Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1618 (2003) 67–78
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

Fluorescence properties of Laurdan in cochleate phases

Karthik Ramani, Sathyamangalam V. Balasubramanian*

Department of Pharmaceutical Sciences, 541 Cooke Hall, University at Buffalo, State University of New York, Amherst, NY 14260-1200, USA

Received 29 May 2003; received in revised form 15 September 2003; accepted 10 October 2003

Abstract

Cochleates are lipid-based delivery system that have found application in drug and gene delivery. They are precipitates, formed as a result of interaction between cations (e.g. Ca^{2+}) and negatively charged phospholipids such as phosphatidylserine (PS). In the present study, we investigated the utility of fluorescent probe Laurdan (6-dodecanoyl-2-dimethylamino naphthalene) to monitor cochleate phase formation. Following addition of Ca^{2+} to Laurdan labeled lipid vesicles comprised of brain phosphatidylserine (BPS), a significant blue shift in the emission peak maximum of Laurdan was observed and the spectral features were distinct from those observed for the gel and liquid-crystalline (LC) phases. This is consistent with the formation of anhydrous cochleate cylinders that was further confirmed by electron microscopy studies. Due to dipolar relaxation, excitation and emission generalized polarization (GP_{Ex} and GP_{Em}) indicate transition from a LC to a rigid and dehydrated (RD) cochleate phase. These spectral changes were utilized to monitor the influence of lipid composition, ionic strength and lamellarity on the formation of cochleate phase. The results indicated that the presence of phosphatidylcholine (PC) and bulk Na^{+} concentration influenced the formation of cochleate structures from small unilamellar vesicles (SUV) and multilamellar vesicles (MLV) composed of PS. The presence of PC and higher bulk Na^{+} concentration stabilized the PS vesicles against collapse and total loss of contents, intermediate molecular events in the formation of cochleate structures. From these studies, we conclude that Laurdan fluorescence is a sensitive and a rapid method to detect cochleate phase formation.

Keywords: Ca^{2+}–PS interaction; Cochleate; Laurdan fluorescence; Generalized polarization; Phosphatidylcholine–phosphatidylserine mixture

1. Introduction

Cochleates were first described by Papahadjopoulos et al. [1] as intermediates during formation of large unilamellar vesicles (LUV). Addition of Ca^{2+} to sonicated phosphatidylserine (PS) vesicles in sodium chloride buffer was found to rupture the vesicles, resulting in the formation of flat bilayer disks, followed by fusion of these disks into sheets and eventual folding of these sheets into large anhydrous cylindrical structures. They are structurally distinct from liposomes and have recently found application in the delivery of drugs, proteins and DNA-based vaccines [2–4].

Several biophysical techniques such as dynamic light scattering (DLS) [5], negative stain and freeze fracture electron microscopy (EM) [1], and infrared spectroscopy (IR) [6] have been used to study this unique lipid system. A fluorescence-based method would be sensitive and rapid to detect the formation of cochleate phases.

Fluorescence spectroscopy has several advantages including high sensitivity, intrinsic time scale and excellent response to physical properties of the membrane [7]. The membrane probe, Laurdan, due to its localization at the hydrophilic–hydrophobic interface of the bilayer is very sensitive to the polarity of its environment [7,8]. In membranes, the probe displays a 50 nm emission red spectral shift during transition from a gel to liquid-crystalline phase (LC). This spectral shift is attributed to Laurdan's sensitivity to the dipolar relaxation phenomenon caused by reorientation of water molecules in the LC phase [8]. Further, the extent of spectral shift is dependent on the dipolar relaxation rate.

Abbreviations: BPS, brain phosphatidylserine; DMPC, dimyristoyl phosphatidylcholine; DLS, dynamic light scattering; EM, electron microscopy; EDTA, ethylenediaminetetraacetic acid; GP, generalized polarization; IR, infrared spectroscopy; Laurdan, 6-dodecanoyl-2-dimethylamino naphthalene; LS, light scattering; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; PC, phosphatidylcholine; PS, phosphatidylserine; SUV, small unilamellar vesicles

* Corresponding author. Tel.: +1-716-645-2842x245; fax: +1-716-645-3693.

E-mail address: svb@acsu.buffalo.edu (S.V. Balasubramanian).

0005-2736/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2003.10.009

The excitation spectra of Laurdan corresponding to the two lamellar phases are also different. While the excitation spectrum in the LC phase has a peak maximum at the blue edge of the spectrum (~360 nm), the gel phase excitation maximum is at the red edge of the spectrum (~390 nm). The excitation band centered at 390 nm is a distinct feature of Laurdan spectroscopy and originates from the stabilization of Laurdan ground state by water molecules, partially immobilized at the level of glycerol backbone [9]. These water molecules have their dipole in a favorable orientation and are relaxed. Selective excitation of Laurdan molecules in this red edge results in emission properties, which depend on the local polarity and the phase state. Therefore, information on the membrane state can be obtained rapidly by analyzing the spectral features [7,8,10]. This unique property of Laurdan has been used to detect changes in membrane properties including drug-induced fluid phase domains in model membranes [11], bilayer-to-hexagonal phase transitions [12], effect of cationic lipids on liposome physical properties [13].

In this study, we first examined the response of fluorescent properties of Laurdan in dehydrated cochleate phases. The influence of lipid composition, ionic strength and lamellarity on the fluorescence properties of Laurdan was also investigated. The results indicated that (a) properties of Laurdan were sensitive to cochleate phase and quite distinct from those observed in gel and LC phases, and (b) Laurdan would be a sensitive probe to

