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Neutron reflectivity study of substrate surface chemistry effects on supported phospholipid bilayer formation on (1 1 2 0) sapphire

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Oxide-supported phospholipid bilayers (SPBs) used as biomimetic membranes are significant for a broad range of applications including improvement of biomedical devices and biosensors, and in understanding biominalization processes and the possible role of mineral surfaces in the evolution of pre-biotic membranes. Continuous-coverage and/or stacked SPBs retain properties (e.g., fluidity) more similar to native biological membranes, which is desirable for most applications. Using neutron reflectivity, we examined the role of oxide surface charge (by varying pH and ionic strength) and of divalent Ca2+ in controlling surface coverage and potential stacking of dipalmitoylphosphatidylcholine (DPPC) bilayers on the (1 1 2 0) face of sapphire (α-Al2O3). Nearly full bilayers were formed at low to neutral pH, when the sapphire surface is positively charged, and at low ionic strength (1 = 15 mM NaCl). Coverage decreased at higher pH, close to the isoelectric point of sapphire, and also at high I = 210 mM, or with addition of 2 mM Ca2+. The latter two effects are not additive, suggesting that Ca2+ mitigates the effect of higher I. These trends agree with previous results for phospholipid adsorption on α-Al2O3 particles determined by adsorption isotherms and on single-crystal (1 0 1 0) sapphire by atomic force microscopy, suggesting consistency of oxide surface chemistry-dependent effects across experimental techniques.

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1. Introduction

The function of supported phospholipid membranes or bilayers (SPBs) as biomimic membranes depends on the SPBs retaining properties of native membranes including fluidity [1], even when immobilized on solid substrates. Greater surface coverage on the substrate surface and the presence of stacked multiple bilayers, where the first bilayer “cushions” the second, impart properties to SPBs that are closer to a true biological membrane [2–4]. Supported phospholipid bilayers with optimized properties are important for many medical and biotechnological applications including physiologically-compatible implants, biosensors, and therapeutic drug delivery devices [1, 5]. Furthermore, the behavior and interactions of phospholipid membranes at solid (e.g., mineral) surfaces are relevant for understanding biologically-mediated mineralization [6], the fate of inhaled or ingested mineral dusts in the body [6–8], and the possible role of mineral surfaces in the evolution of pre-biotic membranes [9].

The structure of SPBs supported on quartz (α-SiO2), silica and oxidized silicon surfaces, which are negatively charged at physiological (i.e., near neutral) pHs, have previously been scrutinized in depth [10–13], but SPBs on alumina surfaces, which are positively charged at pH 7.2, have not been studied in detail. The lateral continuity (extent of surface coverage) of bilayers and the presence of single or stacked multiple bilayers may change with surface charge due to the role of electrostatic interactions at the bilayer–water–oxide interface. In the present study, therefore, we used specular neutron reflectivity to examine the effects of surface charge, charge screening, and the presence of divalent Ca2+ on the lateral continuity and possible stacking of dipalmitoylphosphatidylcholine (DPPC) bilayers supported on the (1 1 2 0) face of sapphire (α-Al2O3).

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Among the many modern techniques for studying SPBs, neutron-based methods including neutron scattering, diffraction, and reflectometry have become increasingly popular. Neutrons have particular advantages for studying biological systems: they are non-destructive and highly penetrating, the wavelengths of neutrons used (angstroms) are suitable for studying structures of sizes relevant to biology (e.g., biomembranes), and neutron scattering by hydrogen and deuterium differs dramatically so that organic-rich structures can be studied in detail by selectively deuterating portions of molecules or the surrounding medium without disturbing the chemistry of the system [14]. Neutron reflectivity is especially useful for studying interactions and interfacial structure of biological materials at solid surfaces, as in SPBs. Previous efforts with this technique have examined the structure of SPBs deposited directly on quartz or oxidized silicon substrates [10] or onto underlying self-assembled films [15], the composition of multi-component membranes [16], and the interactions of other molecules (e.g., protein domains, DNA) with SPBs [17]. Several recent studies have also investigated the stability of stacked bilayers as a potential improvement over single SPBs for biomedical applications [2–4], as well as the effects of external influences such as an applied electric field [13] and UV illumination [18] on the membranes.

The goals of the present study are (i) to determine the lateral continuity (extent of surface coverage) and number of stacked bilayers of liquid–crystalline phase dipalmitoylphosphatidylcholine (DPPC) bilayers deposited from vesicles on sapphire (i.e., α-Al2O3 or corundum) as a function of varying surface charge conditions, (ii) to compare the results to other oxide substrates [19–21], and (iii) to discern other experimental factors (e.g., oxide support geometry) that may affect SPB formation. In detail, we examine DPPC bilayers at 55 °C under a range of pH (4.0, 7.2, and 9.2), ionic strength (15 to ≥ 210 mM) and divalent cation-bearing (0 or 2 mM Ca2+) conditions. The solution conditions are similar to those we have studied previously [19–21]. We quantify DPPC adsorption by estimating extent of surface coverage, and relate this to solution-dependent effects on substrate surface charge. We also discuss differences in bilayer stacking between our current results and our prior work using adsorption isotherms and atomic force microscopy to investigate SPB formation on micron-size particles [19,20] and planar substrates [21], respectively.

