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Hydration repulsion effects on the formation of supported lipid bilayers

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When zwitterionic lipids fuse onto substrates such as silica (SiO2), the water of hydration between the two approaching surfaces must be removed, giving rise to an effective hydration repulsion. Removal of water around the polar headgroups of the lipid and the silanols (SiOH) of SiO2 allows supported lipid bilayer (SLB) formation, although an interstitial water layer remains between the lipid and surface. The importance of hydration repulsion in SLB formation is demonstrated by monitoring fusion of zwitterionic lipids onto silica (SiO2) nanoparticles heat treated to control the silanol group (SiOH) density and thus the amount of bound water. SLB formation, observed by cryo-TEM and nano-differential scanning calorimetry, was found to be slower for the more hydrated surfaces. Although the SiOH density decreased with increasing heat treatment temperature, ζ-potentials were the same for all the SiO2. This arose since at the pH = 8 of the experiments, only isolated silanols, with a pKα = 4.9, and not hydrogen bonded silanols, with a pKα = 8.5, were dissociated/charged. Since there were no differences in double layer forces between the SUVs and SiO2, which are the largest and most important interactions determining lipid fusion onto surfaces,2,3 the slower rate of SLB formation of DMPC onto SiO2 nanoparticles with higher silanol densities and more bound water was therefore attributed to greater hydration repulsion of the more hydrated nanoparticles. For SiO2 heated to 1000 °C, with only a few isolated silanols, little adsorbed water and many hydrophobic Si–O–Si groups, particle aggregation occurred and lipid sheaths formed around the nanoparticle aggregates.

Introduction

Supported lipid bilayers (SLBs) on nanoparticles are of importance as a method of biofunctionalizing solid surfaces for drug delivery and can provide a platform to investigate membrane proteins. They have been formed on micron sized spherical4 and nanoparticle5-7 surfaces, and been investigated by molecular dynamics (MD) simulations.8 SLBs can be formed on planar and curved surfaces by the fusion of small unilamellar vesicles (SUVs)9 on a variety of inorganic and organic supports. Fusion can occur by a process of adorption and rupture, or by a single rupture step, and can depend on factors such as the ionic strength of the medium, type of buffer and solid support.10 While critical densities of SUVs can be required for fusion onto planar surfaces, vesicles appear to rupture one by one on SiO2 nanoparticles.9

The effects of nanotopography of the substrate11 and the substrate type12 have been investigated by techniques such as quartz crystal microbalance with dissipation monitoring (QCM-D), where simultaneous measurements of the shift in frequency and the change in energy dissipation provide information on whether intact vesicle adsorption or vesicle fusion occurs. For zwitterionic lipids, only intact vesicles adsorb onto oxidized gold,13,14 oxidized platinum15 and TiO216 solid supports, and in the low coverage regime where there is only intact adsorption, the flattening deformation due to collapse of the SUVs was much larger on SiO2. Supported lipid bilayer formation occurs for SiO2 and Si3N4 at higher coverage.13,14 Although the most common inorganic support is silica (SiO2), SLBs also form on borosilicate glass, silicon wafers, mica15,16 TiO2 (rutile),17 and SrTiO3 single crystals18 (although this has been suggested to be due to SiO2 impurities19). In the case of Al2O3, deposition does not occur via a vesicle rupture process but SLBs can be formed by bubble collapse deposition.19 The difference between SiO2 and TiO2 was attributed to the higher charge density of the former, due to its lower isoelectric point.20 SLBs also form on planar polymer supports.21,22

Although the substrate itself affects bilayer formation, there are water layers on both the lipid and substrate that must be removed before a SLB can be formed. Not all of the water is removed, since after formation of the SLBs on silica substrates the lipid bilayer is separated from the SiO2 support by a water layer 1.5–2.0 nm thick.24,25 The organization of water on these surfaces may play a role in the adsorption/fusion process of lipids to form SLBs. In particular, as the two surfaces approach each other, the water of hydration must be removed from the polar headgroups of the lipid24 and the surface water on the inorganic substrate, giving rise to an effective hydration repulsion,25 which
can be affected by the nature of the water layers on the lipid and inorganic surfaces. In the case of hydrophilic planar surfaces, phospholipid bilayers spread by sliding over surfaces with a thin lubricating water film.\textsuperscript{26}

NMR and molecular dynamics (MD) simulations of water around fully hydrated DMPC showed both a more mobile, clathrate-like hydration shell of ca. 5–6 hydrogen-bonded water around the positively charged N(CH\textsubscript{3})\textsubscript{4} choline moiety and “frozen”, less mobile, hydrogen-bonded water (1–2 water molecules) with the oxygen of the phosphate.\textsuperscript{27,28} Dynamic AFM studies of SLBs of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) phospholipids showed that in the gel phase, up to 5 ordered water layers (1–3 layers more frequently observed) with spacings of ca. 0.29 ± 0.6 nm each between the carbon nanotube tip and the SLB could be successively removed; the layers close to the lipid required more force to remove. Whether the structured water arose from inherent hydration layers around the lipid and/or tip from induced confinement between the two could not be determined.\textsuperscript{29} Molecular dynamics simulations indicated that one or two ordered water layers extended from the DPPC headgroup.\textsuperscript{30}

Water is believed to have an organized structure with perturbations compared with bulk water about 1.0–1.5 nm from a mineral interface.\textsuperscript{14,32} Up to seven confined water layers with spacings of 0.252 ± 0.048 nm, roughly the diameter of a water molecule, could be measured on a mica surface by off-resonance AFM, with dynamics slowed down as the confining separation was reduced.\textsuperscript{33} The stable molecular water layers on hydrophilic silica\textsuperscript{32} were suggested to be responsible for the hydration repulsion that has been shown to exist below 5 nm from the silica surface.\textsuperscript{34} Thus, the energy penalty for removing water layers from both the lipid headgroup and inorganic surface can affect the rate of SLB formation.

