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USE OF PHOSPHOLIPID-CLAY COMPLEXES FOR DETERMINING VIBRATIONAL SPECTRA OF MEMBRANE RELATED SYSTEMS

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SUMMARY

For determining the infrared and Raman spectra of membrane related systems, a method is developed to incorporate phospholipid bilayer assemblies in a clay matrix to form ultra-thin, self-supporting films. These films, containing stabilized bilayers arranged between the silicate layers of hectorite, are in the shape of discs which measure about 2 cm in diameter and 25 microns thick and require approximately 2 mg of phospholipid for preparation. Although several spectral regions below 1100 cm^{-1} are masked by the host clay, both head group and acyl chain vibrations may be conveniently observed and monitored for phospholipid conformational changes.

INTRODUCTION

In preparing solid and semisolid samples for determining infrared vibrational spectra, the potassium bromide (KBr) pellet method is widely used as a complementary procedure to the oil dispersion (mull) technique [1, 2]. With suitable dies, presses and vacuum equipment, low light scattering, transparent discs are produced which yield high quality spectra that are free of transitions originating from the KBr matrix material. An advantage of the pellet method lies in the capability of storing the halide disc samples in a dry environment for indefinite periods of time [1, 2]. Difficulties arise, however, in applications of the technique in that small quantities of water are always present in the pellets despite careful drying procedures. Further, the grinding or pressing processes often tend to induce physical changes within the sample. At times, exchange of halide ions from the matrix with sample ions becomes a consideration when recording spectra of ionic materials [1, 2].

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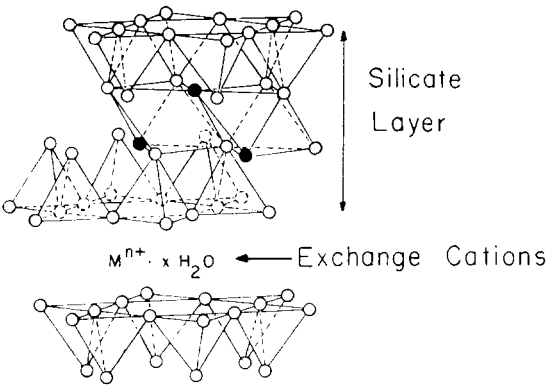

Fig. 1. Structure of a smectite mineral. Open circles are oxygen, closed circles are fluorine or OH. The idealized unit cell formula for hectorite is $Na_{0.66}[Mg_{5.34}Li_{0.66}](Si_8)(OH,F)_4$. Mg^{2+} and Li^{2+} are in octahedral positions, Si^{4+} is in tetrahedral positions.

A spectroscopic sampling technique which obviates some of the difficulties of the KBr pellet method, as well as having advantages of its own, involves the use of hectorite clay as the host, or matrix material. Hectorite is a member of the smectite clay mineral family. These compounds are microcrystalline silicates capable of being swelled dramatically along a single crystallographic dimension by adsorption of various substrates on their large internal surface area ($\approx$ 800 m^2/g) [3]. As a member of this class, hectorite possesses the requisite layer lattice structure in which the silicate sheets are defined by a two-dimensional network of oxygen, fluorine and hydroxyl groups, as shown in Fig. 1. The tetrahedral sites within this network are filled by silicon, while the octahedral sites are occupied primarily by magnesium. Partial substitution of magnesium by lithium in the octahedral positions results in a net negative charge on the sheets. This charge is balanced by hydrated exchange cations which reside in the interlayer regions.

With certain exchange cations, such as sodium or lithium, the smectite structure can be completely dispersed in water to form a colloidal suspension containing separated silicate platelets. Under these conditions much of the theoretical surface area is available for adsorption of a wide variety of organic species, including relatively large biological molecules [4]. Depending on the nature of the adsorbate and the smectite mineral, the silicate sheets may be used to sandwich organic species within the lattice organization.

