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# 1,2-Dielaidoylphosphocholine/1,2-dimyristoylphosphoglycerol supported phospholipid bilayer formation in calcium and calcium-free buffer ☆

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ABSTRACT

Phospholipid membranes are useful in the field of biocatalysis because a supported phospholipid membrane can create a biomimetic platform where biocatalytic processes can readily occur. In this work, supported bilayer formation from sonicated phospholipid vesicles containing 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine and 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] was studied using a quartz crystal microbalance with dissipation monitoring and an atomic force microscope. The molar percentages of DEPC and DMPG were varied to determine the effect of overall lipid composition on supported bilayer formation. This work also explored the effect that calcium ion concentration had on supported bilayer formation. Results show that vesicles with up to 50 mol% dimyristoylphosphoglycerol can form a supported bilayer without the presence of calcium ions; however, supported bilayer formation in calcium buffer was inhibited as the anionic (negatively charged) lipid concentration increased. Data suggest that supported phospholipid bilayer formation in the absence of $Ca^{2+}$ from vesicles containing negatively charged lipids is specific to phosphatidylglycerol.

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## 1. Introduction

In recent years, interest in supported thin films composed of phospholipid bilayers for use in biocatalysis has increased because phospholipid thin films can mimic cellular membrane structure and form functional biomimetic interfaces [1–3]. Two types of thin phospholipid films are used in biocatalysis (tethered and supported). Considerable research has focused on supported phospholipid bilayer (SPB) formation using a quartz crystal microbalance with dissipation monitoring (QCM-D) [4–12]. QCM-D is a highly valuable and effective tool for studying the formation process of thin lipid films supported on surfaces because QCM-D can readily follow the transition from vesicle adsorption to SPB formation. QCM-D is able to simultaneously detect two parameters on the surface, adsorbed mass (seen as a frequency shift) and viscoelastic behavior (seen as a change in dissipation), which makes it possible to distinguish between intact vesicles and a bilayer on the surface [4–14].

Using QCM-D, others have shown that SPB formation is sensitive to the surface chemistry [4,8,11,13,14], buffer species and pH [15], lipid composition [9,15], salt concentration [12], and lipid charge and calcium presence [6,8,14,16–20]. These previous studies demonstrated that increasing the concentration of phosphatidylserine (PS), a negatively charged lipid, slowed SPB formation on silica. In the presence of calcium ions, as the concentration of PS was increased from 20 mol% to 67 mol%, SPB formation time increased from 3 min to 57 min [6]. The effect was more dramatic in the absence of calcium with SPB formation time increasing from 3 min to 80 min for the same PS concentration increase. At a PS concentration of 50 mol% in the absence of calcium, vesicles adsorbed to silica without rupturing; with calcium present, the vesicles ruptured to form a SPB with restructuring of the vesicles occurring afterward. Richter and Brisson [8] showed that the surface chemistry affected the process by which PS-containing vesicles form a SPB. They demonstrated that on mica, vesicles with as little as 10 mol% PS (versus 67 mol% on silica) did not adsorb in the absence of calcium ions and therefore did not form a SPB, but there was slow vesicular rupture beginning at 20 mol% PS with calcium ions present and therefore SPB formation.

Focusing on these two important influences on SPB formation (negatively charged lipids and calcium ions), the goal of this current work was to explore the effect of calcium ions and lipid charge on SPB formation on silica, with the intention of developing a lipid system for use as a biocatalytic system. The binary lipid system chosen for this work was 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine (DEPC) and 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DMPG). Earlier results by Evans demonstrated that DEPC/DMPG at 80/20 and 50/50 molar ratios in 2 mM calcium ions did not form the expected supported phospholipid bilayer on a self-assembled monolayer [21]. Silicon dioxide surfaces used in this work were 5 MHz quartz crystal with a 50-nm thick layer of Si and boron-doped silica wafers.

☆ Product names are necessary to factually report on available data; however, the USDA neither guarantees nor warrants the standard of the product. Also, the use of the name by the USDA implies no approval of the product to the exclusion of others that may be suitable.