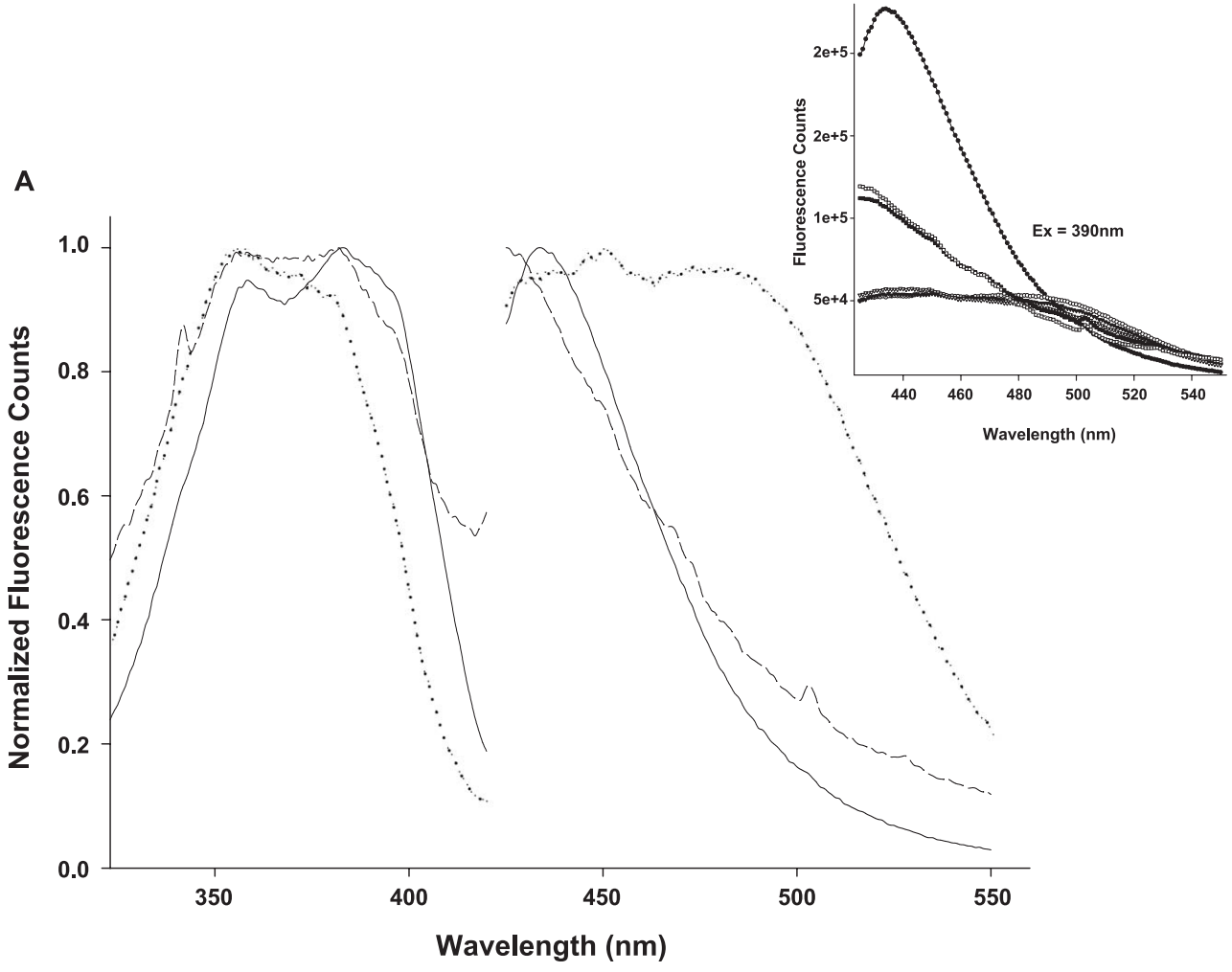


Fig. 1. (A) Excitation and emission spectra of Laurdan. Normalized excitation and emission spectra of Laurdan in SUV composed of BPS (buffer: 300 mM NaCl, 25 mM Tris, pH = 7.0) in the absence (dotted line, LC phase), presence (dashed line, cochleate phase) of Ca^{2+} (~4.95 mM) at 37 °C and in the absence of Ca^{2+} (solid line, gel phase) at 1 °C. For the excitation spectra, the emission was monitored at 440 nm and for the emission spectra the excitation wavelength was 390 nm. Inset: emission spectra of Laurdan in SUV composed of BPS in the absence or presence of varying concentrations of Ca^{2+}; (●) gel phase (no Ca^{2+}); (○) LC (no Ca^{2+}); (▼) ~0.5 mM; (▽) ~1 mM; (■) ~2 mM; (□) ~5 mM. Ex = Em slit width = 2 nm. (BPS) was ~0.5 mM and (Laurdan) was ~0.5 μM (probe/lipid = 1:1000). (B) Negative stain electron micrographs of SUV composed of BPS in the absence of Ca^{2+} (top panel) and following addition of Ca^{2+} (bottom panel, [Ca^{2+}] ~4.95 mM). Electron micrographs were obtained as described in Section 2.

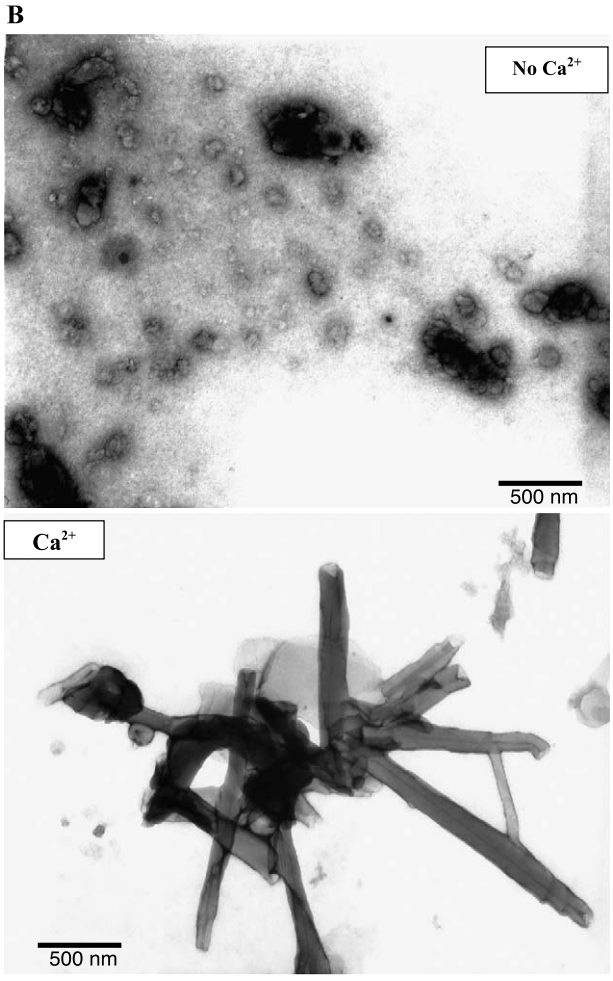


Fig. 1 (*continued*).

follow membrane dynamics in a phosphatidylcholine (PC)/PS system in the presence of Ca^{2+} ions and will be very useful to screen for the existence of non-liposomal lipid structures like cochleate cylinders in this lipid system.

2. Experimental procedures

2.1. Materials

Brain phosphatidylserine (BPS) and dimyristoyl phosphatidylcholine (DMPC) dissolved in chloroform were obtained from Avanti Polar Lipids (Alabaster, AL), stored at −70 °C and used without further purification. Laurdan was obtained from Molecular Probes Inc. (Eugene, OR). Sodium chloride, Tris (hydroxymethyl)-aminomethane hydrochloride, calcium chloride dihydrate and chloroform were obtained from Fisher Scientific (Fairlawn, NJ) and used without further purification. Ethylenediaminetetraacetic acid (EDTA-disodium salt) was from Sigma-Aldrich Corp. (St. Louis, MO) and ethanol (200 proof) was purchased from Pharmaco Products Inc. (Brookfield, CT).

2.2. Preparation of model membrane vesicles

Small unilamellar vesicles (SUV) and multilamellar vesicles (MLV) were used for Laurdan measurements. The required amount of BPS (T_c~6–8 °C) and/or DMPC (T_c~23 °C) was dissolved in chloroform and the solvent was evaporated using a rotaevaporator (Buchi R-200, Fisher Scientific) to form a thin film on the walls of a Kimax glass tube or round-bottomed flask. Residual solvent (if any) was removed from the sample under a stream of dry nitrogen. The lipid film was hydrated immediately or the glass flask/tube with the dry lipid-film were sealed with Teflon and stored in the dark at 4 °C for a duration not exceeding 24 h. The lipid film (for all compositions) was hydrated in 1 ml Tris buffer (300 or 100 mM NaCl, 25 mM Tris, pH = 7.0, prepared in double-distilled/de-ionized Millipore water) at 37 °C with vortexing, forming MLV. The MLV thus formed were subjected to sonication using a bath sonicator (Laboratory Supplies, Hicksville, NY) for ~10–15 min to form SUV. The molar ratios of PS/PC used in the study was 100:0, 70:30, 50:50, 30:70 and 0:100.

2.3. Incorporation of fluorescent probe

Laurdan was incorporated into MLV by adding the probe as a chloroform solution to the lipid, prior to thin-film formation. For SUV, a stock solution of Laurdan was prepared in ethanol (100 μM) and 2.5 or 5 μl of the probe was added to the vesicle suspension and sample equilibrated at 37 °C for ~20 min with intermittent vortexing. The final probe/lipid concentration was typically ~1:1000–2000. Concentration of the lipid ranged from ~500 μM to 4.5 mM. All vesicle preparations were used within minutes of preparation.