Here we investigate the effects of surface water and silanol density of nominal 100 nm SiO\textsubscript{2} nanoparticles on the formation of supported lipid bilayers from 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) small unilamellar vesicles (SUVs). The silanol densities were varied by heat (600 °C and 1000 °C) and piranha treatments and the nanoparticles characterized by dynamic light scattering (DLS), \( \zeta \)-potential measurements, transmission electron microscopy (TEM), FTIR spectroscopy and thermogravimetric analysis (TGA). Unexpectedly, the kinetics of formation of SLBs were faster on nanoparticles heated to 600 °C than on fully hydroxylated (“as-is”) SiO\textsubscript{2} nanoparticles. This was associated with a higher amount of bound water on the fully hydroxylated SiO\textsubscript{2}. The increased affinity of the SUVs for the nanoparticles with less adsorbed water was hypothesized to be due to the different structures of the interfacial water layers on the nanoparticles. Both nanoparticles were suggested to have 1–2 monolayers of an ordered “ice-like” water layer that was hydrogen bonded to the SiOH groups, with a greater amount of disordered water around the “as-is” nanoparticles. Since the \( \zeta \)-potentials of the nanoparticles were all the same (−45 ± 5 mV), indicating similar electrostatic interactions between the SUVs and the nanoparticles, the decreased affinity/fusability of the SUVs for the fully hydroxylated “as-is” SiO\textsubscript{2} was attributed to greater hydration repulsion between them, due to the necessity of removing more disordered water from the more hydrated SiO\textsubscript{2}. The SiO\textsubscript{2} heat treated to 1000 °C had so few SiOH groups that hydrophobic clustering of the nanoparticles occurred.

### Experimental

#### Materials

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC, 14 : 0 PC) was obtained from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Dry colloidal silica beads with 100 nm nominal size, prepared by the Stöber process with densities of 2.0 g cm\textsuperscript{-3} (reported by manufacturer) were purchased from Alfa Aesar Lancaster (Ward Hill, MA) and were used as received or after thermal and/or piranha treatment. SiO\textsubscript{2} nanoparticles in suspension, with nominal 100 nm diameters, densities of 2.4 g cm\textsuperscript{-3} (reported by manufacturer) and prepared by a water glass process, were a gift from Nissan Chemical America (Houston, TX). All solutions/suspensions were prepared with HPLC grade water and chloroform, purchased from Fisher Chemicals (Fairlawn, NJ), as was the H\textsubscript{2}SO\textsubscript{4} : H\textsubscript{2}O\textsubscript{2} (70 : 30) used to make the piranha solution. A 0.1 M, pH 8.0 buffer was made from Na\textsubscript{2}HPO\textsubscript{4} : H\textsubscript{2}O and NaH\textsubscript{2}PO\textsubscript{4} : H\textsubscript{2}O (PBS) and 75 mM NaCl. An Avanti Mini-Extruder from Avanti Polar Lipids was used for extrusion of the lipids, using 50 nm polycarbonate filters.

#### Thermal/piranha modification of SiO\textsubscript{2}

The SiO\textsubscript{2} described as “as is” was used from the supplier as received. The “as is” SiO\textsubscript{2} was thermally modified by heat treatment at 600 and 1000 °C for 7–8 hours. Thermally modified beads were further heated in piranha solution at 100 °C for 2–3 hours, and rinsed with distilled water until a pH of 7.0 was achieved. The “as-is”, “as-is” + piranha, 600 °C and 600 °C + piranha SiO\textsubscript{2} could all be dispersed in buffer. Not all of the 1000 °C or 1000 °C + piranha SiO\textsubscript{2} went into suspension. Only the material that was in suspension was used for subsequent use and characterization. The amount that did disperse was obtained by centrifuging out the non-dispersed beads and measuring (by TGA) the amount of SiO\textsubscript{2} in the supernatant; this weight percent was used to calculate the weight of lipid required for bilayer coverage.

#### Preparation of supported lipid bilayers

Appropriate amounts of lipid were dissolved in chloroform. Dry lipid films were formed after evaporation of the solutions under a stream of nitrogen and then in a vacuum oven overnight to remove any residual solvent. The lipid film was then dispersed in buffer and incubated at a temperature (ca. 50 °C) above the \( T_m \) of the DMPC for a minimum of 2 hours with periodic shaking to form hydrated multilamellar vesicles (MLVs). Small unilamellar vesicles (SUVs) were obtained from MLVs by subjecting them to 5 freeze/thaw cycles followed by extrusion using a polycarbonate filter with a pore size of 50 nm. Approximately 1 ml of a 5–10 mg ml\textsuperscript{-1} lipid solution was passed back and forth for up to 50 times. Although a clear solution was obtained after 20 passes, the vesicles became more monodisperse as the number of passes increased (as determined by dynamic light scattering data, see
below), and which had an average diameter of 60 nm. Assuming no loss of lipid during the extrusion process, additional buffer was added to the extrusion product to yield vesicle solutions of ~2 mg ml⁻¹ lipid. The dry silica particles were dispersed in buffer at a concentration of 2-4 mg ml⁻¹ and were sonicated for 1 hour in order to separate and prepare homogeneous colloidal solutions. The Nissan nanoparticles, which were already dispersed, were diluted in buffer to prepare the necessary concentrations.

Adsorption of the vesicles onto the nanobeads was accomplished by addition of the SiO₂ dispersions to the vesicle solutions held above T_m. The amount of lipid required to achieve single bilayer coverage was calculated using the surface area occupied by the lipid headgroup (0.59 nm² for DMPC) and the total surface area of the nanoparticles, with the assumption that the latter was a planar surface; values for the density of 2.0 g cm⁻³ and 2.4 g cm⁻³ were used for the Lancaster and Nissan SiO₂, respectively. The amount of lipid required for single bilayer coverage of the nanoparticles is achieved when the surface area of the SUVs (SAUV) was equal to the surface area of the SiO₂ (SASiO₂)SAUV/SASiO₂ = 1; other coverages will be referred to as fractions or multiples of this amount. For SiO₂ heat treated to 1000 °C, not all of the SiO₂ could be redispersed; only the fraction that was dispersed was used for the calculations.

