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Infrared and Fluorescence Spectroscopic Studies of a Phospholipid Bilayer Supported by a Soft Cationic Hydrogel Scaffold

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Abstract

Polarized attenuated total reflection (ATR-IR) spectroscopy and fluorescence microscopy techniques were used to characterize a 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) membrane supported on porous, cationic hydrogel beads. Fluorescence microscopy images showed that the DPhPC coated the external surface of the hydrogel scaffold. In addition, a fluorescence assay of the emission intensity of the Tb³⁺/dipicolinic acid complex demonstrated that the DPhPC coating acted as a barrier to Tb³⁺ efflux from the scaffolded vesicle and successfully sealed the porous hydrogel bead. Fluorescence quenching and ATR-IR spectroscopic measurements revealed that the lipid coating has a bilayer structure. The phytanoyl chains were found to exhibit significant trans-gauche isomerization, characteristic of the fluid liquid phase. However, no lipid lateral mobility was observed by fluorescence recovery after photobleaching (FRAP) measurements. The phosphocholine headgroup was found to be well hydrated and oriented such that the cationic choline group tucked in behind the anionic phosphate group, consistent with an electrostatic attraction between the cationic scaffold and zwitterionic lipid. The absence of lipid lateral mobility may be due to the strength of this attraction.

Introduction

Supported vesicle systems have generated much interest because of their desirable properties including structural stability, biocompatibility, modifiable surface properties, and an enclosed internal volume (1-5). These properties make supported vesicles attractive candidates for use in biotechnological applications such as therapeutic drug delivery and as biomimetic membrane platforms (1). Supported vesicles exhibiting good structural stability have been developed on a wide range of materials, including silica, polymer gels, polyelectrolytes, and numerous nanoparticles; and have ranged between tens of nanometers to tens of micrometers in diameter (1, 6-25). The size of the support dictates vesicle size, and significantly impacts their potential applications. Large architectures ($\sim 1\ \mu\text{m}$) are primarily used for studying cell membrane properties, while smaller architectures ($\sim 100\ \text{nm}$) are targeted towards biomedical applications (1). Developing supported vesicles suitable for characterization by surface analytical and electrochemical techniques can provide valuable information about these systems. This work describes the development of a giant supported vesicle accessible to both surface analytical and electrochemical methods, for use as a model membrane. Specifically, the model is designed to be studied simultaneously by attenuated total reflection infrared (ATR-IR) spectroscopy and electrophysiological patch-clamping, which necessitates a scaffold diameter $\geq 100\ \mu\text{m}$ (26). We have investigated similar systems for this purpose as detailed in reference 26 and 27.

In addition to serving as a model membrane, the spherical geometry, and porosity of the hydrogel scaffold in conjunction with the barrier properties of a lipid bilayer can allow for the encapsulation of appropriate molecules. Ref. 26 detailed the permeability characteristics of scaffolded vesicles with a 70:30 DMPC:cholesterol coating. These studies revealed a maximum permeability at the main phase transition temperature of DMPC (26). In that study, a large ($\sim 100\ \mu\text{m}$ diameter) hydrophilic hydrogel beads was used as a structural scaffold for a lipid bilayer

(26). The large scaffold allows for the measurement of lipid orientation by ATR-IR spectroscopy. To improve membrane durability and its barrier properties a subsequent study examined the properties of the lipid membrane supported on a hydrophobically modified hydrogel scaffold. It was found that the durability and barrier properties of these lipid coatings were greatly improved (27).

In references 26 and 27 the Sephadex[®] hydrogel particles were coated by fusion and rupture of unilamellar lipid vesicles producing scaffolded vesicles. The hydrogel particles were coated with lipids, however we did not know if the coating consisted of a single bilayer or a multibilayer system. An objective of the present work is to demonstrate that the vesicle fusion procedure provides a single bilayer coating to the hydrogel particle. To achieve this goal, design changes to both the hydrogel scaffold and lipid membrane were made. The spherical scaffolds used in this work are Sephadex[®] A50 hydrogel beads. Sephadex[®] A50 is an anion exchanger consisting of cross-linked dextran functionalized with positively charged diethyl(2-hydroxypropyl) quaternary aminoethyl groups. The functional groups are attached to the glucose units of the dextran (28). These cationic functional groups provide the hydrogel with a positive charge, which has been shown to promote adhesion and formation of supported lipid bilayers, providing an attractive electrostatic interaction between the support and bilayer (29-33). Electrostatic attractive interactions have been used in various charged polyelectrolyte and microgel systems to promote stable lipid bilayer coating on spherical particles (22-25, 34-38). However, these systems do not exceed ~10 μm in diameter, making them unsuitable for investigation by ATR-IR spectroscopic and patch-clamp techniques.

