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Fabrication of Charged Membranes by the Solvent-Assisted Lipid Bilayer (SALB) Formation Method on SiO_2 and Al_2O_3

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Abstract

In this study, we employed the solvent-assisted lipid bilayer (SALB) formation method to fabricate charged membrane on solid supports. The SALB formation method exploits a ternary mixture of lipid/alcohol/ aqueous buffer, to deposit lamellar phase structures on solid supports upon gradual increase of buffer fraction. Using quartz crystal microbalance with dissipation (QCM-D) technique, we investigated formations of negatively and positively charged membranes via SALB formation method and directly compared with vesicle fusion method on two different oxide films. Bilayers containing an increasing fraction of negatively charged DOPS lipid molecules were successfully formed on both SiO_2 and Al_2O_3 substrate using SALB formation method at physiological pH (7.5). In contrast, the vesicle fusion method did not support bilayer formation on Al_2O_3 and those containing more than 10% DOPS ruptured on SiO_2 only in acidic condition (pH 5). Characterization of the fraction of negatively charge DOPS by in-situ annexin 5A binding assay, revealed that the fraction of DOPS lipid molecules in the bilayers formed on Al_2O_3 is significantly higher than that of formed on SiO_2. This suggests that SALB self-assembly of charged membranes are predominantly governed by the electrostatic interaction. Further, our finding indicates that when multicomponent lipid mixtures are used, the relative fraction of lipids in the bilayer may differ from the fraction of lipids in the precursor mixture.

Introduction

Lipid membranes supported on solid substrates are reductionist platforms which enable studying a wide range of biomembrane-related processes in a rather controlled and simple environment instead of otherwise complex native cell membranes[1, 2]. The surface immobilization of membranes makes them compatible with a range of surface-based analytical techniques such as atomic force microscopy (AFM)[3], total internal reflection microscopy (TIRF)[4], quartz crystal microbalance with dissipation (QCM-D)[5] and

impedance spectroscopy[6]. Despite strong association with the substrate, supported bilayers retain lipid lateral mobility and are capable of hosting membrane-active biomolecules[7, 8]. Vesicle fusion (VF) which involves adsorption and spontaneous rupture of small unilamellar vesicles (≤100 nm diameter) on a solid support is a widely used method to fabricate supported bilayers[9]. However the vesicle fusion method is mainly suitable for use on silica-based materials such as glass and mica[10, 11], as other important surfaces such as Au[12, 13], TiO_2[14] and Al_2O_3[15] are intractable to vesicle fusion. Moreover, application of vesicle fusion is limited to a subset of lipid compositions. For instance, vesicles containing high fractions of cholesterol do not rupture spontaneously on solid supports[16]. These limitations are caused by the high adhesion energy required for vesicles to rupture[17]. In some cases, these limitations can be overcome by optimizing environmental conditions such as solution salinity[18], pH[19], temperature[20], osmotic shock[21], and addition of divalent cations[22]. In addition, to bypass the limiting step of vesicle rupture, other self-assembly methods which do not require lipid vesicles have been developed including: air bubble collapse[23], spin-coating[24], and solvent assisted bilayer (SALB) formation method[25]. The latter has been applied to SiO_2 and surfaces which are intractable to vesicle fusion such as Al_2O_3, Cr, Tin-Oxide, and Au[25-27]. In addition, highly cholesterol rich bilayers have been successfully prepared by SALB formation method[16, 28]. In this method, lipids dissolved in a water-miscible organic solvent (e.g., isopropanol) are first introduced and adsorb to the solid support, then the solvent is gradually replaced by aqueous buffer. As the fraction of water increases; the ternary mixture of lipid/isopropanol/water undergoes several phase transitions, leading to the formation of lamellar phase structures in the bulk solution and assembly of planar bilayer on the solid substrate[29]. The self-assembly on the surface is complicated but it is likely that the surface-adsorbed lipids in the presence of pure organic solvent act as nucleation sites for the formation and growth of complete bilayer. So far SALB formation method has been mainly used to prepare neutral membrane using zwitterionic lipid composition (e.g, Phosphatidylcholines). However, biological membranes are composed of a wide variety of phospholipids[30], including those with negatively charged headgroups such as phosphatidylserines which play key roles in cell signaling and apoptosis[31] along with regulating enzymatic activities (e.g., phospholipase A_2[32]). Cationic bilayers are also important and have been used to develop drug delivery vesicles[33]. In this work, we examined the formation of supported bilayers containing increasing concentrations of anionic (DOPS) and cationic (DOEPC) lipids on SiO_2 and Al_2O_3, using SALB formation method and compared the results with conventional vesicle fusion method.