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## 2. Experimental details

### 2.1. Materials

Sodium chloride (NaCl) and calcium chloride ($CaCl_2$) were purchased from Fisher Scientific at A.C.S grade (Fair Lawn, NJ). Sodium dodecyl sulfate (SDS) and (N-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid) (TES) were obtained from Sigma-Aldrich (St. Louis, MO) and used as received. Chloroform stock of DEPC and powder DMPG were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. Deionized nanopure water at 18.2 MΩ-cm was obtained from a Barnstead Nanopure Diamond model D11911 water purification system. Sensor crystals (5 MHz) were purchased from Q-Sense (Västra Frölunda, Sweden). Any surface modifications were done as described in Section 2.4.

### 2.2. Buffer preparation

A buffer consisting of 10 mM TES, 150 mM NaCl, pH 7.4 was prepared in nanopure water and used for "calcium-free" measurements. For "calcium" measurements, a second buffer consisting of 2 mM $CaCl_2$, 10 mM TES, and 150 mM NaCl, pH 7.4 was used. Both buffers were adjusted to pH 7.4 using sodium hydroxide and filtered at 50 nm.

### 2.3. Vesicle preparation

Stock lipids were prepared at 5 mM final concentration by adding the appropriate amounts of lipids to an amber glass vial and drying under a gentle stream of argon until a thin lipid film formed. DEPC (phase transition temperature of 13 °C) was stored in chloroform; DMPG (phase transition temperature of 23 °C) was stored in 65/35/2 volume ratio of chloroform–methanol–water (solvent mixture suggested by Avanti Lipids technical support). Dried lipid samples of the appropriate molar ratios (DEPC/DMPC at 95/5, 90/10, 80/20, 70/30, 60/40 and 50/50) were then put into a rotary evaporator and heated at 50 °C under a vacuum for an hour to remove residual chloroform. Samples were continuously dried under a vacuum overnight. Afterward, samples were stored under argon at −20 °C until needed. Just prior to use, lipids were rehydrated in 4 mL of the appropriate buffer with or without calcium ions and allowed to equilibrate for at least 45 min with periodic mixing. Lipid samples were then sonicated at 28–35 °C for 20 min using a Misonix (Farmingdale, NY) Sonicator-3000 (at 60% power; pulse set at 30 s on, 15 s off; power output at $12 \pm 3$ W) equipped with a microtip. This formed small, unilamellar vesicles (SUVs). After sonication, SUVs were centrifuged to remove any titanium particulates that come from the tip. Vesicles were determined to have a diameter in the range of 24–35 nm using a Nicomp Submicron Particle Sizer (Particle Sizing Systems, Inc., Santa Barbara, CA). Before use, stock 5 mM vesicles were diluted to 0.125 mM for each measurement.

### 2.4. Silicon dioxide substrate preparation

Crystals were cleaned by exposure to ultraviolet (UV) light and ozone for 10 min cycles per side in a UV/ozone tip cleaner (Bioforce Nanosciences; Ames, IA) [22]. UV-ozone exposure of the crystal was followed by treatment with 2% (m/v; ~69 mM) of sodium dodecyl sulfate in nanopure water for 30 min [23]. Crystals were rinsed thoroughly with nanopure water followed by ethanol. Crystals were dried under a stream of high-purity nitrogen after the rinses and exposed to a second round of UV-ozone exposure just prior to use.

### 2.5. Quartz crystal microbalance with dissipation monitoring

Monitoring of vesicle adsorption onto various surfaces was accomplished using a QCM-D300 electronic system with axial flow (Q-Sense, Inc., Västra Frölunda, Sweden) [24]. As material is adsorbed to the crystal surface, a negative shift in the resonance frequency of the crystal is observed. This frequency shift (Δf) thus correlates with the additional mass attached to the sensor crystal. A change in the dissipation (ΔD) of the resonance frequency is also observed because the adsorbed layer of mass not only causes a shift in the resonance frequency, but also causes the resonance frequency to lose energy through frictional losses. When the adsorbed mass is "soft", there is a large energy loss in the resonance frequency. Therefore, dissipation is high (typically $>1\times10^{-6}$). When the adsorbed mass is stiffly bound, the energy loss in the resonance frequency is low (resulting in low dissipation, $<1\times10^{-6}$).

