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A laser-induced europium(III) ion luminescence study of the interaction of this ion with phospholipid bilayer vesicles above and below the gel to liquid-crystalline phase transition temperature

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Laser-induced europium(III) luminescence spectroscopy was used to investigate the formation and integrity of phospholipid bilayer vesicles produced by ultrasonication and detergent dialysis. Eu(III) ion interactions with these model biological membrane systems were explored. $^7F_0 \rightarrow {}^5D_0$ spectral and excited-state lifetime data reveal two distinct, temperature-dependent binding sites, one involving a weak, 'superficial' interaction with the phosphate moiety of the phosphatidylcholine head group, the other involving a more tightly bound ion in a relatively dehydrated region of the head group. This latter 'sequestered' species appeared only at temperatures equal to or below that of the gel → liquid-crystalline phase transition. Systems containing various amounts of cholesterol showed a decrease in the formation of the sequestered species, indicative of a decrease in ion permeability. The results of this study demonstrate that the Eu(III) luminescence technique is useful for detecting major phase alterations in phospholipid bilayer vesicles.

1. Introduction

A great deal of literature has been devoted to the study of both model and natural cell membranes through the employment of a variety of physical techniques; inconclusive and conflicting data are abundant, particularly concerning the sites and stoichiometries of metal ion interactions. The primary goal of the research presented herein is to demonstrate the utility of laser-induced europium(III) luminescence spectroscopy for the study of model biological membrane systems and to develop a protocol for the eventual exploration of actual cell membranes. These systems are excellent candidates for such an investigation due to the importance of calcium(II) to the function of biological cells, as well as the success of europium(III) (and other lanthanide(III) ions) as a substitutional probe for the spectroscopically and magnetically 'silent' calcium(II) ion [1,2].

The substitution of europium(III) for calcium (II) is a well documented procedure; many macromolecular systems which require Ca(II) for their activity have been shown to retain total or partial activity in the presence of Eu(III) [3,4]. The success of Eu(III) as a surrogate lies in its similarity to Ca(II) in ionic radius and mode of coordination. Eu(III) has radii of 0.95 and 1.07 Å for coordination numbers of six and eight, respectively, while Ca(II) has values of 1.00 and 1.12 Å. Both ions possess a high affinity for oxygen-donor ligands, particularly carboxylate moieties, and are able to accommodate six or more ligating atoms in their coordination spheres with little geometric preference [5]. The difference in charge between trivalent europium and divalent calcium has little or no

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effect on binding modes; it does, however, lead to considerably higher binding constants (a factor of $\sim 10^4$–10^5) and slower ligand exchange rates for Eu(III) [6].

The ability of Eu(III), as well as other lanthanide(III) ions, to luminesce in solution at room temperature provides for a convenient means of analysis. Eu(III) is unique in that it possesses a nondegenerate ground ($^7F_0$) and excited ($^5D_0$) state, incapable of being split by ligand fields due to their J values of zero. This dictates, barring coincidence, the presence of a single band in the $^7F_0 \rightarrow {}^5D_0$ excitation spectrum for each distinct Eu(III) environment. These bands are relatively sharp (full widths at half maximum of the order of 10 cm^{-1}).

A vast amount of structural and binding information can be obtained through a variety of Eu(III) spectroscopic methods. Those methods exploited in this investigation include: (a) measurement of the number of $^7F_0 \rightarrow {}^5D_0$ excitation bands in a spectrum to determine the number of distinct Eu(III) species present in a particular sample; (b) measurement of the $^7F_0 \rightarrow {}^5D_0$ transition energy for each Eu(III) species to elucidate the total electrostatic charge of the coordinated ligands [7]; (c) measurement of $^5D_0$ excited-state lifetimes in protonated and deuterated solvents to determine the number of O-H oscillators (i.e., water molecules) in the primary coordination sphere of Eu(III) [8].

Multi- and unilamellar phospholipid vesicles undergo conformational changes at a particular temperature defined by the composition and charge of the head group, length, and degree of unsaturation of the hydrocarbon chains, and the extent of phospholipid and/or solute mixing within the phospholipid bilayers. The alterations occurring at this 'phase transition' temperature, T_c, do not affect the general features of vesicles – the systems remain closed and spherical – but do result in subtleties which greatly affect their physical properties. Below the phase transition, a highly ordered, solid-like state exists, with the hydrocarbon chains in all-*trans* conformations and tilted with respect to the perpendicular to the tangent plane of the membrane surface. Above the temperature, the phospholipid molecules become fluid-like and less tightly packed due to *gauche* rotations and kinks in the chains [9]; hence, the origin of the term, gel → liquid-crystalline phase transition.

There is a vast amount of literature on the subject of metal ion binding to neutral (zwitterionic) phospholipid bilayer vesicles. A few articles report no detectable metal ion-lipid interaction [10,11], however, most recognize a weak interaction (for Ca^{2+}, $K_b \sim 1$–$100\ M^{-1}$) with the phosphate group [12,13]. Many of the studies reporting metal ion interactions with phospholipid bilayers involve NMR, X-ray diffraction, or electrophoresis experiments which focus on either the gel or liquid-crystalline state. Most of these studies involved relatively high concentrations of di- or trivalent metal ions (> 1 mM), a condition which may perturb the bilayer structure.

In this article, the utility of laser-induced europium(III) luminescence spectroscopy is demonstrated by the examination of phosphatidylcholine vesicles as a function of temperature in both phases, in the presence of submillimolar concentrations of Eu(III). The distinction between metal ion binding to the solid and fluid structures is discussed in detail. Previous studies concerning Eu(III) luminescence in phospholipid systems employed relatively low-powered excitation sources which apparently failed to detect weak interactions [14–16]. One of the reports even neglected to discuss the importance of the phase transition and did not mention the temperature(s) at which the experiments were performed [16].