2. Experimental

2.1. Materials

A single-crystal sapphire wafer (α-Al2O3, Meller Optics, Inc., Providence, RI) of diameter = 76.2 mm and thickness = 3.2 mm was used as the substrate. The working surface of the crystal was parallel to the (1 1 2 0) crystallographic plane, with a root mean square (RMS) roughness of ~4–5 Å as determined by X-ray diffraction and reflection, respectively. Zwitterionic 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, C16H31NO5P); gel–liquid crystalline, Lα=–Lα′, phase transition temperature, Tm = 41.5 °C [20]) of >99% purity in chloroform was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. N-(2-Hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (HEPES, molecular biology grade), NaCl, and CaCl2 were purchased from Fisher Scientific, Inc. (Pittsburgh, PA). Deuterated water (D2O) of 99.8% purity was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Concentrated solutions of HCl (12 N) and NaOH (10 N) (Fisher) in H2O were purchased from Fisher and diluted to 1 M in D2O. Additional materials used to clean the sapphire substrate and to flush the sample cell between experiments (see below) included Triton X-100 detergent (ICN Biomedicals, Inc., Aurora, OH), methanol (HPLC grade from Fisher), H2O2 (Fisher), and water purified to a resistivity of 18.2 MΩ cm with a Milli-Q Synthesis system (Millipore Corp., Billerica, MA).

2.2. Vesicle solution preparation

Buffer solutions containing 50 mM HEPES in D2O were prepared and adjusted to pH 7.2 with 1 M NaOH. Subsequent adjustments to pH 4.0 or 9.2 were made, respectively, with 1 M HCl or additional 1 M NaOH. Appropriate masses of dried NaCl and/or CaCl2 were added as necessary to create high ionic strength (≥ 210 mM) and Ca2+-bearing (2 mM) solutions. Solution conditions for the experiments presented in this work are indicated in Table 1.

Necessary volumes of DPPC in chloroform were dispensed into glass vials, and solvent was removed under a light nitrogen flow to create a thin film on the inside of the vial, followed by continued drying under vacuum for ≥2 h. Dried DPPC portions were rehydrated to ~20 mM (15 mg mL–1) in deuterated buffer solution, heated in a water bath at ~50 °C (i.e., above Tm) for 1.5 h with occasional vortexing, and stored at 0 °C until needed (<2 days). Prior to use, lipid solutions were bath sonicated for 30 min at ~50 °C, then passed 31 times through 50 nm pore-size polycarbonate membranes (Whatman, Maidstone, Kent, UK) to produce small unilamellar vesicles using an Avanti mini-extruder (Avanti Polar Lipids, Inc.). The extruder apparatus was maintained at ~50 °C using a heating block mounted on a hot plate. Extruded vesicle solutions were further diluted with buffer to yield experimental DPPC concentrations of 1.25 ± 0.04 mM (~0.95 mg/mL) as determined by a previously described UV–Vis spectrophotometric assay for phosphorous [19,20].

2.3. Sample cell

Prior to assembly of the sample cell (Fig. 1), the sapphire substrate and the silicon backing puck were cleaned using the following sequence: sonicated 15 min in 0.1% (v/v) Triton X-100 detergent; rinsed and sonicated 5 min in Milli-Q purified water; sonicated 15 min in methanol; sonicated 15 min in ~1% HCl; sonicated 25 min in ~10% H2O2; rinsed and sonicated 10 min in Milli-Q purified DI water; dried thoroughly under N2 gas. Immediately after cleaning, the sample cell was assembled and filled with DI water to prevent substrate contamination. A flow-through brass block, connected via hosing to a Neslab RTE-221 circulating bath (Thermo Scientific, Waltham, MA), was secured to the aluminum block. This allowed for external temperature control, which was monitored using a thermocouple affixed to the sapphire spacer puck (Fig. 1). For additional description of the sample cell, see Section S1.1 in the Supplementary material.

2.4. Sample introduction

Vesicle solutions were introduced and equilibrated in the sample cell for each set of buffer conditions (pH + electrolyte) examined. Roughly 20 mL of sample solution, far greater than the sample cell volume (~0.5 mL), were flushed through the cell to ensure thorough replacement of water by the sample solution. Following introduction of the solution at ~25 °C, the sample cell was subjected to up–down–up temperature cycling between 25 °C and 55 °C (±3 °C). Temperature change occurred at ~1 °C min–1, and after the final cycle, the cell was maintained at ~55 °C for 30 min. Total duration from sample introduction to measurement initiation was about 2 h. A similar sequence of temperature cycling has previously
been shown to induce rupture of adsorbed vesicles on \( \alpha\)-Al\(_2\)O\(_3\) (corundum) particles [20]. Prior to beginning the reflectivity measurement, an additional 10 mL of buffer (without vesicles) was flushed through the sample reservoir to remove excess lipid. Between experiments, the sample reservoir was flushed in the following sequence to clean the substrate and remove lipid: 15 mL methanol, 15 mL Milli-Q purified water, 15 mL 0.1% HCl, 15 mL Milli-Q purified water. All sample and flush solutions were degassed thoroughly in a vacuum oven prior to injection into the cell assembly to minimize introduction of microscopic air bubbles that might otherwise adsorb to the substrate and affect the measured reflectivity. We note that formation of DPPC bilayer(s) on the oxidized surface of the silicon backing block is likely in our experimental setup. The sample reservoir depth (~100 \( \mu \)m) is sufficiently large, however, to make detected reflection from this interface insignificant.

