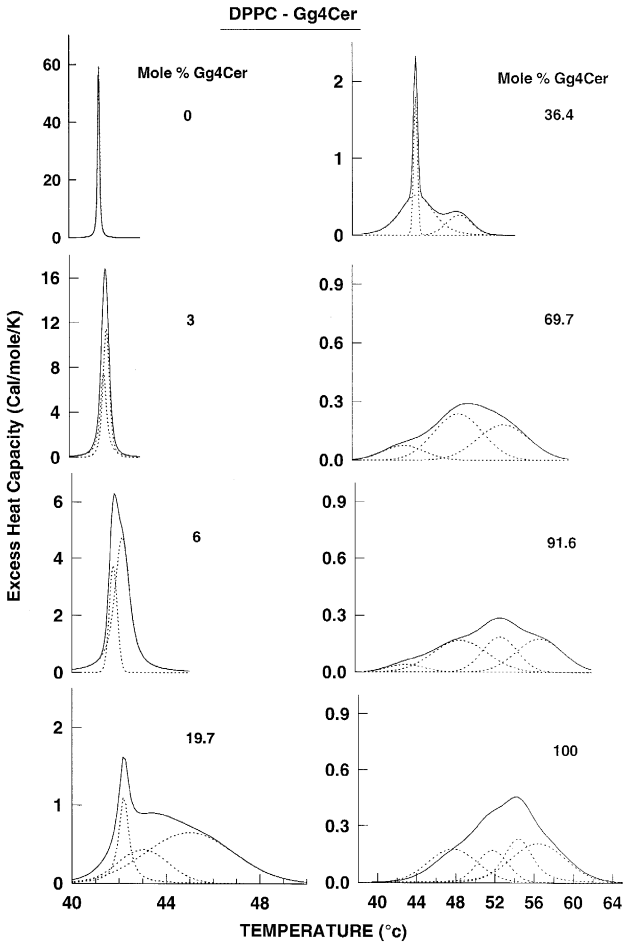

Fig. 4. Calorimetric thermograms of representative mixtures of DPPC/Gg_4Cer with the deconvoluted peaks.

tion. These values are similar to those found for Laurdan in phospholipid vesicles in the liquid crystalline phase state (Table 1).

The behaviour of *GP* values as a function of excitation and emission wavelengths has been used to ascertain the coexistence of phase domains in the lipid aggregate [2,3]. Fig. 3 shows the variation of GP_{ex} (Fig. 3a) and GP_{em} (Fig. 3b) with wavelength for pure Gg_4Cer and G_{M1} aggregates compared to those in pure DPPC vesicles at temperatures below, in the range of and above the phase transition. The GP_{em} and GP_{ex} values decrease as the temperature increases but similar *GP* spectra's profiles were found independent on the phase state of both glycolipids, see Fig. 3. These results are indicative that a 'liquid-crystalline' profile may be detected, independently on the type and phase of the glycolipids tested. This

contrasts with the behaviour described for pure phospholipids, (Fig. 3, see also Refs. [2,17,21]).

3.2. Calorimetric behaviour of Gg_4Cer / DPPC mixtures

In order to ascertain the interpretation of the Laurdan fluorescence characteristics of Gg_4Cer/DPPC mixed aggregate the calorimetric behaviour of the mixture was evaluated. The calorimetric data of representative mixtures of DPPC with increasing proportions of Gg_4Cer is shown in Fig. 4. An increase of the mol% of Gg_4Cer induces a progressive shift and broadening of the transition temperature of the mixture. Beginning at very low proportions of Gg_4Cer the overall transitions can be deconvoluted (to a high statistical significance) into more than one component transition, indicating the coexistence of different phase states. The deconvolution analysis (Fig. 4) indicates the presence of peaks having a higher T_m, increased broadening and lower cooperativity as the composition is enriched in Gg_4Cer.

Table 2 shows the thermodynamic parameters of the deconvoluted components at representative mole fractions of Gg_4Cer. In general, the T_m values of some of these components increases with the proportion of glycolipid up to a certain range of composition after which it remains constant. Together with this effect (Fig. 4 and Table 2), a new transition is gradually established indicating the appearance of a new phase as the mole fraction of Gg_4Cer is increased in the mixture. In general, at each composition, the transition component with the lowest T_m exhibits the lower cooperativity while those occurring at higher temperatures become progressively less cooperative. According to the positions of the peaks in the excess heat capacity-temperature curves the ones having the lower T_m and the higher cooperativity appear always to correspond to the mixture more enriched in DPPC.

