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Gel state surface properties of phosphatidylcholine liposomes as measured with merocyanine 540

Delia L. Bernik and E.A. Disalvo

Area de Biomembranas, Instituto de Investigaciones Fisicoquímicas Teóricas y Aplicadas (INIFTA), Universidad Nacional de La Plata; CC 16 Suc 4 1900 La Plata (Argentina)

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The surface properties of liposomes composed by saturated phosphatidylcholines and their mixtures with cholesterol in the gel state have been studied using merocyanine 540 as a fluorescent and optical probe. A new absorption peak at 450 nm and a new fluorescent band at 630 nm were observed when the dye was added to suspensions of DMPC multilamellar liposomes in the gel state. These peaks were also observed in membranes with different lipid compositions in conditions in which the $P_{\beta'}$ and the $L_{\beta'}$ phases were present. The increase of temperature above the main transition temperature of DMPC or the incorporation of 35% cholesterol into DMPC bilayers at 13°C caused the disappearance of these peaks. The changes in the absorption and fluorescent spectra upon addition of cholesterol resembles very well the phase diagrams reported by Mortensen et al. ((1988) Biochim. Biophys. Acta 945, 221–245) indicating that the corrugated structures characteristic of the $L_{\beta'}$ and the $P_{\beta'}$ phases have different surface properties related to the partitioning of amphiphilic dies.

Introduction

The gel state between the pre and the main phase transition temperature (the $P_{\beta'}$ phase) shows topological characteristics different than that observed below the pretransition (the $L_{\beta'}$ phase). This region is characterized by the presence of rippled structures, whose periodicity and organization is a function of temperature and membrane composition, in particular by the presence of cholesterol in the bilayer [1–7]. The analysis of Mortensen et al. indicates that the sides of the ripples primarily contain gel phase phosphatidylcholine, whereas the apices are enriched in cholesterol and are liquid crystalline. The boundaries separating the $L_{\beta'}$ and $P_{\beta'}$ phases show undulations and defects in the structure which seems to propagate into both phases in contact [5].

A number of experimental methods, giving information on different length and time scales have attempt to understand the structure and dynamics of phospholipid bilayers around the phase boundaries and with the incorporation of cholesterol. The resulting data show still some controversies related to the formation of domains that may determine local properties at different depths in the plane of the membrane with different affinities for amphiphile dies.

Among the dyes used in membrane spectroscopy, merocyanine 540 is able to detect changes in the lipid phase state and in the electrical properties of the surface. This probe has been used as a sensor of molecular events either in model membrane systems or in biological membranes. In addition, merocyanine have different affinities for cholesterol-free and cholestrol-rich regions [8].

Therefore, changes in the MC spectra can be expected in relation to the different phase state of the regions of the membrane in which the dye has the highest probability for partitioning. In addition, the partition of the probe may change the dimer/monomer ratio affecting the fluorescence spectra [15].

Correspondence to: E.A. Disalvo, Area de Biomembranas, Instituto de Investigaciones Fisicoquímicas Teóricas y Aplicadas (INIFTA), Universidad Nacional de La Plata, CC 16 Suc 4, 1900 La Plata, Argentina.

Abbreviations: DMPC, dimyristoylphosphatidylcholine; di-15:0-PC, dipentadecanoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; 14:0/18:0-PC, 1-myristoyl-2-stearoylphosphatidylcholine; di-17:0i-PC, diisomargaroylphosphatidylcholine; MC 540, merocyanine 540.

In the previous studies, assignation between different absorption and emission bands with the probable localization of the probe in the membrane has been done mainly with lipids in the fluid state [9–18]. In contrast, the spectroscopical and fluorescent features of MC 540 in the presence of membranes in the gel state and its dependence with the lipid composition have not been so far analyzed.

In this study, the surface properties of lipid bilayers in conditions in which the phase diagrams of lamellar systems show changes from the $P_{\beta'}$ to the $L_{\beta'}$ as a function of temperature and cholesterol concentration have been analyzed by means of absorption and fluorescence spectroscopy of merocyanine The new features of the optical and fluorescence properties of MC 540 obtained for lipid membranes in the gel state according to the cholesterol ratio, the temperature and the length of acyl chains have been correlated with the topological features and the phase diagrams reported in literature.

Materials and Methods

Dimyristoylphosphatidylcholine (DMPC), dipentadecanoylphosphatidylcholine (di-15:0-PC), dipalmitoylphosphatidylcholine (DPPC) and 1-myristoyl-2-stearoylphosphatidylcholine (14:0/18:0-PC) were obtained from Avanti Polar Lipids (Birmingham, AL). Diisomarganoylphosphatidylcholine (di-17:0i-PC) was a gift from Dr. Mc Elhaney (Edmonton, Canada). All of them showed a single spot on thin-layer chromatography using a solvent mixture chloroform/methanol/water (65:5:5, v/v). Therefore, they were used without further purification. Merocyanine (MC 540) and cholesterol were obtained from Sigma (St. Louis, MO). The purity of MC 540 was checked by high-performance liquid chromatography.