For nano-DSC measurements, mixtures with variable SAUV/SASiO₂ ratios were incubated at 50 °C for 2 h, cooled to RT and stored at 4 °C. For time dependent measurements, mixtures with ratios of SAUV/SASiO₂ = 1.5 were prepared at RT and run immediately at 30 °C (see nano-DSC below). The excess lipid was used to stabilize the suspensions; we have previously shown that flocculation/precipitation of SLBs could be prevented by addition of excess SUVs. TGA samples were prepared with excess SUVs (SAUV/SASiO₂ = 2-5), incubated at 50 °C for 2 h and cooled to RT. They were then centrifuged at 3900 rpm using a Fisher Scientific Marathon 3900 centrifuge, and the supernatant decanted. Additional water was added to the pellet, and the centrifuge/washing steps repeated 3 times to remove excess SUVs and salt/buffer. The pellet was dried overnight in a vacuum oven at RT to quantify the amount of lipid adsorbed on the surface of the silica nanoparticles. For cryo-TEM measurements, the samples were prepared in a slight excess of lipid in order to obtain stable colloidal solutions.

Analysis

Thermogravimetric analysis (TGA). TGA data were obtained on a TA Instruments Hi-Res TGA 2950 Thermogravimetric Analyzer using a ramp rate of 10 °C min⁻¹ under a N₂ atmosphere. The samples were run from 25 to 800 °C. Derivative plots of the TGA data (DTGA) show regions of maximum rate of weight loss as inflection points, and highlight details of the weight loss as separate thermal events.

FTIR spectroscopy. The SiO₂ particles were characterized using Fourier transform infrared spectroscopy (FTIR) in the transmission mode, with 256 scans and a resolution of 2 cm⁻¹ using a Mattson Research Series 1 spectrometer (Mattson Instruments, Madison, WI) equipped with a MCT detector.

DLS and ζ-potentials. Dynamic light scattering (DLS) and ζ-potential measurements were obtained on a Malvern Zetasizer Nano-ZS (Malvern Instrument Ltd. Malvern, UK) at 25 °C, with an electric field strength of 30 V cm⁻¹ for the ζ-potentials. The natural (unbuffered) pH of the aqueous samples was used. The Smoluchowski equation was used to convert electrophoretic mobilities to ζ-potentials.

Nano-differential scanning calorimetry (Nano-DSC). Nano-differential scanning calorimetry (nano-DSC) measurements were obtained on a TA Instruments (New Castle, DE) Nano DSC-6300. Samples prepared using different ratios of SAUV/SASiO₂ (which had been incubated at 50 °C for 2 h and then stored in the refrigerator) were placed in the nano-DSC at RT, cooled to 5 °C and heated/cooled from 5 to 35 °C at 1 °C min⁻¹. For the time-dependent runs, suspensions with SAUV/SASiO₂ = 1.5, that is with a 1.5 excess of SUVs, were used. The nanoparticle (4 mg ml⁻¹) and SUV (2 mg ml⁻¹) suspensions were prepared separately, mixed at RT and immediately placed in the nano-DSC, which was at 27–28 °C. They were ramped (~1 min) to 30 °C (to slow the kinetics), incubated at various times and then the cooling cycles were measured at 1 °C min⁻¹ from 30 °C to 10 °C, to obtain snapshots of the fusion process to form SLBs. The sample was then re-ramped to 30 °C (time approximately 2–3 minutes) and re-cooled to 10 °C. The same procedure was repeated for times up to ca. 11 h to 3 days. The samples were in suspension during the nano-DSC runs due to the presence of the excess SUVs.

BET measurements. BET measurements were made on a model ASAP 2020 Micromeritics (Norcross, GA) Surface Area and Porosity Analyzer. Samples were ramped at 10 °C min⁻¹ to 150 °C or 180 °C and held at those temperatures for 13–14 h and analyzed by both single point and 5 point methods.

Cryogenic transmission electron microscopy (Cryo-TEM). The supported lipid bilayer suspensions were prepared for cryo-TEM at 25 °C using a Vitrobot (FEI Company), which is a PC-controlled robot for sample vitrification. Quantifoil grids were used with 2 μm carbon holes on 200 square mesh copper grids (Electron Microscopy Sciences, Hatfield, PA). The grid was immersed in the sample, blotted to reduce film thickness, and vitrified in liquid ethane. The sample was then transferred to liquid nitrogen for storage. Imaging was performed using a cooled stage JEOL JEM-2100F TEM (Model 915, Gatan Inc., Pleasanton, CA) at 200 kV.

Results

Characterization of nanoparticles

The results of the nanoparticle characterization by DLS, ζ-potential (ζ), BET measurements, TGA and TEM analysis are presented in Table 1, and representative cryo-TEM micrographs of the 600 °C particles are shown in Fig. 1a. The nanoparticles from Lancaster are not monodisperse even before heat-treatment. There was a combination of single particles with diameters of about 125 nm, as well as “doubles”, particles that appeared to have fused during the manufacturing process, with dimensions of...
adsorbed water (which desorbed and affected pressure measurements), so only BET measurements for the 600 °C, 600 °C + piranha, 1000 °C and piranha + 1000 °C SiO2, which had little, or effectively no adsorbed water (vida infra), are reported. Even BET data for the 600 °C + piranha give dimensions that are too small for the same reason. DLS data are reported as the z-average, which assumes single exponential decay of the correlation function and preferentially weights larger size particles. The DLS data were also analyzed and reported by number and surface area averages. The closeness in size between the “singles” and “doubles” makes it difficult to separate them by DLS. The Nissan nanobeads are more uniform, and smaller in size, ca. 110 nm in diameter by DLS, with no “dimers”. When calculating SA_{SUV}/SA_{SiO2} ratios, diameters based on DLS surface areas were used, since surface area is the parameter required for the amount of lipid needed for coverage of the SiO2. Values of 125 nm for the “as-is” and 600 °C SiO2, 185 nm diameter 1000 °C SiO2, with densities of 2.0 g cm$^{-3}$, and 110 nm diameter Nissan SiO2 with densities of 2.4 g cm$^{-3}$.