We have recently been examining general structural characteristics of phospholipid molecules in model membrane systems using Raman spectroscopic techniques. The novel chemical and physical properties of hectorite suggest its use as a host lattice for spectroscopically probing conformational changes within the phospholipid bilayer, as well as for examining possible phospholipid-clay interactions. Several advantages emerge in using the hectorite matrix as an alternative to other sampling methods for membrane related systems. Specifically, the preparation of liposome-clay samples for recording either Raman or infrared vibrational spectra is straightforward and reproducible. The presence of a clay-water dispersion creates no special difficulties with respect to the liposome samples, particularly, as water is necessary to main-

tain the integrity of the phospholipid bilayer. Although water is often an inconvenience in recording infrared spectra, the ultra-thin clay samples preclude many of the spectral difficulties usually encountered with water systems. Further, the weak scattering properties of water pose no problems in applying the Raman technique. Although mild sonication is required to disperse the liposomes in the clay matrix, no extended sample grinding or pressing is necessary to prepare optically clear specimens. The self-supporting clay films produced by this method are durable, easily handled for sampling purposes and may be stored indefinitely undesiccated.

In the present study we assess the applicability of phospholipid-clay complexes toward determining vibrational spectra of membrane related systems. Specifically, dipalmitoyl phosphatidylcholine liposomes and erythrocyte ghosts were dispersed in hectorite and examined with both infrared and Raman spectroscopic techniques.

EXPERIMENTAL

High purity samples of 1,2-dipalmitoyl-DL-phosphatidylcholine and hectorite were obtained from Sigma Chemical Company and N. L. Industries (Baroid Division), respectively. Erythrocyte ghosts were prepared by the method of Dodge et al. [5], except that 8–10 washes of 20 mosM phosphate hypotonic buffer (pH 7.6) and 1–3 final washes with distilled water were used to remove all traces of hemoglobin and phosphate ion, respectively. The ghosts were spun at 15 000 rev./min for 15 min to pellet prior to concentrating to 5–7 % by a stream of dry nitrogen. Outdated whole human blood was generously donated by the National Institutes of Health Clinical Center Blood Bank.

Clay samples suitable for infrared and Raman spectroscopic examination were prepared using the following procedures.

(*a*) *Pure hectorite clay.* 400 μl of a 2 % clay slurry (wt./wt.) in distilled water were diluted to 1.0 ml to give a final slurry concentration of 0.8 %. The mixture was briefly sonicated ($\approx$ 5 W) for 2–3 minutes (Branson Model W-185 Sonifier, equipped with a micro tip) and allowed to evaporate on a polyethylene sheet overnight.

(*b*) *Clay plus erythrocyte ghosts.* 20 mg of hectorite were sonicated in 2 ml of water (1 % slurry). Approximately 0.25 ml of an approx. 7 % whole ghost water mixture and 1 ml water were added to this suspension. The sample was prepared by diluting 1 ml of the above stock slurry with 1 ml water and allowing it to dry on a polyethylene sheet. Fractured ghost samples were similarly prepared, except that the samples were less concentrated and were sonicated about 10 min prior to drying.

(*c*) *Clay plus 1,2-dipalmitoyl-DL-phosphatidylcholine.* A stock solution contaning 0.16 g dipalmitoyl phosphatidylcholine in 1 ml water was sonicated to near clarity, then centrifuged to remove undispersed material and titanium particles. 10 μl of this stock solution were added to 400 μl of a clay slurry prepared by sonicating 80 mg of hectorite in 4 ml water. This dispersion was then sonicated to obtain a thick gel, diluted to a total volume of 1 ml, resonicated and allowed to evaporate on a flat acetate sheet to obtain a self-supporting film. The nearly transparent, circular clay discs, produced from the above procedures ($\approx$ 2 cm in diameter and $\approx$ 25 μm thick), were peeled from their substrates; and although brittle, they were convenient to handle for sampling spectroscopically.

The infrared spectra were recorded from 4000–250 cm^{-1} with a Perkin-Elmer

Model 521 spectrophotometer which was purged of atmospheric water vapor by flushing with dry nitrogen. Infrared polarization measurements were performed on a Perkin-Elmer Model 621 spectrophotometer equipped with a common beam wire grid polarizer. Sample films of dipalmitoyl phosphatidylcholine were prepared by evaporating a solution of the phosphatidylcholine and chloroform onto KBr plates. Spectra resolution was of the order of 1 cm^{-1}. Polystyrene and water vapor were used to calibrate the spectra to ± 2 cm^{-1}.

