On the Effect of the Solid Support on the Interleaflet Distribution of Lipids in Supported Lipid Bilayers

Ralf P. Richter, Nicolas Maury, and Alain R. Brisson*

Laboratoire d'Imagerie Moléculaire et Nano-Bio-Technologie, IECB, UMR-CNRS 5471, Université Bordeaux I, 2 Rue Robert Escarpit, 33607 Pessac Cedex, France

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The adsorption and spreading of lipid vesicles on solid supports has become a popular way to create supported lipid bilayers (SLBs), but little attention has been paid to the possible redistribution of lipid material between the two leaflets of an SLB. We use the technique of quartz crystal microbalance with dissipation monitoring (QCM-D) to follow the adsorption of prothrombin on SLBs formed from sonicated unilamellar vesicles containing mixtures of dioleoylphosphatidylcholine (DOPC) and dioleoylphospatidylserine (DOPS). The specific interaction of prothrombin with negatively charged lipids is quantified and serves as a reporter of the content of accessible DOPS in SLBs. We compare results obtained on silica and mica and find that the underlying support can induce substantial redistribution of lipid material between the two leaflets. In particular, SLBs formed on mica showed a substantially depleted amount of accessible DOPS in the presence of calcium. The mechanisms that lead to the lipid redistribution process are discussed.

Introduction

Supported lipid bilayers (SLBs) have become popular[1,2] as model systems for cell membranes[3,4] and as a building block for biofunctional surfaces.[5−7] The creation of SLBs by adsorption and spreading of vesicles on hydrophilic supports[8] is attractive by its simplicity, and important insights into the nature of this self-organization process have been gained during past years.[9−24] Recent studies by us[21−23] and others[9,12,25,26] provide evidence that the solid support plays a determinant role in the lipid deposition process, giving rise to a multitude of SLB-formation pathways or even preventing SLB-formation.

In addition, the solid support can have pronounced effects on the properties of the SLB, once formed. This may be illustrated by our observation that annexin A5, a protein that adsorbs to DOPS-containing bilayers in a calcium-dependent manner,[27] self-assembles into two-dimensional (2D) crystals on SLBs formed on mica,[28,29] whereas only a close-packed layer of annexin A5 can be found on silica-SLBs under otherwise identical conditions.[30] While the origin of this effect remains unclear, it prompted us to further investigate the properties of DOPS-containing SLBs on mica and silica.

Little attention has so far been paid to the trans-bilayer distribution of lipids in SLBs that are formed from vesicles containing a mixture of different types of lipids. Commonly, the lipid molecules are assumed to be distributed equally between the two SLB-leaflets, neglecting that the solid support may interact differently with the support-facing (proximal) and the bulk-facing (distal) leaflet.

The objective of this study was to develop an assay that allows one to measure the amount of DOPS accessible in the distal leaflet of SLBs formed on silica and on mica. A promising strategy to quantify the amount of accessible lipids is to follow the adsorption of a molecular entity that binds specifically to the lipid in question. An important and, as the example of annexin A5 demonstrates, nontrivial prerequisite for such an assay is that the adsorption and 2D organization of the molecule is not biased by the underlying solid support.

Here, we make use of the quartz crystal microbalance with dissipation monitoring (QCM-D) technique to follow

* Corresponding author. E-mail: a.brisson@iecb.u-bordeaux.fr.

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the adsorption of prothrombin to DOPS-containing SLBs. As annexin A5, prothrombin binds to DOPS, in a calcium-dependent manner.[31,32] However, no membrane-induced clustering has been reported to date. The QCM-D technique is by now established as a versatile tool to characterize interfacial processes such as SLB-formation[12,21,26] or protein adsorption.[33] Recently, we have demonstrated that QCM-D measurements can be performed on mica-coated sensors,[22] opening up for comparative measurements between silica and mica.

After establishing that prothrombin is indeed a suitable reporter for the DOPS-content in SLBs, we demonstrate here that the solid support can indeed have a profound influence on the trans-bilayer distribution of DOPS.

