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RAMAN STUDY OF CALCIUM-INDUCED FUSION AND MOLECULAR SEGREGATION OF PHOSPHATIDYLSERINE/DIMYRISTOYL PHOSPHATIDYLCHOLINE-d_{54} MEMBRANES

SUI-KONG HARK and JOHN T. HO *

Department of Physics and Astronomy, State University of New York at Buffalo, Amherst, NY 14260 (U.S.A.)

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Summary

Raman spectroscopy has been used to study the effect of Ca^{2+} on the molecular properties of model membranes consisting of mixtures of phosphatidylserine and dimyristoyl phosphatidylcholine-d_{54}. The I_{2880}/I_{2935} intensity ratio associated with the C-H stretching modes is used to monitor the phosphatidylserine molecules, while the linewidth at 2103 cm^{-1} associated with the C-^{2}H stretching modes is used for the dimyristoyl phosphatidylcholine-d_{54} molecules. Membranes containing phosphatidylserine and dimyristoyl phosphatidylcholine-d_{54} at a molar ratio of 1 : 2 show evidence of initial immiscibility, which is further enhanced by the addition of Ca^{2+}. Membranes containing phosphatidylserine and dimyristoyl phosphatidylcholine-d_{54} at a molar ratio of 2 : 1 are completely miscible, and fuse with the addition of Ca^{2+}. The phosphatidylserine molecules in the fused product of mixed vesicles have highly rigid acyl chains, and behave identically to those in an earlier Raman study of Ca^{2+}-induced fusion of pure phosphatidylserine vesicles. The dimyristoyl phosphatidylcholine-d_{54} molecules are also solid-like, suggesting a higher degree of miscibility with phosphatidylserine in the presence of Ca^{2+} than dipalmitoyl or distearoyl phosphatidylcholine.

Introduction

The physical properties of model membranes containing both neutral and negatively charged phospholipids have been intensely studied. Of particular

* To whom correspondence should be addressed.

interest is the effect of Ca^{2+} on these properties because of the possible relationship to Ca^{2+}-induced events in biological membranes. A variety of laboratory techniques has been used in recent years to study these phenomena. They include spin label ESR [1—3], differential scanning calorimetry [4—6], freeze-fracture electron microscopy [6—8], and NMR [9,10]. The results have led to speculations concerning an intimate relationship between Ca^{2+}-induced fusion and molecular segregation [5,6]. It has also been found that a combination of several techniques applied to the same membrane system is required to yield detailed information about miscibility and phase diagrams [3,6,8]. A recent dynamic light-scattering study on phosphatidylserine/phosphatidylcholine vesicles has suggested that Ca^{2+}-induced molecular segregation occurs prior to subsequent fusion between relatively pure phosphatidylserine domains [11].

We report here the first study using Raman scattering to examine the effect of Ca^{2+} on mixed membranes containing both neutral and negatively charged phospholipids. Raman spectroscopy has been found to be useful in studying phospholipid conformation in model membrane systems [12—17]. Vibrational modes of the acyl chains are sensitive to the fluidity and packing of the membrane, resulting in significant changes in the intensity, linewidth, and frequency in several regions of the Raman spectra, especially through the gel-to-liquid crystalline phase transition. Apart from being a convenient and sensitive probe of the membrane structure at the molecular level, Raman scattering has a unique advantage over all other techniques in studying membranes composed of a binary mixture of phospholipids. By using deuterated phospholipids as one of the components, the behavior of each phospholipid component can be monitored simultaneously and non-perturbatively. Comparison between the Raman spectra of deuterated and undeuterated phospholipids has suggested that there is sufficient difference between the two spectra to make this approach potentially feasible [18—20]. A study using mixtures of dimyristoyl phosphatidylcholine-d_{54} and distearoyl phosphatidylcholine has demonstrated the applicability of this technique [21].

In this initial test of the usefulness of Raman spectroscopy in elucidating Ca^{2+}-induced effects on negatively charged mixed membranes, we have studied binary mixtures of bovine brain phosphatidylserine and dimyristoyl phosphatidylcholine-d_{54}. This system has been chosen because the broad phase transition in phosphatidylserine and the proximity of the phase transition regions of the two components make it relatively challenging. Furthermore, a Raman study of the effects of Ca^{2+} and Mg^{2+} on pure phosphatidylserine membranes has been reported by us previously [22].