Raman spectra were obtained with a Cary Model 81 spectrophotometer equipped with a Coherent Radiation Model 52 argon ion laser source and a modified external optical system. The laser was typically operated to give 300–600 mW of 514.5 nm or 488.0 nm radiation incident on the sample. Spectral resolution varied between 2–5 cm^{-1}. Spectra, calibrated with atomic argon lines, are reported to ± 2 cm^{-1}. Sampling of the clay discs for Raman spectra was accomplished by attaching a layer of 4 discs to a spinning circular plate to prevent the puncturing of the sample by the incident laser beam. The incident beam impinged upon the sample near the periphery of the rotating discs. Raman spectra were recorded at an angle of about 45° to the plane of the discs. Temperature variations in the spinning sample were achieved by directing toward the sample a stream of temperature regulated dry nitrogen.

RESULTS AND DISCUSSION

Dipalmitoyl phosphatidylcholine-water dispersions are first sonicated to produce liposomes of known size ($\approx$ 250–300 Å diameter) [6–8]. When the sonicates are mixed with hectorite and then dried into discs, a question arises as to whether sufficient water remains within the clay matrix to preserve the integrity of the bilayer

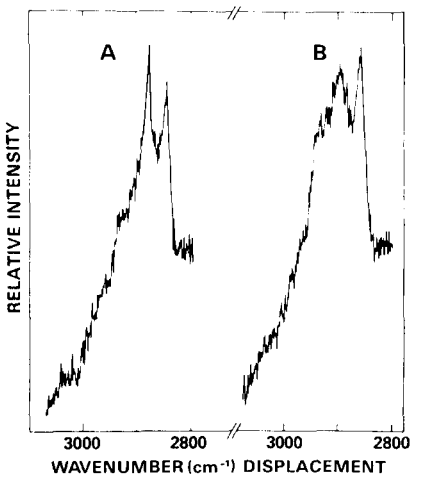

Fig. 2. Raman spectra in the 3100–2800 cm^{-1} region of 1,2-dipalmitoyl phosphatidylcholine liposomes incorporated into hectorite clay discs (4 discs, $\approx$ 100 μm thick), showing the effect of temperature on the carbon-hydrogen stretching vibrations. Excitation at 514.5 nm was used. A. Below gel-liquid crystalline phase transition temperature. B. Above phase transition temperature.

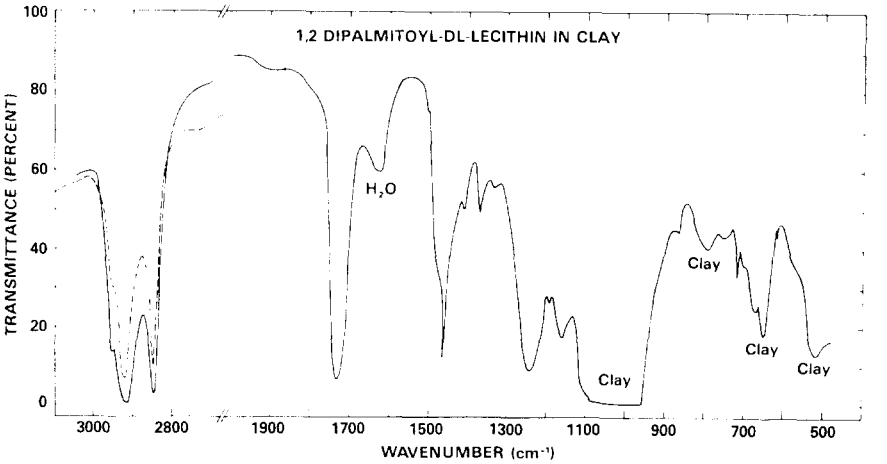

Fig. 3. Infrared spectrum in the 3100–2700 cm^{-1} and 2000–450 cm^{-1} regions of a 25 μn thick hectorite disc containing 1,2-dipalmitoyl phosphatidylcholine liposomes ($\approx$ 16 % phopholipid). The dotted line is a spectrum of a clay disc with a lower concentration of phosphatidylcholine.