The average diameter of the hydrated Sephadex[®] A50 beads is ~225 μm , which is about twice that of the scaffolds used in our previous studies. Sephadex[®] A50 is less cross-linked than

Sephadex[®] G10 and thus it is a more flexible, porous hydrogel. Using a porous hydrogel as the scaffold is intended to provide the inner leaflet of the bilayer with access to an aqueous-like environment. An internal aqueous environment is important for promoting the insertion of membrane proteins into the host lipid matrix, and in investigations of membrane transport processes. The synthetic phospholipid 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) was used for the construction of the biomimetic membrane. The phytanoyl chains of DPhPC are 16 carbons long with branched methyl groups. DPhPC has been shown to form high quality, stable model membranes and has been used as a matrix for the reconstitution of membrane proteins (39-46). This stability has been attributed to the interdigitation of the branched methyl groups of the hydrophobic tails (47). The phosphocholine headgroup has been shown to have an affinity to positively charged supports (14,31). DPhPC does not exhibit a phase transition between -120 and +120 °C, and therefore avoids the permeability spike associated with phospholipid main phase transitions (48). This improvement to the system design will aid further controlled-release studies on scaffolded vesicles.

The scaffolded vesicles have been characterized using fluorescence microscopy and spectroscopy, fluorescence quenching, fluorescence recovery after photobleaching (FRAP) and ATR-IR spectroscopy. Fluorescence microscopy was used to evaluate the coating of the hydrogel scaffold and stability of the lipid membrane. A phytanoyl lipid labeled with a fluorescent tag was used as the fluorescent probe. A fluorescence spectroscopy Tb³⁺/dipicolinic acid assay was implemented to monitor the temporal efflux of Tb³⁺ from the lipid coated hydrogel in order to further corroborate the presence of a complete coating. Fluorescence quenching experiments were performed in order to obtain structural information for the phospholipid coating. By using a positively charged hydrogel and positively charged

fluorescence quencher we would be able to demonstrate that the hydrogel scaffolds can be coated by a single lipid bilayer. The mobility of fluorescently tagged phytanoyl phospholipid within the membrane was probed by FRAP experiments. Lipid mobility is a measure of membrane fluidity, an important feature of biological membranes. ATR-IR spectroscopy, a surface sensitive infrared spectroscopic technique, was used to examine the order and orientation of the hydrophobic phytanoyl chains and polar headgroup. The accessibility of the scaffolded vesicles to ATR-IR spectroscopy is important because these measurements can provide valuable insight into the membrane structure. Furthermore, when studying controlled-release from scaffolded vesicles, it may be possible to correlate the controlled-release properties of the system with the membrane structure. Using this strategy we will provide a complete description of the structure and properties of the lipid coating on a hydrogel particle. These results are significant because they could be used to develop strategies for the controlled-release of the internal contents of scaffolded vesicles, with potential applications in fields such as drug and nutraceutical delivery

Experimental Section

Sample Preparation

Phospholipid Vesicle Preparation

Dry lipid films were prepared by combining DPhPC and 1,2-diphytanoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DPhPE) chloroform solutions in a test tube, at a 99:1 molar ratio. The chloroform solvent was slowly evaporated under a stream of argon while vortexing. After evaporation, the test tubes were stored in a desiccator under vacuum for at least 24 h prior to use. To make vesicles, a Milli-Q water was added to the dry lipid film such that a 5 mg/mL solution was obtained. The mixture was sonicated for 5 min at 45 °C.

Scaffolded Vesicle Preparation

To prepare scaffolded vesicles, phospholipid vesicles and hydrated Sephadex[®] A50 were incubated at 45 °C for 1 h with constant vortexing. The system was then allowed to incubate further at room temperature for 24 h. The scaffolded vesicles were then rinsed thoroughly prior to use.

Fluorescence Microscopy

Fluorescence microscopy was performed on an Olympus BX51 microscope equipped with a FITC filter and a QImaging Retiga 2000R camera. An Olympus U-RFL-T mercury burner served as the light source. Fluorescent scaffolded vesicles were dropped onto a microscope slide and allowed to settle. Fluorescence images were then obtained.

Fluorescence Quenching

The fluorescent scaffolded vesicles used for fluorescence quenching experiments were prepared as described above. Fluorescent scaffolded vesicles were dropped onto a microscope slide and initial fluorescence images were obtained. The CoCl₂ quenching solution was then added to obtain the desired concentration of Co²⁺. After waiting for ~500 s, fluorescence images were collected. Generally, 10-20 scaffolded vesicles were deposited onto the microscope slide. Addition of the quenching solution changed the position of the scaffolded vesicles. However, the pre- and post-quenching images were taken such that all of the scaffolded vesicles were present in the field of view. Therefore, analysis of all of the scaffolded vesicles in an image reflected the average fluorescence intensity, before and after quenching. The fluorescence intensity was quantified using ImageJ software. The average pixel intensity of each scaffolded vesicle was measured before and after quenching. The percent quenching was calculated as follows,

$$\% \text{ quenching} = \left[1 - \frac{F}{F_0} \right] \times 100 \quad \text{Eq. 1}$$

where F_0 and F are the fluorescence intensities measured before and after the addition of Co^{2+} , respectively (15). For analysis of a single scaffolded vesicle, a suction micropipette was used to hold the scaffolded vesicle in place. This prevented any change in position upon the addition of the Co^{2+} quenching solution.

