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Phospholipid Diffusion Coefficients of Cushioned Model Membranes determined via Z-Scan Fluorescence Correlation Spectroscopy

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Abstract

Model cellular membranes enable the study of biological processes in a controlled environment and reduce the traditional challenges associated with live or fixed cell studies. However, model membrane systems based on the air/water or oil/solution interface do not allow for incorporation of transmembrane proteins, or for the study of protein transport mechanisms. Conversely, a phospholipid bilayer deposited via the Langmuir-Blodgett/Langmuir Schaefer method on a hydrogel layer is potentially an effective mimic of the cross-section of a biological membrane, and facilitates both protein incorporation and transport studies. Prior to application, however, such membranes must be fully characterized, particularly with respect to the phospholipid bilayer phase transition temperature. Here we present a detailed characterization of the phase transition temperature of the inner and outer leaflets of a chitosan supported model membrane system. Specifically, the lateral diffusion coefficient of each individual leaflet has been determined as a function of temperature. Measurements were performed utilizing z-scan fluorescence correlation spectroscopy (FCS), a technique that yields calibration-free diffusion information. Analysis via the method of Wawrezinieck and coworkers, revealed that phospholipid diffusion changes from raft-like to free diffusion as the temperature is increased; an insight into the dynamic behavior of hydrogel supported membranes not previously reported.

Introduction

Cellular membranes play an essential role in a vast array of biological functions across a huge cross section of living organisms. Membranes are vital as the mediator between the intra and extracellular environments and further serve as boundaries for organelles. Membrane components such as phospholipids, sphingolipids, cholesterol, proteins, and membrane-associated structures such as lipid rafts, ion channels, water channels, growth factor receptors, cell-cell and cell-substrate adhesion complexes, play crucial roles in cell metabolism, signaling, proliferation, differentiation, and motility.^{1–3} It is clear that understanding the molecular organization of cellular processes requires an in-depth knowledge of the structure and function of cellular membranes. However, the variety of

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structures present in, and complexity of, cellular membranes necessitates model systems that provide a controlled environment to explore individual processes without perturbation by competing factors.⁴ Additionally, model systems enable the application of experimental methods that would not be amenable to fixed or live cell studies. Recently, model cellular membranes have proven valuable for studies of processes as diverse as lipid oxidation⁵ and drug delivery.⁶

A wide variety of model membrane systems exist, ranging from spherical non-supported lipid bilayers (vesicles), to planar lipid bilayer supported on a solid substrate, either directly or via a cushion. In the latter system, the cushion may comprise a lipopolymer tether or a hydrated polymer/hydrogel layer which provides the space required for incorporation of transmembrane proteins and transmembrane transport. The thickness of the cushion may be tailored to suit the needs of a particular study, as may the material employed for the cushion.^{7–15} Hydrated polymers and hydrogels employed to date have included polyethyleneimine,^{8,10,16} polyacrylamide,^{12,17,18} and polysaccharides such as dextran, cellulose, and chitosan.^{11,19–21}

A variety of techniques have been employed to apply hydrated polymers and hydrogels to solid supports. Adsorption of polyelectrolytes has been effective on substrates such as quartz, mica, and silica, however this method inherently lacks control over polymer layer thickness.^{8,10,22} An alternate method for hydrated polymer layer formation is Langmuir-Blodgett (LB) deposition. LB films have inherently well-controlled thicknesses as the substrate may make multiple passes through a hydrophobic polymer at the air/water interface. Once deposited on the substrate, the polymer must be chemically modified to produce a hydrophilic hydrated layer on which the lipid membrane may be formed.²³ While such polymers have been shown to support lipid membranes, they are not readily available and must be specifically synthesized, thereby limiting application of the technique. In lieu of LB deposition, a method employed in a variety of applications is spin coating. Spin coating has the advantage that film thickness may be readily controlled by operational parameters including rotational speed, duration, etc., and is capable of producing extremely smooth films. Indeed high quality cushioned model membranes have recently been successfully fabricated with the polysaccharide chitosan.¹¹