state of the original phospholipid-water system. Attempts to determine the distribution of the dipalmitoyl phosphatidylcholine in the clay by examining fat stained portions (frozen section, transverse cut) with a light microscope were nonproductive. Since the properties of multilamellar lipid systems undergoing either environmental perturbations or phase transition effects are reflected by changes in intensity and frequency of various vibrational transitions [8–14], we examined the Raman spectrum of the phospholipid-clay complex in the 2800–3100 cm^{-1} carbon-hydrogen (C-H) stretching region, which is displayed in Fig. 2. The 2882 and 2847 cm^{-1} methylene asymmetric and symmetric stretching modes, respectively, and the 2930 cm^{-1} methyl symmetric stretching mode of diplamitoyl phosphatidylcholine bilayers are known to be temperature sensitive [10, 11, 13]. From previous work, therefore, we recognize spectrum B as characteristic of the lipid bilayer system in the liquid crystalline state ($>$ 40 °C), while spectrum A, produced by cooling the clay discs, is representative of the system in the gel phase ($<$ 40 °C). Since the phospholipid assembly undergoes a phase transition around 40 °C, we are confident that the bilayer organization is preserved within the clay disc.

Fig. 3 displays an infrared spectrum of 1,2-dipalmitoyl phosphatidylcholine liposomes embedded in hectorite clay. The weight of the phosphatidylcholine in the clay disc was less than 2 mg, which emphasizes that only small quantities of sample need be used in this technique. The infrared spectrum clearly shows the methylene symetric and asymmetric stretches and the methyl asymmetric stretch at 2853, 2926 and 2956 cm^{-1}, respectively. Also, the carbonyl stretching mode and methylene deformation mode at 1728 and 1469 cm^{-1}, respectively, are apparent. Underlying the 1470 cm^{-1} region is some contribution due to carbonate impurity in the clay. No attempt was made to eliminate this interfering ion, although it may be removed through purification with sodium bisulfate. A small amount of water is present in the clay disc as seen by its absorption near 1630 cm^{-1}. Other readily assigned phosphatidylcholine vibrations are evident: the methyl symmetric deformation at 1374 cm^{-1},

the PO_2^- asymmetric stretch at 1246 cm^{-1}, a combination of the CH_2 wag and C-O-C antisymmetric stretch at 1168 cm^{-1} and the CH_2 rocking mode near 720 cm^{-1} [11–15]. This last vibrational mode in the infrared is particularly useful in monitoring the development of gauche structures within the acyl chains [16]. Table I summarizes these observations and assignments. Much of the spectral region below 1100 cm^{-1} is masked by several intense silicate bands. The relative opacity in this range is the most serious disadvantage of using hectorite clay as a host lattice.

TABLE I

COMPARISON OF INFRARED FREQUENCIES AND ASSIGNMENTS FOR 1,2-DIPALMITOYL PHOSPHATIDYLCHOLINE EMBEDDED IN HECTORITE CLAY AND DEPOSITED AS A FILM ON A POTASSIUM BROMIDE PLATE

Frequencies calibrated from polystyrene. Abbreviations: Sh, shoulder; S, strong; m, medium; w, weak.

Dipalmitoyl phosphatidylcholine frequencies (cm^{-1})		Assignments
Clay	Film	
≈ 2956 (Sh)	2947 (Sh)	CH_3 asymmetric stretch
2926 (S)	2919 (S)	CH_2 asymmetric stretch
2853 (S)	2851 (S)	CH_2 symmetric stretch
1728 (S)	1740 (S)	C = O stretch
≈ 1488 (Sh)	≈ 1490 (Sh)	CH_2 deformation
1469 (m)	1470 (m)	
≈ 1420 (w)	1419 (w)	CH_2 def. adjacent to C = O*
1374 (w)	1379 (w)	CH_3 symmetric deformation*
1246 (m)	1254 (m)	PO_2^- asymmetric stretch
≈ 1168 (m)	≈ 1168 (m)	CH_2 wag, C-O-C asymmetric stretch
720 (w)	721 (w)	CH_2 rock

* Ref. 15.