Fluorescence Recovery After Photobleaching (FRAP)

The scaffolded vesicles used for FRAP experiments were prepared as described above. The fluorescent scaffolded vesicles were deposited onto a microscope slide and allowed to settle. A single scaffolded vesicle was selected for study and an initial fluorescence image was obtained using the 5x objective, low source intensity, maximum aperture setting. The photobleaching was carried out using a 50x distance objective, maximum source intensity, and minimum aperture setting. The scaffolded vesicle was typically exposed to these conditions for ~10 s. Photobleaching resulted in a ~60 μm diameter bleached spot. Subsequent images were acquired using the 5x objective, at low source intensity, with a maximum aperture setting.

The average pixel values of the bleached, background, and total scaffolded vesicle regions of interest are obtained using the software program ImageJ. Equation 2 shows the mathematical treatment of this data for the construction of each point (at time, t) on the fluorescence recovery curve ($R(t)$). Normalization is carried out by using the fluorescence intensity values of the initial image (t_i).

$$R(t) = \frac{\text{bleach}(t) - BG(t)}{Tot(t) - BG(t)} \times \frac{Tot(t_i) - BG(t_i)}{\text{bleach}(t_i) - BG(t_i)} \quad \text{Eq. 2}$$

Permeability Studies

Terbium (III) chloride (TbCl_3) and 2,6-pyridinedicarboxylic acid (DPA) were purchased at 99.99 % and 99% purities, respectively, and used without further purification. A solution of 10 mg/mL of Sephadex[®] A-50 beads were incubated in 1.0 mM $\text{TbCl}_{3(\text{aq})}$ for 7 days in order to allow passive loading of the beads with the lanthanide. A 50 μL aliquot of $\text{TbCl}_{3(\text{aq})}$ loaded Sephadex[®] A-50 beads was then incubated with a 2 mg/mL DPhPC vesicle solution in 1.0 mM $\text{TbCl}_{3(\text{aq})}$. The DPhPC vesicle solution was prepared as described above. The mixture of beads and DPhPC vesicles was vortexed for 1 h and allowed to incubate without further agitation at room temperature for 24 h.

The fluorescence emission assays were performed as outlined previously (26). The fluorescence emission of the Tb^{3+} /DPA complex was monitored over time with a Varian Carey Eclipse Spectrophotometer with a temperature control unit (Varian Inc., Mississauga, ON). The first order kinetics of terbium permeability from the DPhPC coated Sephadex[®] A-50 scaffolded vesicle allowed for the calculation of the half-life of efflux.

ATR-IR Spectroscopy

ATR-IR spectra were collected on a Varian 660-IR spectrometer equipped with an MCT-A detector, a Pike MIRacle ATR accessory (Pike Technologies, Madison, WI) and a ZnSe wire-grid polarizer (Pike Technologies, Madison, WI). Scaffolded vesicles were prepared as previously described. A layer of scaffolded vesicles was placed on either a zinc selenide-diamond ATR crystal for collection of the sample spectra. Sephadex[®] A50 was used for collection of the background spectra. A pressure clamp equipped with a concave tip was used to achieve good contact between the sample and ATR crystal. Typically, 5000 scans were collected and averaged using an instrumental resolution of 4 cm^{-1} . Since the bands are plotted by interpolation between these points, the band center position can be determined with uncertainty

0.5 cm⁻¹ (49). The reported spectra are averages of at least four independent experiments, and the standard deviation of the reported orientation angles do not exceed $\pm 3^\circ$.

The spectra are presented in absorbance units defined as $A = -\log(I/I_0)$, where I and I_0 are the single beam reflected intensities for the sample and background, respectively. Absorbance spectra for both p- and s-polarizations (A_p and A_s) were obtained. The absorbance spectra were baseline-corrected, typically with a quadratic function, before further analysis. For quantitative analysis, the dichroic ratio of an absorption band was found by taking the ratio of the p- and s-polarized absorbance spectra ($R=A_p/A_s$) at the band maximum. Table 1 lists the refractive index values used for the quantitative molecular orientation calculations.

Table 1- Refractive index values used for quantitative molecular orientation calculations. The refractive index of the second medium, n_2 , is that of DPhPC at the corresponding wavenumber. The refractive index of the overlying third medium, n_3 , is taken to be that of water at the corresponding wavenumber (50). The refractive index of the IRE (n_1) is 2.4 for the zinc selenide-diamond ATR crystal (51).