Materials and Methods

Materials. Dioleoylphosphatidylcholine (DOPC) and dioleoylphospatidylserine (DOPS) were purchased from Avanti Polar-Lipids (AL). Lyophilized prothrombin (factor II) from human plasma and other chemicals were purchased from Sigma. Ultrapure water with a resistivity of 18.2 MΩ (Millipore, Molsheim, France) was used.

QCM-D sensor crystals (5 MHz) with a final coating of 50 nm reactively sputter-coated silicon oxide were purchased from Q-SENSE (Gothenburg, Sweden). Muscovite mica disks of 12 mm diameter and low viscosity epoxy glue (EPOTEK 377) for the mica-coating of the QCM-D sensors were purchased from Metafix (Montdidier, France) and Gentec Benelux (Waterloo, Belgium), respectively.

A buffer solution made of 150 mM NaCl, 3 mM NaN_3, and 10 mM HEPES, pH 7.4, was prepared in ultrapure water, and 2 mM $CaCl_2$ or EDTA was added, as indicated in the text. Small unilamellar vesicles (SUVs) of desired lipid mixture were prepared by sonication as described earlier.[21] Lipid concentrations were deduced from the mass of the lipids dissolved and checked by phosphorus content determination[34] of the final lipid suspensions. Errors of less than 10% were obtained. Before use, vesicle suspensions were diluted at 0.05 mg/mL, if not otherwise stated.

Lyophilized prothrombin was reconstituted in ultrapure water as described by the manufacturer. Small aliquots were stored at −20 °C. Before use, the prothrombin solution was thawed at 4 °C and used within 2 days. The protein concentration was determined from optical density measurements at a wavelength of 280 nm using $\epsilon_{280} = 1.53\ g^{-1}\ L\ cm^{-1}$.

Substrate Preparation. Silica-coated QCM-D sensors were cleaned by two cycles of exposure to 2% sodium dodecyl sulfate solution for 15 min, rinsing with ultrapure water, blow-drying with nitrogen, and exposure to ultraviolet (UV)/ozone[21] (BHK, Claremont, USA) for 10 min. Cleaned substrates were stored in air and again exposed to UV/ozone (10 min) prior to use. Mica was glued on QCM-D sensor crystals and verified to operate stably according to a previously described protocol.[22]

Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D). QCM-D measurements were performed with the Q-SENSE D300 system equipped with an Axial Flow Chamber (QAFC 302) (Q-SENSE AB, Gothenburg, Sweden) as described in detail elsewhere.[35] Briefly, upon interaction of (soft) matter with the surface of a sensor crystal, changes in its resonance frequency, Δf, related to attached mass (including coupled water), and in its dissipation, ΔD, related to frictional (viscous) losses in the adlayer, are measured with a time resolution of better than 1 s.

The system was operated in slow flow mode. Degassed sample liquid was continuously delivered to the measurement chamber by the aid of a peristaltic pump (ISM832A, Ismatec, Zürich, Switzerland). The T-loop in the measurement chamber was bypassed, and flow rates were kept sufficiently low (40 or 80 μL/min, as indicated in the text) to ensure stable operation at a working temperature of 24 °C. To switch between sample liquids, the flow was interrupted for a few seconds without disturbing the QCM-D signal. A lag time of 2−4 min remained until the sample reached the QCM-D sensor. With this setup, adsorption and interfacial processes can be followed *in situ* while subsequently exposing different solutions to the surface. The system can be operated at relatively small sample concentrations (1 μg/mL and less) as the flow limits depletion (enrichment) of the bulk upon adsorption (desorption) of sample to (from) the sensor surface or the chamber walls.

Resonance frequency and dissipation were measured at several harmonics (15, 25, 35 MHz), simultaneously. If not stated otherwise, changes in dissipation and normalized frequency ($\Delta f_{norm} = \Delta f_n/n$, with n being the overtone number) of the third overtone ($n = 3$, i.e., 15 MHz) are presented. Adsorbed (wet) masses, Δm, were calculated according to the Sauerbrey equation,[36] $\Delta m = -C \cdot \Delta f_{norm}$, with $C = 17.7\ ng\ cm^{-2}\ Hz^{-1}$. The equation has been demonstrated to be a good approximation for lipid bilayers, adsorbed nonruptured SUVs, or globular proteins on the supports investigated here.[12,18,22] The validity of the equation was further verified by comparison with the viscoelastic model[37] as implemented in the software QTools 2 (Q-Sense, Gothenburg, Sweden). Deviations of less than 5% were obtained.