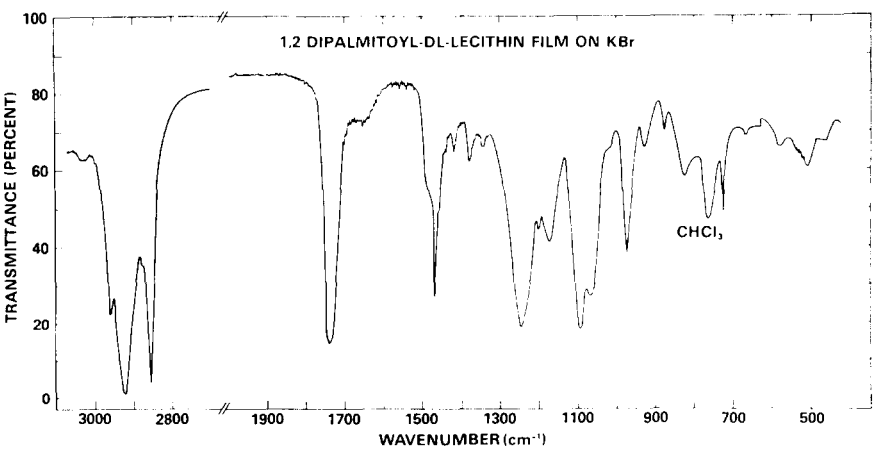

Fig. 4. Infrared spectrum in the 3100-2700 cm^{-1} and 2000–450 cm^{-1} regions of a film of 1,2-dipalmitoyl phosphatidylcholine deposited on a KBr plate.

For comparison, Fig. 4 displays an infrared spectrum of a film of dipalmitoyl phosphatidylcholine on a KBr plate. Above 1100 cm^{-1} both spectra are quite similar. Close examination, however, reveals some differences in the spectral frequencies (see Table I). The symmetric and asymmetric methylene modes are observed at 2851 and 2919 cm^{-1}, respectively, in the film as compared to 2853 and 2926 cm^{-1} in the clay spectrum. The carbonyl stretching mode is shifted to a greater extent to 1740 cm^{-1} and the PO_2^- asymmetric stretch is seen at 1254 cm^{-1}. Interactions between the charged silicate layers and the phospholipid head group, as well as the polar carbonyl group, are quite probable and thus the frequency differences observed between spectra of the film of the phospholipid dispersion and spectra of the phospholipid-clay complexes are not surprising.

With specific reference to the Raman spectra of membrane related systems, Mendelsohn, et al. [17, 18] suggested that the methylene stretching modes reflect intrachain disorder and interchain interactions and are therefore sensitive to the packing characteristics of bilayers. For the infrared spectra we compare the frequency differences Δ between the CH_2 symmetric and asymmetric stretching modes for the dipalmitoyl phosphatidylcholine film, sonicated liposomes and clay systems. Δ values for the three phospholipid assemblies are 67.9 ± 0.5, 67.5 ± 0.5 and 72.6 ± 0.5 cm^{-1}, respectively. In addition, absolute frequency values for the samples in the clay discs are higher, with about a 7 cm^{-1} blue shift for the asymmetric C-H stretching mode. Both of these observations suggest an increase in interchain interaction as they reflect the same trends as the calculation of Tasumi and Shimanouchi [19] in which the frequencies for a single polyethylene chain are compared to those for a crystal. The Raman spectroscopic frequency differences between the C-H stretching modes appear less sensitive to packing arrangements than the infrared Δ values in a comparison between the anhydrous, sonicated liposomes and clay systems. These frequency separations are 34.0 ± 0.5, 34.0 ± 0.5 and 34.7 ± 0.5 cm^{-1}, respectively. The increase in hydrocarbon interchain interaction in the clay complex may arise from the constraint placed upon the charged head group by the silicate sheets. An effect arising from the reduction in mobility of the head group would probably be transmitted to the general packing characteristics of the acyl chains.