### 2.5. Neutron reflectivity

Specular reflection of cold (i.e., low energy) neutrons was measured at fixed neutron wavelength \( (\lambda = 5.00 \pm 0.003 \text{ Å}) \) on the Advanced Neutron Diffractometer/Reflectometer (AND/R) [22] at the National Institute of Standards and Technology Center for Neutron Research in Gaithersburg, MD. The assembled sample cell (Fig. 1) was mounted in a vertical orientation to the instrument on a stainless steel stage, and aligned in the neutron beam to optimize the measured reflectivity signal. The incident beam was twice collimated through slits, which were opened linearly with increasing \( \theta_i \) to keep the illuminated sample surface area approximately constant, and directed through the sapphire substrate before the beam impinged on the sample interface (Fig. 1). The reflected beam was also collimated twice through slits that were similarly opened with increasing \( \theta_i \) to permit detection of all specular scattering. Specular reflectivity was measured from \( \theta_i = 0 \) to 6.52° (\( Q_s = 0 \) to 0.285 \( \text{Å}^{-1} \)), with two measurements typically made for the range below \( \theta_i < 4.00^\circ \), but only one above, due to time and sensitivity limitations. Background scans of off-specular reflection over the same range of \( \theta_i \) were also collected and averaged to interpolate the background signal occurring during the specular scans. Total duration of reflectivity measurements for a given experiment was ~9 h. For a brief discussion of the theory and formalism of neutron reflectivity, see Section S1.2 in the Supplementary material.

### 2.6. Data analysis

The programs of the Reflpak [23] suite were used for reduction and fitting of the data, with the assumption that DPPC vesicles had ruptured and formed planar bilayers on the substrate. Following background subtraction, the specular reflectivity was corrected for varying incident neutron flux due to changing slit size, and for the effect of finite sample size at low \( Q_s \) values. Data that indicated negative reflectivity or that had uncertainty larger than the reflectivity were omitted from the resulting reduced reflectivity curve. The layer-based fitting program in Reflpak uses a Levenberg–Marquardt non-linear least squares algorithm to fit a specified number of parameters. In our study, one fixed and six variable parameters were used in fitting each reflectivity curve (Table 1 and Fig. 2). The SLD of the sapphire was fixed at the well-established value of \( \rho_{\text{sapp}} = 5.71 \times 10^{-6} \text{ Å}^2 \) (Table 2) [24,25]. An explicit solvent layer with SLD = \( \rho_{\text{D}_2\text{O}} \), and thickness = \( l_{\text{D}_2\text{O}} \), was allowed to vary from its ideal value of \( \rho_{\text{D}_2\text{O}} = 6.35 \times 10^{-6} \text{ Å}^2 \) at 25°C [17]. Both the substrate and bulk solvent were treated as semi-infinite layers of indefinite thickness. DPPC bilayers were approximated by a single slab of SLD = \( \rho_{\text{Bil}} \), and thickness = \( t_{\text{Bil}} \). A one-slab approximation has been applied successfully in previous work [18]. In the current work, a one-slab approximation was found to give fits of equal or better quality than...
at the substrate–solvent interface was found to have a negligible impact on the fits, and was, thus, not included as a parameter.

In addition to model fits to the reflectivity data assuming adsorbed planar bilayers, attempts were also made to model the data under the assumptions that DPPC at the substrate was adsorbed either as unruptured vesicles or as a combination of bilayer patches and unruptured vesicles. The latter attempts gave fits of lower quality in all cases compared to fits considering only adsorbed bilayers. We speculate that any unruptured adsorbed vesicles may have been rinsed off the substrate during solvent flushing. A detailed description of the method used to fit the reflectivity data and estimate uncertainty is included in Section S1.3 in the Supplementary material.

3. Results and discussion

3.1. Reflectivity curves

Reduced specular reflectivity data for DPPC adsorbed on the (1 1 2 0) crystallographic face of sapphire at 55°C in D2O–HEPES solutions are reported for varying pH at low ionic strength (Fig. 3), and for different electrolyte conditions at pH 7.2 (Fig. 4). Total reflection of the incident neutron beam below the critical grazing angle is indicated by the initial plateau at low Qz, where R is unity (Figs. 3a and 4a; note that successive curves are offset by a factor of 10). Beyond the critical edge (Qz), which occurs at Qz ≈ 0.0044 Å−1 in agreement with Qc = 4/ðρD2O−fit−ρSapÞ [27], the reflectivity decreases steeply. A minimum occurs at Qz ≈ 0.15–0.17 Å−1 separating a complete first and an incomplete second oscillation. Uncertainty increases with increasing Qz because reflectivity decreases as R ∝ Qz−4 [18,28], whereas the intensity of the incident beam increases only as Qz2 as the collimating slits are widened.