The system was initiated by passing excess buffer (total volume ≥ 3 mL) through a preheated loop (28 °C) and allowing the buffer to thermally equilibrate while acquiring frequency and dissipation changes in air. Once the signal remained stable for several minutes (Δf < 1 Hz) in air, a total of 1.5 mL of buffer was passed over the sensor crystal to ensure all air was forced out of the crystal chamber. Again, the system was equilibrated and maintained a stable signal for several minutes with the desired buffer. After system stabilization, measurements of Δf and ΔD during vesicle adsorption were performed in exchange mode (approximately 0.5 mL of temperature-stable sample liquid was passed through the loop and over the crystal to replace the buffer in the chamber of the sensor crystal) [6,8] with acquisition of 5 MHz and the 3rd, 5th, and 7th overtones (15, 25, 35 MHz). All measurements were conducted at $28.5 \pm 0.8$ °C (above the phase transition temperature of DMPG).

The response of the resonance frequency of the quartz crystal microbalance depends on the total oscillating mass adsorbed to the sensor surface. Adsorption of solutes from the contacting buffer medium results in a decrease in frequency. If the attached mass is thin (≪300 μm) in comparison to the crystal and rigid, then the mass

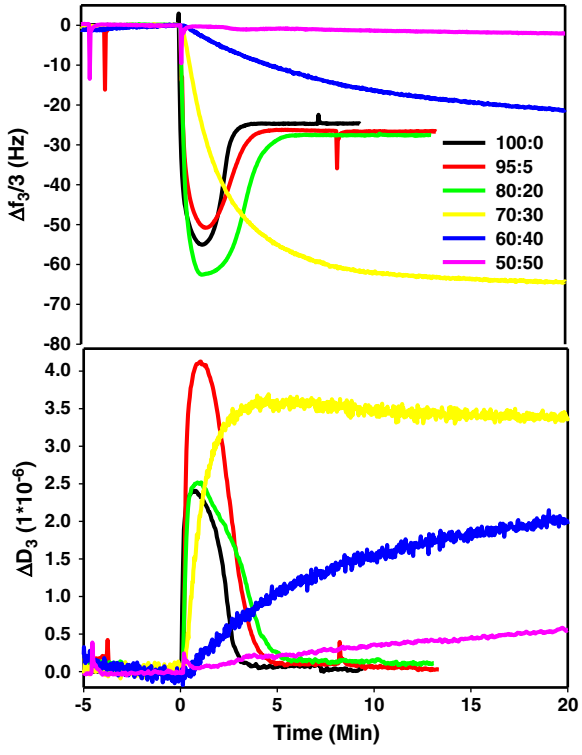


**Fig. 1.** Changes in QCM-D response for the 3rd overtone frequency, 15 MHz, (top) and its corresponding dissipation (bottom) for the adsorption of DEPC:DMPG SUVs at 100:0 (black line), 95:5 (red line), 80:20 (green line), 70:30 (yellow dotted line), 60:40 (blue line), and 50:50 (pink line) molar ratios onto silica in the presence of 2 mM $CaCl_2$, 10 mM TES, and 150 mM NaCl at pH 7.4.