Fabrication of a phospholipid bilayer upon a polymer/hydrogel cushion may be achieved by one of two methods: Langmuir-Blodgett/Langmuir Schaefer (LB/LS) deposition, or vesicle fusion. The relative advantages of the two techniques have been explored elsewhere employing polymeric cushions of polyethylenimine and phosphatidylcholine lipids.¹⁰ While vesicle fusion was established as the simplest method to deposit a lipid bilayer,¹⁰ LB/LS deposition is typically the preferred method due to its inherent control of the constituents of each lipid leaflet.^{11,24} Successful LB/LS deposition however requires that several factors be well controlled, including cushion swelling and roughness¹⁰ which affect membrane fluidity and the mobility of incorporated transmembrane proteins.¹² Additional considerations include polymer/lipid electrostatic interaction, steric forces from polymer chains extending into solution from the cushion, and osmotic stress.^{13,25}

Prior to detailed studies employing a given model membrane system, the membrane itself should be well characterized. Of particular concern is potential perturbation of the phospholipid bilayer phase transition temperature due to the presence of the cushion. The presence of such a perturbation could significantly reduce the physiologic relevance of any subsequent study. Although a number of studies have investigated support-dependent perturbation of lipid mobility and phase transition temperature,^{26–29} they have largely employed indirect methods rather than measurement of phospholipid dynamics, i.e. diffusion. Phospholipid diffusion coefficients may be determined via fluorescence recovery

after photobleaching (FRAP), single particle tracking (SPT), or fluorescence correlation spectroscopy (FCS).³⁰ Unfortunately, FRAP requires an extremely high concentration of fluorophore labeling, potentially resulting in system perturbation.³¹ Conversely, SPT employs a much lower labeling density, but data collection and processing require high capacity computing, and a comparatively small sample population is investigated.³² FCS is typically the technique of choice as it employs extremely low labeling densities (5–100 ppm), is non-computationally intensive when utilizing hardware correlators, and provides an excellent statistical representation of a given sample.³⁰ First developed in the 1970s, FCS is a single molecule technique that yields molecular diffusion coefficients, chemical conversion rates, and photophysical information.^{33,34} A detailed review of FCS may be found elsewhere.³⁵

Unfortunately, determination of absolute diffusion coefficients via FCS relies heavily on knowledge of the size and shape of the observation volume, which is most often approximated as a 3D Gaussian.³⁶ As such, diffusion coefficients are typically determined by reference to a known standard,³¹ although this may be a poor choice.³⁷ Subsequently, a number of calibration-free FCS variants have appeared in the literature over the last decade.^{38–42} Of particular relevance for thin planar systems is z-scan FCS, which alleviates sample positioning issues, and produces calibration-free diffusion information.⁴³ Z-scan FCS employs a confocal FCS geometry with the ability to step the sample through the focal volume axially (similar schemes change the beam waist without axially translating the sample to generate the same effect⁴⁴). A correlation curve is collected at each axial sample position and analyzed with the appropriate planar diffusion model. The resulting diffusion time and, separately, the particle number are then plotted as a function of the relative sample position yielding parabolic dependencies. The diffusion time and particle number versus relative sample position are subsequently fit to models such as those detailed by Benda et al.,⁴³ resulting in a calibration-free diffusion coefficient, the fluorophore concentration, and the beam waist.

In the present work we report the first determination of the diffusion dynamics of a hydrogel cushioned model membrane utilizing z-scan FCS. Indeed to the authors' knowledge this represents the first application of a calibration-free FCS method to characterizing the phospholipid phase transition temperature of a model membrane. Specifically, a model membrane system comprising a spun coat chitosan hydrogel film supporting a phospholipid bilayer deposited via the Langmuir-Blodgett/Langmuir Schaefer method has been investigated. Diffusion coefficients of each individual leaflet have been determined as a function of temperature in order to ascertain their respective phase transition temperatures.