Representative fits of the data based on the best fit parameters (Table 1) are shown in Figs. 3a and 4a. The overall shape of the fits is consistent with a single DPPC bilayer adsorbed to the sapphire substrate (Fig. 2). Comparable data have been interpreted similarly in previous reflectivity studies investigating phospholipid adsorption on oxidized silicon or quartz (α-SiO2) substrates [10,18]. To accentuate the differences among the curves obtained under various experimental conditions, we have replotted the ordinate as RQz2 (Figs. 3b and 4b), which compensates for decreasing reflectivity with increasing Qz according to Fresnel’s Law [18,28]. The disparity in the amplitude of the first oscillation is, thus, clearer. The amplitude decreases with increasing pH as 4.0 ≈ 7.2 > 9.2 (Fig. 3b), and, with changing electrolyte conditions at pH 7.2, as low I > low I + 2 mM Ca2+ > high I + 2 mM Ca2+ > high I (Fig. 4b). Oscillation amplitude is related to the chemical contrast, and, thus, to the variation of ρ across a series of interfaces. Considering the similarity of ρD2O,fit for the various experimental conditions, the difference in oscillation amplitude equates primarily to variations in ρSap,fit (Table 1).

Small peaks centered around Qz = 0.095 Å−1 are found in the reflectivity profiles for the experiments conducted at pH 7.2 with either low (I = 15 mM) or high (I = 210 mM) ionic strength (Figs. 3 and 4), with the peak in the former being more prominent. A similar feature observed with dipalmitoylphosphatidylethanolamine (DPPE) bilayers deposited by the Langmuir–Blodgett and Langmuir–Schaefer methods on SiO2 at T > Tm,DPPE = 64°C [3], was attributed to the formation of isolated, multi-bilayer stacks consisting of 11 bilayers atop a high-coverage DPPE double bilayer. Given that both DPPE and DPPC are zwitterionic and that the systems were at T > Tm when the peaks were observed, this seems a plausible explanation for the small peaks in our data as well. The larger width and smaller height of the peaks in our data suggest,
respectively, that multilamellar islands in our experiments would have comprised fewer stacked bilayers and covered a smaller area compared to the earlier DPPE study [3]. It is not clear from our data how these stacks may form. One possibility is that as the temperature is increased above $T_m$, the increase in molecular area causes the bilayer to rupture and ejects bilayer fragments away from the substrate, forming the multilamellar stacks [3]. Why this would only happen under particular solution conditions as observed here is also unclear. Similar stacks might have initially formed under other conditions tested here, but could have been removed during the solvent flushing step. This would suggest that the stacks were not formed due to unique substrate effects or solution conditions at pH 7.2.

Our attempts to effectively model the data for our pH 7.2 and low $I$ condition including the additional peak yielded poor fits as judged visually and by $\chi^2$ values. If isolated multi-bilayer islands were present and the islands had a diameter larger than the coherence length of the neutron beam (>10 μm), then the reflectivities from distinct sample domains with and without islands would be superimposed in the measured reflectivity profile [3,27]. Reffpak, however, treats layers as homogenous over the entire area analyzed, yielding an average reflectivity for the whole system. Thus, simultaneously modeling two or more superimposed reflectivities with high confidence is quite difficult. Because the additional peak is clearly not consistent with a single bilayer adsorbed on the substrate, best fit parameters (Table 1) for the experiment at pH 7.2 and low $I$ were determined after omitting data in the range of the small peak ($Q_z = 0.081–0.105 \text{ Å}^{-1}$). The smaller peak observed
in the data for the pH 7.2 and high I experiment did not significantly affect best fit parameters, so it was not omitted.

3.2. Scattering length density profiles and interfacial structure

Scattering length density profiles corresponding to the best fits of the reflectivity data in Figs. 3 and 4 are shown as a function of distance from the substrate, z (Fig. 5a and b). The constant SLD of 5.71 × 10^{-6} \text{Å}^{-2} at z < 0 corresponds to the sapphire substrate. At the sapphire–solvent interface (z = 0), the SLD increases to \rho_{2D,fit}, varying slightly from 6.08 to 6.14 × 10^{-6} \text{Å}^{-2} for the experimental conditions investigated (Table 1). These values are lower than the ideal value of 6.35 × 10^{-6} \text{Å}^{-2} for pure D_{2}O at 25 °C (Table 2) due, in part, to the elevated temperature (T = 55 °C) at which the reflection measurements were made. The slight decrease in D_{2}O density at T = 55 °C results in a correspondingly lower value for \rho_{2D} of 6.29 × 10^{-6} \text{Å}^{-2} (www.ncnr.nist.gov/resources/sldcalc.html). Further differences between the ideal value and \rho_{2D,fit} are likely due to addition of H_{2}O during pH adjustment of the sample solutions, as well as to traces of H_{2}O remaining in the sample cell following flushing and sample introduction. The best fit thickness of the initial D_{2}O layer, t_{2D,fit}, is 5–11 Å, depending on the solution conditions (Table 1). Because these values are below the resolution (~12.5 Å) of the measurements given Q_{max} ≈ 0.25 Å^{-1}, the fits are relatively insensitive to t_{2D,fit} as indicated by the large error estimates for this parameter.