Liposomes were prepared by the method described by Bangham et al. [20] A chloroformic lipid solution was evaporated under vacuum in a round bottom flask. The dry film was dispersed in 10 mM Tris-HCl buffer (pH 7.4) at temperatures above the phase transition of the lipids. Liposomes prepared in this way were stabilized at the desired temperatures before an aliquot of MC 540 dissolved in the same buffer solution was added. The liposome dispersion was incubated in the presence of the dye 30 min at the same temperature at which the spectra was run.

The final concentration of lipids was, in all cases, $3.8 \cdot 10^{-4}$ M. The dye was added to the dispersion from a fresh stock solution in order to obtain the different lipid/probe ratios indicated in the figures. Molar concentrations of MC 540 ranged between $1.5 \cdot 10^{-5}$ and $1.9 \cdot 10^{-6}$ M.

The spectra were obtained in a Hitachi 100–60 double beam spectrophotometer using 2 ml glass cuvettes thermostatized within ± 0.2 C° by a circulating bath connected to the cuvette holder. Temperature inside the cuvette was monitored by a thermocouple in the absorption experiments and by a Pt 100 resistor in the fluorescence experiments.

Steady-state fluorescence intensity was measured in a Perkin-Elmer LS5 spectrofluorometer in a 1-ml cubette thermostated in the holder by a circulating bath. Liposome dispersions of the same procedence as the measured samples were used in order to correct for scattering. Samples were excited at 440 nm and the emission spectra were obtained between 400 and 650 nm. The dye/lipid ratio in the fluorescence experiments was 1:200 in all cases.

Results

The spectra of MC 540 in the presence of DPPC liposomes in the gel state (13°C) show a peak at 500 nm and a peak at 530 nm (Fig. 1A). However, DMPC liposomes at the same temperature and lipid/probe ratios show one shoulder at 570 nm and a peak at 450 nm (Fig. 1B). The inclusion of DPPC in the DMPC bilayer (in a 50:50 molar ratio) decreases both peaks with respect to that found with pure DMPC (Fig. 1C).

In Fig. 2, the relative increase of the 450 nm peak taken as a baseline the absorbance at 600 nm, is plotted as a function of the MC/lipid ratio. The peak at 450 nm remarkably increases with the concentration of the probe added to the external solution of liposomes of pure DMPC. This increase is lower in the presence of liposomes composed by 50% and 65% DPPC, respectively, and completely absent in 100% DPPC liposomes (Fig. 2). In the same figure, the influence of the chain length on the absorbance ratio is included for di-15:0-PC and 14:0/18:0-PC. The values of these ratios for a 0.05 MC/lipid ratio are given in Table I. The peak at 450 nm for di-15:0-PC is lower than for DMPC at 14°C. In addition, the replacement of one 14:0 acyl chain by a 18:0 acyl chain in the position 2 produces a significant decrease in the 450 nm absorbance. In contrast, a ramification in the hydrophobic region as it is the case for the analogue of DPPC, diisomarganoylphosphatidylcholine, which has a methyl group in the 15th position, induces the appearance of the peak at 450 nm.

At 30°C, DMPC liposomes are in the fluid state. Under this condition, the spectra obtained when MC is added to liposomes above the main phase transition temperature shows no peak at 450 nm (Fig. 3A). Similar results were obtained with liposomes composed by 65% DMPC/35% DPPC and 50% DMPC/50% DPPC (spectra not shown). However, the mixtures composed by 35% DMPC/65% DPPC, which according to the phase diagram are in the solid state [6,22], shows the peak at 450 nm and the shoulder at 505 nm for the