Methods

Bovine brain phosphatidylserine (greater than 99% purity) and dimyristoyl phosphatidylcholine-d_{54} were obtained from Avanti Biochemicals. To avoid oxidation, the materials were handled under nitrogen or in a vacuum at all times. Vesicles were prepared in a buffer containing 0.1 M NaCl, 2 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, 2 mM L-histidine, and 0.1 mM EDTA, adjusted to pH 7.4. In binary mixtures, the two phospho-

lipid components were mixed thoroughly in $CHCl_3$ prior to evaporation and dispersion. Typically, 25 mg of phospholipid material were dispersed in 0.3 ml of buffer and shaken mechanically for 10 min. Raman spectra in the absence of Ca^{2+} were taken with the dispersion in a sealed 1 mm capillary tube. Sonicated vesicles were used in the study of membranes with 67 mol% phosphatidylserine in the presence of Ca^{2+}. The dispersion was sonicated in a bath-type sonicator under N_2 for at least 2 h at a temperature slightly higher than the phase transition temperature. About 50 μl of the sonicated vesicles in buffer were transferred to a capillary tube and mixed with an equal volume of buffer containing 0.1 M $CaCl_2$. The final concentrations of both the phospholipid mixture and Ca^{2+} are typically 50 mM. The capillary tube was sealed under N_2 and centrifuged to pack the precipitate.

Raman spectra were taken with a Spex 14018 double monchromator at 3 cm^{-1} resolution. The 514.5 nm radiation from a Coherent CR-4 argon ion laser was used as the light source. The typical incident power at the sample was 300 mW. The 90° scattering geometry was used. The temperature of the sample in the capillary tube was controlled above and below ambient temperature to a stability of 0.1°C by means of a Cambion thermoelectric module with a Kepco bipolar power supply as the current source. Temperature measurements were made with a copper-constantan thermocouple. The amount of local heating due to the laser beam was estimated from the data of single-component samples, for which the phase transition temperatures are known, and temperature corrections have been applied to all the data. The optical detector was a cooled Hamamatsu R955 photomultiplier. Photon counting was used to monitor the detector output. All the temperature scans were repeated at least once with samples from a different preparation to test for repeatability. Band intensities were taken as peak heights measured from consistently chosen baselines. For the linewidth at 2103 cm^{-1} of samples containing dimyristoyl phosphatidylcholine-d_{54}, the full width at half-maximum

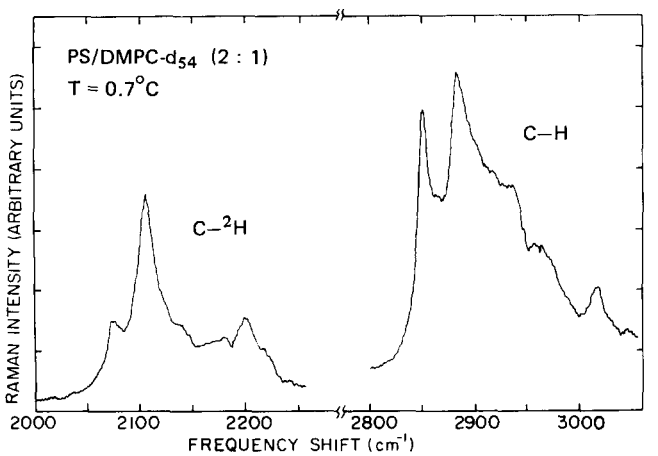


Fig. 1. Raman spectrum in the C-H and C-^{2}H stretch regions at 0.7°C of mixed vesicles containing phosphatidylserine (PS) and dimyristoyl phosphatidylcholine-d_{54} (DMPC-d_{54}) at 2 : 1 molar ratio. The incident light was 514.5 nm at 300 mW. The spectrometer was set at 3 cm^{-1} resolution and a scan rate of 0.2 cm^{-1}/s.

was measured using a baseline between 2000 and 2256 cm^{-1}. A typical spectrum taken from a sample containing mixed vesicles is shown in Fig. 1.