The focus of all studies to be discussed herein is on artificial membrane systems composed of phosphatidylcholine containing pairs of totally saturated 16-, 14- and 12-carbon chains, known as dipalmitoyl-, dimyristoyl- and dilauryloylphosphatidylcholine, respectively, and egg phosphatidylcholine, a mixed-chain system. The effect of cholesterol incorporated into the bilayer is also addressed.

2. Materials and methods

L-α-Dipalmitoylphosphatidylcholine (DPPC, T_c = 41° C, synthetic, ~ 99 + %), L-α-dimyristoyl-

phosphatidylcholine (DMPC, $T_c = 23°C$, 99 + %), L-α-dilauryloylphosphatidylcholine (DLPC, $T_c = -2°C$, synthetic, ~ 99%), L-α-phosphatidylcholine (EPC, type V-E from frozen egg yolk, ~ 99%), cholesterol (5(6)-cholesten-3-ol, 99 + %), L-α-glycerophosphorylcholine (GPC, $CdCl_2$ complex, ~ 98%), EGTA (97–98%), octylglucoside (crystalline), Triton X-100 (octyl phenoxy polyethoxyethanol), Sepharose 4B and Sephadex G-50-80 were obtained from Sigma and used without further purification. Deuterium oxide (^2H_2O, 99.8%) was obtained from Aldrich. H_2O (doubly distilled) and ^2H_2O stock solutions were identically prepared (10 mM piperazine; Sigma, laboratory grade; 150 mM KCl; Fisher, ACS grade, pH 6); the hydrogen ion concentrations for the ^2H_2O stocks were adjusted according to the relationship, $p^2H = pH$ (meter reading) + 0.45. These stocks were used in all experiments, unless otherwise noted.

A typical preparation of small unilamellar vesicles involved exposing 20 mg phospholipid in 2 ml buffer (10 mM piperazine, 150 mM KCl, pH 6, with or without 1 mM $EuCl_3$) to low-frequency ultrasound [17,18] for a continuous 20 min, using a Branson Ultrasonics model 250 sonifier with a titanium double-step micro tip set at approx. 25 W. Sample temperature was initially ambient but increased to above 50°C over the course of sonication due to heating of the immersed tip. Sample appearance changed from a milky dispersion prior to sonication to clear and opalescent. Removal of unincorporated lipid and titanium contaminant from the vesicle preparation was performed with a Fisher Centrific (model 225) fixed-angle, table-top centrifuge set at maximum speed for 30 min (4750 rpm, 3075 × g). In most cases, no more than 1 ml of supernatant was extracted for study. A fresh vesicle sample was prepared and used immediately for each experiment. External $EuCl_3$ was removed from samples using the Sephadex minicolumn technique of Fry et al. [19]. In the mixed DPPC/cholesterol experiments, approx 20 mg DPPC was dispersed in buffer with the appropriate amount of cholesterol to yield samples of 10 and 35 mol% cholesterol. Preparation was identical to that involving pure phospholipid. It should be noted that the 35 mol% cholesterol sample was considerably more turbid than those containing 0 or 10 mol% cholesterol. This was in accord with the finding that above 30 mol%, an asymmetric distribution of cholesterol between the two monolayers occurs, resulting in vesicles of greater diameter [20].

Larger vesicles were prepared by the detergent dialysis method of Philippot et al. [21], using 15 mg EPC and 60 mg octylglucoside in 10 mM piperazine buffer with 150 mM KCl and 1 mM $EuCl_3$ at pH 6. $EuCl_3$ in the external aqueous region was removed by passing the vesicle sample through a Sepharose 4B column equilibrated with buffer at 4°C.

Additions of 10–50 μl $EuCl_3$ were made from a 1 mM stock solution (10 mM piperazine, 150 mM KCl, pH 6) to an aliquot of a vesicle preparation, ranging from 1 ml to a portion diluted to 1 ml. All volumes used in parallel experiments were equal; measurements were made with the appropriate Eppendorf digital pipet (±1%). GPC titrations were performed by adding appropriate aliquots of a concentrated GPC/buffer stock (50 mM, pH 6) to a 1 mM $EuCl_3$/buffer solution.

A pulsed Nd-YAG-pumped tunable dye laser was used to obtain excitation spectra and lifetime data. Europium(III) $^7F_0 \rightarrow {}^5D_0$ spectra were recorded by continuous scanning of a rhodamine 590/610 dye laser through a 3- or 4-nm region centered at 579 nm; the subsequent $^5D_0 \rightarrow {}^7F_2$ emission at 614 nm was monitored as a function of excitation wavelength. Excited-state lifetime data were obtained by monitoring the 614 nm emission intensity as a function of time, while exciting at a single wavelength. The resolution of the dye laser is less than 0.01 nm at 560 nm; an energy of approx. 50–100 mJ per pulse was provided, depending on the age and condition of various components such as flashlamps and the rhodamine 590/610 dye mixture. Complete instrumental detail is reported elsewhere [22]. Sample temperatures were controlled using a Haake D3 water bath (±0.1°C).

Electron micrographs were obtained with a Philips Em 300 transmission electron microscope operating at 80 kV accelerating voltage. Vesicle samples were applied to 400-mesh copper grids, coated with Formvar and deposited carbon as the support, and negatively stained with 1% sodium

phosphotungstic acid. Freeze-etchings were prepared by cooling the sample, contained in a gold carrier, with Freon 22 followed by liquid N_2. Fracturing was performed with a Balzers 360 M freeze-fracture plant; platinum and carbon were applied to the fractures for replication. The actual vesicle sample was dissolved away with 3% sodium hypochlorite and rinsed with doubly distilled water. Etchings were mounted on 200-mesh copper grids and photographed.