Fig. 5 shows the complete temperature-composition phase diagram for the lipid mixture, including the variation with composition of the component peak transitions. Both, miscibility, partial miscibility and immiscibility are apparent among the different phases designated as Peak-1, Peak-2, Peak-3 and Peak-4. This is clearly indicated by the presence of regions of both progressive variation with composition of the T_m of each component (miscibility) and isothermal melting (phase immiscibility) occurring in different ranges of composition. It should be pointed out that the broad thermogram of pure Gg_4Cer (cf. Ref. [18]) can itself be deconvoluted into four components having increasingly higher T_m values (Table 2 and Fig. 4). This can be related to the coexistence of various aggregates of different size such as bilayer vesicles, cylindrical, stacked, and globular micelles found when this lipid is dispersed in aqueous solution. These heterogeneous aggregates contain a different number of molecules having variations in their intermolecular packing [6,19]. This determines the coexistence of different intermolecular interaction energies in the various aggregates [18] and their progressive dissociation with temperature is responsible for the low cooperativity of the phase transition process. Both the phase diagram and the calorimetric curves suggest that a proportion of DPPC and Gg_4Cer becomes immiscible with the rest of the mixture when the lipid proportions are above 90 mol% of DPPC or among 60 and 90 mol% of Gg_4Cer, respectively.

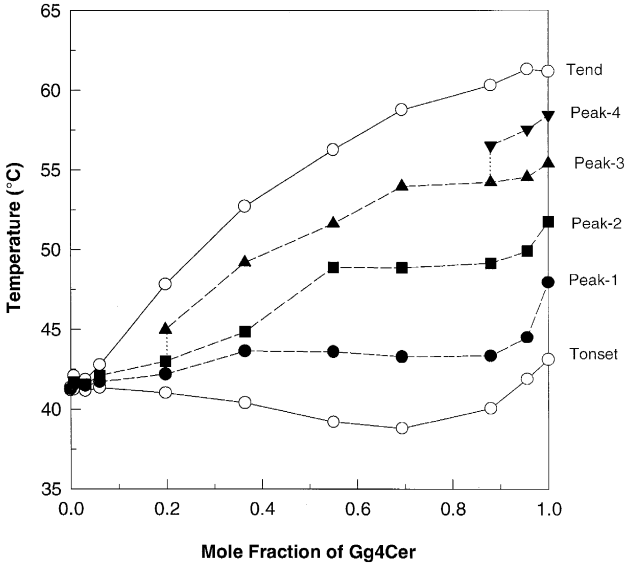

Fig. 5. Complete temperature-composition phase diagram of DPPC/Gg_4Cer mixtures.

3.3. Dependence of fluorescence properties of Laurdan with the phase state in Gg_4Cer / DPPC mixtures

For DPPC-Gg_4Cer mixtures the Laurdan excitation spectra are practically identical to those obtained

in phospholipids vesicles. Only a slight blue shift in the emission spectra occurs below the phase transition of the mixture when the mole fraction of Gg_4Cer increases with respect to DPPC at 20°C, (not shown). This finding is in agreement with a more rigid conformation of the probe's environment when it is inserted in the mixed aggregates.

The Laurdan excitation GP_{ex} and emission GP_{em} values in DPPC-Gg_4Cer vesicles were also measured at different temperatures at mole fractions of 0.06, 0.364 and 0.697 of the glycosphingolipid. The *GP* vs. temperature plot (Fig. 2) shows an increase of T_m when the Gg_4Cer mole fraction increases. These data are in keeping with the calorimetric studies. In the gel phase the Laurdan excitation GP_{ex} values show a slight increase with respect to pure DPPC vesicles when the mole fraction of Gg_4Cer increases (Fig. 2a).

Deconvolution of the calorimetric transitions of the mixture of DPPC-Gg_4Cer, at a the glycolipid mole fraction of 0.364, reveals three transition components with a phase transition centred at about 45°C for the two (superimposed) lower melting components and at 49°C for the higher melting component (see Table 2 and Fig. 4). Fig. 2 shows an abrupt change of Laurdan *GP* values during the phase transition of this mixture. However, Laurdan is not sensitive for discriminating between the different transition components detected by calorimetry (see above) indicating that water penetration and/or relaxation rate are not affected by these transitions. On the other hand, the slope of the transition in Gg_4Cer is rather flat compared to that obtained for pure DPPC (Fig. 2) contrasting with the abrupt changes observed for the DPPC-Gg_4Cer mixtures. Even when a broad transition was found for DPPC-Gg_4Cer, at a mole fraction of 0.697 that tends to resemble that of pure Gg_4Cer (Fig. 4), the Laurdan *GP* for this sample still shows a similar slope than in the pure DPPC.