### Table 1 Dynamic light scattering, z-potential, TEM and TGA weight loss data for SiO2 and SLBs

<table>
<thead>
<tr>
<th>Diameters of SiO2 nanoparticles, [nm]</th>
<th>SiO2 Nanoparticles</th>
<th>SiO2 + SLBs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BET</strong></td>
<td>z \text{Av.} (\pm 10)</td>
<td>PDI</td>
</tr>
<tr>
<td>“as is”</td>
<td>168</td>
<td>0.12</td>
</tr>
<tr>
<td>“as is” + Pr$^c$</td>
<td>600 °C</td>
<td>105</td>
</tr>
<tr>
<td>1000 °C + Pr$^c$</td>
<td>90</td>
<td>163</td>
</tr>
<tr>
<td>1000 °C + Pr$^c$</td>
<td>1000 °C</td>
<td>160</td>
</tr>
<tr>
<td>100 nm Nissan SUVs</td>
<td>116</td>
<td>0.02</td>
</tr>
</tbody>
</table>
| $^a$ BET surface area converted into spherical particles with diameter $D$. $^b$ DLS = dynamic light scattering. $^c$ PDI = polydispersity index. $^d$ SA = surface area, after 2 h incubation. $^*$ Based on 125 nm diameter “as-is” and 600 °C SiO2, 185 nm diameter 1000 °C SiO2, with densities of 2.0 g cm$^{-3}$, and 110 nm diameter Nissan SiO2 with densities of 2.4 g cm$^{-3}$. $^\prime$ Pr = piranha.

Fig. 1 Cryo-TEM images of: (a) SiO2 heat treated to 600 °C + piranha, and (b) with added DMPC after incubation at 50 °C for 2 h; (c and d) 1000 °C + piranha SiO2 with added DMPC incubated at 50 °C for 2 h. Arrows indicate (c) lipid sheaths and (d) vesicles.

about 125 × 200 nm, and occasionally “triples”. This was also observed for the “as-is” SiO2 and SiO2 heat-treated to 1000 °C, with a greater proportion of “doubles” for the 1000 °C SiO2. Not all of the 1000 °C or 1000 °C + piranha SiO2 could be redispersed in water. TGA data indicated that approximately 50% of the original heat-treated sample could be redispersed, and TEM, DLS and z-potential data were obtained only for the dispersed fraction. The water content of the “as-is”, “as-is” + piranha, and Nissan SiO2 precluded accurate BET measurements due to
treatment temperature increases, the silanol density decreases, the ratio of the isolated/H-bonded SiOH increases, and the amount of adsorbed water decreases. Subsequent acid treatment of the heat-treated samples recovers some of the H-bonded silanols, but never to levels comparable to the “as-is” SiO2. The “as-is” SiO2 from Nissan, made by a water–glass process, has no organic component, and has FTIR spectra most comparable to the 600°C/C14C + piranha treated SiO2.

The trends observed in the FTIR data are confirmed by TGA analysis of the SiO2, shown in Fig. 3 and summarized in Table 1. The inset in Fig. 3 shows derivative (DTGA) weight loss data for the SiO2. Weight losses are greatest for the “as-is” nanobeads, and decrease to minimal amounts for the nanobeads heat-treated at 1000°C. The weight loss due to physically adsorbed water, which occurs below ca. 100°C, is greatest for the “as-is” nanobeads and least for the nanobeads heat-treated at 1000°C. Piranha treatment increases the surface silanol density and thus weight loss, at each heat-treatment temperature, as well as the amount of physically adsorbed water. The former statement is true except for the “as-is” SiO2 compared with the “as-is” + piranha SiO2 and is due to the replacement of the higher mass hydrophobic groups with SiOH.

Characterization of SUVs and supported lipid bilayers

The SUVs had z-average diameters of 60 nm ± 5 nm and ζ-potentials of 0 ± 0.5 mV (Table 1). TGA weight loss data for the lipids + nanoparticles (incubated for 2 h at 50°C) are presented in Table 1, and compared with calculated values based on 125 nm (“as-is” and 600°C), 185 nm (1000°C) and 110 nm (Nissan) diameters. Both the 600°C and Nissan SiO2 have the expected weight losses, but the 1000°C has 64–74% less and the “as-is” sample has 70–75% less, than the calculated values, respectively. Derivative TGA data for the nanobeads with adsorbed lipids are shown in Fig. 4, where the peaks originating from water loss clearly show the order “as-is” + piranha > “as-is” > 600°C + piranha > 1000°C + piranha ≈ 1000°C ≈ 0, the same order as for the pure nanoparticles. The peaks at 240–270°C and 300°C in the DTGA traces, attributed to lipid decomposition of the head-group and hydrocarbon tail, respectively, are similar in appearance for the 600°C, 600°C + piranha, 1000°C and 1000°C + piranha. The lipid peaks on the Nissan nanoparticles are also narrow, but shifted down in temperature. For the “as-is” and “as-is” + piranha, the peaks are broader and occur at higher temperature; this cannot be attributed to the underlying SiO2 since the derivative weight loss peaks (inset, Fig. 3) show no features in this temperature region. It is possible that some of the chains, once water is removed, can adsorb to the silica surface, spreading out the range of decomposition temperatures.
Table 2  Phase transition temperatures and enthalpies for DMPC MLVs, SUVs and SLBs

<table>
<thead>
<tr>
<th></th>
<th>$T_m$/°C</th>
<th>$\Delta H_m$/kcal mol$^{-1}$</th>
<th>$T_c$/°C</th>
<th>$\Delta H_c$/kcal mol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLVs</td>
<td>24.0</td>
<td>5.43</td>
<td>23.8</td>
<td>5.41</td>
</tr>
<tr>
<td>SUVs</td>
<td>24.2</td>
<td>5.26</td>
<td>24.4</td>
<td>5.27</td>
</tr>
</tbody>
</table>
| SLBs
| Nissan                   | 22.5     | 3.20                         | 22.1     | 3.24                          |
| As is + piranha          | 21.7     | 2.57                         | 21.0     | 2.75                          |
| 600 °C + piranha         | 21.9     | 2.75                         | 21.4     | 2.77                          |
| 1000 °C + piranha        | 21.6     | 4.89                         | 20.4     | 3.39                          |