Results and Discussion

Dimyristoyl phosphatidylcholine-d_{54}

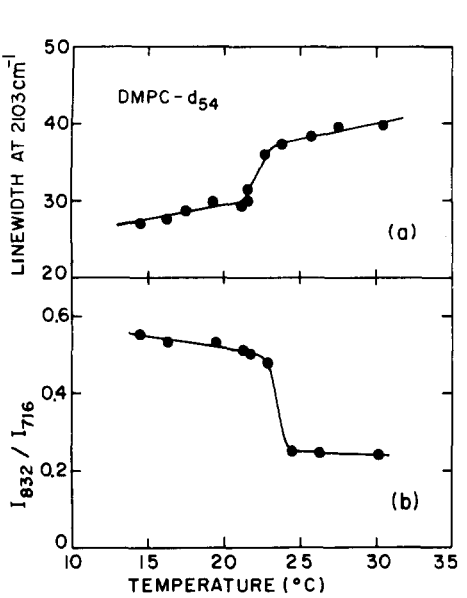
Before mixed membranes were examined, the Raman spectra of the individual phospholipid components were studied. Fig. 2 shows the behavior of two temperature-dependent features of the Raman spectra of dimyristoyl phosphatidylcholine-d_{54} dispersions. Of the Raman modes that are sensitive to membrane fluidity, it is expected that the C-C symmetric skeletal stretching modes at 832 and 1145 cm^{-1} [19,20] would be indicative of the number of *trans* bonds in the acyl chains. For example, the temperature dependence of the intensity at 832 cm^{-1}, relative to that of the temperature-insensitive C-N stretching mode at 716 cm^{-1}, is shown in Fig. 2b. The sharp phase transition at 23°C is apparent. Unfortunately, there is considerable spectral interference at these and most other frequencies in the presence of undeuterated phosphatidylserine.

The most noteworthy spectral feature of deuterated phospholipids occurs at 2103 cm^{-1}, which has been assigned as the C^2H_2 symmetric stretching mode of the acyl chains [19,20]. The linewidth of the mode at 2103 cm^{-1} in dimyristoyl phosphatidylcholine-d_{54}, as shown in Fig. 2a, increases at the phase transition. By choosing to define the linewidth as the full width at half-maximum with respect to a baseline measured between 2000 and 2256 cm^{-1}, we find a considerably larger relative change (50%) between 15 and 30°C than the 12% increase previously reported [21]. This linewidth is therefore a convenient indication of the fluidity of dimyristoyl phosphatidylcholine-d_{54}, even in the presence of phosphatidylserine.

Phosphatidylserine

Two temperature-sensitive intensity ratios in the Raman spectra of phosphatidylserine dispersions are shown in Fig. 3. The intensities of the C-C skeletal stretching modes at 1063 and 1086 cm^{-1} are known to be a measure of the number of *trans* and *gauche* bonds, respectively, in the acyl chains of phospholipids [12,14,23]. Fig. 3b shows the temperature dependence of the I_{1063}/I_{1086} intensity ratio in phosphatidylserine. A broad phase transition ending in a fluidus point at about 11°C can be seen. It is known that bovine brain phosphatidylserine contains a mixture of molecules with different fatty acid composition, which has been determined to be primarily 48.9% of 18 : 0 and 37.2% of 18 : 1, with small amounts of longer chain components [24]. The transition is therefore considerably broader than in synthetic phosphatidylcholines, in agreement with differential scanning calorimetric results [5,25]. These Raman modes, however, cannot be used in the presence of deuterated phospholipids because of spectral interference.