Absorption Band	Wavenumber (cm⁻¹)	n_2	n_3
$\nu_{as}(\text{CH}_2)$	~2926	1.42	1.42
$\nu_s(\text{CH}_3\text{-m})$	~2870	1.45	1.42
$\nu_s(\text{CH}_2)$	~2856	1.47	1.42
$\nu_s(\text{CH}_3\text{-t})$	~2845	1.50	1.42
$\nu_{as}(\text{PO}_2^-)$	~1230	1.40	1.30
$\nu_s(\text{PO}_2^-)$	~1086	1.42	1.30
$\nu_{as}(\text{CN}^+(\text{CH}_3)_3)$	~970	1.50	1.30

†n₃ values from Ref. 23

Results and Discussion

Fluorescence Microscopy

The lipid coating of the scaffolded vesicles was initially evaluated using fluorescence microscopy. The fluorescently tagged phospholipid, 1,2-diphytanoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DPhPE) was used at a concentration of 1 mol%. NBD-DPhPE has phytanoyl chains and a fluorescent tag located on the headgroup. Figure 1 shows a representative fluorescence image of the scaffolded vesicle. There is clearly an association between the phospholipids and hydrogel scaffold. The coronal appearance of the fluorescence suggests a vesicle-like structure, with the phospholipids confined to the outer surface of the hydrogel support. The scaffolded vesicles were able to withstand thorough rinsing and agitation without loss of fluorescence. In Figure 1, the fluorescence "inside the circle" is weak with respect to the fluorescence at the edge of the scaffolded vesicle. The vesicle is a 3D ball. When viewing the 3D ball from above one sees a 2D fluorescence picture. The edges are brighter than the center as a result of the three dimensionality of the scaffolded vesicle (ie, the "sides" of the sphere contribute to the fluorescence intensity at the edge of the fluorescence image).

Figure 1 – Fluorescence image of a scaffolded vesicle containing 1 mol% of the fluorescent probe NBD-DPhPE.

Fluorescence Quenching

Fluorescence quenching studies were performed on the scaffolded vesicles in order to further examine the structure of the phospholipid coating. Again, NBD-DPhPE was used as the fluorescent probe. The fluorescence of the NBD fluorophore is known to be quenched by Co^{2+} (52-54). The NBD fluorophore is located on the headgroup of the phospholipid. Assuming a bilayer structure, the NBD tag of phospholipids located in the outer leaflet will be exposed to the external solution. The NBD tags of phospholipids located in the inner leaflet will be hidden from the external solution. The presence of Co^{2+} ions in the external solution should readily quench the fluorescence of NBD-DPhPE located in the outer leaflet of a phospholipid bilayer.

The fluorescence behaviour of the scaffolded vesicles was observed following the addition of Co^{2+} solution, such that the scaffolded vesicles were exposed to $\sim 30 \text{ mM } \text{Co}^{2+}$. The fluorescence behavior with respect to time for a single scaffolded vesicle is shown in Figure 2. As is evident, there is a rapid decrease in the fluorescence intensity, which then levels off with time. For many scaffolded vesicles, an average fluorescence quenching of $53 \pm 3\%$ was measured. The significance of this value will now be discussed.

Figure 2 – Representative fluorescence quenching profile for a single scaffolded vesicle, with respect to time.

For lipid vesicles of small diameters (on the order of nm's), more lipid molecules will be located in the outer leaflet of a bilayer than in the inner leaflet, due to bilayer curvature considerations. However, for large diameter vesicles, such as scaffolded vesicles, the curvature is less pronounced and the number of lipid molecules in the inner and outer leaflets will be roughly equal. Therefore, the number of fluorophores present in the inner and outer leaflets should also be roughly equal. For a single lipid bilayer with an equal distribution of fluorophores between the inner and outer leaflets, one would expect to observe a 50% quenching of the fluorescence intensity. The observed $53 \pm 3\%$ fluorescence quenching suggests that the phospholipid coating is a single bilayer.

It should be noted that Co^{2+} is slightly membrane permeant with time (15). Therefore, even if a perfect phospholipid bilayer coated the cationic hydrogel, one would expect to eventually see complete fluorescence quenching. However, this was not observed for the scaffolded vesicles. This behaviour may be attributed to the positive charge of the scaffold. Since

both the hydrogel support and the quencher (Co^{2+}) are both positively charged, one may expect an electrostatic repulsion between the two. Therefore, the NBD tags of the inner leaflet phospholipids may be sheltered from the Co^{2+} quencher by the cationic scaffold. This would explain why complete quenching is never observed, even at higher Co^{2+} concentrations.

Fluorescence Recovery After Photobleaching (FRAP)

The mobility of the NBD-DPhPE fluorescent probe was studied using FRAP. FRAP is used to measure the mobility of fluorescent probes in biological membranes. The fluorescence recovery of a photobleached region is monitored with respect to time, and quantitative information can be extracted from the characteristics of the recovery curve. No fluorescence recovery was observed for the scaffolded vesicles. Figure 3 shows a fluorescent scaffolded vesicle with a 60 μm diameter photobleached spot. Figure 4 shows the fluorescence recovery curve. There is no evidence of fluorescence recovery with time. This suggests that the fluorescent probes are not free to diffuse within the bilayer. One possibility is that only NBD-DPhPE is immobile, perhaps due to the fluorescent tag, and the DPhPC may still be free to diffuse. However, lack of lipid mobility in model membranes that utilize electrostatic attraction has been observed in the literature (55). It has been suggested that the electrostatic attraction between the support and lipid can result in the pinning of the membrane to the support (55-58). This in turn prevents the lateral diffusion of the membrane constituents.

Figure 3 – Representative FRAP experiment images a) photobleached, b) after 30 minutes, c) after 60 minutes. No fluorescence recovery is observed. To aid the eye, the Thal colour map from ImageJ software is used. The bleached spot is indicated by the white arrow in each image.