Experimental Section

Cleaning procedures and substrate preparation

Chitosan films were prepared on No. 1.5, square cover glass substrates (Corning, Corning, NY). All glass and liquid sample cell components were rinsed in 18.2 M Ω -cm water (Milli-Q, Millipore, Billerica, MA) followed by overnight immersion in a 2–5% v/v Contrad 70 detergent/18.2 M Ω -cm water solution (Decon, King of Prussia, PA). The immersion was followed by rinsing with copious amounts of 18.2 M Ω -cm water and overnight immersion in 70% nitric acid (Fisher Scientific, Suwanee, GA). A final rinse with copious amounts of 18.2 M Ω -cm water was performed, storage prior to use was in 18.2 M Ω -cm water.

Cushion preparation and application

Following the procedure of Baumgart and Offenhäusser,¹¹ medium molecular weight chitosan was purchased from Sigma-Aldrich (St. Louis, MO) and used without further

purification. A 1% w/w chitosan solution was prepared in a 1% v/v glacial acetic acid solution (Certified ACS, Fisher Scientific) in 18.2 M Ω -cm water. The solution was stirred for 2 days with a magnetic stirrer to facilitate complete chitosan dissolution. The resulting solution was filtered through a 5 μ m pore size syringe filter (Millipore) for storage. Prior to spin casting, 1–2 mL of the stock chitosan solution was centrifuged at 11400 rpm for 30 minutes. A 60 μ L aliquot of the centrifuge supernatant was dispensed onto a cover glass, which was resident on the stationary chuck of a Specialty Coating Systems (Indianapolis, IN) spin coater with a programmable spin cycle. The spin coating parameters were optimized to give complete chitosan coverage of the cover glass. Specifically, a 1 second ramp time from stationary to 5000 rpm was employed, the 5000 rpm speed was maintained for 8 seconds prior to a return to the stationary state. The film-coated cover glass was subsequently immersed in a borate buffer solution for 1.5 hours (Product number 1470953, Hach, Loveland, CO) to neutralize the chitosan film, and finally rinsed with 18.2 M Ω -cm water.

Film characterization

As an initial test for chitosan film uniformity, fluorescence images of chitosan films doped with 1 mM fluorescein (Sigma-Aldrich, St. Louis, MO) were collected via a custom built sample scanning confocal microscope.⁴⁵ Separately, *ex situ* and *in situ* ellipsometry (J.A. Woollam M-2000V spectroscopic ellipsometer, Lincoln, NE) were employed to determine chitosan film thickness in the dry and swollen states, respectively. Gold-coated silicon wafers (Platypus Technologies, Madison, WI) were functionalized with 3-mercaptopropionic acid (Sigma-Aldrich, St. Louis, MO),²² and chitosan films were spin cast onto the surface. Ellipsometric measurements were performed at an incident angle of 75°; *in situ* measurements were facilitated by the use of a 5 mL liquid cell. Additionally, atomic force microscopy (AFM) (Digital Instruments Nanoscope IIIa controller with a Multimode Atomic Force Microscope, Veeco Instruments, Plainview, NY) in tapping mode was performed on chitosan films on mica to determine the rms roughness of the surfaces *ex situ* and *in situ*. Standard silicon tapping mode AFM tips with 10 nm radius of curvature (Veeco Instruments, Plainview, NY) were employed at a scanning rate of 10 μ m/s. No post-processing of the AFM images was performed.

LB/LS deposition

1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (RhoPE) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without purification. The phospholipids were stored at –20°C. Stock solutions of DMPC were prepared with HPLC grade chloroform (Fisher Scientific) and were diluted to a concentration of 1 mg/mL on the day of use. RhoPE stock solutions were received at a concentration of 1 mg/mL in chloroform. RhoPE solutions were prepared on the day of use via dilution of the stock solution with chloroform and mixing with the DMPC stock solution to a final RhoPE concentration of 0.0005 mol% to DMPC, a sufficiently low concentration as to reduce perturbation of the bilayer by the fluorophore.⁴⁶ Stock solutions and subsequent dilutions were sonicated for 15 minutes prior to use. Utilizing a Langmuir-Blodgett trough (Nima, Coventry, England) containing two Wilhelmy balance sensors, a mechanical dipper, and a custom fixed center barrier, two chitosan-coated cover glass were submerged back-to-back in a subphase of 18.2 M Ω -cm water maintained at 15°C. The DMPC solution and the DMPC:RhoPE solutions were spread in a drop-wise manner on the subphase of the appropriate trough compartment. The chloroform was allowed to evaporate for a period of 30 minutes prior to the monolayers being compressed to 35 mN/m. The monolayers were subsequently decompressed, recompressed to the final pressure of 35mN/m, and held for 30 minutes. The LB deposition was performed at a dipper speed of 4 mm/min in one of the two