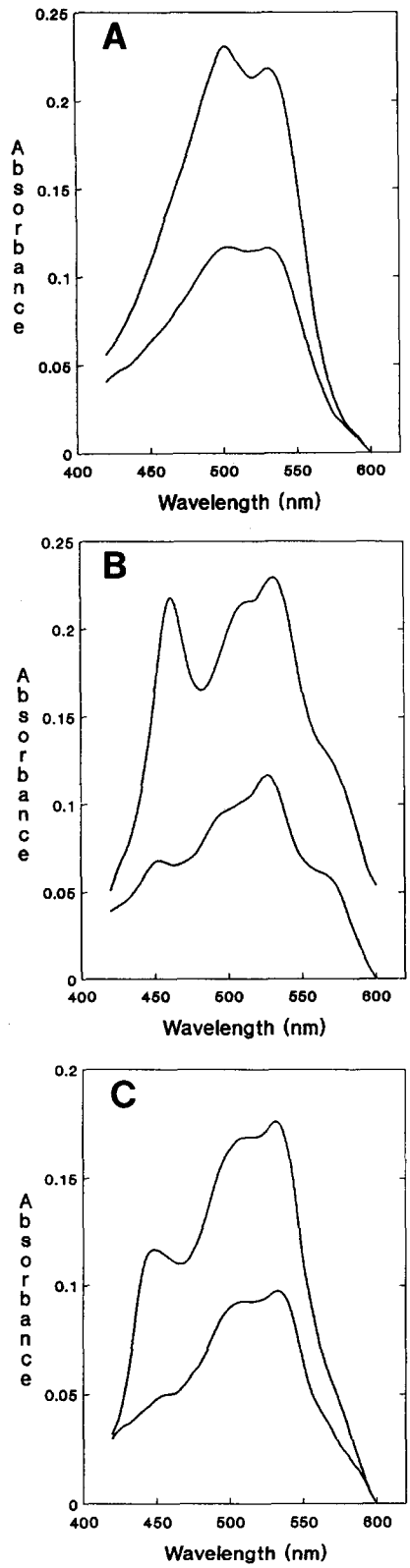


Fig. 1. Merocyanine 540 absorption spectra in the presence of DMPC and DPPC liposomes. Liposomes were composed of (A) 100% DPPC; (B) 100% DMPC; (C) 50% DMPC/50% DPPC. Merocyanine was added to the external solution of the liposome dispersion at 13°C to achieve a lipid/probe ratio of 200:1 (bottom curves) and 100:1 (top curves).

highest probe/lipid ratio (Fig. 3B). It must be noticed, in addition, that 100% DPPC liposomes, which at this temperature are in the $L_{\beta'}$ phase [23], show a small shoulder at 450 nm for the highest probe/lipid ratio (Fig. 3 C).

A comparison of the changes induced by temperature on the absorbance and fluorescence spectra of MC 540 when DMPC dispersions are heated above the main phase transition temperature in the presence of the dye is shown in Fig. 4. It is observed that above the phase transition of DMPC the peak at 450 nm disappears (Fig. 4B).

The fluorescence band observed at 630 nm in the presence of DMPC liposomes in the gel state (Fig. 4D) gradually disappears when temperature increases and above the phase transition temperature is completely absent (Fig. 4E). At 28°C, the peak at 570 nm has been displaced to 590 nm. When the temperature is lowered back to those below the phase transition temperature the peak at 630 nm in the fluorescence spectra and the peak at 450 nm in the absorbance spectra are again observed (compare Figs. 4C and F). However, the fluorescence band at 590 nm is still present and the absorption peak at 450 nm and the band at 639 nm are reduced in comparison to the spectra obtained at 13°C before heating (Figs. 4A and D).

The 450/530 absorbance ratio decreases at 13°C for all the dye/lipid ratios by the inclusion of 5% cholesterol in DMPC membranes (Figs. 5A and B). This is mostly due to the increase in the absorbance at 530 nm. In addition, an increase of the shoulder at 570 nm (Fig. 5B) is apparent. At 10% cholesterol (Fig. 5C) the relative height of the peak at 450 nm with respect to that at 500 nm is greater than in the absence of cholesterol. However, when the DMPC bilayer contains 35% cholesterol (Fig. 5D), the spectrum is comparable to that of 100% DPPC in the gel state (Fig. 1A).

The fluorescence spectra of DMPC/cholesterol liposomes show an increment of the peak at 630 nm for 10% cholesterol (Fig. 6A) in comparison to liposomes of DMPC (Fig. 4D). At 30% cholesterol, the peak at 630 nm disappears leaving only a small shoulder and a sharp peak at 575 nm (Fig. 6B).

Discussion

The present study indicates that the spectra of MC 540 show a new peak at 450 nm in the presence of liposomes in the gel state which depends on the lipid composition of the bilayer, the lipid/dye ratio and the temperature.

The peak at 450 nm disappears when the MC 540 spectra are taken in the presence of liposomes above the main transition temperature of DMPC (21°C) giving the characteristic spectra for MC in non-polar

TABLE I

Effect of acyl chain length and ramification on the MC 540 absorption spectra of liposomes in the gel state
MC 540/lipid ratios were 0.04 mol/mol.

Lipid	Temperature (°C)	450/530 absorbance ratio
Di-14:0-PC	13	2.75
Di-15:0-PC	13	0.61
Di-16:0-PC	25	0.50
18:0/14:0-PC	14	0.62
Di-17:0i-PC	25	0.65

media (Fig. 3A). In this case, MC 540 is not present in the dispersing solution when the lipids go through the phase transition, i.e., it is added to the dispersion when the thermal equilibrium is achieved. Thus, the spectra correspond to the properties of the external bilayer interface. This is confirmed by the experiments of Fig. 4 which show that the temperature increase through the phase transition in the presence of MC 540 promotes both the peak at 450 nm (Fig. 4B) and the band at 630 nm in the fluorescence spectra to disappear (Fig. 4E). The peak and the band are again observed when the DMPC bilayer is cooled down to 13°C or below (Figs. 4C and 4F). However, the intensity of both of them is significantly lower and the band at 590 nm is still observed. This behavior is indicative of the penetration of the dye into the multilamellar liposomes when the bilayer goes through the phase transition [18].