Figure 4 – Representative fluorescence recovery curve. No recovery is observed, which suggests that the phospholipids are not free to diffuse within the plane of the bilayer.

Membrane Permeation Study

The permeability of the Sephadex[®] A50 hydrogel coated with DPhPC demonstrated a half life ($t_{1/2}$) of (40 ± 2) min, which was approximately three times slower than the uncoated hydrogel (Figure 5). The efflux of Tb^{3+} from the Sephadex[®] A50 ($t_{1/2} = (11 \pm 3)$ min) would have included an electrostatic repulsion of cations. However, even with the possibility of electrostatic repulsion between the terbium and Sephadex[®] A50, it was evident from the fluorescence permeability data that the DPhPC was the rate limiting barrier to Tb^{3+} efflux due to the similar half life ($t_{1/2} = (40 \pm 2)$ min) to previous studies (26, 27). This fact was further evidence that the DPhPC coating on the Sephadex[®] A50 hydrogel was complete and akin to coatings on neutral hydrogels, such as Sephadex[®] G10 (26).

The half life of the DPhPC coated Sephadex[®] G10 scaffolded vesicle ($t_{1/2} = (42 \pm 1)$ min) was similar to that of its Sepadex[®] A50 counterpart. The pore sizes of the Sephadex[®] A50 are approximately one order of magnitude larger in diameter than those of Sephadex[®] G10. The similar half lives of Tb^{3+} efflux indicated that the DPhPC membrane was capable of sealing the larger pores of the cationic hydrogel just as effectively as the smaller pores of the neutral hydrogel. This result is in accordance with the stationary nature of the lipids as observed in the FRAP measurements and further demonstrates the affinity of the DPhPC coating for the cationic hydrogel. This affinity could be assessed more completely through an investigation of the exact orientation of the DPhPC on the surface of the cationic hydrogel. Therefore, ATR-IR spectroscopy was implemented in order to gain additional insight into this interaction.

Figure 5 – Normalized fluorescence intensity as a function of time for the DPhPC-Sephadex[®] A50(●), and DPhPC-Sephadex[®] G10 (□) scaffolded vesicles and the uncoated Sephadex[®] A50 hydrogel (○). The half lives of Tb³⁺ efflux were (40 ± 2) min, (42 ± 1) min and (11 ± 3) min for the DPhPC-Sephadex[®] A50, DPhPC-Sephadex[®] G10 and uncoated Sephadex[®] A50, respectively.

Quantitative ATR-IR Spectroscopy

The determination of quantitative molecular orientation data for scaffolded vesicles using polarized ATR-IR spectroscopy has been done previously (26,27). These studies established suitable experimental parameters for quantitative orientation studies. The present work was performed on both Ge and ZnSe-diamond ATR crystals in order to confirm the validity of these previous studies for the present system. These experiments yielded identical results (within experimental error), and therefore only the spectra obtained using the ZnSe-diamond ATR crystal will be shown. The theory used to determine quantitative molecular orientation

information from polarized ATR-IR spectroscopy is described in detail in ref 26 and other sources (51,59,60).

Phytanoyl Chains

The C-H stretching region contains information regarding the order and orientation of the lipid phytanoyl chains. In Ref. 26 and 27, where Sephadex[®] G10 was used as the hydrogel scaffold, it was necessary to use lipids with deuterated hydrocarbon chains in order to avoid spectral interference from the scaffold. However, this is not necessary for the present work because the C-H stretching bands arising from hydrated Sephadex[®] A50 are sufficiently weak and effectively removed by the background spectrum (Figure 7a).

The C-H stretching region of DPhPC is a complex spectral region that contains many absorption bands. The Fourier self-deconvolution and band fitting of the C-H stretching region of DPhPC is shown in Figure 6. The band assignments are made in accordance with Ref. 61. The bands at $\sim 2960\text{ cm}^{-1}$ and $\sim 2954\text{ cm}^{-1}$ correspond to the terminal and middle chain asymmetric methyl vibrations ($\nu_{\text{as}}(\text{CH}_3\text{-t})$ and $\nu_{\text{as}}(\text{CH}_3\text{-m})$). The $\nu_{\text{as}}(\text{CH}_2)$ band is well-defined and located at $\sim 2926\text{ cm}^{-1}$. The C-H stretching vibrations adjacent to the middle chain methyl groups exhibit a band at $\sim 2915\text{ cm}^{-1}$. The bands at 2870 cm^{-1} and 2845 cm^{-1} correspond to the middle chain and terminal symmetric methyl vibrations ($\nu_{\text{s}}(\text{CH}_3\text{-m})$ and $\nu_{\text{s}}(\text{CH}_3\text{-t})$), respectively. The $\nu_{\text{s}}(\text{CH}_2)$ band is located at $\sim 2856\text{ cm}^{-1}$. Two Fermi resonance bands are located at $\sim 2940\text{ cm}^{-1}$ and 2900 cm^{-1} .