Laurdan GP_{em} and GP_{ex} spectra in mixtures of DPPC/Gg_4Cer aggregates are shown in Fig. 6a and b. A wavelength independence of GP_{em} and GP_{ex} are found in the gel phase of the mixed aggregates. In general, the wavelength dependence of Laurdan either in the GP_{ex} or GP_{em} spectra is close to those obtained in pure DPPC vesicles. For the mixtures, the wavelength dependence of Laurdan GP_{ex} and GP_{em} spectra in the phase transition region were similar to those previously reported [3,16]. This suggests that in the DPPC-Gg_4Cer mixture the behaviour of Laurdan probably reflects the phase changes of the domains more enriched in phospholipid. At temperatures above the respective phase transition, the GP_{ex} spectra show

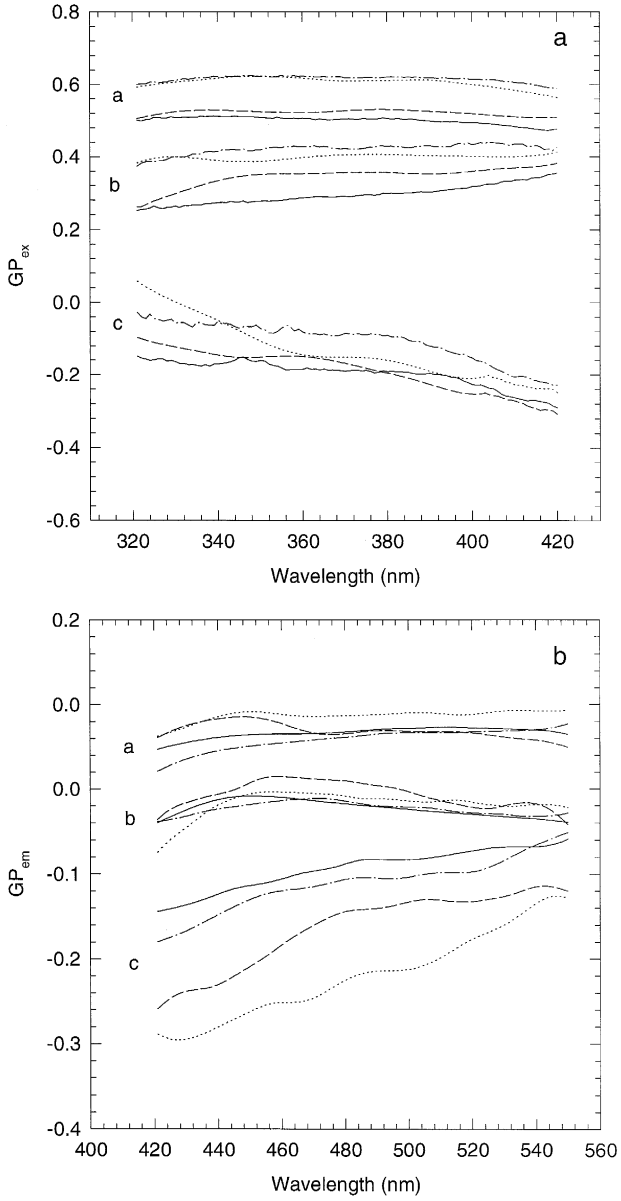

Fig. 6. Excitation (a) and Emission (b) Generalized Polarization values for Laurdan in aggregates of DPPC (————); DPPC/Gg_4Cer mixtures at 6 (---), 36.4 (· · ·) and 69.7 (– · – · –) mol% of Gg_4Cer at below of their respective main phase transition temperature (a); at their phase transition temperature (b) and above of their main phase transition temperature (c). Buffer was 20 mM Tris-HCl-50 mM NaCl (pH 7.4). Laurdan concentration was 0.5 μM, lipid/probe ratio was 400:1.

Table 3
DPH fluorescence lifetime distribution data

Sample	τ (ns) (below phase transition)	fwhm (ns) (below phase transition)	τ (ns) (above phase transition)	fwhm (ns) (above phase transition)
DPPC	10.60	0.98	7.28	0.05
DPPC-Gg_4Cer 6%	10.27	2.13	7.42	0.51
DPPC-Gg_4Cer 36.4%	9.67	2.54	7.13	1.22
DPPC-Gg_4Cer 69.7%	7.98	2.00	7.15	1.46
Gg_4Cer	7.38	3.22	6.43	1.94
G_{M1}	6.15	1.16	5.71	0.91

decreasing values with increasing excitation wavelength whereas the GP_{em} spectra show increasing values as the emission wavelength increases. This behaviour is due to the dipolar relaxation process, observed in the liquid-crystalline phase, extensively discussed before [3].