Experimental work [5], as well as theoretical calculations based on DLVO theory [29], support the consensus view for the presence of a thin water layer between the substrate and the bilayer in SPB systems. There is still, however, some controversy over the explanation for its existence [16] and its possible thickness as evidenced by the range of values determined from neutron reflectivity studies from 1 to ~30 Å [2,3,10,13,16,17], with values of 5–10 Å being the most commonly reported. Inclusion of this water layer was necessary to achieve the best fits in our study, and the values determined (5–11 Å) are in agreement with the previous measurements. However, because the uncertainty in t_{2D,fit} overlaps zero, the existence of a water layer is not certain based on the current results.

Beyond the initial solvent layer, the SLD drops dramatically to \rho_{bulk,fit}, indicating the presence of a DPPC bilayer. Variation in the value of \rho_{bulk} for the different experimental conditions is related to fractional surface coverage, as discussed below in detail. Bilayer thickness, t_{bil,fit}, determined here to range from 36 to 40 Å (Table 1), is in agreement with a thickness of ~40 Å for a DPPC bilayer modeled as a single slab at a quartz surface in D_{2}O [18]. These values are smaller than the known DPPC bilayer thickness of ~46.5 Å at T > T_{m} [30], due to the location of the effective neutron scattering interfaces within the DPPC headgroup regions on either side of the hydrocarbon core. For comparison, three-slab models for bilayers, which explicitly treat the hydrocarbon core and the two head group regions as slabs of different SLD relative to each other and the solvent, typically result in hydrocarbon layers of ~30 Å flanked by headgroup layers of ~8–9 Å [2,17]. The SLD increases back to \rho_{2D,fit} beyond the bilayer and no additional changes in \rho(z) are evident, indicating the bulk D_{2}O reservoir.

Interfacial width, or roughness, is indicated by the vertical sloping portions of the SLD profiles between adjacent layers (Fig. 5). Fit values of \sigma_{ill} and \sigma_{out} were 2–3 Å RMS (root mean square) and 3–9 Å RMS, respectively. The asymmetric bilayer roughness may be due to the greater freedom of motion of the outer DPPC leaflet because it is less coupled to the sapphire surface than the inner leaflet. The values obtained in our study are in reasonable agreement with values derived in earlier reflectivity studies, which tend to range from 2 to 6 Å [2,3,13,17]. The slightly larger values observed in some cases here may arise from using a single-slab rather than a multi-slab treatment of the bilayer. See Section S2.1 in the Supplementary material for further discussion of the roughness values.

Our results indicate the formation of a single liquid–crystalline phase DPPC bilayer of varying surface coverage on the (1 1 2 0) face of sapphire at T = 55 °C in solutions of varying pH and electrolyte composition. The one-dimensional interfacial structure determined from fitting of our reflectivity data is in general agreement with structures determined in previous neutron reflectivity, AFM, and quartz crystal microbalance with dissociation (QCM-D) studies, which have used quartz or oxidized silicon substrates [10–12,18].

3.3. Determination of fractional coverage

If neither bilayer islands nor uncovered (i.e., water–filled) patches of the substrate are larger than the coherence length of the neutron beam, then the signals from these distinct regions should be
averaged in the plane of the sample for the SLD determined from the fits [10]. Differences in $\rho_{\text{bil,fit}}$, thus, imply differences in fractional surface coverage, $\Gamma$, with lower bilayer coverages resulting in values of $\rho_{\text{bil,fit}}$ that are closer to $\rho_{\text{D,fit}}$ due to greater area of uncovered patches (Fig. 2). The variation in $\rho_{\text{bil,fit}}$ with solution conditions (Table 1 and Fig. 5) is the most unequivocal difference among the data collected here.

In order to convert values of $\rho_{\text{bil,fit}}$ to absolute values of surface coverage, we compare $\rho_{\text{bil,fit}}$ to $\rho_{\text{D,fit}}$ and to the expected SLD of the DPPC hydrocarbon core, $\rho_{\text{HC}}$, at $T > T_m$ (Table 2), so that [18],

$$\Gamma = \frac{\rho_{\text{D,fit}} - \rho_{\text{bil,fit}}}{\rho_{\text{D,fit}} - \rho_{\text{HC}}} \quad (1)$$

Calculated values of $\Gamma$ using Eq. (1) range from 0.54 to 0.85 (i.e., 54–85% coverage by the single bilayer proximal to the substrate) for the conditions investigated in this study (Table 1). The SLD for 100% coverage is indicated by the black dashed lines in Fig. 5a and b.