In order to investigate the effect of surface water on lipid fusion to form SLBs, nano-DSC traces were obtained as a function of SA$_{DMPC}$/SA$_{SASiO2}$ for the “as-is” + piranha, 600 °C + piranha, 1000 °C + piranha, and the Nissan “as-is” SiO$_2$. The piranha treated SiO$_2$ were used, since they provided a range of surface properties, the piranha treatment was likely to hydrolyze interparticle silanol condensation of the nanoparticles after heat treatment, and remove ethoxy groups. The gel-to-liquid phase transition temperatures of DMPC on supported lipid bilayers (SLBs) have been previously identified. These transitions on heating ($T_m$) and cooling ($T_c$), and their respective enthalpies, $\Delta H_m$ and $\Delta H_c$, are presented in Table 2. For comparison, the same thermodynamic data are presented for DMPC MLVs and SUVs. The melt and crystallization temperatures of the SUVs are very slightly higher than those of the MLVs, as we have previously observed. The SLBs have $T_m$s decreased by 1.7–2.6 °C and $T_c$s decreased by 2.3–4 °C compared with the MLVs and SUVs. In general, $T_c \approx T_m$ for the MLVs and SUVs, but $T_m > T_c$ for the SLBs; this occurs since equilibrium is reached more quickly for lipids in MLVs and SUVs than lipids on the solid supports. Although the effect is small, $T_m$, $T_c$ (Nissan) > $T_m$, $T_c$ (600 °C + piranha) > $T_m$, $T_c$ (“as-is” + piranha) > $T_m$, $T_c$ (1000 °C + piranha). The average values of the transition enthalpies, $\Delta H_m$ and $\Delta H_c$, are smaller for the SLBs than for the SUVs by 64% and 57% respectively.

Fig. 5 shows nano-DSC traces for the 600 °C + piranha SiO$_2$ incubated for 2 h at 50 °C as a function of SA$_{DMPC}$/SA$_{SASiO2}$ for the 2nd cooling cycle. Below SA$_{DMPC}$/SA$_{SASiO2}$ = 1, only a single transition is observed, which originates from the gel-to-liquid transition of DMPC on supported lipid bilayers (SLBs). For SA$_{DMPC}$/SA$_{SASiO2}$ > 1, the peak due to $T_c$ from DMPC SUVs is observed, which increases with increasing amount of DMPC in the suspension. The peak height or area ratio of the SLB peak, $I_{SLB}$, to that of the total, $I_{Total} = (I_{SLB} + I_{SUV})$ is proportional to the amount of excess SUVs that are in the suspension. However, the enthalpy of the transition for SLBs is 64–57% of that for SUVs. Therefore,

$$I_{SLB}/I_{Total} = \frac{\Delta H_{SLB} (\#SLBs)}{\Delta H_{SLB} (\#SLBs) + \Delta H_{SUV} (\#SUVs)} = \frac{0.6 (\#SLBs)/0.6 (\#SLBs) + (\#SUVs)}{0.6 (\#SUVs)}$$

if an average of $\Delta H_{SLB} = 0.6 \Delta H_{SUV}$ is used. For example, if SA$_{SUV}$/SA$_{SASiO2}$ = 1.5, there would be 1 SLB and 0.5 SUV, so that $I_{SLB}/I_{Total} = 0.56$. This is approximately what is observed for the SA$_{SUV}$/SA$_{SASiO2}$ = 1.5 sample. However, these results are only qualitatively correct for several reasons. In addition to the errors inherent in determining the sizes of the nanoparticles, the SA$_{SUV}$/SA$_{SASiO2}$ ratio was calculated based on planar surfaces. In fact, the particles due to their curvature can accommodate more than the calculated amount of lipid. A plot of $\#SUVs$/#SLBs for the “as-is” + piranha, 600 °C + piranha, 1000 °C + piranha, and Nissan beads obtained after a 2 h incubation at 50 °C is shown in Fig. 6. The intensities were corrected in order to obtain the ratio for the number of SUVs to the number of SLBs ($\#SUVs$/#SLBs). We had first expected that the SiO$_2$ with the highest SiOH density would have the greatest adsorption of DMPC, and the SiO$_2$ with the lowest SiOH density would have the least DMPC adsorption at comparable SUVs/SLBs ratios. The SiO$_2$ heated to 1000 °C with very few silanols did have the least SLB formation. However, the SiO$_2$ heated at 600 °C + piranha had greater adsorbed DMPC than the “as-is” + piranha for the same ratio of #SUVs/#SLBs.

Time dependent adsorption experiments confirmed this trend. Fig. 7 presents nano-DSC plots on cooling of the “as-is” + piranha, 600 °C + piranha, 1000 °C + piranha, with SA$_{SUV}$/SA$_{SASiO2}$ = 1.5 as a function of time. Since excess SUVs were always present, the transition attributed to $T_c$ (SUVs) never disappeared. At fixed SA$_{SUV}$/SA$_{SASiO2}$, the intensity ratio of SUVs/SLBs was greatest for the 1000 °C + piranha SiO$_2$, and was...
I, the ratio of intensities, \( D \), the ratio of SUVs/SLBs by normalization using their respective enthalpies, Measurement of peak height ratios of SUVs and SLBs converted to with nominal 100 nm SiO\(_2\) and the following thermal/chemical treatments: (a) as is + piranha; (b) 600 °C/C\(_14\) + piranha; (c) 1000 °C/C\(_14\) + piranha SiO\(_2\). Nano-DSC data obtained after incubation of SiO\(_2\) with DMPC SUVs for 2 h at 50 °C. Measurement of peak height ratios of SUVs and SLBs converted to #SUVs/#SLBs by normalization using their respective enthalpies, \( \Delta H_m \) (SUVs) and \( \Delta H_m \) (SLBs).

There are other interesting observations that can be made from these data. Much of the SLB formation occurs at relatively short times for both 600 °C + piranha and 1000 °C + piranha SiO\(_2\). SLB formation on the 1000 °C + piranha SiO\(_2\) was small, and changed little with time for periods up to 4 days. Similarly, much of the SLB formation had occurred after 2 minutes for the 600 °C + piranha SiO\(_2\). However, for the as is + piranha SiO\(_2\), although there was significant SLB formation after 2 minutes, more SLB did occur for another 3–4 hours, and then remained constant. This clearly indicates that some SLB formation occurs very quickly for all the SiO\(_2\), but that the rate of SLB formation becomes slower for the as is + piranha SiO\(_2\) compared with the 600 °C + piranha SiO\(_2\).