Figure 6 – Fourier self-deconvolution and band fitting for the C-H stretching region of DPhPC. The $\nu_s(\text{CH}_2)$ and $\nu_{as}(\text{CH}_2)$ bands are found in this region. They contain molecular orientation information for the phytanoyl chains. The middle chain $\nu_s(\text{CH}_3\text{-m})$ band is also present, and contains orientation information. The terminal symmetric methyl stretch is indicated by $\nu_s(\text{CH}_3\text{-t})$. Asymmetric methyl stretching vibrations, $\nu_{as}(\text{CH}_3)$, and bending Fermi resonance bands, $\delta_s(\text{CH}_2)$ and $\delta_s(\text{CH}_3)$ are present in this region.

Figure 7 shows the C-H stretching region of the polarized ATR-IR spectra collected for scaffolded vesicles. The $\nu_{as}(\text{CH}_2)$, middle chain $\nu_s(\text{CH}_3\text{-m})$, and the $\nu_s(\text{CH}_2)$ bands are observed at ~ 2926 , ~ 2870 , and $\sim 2856 \text{ cm}^{-1}$, respectively (61). These bands contain information regarding the order and orientation of the DPhPC phytanoyl chains. The maximum of the $\nu_{as}(\text{CH}_2)$ band is very well defined in the C-H stretching region, and the position of this band may be used to

estimate the conformational order of the phytanoyl chains. The very high wavenumber position (2926 cm^{-1}) for the $\nu_{\text{as}}(\text{CH}_2)$ band suggests that the DPhPC phytanoyl chains are highly disordered with significant trans-gauche isomerization, which is consistent with previous studies of phytanoyl phospholipids (62).

Figure 7 – a) – Raw ATR-IR p-polarized spectra of the C-H stretching region of Sephadex G10, A25, and A50. The reduced C-H stretching band intensity of A50 compared to G10 makes the use of lipids with deuterated hydrocarbon chains unnecessary. b) Polarized ATR-IR spectra of the C-H stretching region for scaffolded vesicles. The p- and s-polarized traces are indicated.

All molecular orientation data (ie. dichroic ratios and orientation angles) were calculated as described in detail in Ref. 26. The standard deviation of the reported orientation angles do not exceed $\pm 3^\circ$. The dichroic ratios for the $\nu_{\text{s}}(\text{CH}_2)$, $\nu_{\text{as}}(\text{CH}_2)$, and $\nu_{\text{s}}(\text{CH}_3\text{-m})$ absorption bands were found to be 1.33, 1.41, and 1.44, respectively. The orientation of the $\nu_{\text{s}}(\text{CH}_2)$, $\nu_{\text{as}}(\text{CH}_2)$, and $\nu_{\text{s}}(\text{CH}_3\text{-m})$ transition dipole moments with respect to the surface normal were thus found to be 61° , 61° , and 60° , respectively. The transition dipole moments of each of these vibrations are oriented perpendicular to the extended all-trans phytanoyl chain molecular axis and can each be used to independently determine the orientation of the DPhPC phytanoyl chains, with respect to

the surface normal. The $\nu_s(\text{CH}_2)$, $\nu_{as}(\text{CH}_2)$, and $\nu_s(\text{CH}_3\text{-m})$ bands yielded phytanoyl chain tilt angles of 44° , 43° , and 46° , respectively. It is important to note that there is very significant trans-gauche isomerization for the phytanoyl chains, and the membrane is in a liquid crystalline state. Therefore, the orientation angles that are found from these experiments are best described as the average tilt angle of the trans fragments of the chains.

The $\nu_s(\text{CH}_2)$ and $\nu_{as}(\text{CH}_2)$ bands can also provide information regarding the twist of the phytanoyl chains. The orientation of the $\nu_s(\text{CH}_2)$ and $\nu_{as}(\text{CH}_2)$ transition dipole moments were found to be the same within experimental error. This suggests that there is no preferential twisting of the phytanoyl chains. The twist angle (θ_{twist}) of the trans segment of the chain is defined as the angle between the -C-C-C- plane and the plane formed by the chain axis and the surface normal (63). The trigonometric relationship shown in Equation 3 may be used to find the twist angle, θ_{twist} (63).

$$\tan \theta_{\text{twist}} = \frac{\cos \theta_{as}}{\cos \theta_s} \quad \text{Eq. 3}$$

The twist angle value was found to be 45° . A twist angle of 45° is referred to as the trivial angle, and corresponds to no preferential chain twist (63). Therefore, there is no preferential twisting of the phytanoyl chains.

Polar Headgroup Region

The polar headgroup region of the ATR-IR spectra contains important information regarding the orientation, conformation, and hydration of the phospholipid molecule. As with the C-H stretching region, potential spectral interference from the hydrogel scaffold must be considered. Figure 8 shows the $1300 - 800 \text{ cm}^{-1}$ region of the raw ATR-IR p-polarized spectra of hydrated hydrogel and scaffolded vesicles collected on a ZnSe-diamond ATR crystal.