trough compartments. Using a custom vacuum sample holder, the LS deposition was performed on one substrate utilizing the second trough compartment. The resultant bilayer was maintained in the subphase while it was mounted in a custom liquid sample holder, described elsewhere.⁴⁷

Fluorescence correlation spectroscopy

Measurements of 3 nM Alexa-Fluor 546 (Invitrogen, Carlsbad, CA) in 18.2 MΩ·cm water were made and fit with the 3D diffusion model for a single, freely diffusing species in solution (Equation 1) to optimize the microscope setup and assess its performance.

$$G(\tau)_{3D} = \frac{1}{N} \left(1 + \frac{\tau}{\tau_D} \right)^{-1} \left(1 + \frac{\tau}{\omega^2 \tau_D} \right)^{-0.5} \quad (1)$$

where N is the particle number in the observation volume, ω , is the ratio of the axial to lateral dimension ($\omega = z_0/r_0$) of the confocal observation volume, τ_D is the diffusion time, and τ is time.^{34,35} An autocorrelation curve and diffusion model fit of Alexa-Fluor 546 in solution is presented in Figure 1. For FCS measurements of planar model membranes, such as those employed in the present work, it is appropriate to utilize a diffusion model for 2D diffusion, given as:

$$G(\tau)_{2D} = \frac{1}{N} \left(1 + \frac{\tau}{\tau_D} \right)^{-1} \quad (2)$$

Equation 2 was utilized for fitting all membrane autocorrelation curves collected in the present work.

Z-scan FCS background and instrumentation

While traditional FCS has been widely employed, determination of absolute diffusion coefficients is dependent upon calibration and is therefore susceptible to error.⁴⁸ As such, an alternative, calibration-free method to collect diffusion coefficients for bilayers, z-scan FCS, was employed in the present work.⁴⁷ Z-scan FCS measurements are accomplished by collecting correlation curves at each of a series of axial positions, fitting each mean correlation curve to the appropriate diffusion model (Equation 2 for the present work), and plotting the diffusion time, τ_D , and the particle number, N , separately, as functions of the relative axial position.^{43,47} The resulting parabolic dependence of τ_D and N on the axial position may be fit with

$$\tau_D = \frac{w_0^2}{4D} \left(1 + \frac{\lambda_0^2 \Delta Z^2}{\pi^2 n^2 w_0^4} \right) \quad (3)$$

and

$$N = \pi c w_0^2 \left(1 + \frac{\lambda_0^2 \Delta Z^2}{\pi^2 n^2 w_0^4} \right) \quad (4)$$

where w_0 is the radius of the beam in the focal plane, c is the average concentration of the fluorophores in the focal plane, λ_0 is the excitation laser wavelength, ΔZ is the relative axial position of the sample, and n is the refractive index of the medium.^{43,47}

A detailed description of the custom microscope, sample holder, and temperature control stage employed in the present work is given elsewhere.⁴⁷ In brief, a 543 nm Helium-Neon