The comparison of the results of Figs. 3A and 4C allow to infer that the merocyanine spectra obtained when the dye is added to dispersions of liposomes maintained after its preparation below the phase transition correspond to the properties of the outer interface. All the subsequent analysis were done in these conditions. Therefore, only the outer bilayer was accesible to the MC 540 molecules and the dye concentration is mainly on the outer interface.

The topological features of membranes in the gel state have been studied by means of X-ray, differential calorimetry and electron microscopy using multilamellar liposomes as model system [2–7]. These studies have shown that the arrangement of the molecules in the gel state depends strongly on the temperature and the hydration of the membrane. For the sake of comparison the absorption and fluorescence spectroscopical studies were done with liposomes for which the phase diagrams have been determined.

The data of Table I denote that the magnitude of the peak at 450 nm depends on the length of the lipid chains. The peak decrease in the sequence 14:0-, 15:0- and 16:0-PC indicates that the increase in the length of both chains hinders the interaction of merocyanine with the membrane. In addition, the increase of the length of only one acyl chain as in the case of 14:0/18:0-PC causes a change in the merocyanine spectra in a lower extent but in the same direction. In contrast, the inclusion of a lateral group in a 16:0 lipid chain as in di-17:0i-PC, results in a spectrum similar to those obtained when the chain length is shortened in one methylene group as it is the case with di-15:0-PC.

When the lipid polar headgroups are sufficiently hydrated, the weakening of the interchain packing results in a lamellar-to-undulated lamellar phase transition ($L_{\beta'} \rightarrow P_{\beta'}$) [24]. DMPC in an excess of water is in the $P_{\beta'}$ phase between 10 and 21°C. [30] and that DPPC at 30°C is in the $L_{\beta'}$ phase [23]. In these

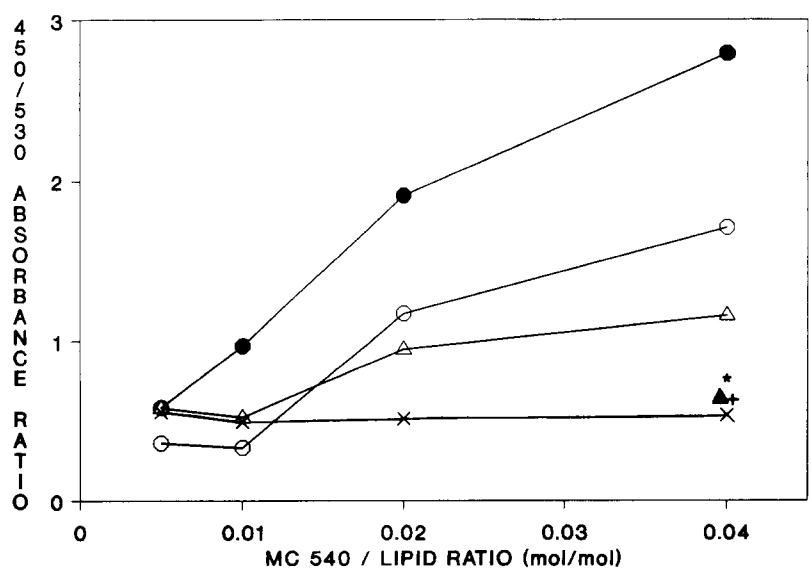
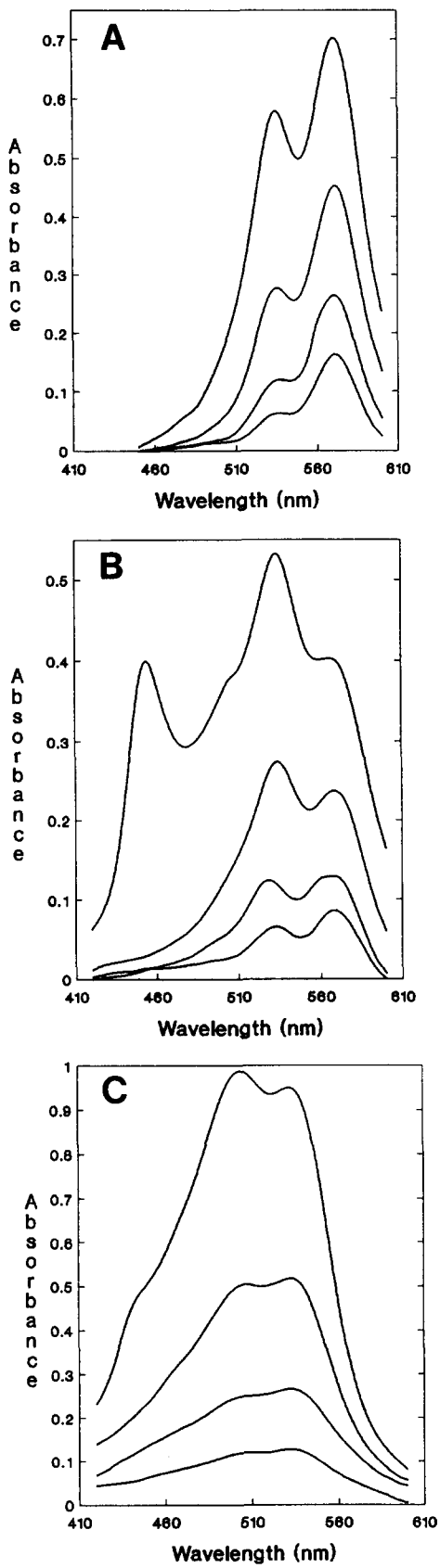