Other useful Raman modes of phosphatidylserine occur at about 2900 cm^{-1}. The CH_2 symmetric stretching modes at 2845 and 2880 cm^{-1} have been found to be sensitive to both the lateral packing of the acyl chains and to the number of *gauche* bonds [12,13,23,26]. It is customary in the



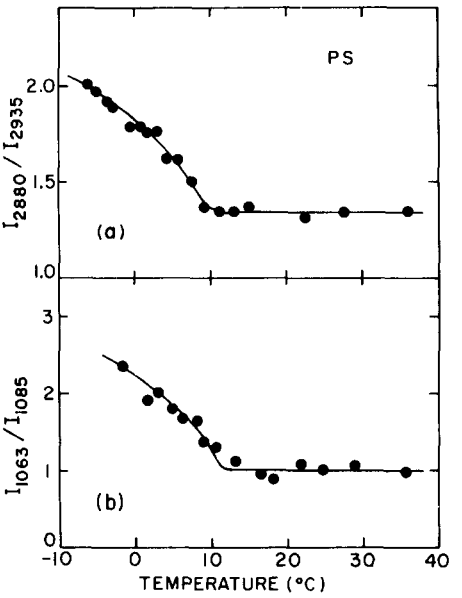


Fig. 2. Temperature dependence of (a) Raman linewidth at 2103 cm^{-1} and (b) I_{832}/I_{716} Raman intensity ratio in dimyristoyl phosphatidylcholine-d_{54} (DMPC-d_{54}) dispersions.

Fig. 3. Temperature dependence of Raman intensity ratios (a) I_{2880}/I_{2935} and (b) I_{1063}/I_{1085} in phosphatidylserine (PS) dispersions.

literature to use the I_{2880}/I_{2850} intensity ratio in melting curves, even though I_{2850} itself is sensitive to temperature. However, the shoulder at 2935 cm^{-1}, which has been identified as a CH_3 stretching mode, is relatively independent of temperature [23,27]. In a separate experiment on dipalmitoyl phosphatidylcholine, we have found that the I_{2880}/I_{2850} intensity ratio increases from 0.97 to 1.20 (24%) upon cooling at the phase transition at 41°C, while the I_{2880}/I_{2935} intensity ratio changes from 1.49 to 2.34 (57%). We have therefore chosen to use the latter ratio in the current study. Fig. 3 illustrates the temperature dependence of the I_{2880}/I_{2935} intensity ratio for phosphatidylserine, showing that it is also a good indicator of the phase transition. Furthermore, the Raman modes in deuterated phospholipids at these frequencies are relatively weak [20]. The C-H stretching modes are therefore suitable monitors for the phosphatidylserine molecules in the presence of dimyristoyl phosphatidylcholine-d_{54}.

Mixed membranes

The results obtained in mixed membranes with phosphatidylserine and dimyristoyl phosphatidylcholine-d_{54} at a molar ratio of 1 : 2 are shown in Fig. 4. From the results with the pure components described above, the I_{2880}/I_{2935} intensity ratio is chosen to monitor the phosphatidylserine molecules, while the linewidth at 2103 cm^{-1} is used to monitor the dimyristoyl phosphatidylcholine-d_{54} molecules. Fig. 4a shows the temperature dependence of these spectral features in the absence of Ca^{2+}. The broad chain-melting transi-

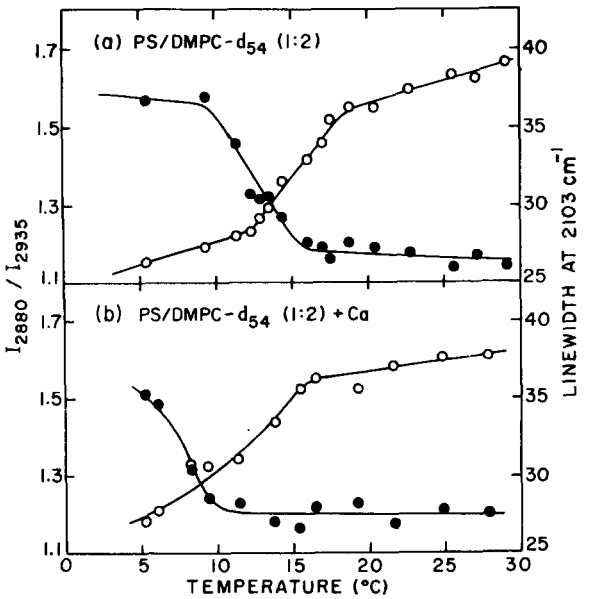


Fig. 4. Temperature dependence of Raman spectral features in mixed membranes containing phosphatidylserine (PS) and dimyristoyl phosphatidylcholine-d_{54} (DMPC-d_{54}) at 1 : 2 molar ratio (a) without Ca^{2+} and (b) with 50 mM Ca^{2+}. •, I_{2880}/I_{2935} intensity ratio; ○, linewidth at 2103 cm^{-1}.