Attempts were made to determine whether the phospholipid molecules were oriented between the silicate layers of the hectorite. Recent work by Lagaly and coworkers showed that *n*-alkanols and *n*-tetradecylammonium ions on beidellite, a clay related to hectorite, were oriented with respect to the silicate layers [20]. Infrared dichroic spectra of partially oriented polypeptides have been determined by Goodman and coworkers using another substrate, polyoxyethylene [21]. The ability to determine orientation in the clay samples was limited by the thinness of the discs; however, the discs were placed in two orientations to the incident infrared radiation; namely, normal and tilted at 45° to the beam. A common beam wire grid polarizer was used to select the same component of the electric vector for the two orientations. The only distinct change in the vibrational intensities for the two sample positions occurred in the 1469 cm^{-1} CH_2 deformation mode in which a 6% increase in intensity was recorded for the tilted sample. Although the evidence is limited, a possibility for a preferred orientation does exist for the hydrocarbon chains. It is interesting to note that the 1470 cm^{-1} infrared band has been used as a monitor for chain fluidity as a function of temperature [10, 22]; thus, it is not unreasonable to expect this mode to

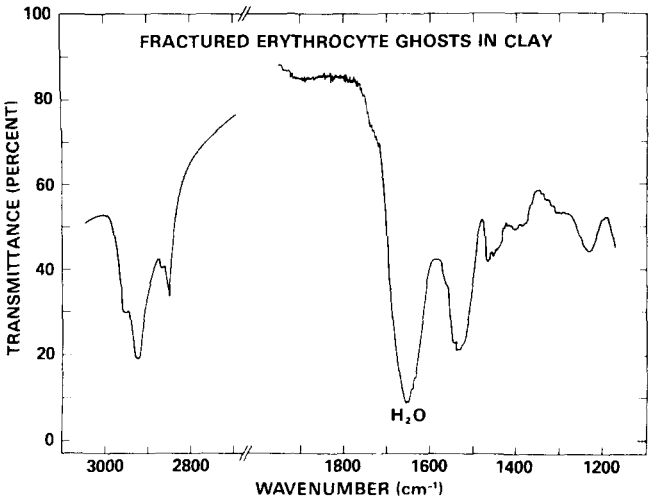

Fig. 5. Infrared spectrum in the 3100–2700 cm^{-1} anc 2000–1200 cm^{-1} regions of an approximate 25 μm thick hectorite disc containing fractured (by sonication) erythrocyte ghosts.

reflect at a given temperature the alignment characteristics of the acyl chains.

The further utility of the hectorite sampling technique is seen in Fig. 5 which displays an infrared spectrum of fractured (by sonication) erythrocytes ghosts embedded in hectorite discs. The 3000–2800 cm^{-1} C-H stretching region reveals differences from the dipalmitoyl phosphatidylcholine spectra in both relative intensities and frequencies of the bands. These changes can be attributed mainly to the addition of protein absorption [23]. The 2000–1200 cm^{-1} region shows that more water was held by the erythrocyte sample than by those containing pure dipalmitoyl phosphatidylcholine. The main feature near 1540 cm^{-1} is the amide II band, which is indicative of protein in the α-helical or random coil conformation [23, 24]. The other major bands in this region near 1460 and 1235 cm^{-1} are attributed to C-H deformation modes and the PO_2^- asymmetric stretching vibrations, respectively. No singificant differences in spectra were observed between sonicated and whole erythrocyte ghosts embedded in the clay discs.

In summary, we have presented an alternative approach to the preparation of membrane related systems for the determination of their vibrational infrared and Raman spectra. Using hectorite clay as a matrix material, stabilized bilayer assemblies are arranged between the silicate layers to form sturdy, easily manipulated discs of about 2 cm in diameter and 25 microns thick. The technique involves mg samples of the phospholipid. Although several regions below 1100 cm^{-1} are masked by the host clay, both head group and acyl chain vibrations may be conveniently monitored.

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