Analysis of Adsorption Kinetics. Dissociation constants, k_D, and maximum adsorbed amounts, Δf_{max}, were determined by fitting the equilibrium frequency shifts, Δf_e, established at various bulk concentrations, c, of prothrombin to the Langmuir isotherm, $\Delta f_e = \Delta f_{max} \times c\ /(k_D + c)$.

Results

SLB-Formation and Prothrombin Adsorption. In a first step toward using prothrombin as a reporter of the content of accessible DOPS in SLBs, we characterized the adsorption of prothrombin on model SLBs of DOPC/DOPS (molar ratio 4:1) on silica and of DOPC/DOPS (1:1) on mica. Both the formation of the SLB and the adsorption/desorption of prothrombin were followed by QCM-D (Figure 1).

The QCM-D response upon exposure of lipid material to the support revealed, as expected, a characteristic two-phase behavior, reflecting the initial adsorption of intact vesicles which is followed by the formation of an SLB.[12,22] The final frequency shift of -25 ± 1 Hz and the low dissipation shift of less than 0.2×10^{-6} confirm the formation of a lipid bilayer that entirely covered the support with no or only minor defects.[18,23]

On a silica-SLB (Figure 1A), stepwise addition of prothrombin solution of increasing concentration induced a stepwise decrease of the frequency, indicating adsorption, until equilibration. Rinsing with prothrombin-free buffer caused desorption of more than 80% of the prothrombin over the time scale of 1 h. Addition of a buffer containing the calcium chelator EDTA resulted in rapid unbinding of the remaining prothrombin. The results confirm that the adsorption of prothrombin is (i) reversible and (ii) calcium dependent, as was previously known.[31,32]

Whereas the frequency provides information on the adsorbed amounts, the dissipation can reveal details about the "pathway" of adsorption taken by the prothrombin. It is interesting to note that, after an initial increase with decreasing frequency, the dissipation decreased (Figure 1A, asterisk) once the amount of prothrombin adsorbed was higher than an amount corresponding to $\Delta f = -28$ Hz. The decrease in dissipation clearly indicates a

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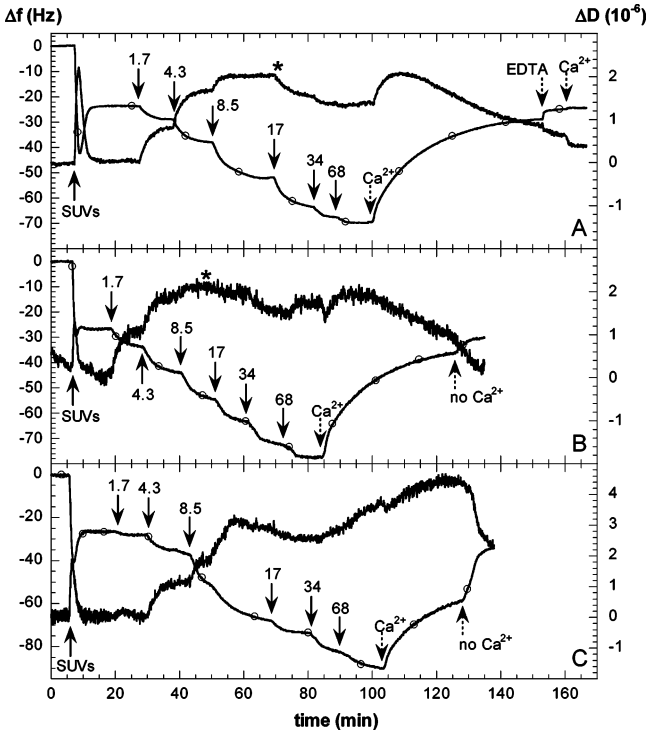