tions of both phospholipid components are apparent. It can also be seen that the two components are not completely miscible. Both the solidus and the fluidus points for dimyristoyl phosphatidylcholine-d_{54} are about 3°C higher

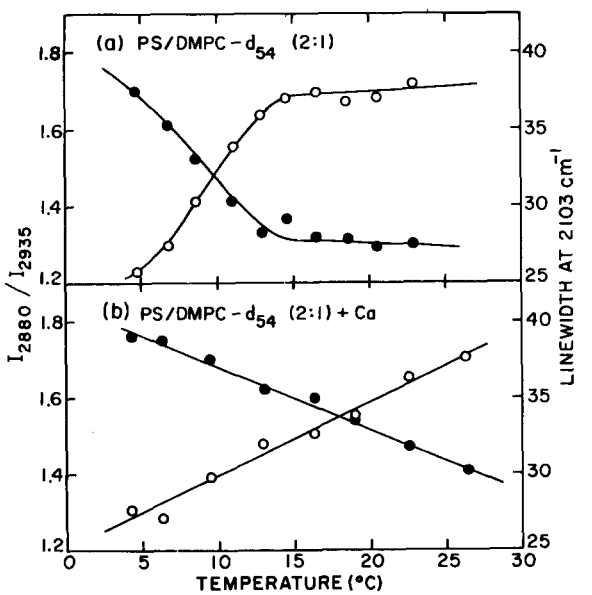


Fig. 5. Temperature dependence of Raman spectral features in mixed membranes containing phosphatidylserine (PS) and dimyristoyl phosphatidylcholine-d_{54} (DMPC-d_{54}) at 2 : 1 molar ratio (a) without Ca^{2+} and (b) with 50 mM Ca^{2+}. •, I_{2880}/I_{2935} intensity ratio; ○, linewidth at 2103 cm^{-1}.

than the corresponding points for phosphatidylserine. This clear indication of immiscibility in the presence of broad transitions illustrates the power of Raman spectroscopy in studying binary systems. Another interesting result is the overall lower values of the I_{2880}/I_{2935} intensity ratio in the mixture compared to those in pure phosphatidylserine. This is probably because this ratio is sensitive to the lateral environment of the acyl chains, which has become more heterogeneous in the mixture.

The effect of Ca^{2+} on mixed membranes with phosphatidylserine and dimyristoyl phosphatidylcholine-d_{54} at a molar ratio of 1 : 2 is shown in Fig. 4b. There is no evidence of significant aggregation or fusion of vesicles induced by Ca^{2+}. The temperature dependence of the Raman spectra shows that the addition of Ca^{2+} causes the difference between the fluidus points of the two phospholipid components to increase from 3 to about 5°C. This indicates an enhancement of the degree of immiscibility due to Ca^{2+}-induced molecular segregation. This result again illustrates the ability of Raman scattering to reveal these subtle changes in the presence of broad transitions. Previous reports of such a Ca^{2+}-induced effect have been performed with both components being synthetic phospholipids, giving much sharper phase transitions which can be studied with differential scanning calorimetry [6].

Fig. 5 shows the results obtained with mixed membranes with phosphatidylserine and dimyristoyl phosphatidylcholine-d_{54} at a molar ratio of 2 : 1. In Fig. 5a, in the absence of Ca^{2+}, both the I_{2880}/I_{2935} intensity ratio and the linewidth at 2103 cm^{-1} show very similar behavior as a function of temperature, with a common fluidus point at 15°C. Thus, both phosphatidylserine and dimyristoyl phosphatidylcholine-d_{54} components melt together, and the membrane behaves as a completely miscible system.