The values of fluorescence lifetimes found for DPPC/Gg_4Cer mixtures below and above the phase transition are close to those obtained for pure DPPC (Table 1). These results and the similar slopes for the changes in *GP* with temperature (Fig. 2) indicate that in the DPPC-Gg_4Cer mixture Laurdan is partitioned preferably into the phospholipid enriched phase.

3.4. Diphenylhexatriene (DPH) studies

For comparison, the lifetime distributions of DPH were measured in pure DPPC, Gg_4Cer, their mixtures and in pure G_{M1}. The average lifetime and the full width of the half maximum (fwhm) of the DPH lifetime distribution values were interpreted to be due to different values of dielectric constant and of the heterogeneity of the probe's environment in the lipid aggregate [22]. Even when Gg_4Cer has a higher T_m than DPPC (Table 2) the DPH average lifetime is lower and the distribution width larger than DPPC (Table 3). Both data indicate that DPH is sensing a less hydrophobic environment with a greater heterogeneity in pure Gg_4Cer aggregates compared to DPPC vesicles. A considerable topological heterogeneity has been reported for this glycosphingolipid [7] and a higher dielectric constant and increased dielectric gradient in glycosphingolipids compared to phospholipids was also reported previously [10–12]. Both of these effects are in agreement with the DPH data obtained.

At temperatures below the main phase transition the DPH lifetime distribution centre decreases as the Gg_4Cer mole fraction increases. The fwhm of the DPH lifetime distribution decreases above the main phase transition in all the mixtures, but these values increase as the mole fraction of Gg_4Cer increases in the mixture (Table 3). This also suggests that DPH is sensing a more heterogeneous environment and a higher dielectric gradient in pure Gg_4Cer than in the DPPC-Gg_4Cer mixed vesicles.

For G_{M1}, DPH was less sensitive in detecting the changes of the lipid phase state. The fwhm of the DPH lifetime distribution indicate a more homogeneous probe environment (Table 3). This is in agreement with a more homogeneous micellar structure for this ganglioside compared to the heterogeneous aggregates formed by Gg_4Cer [20].

4. Discussion

In the present work we studied if the fluorescence properties of Laurdan in glycosphingolipid dispersions are similar to those found in phospholipid aggregates. Laurdan fluorescence in pure glycosphingolipid or in mixtures of phospholipid-glycosphingolipid interfaces shows a particular behaviour respect to phospholipids. This is because of the inherent different molecular and interfacial structure of glycosphingolipids compared to phospholipids. These differences influence concomitantly the type and size of the topological aggregate, the hydration of the interface and the phase transition parameters that, in turn, are reflected in the Laurdan's fluorescence behaviour.

The calorimetric data indicate that the phase transi-

tions in mixtures of DPPC-Gg_4Cer occur in a complex fashion. Several transition components are detected by deconvolution analysis of the excess heat capacity vs. temperature curves obtained by high sensitivity differential scanning calorimetry. Comparing the fluorescence data with the calorimetric results we suggest that Laurdan molecules partition preferentially into one of phospholipid-enriched phases of the mixtures. This interpretation correlates with both the abrupt transition slopes of Laurdan *GP* (Fig. 2) and the Laurdan fluorescence lifetimes (Table 1) in DPPC-Gg_4Cer at mole fractions of the glycolipid of 0.364 and 0.697 (Fig. 2).The slopes found for Laurdan *GP* in the mixtures are similar to those found with pure DPPC vesicles, even when the thermogram is rather complex (Fig. 4) and they particularly contrast with the flat transition slope of the Laurdan *GP* found with pure Gg_4Cer (Fig. 2) that is in agreement with the broad phase transition obtained by calorimetry (Fig. 4). On the other hand, DPH was sensitive to the phase state of DPPC, Gg_4Cer and their mixtures. In contrast to Laurdan, DPH is capable of detecting the extent of phase heterogeneity depending on the proportion of Gg_4Cer in the mixture. This suggests a more homogeneous distribution for DPH probe in the lipid aggregates compared to Laurdan. This observations are in agreement with the results showed by Tillack et al. [23] that measured the DPH polarization vs. temperature in DMPC-Gg_4Cer mixed aggregates. This probe detect the broadening transition of these mixtures indicating an homogeneous distribution in the lipid aggregates.