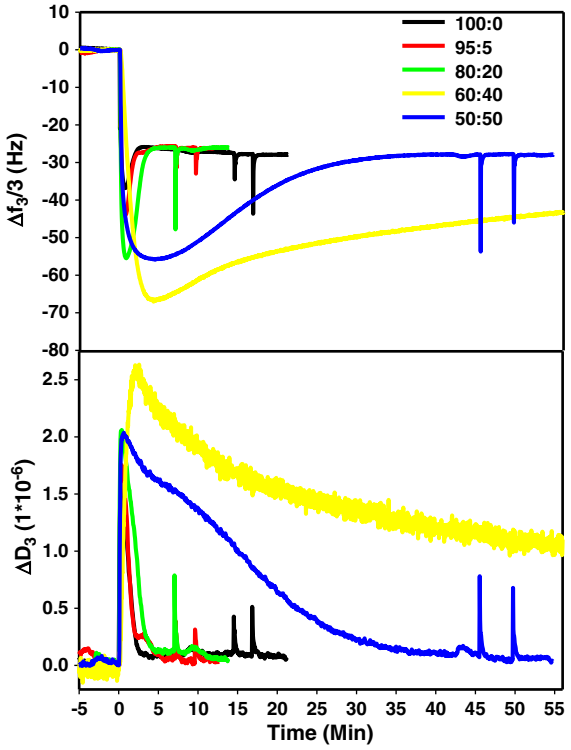


**Fig. 2.** QCM-D response during adsorption of DEPC:DMPG vesicles onto silica in "calcium-free" buffer.

can be calculated by the Sauerbrey equation [25]. The Sauerbrey equation is $\Delta m = -C \times \Delta f/n$, where $C = 17.7$ ng/cm$^2$/Hz for a 5 MHz sensor crystal, $\Delta f$ is change in frequency and n is the overtone number (1 for 5 MHz, 3 and 5 for the 3rd and 5th, overtones respectively). This calculation of adsorbed mass has been demonstrated to be valid for thin lipid bilayers and to just slightly underestimate the mass (~5%) for adsorbed unruptured vesicles of approximately 25 nm in thickness [6,13].

### 2.6. Atomic force microscopy

Images were obtained using a Nanoscope-IV controller equipped with an E-scanner (15 μm). All measurements were conducted in buffer solution. Images were captured in tapping mode. Prior to use, the fluid-flow cell (tubing and O-ring included) was washed by sonication in a mixture of ethanol and nanopure water at 50/50 (v/v) ratio for 5 min. The flow cell assembly was subsequently rinsed thoroughly with nanopure water and dried using a stream of pure nitrogen. Images were acquired with a scan rate of 1–2 Hz and plane-fitted. Tip interactions with adsorbing vesicles were minimized by using a poly(ethylene) glycol (PEG) coated tip [6]. The tip was modified with PEG (8000 kDa) by immersing in a 3% (v/v) PEG solution for 1 h and rinsed thoroughly with nanopure water prior to use (tip was UV/ozone cleaned for 10 min prior to soaking in the PEG solution).

Silica wafers for the atomic force microscopy (AFM) measurements were purchased from Wafer World, Inc. (West Palm Beach, FL) and cut to fit (using a diamond scribe) onto steel pucks. The silica was attached to the pucks with epoxy and allowed to cure (at least 15 min). These silica wafers were then cleaned in the same manner as the QCM-D silica crystals (Section 2.4).