2.4. Steady-state Laurdan fluorescence measurements

The excitation and emission spectra of Laurdan were acquired on a PTI fluorometer (Photon Technology International, Lawrenceville, NJ) equipped with photon counting electronics and a xenon arc lamp. Excitation (Ex) and emission (Em) slit widths were set at either 2 or 4 nm. Stock solutions of $CaCl_2.2H_2O$ (1 M) and EDTA (100 mM) were prepared and required volume of these solutions were added in succession to the vesicle preparation to get a final Ca^{2+} and EDTA concentrations of ~4.95–4.97 and ~9.01–9.05 mM, respectively. The Ca^{2+} concentration was above the threshold cation concentration (typically 1 mM) to induce cochleate phases in PS containing vesicles.[1] The EDTA concentration

[1] This threshold cation concentration of ≥1 mM that results in formation of cochleate structures was initially reported for a bulk Na^+ concentration of 100 mM. See text for more discussion (see Ref. [1]).

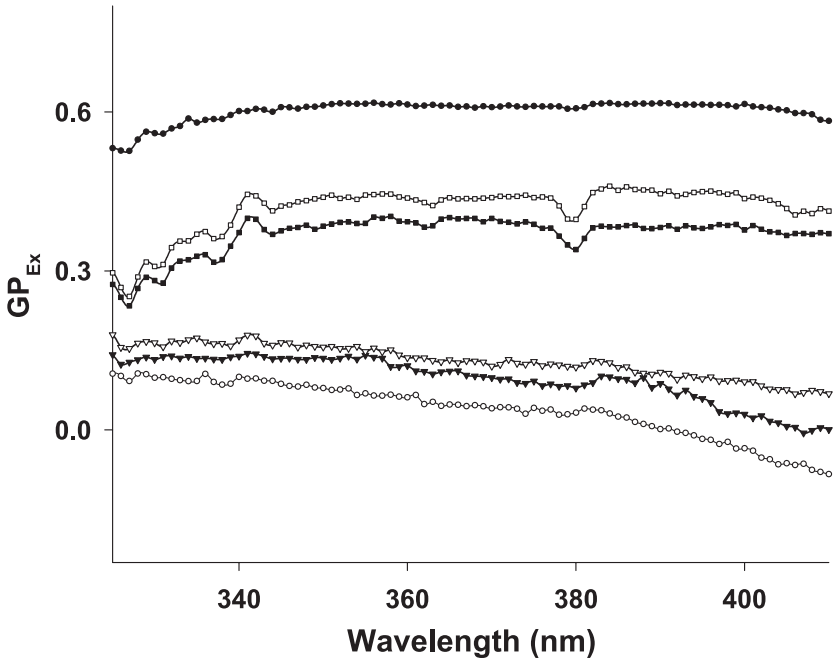


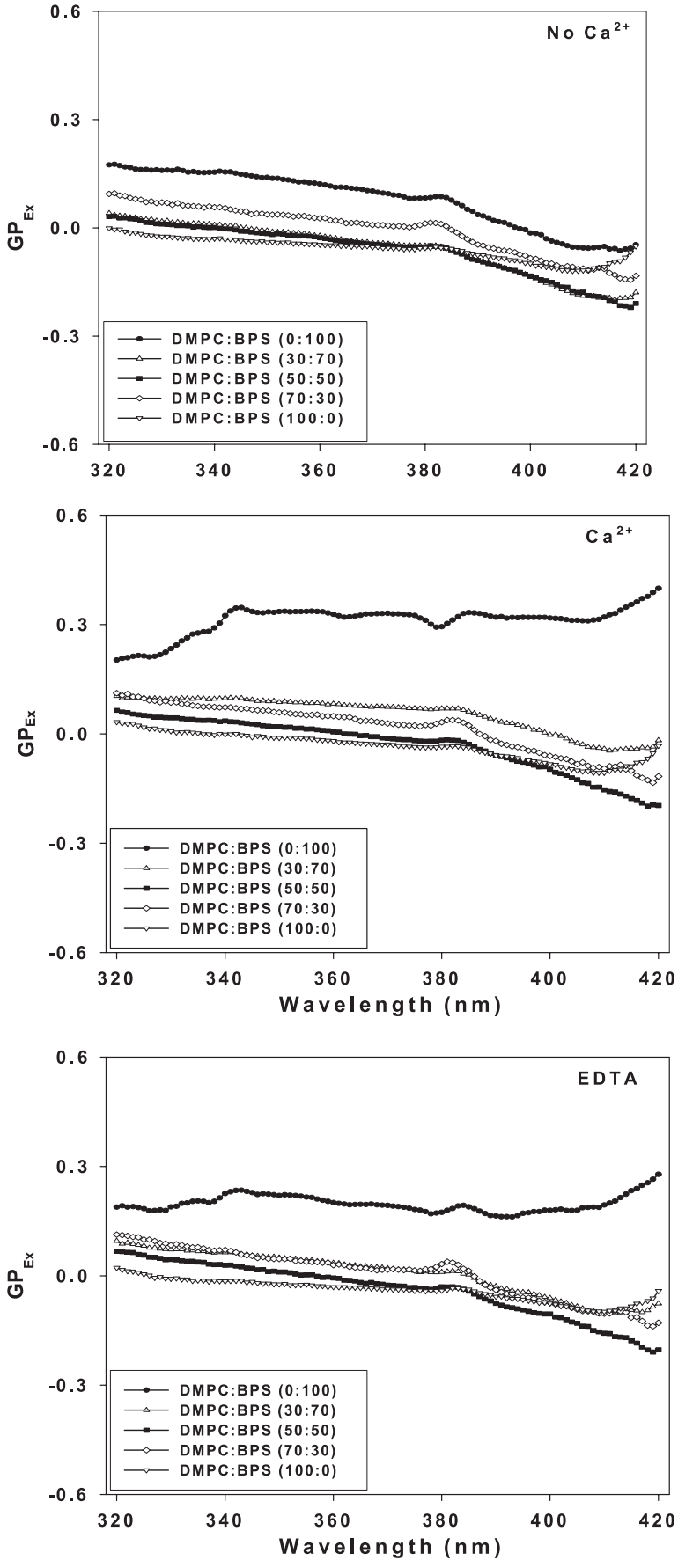
Fig. 2. Generalized polarization as a function of wavelength at various concentrations of Ca^{2+}. Laurdan excitation GP spectra in SUV composed of BPS (in 300 mM NaCl, 25 mM Tris, pH = 7.0) in the absence (○, LC phase), presence of Ca^{2+} (▼, ~0.5 mM; ▽, ~1 mM; ■, ~2 mM; □, ~5 mM) at 37 °C and in the absence of Ca^{2+} (● gel phase) at 1 °C. (BPS) was ~0.5 mM and (Laurdan) was ~0.5 μM (probe/lipid = 1:1000). Ex = Em slit width = 2 nm. The emission was monitored at 440 and 490 nm. Excitation GP values were computed as described in Section 2.

required for chelating the Ca^{2+} ions was also in excess to achieve chelation. Samples were mixed thoroughly after adding required amounts of Ca^{2+} and EDTA, prior to acquiring spectra (dilution of buffer was ~0.5–1% and ~10% following addition of Ca^{2+} and EDTA, respectively). In experiments where the utility of Laurdan was investigated to monitor the effect of different Ca^{2+} concentrations on PS vesicles, required volumes of 1 M $CaCl_2.2H_2O$ were added in succession to get a final Ca^{2+} concentration of ~0.5, 1, 2 and 5 mM. Background contribution due to scattering was not corrected for the studies reported here, as the effect of scattering on the spectral features of the probe was minimal. This was confirmed by monitoring the shape of the spectra following repeated scans. Further, a variable pathlength cuvette (1 cm towards the excitation path and 0.4 cm towards the emission path) was used to minimize the inner filter effects [14]. Measurements were carried out at either 37 °C (± 0.1 °C) or ~1 °C and the temperature of the cuvette was maintained in a temperature-controlled chamber using a Peltier unit. The emission generalized polarization (GP_{Em}) was evaluated from two sets of emission spectra, one with excitation at 340 nm and the other at 390 or 410 nm, using the expression $GP_{Em}=(I_{390\ or\ 410}-I_{340})/(I_{390\ or\ 410}+I_{340})$, where $I_{390\ or\ 410}$ and I_{340} are the emission intensities at each wavelength from 425 or 430 to 550 nm using a fixed excitation wavelength of 390 or 410 and 340 nm, respectively. Similarly, the excitation generalized polarization (GP_{Ex}) was evaluated from two sets of excitation spectra, one with emission wavelength of 440 nm and the other with 490 nm using the expression $GP_{Ex}=(I_{440}-I_{490})/(I_{440}+I_{490})$, where I_{440} and I_{490} are the intensities from 320/325 to 420/410 nm using fixed emission wavelengths of 440 and 490 nm, respectively [7,10,11].