(HeNe) beam was adjusted such that it filled approximately 60% of the back aperture of the microscope objective (60X, 1.2 NA UPlanApo/IR water immersion, Olympus, USA). Emission was collected with a 50 μm diameter silica core multimode fiber optic cable (Thorlabs, Newton, NJ). The fiber was connected to an avalanche photodiode (APD) (SPCM-AQRH-13-FC, Perkin Elmer, Waltham, MA) which was subsequently connected to an external hardware correlator (Flex031q, Correlator.com, USA). The signal from the APD was autocorrelated, in hardware, while the intensity as a function of time was simultaneously recorded. The objective correction collar was adjusted for the cover glass thickness and to minimize spherical aberrations. The sample was initially positioned to produce the maximum count rate utilizing $\sim 1.5 \mu\text{W}$ at the sample. Measurements were taken from 20°C to 44°C. At each temperature, 12 different axial positions (centered around the pre-determined maximum count rate) were employed, with ten 3 second correlation curves collected at each position. An axial step size of 300 nm was employed covering a total axial range of 3.3 μm . A custom Matlab (MathWorks, Natick, MA) program was employed to control the hardware and acquire the data. Correlation curves whose corresponding time courses showed events greater than three standard deviations above the mean were removed and the remaining curves were averaged.⁴⁹ Data was analyzed via Mathematica 7.0.1 (Wolfram Research, Champaign, IL) where a weighted non-linear Levenberg-Marquardt fit routine was applied to the mean correlation curve with $1/\sigma^2$ of each point serving as the fitting weight. The mean correlation curve at each position was fit to Equation 2, and the resultant τ_D and N plotted with respect to position. Finally, the z-scan FCS plots of diffusion time and particle number, each with respect to the relative position, were fit to Equations 3 and 4, respectively, in order to extract the diffusion coefficient of RhoPE in each of the DMPC bilayer leaflets.

Results and Discussion

Prior to bilayer deposition, spin cast chitosan films were characterized to assess spatial uniformity, thickness, and surface roughness. Fluorescence images of chitosan films doped with 1 mM fluorescein (Figure 2A and 2B) indicated that the fluorophore was uniformly distributed throughout the film, and further, that the films were relatively featureless. The thickness of the films, and the uniformity of their thickness as a function of spatial position, was determined by ellipsometry. Thickness measurements from the center of a given film were routinely within 1 nm of the thickness measured at the periphery. *Ex situ* and *in situ* ellipsometry were employed to determine the extent of swelling of the chitosan films when equilibrated in both water and a buffer solution (10 mM HEPES, pH 7.4). Film thickness was demonstrated to increase by a factor of 2.3 with a standard deviation of 0.1, from the dry to the fully hydrated state, independent of the solution employed. Indeed, employing the spin coating parameters provided above, film thicknesses ranged from 55–60 nm in the dry state to 125–140 nm in the fully hydrated state. Atomic force microscopy was utilized to determine the surface roughness of mica supported chitosan films both *ex situ* and *in situ*. Root mean square roughness (rms) values of 1.1 nm and 1.2 nm, respectively, were determined, as evident in Figure 2C and 2D. It is noted that the rms roughness values of the chitosan films were significantly smaller than the thickness of the phospholipid bilayers to be deposited upon them ($\sim 5 \text{ nm}$ ⁵⁰). In accordance with the work of Richter et al.,⁵¹ and separately, Smith et al.,¹² it was not anticipated that the chitosan hydrogel film would propagate defects or roughness to the bilayer.

Phospholipid bilayers were deposited on chitosan films with LB transfer ratios of unity, within uncertainty. Bilayers prepared in the absence of a fluorophore were found to have a very low autofluorescence, and as such were excellent candidates for characterization by z-scan FCS (once doped with an appropriate fluorescent probe). Consequently, the dynamics of each leaflet of the membrane were characterized independently via incorporating RhoPE

in the lipid solution employed in the LB deposition of the inner leaflet, or in the LS deposition of the outer leaflet (Figure 3). Diffusion of RhoPE was confirmed visually at room temperature for all samples and subsequently, the sample temperature was reduced to 20°C and allowed to equilibrate. Data collection occurred over a temperature range of 20°C – 44°C, from low to high temperature, in increments of 1–2°C. Separate experiments (data not shown) revealed that data collected from high temperature to low temperature yielded comparable results. For each temperature, correlation curves were collected at each of 12 axial positions. A weighted Levenberg-Marquardt fit routine was applied to the correlation curve collected at each axial position, with the standard deviation serving as the fitting weight. Sample autocorrelation curves from a single axial position fit by this method for the inner and outer leaflets of a DMPC bilayer supported on chitosan at 36°C are given in Figure 4A and 4C, respectively. The model employed in the fits was the 2D diffusion model as presented in Equation 2. Employing the data from all 13 fitted autocorrelation curves recorded as a function of axial position at a given temperature, plots of diffusion time, and separately particle number, as a function of axial position were generated, (see sample data presented in Figure 4B and 4D). Equations 3 and 4 were used to fit the diffusion time and particle number z-scan FCS data, respectively, in order to extract the diffusion coefficient of the RhoPE. By employing RhoPE, a phospholipid chemically labeled with a fluorescent molecule, at a suitably low concentration, the results are expected to reflect the mobility of DMPC in the bilayer leaflets.^{35,46}