^{1}H-NMR spectra were recorded on a Varian Em 360 60 MHz continuous-wave spectrometer. Vesicle samples for NMR studies were prepared as described above, except in ^2H_2O.

Spectral peak deconvolution and excited-state lifetime exponential decay analyses were performed using computer programs developed in this laboratory, which are based on the nonlinear regression method of Marquardt [23]. The programs are self-consistent in that improved computer-generated guesses are continuously entered until the calculated and experimental data meet specific convergence criteria. A complete discussion of this analysis has been previously documented [24]. The spectral deconvolution program utilizes both the Gaussian and Lorentzian equations to fit the experimental values for intensity, peak position and halfwidth [25]. The lifetime program fits data composed of up to three exponentials by varying initial intensities and lifetimes. Goodness of fit difference plots of actual vs. theoretical data were deemed acceptable when symmetric deviations from zero were obtained [26].

3. Results and discussion

3.1. Proof of vesicle formation

The formation of closed, spherical structures was demonstrated by the successful encapsulation of Eu(III) within the internal aqueous cavity. The buffer systems of both small and large vesicle preparations included 1 mM $EuCl_3$, allowing the vesicles to form in a Eu(III)-containing environment. If structures other than closed spheres were produced, then removal of Eu(III) from the external aqueous region (as described in section 2) would lead to rapid leakage of the internal contents and its subsequent removal.

EGTA is a metal chelator with a high affinity for Eu(III) ($K_b \approx 10^{17}$ M^{-1}) [27] and a $^7F_0 \rightarrow {}^5D_0$ band at 580.1 nm, well-removed from the region where Eu(III)-vesicle interactions appear (section 3.3). This ligand was used to aid in the detection of Eu(III), owing to its characteristic spectrum. In the presence of excess EGTA, a vesicle sample (produced by the detergent dialysis method), which has had the external Eu(III) removed, has no appreciable $^7F_0 \rightarrow {}^5D_0$ EuEGTA signal. However, upon addition of the detergent Triton X-100, which is known to disrupt vesicle structures by solubilizing the lipid, a significant increase in the EuEGTA peak intensity was observed. A similar result was obtained for the vesicles prepared by ultrasonication.

Electron micrographs and a freeze-etching of various vesicle preparations are shown in figs. 1 and 2. It is evident from these photographs that spherical structures were indeed produced. ^{1}H-NMR was also used to analyze the results of ultrasonication. Paramagnetic lanthanide(III) ions have been used extensively with NMR to probe vesicle systems [28]. Ions with unpaired electrons have the ability to shift and/or broaden the resonances of those species to which they are near. The effects, which may be through-bond or through-space, have magnitudes inversely proportional to the distance between the paramagnetic ion and the nuclei being observed. A closed bilayer system would be expected to have virtually indistinguishable inner and outer monolayer resonances in an NMR spectrum. Addition of a paramagnetic species to the external aqueous region of a vesicle sample should separate inner from outer resonances through their interaction with the atoms of the outer head group only. This is precisely what was observed when Eu(III) was added to the external aqueous region – the splitting of the sharp *N*-methyl proton resonance into two peaks ($\delta = 3.2$, 3.4 ppm) [29].

3.2. Vesicle integrity

The tendency for phospholipid vesicles to aggregate in the presence of metal ion, or simply

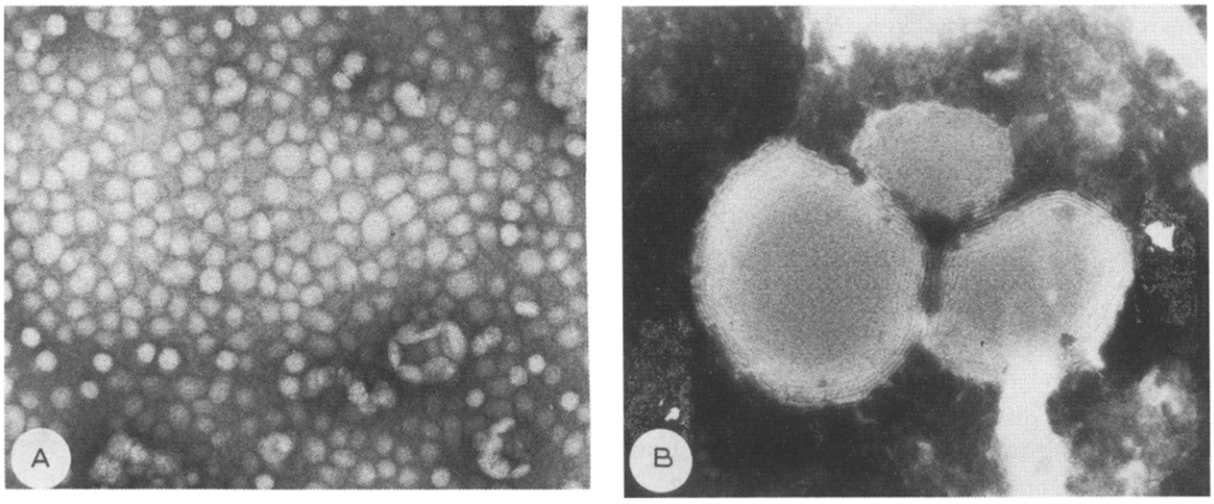


Fig. 1. Electron micrographs of phospholipid bilayer vesicles prepared by (A) ultrasonication and (B) detergent dialysis. Magnification, ×112674 (A), ×110970 (B), average diameter = 250 Å (A), 3000 Å (B).