Materials and Methods

Lipid preparation

Zitterionic lipid, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), cationic lipid 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (chloride salt) (DOEPC) and anionic lipid 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt) were purchased from Avanti Polar Lipids (Alabaster, AL). To prepare the lipid samples for vesicle preparation, chloroform solutions of lipids with desired composition were first dried using a gentle stream of nitrogen gas and subsequently stored under vacuum overnight to remove traces of chloroform. Vesicles were prepared in tris buffer (10 mM Tris, 150 mM NaCl, pH 7.5), via extrusion through 50 nm diameter, track-etched polycarbonate membranes as previously described[34]. For SALB experiments, the lipid powders were first dissolved in isopropanol. The stock solutions were mixed to gain the desired composition and diluted to a 0.5 mg/ml lipid concentration. To improve the solubility of negatively charged lipids, the lipid powder was first dissolved in ethanol at 40 °C at a nominal lipid concentration of 1 mg/ml, and then diluted in isopropanol.

Substrate preparation

QCM-D substrates with different coatings (Q-sense AB, Gothenburg, Sweden) were cleaned by immersion in 10% sodium dodecyl sulfate (SDS) followed by extensive rinsing with Milli-Q water (Millipore) and drying with N_2. All samples were treated with oxygen plasma at 180 W for 30 sec (March Plasmod Plasma Etcher, March Instruments, California) immediately before use.

Quartz Crystal Microbalance-Dissipation (QCM-D)

A Q-Sense E4 (Q-Sense AB, Gothenburg, Sweden) set up was used to monitor the lipid deposition process in real time and to evaluate the viscoelastic properties of the lipid film. The frequency and dissipation changes of an AT-cut piezoelectric quartz crystal resonating at 5 MHz were followed at 3, 5, 7, 9 and 11 overtones. All measurements were performed under flow-through conditions at a flow rate of 100 μL/min, using a peristaltic pump (Ismatec Reglo Digital M2-2/12). The temperature of the flow cell was fixed at 24.0 ± 0.5°C. If not mentioned otherwise, all the QCM-D data presented here was measured at the third overtone (15 MHz) and normalized based on the overtone number. QCM-D experimental data collected at the 3rd, 5th, 7th, and 9th overtones were analyzed by using the Sauerbrey model, which is valid for rigid films ($\Delta D/\Delta f < 1 \times 10^{-8}$ Hz^{-1}) as previously reported[35]. Details of QCM-D principles and operation can be found elsewhere[36].

Results and Discussion

Using the SALB formation method, we first examined the formation of DOPC/DOPS lipid bilayers with varying fraction of DOPS on SiO_2 and Al_2O_3. **Fig.1a and b** exhibit the time course of QCM-D frequency

(Δf, top panels) and dissipation (ΔD, bottom panels) shifts during SALB formation and subsequent annexin A5 binding on SiO_2 and Al_2O_3, respectively. As depicted in **Fig.1a**, a typical SALB formation experiment involved the following steps: base line recording in the aqueous buffer (10 mM Tris, 150 mM NaCl, pH 7.5) solution (arrow 1), injection of the organic solvent (arrow 2), addition of lipid mixture in organic solvent (arrow 3) and solvent exchange back to the initial buffer solution (arrow 4). Similar steps were performed using lipid mixture of DOPC with increasing fraction of DOPS (10-40 mol %).

For all lipid compositions, in both SiO_2 and Al_2O_3, the average final frequency shifts were in the range of 24-26 Hz and the dissipations were less than 0. 3×10^{-6} (detailed values are summarized in **Fig. 1e**). These results are in agreement with previous QCM-D shifts observed upon formation of planar bilayers[35], typically yielding a Δf of around −25 Hz and ΔD of less than $<0.2 \times 10^{-6}$. In order to check the integrity of bilayers, we used bovine serum albumin (BSA) protein binding as a probe. Since planar bilayers are resistant to nonspecific adsorption of proteins such as BSA, the adsorption of protein to the defect free bilayer would be negligible[37]. A solution of BSA protein (0.1 mg/ml) (**arrow 5, Fig. 1a**) was injected which led to a change in frequency of $\leq$ 1 Hz indicating presence of no or negligible defects in the bilayers. Previously, the SALB formation method has been used to fabricate zwitterionic POPC lipid bilayers on Al_2O_3[26]. Noticeably, a QCM-D frequency change of around -36 Hz was reported for such bilayers, which is significantly higher than that obtained for POPC lipid bilayers on SiO_2 (~ -25 Hz). This was assigned to be due to a thicker hydration layer and therefore greater hydrodynamically-coupled mass for bilayers on Al_2O_3. In addition, the thickness of a supported zwitterionic lipid bilayer on Al_2O_3 was determined to be around 6.5 nm, as measured by AFM[38] which is nearly 2 nm thicker than that of a bilayer on SiO_2 (~4.5 nm)[39]. Interestingly, in the present study, negatively charged lipid bilayers on Al_2O_3 did not exhibit appreciably thicker hydration layers, suggestive of an interplay between the hydration force and additional interfacial forces[40]. Indeed, this difference could be due to the fact that the surface charge of Al_2O_3 is slightly positive at pH 7.5, and therefore electrostatic attraction between the anionic bilayer and positively charged substrate overcomes the repulsive hydration force, resulting in a thinner hydration layer for anionic lipid bilayers in comparison to zwitterionic lipid bilayers on Al_2O_3.