Fig. 2. Influence of the dye/lipid ratio on the 450/530 absorbance ratio in the MC 540 absorption spectra in the presence of liposomes of different DMPC/DPPC ratios. Liposomes were composed of 100% DMPC (●); 50% DMPC/50% DPPC (○); 65% DPPC/35% DMPC (△); 100% DPPC (×); 14:0/18:0-PC (▲); di-15:0-PC (+); di-17:0i-PC (*). All assays were done at 13°C under the same conditions as in Fig. 1.



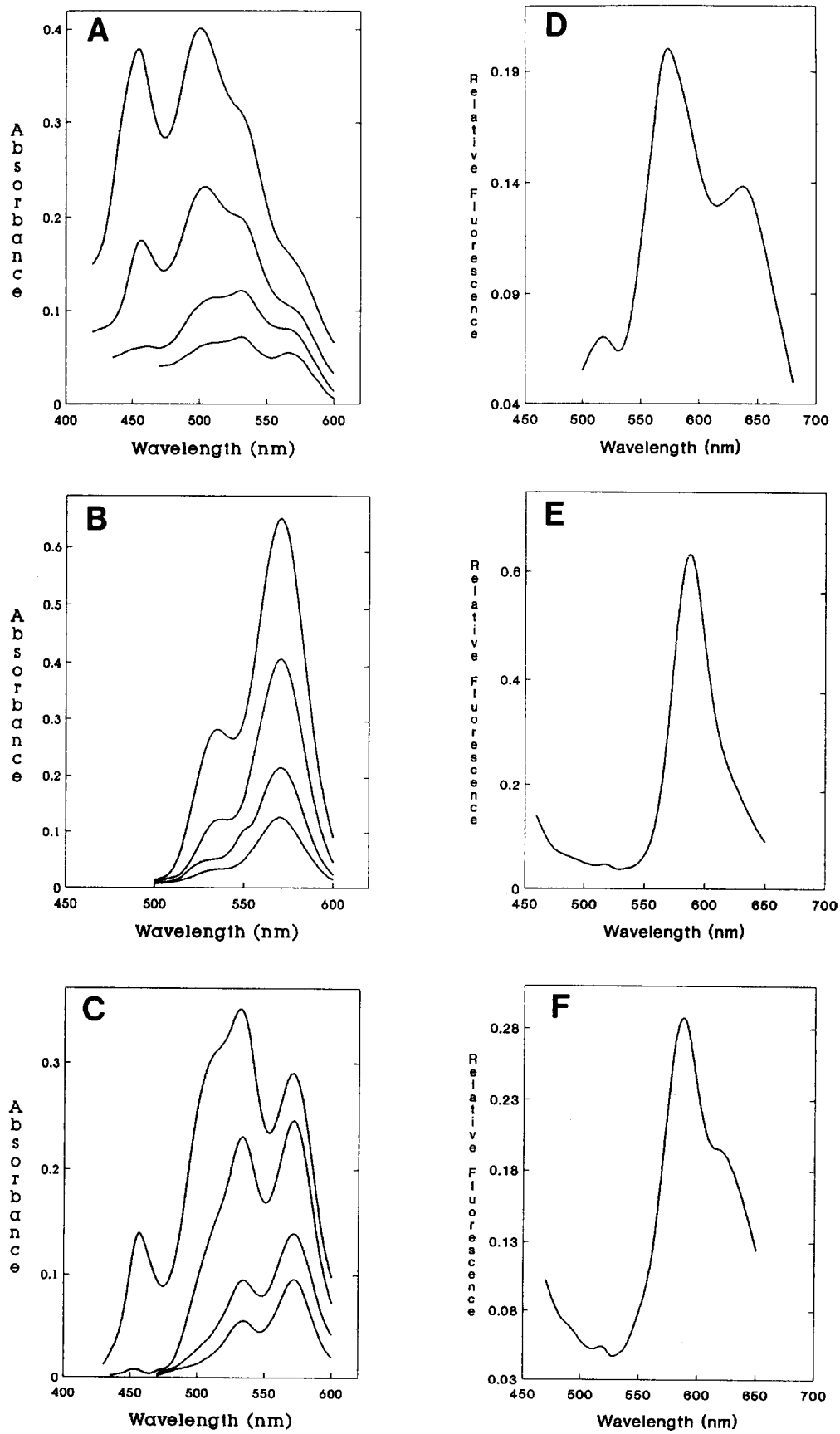
conditions the peak at 450 nm is clearly observed in the presence of DMPC at 13°C but not at 30°C (Figs. 1B and 3A). In addition, at low dye/lipid ratios the absorbance at 450 nm is absent for DPPC in the $L_{\beta'}$ phase (Fig. 1A).