## 3. Results and discussion

### 3.1. Results in the presence of calcium ions

To establish the basic QCM-D properties of the DEPC/DMPG supported bilayer, two parameters were examined: 1) vesicle adsorption and 2) bilayer formation on the $SiO_2$ surface, which is known to interact with lipid vesicles to form a supported bilayer [6,9,11,13,26]. Fig. 1 displays the typical frequency and dissipation shifts for vesicles interacting with a $SiO_2$ surface in the presence of 2 mM calcium ions. Vesicles containing up to 30 mol% DMPG readily adsorbed to the surface. With up to 20 mol% DMPG present, the vesicles exhibited a two-step adsorption process similar to that observed with other phosphatidylcholine/anionic lipid systems in buffer solutions containing 2 mM calcium ions [6,8,16]. A maximal shift in the frequency (~50 to 63 Hz) occurred initially, followed by a reduction in the frequency to produce a final shift of 25 to 27 Hz (Fig. 1, top; Table 1). This frequency shift pattern is typical of vesicles that are adsorbed up to a maximum amount onto $SiO_2$ (called critical mass coverage of the surface) just prior to rupturing and forming a single thin bilayer (second step of process) [4,6–9,11,13,16,26–28]. The dissipation values also reflect a two-step process (Fig. 1, bottom) that is consistent with "soft" lipid material ($\Delta D > 1 \times 10^{-6}$) adsorbing to the sensor surface and converting to a more rigid layer ($\Delta D < 1 \times 10^{-6}$) [4,6–9,11,13,16,26–28] as water is expelled from the vesicles on the surface. The Sauerbrey equation is valid for rigid adsorption ($\Delta D < 1 \times 10^{-6}$), and applying the model over the final frequency shift found that a mass equivalent of ~450 to 500 ng/cm$^2$ (Table 1) was adsorbed on the surface. This result was consistent with literature values for a single hydrated phospholipid bilayer [6,13]. A slight increase in adsorbed mass was observed after the vesicles ruptured and formed a SPB (Table 1) as the mole percent of DMPG increased (up to 20 mol%). Two factors may account for this increase in adsorbed mass with increasing percent of DMPG. First, phosphatidylglycerol is more hydrated than

**Table 1**
QCM-D parameters for vesicle adsorption or SPB formation on silica. Values are in the average of 2–3 experiments.

| Lipid mol.% | | $Ca^{+2}$ (mM) | $\Delta f_3/3$ (Hz) | $\Delta D_3$ ($10^{-6}$) | Mass per area (ng/cm$^2$) | $t_{SLB}$ (min) |
|---|---|---|---|---|---|---|
| DEPC | DMPG | | | | | |
| 100 | 0 | 2 | −25.0 ± 0.3 | 0.25 ± 0.23 | 442 ± 6 | 2.12 ± 1.58 |
| 95 | 5 | 2 | −26.0 ± 0.9 | −0.04 ± 0.24 | 461 ± 15 | 4.91 ± 0.58 |
| 80 | 20 | 2 | −27.3 ± 0.4 | 0.14 ± 0.12 | 483 ± 6 | 4.16 ± 1.54 |
| 70 | 30 | 2 | −70.5 ± 4.95 | 3.49 0.25 | 13587 ± 362[a] | – |
| 60 | 40 | 2 | −62.60 ± 6.66 | 4.34 ± 0.84 | 13534 ± 1541[a] | – |
| 50 | 50 | 2 | −5.1 ± 3.1 | 0.82 ± 0.20 | 1208 ± 104[a] | – |
| 100 | 0 | 0 | −27.5 ± 0.5 | 0.14 ± 0.09 | 486 ± 9 | 2.51 ± 0.28 |
| 95 | 5 | 0 | −26.3 ± 0.1 | 0.11 ± 0.07 | 475 ± 17 | 2.17 ± 0.28 |
| 80 | 20 | 0 | −26.5 ± 0.7 | 0.05 ± 0.004 | 470 ± 12 | 3.27 ± 0.71 |
| 70 | 30 | 0 | – | – | – | –[b] |
| 60 | 40 | 0 | −30.7 ± 4.4 | 0.40 ± 0.32 | 539 ± 76 | >60 |
| 50 | 50 | 0 | −26.9 ± 1.6 | 0.09 ± 0.03 | 476 ± 29 | 20.96 ± 8.56 |

[a] Voigt mass calculation.
[b] Adsorption not detected.

phosphatidylcholine, which results in an increased amount of bound water molecules [17]. Second, salt (calcium and/or sodium) ions are able to penetrate deep into the bilayer and bind to lipid molecules [29]. However, it is unclear which one would take precedence over the other.

Also, it was observed that the time ($t_{SPB}$) for a rigid bilayer to form (Table 1) increased as the amount of DMPG increased, consistent with prior systems [6]. An increase in the $t_{SPB}$ indicates two steps are occurring. First, vesicles are remaining intact on the surface longer as the percentage of DMPG present increases. Second, a higher critical surface coverage of vesicles is required before a SPB will form as DMPG concentration increases.