To evaluate presence of DOPS lipid molecules in the bilayers, a solution of 5 µg/ml annexin A5 (**arrow 6, Fig. 1a**) in the presence of 2 mM Ca^{2+} (10 mM Tris, 150m M NaCl, 2 mM $CaCl_2$, pH 7.5) was added to the bilayers. Annexin A5[41] is a water-soluble protein which in the presence of Ca^{2+} ions, binds specifically to negatively charged phospholipids[42]. Injection of annexin 5A resulted in a decrease in the resonance frequency, suggesting an increase in the mass of membrane due to the adsorption of the protein (see insets in **Fig. 1a and b** for magnified view of frequency change upon annexin 5A injection). However, the dissipation did not change during annexin 5A binding, indicating tightly packed structure of protein

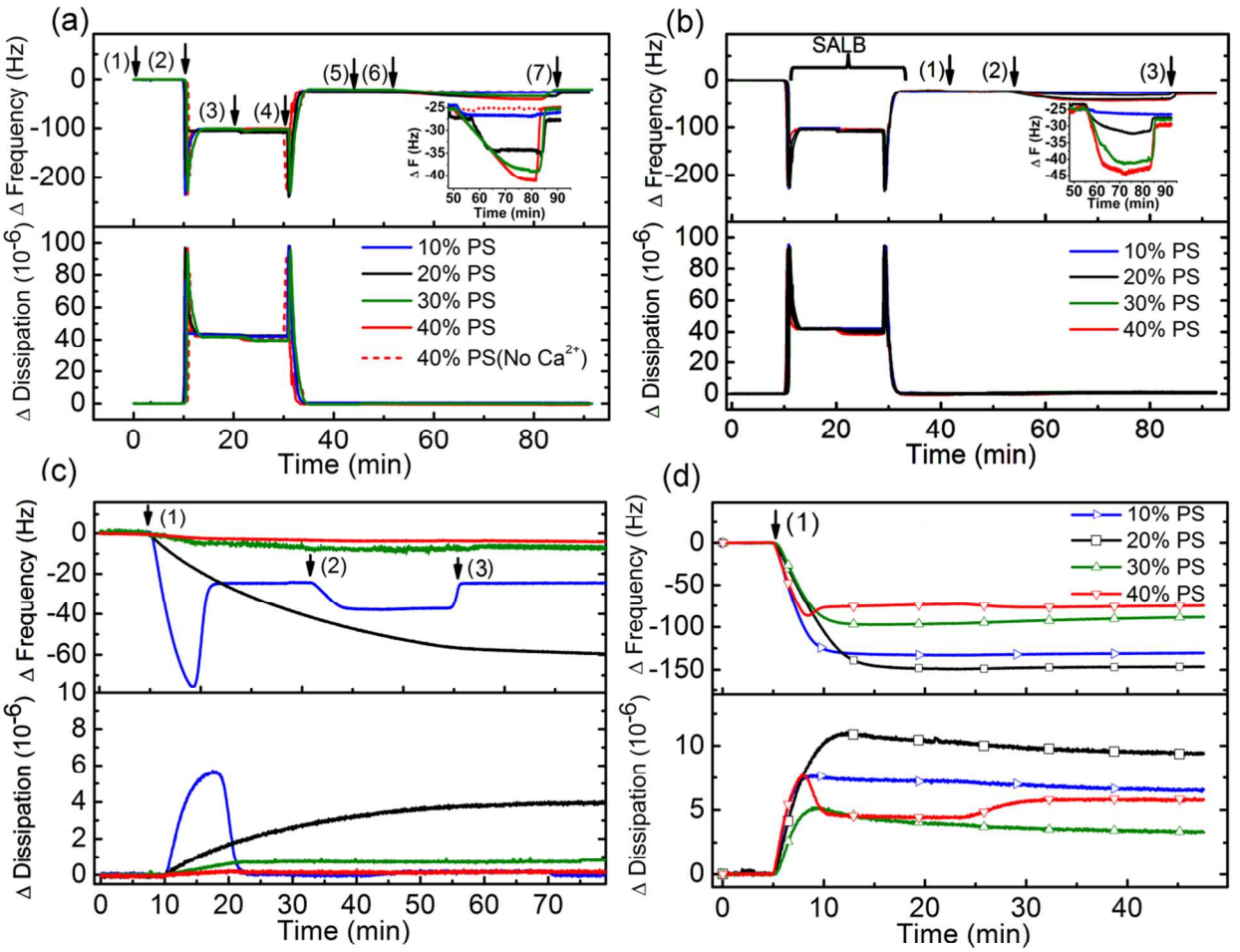
molecules on the bilayer surface. To ensure that protein binding was specific (i.e. mediated by Ca^{2+} ions), buffer with no calcium was injected after protein binding (**arrow 7, Fig. 1a**). Immediately after injection of buffer with no Ca^{2+}, the frequency increased and stabilized at the same value as before protein binding step, indicating detachment of annexin 5A from the bilayer surface. As for control experiment, annexin 5A was injected in the absence of Ca^{2+} ions and no change in frequency was observed as we expected[42] (see dotted line in **Fig. 1a**).