2.5. Electron microscopy studies

Samples were prepared by air-drying the lipid dispersion on formvar-coated grids and negatively staining them with 2% uranyl acetate for approximately 1 min. The samples were analyzed and photographed with a Hitachi H500 TEM operating at 75 kV. Negatives were scanned at 300 dpi with an Agfa Duoscan T1200 scanner.

Fig. 3. Laurdan excitation GP spectra in SUV composed of varying amounts of DMPC and BPS (buffer: 300 mM NaCl, 25 mM Tris, pH = 7.0) at 37 °C in the absence (top panel) and presence (middle panel) of Ca^{2+} ions ([Ca^{2+}] ~4.95–4.96 mM) and following addition of EDTA (bottom panel, (EDTA) ~9.01–9.03 mM). (Lipid) was ~0.5 mM and (Laurdan) was ~0.25 μM for (PS-100 and 70 mol%, probe/lipid = 1:2000) and ~0.5 μM for (other PC/PS mixtures and PC-100 mol%, probe/lipid = 1:1000). Ex = Em slit width = 4 nm. The emission was monitored at 440 and 490 nm. Excitation GP values were computed as described in Section 2. (●, PC/PS (0:100); △, PC/PS (30:70); ■, PC/PS (50:50); ◇, PC/PS (70:30); ▽, PC/PS (100:0)).


No Ca2+
Ca2+
EDTA
GPEx
Wavelength (nm)
DMPC:BPS (0:100)
DMPC:BPS (30:70)
DMPC:BPS (50:50)
DMPC:BPS (70:30)
DMPC:BPS (100:0)
0.6
0.3
0.0
-0.3
-0.6
320
340
360
380
400
420

3. Results and discussion

3.1. Effect of Ca^{2+} on membrane vesicles comprised of PS

3.1.1. Emission and excitation spectra

Cochleate phases are formed following the addition of Ca^{2+} to SUV composed of PS in LC state [1]. Fig. 1A shows the normalized emission and excitation spectra of Laurdan in SUV containing PS. In the absence of Ca^{2+} ions (dotted line), the emission spectrum of Laurdan has a broad peak centered at about 490 nm and this red shift is consistent with the existence of a LC phase. The addition of Ca^{2+} (5 mM) to the vesicles in LC phase resulted in significant changes in the emission spectrum; the peak centered at ~490 nm shifted to the blue edge of the spectrum (dashed line, peak at 425 nm) indicating transition from a LC phase to a rigid and dehydrated (RD) phase. Spectral analysis indicated that the extent of blue shift observed in this RD phase was distinct from the one observed in the gel phase (solid line, peak centered ~440 nm). The above samples were subjected to EM analysis and are shown in Fig. 1B. In the absence of Ca^{2+}, as expected, the electron micrograph showed the presence of small vesicles (top panel). The addition of Ca^{2+} resulted in the formation of elongated cylindrical-shaped structures (bottom panel) and is consistent with the formation of cochleate phase. Thus, the RD phase defined above represents cochleate phase and the terms will be used interchangeably in the remainder of the discussion.

The excitation spectrum of Laurdan was also sensitive to the formation of cochleate phases. In the absence of Ca^{2+} (Fig. 1A, dotted line, 37 °C), the excitation spectrum displayed two peaks; one at ~360 nm and the other at ~390 nm and the ratio of the intensities of the peaks (390:360) in the LC phase was <1, while in the gel phase (solid line, 1 °C), the ratio was >1. The band centered at ~390 nm is considered a distinct feature of Laurdan spectroscopy and has been reported to be prominent in polar solvents while absent in non-polar solvents [9]. In the gel phase, it has its origin from the stabilization of ground state of Laurdan by a local polar environment provided by partially immobilized water molecules. However, in the cochleate phase (dashed line), the ratio of the intensities of the 390:360 was ~1. It is possible that the number of water molecules available to stabilize Laurdan ground state may be significantly less and in an unrelaxed state. This is consistent with the highly dehydrated environment of the cochleate phase.

In order to investigate the utility of the probe in detecting phase changes in PS vesicles as a function of Ca^{2+} concentration, emission spectra was obtained at various Ca^{2+} concentrations (Fig. 1A, inset). In the gel state (● no Ca^{2+}), Laurdan displayed an emission peak at ~440 nm while in the LC state (○, no Ca^{2+}), a broad band centered ~490 nm was observed as discussed above. Following addition of 0.5 (▼) and 1 mM Ca^{2+} (▽) to the LC phase, the broad band centered ~490 nm displayed a small decrease in intensity that was also a accompanied by a small blue shift, suggesting that the probe detects an environment with less water, though the above concentrations of Ca^{2+} were insufficient to promote complete collapse of vesicles and formation of cochleates (see footnote 1). This was consistent with the observations of Duzgunes et al. (see Section 3.5) where the presence of increased concentration of Na^{+} ions was shown to interfere with the destabilization effect of Ca^{2+}. On increasing the Ca^{2+} concentrations to 2 (■) and 5 mM (□), the changes observed in the emission profile of Laurdan were consistent with the formation of cochleate phases as discussed above.

3.2. Generalized polarization (GP)

GP values were calculated using excitation (320/325–420/410 nm) or emission (425 or 430–550 nm) spectra to obtain GP_{Ex} or GP_{Em} spectra as described in Section 2.

3.3. Wavelength dependence of GP spectra

3.3.1. Excitation GP spectra

Representative excitation GP spectra of Laurdan under various experimental conditions are shown in Fig. 2. In the absence of Ca^{2+} (at 37 °C), the excitation GP spectrum of Laurdan labeled SUV comprised of PS showed wavelength dependence [7,11]. This wavelength dependence is a characteristic property of Laurdan partitioned in LC phases and is attributed to the dipolar relaxation phenomena [7,11]. Excitation at lower wavelengths results in the photoselection of unfavorably oriented ground state molecules. Therefore, by moving the excitation spectrum towards the lower wavelength region, more molecules emitting at the blue end of the spectrum are preferentially excited. As the GP_{Ex} values depend on the differences in the emission intensities at 440 and 490 nm, GP_{Ex} values are higher at shorter excitation wavelengths but decreases at longer wavelengths.