Cryo-TEM data for the 600 °C + piranha SiO\(_2\) are presented in Fig. 1b and show SLB formation around the nanoparticles with no SUVs present. The 1000 °C + piranha sample (Fig. 1c and d) shows what appears to be clusters of nanoparticles surrounded by a lipid membrane sheath, as well as 50–60 nm SUVs, and larger SUVs adsorbed but not fused to the nanoparticles. Comparatively, the 600 °C + piranha shows individual nanoparticles surrounded almost completely by SLBs with little evidence of free SUVs. This comparison is consistent with Fig. 6 and 7, which show greater SLB formation and fewer free SUVs for 600 °C + piranha than for 1000 °C + piranha.

Discussion

The effect of surface water on nominal 100 nm SiO\(_2\) nanoparticles, which had been heated to various temperatures and treated with piranha solution, on the adhesion/fusion of SUVs of zwitterionic DMPC, was investigated. Thermal/chemical treatments affected the silanol densities and thus the amount of bound water to the silica surface. Although we expected that both the amount and rate of SUV fusion to form SLBs would increase with increasing silanol density, it was found instead that the surface with a lower silanol density (600 °C + piranha) had a higher rate of vesicle fusion compared with the surface with higher (“as-is” + piranha) silanol density, and had higher coverage, as monitored by TGA, after a 2 h incubation, as well as by time dependent nano-DSC thermograms. If only the number of “contact points,” in this case charged silanol groups, between the SUVs and surface determined vesicle rupture\(^4\) the order would be reversed. This suggests that the organization of water next to a solid surface plays an important role in lipid fusion onto the surface. The water around the lipid headgroup is expected to be the same in all cases.

In order to understand the effects of water, it is useful to review the interactions that exist between SUVs–SUVs, SiO\(_2\)–SiO\(_2\), and SUVs–SiO\(_2\). Zwitterionic SUVs interact with each other through weak van der Waals (attractive) and thermal undulation/protrusion (repulsive) forces. The dominant interaction between negatively charged SiO\(_2\) is electrostatic in origin, but hydrophobic attraction of Si–O–Si groups can occur on highly dehydrated surfaces. In the case of SUV–SiO\(_2\) interactions, the attractive van der Waals and thermal undulation/protrusion repulsive forces would be expected to be similar, but electrical double layer and hydration forces might be expected to be different as the SiO\(_2\) surface properties changed.
The fusion of zwitterionic lipids to form supported lipid bilayers may thus be affected by both electrical double layer and hydration forces. In fact, the major driving force for adhesion between SUVs and silica was suggested to be the electrostatic attraction between the two surfaces, which for zwitterionic lipids (with zero potential) was proportional to the square of the ζ-potential, ζ, or charge density, σ, of the silica. Although the isoelectric point of zwitterionic egg phosphatidylcholine (PC), \( pI = 4.13,^{45} \) might suggest that the DMPC would have a slightly negative charge at \( pH = 8 \), and thus be repelled by the negatively charged SiO\(_2\) surface, the measured ζ potentials of the DMPC SUVs investigated here were zero within experimental error, and thus should not affect the electrostatic interaction. More importantly, we do not see much difference between ζ of the “as-is” or heat treated silica, with or without piranha treatment, which were all ca. \(-45 \pm 5\) mV.

The same ζ-potentials were measured although the total silanol densities were very different, as determined both by FTIR and TGA data. Similarity in ζ could occur if only the isolated SiOHs contributed to the ζ-potential, which would happen if only the isolated SiOH, and not the hydrogen-bonded silanols, dissociated to give a negatively charged surface.

Evidence that this is the case comes from second harmonic generation (SHG) data. Silanol densities on fully hydroxylated silica surfaces are reported to be about 4.9 SiOH per nm\(^2\),\(^{36,40}\) composed of isolated silanols and hydrogen-bonded silanols, where the latter can be directly hydrogen bonded with each other (46%), or through a bridging water molecule (35%).\(^{1}\) The surface populations of the isolated and hydrogen bonded SiOH inferred from SHG data were estimated to be 19% and 81%, respectively, with pK\(_a\) values of 4.9 and 8.5;\(^{1}\) consistent with the view that isolated silanols can more readily dissociate compared with the hydrogen-bonded silanols.\(^{46}\) In the current investigation, at the \( pH = 7, \) where the ζ-potentials of the SiO\(_2\) in pure water were obtained or at \( pH = 8.0, \) where the fusion experiments were performed, only the isolated silanols would be expected to dissociate on any of the SiO\(_2\) surfaces, resulting in similar charge densities. In addition, for the buffered solutions at \( pH = 8, \) there is charge shielding of the nanoparticles. The surface charge density at this pH has been reported as 0.2C m\(^{-2}\).\(^{1,36}\)

Since surface charge density differences, and thus electrostatic interactions between the neutral SUVs and similarly charged SiO\(_2\) surfaces, are therefore not expected to play a major role in the differences between the adsorption/fusion processes, what is left is only hydration repulsion between the SUVs and SiO\(_2\) or hydrophobic attraction between the SiO\(_2\). The former interaction is critical to explain the difference between the “as-is” SiO\(_2\) and the SiO\(_2\) heat treated to 600 °C. For the SiO\(_2\) heat treated to 1000 °C, the silica has many hydrophobic surface Si–O–Si groups. In fact, only approximately 50% of the 1000 °C SiO\(_2\) could be resuspended, indicating that the remainder was so hydrophobic that it could not be wet by water. The SiO\(_2\) that could be resuspended was found to form clusters due to hydrophobic interactions between the nanoparticles, with some silanols on the exterior of the clusters promoting SLB formation around the whole cluster. On fully hydrated planar silica surfaces, it has been shown that isolated silanols separated by >120 Å were about 12% of the isolated silanols.\(^{44}\) After heat treatment, this number should increase leaving large hydrophobic areas that can associate. It has also been suggested, and may be the case here, that for silica heated above 650 °C, the adsorption/fusion of DMPC does not occur through a water-mediated interaction. Instead, there is direct adsorption of DMPC headgroups to defect sites, which have been observed as pits on a planar SLB silica surface that disrupted lateral assembly of the bilayer.\(^{42}\)