Z-scan FCS measurements and analysis were performed on three separate DMPC bilayers over the temperature range stated for each leaflet. Plotting the resultant mean diffusion coefficients with respect to temperature generated a phase transition curve, Figure 5. It is noted that since the fluorophore was incorporated into either the inner or outer leaflet of the membranes, phase transition curves for the two leaflets were obtained independently. The mean diffusion coefficients and uncertainties were determined from a weighted average, with error propagation based on the fitting of the correlation curves and resultant z-scan FCS plot analysis.

The phase transition temperature was determined via a sigmoidal fit method employed elsewhere.^{52,53} Specifically, the phase transition curves were fit with the Boltzmann sigmoidal line-shape, given as

$$D(T) = \frac{D_0 - D_f}{1 + e^{(T - T_m)/\Delta T}} + D_f \quad (5)$$

where D_0 is the initial (lowest) diffusion coefficient, D_f is the final (highest) diffusion coefficient, T_m is the phase transition temperature, and ΔT is the change in temperature over the range in which D varies the most. Employing Equation 5 yielded an inner leaflet phase transition temperature, T_m of 28.04±0.08°C and an outer leaflet phase transition temperature, T_m of 28.10±0.07°C as shown by the vertical dashed line in Figure 5.

The diffusion coefficients and resultant phase transition temperatures for DMPC bilayers supported on chitosan in the present work were found to be essentially the same for the inner and outer leaflets. Similar results have been reported for glass supported and polymer supported bilayers.^{24,54,55} In light of the work of Zhang et al. on similar supported bilayer systems, and translocation rates reported by Kol et al. for a variety of vesicle systems, it is not expected that fluorophore translocation or ‘flip-flop’ across the bilayer occurred on the experimental time scales (~4 to 5 hours) employed in the present work.^{55,56} The diffusion coefficients determined in the present work are best compared to those reported by Baumgart et al.¹¹ for FRAP measurements of a chitosan supported DMPC bilayer. Baumgart et al.¹¹ report diffusion coefficients in the range of 0.004 μm²s⁻¹ to 1.02 μm²s⁻¹ over a

temperature range of 14°C to 35°C. It is noted that these values are significantly lower than those determined in the present work of $0.825 \mu\text{m}^2\text{s}^{-1}$ to $3.98 \mu\text{m}^2\text{s}^{-1}$ over a comparable temperature range. Potentially, the discrepancy may be attributed to the fact that two different measurement techniques were employed. Indeed, comparative measurements of diffusion coefficients obtained via FRAP and FCS by Guo et al.³¹ revealed that FRAP derived values are often lower. It is hypothesized that the source of the difference is the much greater degree of fluorophore doping employed for FRAP measurements and consequent perturbation of the membrane (1 mol% in both leaflets by Baumgart and Offenhäusser¹¹ vs. 0.0005 mol% in an individual leaflet for the present work). It is noted that Baumgart and Offenhäusser¹¹ employed a different fluorophore to that employed in the present work, a factor which may also contribute to the differences in the diffusion coefficients measured. Interestingly, the phase transition temperatures determined in the present work for the inner and outer leaflets are within the range of values reported by Baumgart et al. (26.4–30.0°C¹¹), although higher than the often reported main phase transition temperature of DMPC of 23°C.⁵⁰ It was postulated by Baumgart and Offenhäusser¹¹ that diffusion may be perturbed or hindered in this particular cushion/lipid system by electrostatic interaction between the phosphate of the lipid headgroup and the amine of the chitosan. Indeed, it has been reported elsewhere that both the charge of a cushion and its surface roughness may affect diffusion of the bilayers it supports.^{12,57,58} Interestingly, the fact that the diffusion coefficients of the inner and outer leaflets determined in the present work are nearly identical suggests that either minimal perturbation exists, or both leaflets are affected to a similar extent.