Since MC 540 has a low partition in the gel state and, as denoted before only the external surface of the liposomes is accesible to the dye, the peak at 450 nm should be related to specific features of the bilayer surface influenced by the chain packing. It has been suggested that the increase in the chain–chain attraction promotes a decrease in the area per molecule and hence in the headgroup hydration. The resulting structure, characteristic of the $P_{\beta'}$ phase, is a corrugated surface profile which would have noticeable different microenvironments from those present in the $L_{\beta'}$ or the L_{α} phases [1,2]. These may be caused by the headgroup orientation and the hydration degree as it will be discussed below.

The appearance of large periodic ripples in the plane of the bilayer can also be induced by the incorporation of 2 to 3% of cholesterol. In DMPC/cholesterol bilayers at low cholesterol concentrations (8 mol%), Knol et al. [5] and Mortensen [3] found ripples whose wavelength is a function of temperature. For example, at 2 mol% cholesterol, Mortensen observed a wavelength of 360 Å at 4°C which decreases to 220 Å at 14.5°C and Knoll reported a smaller wavelength of about 140 Å at 17°C. At higher concentrations (up to 24% mol) the ripple texture is completely distorted. That is, the vesicle surface exhibits elongated smooth patches separated by dam-like protrusions. At cholesterol concentrations greater than 20 to 24 mol% the ripples disappear and the bilayer becomes flat.

It must be noticed that above this ratio the 450 nm peak and the band at 630 nm are not observed (Figs. 5D and 6B). For 35% cholesterol in DMPC bilayers at 13°C and when the DMPC bilayer reaches the L_{α} phase by the increase in temperature, both conditions in which the undulations disappear, the peak at 450 nm is not observed. Thus, there is a strong correlation between the induction of ripples in the plane of the bilayer and the observed spectroscopical features of merocyanine, notwithstanding that the molecular origin and nature of the corrugations may be entirely different.

Fig. 3. Absorption spectra of merocyanine in the presence of liposomes composed by DMPC/DPPC mixtures at 30°C. Liposomes were composed by (A) 100% DMPC; (B)35% DMPC/65% DPPC; (C) 100% DPPC. Lipid/dye ratios were in all cases 200:1, 100:1, 50:1 and 25:1 from bottom to top, respectively. Merocyanine was added to the lipid dispersion after its equilibration at the indicated temperatures.


A
Absorbance
Wavelength (nm)
B
Absorbance
Wavelength (nm)
C
Absorbance
Wavelength (nm)
D
Relative Fluorescence
Wavelength (nm)
E
Relative Fluorescence
Wavelength (nm)
F
Relative Fluorescence
Wavelength (nm)