When 50 mM Ca^{2+} is added to sonicated vesicles of this 2 : 1 mixture of phosphatidylserine and dimyristoyl phosphatidylcholine-d_{54} at a phospholipid concentration of 50 mM, the resultant Raman spectra are shown in Fig. 5b. There are dramatic changes in the temperature dependence of the spectra as a result of the Ca^{2+}-induced fusion of the vesicles. The I_{2880}/I_{2935} intensity ratio has values considerably higher than those without Ca^{2+}. There is only a gradual increase with temperature with no sign of a phase transition in the temperature range studied. The behavior of the CH_2 stretching modes is almost the same as that in pure phosphatidylserine in the presence of Ca^{2+} [22]. This result indicates that Ca^{2+} causes the phosphatidylserine molecules to attain the same high degree of rigidity in the acyl chains in these mixed membranes as in pure phosphatidylserine membranes. X-ray and NMR measurements in pure phosphatidylserine vesicles have provided evidence for the formation of an anhydrous intermolecular Ca^{2+}-phosphatidylserine complex [25,28,29]. The Raman data suggest that a similar fused structure is obtained even in the presence of 33 mol% of dimyristoyl phosphatidylcholine-d_{54}. The behavior of the linewidth at 2103 cm^{-1} after the addition of Ca^{2+} is equally interesting. It shows that the dimyristoyl phosphatidylcholine-d_{54} molecules are also highly rigid and do not undergo a phase transition. This uniform behavior of both the phosphatidylserine and dimyristoyl phosphatidylcholine-d_{54} components after the addition of Ca^{2+} is in contrast to earlier results obtained by differential scanning calorimetry on similar 2 : 1 mixtures

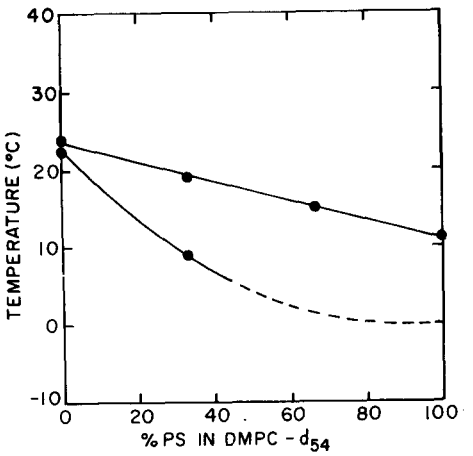


Fig. 6. Phase diagram of mixtures of phosphatidylserine (PS) and dimyristoyl phosphatidylcholine-d_{54} (DMPC-d_{54}). The dashed line represents an extrapolation.

containing phosphatidylserine and dipalmitoyl or distearoyl phosphatidylcholine [5]. In the presence of Ca^{2+}, the calorimetric data show evidence of both fusion and phase separation, with a new thermogram peak indicative of segregated dipalmitoyl or distearoyl phosphatidylcholine regions. The difference between mixtures containing dimyristoyl phosphatidylcholine and those containing dipalmitoyl or distearoyl phosphatidylcholine may be related to the closer proximity of the phase transition temperature region of dimyristoyl phosphatidylcholine to that of phosphatidylserine. It may also indicate a higher miscibility of the shorter chain phosphatidylcholine with phosphatidylserine, which has been suggested by a recent study of the extent of Ca^{2+}-induced fusion in mixed vesicles [11].

Finally, the melting transitions of the two mixtures of phosphatidylserine and dimyristoyl phosphatidylcholine-d_{54} in the absence of Ca^{2+} as well as the pure components are summarised in the phase diagram in Fig. 6. For the 1 : 2 mixture, the fluidus point of the dimyristoyl phosphatidylcholine-d_{54} Raman signal and the solidus point of the phosphatidylserine signal are used. The closeness of the fluidus points of the pure components results in an almost linear fluidus line for the mixtures. Otherwise, the phase diagram is qualitatively similar to that for mixtures containing phosphatidylserine and dipalmitoyl phosphatidylcholine [8]. It should be mentioned that we have not investigated the complicated phase changes that have been observed in association with the pretransition in synthetic phosphatidylcholines [3,8].

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