Due to the noted difference between the reported main phase transition temperature of DMPC and the phase transition temperature obtained for DMPC in the present work, further detail regarding the type of perturbation that may be induced by chitosan on the individual phospholipid leaflets, was required. Baumgart and Offenhäusser¹¹ postulated that closer packed phospholipid headgroups, stemming from the chitosan/phospholipid interaction, may cause an increase in the main phase transition temperature. That is to say, the chitosan may be acting as a mesh, and hindering the diffusion of the phospholipids. A similar phenomenon was reported by Wawrezinieck et al.⁴⁴ for actin meshworks. Indeed, Wawrezinieck et al.⁴⁴ found that performing FCS measurements at varying spatial scales (observation areas) resulted in different measured dynamic behavior. As a result, Wawrezinieck et al.⁴⁴ developed a submicron confinement model of diffusion (the so-called FCS diffusion laws). Specifically, Wawrezinieck et al.⁴⁴ defined three types of diffusion: free diffusion, diffusion within microdomains or so-called lipid rafts, and diffusion confined by a meshwork such as the actin cytoskeleton.

In order to account for the effect of crossing a spatial barrier (such as that posed by a microdomain boundary, or meshwork) on the measured diffusion coefficient, Wawrezinieck et al.⁴⁴ defined an apparent diffusion time, τ_D^{app} , as

$$\tau_D^{\text{app}} = t_0 + \frac{w_0^2}{4D_{\text{eff}}} \quad (6)$$

where D_{eff} is the apparent diffusion constant, w_0 is defined by Equations 3 and 4, and t_0 is a constant that is zero for free diffusion, positive for diffusion in isolated microdomains/rafts and negative for diffusion in a meshwork. Further, t_0 is proportional to the confinement time, or the average time required for a molecule within a domain to exit the domain.⁴⁴

Humpolíková et al.⁵⁹ recently demonstrated that FCS data collected by the z-scan technique may be readily analyzed via the methodology established by Wawrezinieck et al.,⁴⁴ with the simple modification of employing the effective fluorophore concentration as

the metric for a change in the area probed. Specifically, Humpolíková et al.⁵⁹ expressed the apparent diffusion time, τ_D^{app} , as

$$\tau_D^{\text{app}} = t_0 + \frac{w_0^2}{4D_{\text{eff}}} \frac{N}{N_0} \quad (7)$$

where N_0 is the particle number at the minimum beam waist, and N is defined by Equation 4. It is evident from examination of Equation 7 that if one plots the measured diffusion time at each axial position as a function of N/N_0 , a linear fit provides a value for t_0 and hence, a measure of the nature of diffusion. The submicron confinement model was applied to the z-scan FCS data collected in the present work to elucidate the nature of diffusion of each of the DMPC leaflets on the chitosan cushion as a function of temperature. Figure 6 presents the analysis for the inner and outer leaflets of a DMPC bilayer on chitosan at 36°C.

Figure 7, presents a plot of t_0 as a function of temperature for both the inner and outer leaflets of a DMPC bilayer on chitosan. It is evident from examination of Figure 7 that t_0 is greater than zero at temperatures less than approximately 27°C (approximately the phase transition temperature determined by fitting with the Boltzmann sigmoidal lineshape), indicating diffusion hindered by microdomains. Since the bilayer leaflets in the present work are essentially a homogeneous system, the microdomains are likely gel (solid-like, reduced mobility) phase separated domains of DMPC that have been reported to coexist with a fluid (liquid-like, higher mobility) phase of DMPC over a range of temperatures.⁶⁰ At temperatures equal to and greater than approximately 27°C, both leaflets have t_0 values closer to zero, indicative of free diffusion. These findings further support the diffusion coefficient results above, suggesting similar behavior for the two leaflets of the chitosan supported bilayer. It may also be concluded that the type of perturbation, if any, induced by the chitosan on the phospholipid leaflets may not be attributed directly to the meshwork of the chitosan since the resultant t_0 values are non-negative. As reported in the literature, a variety of methods, (e.g. differential scanning calorimetry, electronic spin resonance, and fluorescence recovery after patterned photobleaching), and a variety of substrates (e.g. multilamellar vesicles, planar bilayers formed by vesicle fusion or LB/LS) have been employed to measure the phase transition temperature of DMPC membranes, yielding varying results.^{50,58} The values reported in the present work (27–28°C) are within the range of values reported by Baumgart et al. (26.4–30.0°C¹¹), both of which are higher than values reported in the same article for a substrate with no chitosan (glass supported planar DMPC bilayers). The present work reinforces the conclusion that the use of a chitosan cushion for the support of a DMPC bilayer elevates the phase transition temperature relative to that observed in the absence of a chitosan support.¹¹