over time, is a problem that must be contended with when a uniform, well-defined system is desired for analysis. To minimize the formation of aggregated species, freshly sonicated vesicles were used in each experiment. The following experiment was performed in order to determine the

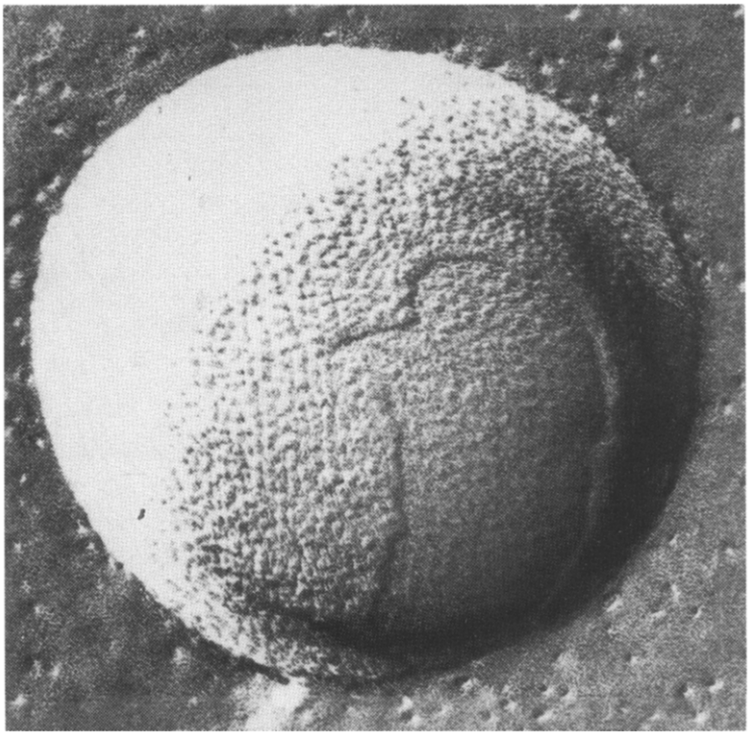


Fig. 2. Freeze-etching of vesicle prepared by detergent dialysis. Magnification, ×107000; diameter = 6200 Å.

integrity of a typical vesicle preparation, containing 50 μM Eu(III) in the external aqueous region, over the time course of a typical luminescence experiment.

Eu(III) was added to a system of pure DPPC vesicles at 65° C; data were collected 30, 105 and 108 min later while maintaining the sample at this temperature. The $^7F_0 \rightarrow {}^5D_0$ excitation spectra recorded over time showed no observable change in peak maximum, consistent with vesicles of reasonable stability. The excited-state lifetime data also support this finding by reflecting an unchanging Eu(III) environment over the course of the experiment; an unchanging value of 180 μs was obtained [29].

3.3. Europium(III) interactions with phosphatidylcholine vesicles

3.3.1. Above the main phase transition temperature – evidence for a weak ('superficial') head group interaction

Upon addition of $EuCl_3$ to the external aqueous volume of vesicles prepared with pure DPPC, DMPC, or DLPC, at a temperature greater than T_c, a single, broad excitation peak is observed (DPPC, fig. 3). The position of this peak is temperature-dependent, its intensity maximum red-shifting monotonically from 578.90 to 579.00 nm as the temperature is decreased from 70 to 12° C (fig. 4; NB: a second peak appears at and below T_c – see section 3.3.2). The temperature-dependent peak is attributed to a weak or superficial association of Eu(III) with the phosphate moiety of the head group. The rapid equilibrium between hydrated Eu(III) and Eu(III)/PO_4 is demonstrated by a closer examination of the excitation peak. The lowest value in the range (578.90 nm) is in reasonable agreement with the position of hydrated Eu(III) (578.85 nm), while the upper extreme (579.00 nm) is consistent with weak inorganic phosphate binding. The width at half maximum (20 cm^{-1}) is significantly greater than that of hydrated Eu(III) (16 cm^{-1}), which is considered to possess one of the broadest $^7F_0 \rightarrow {}^5D_0$ excitation bands. Despite the temperature dependence and breadth of the spectrum, attempts to curve resolve it into two or more peaks proved

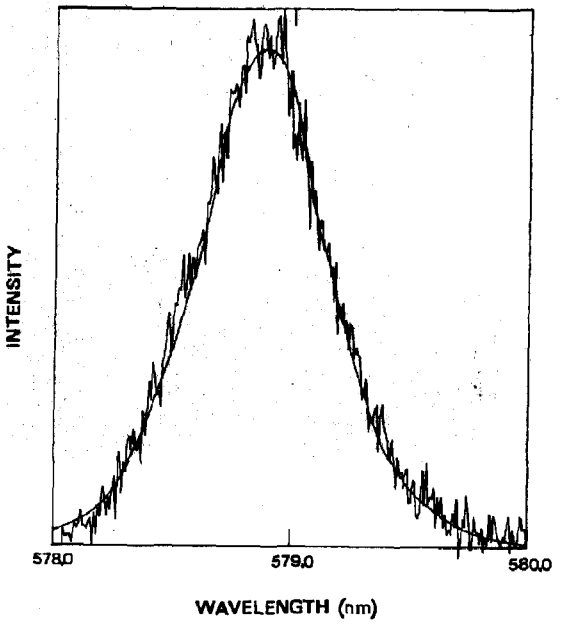


Fig. 3. $^7F_0 \rightarrow {}^5D_0$ resolved excitation spectrum of DPPC vesicles with 30 μM external Eu(III) at 56° C. $\lambda_{max} = 578.87$ nm.