The hydrogel scaffold exhibits a broad band extending from $\sim 1190\text{ cm}^{-1}$ to 970 cm^{-1} , which presents a difficulty in the analysis of the polar head group region of DPhPC. The band displays a flat ledge on the large wavenumber side, with a more intense peak occurring at the low wavenumber side of the band. The maximum of this peak is observed at 1014 cm^{-1} . A weak band is observed at 1014 cm^{-1} in the scaffolded vesicle spectrum, which arises from the incomplete cancellation of the hydrogel. This peak can act as a measure of the degree of cancellation. The s-polarized spectra exhibit similar features.

Figure 8 – Top trace: raw p-polarized spectrum of Sephadex® A50 using water as the background spectrum. Bottom trace: raw p-polarized spectrum of scaffolded vesicles using Sephadex® A50 as the background spectrum. The $\nu_{as}(\text{PO}_2^-)$, $\nu_s(\text{PO}_2^-)$, and $\nu_{as}(\text{CN}^+(\text{CH}_3)_3)$ bands are indicated. The dashed lines highlight the contribution from uncanceled Sephadex® A50 bands, in the bottom trace.

For the scaffolded vesicles, three IR bands of interest are observed close to this region: the $\nu_{\text{as}}(\text{PO}_2^-)$, $\nu_{\text{s}}(\text{PO}_2^-)$, and $\nu_{\text{as}}(\text{CN}^+(\text{CH}_3)_3)$. It should be noted that the $\nu_{\text{as}}(\text{COC})$ is also expected in this region at $\sim 1180\text{cm}^{-1}$, but is not clearly resolved, most likely due to its relatively weak intensity and Sephadex[®] A50 band overlap (51,64). There are also convoluted $\nu(\text{PO}(\text{C}))$ vibrations which overlap with the $\nu_{\text{s}}(\text{PO}_2^-)$ band which are not analyzed (64).

The $\nu_{\text{as}}(\text{PO}_2^-)$ band is observed at $\sim 1230\text{ cm}^{-1}$, and tails into the large wavenumber side of the Sephadex[®] A50 band (33). The $\nu_{\text{as}}(\text{CN}^+(\text{CH}_3)_3)$ band is observed at $\sim 970\text{ cm}^{-1}$, and tails into the low wavenumber side of the Sephadex[®] band (51). Given that the intense peak of the Sephadex[®] band is observed as a weak band in the scaffolded vesicle spectra, it is reasonable to neglect this band overlap in the analysis of the $\nu_{\text{as}}(\text{PO}_2^-)$ and $\nu_{\text{as}}(\text{CN}^+(\text{CH}_3)_3)$ bands.

The $\nu_{\text{s}}(\text{PO}_2^-)$, band is observed at 1086 cm^{-1} , and predominantly occurs on the flat ledge of the Sephadex[®] band (51). The intensity of the peak at 1014 cm^{-1} suggests that this ledge will be very weak. Furthermore, upon baseline correction, the flat ledge will not contribute to the absorbance (and therefore the dichroic ratio) of the $\nu_{\text{s}}(\text{PO}_2^-)$ band.

Phosphate Group

The $\nu_{\text{as}}(\text{PO}_2^-)$ and $\nu_{\text{s}}(\text{PO}_2^-)$ bands of the phosphate group provide hydration and orientation information. Figure 9 show the ATR-IR spectra of the $\nu_{\text{as}}(\text{PO}_2^-)$ and $\nu_{\text{s}}(\text{PO}_2^-)$ vibrations obtained for the DPhPC scaffolded vesicles. The position of the $\nu_{\text{as}}(\text{PO}_2^-)$ band can vary greatly depending on the degree of hydration (53). Increasing hydration of the phosphate results in a decrease in the frequency of the $\nu_{\text{as}}(\text{PO}_2^-)$ band due to hydrogen bonding with water (51, 64). The $\nu_{\text{as}}(\text{PO}_2^-)$ band is observed at 1230 cm^{-1} , which suggests that the phosphate group is well hydrated (64). The position of the $\nu_{\text{s}}(\text{PO}_2^-)$ band also depends on the degree of hydration,

but the variation is not as pronounced as with the $\nu_{as}(\text{PO}_2^-)$. The $\nu_s(\text{PO}_2^-)$ band is observed at $\sim 1086 \text{ cm}^{-1}$, which is also consistent with a high degree of hydration (64).

Figure 9 - Polarized ATR-IR spectra of the a) $\nu_{as}(\text{PO}_2^-)$ and b) $\nu_s(\text{PO}_2^-)$ regions.

The $\nu_{as}(\text{PO}_2^-)$ and $\nu_s(\text{PO}_2^-)$ transition dipole moments were found to be 54° and 58° , respectively. The $\nu_{as}(\text{PO}_2^-)$ and $\nu_s(\text{PO}_2^-)$ bands may each be used to calculate the tilt angle of the vector of the O-P-O esterified phosphate group (θ_{OPO}), with respect to the surface normal. The transition dipole moments of these vibrations are oriented perpendicular to the O-P-O esterified phosphate segment. The $\nu_s(\text{PO}_2^-)$ and $\nu_{as}(\text{PO}_2^-)$ bands yield θ_{OPO} values of 56° and 48° , respectively. These angles are close to the angle of random orientation (55°).