The expected SLD of the DPPC headgroup region, $\rho_{\text{HC}} = 1.73 \times 10^{-6}$ Å$^{-2}$ (Table 2), is higher than that of the hydrocarbon core, $\rho_{\text{HC}} = -0.36 \times 10^{-6}$ Å$^{-2}$. Headgroups at the edges of supported bilayer patches (Fig. 2) are located within the hydrocarbon layer as it is modeled here and will increase the minimum possible value of $\rho_{\text{bil,fit}}$ that corresponds to 100% coverage. This effect should be of more significance for lower coverage and/or patchier bilayers, but it is still expected to contribute only minimally because the volume of these edge headgroups should be small compared to the volume of the hydrocarbon layer. Inclusion of a portion of the headgroup region on either side of the hydrocarbon core, considering $\rho_{\text{bil,fit}}$ is greater than the expected thickness $t_{\text{HC}} = 28.5$ Å [30], may also increase the minimum possible value of $\rho_{\text{bil,fit}}$ that corresponds to 100% coverage. Estimates based on Eq. (1), thus, provide only a lower bound for actual surface coverage.

3.4. Effect of pH on surface coverage

High surface coverage of liquid–crystalline phase DPPC on the (1120) face of sapphire was observed at both pH 4.0 (85%) and pH 7.2 (83%) at low ionic strength, but coverage at pH 9.2 was significantly decreased by 14% (Table 1). A similar trend in the bulk amount of DPPC adsorbed has been noted previously by determining adsorption isotherms on $\alpha$-Al$_2$O$_3$ (corundum) particles at pH 5.0, 7.2, and 9.0 [19]. The isoelectric point (IEP) or point of zero charge (PZC), for $\alpha$-Al$_2$O$_3$ particles is typically found to occur at pH ~9 [31], so that the particles are positively charged at pH 5.0 and 7.2, but bear little or no net charge at pH 9.2. Increased adsorption at pH < PZC was, thus, explained by a more favorable electrostatic interaction on the positively charged oxide particles, compared to net neutral or negatively charged particles at pH $\geq$ PZC [19]. Similar behavior was found for DPPC on $\alpha$-SiO$_2$ (quartz, PZC $\approx$ 3) and $\alpha$-TiO$_2$ (rutile, PZC $\approx$ 6) particles at pH 5.0, 7.2, and 9.0 [19]. These results were interpreted to indicate that the phospholipid headgroups behaved as though they were at least slightly negatively charged [19], in agreement with other work [32,33]. This explanation was corroborated by comparison of bulk adsorption isotherms for DPPC and DTPC (C13-chain ditridecanoylphosphatidylcholine) on the same oxide particles over a range of pHs, and by AFM of DTPC on planar surfaces of all three oxides at constant pH [19,21].

The trend of decreasing coverage with increasing pH obtained here is consistent with a PZC for (1120) $\alpha$-Al$_2$O$_3$ of ~9. Measurements by streaming potential, force microscopy, and second harmonic generation methods, however, tend to indicate PZCs in the vicinity of 5–6 for individual crystallographic faces of $\alpha$-Al$_2$O$_3$, including the (1120) face [31,34], rather than ~9 as for particles. The higher PZC of particles relative to single faces has been suggested to be due to the increased prevalence of singly-coordinated, high pK$_a$ surface hydroxyl groups owing to irregular surface structure [31]. In contrast, most surface hydroxyls on planar single faces have been proposed to be multiply-coordinated, and thus possess lower pK$_a$. If the PZC of the sapphire substrate used here was indeed 5–6, then the surface should have been positively charged at pH 4.0 and negatively charged at pH 7.2 and 9.2. Our observation of similar surface coverage at pH 4.0 and 7.2 and decreased coverage at pH 9.2 is, thus, inconsistent with a PZC for (1120) $\alpha$-Al$_2$O$_3$ of 5–6.

3.5. Effect of ionic strength and Ca$^{2+}$ on surface coverage

When the ionic strength was increased from 15 to 210 mM at pH 7.2 (without Ca$^{2+}$), the surface coverage dropped from 83% to 54% (Table 1), a 29% change. The significant decrease in coverage is likely a result of increased charge shielding at higher ionic strength leading to a less favorable interaction or adhesion energy between sapphire and adsorbed vesicles. Surface charge on the sapphire substrate increases with increasing ionic strength [35,36], which might seem to suggest a more favorable electrostatic interaction energy. The surface potential, however, is unaffected by changes in indifferent electrolyte (e.g., NaCl) concentration [35,36], and the increased charge shielding at higher ionic strength leads to decreased magnitude of both the electrostatic [12,37] and van der Waals [38] contributions to total interaction energy. Furthermore, a less favorable interaction energy between the substrate and a bound vesicle leads to decreased vesicle deformation and, thus, to decreased rupture [12,37,39]. Decreased rupture of the DPPC vesicles on sapphire at high ionic strength (and subsequent removal of unruptured vesicles by solvent flushing) may, thus, explain the lower surface coverage observed here.