For comparison, we also attempted to prepare DOPC/DOPS membranes on the same substrates through conventional VF method. For VF experiments, DOPC lipid vesicles (ca. 70 nm in size) containing varying fraction of DOPS (10-40 mole %), were added onto SiO_2 or Al_2O_3 surfaces. On SiO_2, the adsorption of vesicles with 10 mol% DOPS, at pH 7.5, shows a two-step kinetic behavior with final QCM-D frequency and dissipation of -24.7 ± 0.7 Hz and $0.5 \pm 0.2 \times 10^{-6}$ respectively, corresponding to the formation of planar bilayer[12]. However, at 20% DOPS, upon injection of vesicles, QCM-D shows a simultaneous monotonic decrease in frequency and increase in energy dissipation change due to vesicle adsorption until it reaches the final Δf and ΔD of about -60 Hz and 4×10^{-6}, suggesting that vesicles did not rupture and remained intact[12]. Vesicles remain intact as the adhesion energy between negatively charged vesicles and negatively charged SiO_2 is sufficient for vesicle adsorption but not to induce rupture. The adsorption of vesicles containing higher fraction of DOPS (30 and 40%) were almost negligible due to stronger electrostatic repulsion between negatively charged vesicles and the SiO_2 surface, which is in good agreement with previous observations[43].

It has been previously shown that pH of the buffer has great effect on the vesicle-vesicle as well as vesicle-substrate interaction by altering the vesicle and substrate surface charge[10, 19]. To reduce the negative surface charge of DOPS containing vesicles, pH of the solution was lowered from 7.5 to 5. Changing the pH to 5 also reduces the negative surface charge of SiO_2 surface[44]. Kinetics of DOPC/DOPS vesicle adsorption on SiO_2 at pH 5 is shown in supporting **Fig. S1a**. A two-step kinetic behavior indicating initial vesicle adsorption followed by rupture, after reaching a critical coverage, was observed for all vesicle compositions examined. Noticeably, with increasing DOPS fraction of vesicles, the kinetics of vesicle adsorption and subsequent rupture became slower due to increasing repulsion among vesicles and substrate. Interestingly, the final frequency change was found to increase with increasing DOPS fraction. The higher frequency is indicative of larger amount of mass coupled to the surface. Since the dissipation is low, the extra mass cannot be due to the presence of intact vesicles. One possible explanation would be that the spacing between the substrate and the charged membrane may increase due to electrostatic repulsion between membrane and substrate. Therefore the extra mass detected by QCM-D might account for the thicker hydration layer between the membrane and substrate at higher DOPS fractions.

The subsequent injection of annexin 5A to DOPC/DOPS bilayers prepared in this condition was performed after exchange to pH 7.5 buffer in the presence Ca^{2+} (supporting **Fig. 1a, arrow 6**), which resulted in protein binding in a charge dependent manner.

In agreement with previous reports[26, 45], vesicles independent of the DOPS fraction did not rupture on Al_2O_3 (**Fig. 1d**), indicating that the adhesion energy between vesicles and the substrate is insufficient to trigger vesicle rupture. The rupture of vesicles on Al_2O_3 is hindered by the strong repulsive hydration force between membrane and the solid surface[26] due to the highly hydrated state of Al_2O_3 in aqueous environments[46].