Figure 1. Changes in frequency, Δf (-○-), and dissipation, ΔD (- - -), for the formation of SLBs and the subsequent adsorption of prothrombin. SLBs were formed from SUVs of a DOPC/DOPS-mixture with molar ratio of 4:1 on silica (25 MHz) (A), 1:1 on mica (15 MHz) (B), and 2:1 on mica (15 MHz) (C). Solid arrows indicate the exposure of SUVs and prothrombin; associated numbers denote the prothrombin concentration in μg/mL. Dashed arrows indicate the rinse with buffer containing 2 mM Ca^{2+}, no Ca^{2+}, or 2 mM EDTA, as denoted. The decrease in dissipation at an adsorbed of prothrombin corresponding to −28 Hz (asterisks in (A) and (B)) indicates a rigidification of the prothrombin layer with increasing coverage. The flow speed was 40 μL/min for the exposure with lipids and prothrombin at a concentration of ≥17 μg/mL and 80 μL/min for all other adsorption and desorption steps.

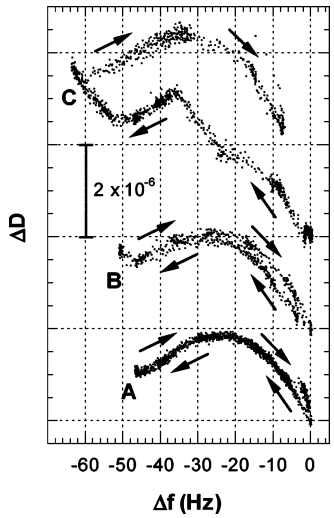

Figure 2. Plots of ΔD versus Δf for the adsorption (desorption) of prothrombin on (from) SLBs. Whereas the plots for the adsorption of prothrombin on DOPC/DOPS (4:1) on silica (A) and on DOPC/DOPS (1:1) on mica (B) are almost identical, anomalous responses are observed for a measurement with DOPC/DOPS (2:1) on mica (C). The data correspond to the measurements shown in Figure 1. The time course, starting at $\Delta f = \Delta D = 0$, is implicit in this plot and is indicated by solid arrows.

rigidification of the adsorbed layer when approaching high protein coverage.

To further characterize the state of the prothrombin layer, we make use of the $\Delta D-\Delta f$-plot (graph A in Figure 2). The plot relates the changes in dissipation (i.e., the rigidity of the adlayer) to changes in frequency (i.e., the adsorbed mass). Consequently, such a graph gives a qualitative fingerprint of how the conformational state (rigid/nonrigid) of the adsorbed layer evolves with coverage.[33,38] As time is not explicit in this plot, it allows for the direct comparison of adsorption and desorption data of several measurements with varying kinetics. Notably, the $\Delta D-\Delta f$-plots corresponding to both the adsorption phase and the desorption phase superposed exactly. In addition, the $\Delta D-\Delta f$-plots were identical, both when the bulk prothrombin concentration was increased stepwise (cf. Figure 1A) and when it was maintained at maximum (68 μg/mL) throughout the entire adsorption process (data not shown).

These observations, together with the observed complete reversibility, confirm that the adsorbed amounts of prothrombin are determined entirely by the equilibrium between the prothrombin concentration in the bulk and on the surface and do not dependent on the history of adsorption. This corroborates the validity of our working hypothesis that prothrombin binding can be characterized by the amounts of prothrombin adsorbed at equilibrium for several bulk concentrations.

On SLBs formed on mica (Figure 1B), an adsorption curve for prothrombin similar to that observed on silica-SLBs was obtained. Again, the adsorption is almost completely reversible,[39] and the $\Delta D-\Delta f$-plots (graph B in Figure 2) for adsorption and desorption follow (i) closely each other as well as (ii) the plots for the silica-SLB (graph A in Figure 2). This indicates that the adsorption processes on mica and silica are identical and thus that the adsorbed amounts on mica and silica can be strictly compared to deduce the amount of accessible DOPS.

Dependence of Prothrombin Adsorption on the DOPS-Content. In a second step, we measured the adsorption of prothrombin to SLBs over the range of DOPS-contents for which SLBs could be obtained, and we systematically investigated the adsorbed amounts at (or close to) equilibrium for various bulk prothrombin concentrations. We employed the $\Delta D-\Delta f$-plot to distinguish between typical and anomalous adsorption behavior. Indeed, most adsorption curves resulted in the typical $\Delta D-\Delta f$-plots (cf. graphs A,B in Figure 2), with some exceptions (described below in the paragraph "Reproducibility", Figure 1C, and graph C in Figure 2) that were discarded. The results for silica and mica are shown in Figure 3.