Addition of 2 mM CaCl$_2$ at low ionic strength and pH 7.2 caused a small decrease of 7% in coverage on the (1120) sapphire surface (Table 1). Low concentrations of Ca$^{2+}$ (e.g., 2 mM) have usually been found to enhance the rate of vesicle rupture and SPB deposition as well as the resulting surface coverage on negatively charged planar surfaces of quartz or mica, as determined by AFM and QCM-D [11,12]. Calcium ions bind to negatively charged substrates thereby decreasing the negative surface charge [38], and also bind to the phosphatidylcholine (PC) headgroup resulting in positively charged bilayers [40]. The latter leads to a favorable electrostatic interaction between the PC bilayers and the negative substrates, thus promoting SPB formation. If sapphire is positively charged, however, then the electrostatic interaction with the bilayers should, if anything, be less favorable with Ca$^{2+}$ binding to the DPPC headgroups, resulting in decreased SPB formation as observed here. This result again suggests that the (1120) face is positively charged at pH 7.2, in agreement with a PZC closer to 9 rather than to 5–6.

At high ionic strength (210 mM) and pH 7.2, addition of 2 mM Ca$^{2+}$ causes a 19% increase in coverage (Table 1). This result suggests that Ca$^{2+}$ mitigates the effect of the increased ionic strength. The same inference is drawn by noting that increasing the ionic strength caused a 29% drop in coverage in the absence of Ca$^{2+}$, but no significant drop in the presence of Ca$^{2+}$ (Table 1). The effects of increasing the ionic strength and of adding Ca$^{2+}$ thus appear to be competitive, rather than additive, with the Ca$^{2+}$ ions providing the dominant control.

A possible explanation for the Ca$^{2+}$ control observed here relates to its role in promoting vesicle membrane fusion. This role has been noted previously in studies of cell–cell interactions and model membrane fusion involving anionic and zwitterionic phospholipids [11,41,42]. Increased fusion results in larger vesicles that...
rupture more easily when bound to a surface compared to small vesicles due to greater elastic tension at the deformed vesicle edge [39]. This effect is expected to be amplified at higher ionic strength because increased charge shielding decreases vesicle-vesicle repulsion in the same manner that it decreases vesicle–sapphire attraction. With respect to bilayer surface coverage, addition of Ca\textsuperscript{2+} thus compensates for decreased substrate–vesicle interaction due to increased ionic strength. For additional discussion, see Section S2.2 in the Supplementary material.

3.6. Bilayer coverage and stacking behavior on \(\alpha\)-Al\textsubscript{2}O\textsubscript{3} substrates

The trends in the amount of DPPC adsorbed to the planar \(\alpha\)-Al\textsubscript{2}O\textsubscript{3} substrate (i.e., surface coverage) observed here go as pH 4.0 \(\approx \) 7.2 \(\approx \) 9.2 at low ionic strength, and, at pH 7.2, low ionic strength, \(I + 2\) mM Ca\textsuperscript{2+} \(= \) high \(I + 2\) mM Ca\textsuperscript{2+} \(> \) high \(I\) (Table 1). These trends are consistent with our previous results from adsorption isotherms using \(\alpha\)-Al\textsubscript{2}O\textsubscript{3} particles, as well as from AFM using planar (10 10) \(\alpha\)-Al\textsubscript{2}O\textsubscript{3} [19–21]. Using Derjaguin–Landau–Verwey–Overbeek (DLVO) theory, we have previously calculated the equilibrium forces between \(\alpha\)-Al\textsubscript{2}O\textsubscript{3} (i.e., sapphire) and \(\alpha\)-SiO\textsubscript{2} (quartz) and adjacent DPPC bilayers at solution conditions similar to those studied here [29]. For all conditions, the oxide-bilayer attraction is expected to result in a complete first bilayer, regardless of how favorable the electrostatic interaction is [29]. The present neutron reflectivity data, however, provide no definitive evidence of a complete first bilayer (although, the coverage estimates presented here may be low as noted in Section 3.3) nor of multiple bilayer deposition. The surface area-normalized amount of lipid adsorbed is, thus, lower under all experimental conditions examined.

There are several potential explanations for the difference in multiple bilayer adsorption seen in our past work and current study. Different crystallographic faces of sapphire were used for our neutron reflectivity and AFM studies: (11 20) and (10 10), respectively. Given that the surface charging behavior for these faces is expected to be similar [31], however, this distinction does not explain our results. Also, the measured roughness of these same two substrates differed by an order of magnitude: the (11 20) face used here had a roughness of \(\approx 4–5\) Å, compared to \(\approx 0.7\) Å for the (10 10) surface [21]. Roughness in the nm range, however, has been found to affect SPB formation only minimally [11].