The increased attraction between the SUVs and 600 °C SiO\(_2\) compared with the “as-is” SiO\(_2\) can be explained by increase of hydration repulsion in the latter case, due to the differences in adsorbed water on the two SiO\(_2\) surface. Hydration repulsion is a short range force that exists between two approaching hydrophilic surfaces in water, and has been attributed to the energy needed to remove the water of hydration between interacting surfaces that contain ionic or polar species.\(^{33,24}\) Water near a silica surface is known to be different than that of bulk water, and this interfacial water layer, which has been extensively investigated at both the solid/air and solid/liquid interface,\(^{44}\) can be affected by the properties of the underlying silica surface,\(^{45,46}\) in particular by the type, number and distribution of silanol groups. Using \( ab \) \( initio \) calculations of cluster models of SiO\(_2\), with one or two (neighboring) SiOH on the surface as surrogates for isolated and hydrogen bonded silanols, a stable water layer was found with two SiOH, which disappeared when there was only one SiOH, although water molecules were still found around the isolated SiOH.\(^{42}\)

Optical SFG has been extremely useful in characterizing the adsorbed water layer on SiO\(_2\). Both disordered and quasi-ice like or ice-like properties of water have been observed near amorphous silica\(^{45}\) and crystalline quartz surfaces.\(^{46}\) Fused quartz or silica, with a pK\(_a\) of 3, can have varying degrees of ionization, depending on the pH.\(^{46}\) When the silanols of quartz are neutral (undissociated, \( pH < 2–3 \)) or fully ionized (all dissociated, \( pH > 10 \)), the structure of water at the surface is ice-like, as determined by optical SFG, with fewer ordered H\(_2\)O layers (1–2) when the weaker hydrogen bonding compared with the stronger electrostatic field force (3–5 ordered water layers) provides the ordering of the interfacial water layer.\(^{47}\) Since the orientation of water at the surface was found to be opposite for the two extreme cases, with the dipoles of water oriented into the solid (oxygens pointing away from the surface) at high pH, and with the oxygens oriented towards the surface at low pH, at intermediate pHs, disordering of the water occurred as a function of the degree of ionization, due to the competing alignment effects.\(^{48}\) The more disordered water had a maximum at \( pH = 8, \) so that at \( pH = 8 \) of the current experiments, the effects of the “water-like” H\(_2\)O are expected to be most pronounced. We postulate that it is this disordered layer that must be removed before fusion of the DMPC SUVs to the SiO\(_2\) surface can occur, and that there is
a greater amount of disordered water on the fully hydrated (as-is) than on the partially condensed (600 °C) SiO₂. This is shown schematically in Fig. 8.

Thus, although the substrate material affects the formation process and character of adsorbed/fused bilayers, the interaction must be mediated by the structure of this water layer, which in turn is affected by the underlying substrate. The decrease in hydrogen-bonded compared with isolated silanols and the corresponding decrease in adsorbed water with increase in heating treatment temperature are clearly observed for the SiO₂ nanoparticles in the current investigation by both TGA and FTIR spectroscopy. This effect has also been observed for Stöber SiO₂ (8 and 260 nm) nanoparticles, where the ratio of hydrogen bonded to isolated silanols decreased with decreasing particle size, and where the amount of hydrogen bonded water to the silicas (as measured using NIR spectroscopy) also decreased. This effect was explained by polarization differences between silanols: hydrogen bonding between SiOH on the silica resulted in polarization in other SiOH, promoting hydrogen-bonding with water molecules. Since polarization of isolated silanol groups was less than for hydrogen-bonded SiOH, there was less hydrogen bonded water.

The increased hydration repulsion between the SUVs and the “as-is” compared with the 600 °C SiO₂ can thus be explained by a model in which both contain a more tightly bound “ice-like” water layer, with a more disordered water layer that is greater for the “as-is” SiO₂. When the SUVs approach the SiO₂ surface, more energy must be expended to remove this greater amount of water from the “as-is” SiO₂. Although it might be expected that the entropy gain would be greater for the more hydrated silica surfaces, and thus the interaction should be more favorable for these surfaces, we observed the opposite. Therefore, this entropy gain is not sufficient to offset the unfavorable hydration repulsion for the more hydrated surfaces.

The water from the DMPC headgroups must also be removed. However, removal of this water would be the same in all cases considered here. Further, it has been shown by dynamic AFM measurements that the water surrounding lipid bilayers is more disordered in the liquid crystalline compared with the gel phase, and requires lower force for removal. This may partially explain why SLB formation is typically accomplished (as has been done in this investigation) by incubating the DMPC with the SiO₂ nanoparticles above the Tₘ of the lipids.

The proposed model is consistent with SHG data of neutral lipid egg phosphatidylethanol (egg PC) on SiO₂ at all pH values, which showed that the strength of the ice-like mode was very similar to the bare quartz surface, but the water-like mode oscillator strength was suppressed. The data were interpreted as...
resulting from PC replacement of the liquid-like interfacial water, leaving the 1–2 layers of ice-like water.\textsuperscript{50,51} The current results are also in agreement with a study of DMPC adsorption onto planar hydrophilic SiO\textsubscript{2} surfaces in which the silanol density was reduced by temperature induced dehydroxylation. In this case, water contact angles were between <5° (as prepared) and 67° (700 °C per 1 h), where 90° is regarded as the boundary between “hydrophilic” and “hydrophobic” surfaces. The SLBs had a higher affinity for the less hydrophilic surface and the formation of SLBs of DMPC was accelerated as the SiOH density decreased.\textsuperscript{52,53}