In the gel phase (●, 1 °C), the excitation GP spectra showed more or less wavelength independence and the GP value was ~0.62.[2] However, in the presence of Ca^{2+} at 37 °C, the GP spectra displayed features that were distinctly different from homogenous lamellar phases such as gel and LC phases. At Ca^{2+} concentrations of 0.5 (▼) and 1 mM (▽), the GP_{Ex} values still showed wavelength dependence

[2] Typically, one encounters wavelength independency for phospholipids system in a purely gel state. The limited wavelength dependency observed at lower wavelengths could be due to the following reasons: (a) heterogeneous nature of BPS that promotes the coexistence of gel and LC phases; (b) packing defect in the vesicles caused by charge repulsion of head groups (PS head group will be negatively charged at pH = 7.0), which in turn may allow enhanced penetration of water molecules in the gel state; (c) presence of trace Ca^{2+} in NaCl salt that can promote fusion of PS vesicles to a very limited extent; (d) SUV at temperatures of ~1 °C may have some LC phase due to their broad transition.

similar to that of the LC state, though the GP_{Ex} values were marginally higher than that of the LC state. While the GP_{Ex} values over the excitation spectrum ranged from ~0.04 to − 0.067 for the LC phase, it increased to ~0.10 to 0.07 and ~0.14 to 0.17 following the addition of 0.5 and 1 mM Ca^{2+}, respectively. On increasing the Ca^{2+} concentration to 2 (■) and 5 mM (□), the GP_{Ex} values displayed wavelength dependence that was opposite to that observed in the pure LC phase, with the GP spectrum showing lower GP values at lower wavelengths and increasing with increasing wavelength. Further, the magnitude of the GP_{Ex} values also increased significantly relative to values observed in the LC phase (GP_{Ex} ranged from ~0.29 to 0.43 for the cochleate phase).

3.3.2. Emission GP spectra

The emission GP spectra in a LC environment showed wavelength dependence (○, 37 °C, in the absence of Ca^{2+}) (data not shown). While excitation at 410 nm preferentially photoselects dipoles oriented favorable to relaxed orientation, excitation at 340 nm photoselects dipoles that are unfavorably oriented to a small extent. This results in greater intensity originating from excitation at 410 nm to be observed at 490 nm. Therefore, GP_{Em} value in a homogenous LC phase decreases towards the lower end of the spectrum to ~ -0.55 [9,11]. In a purely gel phase, GP_{Em} spectrum is typically wavelength-independent [7]. However, following addition of Ca^{2+} (at 37 °C), GP_{Em} spectra showed changes that were distinct from that of the LC and gel phases. When the Ca^{2+} concentration was 0.5 or 1 mM, the GP_{Em} values displayed wavelength dependence similar to that of the LC state though the magnitude of the GP values at the blue edge of the spectrum were slightly higher (~ -0.48 and ~ -0.39 at 0.5 and 1 mM Ca^{2+}, respectively). When the Ca^{2+} was increased to 2 or 5 mM (□), emission GP values again showed wavelength dependence that was opposite to that observed in a homogenous LC phase, with GP values decreasing at longer wavelengths. Further, the magnitude of the GP values increased considerably in comparison to the LC phase especially towards the blue edge of the emission spectrum (~ -0.17 and ~ 0.03 at 2 and 5 mM, respectively).

The mechanistic origin of the wavelength dependence of GP_{Ex} and GP_{Em} for Laurdan in cochleate phases may be due to the following two reasons: (i) coexistence of phospholipid phase domains, LC and cochleate phases. This brings forth the possibility of Laurdan exhibiting similar preference for the LC and cochleate phases.[3] However, analysis of the emission spectra (after addition of Ca^{2+}) following excitation at two different wavelengths of 340 and 410 nm did not display significant changes in the emission characteristics (data not shown) and was consistent with the existence of a single phase. Therefore, coexistence of phase domains may be unlikely; (ii) alternatively, with the environment around Laurdan being quite non-polar, the number of water molecules surrounding the probe is limited to a few, which are specifically associated with the Ca–PS complex [15]. Further, it is possible that these water molecules are not relaxed and have their dipole unfavorably oriented due to which stabilization of Laurdan ground state does not occur as discussed previously (Section 3.1.1). Excitation at 410 nm probably results in photoselection of these unfavorably oriented dipoles, which will not show a relaxed emission. Consequently, due to reduced dipolar relaxation process, the GP_{Em} trend is opposite to that observed in a LC phase. Similarly, in the case of GP_{Ex}, preferential photoselection of the unfavorably oriented dipoles towards the red end of the spectrum over the randomly oriented dipoles in the blue end of the spectrum give rise to an opposite trend. Overall, the spectral properties of Laurdan are sensitive to the formation of cochleate phases.

Based on fluorescence and EM studies, it can be concluded that the blue-shifted emission spectrum of Laurdan and changes in its excitation spectrum in PS containing vesicles following addition of Ca^{2+} ions was due to the formation of anhydrous cochleate cylinders. It is appropriate to mention here that it is important to evaluate composite spectral information obtained from the excitation, emission and GP spectra to differentiate between the existence of cochleate phases and ordered lamellar phases such as gel phase and is summarized below: (a) Laurdan emission profile in cochleate phases is very sensitive to excitation at 390 nm. In the gel phase, the emission spectrum displays an emission peak centered at ~440 nm, while in the cochleate phase, the emission peak blue shifts to 425 nm; (b) in the gel phase, the excitation spectra has two peaks (~360 and ~390 nm) with the ratio (390:360) of the intensities of these peaks being >1. However, in cochleate phases, the ratio of the intensities of 390:360 is ~1, and possibly reflects the polarity changes near the head group region; (c) while the GP_{Ex} spectra of Laurdan is more or less independent of wavelength in the gel phase (GP_{Ex} ~0.6), it shows wavelength dependency in the cochleate phase and the magnitude also decreases (GP_{Ex} increases from ~0.29 at 325 nm to ~0.41 at 410 nm). The observed wavelength dependency of the GP_{Ex} values is opposite to that observed for the LC phase (GP_{Ex} decreases from ~ 0.07 at 325 nm to ~ -0.11 at 410 nm); (d) GP_{Em} spectrum of Laurdan also appears to be sensitive to formation of cochleate phases. GP_{Em} values in cochleate phase display wavelength dependency that is opposite to that of the LC phase and the magnitude of the values were also significantly higher especially at the blue edge of the spectrum (GP_{Em} ~ -0.53 for LC and ~0.023 for cochleate phase at 5 mM Ca^{2+}). The above changes in the cochleate phase are different from the more

[3] It is known that Laurdan partitions equally between the LC and gel phases (see Refs. [7,9]). However, not much is known about the partitioning behavior of the probe between LC state and highly anhydrous state such as cochleate phases.

or less wavelength independence of the GP_{Em} values observed for the gel phase.

3.4. GP of Laurdan in SUV containing PC/PS

The above studies suggest that Laurdan is a sensitive probe to monitor the formation of cochleate phases induced by PS–Ca^{2+} interaction. To further explore the usefulness of the probe as a fluorescence spectroscopic marker of cochleate phase formation, studies using PC/PS mixtures were carried out. It has been documented that the presence of PC interferes with divalent cation-induced fusion and collapse of PS vesicles, thereby preventing formation of cochleate structures [16].

DMPC/BPS vesicles with molar ratios (100:0, 30:70, 50:50, 70:30 and 0:100) were labeled with Laurdan and analyzed as described in Section 2. Fig. 3 shows GP_{Ex} profiles in the absence (top panel), presence (middle) of Ca^{2+} and following addition of EDTA (bottom panel). The GP_{Ex} spectrum with only vesicles composed of 100 mol% PS (●) displayed wavelength dependence that was consistent with the transition to cochleate phases following addition of Ca^{2+}. However, for lipid vesicles comprised of other PC/PS ratios, there were no significant changes before and after the addition of Ca^{2+} suggesting that Ca^{2+} do not promote cochleate cylinders in PC/PS mixtures. This is in agreement with observations of Duzgunes et al., where the presence of PC (in tandem with higher Na^+ concentration, see Section 3.5) in the system interfered with the divalent cation mediated destabilization of PS vesicles that lead to the formation of cochleate structures [16].