(e)

Method	Surface	Composition	Δ*f* (Hz)	Δ*D* (x 10⁻⁶)	Method	Surface	Composition	Δ*f* (Hz)	Δ*D* (x 10⁻⁶)
SALB	SiO_2 pH 7.5	10% PS	24.4 ± 0.2	< 0.2	Vesicle Fusion	SiO_2 pH 7.5	10% PS	24.8 ± 0.4	< 0.2
		20% PS	25.5 ± 0.2	< 0.1			20% PS	60 ± 5	~ 5
		30% PS	24.5 ± 1.5	< 0.1			30% PS	10 ± 1	~ 1
		40% PS	25.5 ± 0.5	< 0.2			40% PS	5 ± 1	~ 0.5
	Al_2O_3 pH 7.5	10% PS	24.8 ± 0.8	< 0.3		Al_2O_3 pH 7.5	10% PS	133 ± 7	~ 7
		20% PS	24.5 ± 1.5	< 0.1			20% PS	147 ± 6	~ 10
		30% PS	25.5 ± 0.5	< 0.2			30% PS	95 ± 5	~ 5
		40% PS	23 ± 1	< 0.1			40% PS	75 ± 5	~ 5

Fig. 1 QCM-D monitoring of DOPS (anionic) containing supported bilayer and subsequent annexin A5 binding on two different substrates; SiO_2 and Al_2O_3. (a) Temporal shifts in frequency, Δ*f* (top) and energy dissipation, Δ*D* (bottom) during SALB formation of DOPC with increasing DOPS fraction (10-40 mol %) on SiO_2. Arrows indicate the injection of (1) tris buffer (10 mM Tris, 150 mM NaCl, pH 7.5), (2) isopropanol, (3) lipid mixture (0.5 mg/mL DOPC/DOPS lipid in isopropanol), (4) tris buffer solution, (5) BSA solution (0.1 mg/ml), (6) annexin 5A (5 µg/ml in10 mM Tris, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.5), and finally, (7) tris buffer. The dashed curve in panel A represents the control experiment in which Ca^{2+} excluded from annexin A5 solution. The inset in panel A represents the magnified view of frequency change during annexin 5A binding (step 6). (b) Change in QCM-D frequency and energy dissipation response for SALB formation method of DOPS containing bilayer on Al_2O_3. The baseline was established in tris buffer (10 mM Tris, 150 mM NaCl, pH 7.5), followed by identical steps as in (a). Arrows (1-3) correspond to injection of BSA, annexin 5A and tris buffer solution respectively. (c) The adsorption kinetics of vesicles composed of DOPC/DOPS at varying fraction (10-40 mol %) onto SiO_2 at pH 7.5. Arrows indicate the injection of (1) vesicles in tris buffer (10 mM Tris, 150 mM NaCl, pH 7.5), (2) annexin 5A solution (5 µg/ml in 10 mM Tris, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.5) and (3) buffer wash (10 mM Tris, 150 mM NaCl, pH 7.5). (d) Kinetics of DOPS containing vesicle

adsorption to Al_2O_3. The arrow indicates injection of 0.1 mg/ml vesicles in tris buffer (10 mM Tris, 150 mM NaCl, pH 7.5). (e) Summary of QCM-D frequency and dissipation shifts after formation of bilayer on SiO_2 and Al_2O_3 via SALB formation method and vesicle fusion method.

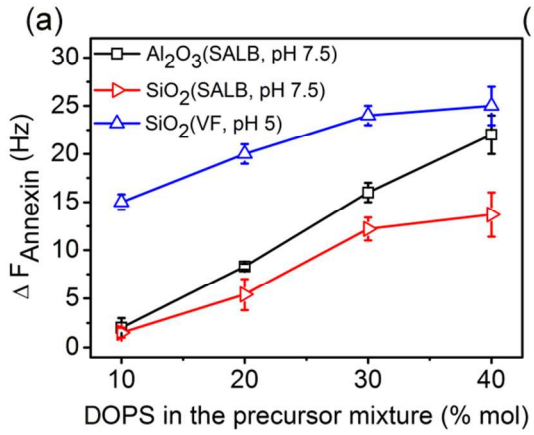
Further to explore the relative fraction of DOPS lipid molecules in formed bilayers, we employed the binding of annexin 5A in the presence of divalent cation, Ca^{2+}. Since annexin 5A binds specifically to DOPS molecules, the frequency shifts caused by its binding at equilibrium can be used as a probe to compare the relative amount of DOPS incorporated in different bilayers as shown in **Fig. 2a**.