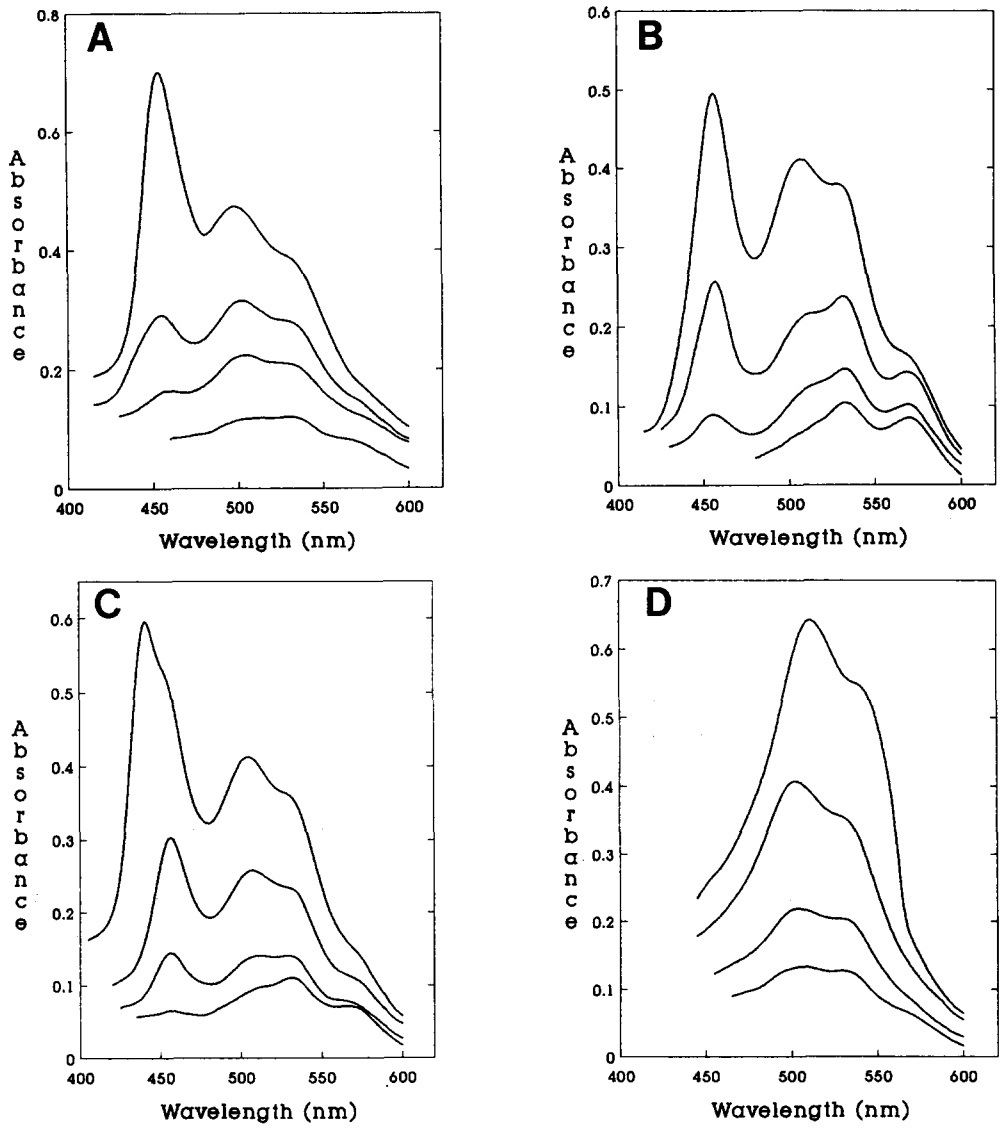


Fig. 5. Effect of cholesterol on the absorption spectra of DMPC liposomes DMPC liposomes contained (A) 0%; (B) 5%; (C) 10% and (D) 35% cholesterol. Lipid/dye ratio were the same as in Fig. 3 from bottom to top. Merocyanine was added to the lipid dispersion after its equilibration at 13°C.

The peak at 450 nm appears at the conditions of temperature and composition in which the cholesterol-induced surface corrugation changes dramatically with the total cholesterol concentration in the bilayer. However, the correlation of the MC-spectra features with the phase diagrams for PC and PC/cholesterol mixtures reported in literature show that, although the highest probability for ripples is in the $P_{\beta'}$ phase the appearance of the 450 nm peak cannot be ascribed only to the presence of that phase. As discussed elsewhere different kinds of ripples can be found within the $L_{\beta'}$ and the $P_{\beta'}$ phases.The phase diagrams for DPPC and 14:0/18:0-PC indicate that the bilayers are in the $L_{\beta'}$ phase at 13°C [23,26]. In the presence of mixed liposomes composed by 35% DMPC/65% DPPC, which according to the phase diagram are in the solid state at 30°C [22] the peak at 450 nm is clearly observed at the highest dye ratio and in 18:0/14:0-PC is small but significant. Moreover, the appearance of a shoulder at 450 nm in DPPC at 30°C for the highest dye/lipid ratio (Fig. 3C) suggests that part of the structures giving place to the peak at 450 nm are

Fig. 4. Changes in the absorption and fluorescence spectra of merocyanine produced by the heating and cooling of a DMPC dispersion across the phase transition in the presence of merocyanine. Absorption spectra were obtained at (A) 13°C; (B) 26°C; (C) 13°C after cooling the sample from 26°C. Lipid:dye ratio same as in Fig. 3 from bottom to top. Fluorescence spectra were obtained at (D) 8°C; (E) 28°C; (F) 7°C after cooling the sample from 28°C. DMPC liposomes were irradiated at 440 nm in the presence of merocyanine 200:1 lipid /dye ratio.

present. Therefore, the present results indicate that the limits between phases are not a sharp boundary. A region of mixed structures belonging to the adjacent phases coexist giving place to defects in the packing. It seems that these defects of molecular order can be detected by MC 540.

The appearance of such undulations can also be detected by the fluorescence band at 630 nm. It must be noticed that this band is observed in DPPC bilayers in conditions in which the peak at 450 nm is not observed (data not shown). However, at high dye/lipid ratio a shoulder at 450 nm for 100% DPPC at 30°C is noticeable (Fig. 3C). Thus, the sensitivity of fluorescence to detect an amount of merocyanine adsorbed to the surface is much higher than visible absorption.spectroscopy.