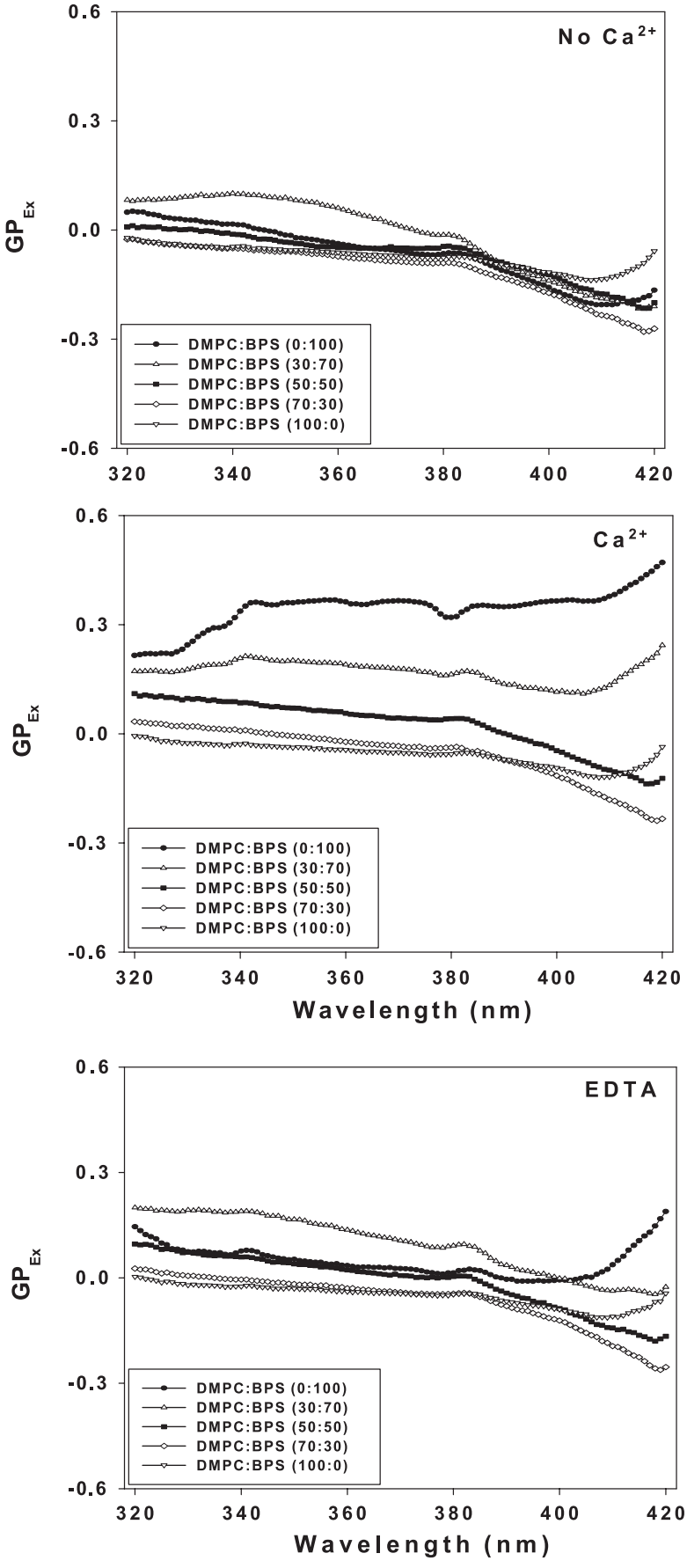
3.5. Effect of ionic strength on GP of Laurdan in SUV containing PC/PS

It has been shown experimentally and theoretically that a critical Ca/PS ratio of ~0.35 is necessary for the destabilization effect of divalent cations and the formation of cochleate phases [16]. However, this destabilization is dependent on Na^+ concentration. High Na^+ concentration was found to inhibit Ca^{2+}-induced fusion of PS vesicles by reducing the critical ratio of bound Ca per molecule of PS (Ca/PS) required for cochleate formation. In order to investigate the effect of Na^+ concentration on cochleate formation and GP of Laurdan, we prepared lipid vesicles (SUV) in 100 mM NaCl, 25 mM Tris, pH, 7.0. In this buffer system, the concentration of Na^+ was reduced by three times compared to experimental conditions discussed in Fig. 3. Shown in Fig. 4 are the GP_{Ex} profiles in the absence (top panel) and presence (middle panel) of Ca^{2+}. In Ca^{2+}-free preparations, the GP_{Ex} spectra (Fig. 4) were similar to profiles obtained when the bulk Na^+ concentration was 300 mM (Fig. 3) for all PC/PS compositions. However, it was of interest to observe that the response of GP_{Ex} spectrum for the PC/PS ratio (30:70) in the presence of Ca^{2+} (△, middle panel, Fig. 4) was different from previously discussed results (△ middle panel, Fig. 3). GP_{Ex} spectrum for this composition showed wavelength dependence and the response was quite similar to that observed with 100 mol% PS, though the effect was less pronounced. This was possibly due to an increase in ratio of bound Ca/PS as a result of reduced Na^+ concentration, leading to increased destabilization of the membrane vesicles but does not result in cochleate formation [16]. It is appropriate to mention here that the observations of Duzgunes et al. [16], based on fusion studies, reported that Ca^{2+} at a concentration of 3 mM was unable to induce fusion in 4:1 PS/PC vesicles when the bulk Na^+ concentration was 500 mM. However, reduction of Na^+ to 100 mM was found to induce considerable fusion in the above system. Thus in our experimental system, reduction of bulk Na^+ to 100 mM, could have resulted in an increase in Ca^{2+}-mediated fusion. However, the presence of PC probably prevented the complete collapse of internal aqueous space of the fused vesicles (see Section 3.4) causing only limited leakage of contents, but not leading to cochleate phases.

3.6. Effect of lamellarity on Laurdan GP

The mechanism of formation of cochleate cylinders from SUV composed of PS has been investigated in detail and is well-understood [1]. However, not much information is available with respect to the role of MLV in the formation of these complexes. Therefore, the aim of this study was to examine the influence of lamellarity, SUV versus MLV on the response of Laurdan fluorescence. Shown in Fig. 5 are the excitation (top panel) and emission spectra (bottom panel) of Laurdan labeled MLV composed of only PS in buffer containing 300 mM NaCl in the absence and presence of varying amount of Ca^{2+}. Both the excitation and emission spectral features clearly indicated the formation of cochleate structures as discussed previously (Section 3.1.1) following addition of Ca^{2+} (at 2, ■ and 5 mM, □). However, a distinct feature of Laurdan excitation profile in cochleate phases, in addition to the ratio of the intensities of the peaks (~390:360) being ~1was the occurrence of a significant blue shift of the 390 nm band (not observed for the SUV). While the precise reason for such a shift is unclear, this observation suggests the possibility that the cochleate phase formed from MLV may be relatively dehydrated to a greater extent than those formed from SUV. Changes in the excitation and emission spectra were

Fig. 4. Laurdan excitation GP spectra in SUV composed of varying amounts of DMPC and BPS (buffer: 100 mM NaCl, 25 mM Tris, pH = 7.0) at 37 °C in the absence (top panel) and presence (middle panel) of Ca^{2+} ions ([Ca^{2+}] ~4.95 mM) and following addition of EDTA (bottom panel, (EDTA) ~9.01 mM). (Lipid) was ~0.5 mM and (Laurdan) was ~0.5 μM for (probe/lipid = 1:1000). Ex = Em slit width = 4 nm. The emission was monitored at 440 and 490 nm. Excitation GP values were computed as described in Section 2. (●, PC/PS (0:100); △, PC/PS (30:70); ■, PC/PS (50:50); ◇, PC/PS (70:30); ▽, PC/PS (100:0)).