The $\nu_s(\text{PO}_2^-)$ and $\nu_{as}(\text{PO}_2^-)$ transition dipole moments exhibit the same geometric relationship to one another and the surface normal as the $\nu_s(\text{CH}_2)$ and $\nu_{as}(\text{CH}_2)$ dipoles. Therefore, a twist angle, which is defined as the angle between the O-P-O plane and the plane formed by the chain axis and the surface normal, may be calculated using Equation 4.

$$\tan \theta_{\text{twist}} = \frac{\cos \theta(\nu_{as}(\text{PO}_2^-))}{\cos \theta(\nu_s(\text{PO}_2^-))} \text{ Eq. 4}$$

The, twist angle was found to be 42° . A twist angle of 45° corresponds to no preferential chain twist (53). Therefore, there is no preferential twisting of the O-P-O plane of the phosphate group.

Choline Group

Figure 10 shows the $1000 - 800\text{ cm}^{-1}$ region of the scaffolded vesicles ATR-IR spectrum.

Figure 10 – ATR-IR spectra of the $\nu_{as}(\text{CN}^+(\text{CH}_3)_3)$ and $\nu_s(\text{CN}^+(\text{CH}_3)_3)$ bands for the scaffolded vesicles. The p- and s-polarized spectra are indicated.

The $\nu_{as}(\text{CN}^+(\text{CH}_3)_3)$ and $\nu_s(\text{CN}^+(\text{CH}_3)_3)$ bands are observed in this region (64, 65). The θ_{dipole} orientation angle for the $\nu_{as}(\text{CN}^+(\text{CH}_3)_3)$ was found to be 54° , which is consistent with randomly oriented transition dipole moments.

The band position of the $\nu_s(\text{CN}^+(\text{CH}_3)_3)$ provides information regarding the conformation of the O-C-C-N framework of the choline group. The trans conformation of the O-C-C-N framework exhibits bands at $\sim 925\text{ cm}^{-1}$ and 875 cm^{-1} (51, 64, 65). In contrast, the gauche conformation exhibits bands at $\sim 900\text{ cm}^{-1}$ and $\sim 860\text{ cm}^{-1}$ (51, 64, 65). Figure 10 clearly shows a band at $\sim 925\text{ cm}^{-1}$. No band is observed at 875 cm^{-1} , likely due to an insufficient signal to noise ratio. This suggests that the choline O-C-C-N framework adopts a trans conformation.

The trans conformation of the O-C-C-N chain may suggest that the positively charged choline group is tucked back behind the negative phosphate group, away from the positively charged hydrogel support. Such a conformation would maximize the electrostatic attraction between the negative phosphate group and the positive scaffold, while minimizing the electrostatic repulsion between the positive choline group and the positive scaffold. This conformation is consistent with literature studies which suggest that the phosphate group, not the choline group, is predominantly responsible for electrostatic interactions with charged surfaces (31). This behavior may be due to the greater negative charge density on the smaller phosphate group compared to the lesser positive charge density on the larger choline group (31). Furthermore vesicles composed of phosphocholine (PC) headgroup phospholipids (such as DMPC and DPPC), have been shown to have negative zeta potentials in solutions of low ionic strengths over a wide range of temperatures (66). A negative zeta potential for the zwitterionic PC head group suggests that the positive choline group is tucked back behind the negative phosphate group. This orientation of the phosphocholine group is consistent with an electrostatic attraction between the positively charged Sephadex[®] A50 scaffold and the zwitterionic DPhPC.

Conclusions

The corona observed in fluorescence microscopy images suggest that the scaffolded vesicles have a durable, vesicle-like structure, with the phospholipid coating confined to the outer surface of the hydrogel scaffold. In addition the presence of a durable and complete DPhPC coating was verified with the Tb³⁺/DPA fluorescence assay which demonstrated the barrier properties to Tb³⁺ efflux. Fluorescence quenching experiments have shown that the lipid coating is a single phospholipid bilayer. FRAP experiments have shown no evidence of phospholipid lateral mobility. This may be due to the strength of the electrostatic attraction between the

membrane and scaffold. This has been observed for biomimetic systems which exploit electrostatic interactions in the construction of model membranes (55-57). Future work will focus on improving membrane mobility and flexibility to achieve this goal.

ATR-IR spectroscopic experiments have examined the phospholipid tails and headgroup of the DPhPC bilayer. The molecular order and orientation of the phytanoyl chains are consistent with previous IR spectroscopic studies on DPhPC bilayers (62, 67) and are summarized in Figure 11. This supports the proposed bilayer structure. Analysis of the headgroup suggests that the bilayer is well hydrated and that the positively charged choline group is tucked behind the phosphate group, which is consistent with literature studies on phosphocholine lipids (66). This orientation is also consistent with an attractive electrostatic interaction between the cationic hydrogel support and the phosphocholine headgroup of DPhPC.

Figure 11– The orientation of specific functional groups of DPhPC in the scaffolded vesicle bilayer coating. No preferential twisting of either the phytanoyl chains or phosphate group is observed.

Table of Contents Graphic

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