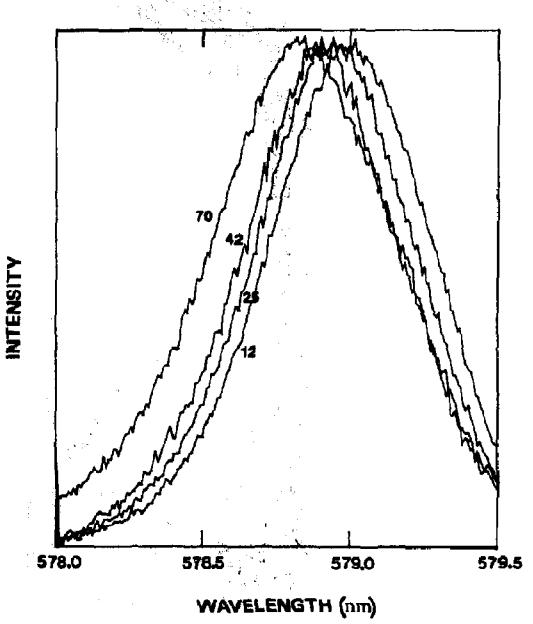
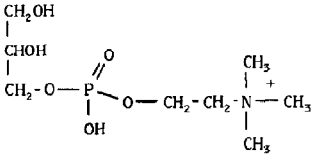


Fig. 4. Overlay of $^7F_0 \rightarrow {}^5D_0$ excitation spectra of DLPC ($T_c = -2°$ C) vesicles with 30 μM external Eu(III) (70–12° C).

unsuccessful; indeed, an excellent single Gaussian/Lorentzian fit was obtained (fig. 3).

Excited-state lifetime data are also consistent with a weak phosphate interaction. Excellent single-exponential decay curves were repeatedly obtained, yielding temperature-dependent values within the 160–200 μs range; lifetimes remained constant for each individual sample at a given temperature when measured at different wavelengths across the band profile. Weak association of a ligand such as phosphate to the hydrated Eu(III) species could displace one or more water molecules from the inner coordination sphere, thereby reducing the overall efficiency of the nonradiative deexcitation provided by the O-H oscillators, thus increasing the magnitude of the lifetime (NB: the lifetime of totally hydrated Eu(III) is 110 μs). A minor dependence of the lifetime upon temperature is not unexpected, as the spectral results indicate that the equilibrium shifts toward the Eu(III)/phosphate species with decreasing temperature.

A study of GPC (see structure) with Eu(III)



was carried out with the intention of modeling a weak Eu(III)-phosphate interaction. Lifetime values between 120 and 130 μs were obtained for solutions containing GPC/Eu(III) ratios in the 1–10 range ([GPC] $\geq$ 1 mM), while the excitation spectra, each consisting of a single band, exhibited minor red shifts with respect to hydrated Eu(III) with increasing GPC concentration. These results, in conjunction with the vesicle data, implicate monodentate phosphate coordination of GPC to Eu(III) and multidentate phosphate coordination of vesicle-incorporated phosphatidylcholine to Eu(III).

Consideration of the model for the most electrostatically favorable head group orientation of phosphatidylcholine also demonstrates the feasibility of the proposed Eu(III)–phosphate interaction [30]. The head group lying parallel to the bilayer surface enables two of the phosphate

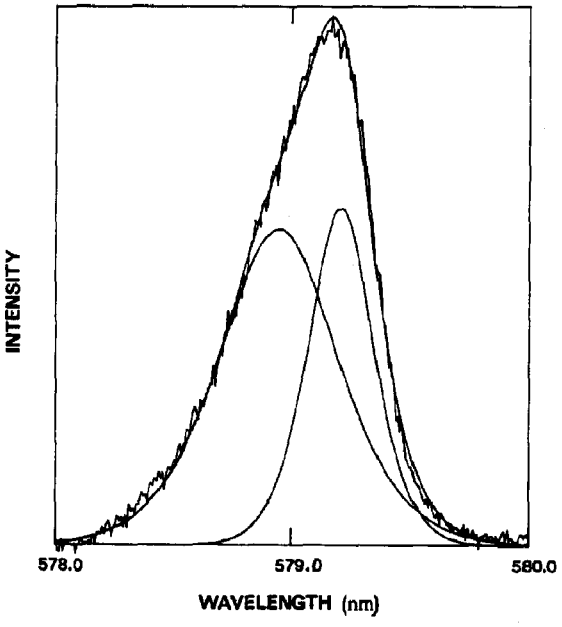


Fig. 5. $^7F_0 \rightarrow {}^5D_0$ resolved excitation spectrum of DPPC vesicles with 30 μM external Eu(III) at 41°C. λ_{max} = 578.91, 579.25 nm.

oxygens to be accessible to the external aqueous region and allows for the partial neutralization of the positively charged choline moiety by adjacent phosphates.