(Almost) no prothrombin adsorbed to pure DOPC, demonstrating the specificity of prothrombin for DOPS. As expected from previous studies,[31,32] adsorbed amounts increased with increasing DOPS-content. Importantly, the adsorbed amounts vary widely over the entire range of accessible DOPS-concentrations, confirming that prothrombin is a suitable reporter of the DOPS-content. The maximum amounts of bound prothrombin were identical for silica and mica ($\Delta f_{max} = -52 \pm 2$ Hz), corresponding to a mass of 920 ± 35 ng cm^{-2}. Comparing the QCM-D

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(39) Adsorption was reversible to within less than 10% of the maximum coverage; the remaining frequency shift of −3 Hz may be attributed to minor drifts on the generally less stable mica surfaces.

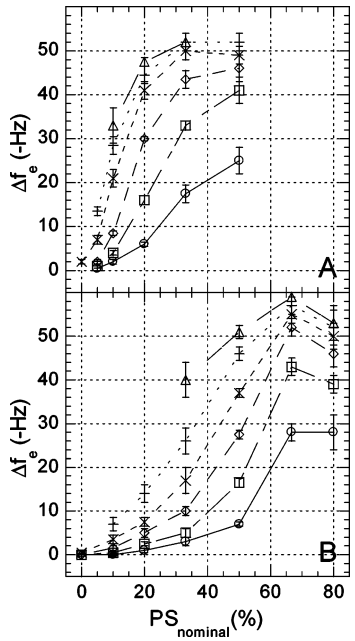

Figure 3. Equilibrium frequency shifts, Δf_e, for the adsorption of prothrombin on SLBs of varying nominal DOPS-content on silica (A) and mica (B). Bulk concentrations of prothrombin were 1.7 (○), 4.3 (□), 8.5 (◇), 17 (×), 34 (+), and 68 μg/mL (△), respectively. Lines are included to guide the eye.

mass, that includes coupled water, with reported values of the dry protein mass ($\sim 350 \pm 60$ ng cm^{-2} [31]), we obtain an effective water content of 55−70%, which is in the upper range reported for (sub)monolayers of globular proteins. The resulting density of ~ 1.13 g cm^{-3} (assuming a protein density of 1.35 g cm^{-3}) gives an effective thickness of the prothrombin layer of around 8 nm, in agreement with available structural data for the prothrombin molecule.[42]

Intriguingly, the amounts of adsorbed prothrombin for a given nominal DOPS-content (i.e., the content determined by the molar mixing-ratio of DOPC and DOPS) differ markedly between silica-SLBs and mica-SLBs. For example, at a nominal DOPS concentration of 20%, the exposure of prothrombin at a concentration of 8.5 μg/mL led to frequency shifts of −30 Hz with a silica-SLB and −5 Hz with a mica-SLB (Figure 3). For a given DOPS-content, the adsorption of prothrombin was systematically higher on silica than on mica, indicating that the amount of accessible DOPS, that is, the DOPS in the distal lipid leaflet, was higher on silica than on mica. The data demonstrate that the underlying support has a profound influence on the amount of DOPS that is accessible to the protein and provide indications for an unequal distribution of DOPS molecules between the distal and the proximal leaflet in the SLB on at least one of the solid supports.

To investigate which of the solid supports induces an asymmetry in the lipid distribution, we hypothetically assumed a balanced lipid distribution between the two leaflets of the SLB on either silica or mica, taken as a reference, and estimated the amount of accessible DOPS on mica (silica), by comparing the adsorbed amounts of prothrombin with the corresponding "reference" (Table 1). The ratio between the nominal and the accessible DOPS-content obtained for silica was smaller than 0.5, for the nominal DOPS-contents of 5%, 10%, and 20%. A ratio of 0.5 corresponds to the extreme situation, for which the entire DOPS-content in the bilayer is contained in the distal leaflet. Values below 0.5 are thus physically not reasonable. This inconsistency disproves that mica can be a proper reference and, in consequence, indicates that the DOPS-distribution is asymmetric in mica-SLBs.