In all cases, with increasing fraction of DOPS in the precursor mixture, $\Delta f_{\text{Annexin 5A}}$ at equilibrium increased proportionally in agreement with previous reports[42]. However, the amount of adsorbed annexin 5A on the bilayer interface was found to be governed by the substrate material and how lipid molecules self-assembled on the substrates. The amount of annexin 5A adsorbed to the bilayer prepared on SiO_2 via vesicle fusion method was higher than of that adsorbed to the bilayers prepared via the SALB formation method on both SiO_2 and Al_2O_3 as shown in **Fig. 2a**. Therefore the fraction of DOPS molecules incorporated in the bilayers prepared by SALB formation method was lower that the fraction of DOPS molecules in the bilayer prepared by vesicle fusion. These results suggest that the fraction of DOPS assembled in the supported bilayers were lower than its fraction which initially included in the precursor lipid mixture used in the SALB formation protocol. In the course of bilayer formation by SALB formation method, lipid molecules first adsorb to the solid surface in the presence of pure organic solvent (see **Fig. 1a, arrow 3**). These adsorbed lipid molecules are in equilibrium with monomeric lipids in the organic solution and act as nucleation sites for further self-assembly on the solid surface, upon gradual increase in the water content of the ternary (alcohol-water-lipid) mixture[27]. Due to the electrostatic repulsion among negatively charged DOPS lipid molecules and the substrate, it is likely that the lipid islands formed in the presence of pure alcohol are preferentially populated with zwitterionic DOPC lipid molecules. Likewise, the electrostatic repulsion affects other steps of the self-assembly in a molecular level, suppressing accumulation of charged lipids at the interface.

The isoelectric point of the SiO_2 and Al_2O_3 are 3.9 and 8.7, respectively[47]. Thus, at pH 7.5, SiO_2 is negatively charged and Al_2O_3 possesses a slight positive charge. Consequently, the amount of negatively charged lipids in the bilayer prepared on Al_2O_3 should be higher than that of SiO_2, which is indeed validated by our results (**Fig. 2a**). The adsorption of annexin to bilayer surfaces was further characterized by converting the corresponding $\Delta f_{\text{annexin 5A}}$ of each bilayer to the surface mass density and calculating the protein surface coverage. The energy dissipations corresponding to the protein binding were very low ($\Delta D_{\text{annexin 5A}} < 0.2 \times 10^{-6}$), indicating rigidity of the protein adlayer. Therefore, Sauerbrey relationship can be employed to convert the $\Delta f_{\text{annexin 5A}}$ to the surface mass density (**Fig. 2b**). The protein surface coverage was also calculated using a multiplying factor (P = -94 Hz)[48] which converts the $\Delta f_{\text{annexin 5A}}$ to the surface coverage (θ), taking into account a jamming limit of 54.7% according to the following equation:

$$\theta = \frac{\Delta f}{(0.547)\times P} \quad (1)$$

The multiplying factor (P) had been calculated by correlating a known protein surface coverage which was obtained from scanning force microscopy images to the corresponding QCM-D frequency change caused by annexing 5A binding to a similar membrane.



(b)

Method	Surface	Composition	Annexin V: Δf (Hz)	Annexin V: Surface Mass Density (ng/cm²)	Annexin V: Fractional Surface Coverage (%)
SALB (pH 7.5)	SiO_2	10% PS	1.5 ± 0.5	26.6 ± 8.9	2.9 ± 1
		20% PS	5.4 ± 1.6	95.6 ± 28.3	10.5 ± 3.1
		30% PS	12.2 ± 1.2	216 ± 21.2	23.7 ± 2.3
		40% PS	13.7 ± 2.3	242.5 ± 39.8	26.6 ± 4.4
	Al_2O_3	10% PS	2 ± 1	35.5 ± 17.7	3.9 ±1.9
		20% PS	8.3 ± 0.3	147.2 ± 5.3	16.1 ± 0.6
		30% PS	16 ± 1	283.7 ± 17.7	31.1 ± 1.9
		40% PS	22 ± 2	390.1 ± 35.5	42.8 ± 3.9
Vesicle Fusion (pH 5)	SiO_2	10% PS	15 ± 0.8	265.5 ± 14.2	29.2 ± 1.6
		20% PS	20 ± 1	354 ± 17.7	38.9 ± 1.9
		30% PS	24 ± 1	424.8 ± 17.7	46.7 ± 1.9
		40% PS	25 ± 2	442.5 ± 35.4	48.6 ± 3.9