At 30 mol% DMPG, vesicles began to slowly adsorb onto the surface, taking approximately 10 min to stabilize. This is shown by a shift in dissipation values to well above 2 which is indicative of a highly dissipative system, and can be interpreted as vesicles adsorbing and remaining intact on the surface. At 40 mol% DMPG, vesicle adsorption required at least 2 h for the frequency shift to stabilize to levels approximately the same as those achieved by vesicles containing 30 mol% DMPG (no rupture was detected up to 16 h — data not shown). When the vesicles contained 50 mol% DMPG, only a slight frequency shift was observed after 20 min. After an hour, enough vesicles were adsorbed to give a total mass on the silica of about 100 ng/cm$^2$ using Sauerbrey analysis (~1200 ng/cm$^2$ using Voigt analysis). Furthermore, the change in dissipation values showed a slow increase over time, indicating that the adsorbing mass was not well coupled to the oscillations of the crystal (well-coupled mass exhibits low dissipation values). Thus, the indications are that few vesicles were adsorbed, which is consistent with previous studies that showed that vesicles containing 20 to 50 mol% of an anionic lipid were not adsorbed well. Lack of a critical mass of vesicles containing up to 50 mol% DMPG was enough to slow and reduce bilayer formation [6,8], presumably by electrostatic repulsion between the anionic lipids and the negatively charged silica surface. At 30 to 50 mol% DMPG, dissipation and frequency shifts for all frequencies exhibited clear separation which is consistent with highly dissipative adsorption. Therefore, this further suggests that vesicles were a highly viscoelastic system and require mass estimates to be determined using Voigt analysis (Table 1); (Sauerbrey analysis underestimates the mass values of a highly viscoelastic system).

### 3.2. Results in the absence of calcium ions

To better understand the details of SPB formation by DEPC/DMPG vesicles on silica, the effects of DMPG concentration were studied in the absence of $Ca^{2+}$. In the "calcium-free" buffer, DEPC/DMPG SPB formation exhibited the typical two-step process, that is, adsorption of vesicles to a critical mass and then rupture to form a bilayer (Fig. 2), as observed for most lipid systems on silica [4,6–9,11,13,16,26–28]. As in the case with calcium ions present, SPB formation in the absence of calcium occurred with a similar final Δf in the range of 26 to 31 Hz (in the presence of 2 mM calcium ions the final frequency shift was 25 to 27 Hz). Additionally, and the final ΔD remained below one indicating a rigid adsorbed layer, and the final adsorbed mass was 470 to 540 ng/cm$^2$ (450 to 500 ng/cm$^2$ in the presence of $Ca^{2+}$), and the time for SPB formation ($t_{SPB}$) increased as the amount of DMPG increased (Table 1). However, SPB formation occurred at 50 mol% DMPG, which was not observed previously on silica for a lipid system with the same mole percent of an anionic lipid in the absence of $Ca^{2+}$ [6].

To verify SPB formation from DEPC/DMPG at 50/50 molar ratio, AFM images were taken under the same conditions (calcium-free buffer and silica surface). After the vesicles were injected into the fluid cell and incubated for 15–25 min, the vesicles were observed adsorbing clustered together on the surface with a height of approximately 8 nm (Fig. 3, white arrow). This height is well within the height range of vesicles that are in the act of fusing to form a SPB on a surface [6].

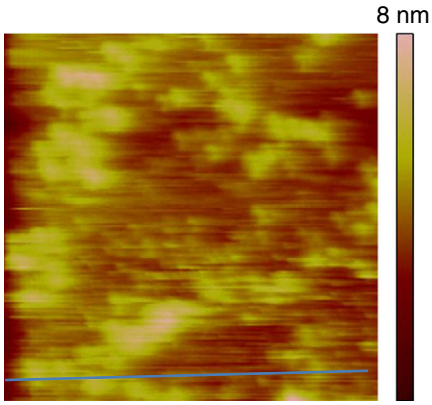


**Fig. 3.** AFM tapping-mode "snapshot" height image of 50:50 DEPC:DMPG vesicles adsorbed on a silica wafer and fusing to form a SPB in "calcium-free" buffer after rinsing. Exposure time was 15 min. SUV concentration was 0.125 mM. Image size was 1.3 μ. Vesicles (circular objects) and bilayers (flat areas) were observed.