Factors such as the duration of oxide–DPPC interaction (i.e., kinetic control), the macroscopic substrate morphology and the specific experimental procedure used can also impact DPPC adsorption on oxides [20,21,29], which may explain the lack of bilayer stacking observed here. Given that the observed coverages here indicate less than one complete bilayer and are not all equivalent, it is likely that adsorption is controlled not only by the thermodynamics but also by the kinetics of vesicle adsorption and rupture. Adsorption isotherms for DPPC on \(\alpha\)-Al\textsubscript{2}O\textsubscript{3} particles at pH 7.2 and low ionic strength (17 mM) show a single adsorbed bilayer after 1.5 h of interaction time between vesicles and particles [29], in contrast to the multiple bilayers obtained after 12–24 h of interaction [20]. This shorter time is similar to the 2 h of interaction time prior to solvent flushing in this study. The multiple bilayers obtained after longer interaction times are consistent with predictions from DLVO calculations [29].

The role of kinetics in our experiments is further supported by the QCM-D results of Seantier et al., who found that longer times were required for supported bilayers to form from dimyristoylphosphatidylincholine (DMPC) vesicles on SiO\textsubscript{2} at higher ionic strength, both in the absence and presence of 2 mM Ca\textsuperscript{2+} [12]. Their observations are consistent with the trend in our data of decreased surface coverage at increased ionic strength. For further discussion of the role of kinetics in this study, see Section S2.3 in the Supplementary material.

Greater bilayer stacking on oxide particles compared to planar substrates may result from the three-dimensional morphology of a particle, which allows bilayers to wrap around the entire particle and form enclosed stable supported bilayers. Two-dimensional planar substrates, as used in the present study, do not allow similar enclosed bilayers to form, so stacked bilayers may be more easily dislodged by perturbations during temperature cycling or solvent flushing. Adsorbed second, or “free,” bilayers of DPPC and similar lipids have previously been observed with neutron reflectivity on oxidized silicon after being manually deposited by Langmuir–Blodgett and Langmuir–Schaeffer techniques [2,3,13]. The free bilayers typically remained stable until perturbed by application of an electric field [13], or inclusion of cholesterol into the membrane [3]. Similarly stacked bilayers formed via spontaneous vesicle adsorption and rupture on oxides have not, however, been observed with neutron reflectivity. Two stacked DPPC bilayers were observed on planar (10 10) sapphire by AFM after only 30 min interaction when the vesicles were physically induced to rupture [21]. But when rupture was not physically induced, only single DPPC bilayer patches were observed [21].

4. Summary and conclusions

The effects of surface charge and Ca\textsuperscript{2+} ions on the formation of supported liquid–crystalline phase DPPC bilayers were observed on the (11 20) face of sapphire (\(\alpha\)-Al\textsubscript{2}O\textsubscript{3}) for the first time by neutron reflectivity. Adsorption (i.e., extent of surface coverage) increased as the negative (positive) surface charge on sapphire decreased (increased), suggesting a role for substrate–bilayer electrostatic interactions. Increased charge shielding with higher ionic strength and Ca\textsuperscript{2+} binding to DPPC decreased the substrate–bilayer attraction, resulting in decreased surface coverage. The effects of increasing ionic strength and addition of Ca\textsuperscript{2+} are not additive, however, with a small concentration of Ca\textsuperscript{2+} compensating for the substantial decrease in adsorption observed at high ionic strength. The adsorption trends with surface charge and presence of Ca\textsuperscript{2+} observed here are consistent with adsorption isotherms for DPPC on oxide particles, and the results of AFM studies on planar oxide substrates determined under similar solution conditions.

Observation of high bilayer surface coverage under a variety of experimental conditions (e.g., 73% at the physiologically relevant conditions of pH 7.2, \(I = 216\) mM, and 2 mM Ca\textsuperscript{2+}) indicates the prospective utility of Al\textsubscript{2}O\textsubscript{3} as a substrate in biomedical applications such as in implant devices or in therapeutic drug-delivery mechanisms. Additionally, it is apparent that SPBs are sensitive to oxide substrate surface charge, indicating that SPB-based devices could be tuned by adjusting solution conditions. The observations here are also relevant for origin-of-life studies, which have used lipid vesicles as model “proto-cells” [9]. Shifting environmental conditions (e.g., ocean acidity, ionic strength and/or composition) could have led to oxide mineral surfaces being more or less favorable substrates on which amphiphilic molecules could accumulate. Also, faster rupture of vesicles, along with the smaller bilayer adhesion energy, on quartz vs. corundum surfaces [20,29] suggests that early proto-cells composed of lipids or lipid-like amphiphilic precursors may have been more stable in contact with corundum or similar positively charged minerals compared to quartz or other minerals with a negative surface charge.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jcis.2011.12.031. Additional description of the sample cell, a brief summary of the formalism of neutron reflectivity and further information about data fitting and analysis are included. Further discussion of interfacial roughness in our reflectivity profiles, the role of Ca\(^{2+}\) in promoting vesicle membrane fusion and the role of kinetics in this study are also included.

References