Fig. 2 Summary of QCM-D response of annexin 5A binding to DOPS-containing bilayers formed on SiO_2 and Al_2O_3 via SALB formation and vesicle fusion method. (a) Equilibrium QCM-D frequency shift of annexin 5A (5 µg/ml) adsorption to bilayers prepared on SiO_2 and Al_2O_3 as function of DOPS molar fraction in the precursor lipid mixtures. The method, substrate and conditions of bilayer formation are specified in the graph legend. (b) The amount of membrane-bound annexin 5A for each membrane composition was determined from its corresponding $\Delta f_{\text{annexin 5A}}$. The surface mass density of proteins was calculated using Sauerbrey's equation (assuming that the frequency change is proportional to the adsorbed protein mass) [$\Delta m = - C_{QCM} (\Delta f)$, C_{QCM} = 17.7 ng cm^{-2} Hz^{-1}]. The fractional surface coverage was estimated using a multiplying factor (P = - 97 Hz) converting the measured frequency shift to the surface coverage, taking into account a jamming limit of 54.7%.

We further used SALB formation to prepare positively charged membranes on SiO_2 and Al_2O_3 and compared the results with VF method (**Fig. 3**). Bilayers were prepared using DOPC mixed with different fractions of positively charged DOEPC (10-40 mol %). For all DOEPC fractions examined, SALB resulted in the formation of bilayer on SiO_2 (**Fig. 3a**) with final frequency shifts of about -25 Hz and energy dissipation of < 0.7 (**Fig. S2a and b** of the Supporting Information).

In contrast to negatively charged vesicles, the adsorption of cationic vesicles led to formation of bilayer (**Fig. 3b**). In this case the vesicle rupture was promoted by attractive electrostatic force between positively charged membrane and negatively charged SiO_2 surface. The final frequency and dissipation shifts of the bilayers prepared by vesicle fusion were similar to that prepared by SALB (**Fig. S2a and b** of the Supporting Information).

On Al_2O_3 positively charge vesicles remained intact (**Fig. 3d**) as expected, with the final frequency change of around -200 Hz and the energy dissipation of greater than 10×10^{-6} (**Fig. S2c and d** of the Supporting Information). Formation of high quality positively charged bilayer on Al_2O_3 substrate using SALB was also found to be difficult. QCM-D results showed that the surfaces were covered by lipid;

however, further BSA protein adsorption study showed those bilayers are defective (**Fig. S3** of the Supporting Information). The difficulty of formation of positively charged bilayer on Al_2O_3 might be due to the repulsive force between positively charged lipids and the slightly positively charged Al_2O_3 surface at pH 7.5.