3.3.2. At and below the main phase transition temperature – evidence for two europium(III)-binding sites

In pure DPPC and DMPC systems, T_c = 41 and 23°C, respectively, a second Eu(III) complex appears at temperatures corresponding to the gel → liquid-crystalline phase transitions and persists at lower temperatures. This species, which coexists with the species described in section 3.3.1, has an excitation peak centered at 579.25 nm, a width at half maximum of 10 cm^{-1}, and shows no temperature dependence in its wavelength maximum (DPPC, fig. 5; a similar spectrum, not shown, was obtained for DMPC) [29]. The excited-state lifetime data, presented in table 1, are further evidence for the distinction between the two Eu(III) environments. The lifetime of the higher wavelength species at T_c is 330 μs, significantly longer than that assigned to the superficial phosphate

Table 1

Excited-state lifetime data of DPPC (with normalized intensities) and DMPC vesicle systems with external Eu(III), as a function of temperature (λ_{ex} = 579.10 nm)

T (° C)	DPPC		DMPC
	τ (μs) [a]	I_{norm} [a]	τ (μs) [a]
72	171	28800	–
64	177	31200	–
50	182	30400	173
41	166(330)	20400(21100)	–
30	187(450)	19600(13400)	179
20	197(500)	20500 (9500)	173(330)
12	–	–	173(450)

[a] Superficial species (sequestered species).

interaction, and this lifetime increases with decreasing temperature. Examination of the normalized initial intensities (amplitudes of exponential decay functions) for DPPC (table 1), which are proportional to the concentration of the absorbing species, shows that the superficial complex is the only complex present at temperatures greater than T_c. At T_c, a substantial decrease of the superficial complex concentration is observed, but this remains constant with further lowering of the temperature. The concentration drop is accounted for by the appearance of the other Eu(III)-containing species, henceforth referred to as the 'sequestered' species.

It is well documented that phospholipid bilayer vesicles in the liquid-crystalline state are virtually impermeable to metal ions; however, a marked increase in permeability occurs at and below the main phase transition temperature [31]. This phenomenon is the result of the major conformational adjustments which take place during the course of the transition. More specifically, it has been postulated that 'regions of disorder' are formed, regions where 'liquid' and 'solid' domains coexist within the membrane, between which metal ions may seep [32]. Once the transition is complete, a uniform, less permeable, solid-like structure remains. With this in mind, the longer-lived sequestered species is assumed to be due to Eu(III) penetration deeper into the head group region, approaching an area of increased hydrophobicity. It should be noted that in this study, similar data were obtained regardless of whether Eu(III) was added to a vesicle system above T_c and then cooled, or first added to the system at each temperature below T_c. However, in the latter experiment, the 330 μs lifetime appeared at T_c, but did not increase in magnitude with decrease in temperature. This is interpreted as follows. In the latter case, below T_c, permeability is attributed to irregularities in the packing of the gel phase, dependent upon the cooling rate [33], and further structural adjustments are made prior to addition of metal ion. In the former experiment, once the membrane is permeated at T_c, further structural adjustments take place around the metal ion. It is reasonable to assume, from the evidence presented here, that the complexes formed in each experiment are similar.

Further examination of these data and additional experiments suggest that the binding site of sequestered Eu(III) is in an oxygen-rich region of the outer monolayer: the phosphate in the head group, the oxygen atoms of the ester linkage at the base of the hydrocarbon chains, or both.

A relationship exists between the energy of the $^7F_0 \rightarrow {}^5D_0$ transition and the total charge of the ligands coordinated to Eu(III) [7]. The peak maximum, 579.25 nm, corresponds to an energy of 17264 cm^{-1} and calculated ligand charge of -2.2. This immediately suggests that participation of two negatively charged phosphate groups, more tightly bound to Eu(III) than those associated with the superficial complex described in section 3.3.1. It is true that this energy/charge relationship is based on a study of compounds containing carboxylate oxygens as donors and that there are examples of phosphate ligands which fail to follow it (e.g., nucleotides) [34], most likely due to delocalization of the negative charge. However, given a situation such as with phospholipid bilayer vesicles where the charge is more localized and a rigid, tightly packed structure is involved, the correlation may be applicable.

The number of water molecules associated with the sequestered species was determined by measuring the magnitude of the isotope effect on the reciprocal excited-state lifetimes [8]. The data collected from identical experiments performed in H_2O and ^2H_2O (table 2) shows that a minor

Table 2

Temperature-dependent excited-state lifetime data for Eu(III)/DPPC in H_2O and ^2H_2O

T (°C)	τ_H (μs) [a]	τ_D (μs) [a]
70	170	1295
52	180	1280
41	165(330)	1315(340)
22	195(500)	1430(595)

[a] Superficial species (sequestered species).

isotope effect exists, corresponding to a hydration number of less than 0.5 ± 0.5. This result is consistent with a neutron diffraction study which revealed a highly dehydrated state, particularly in the region of the glycerol backbone, below T_c [35].

Focusing on the lifetime trend noted for the sequestered species in section 3.3.2 (table 1), it is clear that temperature alone is not responsible for this phenomenon, since lifetime magnitudes of most simple Eu(III) complexes in H_2O show no temperature dependence. Consequently, this phenomenon can be attributed to one of the following: (1) a change in the number of inner sphere coordinated water molecules; (2) a change in the ligands binding, affecting symmetry; (3) rearrangement of the ligands binding, affecting symmetry. The first situation is not likely, as discussed in the previous paragraph, due to the minimal isotope effect at each temperature below T_c.

The fact that the excitation peak maximum wavelength of the sequestered species exhibits no appreciable temperature dependence indicates that the phosphate ligands continue to bind Eu(III) as the temperature decreases (energy/charge correlation). This reduces the likelihood that the second explanation applies to this situation. Further, structural changes which occur with gel → gel transitions are not severe, but involve conformational adjustments of the hydrocarbon chains with a concomitant expulsion of water, allowing for increasingly more ordered and tightly packed arrangements [36,37]. For DPPC, the pre- and subtransition occur at 36 and 22 °C, respectively. With this in mind, the third explanation becomes most likely. Once Eu(III) permeates the membrane at T_c, minor structural adjustments allow the metal ions to become more tightly 'frozen' into the lattice with no change in binding site occurring. Europium's sensitivity to these adjustments is reflected in the magnitude of the lifetime, which varies with ligand orientation, i.e., symmetry.