In contrast, the results obtained for mica with the silica-"reference" remain consistent in the frame of this comparison. Thus, while the presentation in Table 1 allowed us to determine that the distribution of DOPS in mica-SLBs must be asymmetric, it does not enable us to draw any conclusions about the distribution on silica. In principle, the lipid distribution may or may not be asymmetric on silica, and independent reference data would be needed to settle this question.[43]

Analysis of the Kinetics of Adsorption of Prothrombin. The full reversibility of prothrombin adsorption implies that one important prerequisite for a simple kinetic model, the Langmuir model, is fulfilled.[47] Plotting the equilibrium adsorbed amounts against the bulk concentration of prothrombin (Figure 4), however, reveals sigmoid-shaped (and not Langmuir-isotherm-like) curves, that become particularly apparent for low DOPS-content. To compare our data with kinetic constants reported in the literature, we have, though, attempted to fit our results to the Langmuir isotherm by neglecting the equilibrium adsorbed amounts at low protein concentration (Table 2). The resulting effective dissociation constants, k_D, vary between 0.2 and 0.02 μM over a range of 10−50% DOPS. These values are in the range of 0.3−0.01 μM reported previously for less than 50% DOPS.[31,48−50] A more rigorous comparison of our results with those reported in the literature is obscured by variations in the experimental conditions (such as calcium content and temperature) and the methods used to determine k_D. We therefore did not attempt to employ literature data as an independent reference to evaluate the DOPS-distribution.

Influence of the History of the SLB on the Amount of Accessible DOPS. To investigate the influence of the SLB-formation process on the amount of accessible DOPS in mica-SLBs, we performed the prothrombin test on SLBs that were formed from vesicles (DOPC/DOPS (4:1)) at extreme concentrations of 0.5 and 0.015 mg/mL, which mark the limits of the experimentally accessible time range for SLB-formation ($\sim$30 s to $\sim$20 min). The prothrombin adsorption behavior was identical in both cases (data not

(40) For comparison, albumin, ferritin, and fibrinogen, all adsorbing at submonolayer coverage on a solid support, showed a solvent content of ~43%,[41a] ~56%,[41b] and ~64%,[41a] respectively. For streptavidin, coupled to a biotinylated-SLB, a solvent content of ~55% was reported.[41c]

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Table 1. Comparison of the Nominal and the Accessible DOPS-Content for SLBs on Mica (A) and on Silica (B), As Obtained by the Prothrombin Assay (Figure 3), Considering as References Either Silica (A) or Mica (B)

			DOPS-content			
	support	hypothetical reference	nominal (%)	accessible[a] (%)	nominal/ accessible	proximal leaflet/ distal leaflet[b]
A	mica	silica	0	0	–	–
			10	3 ± 1	3.3 ± 1.1	5.7 ± 3.4
			20	7 ± 1	2.9 ± 0.5	4.7 ± 1.0
			33	13 ± 2	2.5 ± 0.5	4.1 ± 1.0
			50	20 ± 2	2.5 ± 0.3	4.0 ± 0.6
			67	>55	<1.2	<1.4
			80	>60	<1.3	<1.7
B	silica	mica	0	0	–	–
			5	16 ± 4	0.31 ± 0.11[c]	–[c]
			10	32 ± 4	0.31 ± 0.05[c]	–[c]
			20	50 ± 2	0.40 ± 0.02[c]	–[c]
			33	65 ± 3	0.51 ± 0.03	0.02 ± 0.06
			50	72 ± 3	0.69 ± 0.03	0.39 ± 0.06

[a] The amount of accessible DOPS was determined by fitting the set of equilibrium frequency shifts, Δf_e, obtained on a mica-SLB (A) (a silica-SLB (B)) of given nominal DOPS-content to the set of (hypothetical) reference curves for the silica-SLB (mica-SLB). [b] Distribution of DOPS between the proximal and the distal leaflet of the SLB, given by 2 × nominal DOPS-content/accessible DOPS-content − 1. It is assumed that the nominal DOPS-content corresponds to the overall DOPS-content in the SLB. [c] Values for the ratio between nominal and accessible DOPS-content below 0.5 are physically unreasonable (see text for details), and no meaningful values for the DOPS-distribution between the bilayer leaflets can therefore be obtained.