Conclusion

The chitosan supported DMPC membranes constructed in the present work are a highly reproducible model membrane system that is attractive for the study of membrane processes such as protein transport in a controlled environment. Prior to application however, such membranes must be fully characterized, particularly with respect to the bilayer phase transition temperature and phospholipid dynamics. In order to characterize the lateral diffusion coefficient of each individual leaflet of chitosan supported DMPC membranes, z-scan FCS was employed. It was determined that the diffusion coefficients of each leaflet as a function of temperature were comparable. The diffusion coefficients of the membrane leaflets were found to be higher than reported by similar studies, although it is noted that the measurements performed in the present work were made at a much lower fluorophore concentration and, as such, were likely of a less perturbed membrane system. The phase transition temperatures of the inner and outer phospholipid leaflets were found to be nearly

identical and within range of values reported in the literature for a similar chitosan supported DMPC membrane. It is noted however that the phase transition temperature determined in the present work is elevated in relation to values reported for DMPC supported on glass substrates. Additional analysis of the diffusion mode as a function of temperature revealed that phospholipid diffusion changes from raft-like/microdomain to free diffusion as the temperature approaches the phase transition temperature; an insight into the dynamic behavior of hydrogel supported membranes not previously reported.

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Figure 1. Autocorrelation curve of 3 nM Alexa-Fluor 546 in 18.2 M Ω ·cm water fit to the 3D diffusion model of Equation 1.

Figure 2. (A) Uniformity of fluorescein distribution within a spin cast chitosan film (scale bar 5 μm). (B) Stepwise photobleaching of a spin cast chitosan film doped with 1 mM fluorescein (scale bar 5 μm). (C) *Ex situ* AFM micrograph of a spin cast chitosan film, rms roughness of 1.1 nm (scale bar 1 μm). (D) *In situ* AFM micrograph of a spin cast chitosan film, rms roughness of 1.2 nm (scale bar 1 μm).

Figure 3.
Schematic representation of the model membrane system (not to scale).

Figure 4.

FCS autocorrelation curves and z-scan FCS analyses for a DMPC bilayer supported on a chitosan film at 36°C. Figures A and C; sample autocorrelation curves and 2D diffusion model fits for the inner and outer leaflets of the membrane, respectively. Figures B and D; diffusion time vs. relative axial position and the particle number vs. relative axial position for the inner and outer leaflets, respectively. The data of Figures B and D were fit to the appropriate z-scan FCS equation to yield the diffusion coefficient and the effective concentration.

Figure 5.

Diffusion coefficient as a function of temperature for the inner and outer leaflets, independently, of a DMPC membrane on a chitosan support. The phase transition temperatures (determined from a Boltzmann sigmoidal line fit) were determined to be 28.04°C and 28.10°C for the inner and outer leaflets, respectively (indicated by the vertical dashed line).

Figure 6.

The apparent diffusion time as a function of the ratio of the particle number, N , to the particle number at the minimum waist, N_0 , fit via a linear expression for a DMPC membrane on a chitosan support at 36°C, (A) inner leaflet and (B) outer leaflet.

Figure 7.
The ordinate intercept, t_0 , of Figure 6 for each leaflet of a DMPC membrane on a chitosan support as a function of temperature.