No Ca2+
Ca2+
EDTA
GPEx
0.6
0.3
0.0
-0.3
-0.6
320
340
360
380
400
420
Wavelength (nm)
DMPC:BPS (0:100)
DMPC:BPS (30:70)
DMPC:BPS (50:50)
DMPC:BPS (70:30)
DMPC:BPS (100:0)

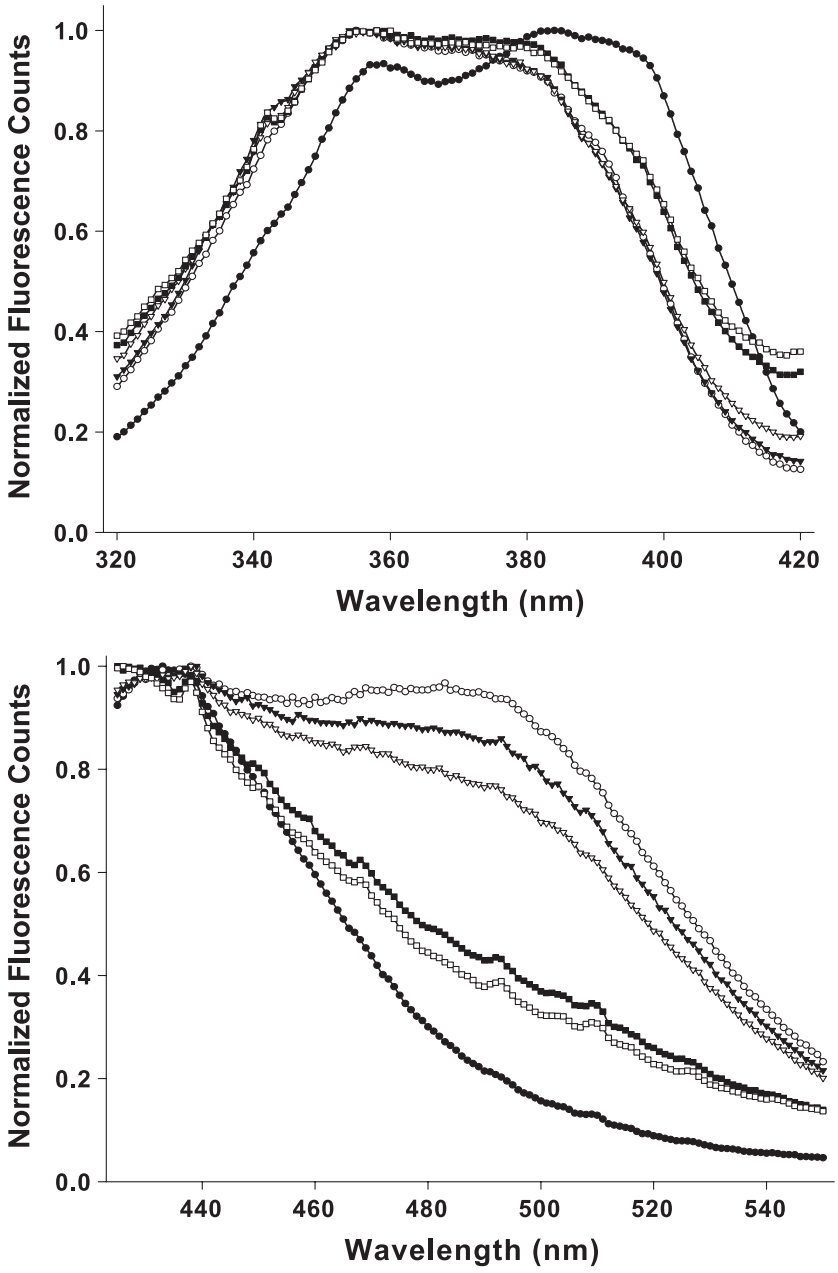
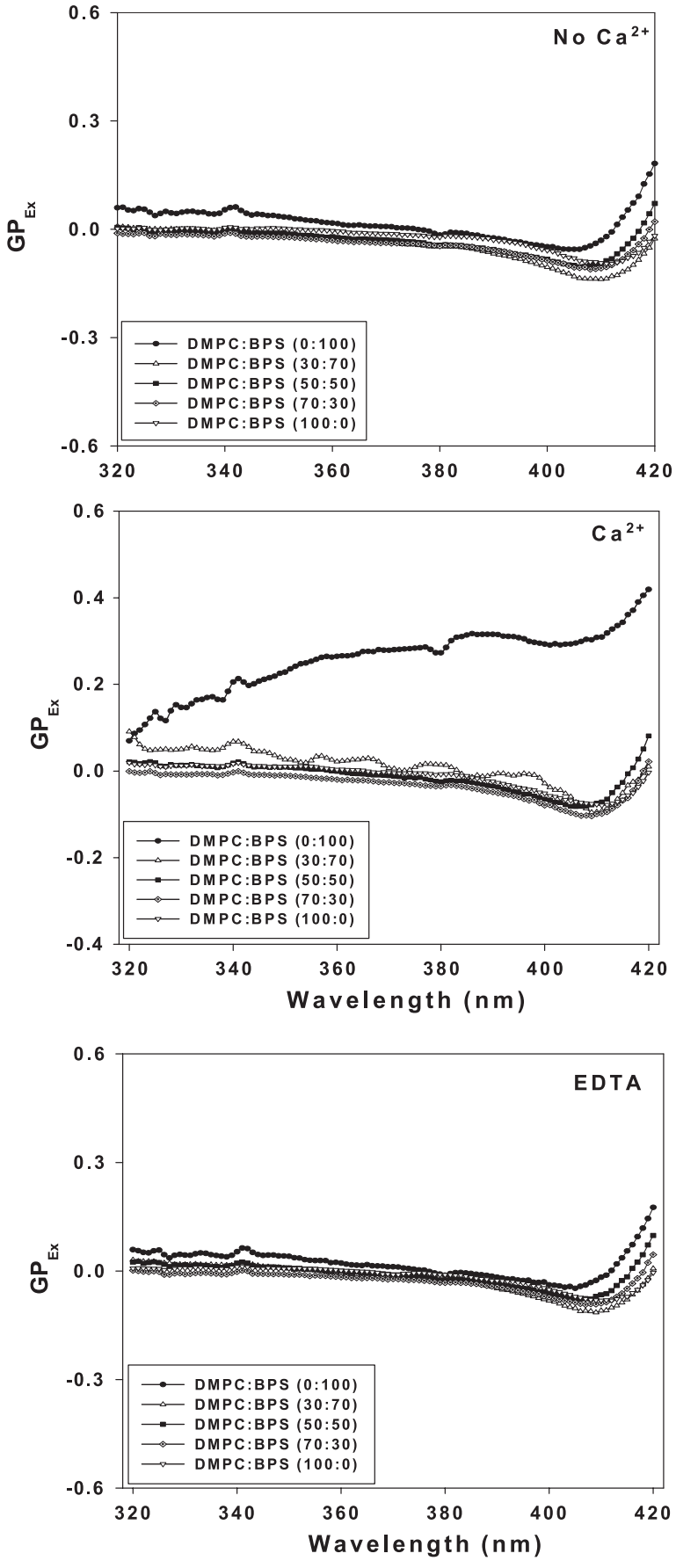