Worthy of comment at this time is the reversibility of the binding of the sequestered species. This complex dissociates beyond the point of detection when the temperature of the system is increased above T_c, regardless of whether Eu(III) is initially added above or below T_c. This is consistent with a previously reported finding involving DPPC in the presence of praseodymium chloride, in which the original ^{1}H-NMR spectrum was obtained when the temperature of the system was increased to 60 °C again, after being cooled below T_c [38].

In summary, the sequestered complex is believed to involve the coordination of two tightly bound, adjacent phosphate moieties in the external monolayer, with a single water of hydration at most, and, based on their close proximity and europium's ability to extend its coordination number well beyond six, one or more ester groups. With regard to the terminology used here, this species is indeed sequestered within the head group region with respect to the superficial phosphate complex, but is not likely to be located beyond the ester linkage because of its reversibility and the energetic unfavorability of that situation.

3.4. Determination of the gel → liquid-crystalline transition temperature

It is appropriate to point out the utility of laser-induced Eu(III) luminescence spectroscopy for the determination of the temperature of this phase transition in phospholipid bilayer systems. A variety of techniques have been employed for this purpose [9], differential scanning calorimetry being the most notable; however, the use of fluorescence has been rather limited [39,40]. In the previously reported fluorescence experiments, molecular probes were incorporated into the bilayer and fluorescence intensities measured as a function of temperature. A question arises, however, given the size and indefinite location of the

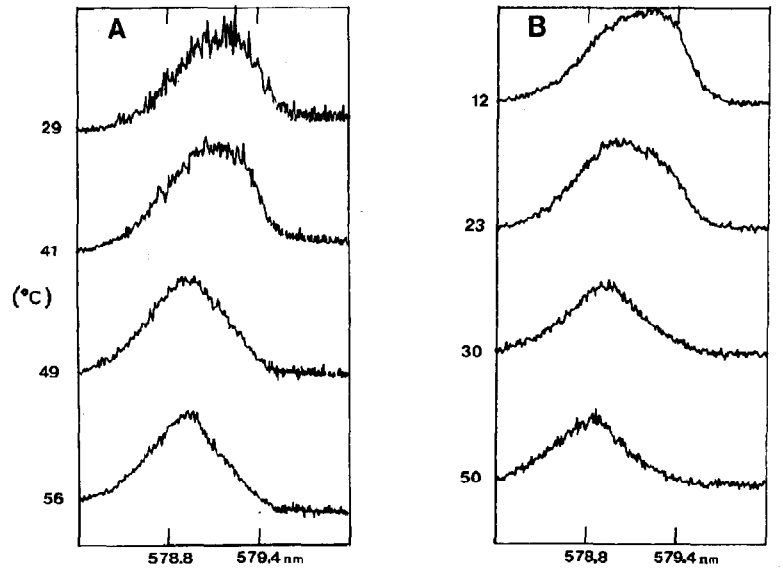


Fig. 6. $^7F_0 \rightarrow {}^5D_0$ excitation spectra of (A) DPPC ($T_c = 41°C$) and (B) DMPC ($T_c = 23°C$) vesicles with 30 μM external Eu(III) vs. temperature.

probes, as to the extent to which the membrane environment was perturbed.

Europium(III) luminescence induced by a high-powered laser is a very sensitive tool for studying model membrane systems, requiring only micromolar concentrations of the metal ion. Under these conditions, only a weak interaction between Eu(III) and vesicles is observed above the phase transition temperature (vide supra). A control experiment showed that the system remains stable above T_c in the presence of submillimolar concentrations of Eu(III) – no evidence of aggregation or fusion was observed – and that the change occurring at T_c is due to ion permeation as a function of temperature, not leakage over time. Figs. 6 and 7 display the spectral and lifetime data from the temperature studies for DPPC and DMPC. The major alterations which occur at T_c are quite prominent, for it is at this temperature that the excitation peak at 579.25 nm first appears

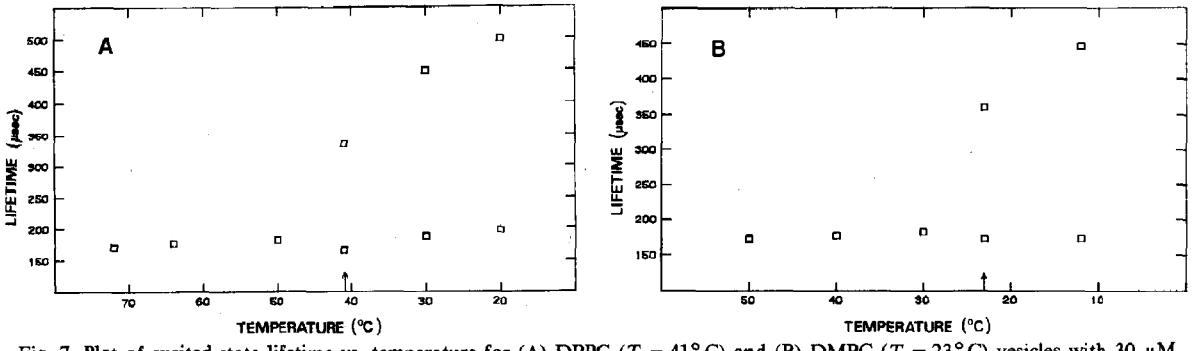


Fig. 7. Plot of excited-state lifetime vs. temperature for (A) DPPC ($T_c = 41°C$) and (B) DMPC ($T_c = 23°C$) vesicles with 30 μM external Eu(III).