Hydration repulsion has been shown to be important in vesicle–vesicle and vesicle–surface interactions. The hydration shells in zwitterionic lipid MLVs, where water layers alternate between the bilayers, were estimated to occupy a layer about 0.5 nm thick around the polar headgroups, leaving a layer of ca. 1.7 nm of “free water” in the center of the inter-bilayer space free.\textsuperscript{23} The overall structure was shown to be determined by a balance between long-range attractive forces, van der Waals interactions between the lamellae, and repulsive hydration forces,\textsuperscript{23} which decayed exponentially with a decay distance of ca. 0.2 nm.\textsuperscript{23} Hydration repulsion was also used to account for the decrease in aggregation of Stöber SiO\textsubscript{2} (8 and 260 nm) with increasing particle size, where there was an increase in the hydrogen-bonded water layer for the larger particles that increased the repulsive hydration force between them.\textsuperscript{49}

A similar effect, in which ions instead of water needed to be removed from the space between SUVs and a surface, has been invoked to explain the absence of SLB formation of zwitterionic lipids on charged surfaces when the large molecular ion of the buffer was of opposite charge to that of the surface. In this case, there was high entropic or osmotic repulsion (electric double layer repulsion) as the two surfaces approached each other, since the counterions had to be squeezed into a smaller and smaller space, which was more difficult the larger the counterion.\textsuperscript{49}

Although the current investigation has pointed out the effects of the water layer on SiO\textsubscript{2} substrates on the formation of SLBs, most silica used to form SLBs has sufficient adsorbed water that SLB formation occurs readily, although the formation times may be different depending on the type of SiO\textsubscript{2} used. The water structure on other substrate materials may play a similar role. Lastly, there are slight differences in transition temperatures between the Nissan (made by a water–glass process) and Lancaster (made by a Stöber process) SiO\textsubscript{2}. The higher \(T_m\) and \(T_c\) of the Nissan nanoparticles by 0.6 to 0.9 °C and 0.7 to 1.7, respectively, suggest better lipid packing for the Nissan nanoparticles, but we are not sure of the reason. The FTIR spectra of the Nissan SiO\textsubscript{2} and the Lancaster 600 °C + piranha treated SiO\textsubscript{2} are close in appearance, and these two samples in fact behave most similarly (e.g. their calculated and observed lipid TGA weight losses are equal, the \(T_a\) and \(T_c\) values and the amount of adsorbed water are closest), confirming the importance of the underlying surface and water structure. The slight decrease in \(T_a\) and \(T_c\) for 600 °C > “as is” > 1000 °C SiO\textsubscript{2} may reflect the decreased amount of lipid on the nanoparticles, which can result in defects at the edges of regions without lipids. The DTGA data suggest that whatever lipid is on the 1000 °C is similar to what was on the other samples, i.e. in SLB form. This was also confirmed by FTIR data for DPPC on the four types of SiO\textsubscript{2}, which showed the all-trans symmetric and asymmetric CH\textsubscript{2} stretches at the same wavenumbers, albeit weaker, for DPPC on the 1000 °C SiO\textsubscript{2}.\textsuperscript{53}

Conclusions

Silica nanoparticles of nominal 100 nm diameter size were characterized by dynamic light scattering, FTIR and \(\zeta\)-potential measurements. They were heat or heat and piranha treated up to 1000 °C to vary the silanol density. Increasing heat-treatment temperature decreased the total silanol density, increased the relative amount of isolated compared with hydrogen bonded silanols and decreased the amount of adsorbed water. Piranha treatment recovered some but not all the silanols condensed at a particular temperature. \(\zeta\)-Potential measurements were similar for all the SiO\textsubscript{2}, consistent with SHG data from the literature indicating that at pH = 8, ionization occurred only for the isolated silanols. Adsorption/fusion of 60 nm DMPC SUVs onto fully hydrated SiO\textsubscript{2} (“as-is”), and SiO\textsubscript{2} dehydroxylated by heat treatment, was measured after 2 h incubation with the SUVs and as a function of time. The fusion process was measured by monitoring the intensities of the gel-to-liquid crystal phase transition temperatures on the cooling cycle \((T_c)\) for the SUVs and SLBs, which differed in temperature by approximately 2 °C. Since the enthalpy of the transition \(\Delta H (SLB) = 0.6 \Delta H (SUV)\), the ratio of the #SUVs/#SLBs was corrected by this amount. SLB formation occurred more slowly on the fully hydrated SiO\textsubscript{2} than on SiO\textsubscript{2} heated/piranha treated at 600 °C. Since the two SiO\textsubscript{2} were similar in other respects, in particular their charge (ionization), as determined by \(\zeta\)-potential measurements, differences in electrostatic interactions between the neutral DMPC and SiO\textsubscript{2} could not account for the difference. Therefore, the increased rate for the less hydroxylated surface was attributed to decreased hydration repulsion. Cryo-TEM data confirmed SLB formation. The decreased amount of SLB formation for the 1000 °C + piranha SiO\textsubscript{2} was attributed to hydrophobic association of the drastically dehydroxylated SiO\textsubscript{2} surfaces that contained Si–O–Si hydrophobic patches. Cryo-TEM images showed SiO\textsubscript{2} aggregates surrounded by lipid sheaths, SUVs and SUVs adsorbed but not fused to the nanoparticles.

Abbreviations

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<tr>
<th>SLBs</th>
<th>supported lipid bilayers</th>
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<tr>
<td>MD</td>
<td>molecular dynamics</td>
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<tr>
<td>SUVs</td>
<td>small unilamellar vesicles</td>
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<tr>
<td>MLVs</td>
<td>multilamellar vesicles</td>
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<td>DMPC</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphocholine</td>
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<td>DLS</td>
<td>dynamic light scattering</td>
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<td>PBS buffer</td>
<td>Na\textsubscript{2}HPO\textsubscript{4}--7H\textsubscript{2}O and NaH\textsubscript{2}PO\textsubscript{4}--H\textsubscript{2}O</td>
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<td>nano-differential scanning calorimetry</td>
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<td>thermogravimetric analysis</td>
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<td>silica</td>
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<td>Pr</td>
<td>piranha</td>
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References

1 S. W. Ong, X. L. Zhao and K. B. Eisenthal, Polarization of water-molecules at a charged interface—2\textsuperscript{nd} harmonic studies of the