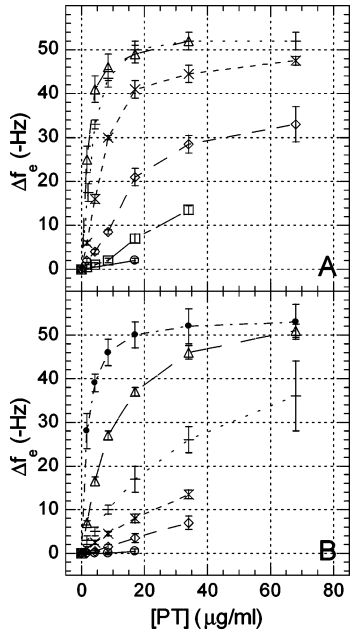

Figure 4. Equilibrium frequency shifts, Δf_e, as a function of bulk concentrations for the adsorption of prothrombin on SLBs on silica (A) and mica (B). SLBs were formed from SUVs of DOPC (○) and DOPC/DOPS-mixtures (molar ratio) 19:1 (□), 9:1 (◇), 4:1 (×), 2:1 (+), 1:1 (△), and 1:4 (●). Lines are included to guide the eye.

Table 2. Effective Kinetic Constants Obtained for the Adsorption of Prothrombin on Silica-SLBs (Data from Figure 4A, the Regime of Low Coverage ($|\Delta f| < 20$ Hz) Was Neglected)

DOPS (%)	k_D (10^{-8} M)	Δf_{max} (−Hz)
10	21.9 ± 6.9	40.5 ± 3
20	5.6 ± 2.3	50 ± 2
33	2.9 ± 1.0	54.5 ± 2
50	1.9 ± 0.8	53.5 ± 2

shown), indicating that the SLB-formation time does not influence the amount of accessible DOPS.

Also, varying the delay time from SLB-formation to prothrombin exposure over a range of 5−60 min did not significantly alter the prothrombin adsorption, indicating that slow lipid redistribution between the two leaflets of the mica-SLB is not responsible for the varying amount of accessible DOPS.

Reproducibility. Most of the QCM-D responses obtained for the adsorption and desorption of prothrombin resulted in the typical $\Delta D-\Delta f$-plot, shown for graphs A and B in Figure 2. In some cases, however, significantly different responses were observed. An example is given in Figure 1C, corresponding to a DOPC/DOPS (2:1)-SLB on mica. At a prothrombin concentration of 8.5 μg/mL, the frequency decreased surprisingly (to around −40 Hz of adsorbed prothrombin), whereas the dissipation increased to anomalous levels. A similar phenomenon could be observed at 68 μg/mL, and both effects lead to characteristic changes in the slope in the $\Delta D-\Delta f$-plot (graph C in Figure 2). In accordance with these results, the dissipation does not decrease to values close to zero after close-to-complete desorption of prothrombin. Such an anomalous behavior was occasionally observed for SLBs both on silica and on mica, and the reason for this behavior is at present not clear. The analysis of freshly prepared prothrombin stock solution by SDS-PAGE analysis revealed some faint bands in addition to the main band, which became more pronounced when the protein was stored at 4 °C for a few days (and even faster when kept at room temperature), and thus suggests protein degradation as a possible reason for the observed anomalies. It is notable that the $\Delta D-\Delta f$-plot allows one to discriminate between different types of adsorption phenomena and we used this plot as a quality control of our experimental data.

Discussion

Lipid Redistribution Induced by the Nature of the Solid Support. The prothrombin assay described here provides evidence for substantial differences in the amount of DOPS present in the distal SLB-leaflet between SLBs formed on mica and on silica, from SUVs with identical DOPS-content. Our results allow us to attribute these differences to a surface-induced redistribution of DOPS between the two SLB-leaflets.

In particular, the comparison between mica and silica provides indications that the distal leaflet in mica-SLBs is depleted in DOPS. As no indications were found for significant changes in the amount of DOPS in the distal leaflet after completion of SLB-formation, the lipid

redistribution must occur during the SLB-formation process. Furthermore, as changes in the SLB-formation kinetics over the experimentally accessible time range (in the order of $10-10^3$ s) did not significantly alter the amount of accessible DOPS, we conclude that the lipid redistribution process takes place on time scales below 10 s.