(proceeding from high to low temperature) and the excited-state lifetime decay curves shift from single to double exponentials.

3.5. Effect of cholesterol

The importance of cholesterol to the health of the human body has never been more evident than in recent years. The deposits that accumulate inside arteries, a condition known as atherosclerosis which increases one's chances of developing heart disease and strokes, are associated with high blood-cholesterol levels. Cholesterol is a steroid and, like the phospholipid, is amphiphilic (most of its surface being hydrophobic) and relatively abundant in human red blood cell membranes (40–50 mol%, 25% by mass of total lipid). Previous studies with model phospholipid membrane systems have demonstrated significant phospholipid-cholesterol interactions with potential importance regarding the physiology of cholesterol in actual cell membranes. For instance, it is well documented that cholesterol affects the fluidity of the hydrocarbon chains at temperatures near that of the phase transition and generally reduces bilayer permeability to ions [38,41]. According to a differential scanning calorimetry study [42], cholesterol concentrations greater than 10% completely remove the pretransition endotherm. The presence of 20 mol% or more causes a broadening of the gel → liquid-crystalline phase transition endotherm and a concomitant decrease in the enthalpy of transition; above 50 mol%, the transition is completely abolished.

In an effort to observe these physicochemical properties by exploiting Eu(III) luminescence, three systems composed of DPPC with 0, 10 and 35 mol% cholesterol were examined and compared. The resolved excitation spectrum for the 90% DPPC/10% cholesterol system with Eu(III) added at 32°C is shown in fig. 8. Of particular interest is the relative insignificance of the sequestered species at 579.25 nm, indicative of a reduction in ion penetration into the sequestered sites. This phenomenon is also reflected in the lifetime data (table 3), in which the initial intensity values (I_0) reported for each sample were obtained by

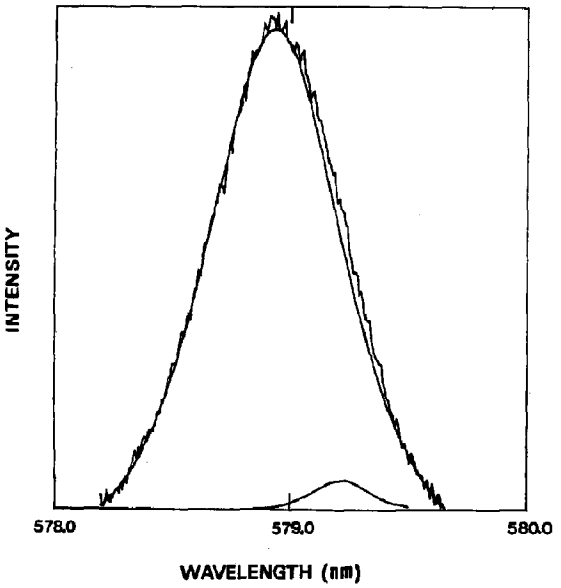


Fig. 8. $^7F_0 \rightarrow {}^5D_0$ resolved excitation spectrum of vesicles containing 90% DPPC/10% cholesterol with external Eu(III) at 32°C. λ_{max} = 578.93, 579.21 nm.

holding the 330 μs lifetime constant during analysis for a double exponential, while allowing all other parameters to float. The intensities of the sequestered and superficial species are presented as I_{seq}/I_{sup}. Comparison of this ratio for each system at a particular temperature reveals an inverse relationship between the amount of sequestered Eu(III) and cholesterol concentration. These results are consistent with the finding that a state of 'intermediate fluidity' is produced at temperatures near and below that of the phase transition. The presence of cholesterol reduces intermolecular interactions between neighboring hydrocarbon

Table 3

Temperature-dependent excited-state lifetime initial intensity ratios (I_{seq}/I_{sup}) of Eu(III)/DPPC as a function of percent cholesterol

T (°C)	% cholesterol		
	0	10	35
42	0.19	0.092	0.067
32	1.03	0.32	0.12
22	1.66	0.85	0.25

chains, preventing them from crystallizing into the rigid gel phase [43].

4. Conclusions

Laser-induced europium(III) luminescence spectroscopy was employed for the study of small and large phospholipid vesicle formation and integrity, as well as metal ion interactions with small unilamellar vesicles at pH 6. Two distinct, temperature-dependent Eu(III)-binding sites in the phosphatidylcholine (PC) head group region of DPPC, DMPC, and DLPC are proposed. One interaction, which is the only interaction present at temperatures above that of the gel → liquid-crystalline phase transition, involves a weak, superficial association with the negatively charged phosphate substituent and is observed as a rapid equilibrium between the free hydrated ion and the phosphate-bound ion. The second interaction is detectable only at temperatures equal to or below that of the phase transition. The Eu(III) $^7F_0 \rightarrow {}^5D_0$ excitation spectra and excited-state lifetime data implicate the binding of Eu(III) to two phosphate moieties in a relatively dehydrated head group region; the binding of one or more ester oxygens is also suggested. The accessibility of this sequestered site was shown to be strictly a temperature (i.e., phase change) effect, not the result of a time-dependent breakdown of vesicle integrity. The incorporation of cholesterol into the phospholipid bilayer leads to a significant reduction in the amount of sequestered Eu(III) at and below the phase transition temperature, consistent with literature reports of decreased ion permeability in the presence of cholesterol. The utility of the Eu(III) luminescence technique in detecting phase transitions is also demonstrated.

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