In pure Gg_4Cer aggregates the emission maximum of Laurdan in the gel phase is red shifted and the emission half width is higher respect to phospholipids (Fig. 1a). Also Laurdan senses a heterogeneous environment probably due to different sizes and shapes of the vesicles and aggregates as reported previously by Maggio et al. [20]. On the other hand, for G_{M1} micelles the emission spectrum of Laurdan shows a dramatic red shift at temperatures below the phase transition (Fig. 1a) and Laurdan *GP* vs. temperature plot does not sense the G_{M1} phase transition centred at 19°C (Fig. 2, see also Ref. [18]). In addition, the GP_{ex} and GP_{em} spectra show a wavelength dependence in the pure G_{M1} micelles that is independent on the phase state. Similar results are found for Gg_4Cer aggregates. Even when the value of GP_{ex} falls in an intermediate value between those found for DPPC and G_{M1}, the Laurdan fluorescence is sensitive to the broad phase transition of pure Gg_4Cer.

All these findings plus the Laurdan lifetime values measured in these pure aggregates below the main phase transition temperature (Table 1) indicate that a progressive dipolar relaxation of Laurdan occurs when it is inserted in the glycosphingolipid aggregates depending on complexity of the polar head group. The enhanced dipolar relaxation in the gel state of complex glycosphingolipids may result from an increased amount of water molecules in the polar head group region [10,11] and to the higher polarity gradient of these interfaces [12]. This Laurdan behaviour in the glycolipid interface may be due to a fast dipolar relaxation of the solvents molecules around the fluorophore in spite of the aggregates being below their phase transition temperature. The insensitivity in detecting the G_{M1} transition is probably due to a preexisting more extensive dipolar relaxation in ganglioside micelles compared to Gg_4Cer aggregates at temperature below the phase transition. This is in keeping with the differences introduced by the incorporation of a sialic acid in the polar head group of the glycolipids [8,9].

It is known that the ganglioside G_{M1} has a bulky polar head group that confers a more hydrated interface with a higher molecular area than phospholipids [4,9–11] which, in turn, influences the topological and thermotropic behaviour of the aggregate [6–8,18,20]. The dipolar relaxation in a given phase physically corresponds to the reorientation of the surrounding dipoles around the Laurdan excited dipole [2]. This fact together with the stabilization of Laurdan molecules in the $L\ \alpha$ conformation by oriented solvent dipoles are responsible of the intensity of the red band in the Laurdan excitation spectrum. Thus, when Laurdan is inserted in phospholipid aggregates the red excitation band depends on both the polarity of the probe's environment and also on the phase state of the interface. Lower values of the intensity of the red band were found in pure glycosphingolipids aggregates compared to DPPC vesicles. Parassasi et al. [2] have proposed that for phospholipids in the liquid crystalline phase the dipolar relaxation is caused by the presence of water molecules in the bilayer having a restricted mobility compared to bulk water molecules. These water molecules have a relaxation

time in the nanosecond time scale. Our results show that in G_{M1}, and to a lesser extent in Gg_4Cer, the dipolar relaxation process is present even at temperatures below the phase transition. This is indicating that the state of relaxation of Laurdan not only is influenced by the physical state of the hydrocarbon core but rather appears to be sensitive to the extent of hydration of the interface. According to previously reported data from our laboratory both the polarity at the interface and the micropolarity gradient from the polar head group to the hydrophobic core of glycosphingolipid aggregates are higher than in phospholipids [10–12] in agreement with the DPH fluorescence lifetime distribution data (Table 3). Therefore, the detection of the coexistence of phase domains through the Laurdan *GP* spectra in complex glycolipids becomes completely masked by the enhanced relaxation process due to the presence of water molecules in an interface of higher polarity. Probably Laurdan inserted in a more complex interface such as natural membranes should also be useful for detecting *state of hydration* through its relaxation state.

The tremendous influence of the oligosaccharide polar head group of the glycosphingolipid on their physical state, topological properties and interfacial hydration [8] may have consequences on Laurdan behaviour, contrasting with the independence found for the Laurdan with phospholipids that differ in the polar head group [2]. The present study is being extended using other related glycosphingolipids to ascertain the behaviour and the usefulness of Laurdan in detecting phase domains in pure glycosphingolipids and in mixed phospholipid systems.

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