The peak at 450 nm appears at low dye/lipid ratios and increases with the dye concentration in a less pronounced way with the increase of DPPC in the bilayer. The curves of Fig. 2 show a saturation of the bilayers with the external dye concentration. The maximum level achieved is dependent on the DMPC/DPPC ratio in the bilayer. The different affinity for the dye can be due to a particular affinity of the dye by the DMPC molecules or for a particular supramolecular arrangement of the phase influenced by the presence of DMPC at a given temperature. However, at high MC ratio DPPC bilayers show a small shoulder at 450 nm. This suggests that some of the structural features giving place to the peak at 450 nm in DMPC are present in DPPC bilayers in a lower extent than in DMPC.

Calorimetric studies suggest that MC 540 is probably weakly attached to the surface or headgroup area of the bilayer in the gel phase [34]. In addition, MC 540 causes no chemical shift line broadening or changes in the relaxation time of the phosphate groups as seen by ^{31}P-NMR. The charge repulsion between the dye and the phosphate group would prevent the probe from penetrating the bilayer to a depth sufficient to alter the local motion of the phosphate moiety [33]. Thus, it is unlikely that the dye can perturb the membrane in the gel state in the present conditions.

Membranes in the gel state has a surface potential higher than that in the liquid-crystalline state as measured in monolayers [28]. In this regard, the interaction of MC with lipid membranes has been shown to obey a Langmuir isotherm in which the binding constant is influenced by the surface potential. Part of this surface potential is determined by the orientation of the phosphocholine dipole groups and water dipole at the membrane interface. Interestingly, the incorporation of cholesterol decreases the surface potential and the hydration layer [29–31] under conditions in which the fluorescence spectra indicate that the incorporation of cholesterol to DMPC at 13°C produces the same spectra than that found for DPPC without cholesterol at 30°C (Figs. 5D and 1A).

It has been described previously that the spectra corresponding to MC in the presence of liposomes below the phase transition temperature is similar to that shown by merocyanine in water which is composed by a peak at 500 nm corresponding to the dimer in water and another at 530 nm corresponding to the sum of the signals of the monomer in water (533 nm) and the dimer in membrane (530 nm). As the concentration of cyanine dyes is increased, aggregation in aqueous solution may proceed beyond the dimeric stage (see Fig. 2). The bands attributed to a polymer of greater complexity than the dimer is sometimes not resolved from the dimeric maximum, but sometimes a definite H-peak can be observed [27]. This may be the case in

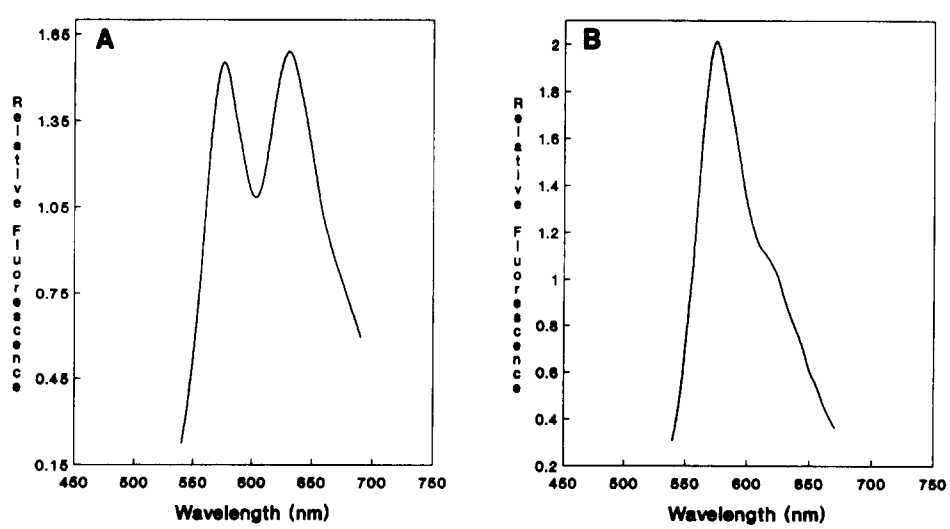


Fig. 6. Effect of cholesterol on the fluorescence spectra of DMPC liposomes. DMPC liposomes contained (A) 0%; (B) 30% cholesterol. The sample was irradiated at 440 nm in the presence of MC (200:1 lipid/dye) at 8°C.

the presence of DMPC bilayers in the gel state. These spectral changes can be attributed to aggregation of the dye molecule in water under the influence of strong dispersion forces associated with the high polarizability of the chromophoric chain. The high dielectric constant of the water acting as a solvent would play a role in reducing the repulsive force between the dye charges. As the appearance of the peak depends on the lipid composition of the bilayer the changes in the spectra would be reflecting the topological changes and redistribution of dipoles in the adjacencies of the bilayer.

In conclusion, visible absorption and fluorescence spectroscopy of merocyanine show that topologically different regions of lipid bilayers have different surface properties probably due to the distribution and orientation of dipole groups and molecules. These characteristics may be important in determining the membrane–membrane or membrane–protein interactions.

Acknowledgements

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