We have previously shown that bilayer patches of sizes ranging from 10^{-4} to 1 μm^2 present an intermediate state in the SLB-formation.[15,21,23] The flip-flop of a lipid molecule across a closed bilayer is commonly considered a slow process (hours and more),[51,52] due to the barrier associated with shuttling the hydrophilic lipid headgroup through the bilayer's hydrophobic interior. On the other hand, a lipid transfer across the edge of the bilayer patches would not be hampered by this barrier and would therefore be much faster. Given that the area covered by a diffusing lipid molecule is on the order of 1 μm^2 s^{-1},[53] we obtain a time of less than 1 s for the lipid transport toward the edge. Assuming that the transfer across the edge occurs at similar time scales or faster,[54] this mechanism of lipid redistribution is consistent with the limit experimentally observed for the redistribution time.

Importantly, the proposed mechanism of lipid redistribution implies that the SLB-formation time is sufficient to install an equilibrium state in the lipid distribution with respect to the interaction of the involved lipid species with the underlying support. Consequently, the lipid distribution in the final SLB would be determined by the properties of the underlying support rather than the genuine lipid distribution in SUVs. In particular, the observed lipid redistribution adds a new aspect to the controversial question of how the lipid leaflets of a rupturing vesicle are oriented on a solid support.[3,16,55]

The Adsorption Behavior of Prothrombin: Implications for the DOPS-Assay. It has already been reported that the adsorption of prothrombin[31,48,56] or prothrombin fragment 1[32] is not satisfactorily described by the Langmuir model with independent and identical binding sites, even though the adsorption is fully reversible. The sigmoidal shape of the curves shown in Figure 4 and the dissociation constants obtained in our study (Table 2) are consistent with previously reported results. To avoid interpretational artifacts, we refrained from a more detailed analysis in this study and used the equilibrium frequency shifts to quantitatively characterize the adsorption of prothrombin with respect to the DOPS-content in the SLBs. We interpret the similitude in the $\Delta D-\Delta f$-plots (cf. Figure 2) for most of our measurements over a large range of DOPS-content and for the different supports as a strong indication that the adsorption pathways are identical. Equilibrium adsorbed amounts of prothrombin (cf. Figure 3) can thus serve as a reliable reporter for the accessible DOPS-content.

Comments on the Dissipation Signal. Phenomenologically, the results shown in Figures 1 and 2 demonstrate that the dissipation, in particular the $\Delta D-\Delta f$-plot, is a sensitive mean to distinguish between different adsorption pathways and/or to identify anomalies in the adsorption behavior.[33] The dissipation signal thus constitutes a relatively simple tool to validate the quality of a measurement and to identify artifacts. Such information is often more difficult to access with other techniques that are commonly used to characterize adsorption phenomena, such as surface sensitive optical techniques (e.g., surface plasmon resonance[57] or ellipsometry) or quartz crystal microbalance without dissipation monitoring.[58]

The lateral interaction of neighboring proteins is likely to be at the origin of the coverage-dependent rigidification of the prothrombin layer, evidenced by the response in dissipation (cf. asterisks in Figure 1A,B). It remains unclear, however, whether the observed decrease in dissipation results from the dense overall packing of prothrombin molecules or from their two-dimensional clustering or ordering. Complementary studies, for example, by AFM, may answer this question but are outside the scope of this work.

Conclusion and Perspectives

Our results show that the support has a determining influence on the distribution of lipids between the two leaflets of an SLB that is formed by the spreading of SUVs. In particular, the distal leaflet of mica-SLBs can be considerably depleted in DOPS. This work thus stresses the importance in understanding the details of the SLB-formation process, in particular the interaction between the solid support and the lipids, to be able to predict and control the properties of SLBs, an important step toward designing biofunctional surfaces.

The QCM-D technique was shown to be a reliable tool in characterizing the adsorption of prothrombin to SLBs. The dissipation served as an important parameter in distinguishing different adsorption pathways and/or identifying anomalies in the adsorption behavior.

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