Several reasons might explain the unexpected SPB formation on silica of DEPC/DMPG at 1/1 molar ratio in solution in the absence of calcium ions. One explanation may be that phosphatidylglycerol (PG) lipids may overcome the electrostatic repulsion of the headgroups and form stable ion-lipid clusters in the presence of NaCl as shown in molecular dynamic simulations for 1-palmitoyl-2-oleoyl phosphatidylglycerol [30]. The PG cluster may allow areas somewhat devoid of negative charges to form and these areas may interact favorably with the silica. Second, TES may facilitate supported phospholipid bilayer formation and stability on silica, as it has been demonstrated by other researchers that buffers influenced the pathway and kinetics of SPB formation differently [31]. In the presence of 2 mM calcium ions, DEPC/DMPG vesicles containing 30 or more mol% of DMPG did not form a SPB possibly because the calcium ions somehow disrupt the PG clusters and the stabilizing interaction of TES. With the PG clusters destabilized, the anionic lipids can redistribute such that the electrostatic interactions between the vesicles and the silica surface are strong enough that only a few vesicles are adsorbed onto the surface. Thus, no critical surface coverage of vesicles was obtained and no SPB was formed within the measurement times in this work. Greater packing in the hydrophobic region of the bilayer may have further enhanced the poor vesicle adsorption on silica in the presence of calcium ions for DEPC and DMPG that have phase transition temperatures at 13 and 23 °C, respectively. A similar affect on $t_{SPB}$ for bilayer formation on silica was demonstrated by Viitala et al. for vesicles composed of 80 mol% 1-palmitoyl-2-oleoyl phosphatidylcholine and 20 mol% L-α-phosphatidylglycerol (PG) [17].

In addition, other considerations may explain the SPB formation of DMPG-doped vesicles. The SPB behavior demonstrated here may be specific to silica surfaces that have been layered to 50 nm [17]. However, this is not likely, since the AFM image was captured on a silicon wafer several microns thick. Another explanation may be that the amount of SDS (~69 mM) used to clean the crystals somehow altered the surface. However, the SDS concentration used in this work is well-below the amount (0.1 M) recommended by Cho et al. for cleaning surfaces for SPB formation studies (0.1 M) [31]. Additionally, unpublished data exploring DOPC and 80/20 molar ratio DOPC/DOPS vesicle adsorption resulted in net mass adsorption comparable to published data [6,13], suggesting that such surface alterations do not change lipid adsorption behavior. Therefore, the adsorption behavior of DMPG-doped SUVs is more likely specific to the presence of PG.

## 4. Conclusions

By using a QCM-D and AFM, characterization of the absorption behavior for DEPC/DMPG on a silica surface was demonstrated. Vesicles containing 0 to 50 mol% DMPG were adsorbed onto silica and formed SPBs in the absence of $Ca^{2+}$. In the presence of 2 mM $Ca^{2+}$, vesicles containing 30 or more mol.% DMPG adsorbed onto the silica surface but did not form SPBs. This suggests that $Ca^{2+}$ is not required to form a SPB from a mixture of DEPC and DMPG lipids, which is opposite to the findings of similar previous studies using other anionic lipids, such as phosphatidylserine, where $Ca^{2+}$ was required for SPB formation. The ability of vesicles containing phosphatidylglycerol (PG) to form SPB in the absence of $Ca^{2+}$ appears to be specific to phosphatidylglycerol. Direct comparison of QCM-D and AFM demonstrated SPB formation on silica in the absence of calcium ions. The combined use of these in situ techniques provided helpful insight into vesicle fusion and SPB formation of a lipid system that has potential use as a biomimetic interface in biocatalysis.

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