Fig. 5. Excitation and emission spectra of Laurdan. Normalized excitation (top panel) and emission spectra (bottom panel) of Laurdan in MLV composed of BPS (buffer: 300 mM NaCl, 25 mM Tris, pH = 7.0) in the absence or presence of varying concentrations of Ca^{2+}. For the excitation spectra, the emission was monitored at 440 nm and for the emission spectra the excitation wavelength was 340 nm. (●) gel phase (no Ca^{2+}); (○) LC (no Ca^{2+}); (▼) ~0.5 mM; (■) ~2 mM; (□) ~5 mM. Ex = Em slit width = 2 nm. (BPS) was ~0.5 mM and (Laurdan) was ~0.5 μM (probe/lipid = 1:1000).

also reflected in the GP excitation and emission spectra (data not shown). As observed with SUV, in the gel state, the excitation GP values were more or less independent of wavelength (GP_{Ex} ~0.6), while in the LC state, GP_{Ex} displayed wavelength dependence (GP_{Ex} decreased from ~0.02 at 325 nm to ~ -0.1 at 410 nm). At concentrations of 0.5 or 1 mM Ca^{2+}, the excitation GP values of Laurdan showed a trend similar to that of the LC state though the values were marginally higher. However, on increasing the concentration of Ca^{2+} to 5 mM, the excitation GP values increased and showed wavelength dependence that was not only more pronounced than the gel phase (Fig. 6, ●, GP_{Ex}

Fig. 6. Laurdan excitation GP spectra in MLV composed of varying amounts of DMPC and BPS (buffer: 300 mM NaCl, 25 mM Tris, pH = 7.0) at 37 °C in the absence (top panel) and presence (middle panel) of Ca^{2+} ions ([Ca^{2+}] ~4.97 mM) and following addition of EDTA (bottom panel, (EDTA) ~9.05 mM). (Lipid) was ~4.5 mM and (Laurdan) was ~2.25 μM (probe/lipid = 1:2000 for all compositions except for PS, 70 mol %; Ex = Em slit width = 2 nm). For PS, 70 mol % (Lipid) ~0.5 mM and (Laurdan) ~0.5 μM (probe/lipid = 1:1000; Ex = Em slit width = 4 nm). The emission was monitored at 440 and 490 nm. Excitation GP values were computed as described in Section 2. (●, PC/PS (0:100); △, PC/PS (30:70); ■, PC/PS (50:50); ◇, PC/PS (70:30); ▽, PC/PS (100:0)).


No Ca2+
Ca2+
EDTA
GPEx
Wavelength (nm)
DMPC:BPS (0:100)
DMPC:BPS (30:70)
DMPC:BPS (50:50)
DMPC:BPS (70:30)
DMPC:BPS (100:0)
0.6
0.3
0.0
-0.3
-0.6
0.4
0.2
-0.2
-0.4
320
340
360
380
400
420

values ranged from ~0.07 to 0.42 for the cochleate phase) but was also opposite to that observed for the LC state as discussed previously (Section 3.3.1). Similar conclusions were drawn from the GP_{Em} spectra with the profiles being comparable to that of the SUV (at the blue edge of spectrum $GP_{Em} \sim -0.5$ for LC and ~ -0.15 for cochleate phase). Further, analysis of GP_{Ex} spectra of various PC/PS mixtures (Fig. 6) indicated that the results observed were similar to that of SUV (Fig. 3).

The formation of cochleate phases in MLV is intriguing as the calcium added externally to MLV will have only limited access to PS in the inner bilayers because of reduced permeability of Ca^{2+} across the bilayer. This may lead to insufficient destabilization of MLV to form cochleates. However, we speculate that the observed formation of cochleate cylinders from MLV may be via a "peeling effect". Ca^{2+} by initiating the destabilization on the outer bilayer will cause collapse of this outer bilayer, followed by loss of contents, thereby increasing the accessibility of Ca^{2+} to the inner bilayers.

In conclusion, we have presented a new fluorometric method for monitoring the formation of cochleate phases and to probe membrane dynamics in PC/PS mixtures. The studies reported here demonstrate that Laurdan fluorescence can be successfully used as a sensitive and a rapid method and detect formation of cochleate structures.

Acknowledgements

This work was supported by NHLBI, National Institute of Health grant R01 HL 70227 to SVB. KR was supported by a Pfizer Fellowship. The authors thank the Pharmaceutical Sciences Instrumentation Facility, University at Buffalo, for the use of Spectrofluorometer. We gratefully acknowledge Mr. Alan Siegel of the Department of Biological Sciences, University at Buffalo for performing the electron microscopy studies.

References

[1] D. Papahadjopoulos, W.J. Vail, K. Jacobson, G. Poste, Cochleate lipid cylinders: formation by fusion of unilamellar lipid vesicles, Biochim. Biophys. Acta 394 (1975) 483–491.

[2] R.J. Mannino, M. Canki, E. Feketeova, A.J. Scolpino, Z. Wang, F. Zhang, M.T. Kheiri, S. Gould-Fogerite, Targeting immune response induction with cochleate and liposome-based vaccines, Adv. Drug Deliv. Rev. 32 (1998) 273–287.

[3] L. Zarif, J.R. Graybill, D. Perlin, L. Najvar, R. Bocanegra, R.J. Mannino, Antifungal activity of amphotericin B cochleates against *Candida albicans* infection in a mouse model, Antimicrob. Agents Chemother. 44 (2000) 1463–1469.

[4] I. Segarra, D.A. Movshin, L. Zarif, Pharmacokinetics and tissue distribution after intravenous administration of a single dose of amphotericin B cochleates, a new lipid-based delivery system, J. Pharm. Sci. 91 (2002) 1827–1837.

[5] E.P. Day, J.T. Ho, R.K. Kunze Jr., S.T. Sun, Dynamic light scattering study of calcium-induced fusion in phospholipid vesicles, Biochim. Biophys. Acta 470 (1977) 503–508.

[6] C.R. Flach, R. Mendelsohn, A new infrared spectroscopoic marker for cochleate phases in phosphatidylserine-containing model membranes, Biophys. J. 64 (1993) 1113–1121.

[7] 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.

[8] T. Parasassi, G. De Stasio, A. d'Ubaldo, E. Gratton, Phase fluctuation in phospholipid membranes revealed by Laurdan fluorescence, Biophys. J. 57 (1990) 1179–1186.

[9] 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.

[10] T. Parasassi, M. Di Stefano, M. Loiero, G. Ravagnan, E. Gratton, Cholesterol modifies water concentration and dynamics in phospholipid bilayers: a fluorescence study using Laurdan probe, Biophys. J. 66 (1994) 763–768.

[11] S.V. Balasubramanian, R.B. Campbell, R.M. Straubinger, Propofol, a general anesthetic, promotes the formation of fluid phase domains in model membranes, Chem. Phys. Lipids 114 (2002) 35–44.

[12] R.M. Epand, B.T. Leon, Hexagonal phase forming propensity detected in phospholipid bilayers with fluorescent probes, Biochemistry 31 (1992) 1550–1554.

[13] R.B. Campbell, S.V. Balasubramanian, R.M. Straubinger, Influence of cationic lipids on the stability and membrane properties of paclitaxel-containing liposomes, J. Pharm. Sci. 90 (2001) 1091–1105.

[14] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Kluwer Academic Publishing/Plenum, New York, 1999.

[15] A. Portis, C. Newton, W. Pangborn, D. Papahadjopoulos, Studies on the mechanism of membrane fusion: evidence for an intermembrane Ca^{2+}–phospholipid complex, synergism with Mg^{2+}, and inhibition by spectrin, Biochemistry 18 (1979) 780–790.

[16] N. Duzgunes, S. Nir, J. Wilschut, J. Bentz, C. Newton, A. Portis, D. Papahadjopoulos, Calcium- and magnesium-induced fusion of mixed phosphatidylserine/phosphatidylcholine vesicles: effect of ion binding, J. Membr. Biol. 59 (1981) 115–125.