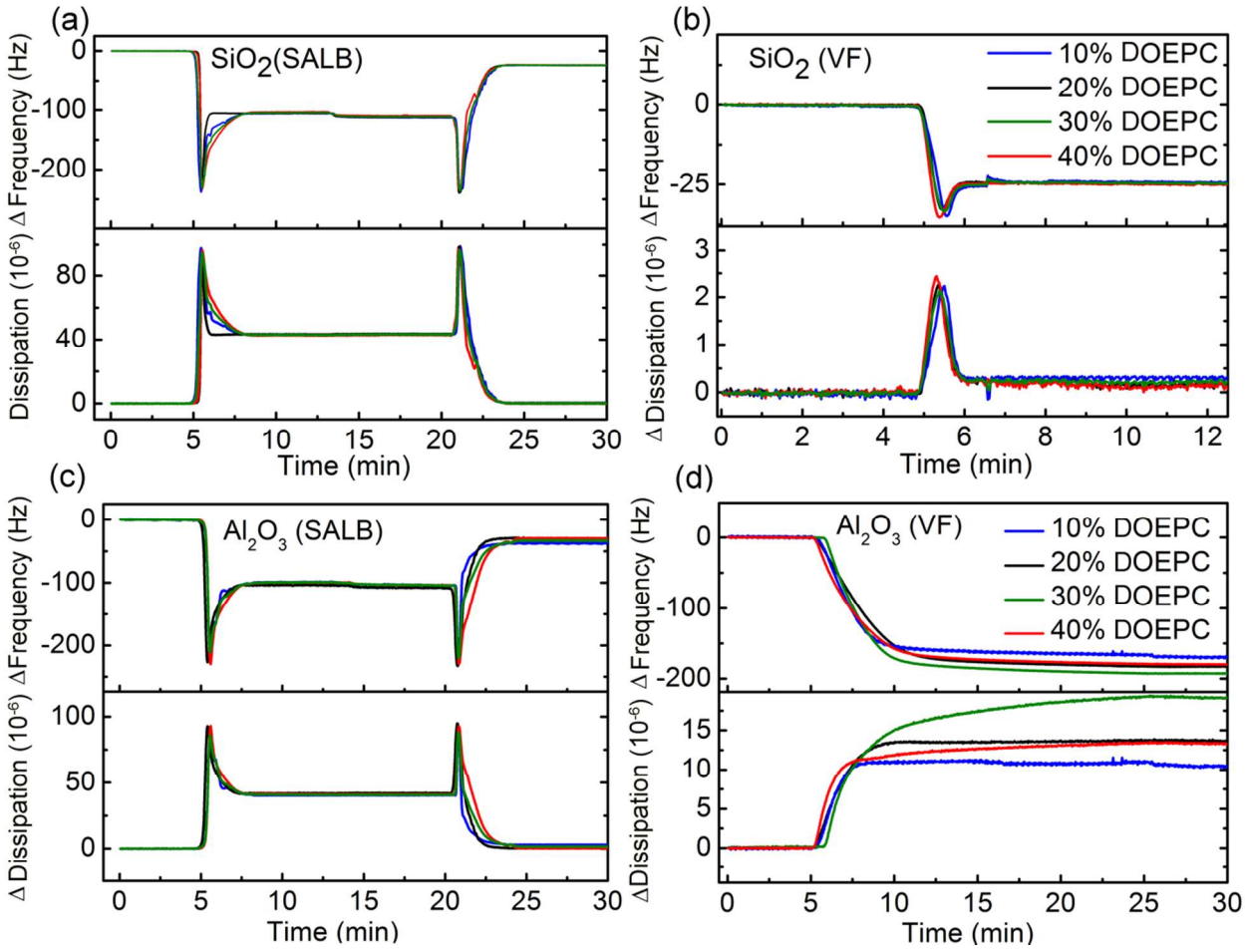


Fig. 3 QCM-D analysis of DOEPC-containing bilayer formation via vesicle fusion and the SALB formation method on SiO_2 and Al_2O_3. Change in the frequency (top) and energy dissipation (bottom) were measured as a function of time during bilayer formation onto (a) SiO_2 and (c) Al_2O_3 via the SALB method using varying mole fractions of DOEPC in the precursor mixture (between 10 and 40 mol%). Panel (b) and (d) correspond to the adsorption of DOPC vesicles with varying fraction of DOEPC (10-40%) on SiO_2 and Al_2O_3 respectively. The arrow indicates the injection of vesicle solution (0.1 mg/ml in 10 mM Tris, 150 mM NaCl, pH 7.5).

Conclusion

In summary, we demonstrated the formation of anionic and cationic bilayers on SiO_2 and Al_2O_3 using SALB method and compared the results with the conventional method of bilayer formation; vesicle fusion. Negatively charged lipid bilayers were successfully formed on both SiO_2 and Al_2O_3 substrate using SALB formation method at physiological pH (7.5). The fraction of anionic lipids incorporated in the bilayers was evaluated by specific annexin 5A binding. The annexin 5A binding results revealed that

the fraction of DOPS lipid molecules incorporated into the bilayers prepared by SALB method on SiO_2 is lower than its fraction in the parent lipid mixture, presumably due to the repulsive force among anionic lipids and negatively charged SiO_2. In agreement with this argument, the fraction of DOPS in the bilayers formed on a slightly positive surface of Al_2O_3 was found to be higher than those prepared on negatively charged SiO_2. These findings indicate that when multicomponent lipid mixtures are used, the relative fraction of lipids in the bilayer prepared by SALB formation method might differ from the fraction of lipids in the precursor mixture.

In case of vesicle fusion method, only vesicles containing 10% DOPS ruptured on SiO_2 and the rest of the examined composition did rupture on neither SiO_2 nor Al_2O_3 due to inability of charged vesicle to adsorb to the substrate and fuse. However it was possible to promote the vesicle rupture on SiO_2 by lowering the pH to 5 and hence reducing the surface charge of SiO_2 and vesicles.

Both the SALB and vesicle fusion method could be used to form positively charged bilayers on SiO_2 at all examined DOEPC fractions. In contrast, neither the SALB nor vesicle fusion method resulted in the formation of uniform, positively charged bilayer on Al_2O_3, presumably due to the repulsive force between membrane and the substrate.

Taking into account the simplicity of the SALB formation method, it is suited for preparing charged supported bilayers in particular on substrates which are intractable to vesicle fusion (e.g., Al_2O_3). Such bilayers can serve as a simple platform to track the interaction of proteins and other biomolecules with charged membranes.

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